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A Role for the Ste20-Like Kinase, SLK, in Cell Cycle Progression
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A Role for the Ste20-Like Kinase, SLK, in Cell Cycle Progression

by

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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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ISBN: 978-0-494-66262-5
Our file Notre référence
ISBN: 978-0-494-66262-5

Preface

At the time of printing this thesis, an article based on chapters 3 and 4 has been published in the Journal of Biological Chemistry as:

> The Ste20-like kinase SLK is required for cell cycle progression through G2.
> O'Reilly PG, Wagner S, Franks DJ, Cailliau K, Browaeys E, Dissous C, Sabourin LA.
> J Biol Chem. 2005 Dec 23;280(51):42383-90

Abstract

The Ste20-like kinase, SLK, is a serine/threonine kinase that has been shown to have a role in migration (Roovers et al., 2009; Storbeck et al., 2009; Wagner et al., 2002; Wagner et al., 2008) and cell cycle progression (Ellinger-Ziegelbauer et al., 2000). The latter of these two observations prompted us to investigate the functional role of SLK during G_2/M.

Consistent with the findings of Ellinger-Ziegelbauer *et al.,* 2000, we observed an elevation in SLK activity when synchronized populations of murine fibroblasts enter the G_2/M compartment of the cell cycle but report a 3-4 fold increase in activity in contrast to the modest 1.3 fold increase previously reported. The expression of a catalytically inactive truncation of SLK ($SLK^{1\text{-}373K63R}$, termed KΔC) results in delayed G_2 kinetics. This was evidenced by KΔC-expressing cultures that fail to down regulate cyclin A, to activate cdc2, and to undergo H3 phosphorylation at appropriate times. Supporting this observation, microinjection of a constitutively active truncation of SLK ($SLK^{1\text{-}373}$, termed YΔC) resulted in ectopic mitotic spindle formation in fibroblasts and induced cell cycle re-entry in *Xenopus* oocytes.

During the course of a yeast two hybrid screen we identified MARK3, a kinase that has links to G_2 progression through Cdc25C phosphorylation (Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997), as a SLK interacting protein. We show that SLK is capable of directly binding MARK3 and is capable of phosphorylating a central domain fragment of MARK3 *in vitro* but could not demonstrate a functional role for this phosphorylation event *in vivo.*

Finally, we show that SLK phosphorylates the focal adhesion adaptor protein paxillin *in vitro* in its amino-terminal LD domain. In addition, we followed up on the previous observation

that MARK3 interacts with paxillin (Migration Gateway, 2009) and show that this interaction is direct. We then present evidence that SLK, MARK3, and paxillin form a complex that is most evident during the G2/M compartment of the cell cycle. We propose that MARK3 is responsible for localizing SLK to paxillin during G_2/M where it phosphorylates paxillin leading to a "loosening" of the cells which is required for cell rounding and division.

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Abbreviations

aa	amino acid
AD	activation domain
Arg	arganine
ATP	adenosine triphosphate
ATH	(AT-146 homology) $SLK^{856-1202}$
Cdk	cyclin dependent kinase
C-TAK1	Cdc25C associated kinase 1
Δ3'	SLK^{1-975}
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ΔN	$SLK^{373-1202}$
ECM	extracellular matrix
ERK	extracellular signal response kinase
FA	focal adhesion
FAK	focal adhesion kinase
FBS	fetal bovine serum
GCK	germinal centre kinase
GST	glutathione S-transferase
GVBD	germinal vesicle breakdown
HA	hemagglutinin-A
HCL	hydrochloric acid
IP	immunoprecipitate
IVT	*in vitro* translated
kb	kilobase
KΔC	$SLK^{1-373K63R}$
LOK	lymphocyte oriented kinase

Lys	lysine
MAPK	mitogen activated protein kinase
MARK3	microtubule affinity regulating kinase 3
μg	microgram
μL	microlitre
mL	millilitre
mM	millimolar
MOI	multiplicity of infection
MPF	mitosis/maturation promoting factor
MST1	mammalian sterile twenty kinase
ng	nanogram
nM	nanomolar
PBS	phosphate buffer saline
pH3	phospho-H3
Plk	polo-like kinase
PVDF	polyvinylidene difluoride
LacZ	β-galactosidase
PAK	p21 activated kinase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
siRNA	short interfering RNA
SLK	Ste20-like kinase
Thr	threonine
TM	transmembrane
Tyr	tyrosine
UAD	ubiquitination association domain
WB	Western blot
xPlk	*Xenopus* polo-like kinase
YΔC	SLK^{1-373}

Acknowledgments

First I would like to thank Luc for taking me on as a graduate student. He was extremely generous and always took time out of his schedule to help with any issues I had. I am very fortunate to have been able to learn under him and had more interaction with him than most graduate students have with their supervisors. He kept the graduate experience interesting. He also helped me improve my wrist shot and taught me some French.

I would like to acknowledge all the friends and colleagues that I made during my graduate studies that worked with me and helped me along the way. They include Simona Wagner, Kate Daniel, Phillip Pelletier, Trevor Flood, Ian Hester, Marlene McKay, Jennifer Quizi, Ziad Chaar, Charlie Thompson, Yi-Hong Zhang, and Vivian Lo. I would also like to especially thank Chris Storbeck for his ideas, motivation, and for the life perspectives he instilled in me. I am very lucky for having and an opportunity to work with him.

I would like to thank my parents and my uncle Ben for moulding me into the person I am today; I hope they're proud. Lastly, I would like thank Nicole Burns who kept me inspired and also made sacrifices while I was completing this degree. I don't know where I'd be without her.

Chapter 1

General Introduction

1.1: The Cell Cycle; G_1 Through M.

Cell cycle progression is monitored through kinase mediated signal transduction and the binding of various cyclin proteins to their respective cyclin-dependent kinases (Cdks). Cyclins were termed so due to their characteristic fluctuating levels throughout the cell cycle (Elvin and Evans, 1983) and Cdks are a conserved family of protein kinases (Pines, 1995) that are generally expressed abundantly throughout the cell cycle. The activity of a cyclin/Cdk complex is regulated by cycles of expression and destruction of the cyclin subunit (Reviewed in Smits and Medema, 2001).

G_1 progression is regulated, in part, by cyclins D and E and their respective cyclin dependent kinases by working together in a complex pathway that results in the retinoblastoma (Rb) protein phosphorylation and consequently the production of cyclin A leading to S phase entry (Figure 1.1)(Reviewed in Sherr and Roberts, 1999). Cyclin B synthesis initiates at the end of S phase (Piaggio et al., 1995; Pines and Hunter, 1989) and forms a complex with $p34^{cdc2/cdk1}$. This complex has been termed MPF (maturation promoting factor or mitosis promoting factor) and is required for mitotic entry (reviewed in Nurse, 1990). During interphase, cytosolic MPF is kept inactive by phosphorylation of cdc2 on threonine 14 (Thr14) and tyrosine 15 (Tyr15) by Myt1 and Wee1, respectively (Booher et al., 1997; Lee et al., 1992; Liu et al., 1997). Activation of this complex is triggered by the Cdc25C phosphatase through cdc2 dephosphorylation of Thr14 and Tyr 15 (Dunphy and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991a). Following dephosphorylation of these residues, MPF is believed to phosphorylate and further activate Cdc25C resulting in full activation of MPF through an autocatalytic feedback loop (Hoffmann et al., 1993; Izumi and Maller, 1993). This results in the translocation

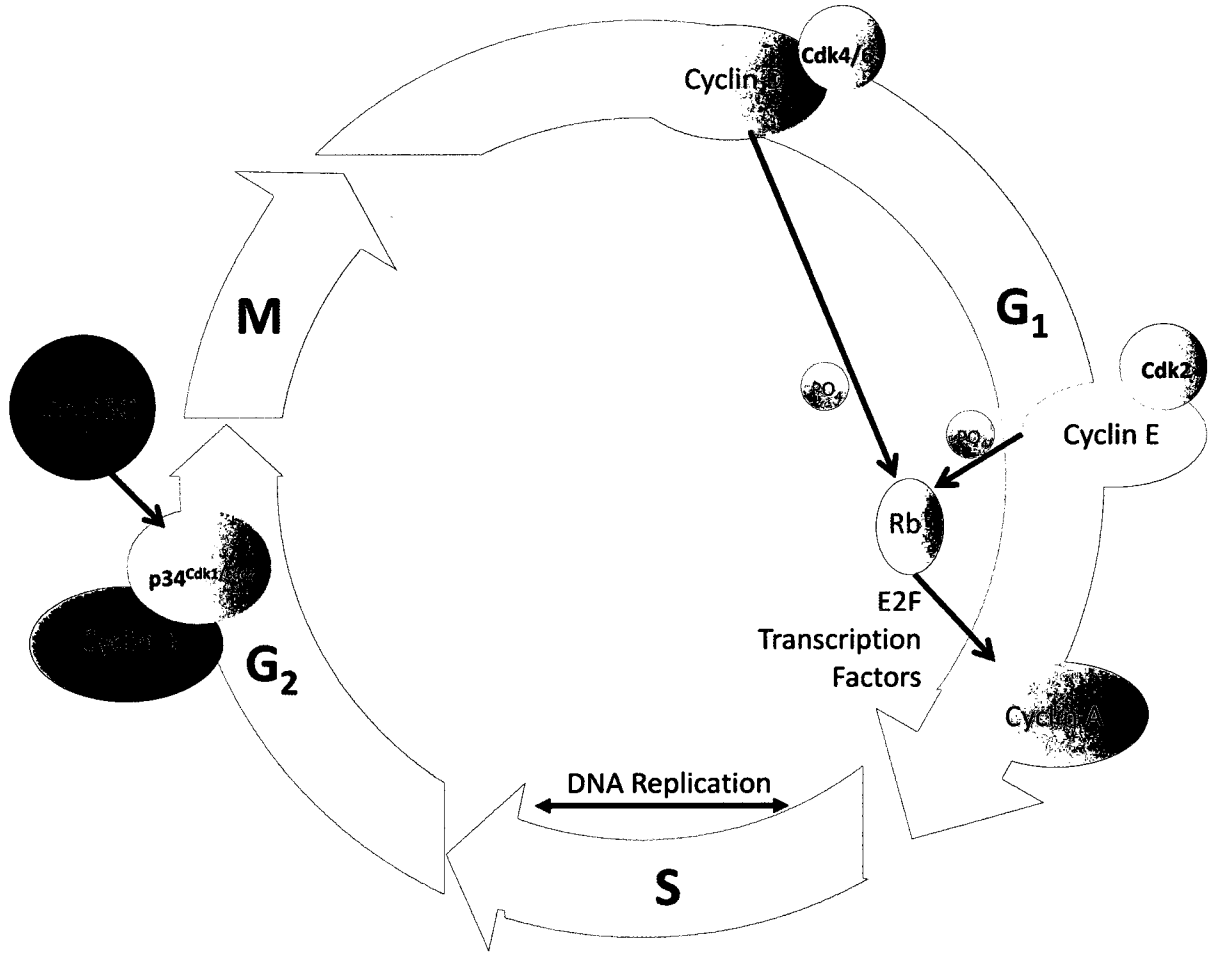

Figure 1.1. Cell Cycle at a Glance: Cell cycling is dependent on the fluctuating expression of cyclins and their interaction with their respective cyclin dependent kinases (Cdks). During G_1, cyclin D interacts with Cdks4/6 while cyclin E interacts with Cdk2. These complexes phosphorylate the retinoblastoma protein which leads to the production of E2F transcription factors and cyclin A expression. Cyclin A expression initiates late in G_1 and leads to S phase entry and cyclin B expression. Late in G_2, the cyclin B-cdc2 complex is activated by the Cdc25C phosphatase which removes inhibitory phosphate groups from cdc2 activating it which ultimately leads to mitosis.

of MPF from the cytoplasm to the nucleus at the beginning of mitosis (Pines and Hunter, 1991) where it phosphorylates histone H1 (Roth et al., 1991) and induces changes in the microtubule network (Blangy et al., 1995) and actin filaments (Sanger and Sanger, 1976; Schoroeder, 1976; Yamashiro et al., 1990).

In *Xenopus*, polo like kinase (Plx1) has been shown to phosphorylate and activate Cdc25 (Kumagai and Dunphy, 1996) and polo like kinase kinase (xPlkk1) has been shown to be a direct activator of Plx1 (Qian et al., 1998). However, this may be an organism specific phenomenon since depletion of mammalian polo like kinase (Plk1) results in elevated activity of Cdc2 (Liu and Erikson, 2002) suggesting a role for Plk in mitotic progression rather than mitotic entry. To date, a *bona fide* upstream activator of mammalian Cdc25C has not been identified.

During mitosis, chromosome condensation is accompanied by the hyperphosphorylation of histone H1 (Bradbury et al., 1973) and phosphorylation of H3 on serine 10 (Ser10) (Allis and Gorovsky, 1981; Hendzel et al., 1997). Microtubules then organize into a bipolar spindle and attach to the kinetochores of each sister chromatids. Chromosome attachment prior to segregation is monitored by the spindle check point protein MAD2 (mitotic arrest deficient) which binds kinetochores lacking microtubule attachment generating a "wait-anaphase" signal (Reviewed in Shah and Cleveland, 2000). Following the attachment of the mitotic spindle to the correct number of kinetochores, the "wait anaphase" signal is silenced and the anaphase-promoting complex (APC) in association with Cdc20 initiates chromosome segregation (Reviewed in Peters, 2002) and culminates into cytokinesis (Reviewed in Scholey et al., 2003).

1.2: Integrins and Focal Adhesions; the Fundamental Components for Signalling Through the Extracellular Matrix.

Focal Adhesions (FAs) are protein complexes that were first described in the 1970's as dark areas observed by interference reflection microscopy responsible for attaching a cell to its extracellular matrix (ECM) substratum (Abercrombie et al., 1971; Couchman and Rees, 1979; Heath and Dunn, 1978). Further research has indicated that actin filaments are anchored to the integrin family of transmembrane receptors at FA's through a multi-molecular protein complex and this protein complex is responsible for linking integrins to the actin cytoskeleton and for transducing signals in both directions across the membrane following the ligation of integrin heterodimers (Reviewed in Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Howe et al., 1998; Jockusch et al., 1995; Moser et al., 2009; Wegener and Campbell, 2008; Yamada and Geiger, 1997).

Integrins are central components of FAs and were first recognized in the 1980's as a large family of transmembrane receptors that are functionally conserved and are formed by noncovalently bound α and β subunits that heterodimerize (Hynes, 1987). They are responsible for connecting the interior of a cell with its extracellular environment by binding to ECM ligands such as collagen and fibronectin which in turn leads to integrin clustering, FA formation, and messages being transmitted across the cell membrane that can stimulate proliferation, survival, and migration (Cluzel et al., 2005; Gabarra-Niecko et al., 2003). In mammals, 18 α and 8 β subunits heterodimerize and are capable of dimerizing to form only 24 of the possible 144 combinations. Each specific dimer will display different ligand binding specificities but dimmers may display overlapping functions (Hynes, 2002). They exist in a low, intermediate, and high ligand affinity state which is determined by poorly understood conformational changes

in the extracellular head domain (Arnaout et al., 2007; Lu et al., 2001; Luo et al., 2007; Shimaoka et al., 2001; Shimaoka et al., 2002; Xiong et al., 2000; Xiong et al., 2003). The cytoplasmic tails of integrins are responsible for linking integrins to the actin cytoskeleton through adaptor proteins such as talin and filamin and for relaying messages to integrin signalling partners. When the interaction between the α and β cytoplasmic tails dissociates integrins are activated (Hughes et al., 1996; Hughes et al., 1995; Kim et al., 2003; Luo et al., 2004; O'Toole et al., 1994).

There are upwards of 125 identified proteins that are recruited to FAs following integrin clustering (Turner, 2000; Zamir and Geiger, 2001; Zimerman et al., 2004) and for the purposes of this thesis it is not necessary to discuss them all. The remainder of this section will introduce the more prominent and better understood proteins that constitute FAs.

Talin is a structural component of FAs and is one of the first proteins recruited to early sites of adhesion (DePasquale and Izzard, 1991; Horwitz et al., 1986). It binds the cytoplasmic tails of integrins and this disengages the cytoplasmic tails resulting in increased integrin affinity for their ligands (Figure 1.2)(Calderwood et al., 2002; Calderwood et al., 1999; Kim et al., 2003). Its importance has been highlighted by studies that have demonstrated that the absence of talin results in failure to activate the integrins and cells devoid of talin expression incur major adhesion defects (Monkley et al., 2000; Nieswandt et al., 2007; Petrich et al., 2007; Priddle et al., 1998; Tadokoro et al., 2003). In addition to the cytoplasmic domains of integrins, talin binds actin filaments and vinculin (Burridge and Mangeat, 1984). Vinculin is also a structural protein that localizes to sites of adhesion (Geiger, 1979) and binds actin filaments (Menkel et al., 1994) and paxillin (Figure 1.2)(Turner et al., 1990). The importance of vinculin is highlighted by the fact that its levels affect adhesion dynamics by stabilizing adhesions when over-expressed and by

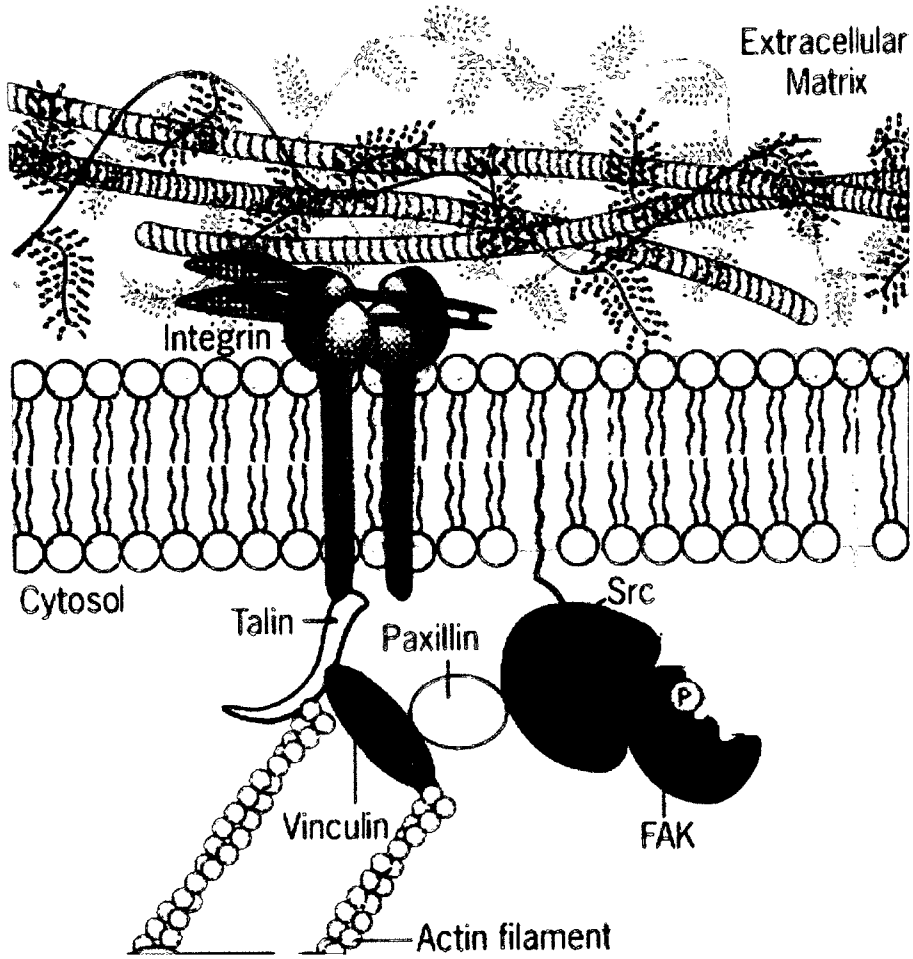

Figure 1.2. Focal Adhesion Structure; the Basics: The proteins depicted here are some of the earliest proteins recruited to focal adhesions. Talin is an adaptor protein that binds to the cytoplasmic tails of integrins and vinculin which binds paxillin. The FAK-Src complex localizes to focal adhesions and has a role in focal adhesion turnover by phosphorylating paxillin. Following the formation of focal adhesions, actin filaments extend from talin and vinculin into the cytosol in which they provide structural support or act as tracks for cargo transport.

de-stabilizing adhesions when its levels are reduced (Rodriguez Fernandez et al., 1992; Rodriguez Fernandez et al., 1993).

Src is a non-receptor tyrosine kinase that was first identified while studying the transforming protein product (termed v-src for viral-src) of the oncogenic retrovirus Rous sarcoma virus (RSV) (Purchio et al., 1978) and has been shown to be important for FA processes (Hall et al., 1996; Hirst et al., 1986; Kaplan et al., 1995; Rodier et al., 1995; Tarone et al., 1985). Src localizes to FAs (Rohrschneider, 1980) and FA components such as β1 integrins, paxillin, and FAK (Figure 1.2) which are highly phosphorylated in v-src transformed cells suggesting a role for Src in events pertaining to FAs signalling. Focal adhesion kinase (p125FAK) is a non-receptor cytoplasmic tyrosine kinase that localizes to sites of adhesion (Hanks et al., 1992; Schaller et al., 1992) and interacts with paxillin (Hildebrand et al., 1995). The kinase activity of FAK increases following integrin activation (Burridge et al., 1992; Guan and Shalloway, 1992) through an autophosphorylation event in which FAK phosphorylates itself on Tyr397 and creates a SH2 binding site for Src (Schaller et al., 1993) allowing it to phosphorylate FAK on Tyr576, Tyr577, Tyr861, and Tyr925. Phosphorylation of these residues regulates the kinase activity of FAK (576, 577) generates additional docking sites (861, 925)(Calalb et al., 1995). Several studies have been conducted that demonstrate a requirement for FAK during FA turnover (Cary et al., 1996; Frisch et al., 1996; Ilic et al., 1995).

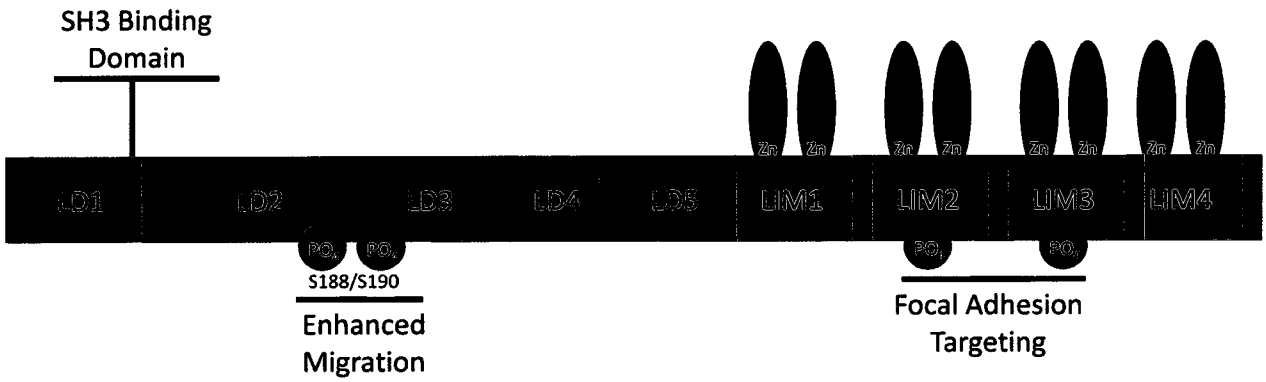

Figure 1.3. Paxillin Structure: Paxillin is composed of five N-terminal leucine/aspartic acid rich motifs (LD) which are responsible for the signalling events that go through paxillin. In addition to the LD motifs, the N-terminal contains a proline rich region which is responsible for binding the SH3 domain of Src and S188/S190 which, when phosphorylated, enhance migration. The C-terminal half of paxillin consists of four LIM zinc finger domains that are responsible for protein-protein interactions. Phosphorylation of LIMs 2 and 3 is required for focal adhesion targeting.

Paxillin is a multi-domain adaptor protein that was initially identified as a tyrosine phosphorylated protein in v-src transformed cells (Glenney and Zokas, 1989). It is one of the earliest proteins to localize to primordial FAs (Digman et al., 2008; Turner et al., 1990) and associates with vinculin (Turner et al., 1990) and the cytoplasmic tail of α4 and β1 integrins (Figure 1.2)(Liu et al., 1999; Schaller et al., 1995). The cloning of paxillin has revealed that it comprises numerous discrete structural domains (Brown et al., 1996; Turner and Miller, 1994) in which the C-terminal half contains four LIM domains (termed LIM1-4) which are double zinc finger motifs that mediate protein-protein interactions (Perez-Alvarado et al., 1994; Schmeichel and Beckerle, 1994). LIMs 2 and 3 are required for FA targeting (Brown et al., 1996) and this targeting is dependent on phosphorylation events in these domains (Brown et al., 1998). The N-terminal half of paxillin contains a proline rich sequence that serves as a docking site for the SH3 domain of c-Src (Weng et al., 1993). The five leucine and aspartic acid rich motifs (termed LD1-5) are responsible for most of the signalling that occurs through paxillin (Figure 1.3)(Brown et al., 1996; Turner and Miller, 1994). Paxillin has several phosphorylation sites and its phosphorylation status dictates the composition of its interacting proteins (Brown and Turner, 2004). It has been shown to be a substrate for several kinases including, but not limited to, the FAK-Src complex (Thomas et al., 1999), PAK (Nayal et al., 2006), JNK (Huang et al., 2004b; Huang et al., 2003), ERK (Ishibe et al., 2003; Ku and Meier, 2000), and MAPK (Huang et al., 2004a). Cell lines devoid of paxillin display migration defects (Hagel et al., 2002; Webb et al., 2004). Supporting a role for paxillin phosphorylation during cell physiological processes, migration and mobility are stimulated when paxillin is phosphorylated on S188 and S190 (Bellis et al., 1997). Paxillin has also been demonstrated to be hyperphosphorylated on serines by a yet

to be identified kinase and degraded during mitosis (Yamaguchi et al., 1994; Yamaguchi et al., 1997) suggesting that it may receive post-translational modifications important for cell division.

1.3: Focal Adhesion Turnover; Assembly and Disassembly.

For a cell to be able to migrate or divide and proliferate, FAs must disassemble to allow the cell to detach from its substratum and then reform when the cell becomes adherent again; this process is know as focal adhesion turn over (Ballestrem et al., 2001; Laukaitis et al., 2001; Webb et al., 2002; Wiseman et al., 2004). Because FAs are involved in migration and proliferation, two of which processes are altered in metastatic cell lines, they have caught the attention of the oncology research community and the mechanism surrounding FA turnover has been documented but is not completely elucidated. FA formation begins when integrins are activated and begin to cluster in the plane of the membrane to form early sites of adhesion in which a large number of proteins are recruited (Duband et al., 1988; Felsenfeld et al., 1996; Schmidt et al., 1993). FA formation relies on the presence of α and β integrin cytoplasmic tails (Briesewitz et al., 1993; Hayashi et al., 1990; Solowska et al., 1989; Ylanne et al., 1993) and once activated, integrins will bind their ligands to induce the recruitment of FAK, Src, paxillin, and vinculin to primordial FAs (Miyamoto et al., 1995a; Miyamoto et al., 1995b). This event is highly dependent on tyrosine phosphorylation (Burridge et al., 1992; Craig and Johnson, 1996; Miyamoto et al., 1995a; Nobes et al., 1995). As FAs mature, the Rho family of GTPases are recruited to sites of adhesion where they initiate the formation of actin fibres, stabilizing the newly formed FAs (Allen et al., 1997; Hotchin and Hall, 1995; Nobes et al., 1995; Ridley and Hall, 1992).

When a cell migrates or divides these protein complexes must disassemble through a pathway that is not fully characterized. However, the studies discussed above relating to the over expression or absence of specific FA proteins provide insights as to the potential mechanism regulating FA turn over. It is well documented that FA breakdown requires activity of the FAK-Src complex (Reviewed in Mitra and Schlaepfer, 2006), and the inactivation of Rho (Paterson et al., 1990; Schoenwaelder and Burridge, 1999). Breakdown is also enhanced when Src substrates, such as FAK and paxillin, are phosphorylated and degraded (Bellis et al., 1997; Fincham et al., 1995) but the manner in which FA breakdown takes place is unclear. The vast majority of the literature relating to focal adhesion turnover pertains to turnover during migration or cell spreading which are not the only cellular processes in which there are alterations in FA structure. When a cell divides there is a "loosening" of adhesiveness in which there are fewer points of contact between the cell and its substratum (Elvin and Evans, 1983; Zwanenburg, 1983) which is highlighted by the ability to shake cells off of their substratum when arrested in metaphase of mitosis (Morla et al., 1989; Pagano et al., 1992; Pines and Hunter, 1989). It is generally accepted that there is a degree of FA disassembly during mitosis in order to allow cells to divide which is followed by FA re-assembly. FA properties and the signals they transduce to promote interphase progression have been well characterized (Reviewed in Assoian, 1997; Margadant et al., 2007; Schwartz and Assoian, 2001) and there have been studies conducted in which the status of some FA proteins have been investigated during mitosis (Curtis et al., 2002; Yamaguchi et al., 1994; Yamaguchi et al., 1997; Yamakita et al., 1999) but literature on the changes taking place in FAs during mitosis is scarce.

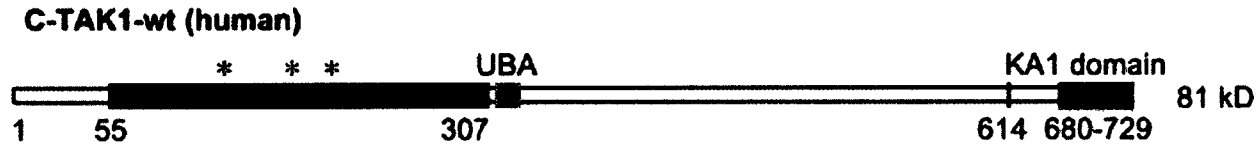

Figure 1.4. Structure of Microtubule Affinity Regulating Kinase 3 (MARK3): MARK3 is an 81 kDa protein that contains an amino terminal kinase domain, a central ubiquitin binding domain (UBA), and a carboxyl terminal kinase associated (KA) domain (Adapted from Bachmann et al., 2004).

1.4: Current Understanding of Cdc25C Associated Kinase 1 (C-TAK1)/Mircrotubule Affinity Regulating Kinase 3 (MARK3)

Members of the microtubule affinity regulating kinase family are of interest because they are involved in cell polarity, cell cycle progression, and microtubule dynamics (Reviewed in Drewes, 2004; Tassan and Le Goff, 2004). Microtubule affinity regulating kinase 3 (MARK3), also termed Cdc25C associated kinase 1 (C-TAK1), is a serine/threonine kinase that is ubiquitously expressed (Peng et al., 1998) and contains an amino terminal kinase domain, acentral ubiquitin binding domain (UBA), and a carboxyl kinase associated domain (KA)(Figure 1.4)(Bachmann et al., 2004). The *MARK3* gene encodes an 81 kDa protein that was first demonstrated to bind to and phosphorylate Cdc25C on its inhibitory site (serine 216) (Ogg et al., 1994; Peng et al., 1998). The Cdc25C phosphatase initiates mitosis by dephosphorylating threonine 14 and tryrosine 15 of $p34^{cdk1/cdc2}$ leading to the activation of p34 (Dunphy and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991b) which is one of the final steps leading to mitotic entry (Figure 1.1)(reviewed in Nurse, 1990). Limited studies have been conducted on MARK3 but it has been suggested to play a role in the cell cycle by phosphorylating Cdc25C on serine 216 which promotes the binding of 14-3-3 (Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997), leading to its cytosolic retention and inhibition (Dalal et al., 1999; Peng et al., 1997). It has also been postulated to be a regulator of cytoskeletal dynamics due to its homology to other members of the MARK family and a recent mass spectrometry study aimed at identifying paxillin interacting proteins has indicated that paxillin forms a complex with MARK3 (Migration Gateway, 2009).

1.5: The Sterile20-Related Kinase Family and the Role of The Ste20-Like Kinase, SLK.

Sterile 20 (ste20) was identified in *Saccharomyces cerevevisia* as a kinase that acted upstream of MAPK (termed MAP4K) in mating. Its homologues in various organisms are called the ste20-related kinases (Manning et al., 2002). A family of Ste20 homologues exist in mammals and this family is subdivided into two structurally distinct subfamilies that include the p21-activated kinase family (PAK) and the germinal center kinase (GCK) family (Reviewed in Ling et al., 2008) . Ste20 kinases of the PAK subfamily contain a carboxyl kinase domain and an amino terminal p21 GTPase-binding domain that facilitates the binding of small Rho GTPases. PAK Ste20-related kinases are well established regulators of migration, growth, and apoptosis (Hofmann et al., 2004). The members of the GCK subfamily contain an amino terminal kinase domain and lack a GTPase binding domain. Members of this subfamily are involved in cell volume regulation and immune response (Shui et al., 2007; Strange et al., 2006).

The Ste20-like kinase, SLK, is a 220kDa serine/threonine kinase of the Ste20 GCK kinase family that spans 1202 amino acids and was initially demonstrated to be involved in cytoskeletal remodelling and apoptosis in a variety of cell lines (Sabourin and Rudnicki, 1999; Sabourin et al., 2000). SLK appears to be activated following caspase 3 mediated cleavage which yields an N-terminal domain that is responsible for inducing apoptosis and cytoskeletal remodelling and a C-terminal domain that initiates actin dissolution (Sabourin and Rudnicki, 1999; Sabourin et al., 2000). SLK is ubiquitously expressed in adult tissue (Sabourin and Rudnicki, 1999) and is restricted to neural and muscle precursors in the early murine embryo (Storbeck et al., 2004; Zhang et al., 2002). A review of the primary structure of SLK indicates that it shares extensive homology with lymphocyte oriented kinase (LOK) and MST1 which are other members of the Ste20 kinase family, involved in lymphocyte adhesion and apoptosis,

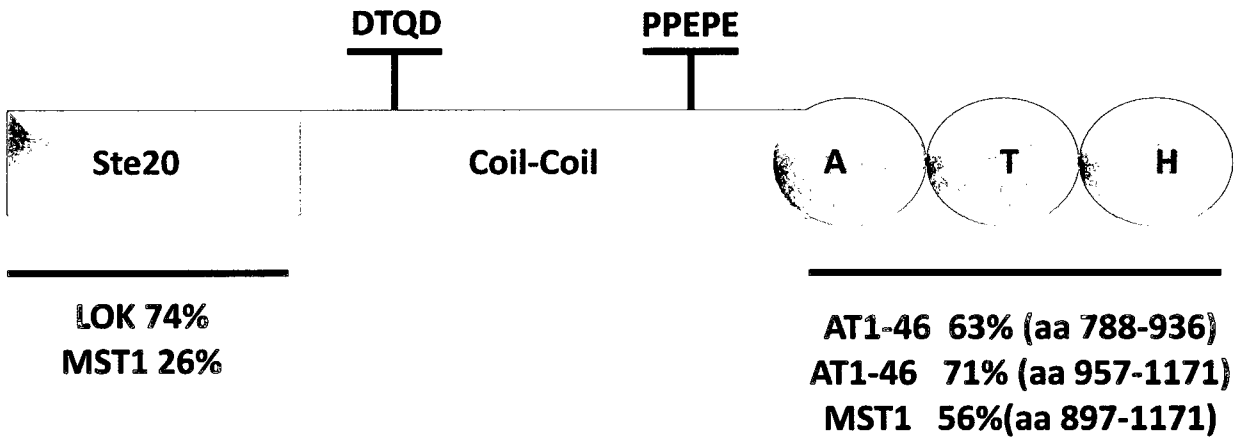

Figure 1.5. The Structural Domains of the Ste20-like Kinase, SLK: SLK contains an amino terminal Ste20 kinase domain that is homologous to the kinase domains of LOK and MST1. The central region is a coil-coil domain that contains a caspase 3 cleavage site and an SH3 binding domain. SLK's carboxyl terminal shares homology with AT1-46 and the carboxyl terminal of LOK.

respectively (Endo et al., 2000; Kuramochi et al., 1997). SLK contains an amino terminal kinase domain that is homologous to LOK and MST1 and a central coil-coil domain. This central domain contains a caspase 3 cleavage site and a proline rich region encoding a putative SH3 binding site (Pawson and Scott, 1997). The carboxyl terminal region of SLK bears homology to the AT1-46 protein and the carboxyl terminal of LOK. This region of homology overlaps with the coil-coil domain and has been termed the ATH domain for **AT**1-46 **h**omology (Figure 1.5)(Sabourin and Rudnicki, 1999; Sabourin et al., 2000). The function of AT1-46 is currently unknown.

To date, the function of SLK is poorly understood but its activity and sub cellular localization appears to be regulated in part by Lbd 1 and 2 which tether SLK to the microtubule (Storbeck et al., 2009). SLK has been implicated in a number of processes that make it a regulator of cytoskeletal reorganization. SLK has been shown to be negatively regulated by v-src and this regulation is mediated through casein kinase 2 (CKII) phosphorylation of SLK (Chaar et al., 2006). It has also been demonstrated to be involved in actin reorganization upstream of Rac1 (Wagner et al., 2002). Furthermore, SLK is capable of phosphorylating the actin capping protein ezrin *in vitro* (Sabourin lab, unpublished), localizes to paxillin and vinculin positive leading edges during migration (Wagner et al., 2002; Wagner et al., 2008), and is activated in metastatic cell lines expressing the ErbB2 oncogene (Roovers et al., 2009).

Initial studies on SLK suggest that it is required for cytoskeletal reorganization (Sabourin and Rudnicki, 1999; Sabourin et al., 2000) which is further supported by the finding that it forms a complex with the microtubule. Supporting this, SLK co-localizes with the microtubule network during interphase (Wagner et al., 2002). SLK knock down or the expression of a

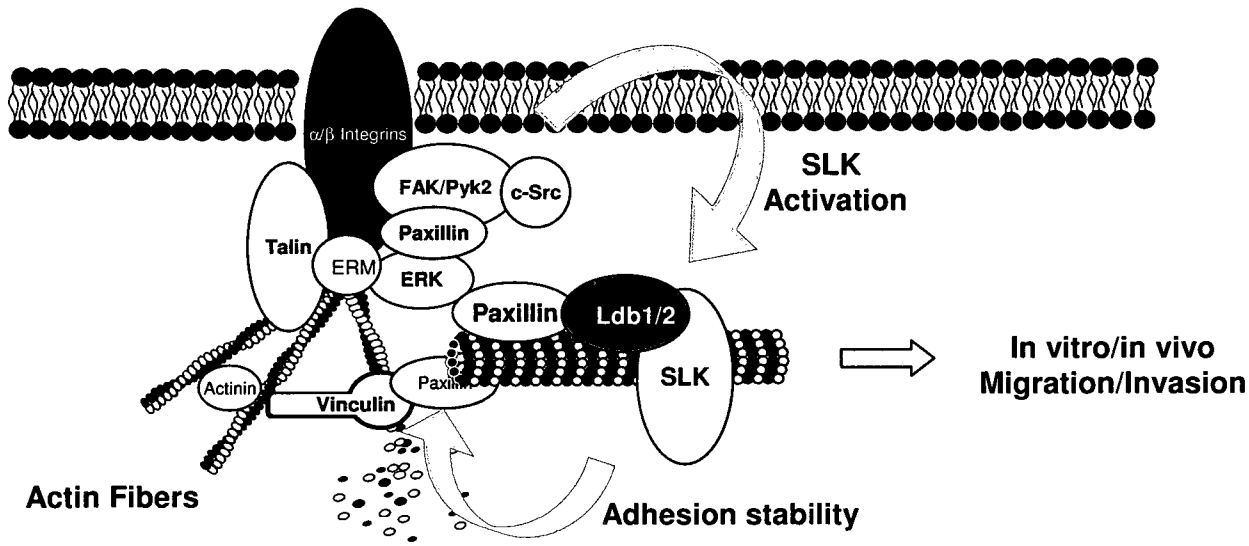

Figure 1.6. Working Model of SLK Mediated Focal Adhesion Turnover: Our current understanding of SLK suggests that SLK activity at the leading edge of migrating cells is dependent on FAK and c-src signalling. The delivery of SLK to sites of adhesion is facilitated by the microtubule to which SLK may be bound through Lbd 1 and 2. We believe that, when active at sites of adhesion, SLK is responsible for phosphorylating paxillin which leads to the instability of adhesions allowing for adhesion turnover and migration (Adapted from Wagner and Sabourin, 2009).

catalytically inactive form results in the failure of the microtubule network to polarize correctly (Burakov et al., 2008). The microtubule network is required for focal adhesion turnover during migration (Kaverina et al., 1999) and a requirement for SLK in focal adhesion turnover has been demonstrated. The activation of SLK at the leading edge of migrating cells is dependent on FAK/src/MAPK signalling (Wagner et al., 2008). Our laboratory is continuing to characterize the function of SLK and is doing so with the belief that SLK is required for focal adhesion turnover. Our working model involves the transport of SLK to sites of adhesion *via* the microtubule where it phosphorylates paxillin. We believe that this paxillin modification will result in the instability of focal adhesions and allow for adhesion turnover and migration (Figure 1.6)(Wagner and Sabourin, 2009). In the case of cell cycle progression, it may allow adhesion "loosening" and cytokinesis.

1.6: Hypothesis, Objectives, Rationale, and Main Findings of this Study.

One of the earliest reports on SLK indicated that it interacts with and phosphorylates polo-like kinase 1 (Plk1) (Ellinger-Ziegelbauer et al., 2000) and this observation prompted us to investigate a possible role for SLK in cell cycle progression. Plk1 been reported to be an upstream activator of Cdc25C (Roshak et al., 2000). However, this Plk1-Cdc25C phosphorylation may be species specific and has been disputed and difficult to reproduce in a mammalian model (Stambrook, P.J, University of Cincinnati, personal conversation). Literature that has been published since the initiation of our study indicates that Plk1 has a role in mitotic progression rather than interphase or mitotic entry (Reviewed in Archambault and Glover, 2009). As for others, we were unable to observe a functional relationship between SLK and Plk1. However, we observed that the expression of a catalytically inactive truncation of SLK, $SLK^{1-373K63R}$ which we have termed KΔC, inhibits G_2 exit of synchronized populations of murine

fibroblasts upstream of Cdc25C activation. Due to complex SLK regulation and an absence of SLK interacting proteins, we could not provide any mechanistic insights as to why dominant negative SLK expression resulted in compromised cell cycle kinetics.

During the course of a yeast-two hybrid screen we identified C-TAK1/MARK3 as an SLK interacting protein and at the time of this discovery the only literature that existed on MARK3 indicated that it phosphorylated and inhibited the Cdc25C phosphatase (Dalal et al., 1999; Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997). Interestigly, the activity of Cdc25C is required for mitotic entry (Dunphy and Kumagai, 1991; Hoffmann et al., 1993; Izumi and Maller, 1993; Lee et al., 1992; Millar et al., 1991b). If Cdc25C fails to activate, one would observe a G_2 block identical to the one observed when KΔC is expressed. Combined with the observation that MARK3 phosphorylates Cdc25C on its inhibitory residue, these data prompted us to investigate if there was an interaction between SLK and MARK3 that was required for the activation of Cdc25C. Using several strategies and after numerous attempts we were unable to identify a pathway in which SLK and MARK3 interacted with each other to release the MARK3 mediated inhibitory phosphorylation of Cdc25C.

While the study involving SLK and MARK3 as upstream activators of Cdc25C was being conducted we found that SLK could phosphorylate paxillin in its N-terminal LD domains and at the time we did not consider that there could be a functional link between SLK, MARK3, and paxillin. More recently, a large scale mass spectrometry study was conducted in which MARK3 was identified as a paxillin interacting protein (Migration Gateway, 2009) which we have reproduced here. We show that there is a weak interaction between paxillin, MARK3, and SLK throughout interphase and these three proteins have a strong association during mitosis. The events leading up to the G_2/M boundary and the end result of mitosis are well established

but the defining moment to consider a cell to be mitotic is undefined. We show that MARK3 and SLK form a complex late in G_2, where MARK3 tethers SLK to paxillin, allowing SLK to phosphorylate paxillin. We believe the consequences of this phosphorylation event results in FA disassembly similar to our working model presented in Figure 1.6 which leads to the characteristic cell rounding observed in mitotic cells.

Chapter 2

Materials and Methods

2.1: Cell Lines, Culture, and Adenovirus Infection.

The mouse fibroblast lines MEF-3T3 (MEF Tet-Off, C3018, Clontech) and C3H10T½ (ATCC number CCL-226) were used in all experiments. Similar results were obtained for both cell lines. The GFP-tubulin expressing cells (LLCKP-1) were a kind gift from Patricia Wadsworth (Rusan et al., 2001). Cell lines were maintained at 37 ^{0}C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Bio-Whitaker) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin. For cell cycle experiments, fibroblasts were arrested by 48 hour incubations in 0.25% FBS-DMEM and released from quiescence by the addition of 20% FBS-DMEM. The epitope-tagged kinase dead or activated versions of SLK used in these studies (HA-KΔC or HA-YΔC) have been previously described (Wagner et al., 2002) and consist of an amino-terminal truncation (aa 1-373) with or without an ATP-binding site (Lys63 to Arg) mutation. To monitor the effect of kinase deficient SLK on cell cycle kinetics, adenoviral vectors expressing HA-KΔC or a β-galactosidase (LacZ) control were used to infect quiescent cultures. Cells were infected at a MOI of 100 by the addition of the adenovirus directly to cells in 0.25% FBS-DMEM 16 hours before the addition of 20% FBS-DMEM and analysis. To isolate fibroblast populations synchronized at M phase, the cultures were treated overnight with nocodazole (40 ng/mL, Sigma-Aldrich) and shaken off into PBS by aggressive tapping against a solid surface. Detached cells floating in PBS were then collected by centrifugation.

For mitotic spindle induction experiments, LLCKP-1 cells were microinjected with HA-YΔC expression plasmid as described previously (Wagner et al., 2002). *Xenopus* oocytes were prepared and injected with cRNA as previously described (Vicogne et al., 2004).

2.2: Western Blotting, Immunoprecipitations, and *in vitro* Kinase Assays.

For protein analysis, the cells were lysed in modified RIPA buffer as previously described (Wagner et al., 2002). Equal amounts of extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Perkin Elmer Life Sciences Inc.) which were then probed with the following antibodies. Anti-Cdc2, cyclins D, E, A, and B, and anti-phospho histone H1 (Upstate Biotechnology). The SLK antibody described previously (Sabourin and Rudnicki, 1999) and the MARK3 was detected with a rabbit polyclonal antibody (Epitomics). Primary antibodies were detected using either a goat anti-rabbit IgG or goat anti-mouse IgG horseradish peroxidase-labeled secondary antibody (BioRad) and visualized using Western Lightning chemiluminescence reagent (Perkin Elmer Life Sciences Inc.) and exposure to X-ray film. SLK immunoprecipitations and kinase assays were carried out essentially as previously described (Sabourin et al., 2000). Briefly, equal amounts of lysate were immunoprecipitated for 2 hours at 4 ^{0}C using 1 μg of SLK antibody and 20 μL of protein A sepharose (4 fast flow, Amersham Biosciences). Immunoprecipitates were then washed three times with NETN (50 mM Tris-HCl [pH 7.5], 150mM NaCl, 1mM EDTA, 0.1% Nonidet P-40) and once with kinase buffer (20mM Tris-HCl [pH 7.5], 15mM MgCl2, 10 mM NaF, 10 mM β-glycerophosphate, 1mM orthovanadate). Kinase reactions (20 μL) were then initiated by the addition of 5 μCi of [γ^{32}P]ATP and incubated at 30 ^{0}C for 15 minutes for SLK or 37 ^{0}C or 30 minutes for MARK3. These reactions were terminated by the addition of 4x SDS loading buffer and then resolved by SDS-PAGE, transferred to PVDF membranes, and exposed to X-ray film. Membranes were then probed for SLK or MARK3 to assess immunoprecipitation efficiency. SLK autophosphorylation was used as an indicator of its kinase activity or ^{32}P incorporation into

Cdc25C for MARK3. In experiments involving paxillin phosphorylation by SLK, recombinant GST-paxillin was obtained from a bacterial expression system.

2.3: Immunofluorescence and Flow Cytometry.

Immunofluorescence studies were carried out by fixation of cells growing on glass cover slips in 4% paraformaldehyde for 10 minutes. The cells were then washed twice with PBS and incubated with primary antibodies for 1 hour. The primary antibodies used in our immunofluorescence studies were as follows: anti-α–tubulin (clone DM1, Sigma), anti-phospho-H3 (Ser10) (Cell Signaling Technology), anti-beta-galactosidase (Promega), anti-HA (12CA5 or sc-805, Santa Cruz Biotechnology Inc.) and anti-SLK (Sabourin and Rudnicki, 1999). Antibodies were detected with either anti-mouse or anti-rabbit antibodies conjugated to either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Sigma). DAPI (4', 6-diamidino-2-phenylindole, 0.25μg/mL) was used to stain DNA and the samples were visualized with a Zeiss Axioscope100 epifluorescence microscope equipped with the appropriate filters and photographed with a digital camera (Sony Corporation HB050) using the Northern Eclipse software package. Cells assayed by flow cytometry were trypsinized and washed once in 10% FBS-DMEM. Cells were then washed twice in PBS supplemented with 1mM EDTA and then fixed in 1 mL of PBS by the drop wise addition of 2 mL of 80% ethanol pre-chilled to –20 ^{0}C. These samples were then stored at –20 ^{0}C for a minimum of 2 hours, washed once in PBS, and resuspended in DNA content staining buffer (1.1% citrate buffer, 10 μg/mL propidium iodide, 1 mg/ml RNase) and incubated for 30 minutes at 37 ^{0}C before being analyzed on a Beckman-Coulter flow cytometer using the Expo 32 software package. BrdU pulse labeling was performed using a BrdU Flow Kit (BD Biosciences) according to the

manufacturers instructions. Briefly, 16 hours before labeling, cultures were infected with either HA-KΔC or LacZ adenovirus at a MOI of 100 for 90 minutes in unsupplemented DMEM and then grown in 10% FBS-DMEM. The cultures were then labeled with BrdU for 1 hour and collected at various times. The DNA content of BrdU positive cells was then analyzed flow cytometrically.

2.4: Cell Counts, Cloning, and Transfections.

To monitor cell proliferation, the cells were counted after infection on day 0 at a MOI of 100 as described above. Cell populations were trypsinized and scored by trypan blue exclusion over time. Cell counts were performed in triplicates in three independent experiments. For anti-sense SLK expression, a 5-prime 300 base pair SLK fragment was cloned into the pPGK-puro expression vector in the reverse orientation. MARK3 and paxillin constructs were cloned into mammalian or bacterial expression vectors using convenient restriction sites or in their absence using standard PCR based amplification using custom primers designed with appropriate restriction sites. Transfections were preformed using LipofectAMINE^{TM} and plus reagent (Invitrogen) according to the manufacturers protocol and, when crating stable transfectants, the cultures were selected in puromycin (100 μg/mL) over a two-week period. Following selection, stable transfectants were scored by colony forming assays using CYTO-QUIK staining (Fisher Health Care) followed by several PBS washes. SLK *Smart Pools* siRNAs were obtained from Dharmacon against the following murine SLK target sequences: 5'-GGTTGAGATTGACATATTA-3'; 5'-GAGAATGCCTGAATAACAA-3'; 5'-AGAAGC GACAATATGA-3'; 5'-GATAAATTTAGCAACA-3'. Control siRNA consisted of siCONTROL (Dharmacon; 5'-UAGCGACUAAACACAUCAAUU-3'), having no perfect match

to known human or mouse sequences. All siRNAs were transfected using the Transit-TKO reagent (Mirrus Corp.) according to the manufacturer's instructions.

2.5: Phosphoamino Acid Analysis.

Phosphoamino acid analysis was conducted as previously described (Hardie, 1999). Briefly, paxillin was phosphorylated by SLK *in vitro* and resolved as described above. ^{32}P labeled paxillin was then retrieved from a PVDF membrane and hydrolyzed in 6M HCl at 110 ^{0}C for one hour. The sample was then desiccated and resuspended in pH 1.9 buffer containing phospho amino acid standards and spotted onto thin layer cellulose plates. Electrophoresis was then conducted in pH1.9 buffer for 20 minutes at 1.5 kV in the first dimension and then for 20 minutes in pH 3.5 buffer at 1.5 kV in the second dimension. Phosphoamino acid standards were visualized using ninhydrin and the plates were exposed to X-ray film.

2.6: Binding Assays and Recombinant Proteins.

Proteins were *in vitro* translated and labeled with ^{35}S-methionine using a TnT *in vitro* translation kit according to the manufacturers protocol (Promega). They were then inverted with recombinant proteins of interest that were bound to agarose beads for 15 minutes at 25 ^{0}C in a final volume of 500 µL in NETN. The beads were then washed three times in NETN and resolved by SDS-PAGE. Gels were dried under a vacuum and bound proteins were visualized by autoradiography following exposure of the dried gels to X-ray film. Recombinant proteins were obtained from bacterial cultures grown over night then diluted by a factor of 10 the following day. Following one hour of growth, these diluted cultures were induce with 1mM IPTG for two hours and collected by centrifugation. The bacterial pellets were lysed as described above and subjected to pulses of sonication. Cleared lysates were then inverted with

glutathione conjugated beads for half and hour, washed three times in NETN, then used for binding or kinase assays.

Chapter 3

SLK is Required for Proliferation

3.1: Introduction and Rationale.

Microtubules are essential cytoskeletal structures in all eukaryotes and act as structural supports and highways for trafficking cargo throughout interphase. They are also the main structural component of the mitotic spindle during mitosis (Reviewed in Alberts, 2008). We have previously shown that a proportion of SLK associates with the microtubule network of exponentially growing fibroblasts (Wagner et al., 2002) and SLK has been implicated in cell cycle progression by displaying a modest increase in catalytic activity when synchronized populations reach the G_2/M boundary (Ellinger-Ziegelbauer et al., 2000). Due to a requirement of the microtubule network in mitosis, the localization of SLK, and its increased activity during G_2/M, we set forth to address if SLK contributes to cell cycle progression.

3.2: SLK is a Component of the Mitotic Spindle.

We have previously demonstrated that a proportion of SLK localizes to the microtubule network of exponentially growing fibroblasts (Wagner et al., 2002) and during these studies we observed rare events in which SLK and αtubulin co-localization resembled the mitotic spindle. To further investigate the possibility that SLK might co-localize with the mitotic spindle we preformed confocal microscopy. Although some SLK was found outside of the mitotic spindle, confocal analysis of DAPI-stained cells in combination with anti-SLK and anti-αtubulin immunofluorescence shows that most of the SLK staining co-localized with αtubulin during metaphase (Figure 3.1, A and B). The observation that SLK associates with the mitotic spindle suggests that SLK may play a role in mitotic progression possibly by contributing towards proper functioning or formation of the mitotic spindle.

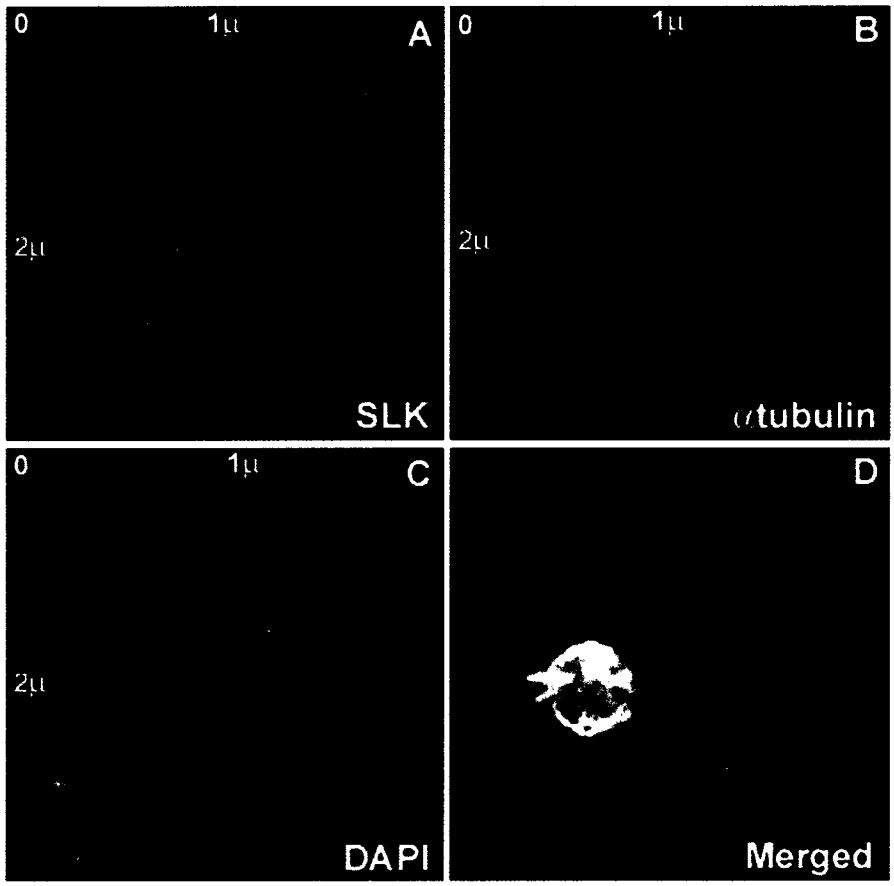

Figure 3.1. SLK Localizes to the Mitotic Spindle: Double labelling and confocal analysis displays that SLK (A) co-localizes with αtubulin (B) when the mitotic spindle is formed. Chromosomes were visualized with DAPI (C). An overlay of these images indicates that SLK localizes to the mitotic spindle (D).

3.3: SLK Activity is Elevated During G_2/M.

To further investigate the potential role of SLK in cell cycle progression we monitored its activity throughout the various phases of the cell cycle (Figure 1.4) using synchronized populations of C3H 10T1/2 fibroblasts. Serum starved populations were released from G_0 by the reintroduction of serum, collected at various times, and monitored for SLK activity using *in vitro* kinase assays in parallel with cell cycle phase determination based on DNA content as assessed by flow cytometric analysis. Figure 3.2A displays the cell cycle phase as determined by flow cytometric measurements of DNA content in these synchronized populations following serum stimulation and indicates that 24 hours post serum stimulation 60% of the population is in G_2/M. During this time point SLK shows a consistent and reproducible 3-4-fold increase in activity (Figure 3.2B) in contrast to the modest 1.3-fold increase in activity previously reported (Ellinger-Ziegelbauer et al., 2000). Interestingly, as the cells exited M phase and re-entered G_1, a marked reduction in kinase activity was observed. SLK levels did not change during the time course. These results suggest that SLK activity is up-regulated at a time when the majority of the population contains a 4N DNA content.

3.4: SLK Activity is Required for Proliferation.

To investigate the role of SLK in proliferation, an expression vector bearing an anti-sense SLK fragment was transfected into MEF-3T3 fibroblasts and subjected to 14 days of puromycin selection following which time stable clones were visualized using Cyto-Quick stain. As shown in Figure 3.3, antisense SLK-transfected cultures displayed a marked reduction in colony

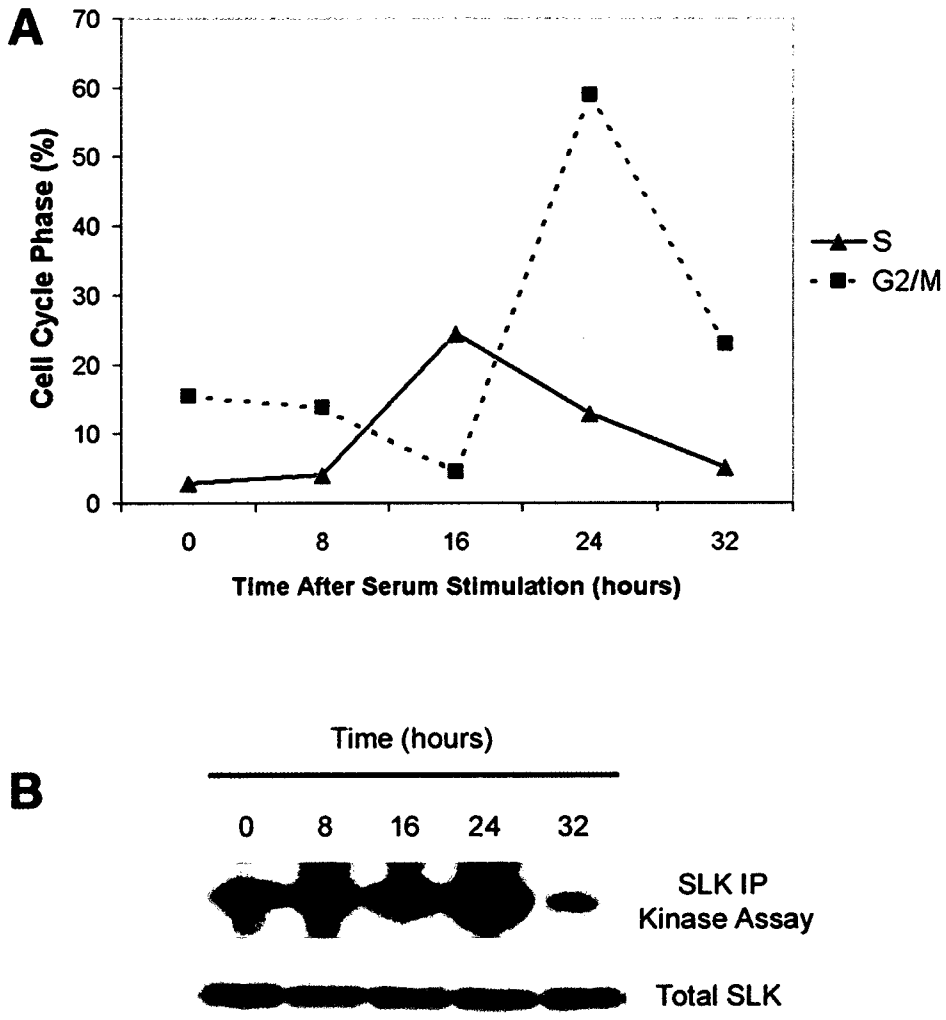

Figure 3.2. SLK Activity is Elevated During G_2/M: C3H 10T1/2 murine fibroblasts were synchronized in G_0 by 48 hours of serum deprivation and stimulated to grow by the addition of 20% FBS. Cells were then collected at various times following the reintroduction of serum and monitored by flow cytometric analysis for cell cycle phase determination (A) and an assessment of SLK activity was conducted in parallel (B). SLK levels remain constant through out cell cycle but its activity increases when 60% of the population is in G_2/M.

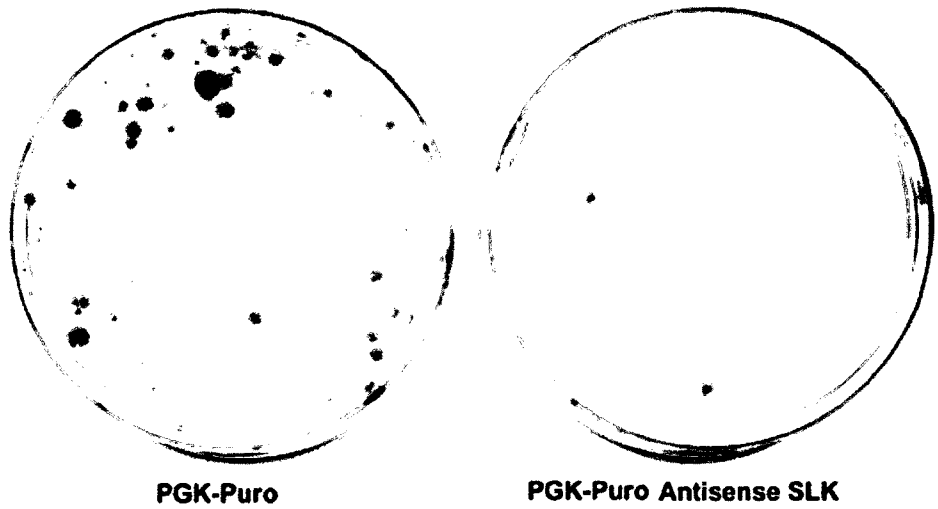

Figure 3.3. SLK is Required for Proliferation: PGK-Puro and PGK-Puro Antisense SLK were stably transfected into MEF 3T3 fibroblasts by selection for puromycin over a 14 day period. Stable clones were then visualized by staining with Cyto-Quick. Cells transfected with antisense SLK display a marked decrease in stable colony numbers suggesting a role for SLK in proliferation.

numbers when compared with the pPGK-puro control vector. Furthermore, expansion of colonies that survived the selection process indicated that they did not down regulate SLK suggesting that the antisense RNA was not expressed in those clones (data not shown).

To complement the antisense studies and to further investigate the impact of SLK on proliferation, we took advantage of an adenoviral system to express either a kinase dead truncation of SLK ($SLK^{1-373K63R}$ termed KΔC) or a LacZ control in exponentially growing populations of C3H10T1/2. We then monitored their proliferation rates over a four day period by counting viable cells. Populations expressing KΔC display reduced proliferation rates compared to LacZ control populations 24 hours post infection in contrast to the LacZ control populations which display a typical exponential growth curve (Figure 3.4). Further examination of later time points indicate that populations expressing KΔC fail to proliferate following the 24 time point and for the remainder of the four day time course Taken together, these results indicate that SLK activity is required for cell proliferation and that KΔC is capable of interfering with SLK dependent pathways.

3.5: Expression of Dominant Negative SLK Results in a G_2/M Block.

To further investigate the mechanism by which KΔC inhibits proliferation, quiescent C3H10T1/2 populations were infected with a KΔC expressing adenovirus or a LacZ control virus, serum stimulated and subjected to DNA content analysis over time. As shown in Figure 3.5, cultures expressing LacZ proceed through the cell cycle and exited the G_2/M compartment by 32 hours post serum stimulation. In contrast, KΔC expressing populations displayed a marked increase in the proportion of cells in the G_2/M compartment suggesting that KΔC

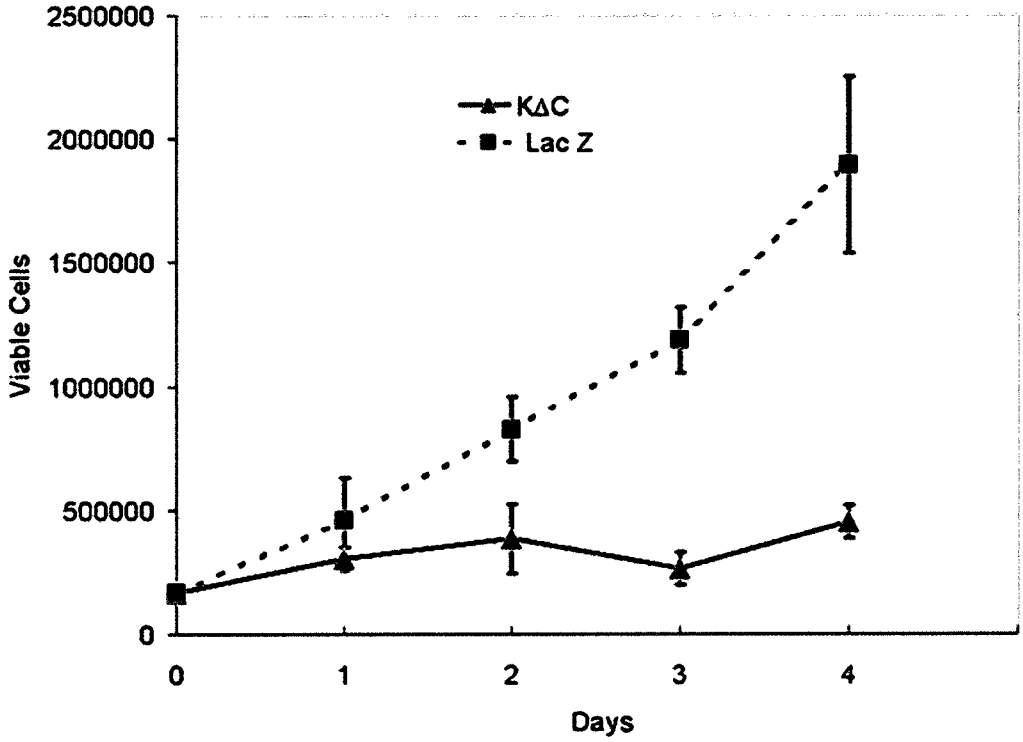

Figure 3.4. SLK Activity is Required for Proliferation: Cultures of fibroblasts infected with an adenovirus bearing KΔC or Lac Z were collected over time and viable cells were counted. Cells expressing KΔC displayed a marked reduction in proliferation.

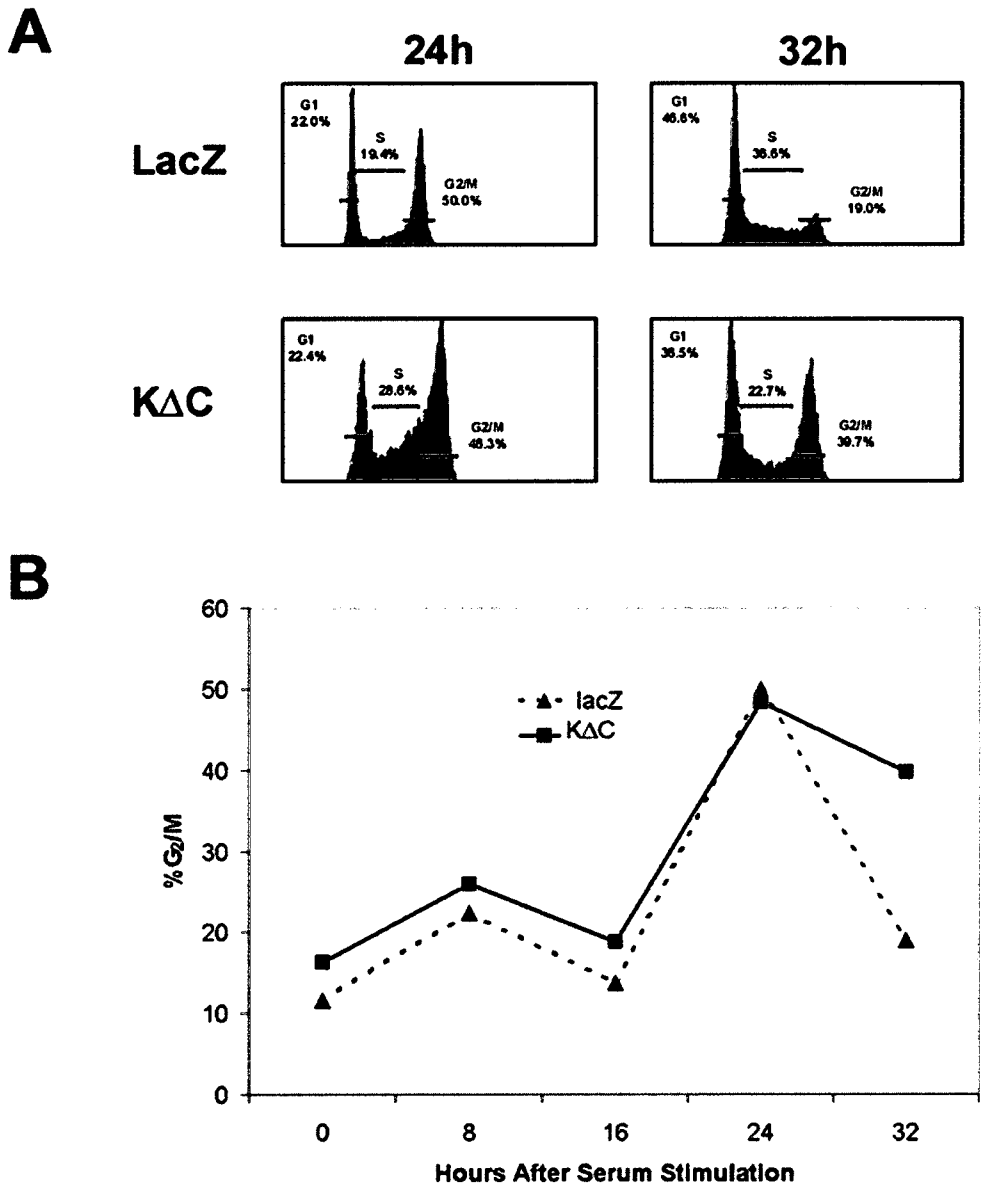

Figure 3.5. Expression of KΔC Results in a G_2/M Block: C3H10T1/2 cells infected with adenovirus carrying LacZ or KΔC were synchronized in G_0 by serum deprivation and stimulated to grow by the reintroduction of serum. Cells were monitored by flow cytometry for DNA content (A) and after 32 hours of serum stimulation, KΔC expressing populations display a delay in G_2/M transit time when compared with LacZ control populations (B).

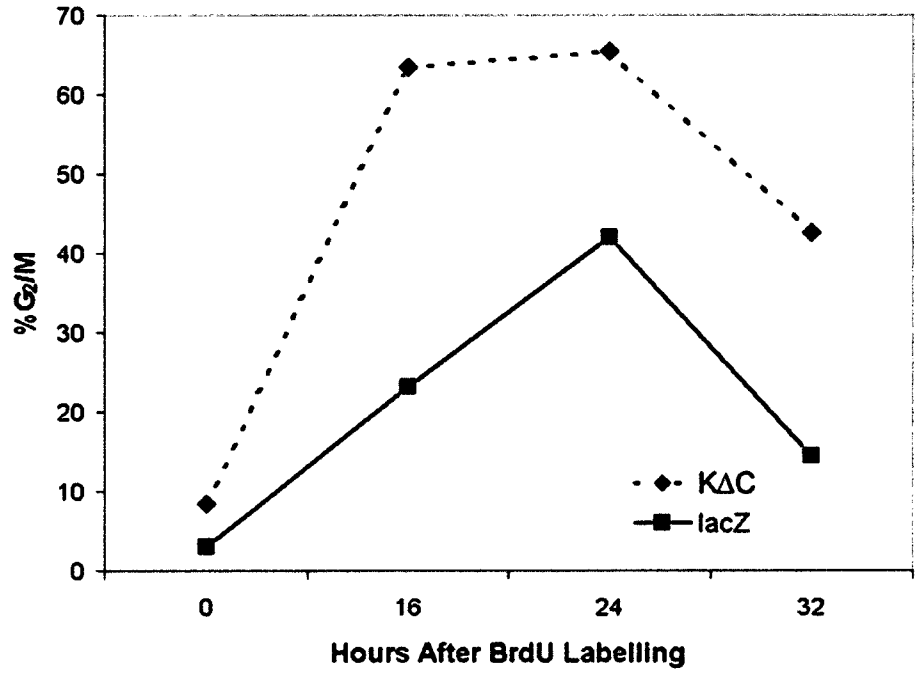

Figure 3.6. Exponentially Growing Populations Expressing KΔC Display Delayed G_2/M Kinetics: Populations of C3H10T1/2 were infected with a HA-KΔC or LacZ adenovirus and were pulsed with BrdUrd 16 hours post infection and BrdUrd positive cells were monitored for DNA content by flow cytometry. KΔC expressing cells proceed through G_2/M with delayed kinetics.

expression delays progression through G_2 or M phase by inducing a specific block in one of these phases. Supporting this finding, flow cytometric tracking of BrdUrd-positive KΔC-infected cultures showed that they proceed through the G_2/M compartment with delayed kinetics when compared with LacZ infected populations (Figure 3.6). Collectively this indicates dominant negative SLK results in delayed progression through G_2/M.

3.6: Discussion and Interpretation.

A proportion of SLK was previously reported to associate with the microtubule network during interphase (Wagner et al., 2002) and we confirm here that SLK is a component of the mitotic spindle (Figure 3.1). Since the mitotic spindle is a very cell cycle specific structure we addressed if there were changes in SLK activity throughout the cell cycle and observed a 3-4 fold increase in its activity when synchronized populations are predominantly in the G_2/M compartment of the cell cycle (Figure 3.2) in contrast to the modest 1.3 fold increase in SLK activity that was previously reported (Ellinger-Ziegelbauer et al., 2000). Taken together these findings suggest that SLK may play a role in cell cycle progression. We investigated this by attempting to stably express antisense-SLK in MEF 3T3 fibroblasts. The fibroblasts that survived the selection period failed to down regulate SLK expression levels likely because antisense RNA was not transcribed and only the selection marker was incorporated stably into the genome. It is reasonable to hypothesize that cells that transcribed antisense SLK failed to proliferate suggesting that SLK is required for normal growth. Similarly to the antisense findings, we observed that KΔC-expressing populations do not proliferate normally over a four day period (Figure 3.4). Taken together, these findings indicate that SLK is required for proliferation and mitotic progression.

To address why there is an absence of proliferation in populations expressing KΔC we conducted further studies. We hypothesized that we would observe a mitotic block in KΔC expressing populations due to SLKs increased activity in populations that are largely in the G_2/M compartment of the cell cycle (Figure 3.2). Consistent with our hypothesis, we observed that synchronized (Figure 3.5) and exponentially (Figure 3.6) growing KΔC-expressing populations displayed delayed G_2/M kinetics based on their DNA profiles when compared to LacZ control expressing populations.

Chapter 4

SLK is Required for Progression Through G_2

4.1: Introduction and Rationale.

Cell cycle progression is a tightly monitored process that is, in part, facilitated by cell cycle phase specific expression of cyclins and their interaction with their respective cyclin dependent kinases (Cdks). The activity of these complexes is regulated by the expression and destruction of the cyclin subunit (Reviewed in Smits and Medema, 2001). $p34^{cdc2/Cdk1}$ interacts with cyclin B late in G_2 and cdc2 is activated by the removal of inhibitory phosphorylation on Thr-14 and Tyr-15 (Dunphy and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991b) and its activation is a critical step for initiating mitosis. During mitosis chromosomes condense which is accompanied by the hyperphosphorylation of H1 (Bradbury et al., 1973) and the phosphorylation of H3 on Ser-10 (Allis and Gorovsky, 1981; Hendzel et al., 1997). Along with varying expression levels of the various cyclins required for cell cycle progression, DNA content will also vary in which cells will contain 2N or 4N DNA content when they are in G_1 or G_2/M respectively. Since SLK displays elevated activity in populations that are largely in the G_2/M compartment of the cell cycle (Figure 3.2) and because KΔC-expressing cultures fail to proliferate (Figure 3.6), we hypothesized that SLK is required for mitosis. Since DNA profiles are not sufficient to determine which phase of the cell cycle displays delayed kinetics because both G_2 and M phase populations have a 4N DNA profile, we can not indicate if the delayed kinetics induced by KΔC expression occur during G_2 or M. Thus, we monitored the expression levels and phosphorylation status of cell cycle specific markers to address where the delay in cell cycle progression occurs.

4.2: Dominant Negative SLK Does Not Repress Cyclin Expression but Inhibits Cyclin A Degradation.

The findings presented in chapter 3 indicate that KΔC induces a G_2/M block but DNA profiles do not provide sufficient information to determine if the block is in G_2 or M. Cyclin D has been observed to be induced prior to S phase and remains elevated in proliferating cells whereas cyclin E is transiently up-regulated at the G_1/S boundary. Cyclins A and B are induced at the G_1/S transition as well but are down-regulated just after the onset and at the end of mitosis respectively (Reviewed in Sherr and Roberts, 1999). To better understand the cell cycle block observed in populations expressing KΔC we performed cyclin profiling on synchronized populations of C3H10T1/2 expressing KΔC or LacZ following release from G_0. The infection efficiency was 80% on average for both viruses and HA-KΔC was observed at levels similar to that of endogenous SLK. Figure 4.1 shows that, relative to actin, cyclin D was slightly up-regulated following serum stimulation in both the LacZ and KΔC infected cultures indicative of cell cycle re-entry. Similarly, cyclin E levels were up-regulated 16 hours post-serum stimulation when a significant proportion of the population entered S phase (Figure 3.2) and down-regulated thereafter suggesting that both cultures entered and exited S phase with similar kinetics. In addition, both cultures induced cyclin A and B expression between 8-16 hours post-serum stimulation during the G_1/S transition. However, only LacZ infected cultures showed a marked and reproducible down-regulation of cyclin A 32 hours post-serum stimulation suggesting that KΔC expressing cultures fail to down-regulate cyclin A and halt the cell cycle before prometaphase since this is the phase of mitosis in which cyclin A is degraded (den Elzen and Pines, 2001; Geley et al., 2001).

4.3: Dominant Negative SLK Induces a G_2 Block.

During interphase, cdc2 is kept inactive by inhibitory phosphorylation on Thr-14 and Tyr-15 (Booher et al., 1997; Liu et al., 1997; Parker and Piwnica-Worms, 1992) and its activation is triggered by the removal of this inhibitory phosphorylation by the Cdc25C phosphatase (Dunphy and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991b). In agreement with elevated cyclin A levels, KΔC infected cultures in the G_2/M compartment of the cell cycle did not significantly up-regulate Cdc2 activity as evidenced by high levels of Cdc2 tyrosine 15 phosphorylation at times when this site is not phosphorylated in LacZ control populations that have a similar cell cycle profile (Figure 4.2). Supporting this, KΔC positive cells did not assemble mitotic spindles 24 hours after serum stimulation at which point over 60% of the populations were in the G_2/M compartment of the cell cycle (not shown).

Chromosome condensation initiates in G2 (Pines and Rieder, 2001) and is accompanied by the hyperphosphorylation of histone H1 (Bradbury et al., 1973) and phosphorylation of H3 on Ser-10 (Allis and Gorovsky, 1981; Hendzel et al., 1997). To further refine the cell cycle block induced by KΔC expression, adenovirus infected cultures were stained for both KΔC expression and phospho-H3 (pH3)(Figure 4.3A-F). Double immunostaining of serum stimulated fibroblast cultures 24 hours post stimulation showed that H3 phosphorylation was markedly reduced in KΔC expressing cells in comparison with control infected cultures. Although the proportion of KΔC and pH3 positive cells slightly increased at 28 and 32 hours following serum stimulation, their numbers were significantly lower than control cells (Figure 4.3G) suggesting that KΔC expressing cells are delayed in G_2.

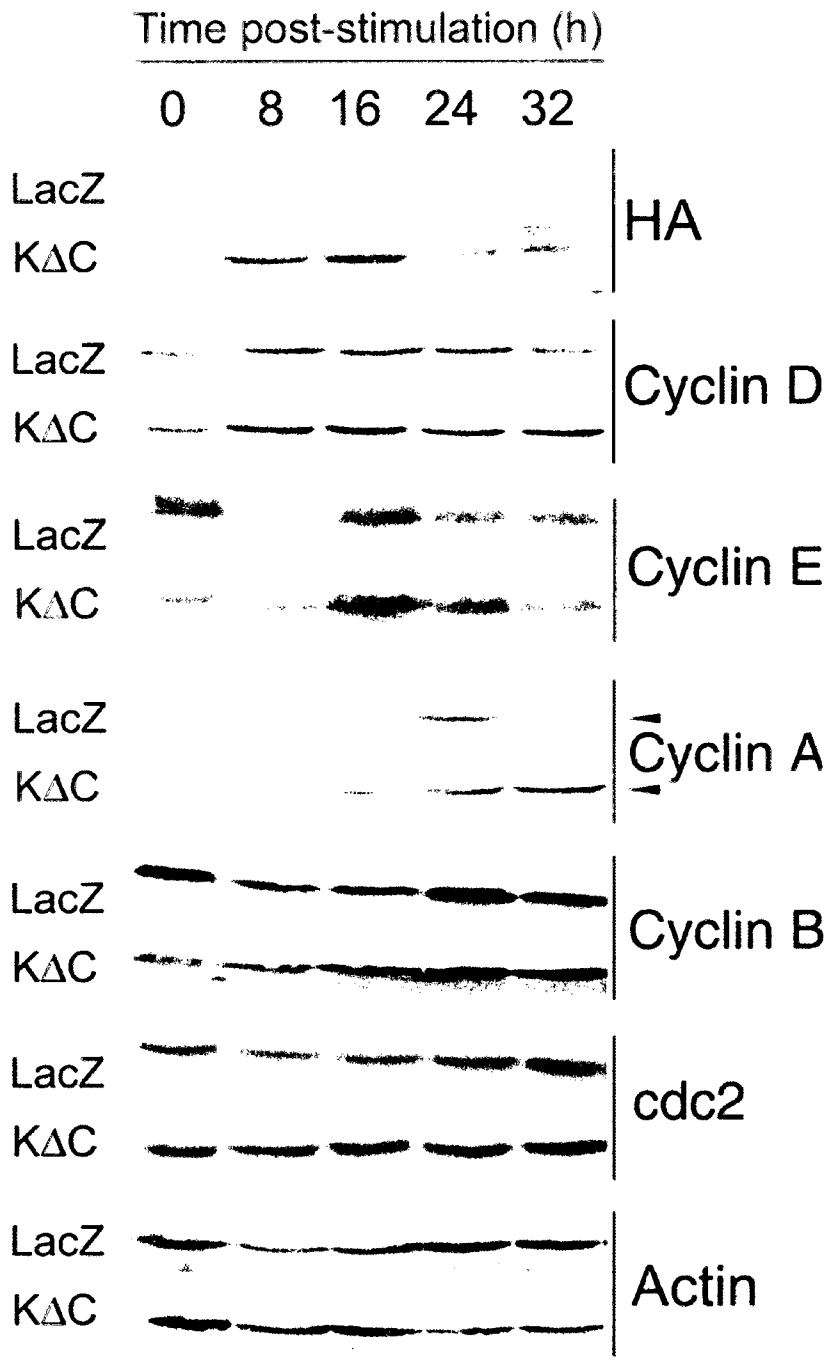

Figure 4.1. Kinase Inactive SLK Alters Cyclin A Expression Pattern: C3H10T1/2 fibroblasts were brought to quiescence by 48 hours of serum deprivation and infected with KΔC or LacZ viruses and monitored for cell cycle-regulating proteins after serum stimulation by Western blot analysis. The expression patterns of cyclins D, E, and B were not found to differ markedly btween LacZ of KΔC infected cultures but KΔC expressing cultures fail to down regulate cyclin A.

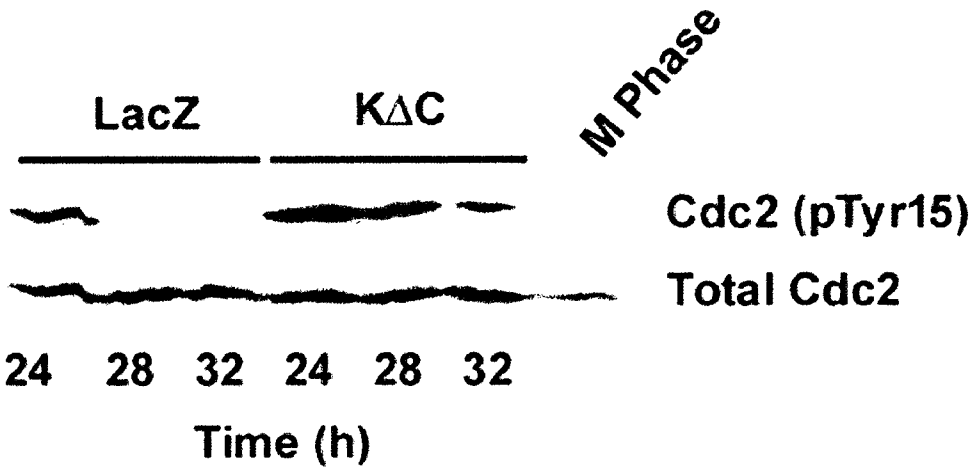

Figure 4.2. Kinase Inactive SLK Inhibits cdc2 activation: C3H10T1/2 fibroblasts were brought to quiescence by 48 hours of serum deprivation and infected with KΔC or LacZ viruses and monitored for cell cycle-regulating proteins after serum stimulation by Western blot analysis. The expression pattern of cdc2 does not differ between KΔC and LacZ expressing cultures but cdc2 fails to activate in KΔC infected cultures at times when it is activated in LacZ expressing cultures as evidenced by an absence of Tyr15 dephosphorylation in KΔC infected cultures indicating a G_2 block. The right-hand lane represents a control M phase synchronized extract to validate the integrity of the pTyr15 cdc2 antibody.

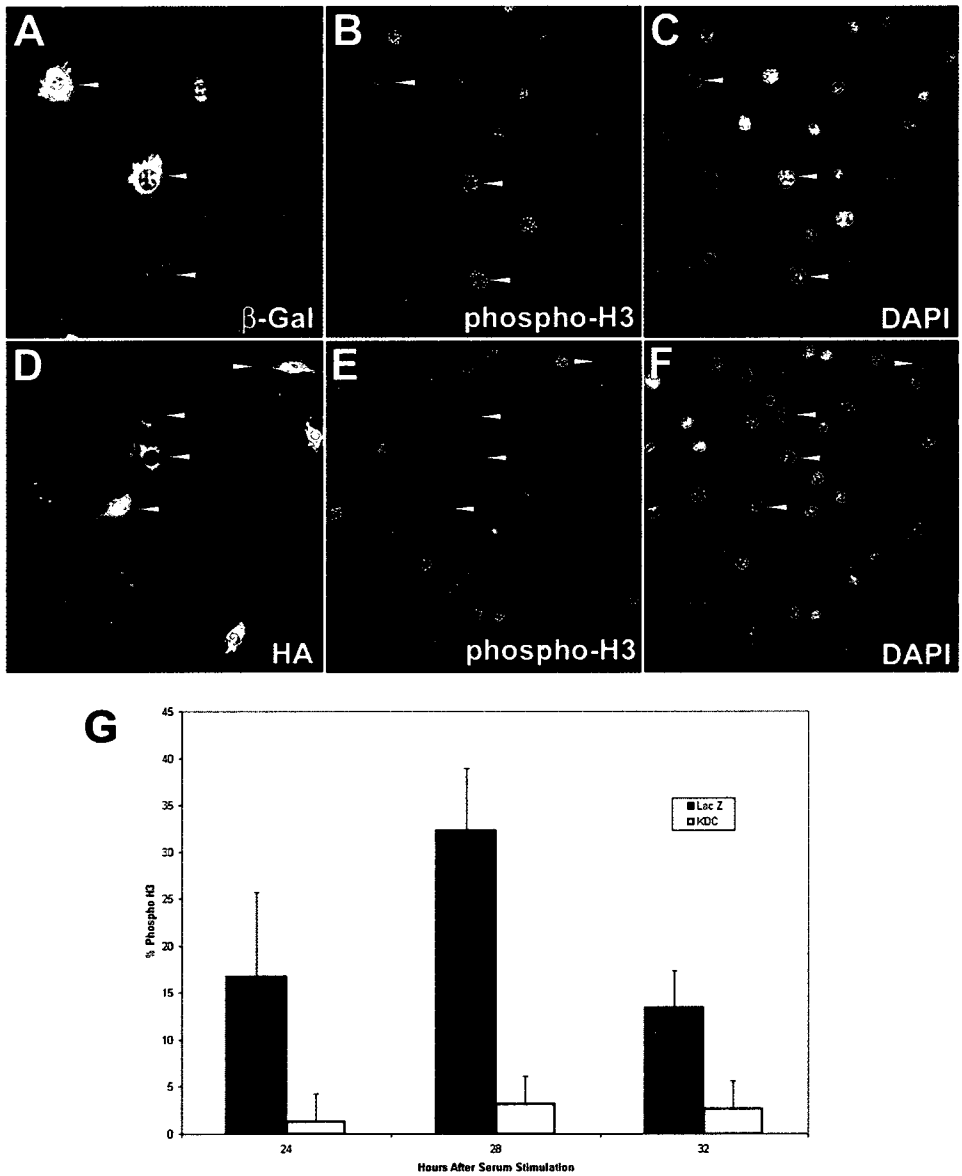

Figure 4.3. KΔC Expression Inhibits Histone H3 Phosphorylation: Synchronized populations of C3H10T1/2 fibroblasts expressing LacZ (A-C) or KΔC (D-F) were monitored for H3 phosphorylation following 24, 28, and 32 hours of serum stimulation. Cells were monitored for HA (D) or B-galactosidase (B-Gal)(A) in conjunction with anti-phospho H3 (B and E). Nuclei were visualized by DAPI counterstaining (C and F). A marked reduction in pH3 staining was observed in KΔC expressing cultures when assessed 24, 28, and 32 hours following serum stimulation (G).

Our results show that expression of a kinase-inactive truncation of SLK is sufficient to induce a G_2 cell cycle block in cycling fibroblasts suggesting that an SLK-dependent pathway is required during G_2 for progression into mitosis. To determine whether SLK plays a central role in G_2 and to rule out potential nonspecific effects by KΔC over-expression, exponentially growing fibroblasts were transfected with an SLK siRNAs pool and subjected to DNA content analysis. Transfection of SLK siRNAs down-regulated SLK protein levels by 80-90% within 48 hours (Figure 4.4A). No effect was observed in the siRNA control samples. DNA content analysis 48 hours post siRNA transfection showed that SLK knockdown resulted in a marked G_2/M accumulation (92% 4N DNA content, Figure 4.4B), an inhibition of proliferation, and increased cyclin A levels (data not shown). These data further support a role for SLK in cell cycle progression and rule out potential nonspecific effects by KΔC overexpression.

4.4: SLK Expression Induces Spindle Formation and Mitotic Entry.

Supporting a role for SLK in G_2 progression, microinjection of activated SLK (SLK 1-373 termed YΔC) in GFP tubulin labelled cells induced ectopic mitotic spindles in the injected cells within 3-6 hours ultimately resulting in cell death (Figure 4.5 I and III)(Wagner et al., 2002). In *Xenopus,* Plk1 becomes hyperphosphorylated during mitosis or when oocytes are treated with progesterone to induce meiosis and this can be visualized by a mobility shift (Descombes and Nigg, 1998) and is often used as a mitotic/meiotic marker when *Xenopus* oocytes are used as a model system for studying the cell cycle. Similar to the presence of ectopic mitotic spindles in YΔC expressing cells, injection of activated SLK, but not kinase dead mRNA in *Xenopus* oocytes resulted in germinal vesicle break down (GVBD) without progesterone induction and the hyperphosphorylation of xPlx1 suggesting that the injected eggs

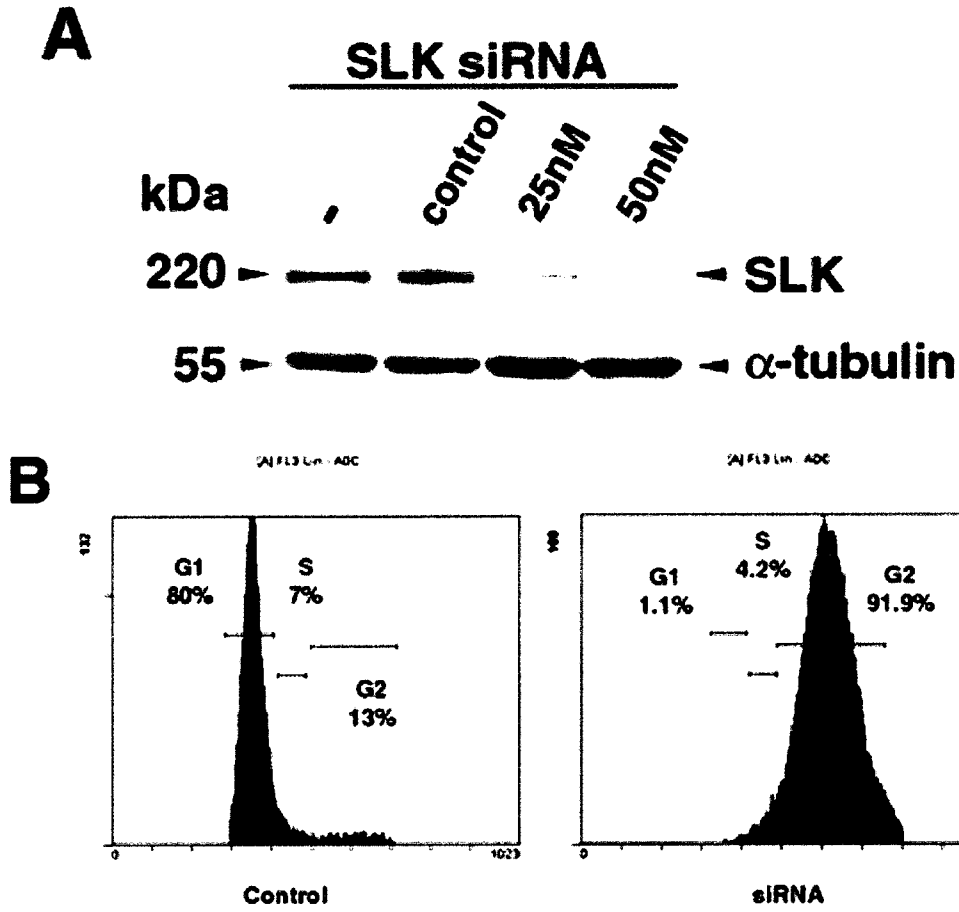

Figure 4.4. SLK is Required for G_2 Progression: Exponentially growing C3H10T1/2 cultures were transfected with the SLK siRNA pool or siCONTROL and analyzed by Western blot for SLK expression (A) and by flow cytometry for a cell cycle profile (B) 48 hours post transfection. A marked down regulation of SLK at 50 nM of siRNA resulted in the accumulation of the cells in the G_2/M compartment of the cell cycle further supporting a requirement for SLK in progression through G_2.

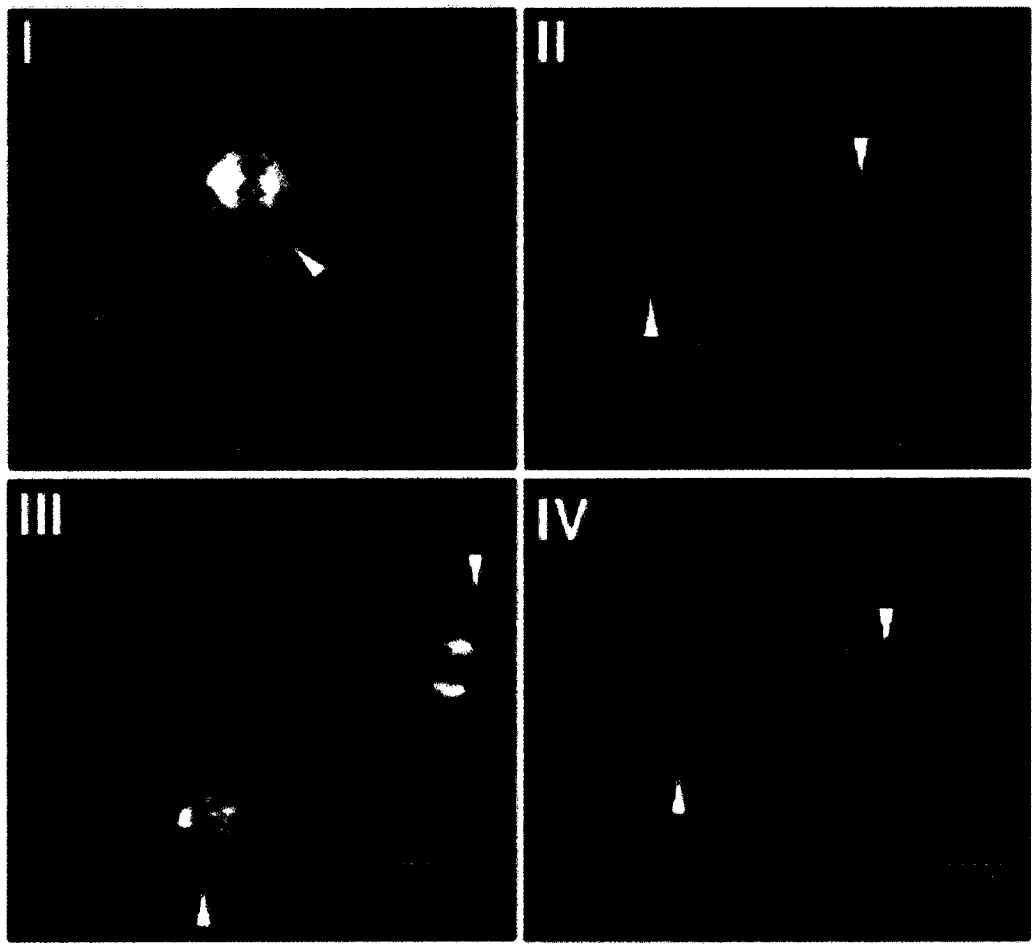

Figure 4.5. YΔC Expression Induces Ectopic Spindle Formation and Death: Exponentially growing LLCPK-1 fibroblasts expressing GFP-tubulin (green) were microinjected with YΔC (I and III) and all injected cells (arrowheads, n=25) displayed ectopic spindle formation suggesting that SLK induces mitotic entry. No spindle formation was observed when cells were injected with KΔC (II and IV).

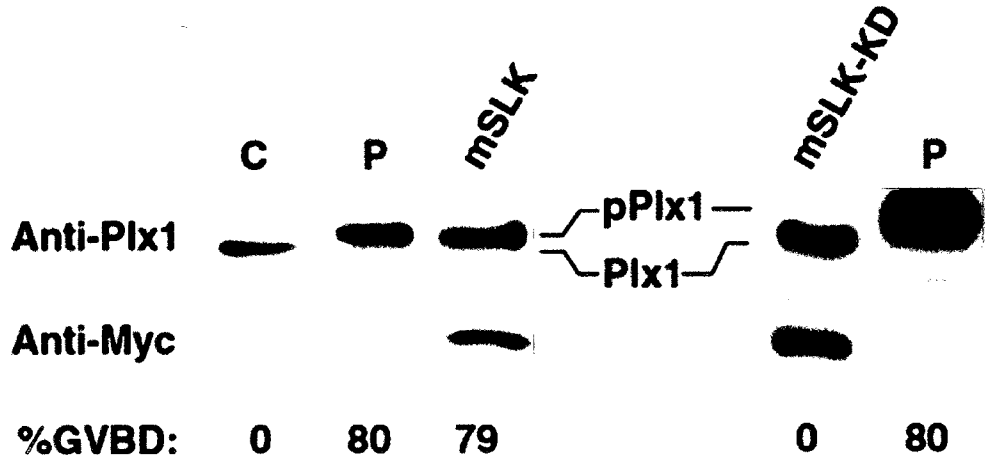

Figure 4.6. YΔC Expression Causes Meiotic Entry in *Xenopus* oocytes: Oocytes injected with YΔC (lane mSLK) re-entered the cell cycle as evidenced by germinal vesicle breakdown (GVBD) and a mobility shift in Plx1 similar to the mobility shift observed when treated with progesterone (lane P). Expression of KΔC (mSLK-KD) could not induce oocyte maturation. The GVBD data represents the average of four independent experiments and Western blot analysis shows the expression of the Myc-tagged SLK truncations.

re-entered the cell cycle as for progesterone treated oocytes (Figure 4.6). Interestingly, expression of a kinase dead version followed by progesterone treatment did not inhibit cell cycle re-entry and GVBD (not shown), suggesting that alternative pathways such as the mitogen-activated protein kinase (MAPK) pathway may be sufficient to promote maturation (Pines and Rieder, 2001).

4.5: Discussion and Interpretation.

Our initial studies indicated that SLK is a component of the mitotic spindle, up regulated during G_2/M, and required for proliferation. To address why there is an absence of proliferation in populations expressing KΔC we conducted further tests and hypothesized that we would observe a mitotic block in KΔC-expressing populations. Consistent with our hypothesis, we observed that synchronized (Figure 3.5) and exponentially (Figure 3.6) growing KΔC expressing populations displayed delayed G_2/M kinetics based on their 4N DNA profiles when compared to LacZ control expressing populations. We confirmed that this was not the result of nonspecific effects by KΔC overexpression by conducting similar tests following the down regulation of SLK by siRNA technology (Figure 4.4). Since DNA profiles are not sufficient to determine which phase of the cell cycle displays reduced kinetics we investigated the status of cell cycle proteins that either vary in their expression levels or their phosphorylation status throughout the various phases of the cell cycle.

We characterized the expression patterns of cyclins D, E, A, and B in synchronized populations from G_0 through M and back to G_1. We observed normal expression patterns of cyclins D, E, and B in KΔC and LacZ expressing populations suggesting that both populations

re-enter the cell cycle and pass through S phase with similar kinetics. However, cyclin A degradation is absent in KΔC expressing cultures at times when it is degraded in LacZ expressing cultures (Figure 4.1) indicating that the cell cycle is blocked before prometaphase, a phase of mitosis where cyclin A is degraded (den Elzen and Pines, 2001; Geley et al., 2001) but does not provide enough information to confirm a G_2 or M block.

To determine which phase of the cell cycle KΔC induces a block we monitored the phosphorylation status of cdc2 and histone H3. Late in G_2, cdc2 is activated by the removal of inhibitory phosphorylation on Thr-14 and Tyr-15 (Dunphy and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991b) and H3 is phosphorylated on Ser-10 (Hendzel et al., 1997) which we used as markers to determine which phase of the cell cycle cultures expressing KΔC fail to progress through. We observed Tyr-15 phosphorylation of cdc2 and an absence of H3 phosphorylation in KΔC expressing cultures at times when LacZ control cultures show cdc2 activation and H3 phosphorylation (Figures 4.3 and 4.4). Based on this finding and the inability for KΔC expressing cultures to degrade cyclin A (Figure 4.1) we can conclude that KΔC induces a cell cycle block in G_2 in contrast to our original hypothesis of SLK regulating mitosis due to its localization to the mitotic spindle (Figures 3.1 and 3.2).

Supporting the findings that SLK activity is required for G_2 progression we observed that YΔC microinjection induces ectopic spindle formation suggesting that these cells are thrust into mitosis. Furthermore, we observed that *Xenopus* oocytes expressing YΔC undergo GVBD and display hyperphosphorylation of Plx1 which are characteristics of mitosis (Figure 4.6), suggesting that they have re-entered the cell cycle. Collectively, the data presented here suggests

a role for SLK upstream of cdc2 activation and demonstrates that interfering with SLK-dependent pathways leads to cell cycle arrest in the G_2 phase of the cell cycle.

Chapter 5

SLK Phosphorylates and Interacts with MARK3

5.1: Introduction and Rationale.

Microtubule affinity regulating kinase 3 (MARK3), also termed Cdc25C associated kinase 1 (C-TAK1), is a serine/threonine kinase that is ubiquitously expressed (Peng et al., 1998) and contains an amino terminal kinase domain, a central ubiquitin binding domain (UBA), and a carboxyl kinase associated domain (KA)(Bachmann et al., 2004). MARK3 was first identified as a kinase that associates with the Cdc25C phosphatase and phosphorylates Cdc25C on Ser-216 leading to its inactivation. The Cdc25C phosphatase initiates mitosis by dephosphorylating threonine 14 and tryrosine 15 of cdc2 leading to its activation (Dunphy and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991b). MARK3 has been implemented as a cell cycle regulator due to its relationship with Cdc25C (Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997). Altering MARK3 should theoretically have similar effects on G_2 kinetics as those observed when KΔC is expressed since MARK3 is implicated in G_2 upstream of cdc2 by holding Cdc25C in an inactive state. Since SLK and MARK3 are involved in G_2 progression upstream of cdc2 activation it is possible that they interact with each other to activate Cdc25C which is a hypothesis that was tested following the identification of MARK3 as an SLK interacting protein.

5.2: MARK3 is an SLK Interacting Protein.

To gain further insight into the function of SLK we conducted a yeast two-hybrid screen in which the strains that were mated expressed the SLK bait or a brain cDNA library. SLK^{K63R} was fused to the gal4 DNA binding domain (DBD) and used to screen a fetal human brain library that was fused to the gal4 activation domain (AD). Following the mating of the bait strain against the library strain we identified several clones that were able to grow on histidine drop-out plates indicating that we had identified several SLK interacting proteins. These clones expressed

the HIS and LacZ reporter genes but for the purposes of this study we will only discuss clone A19 that contained a 1.6kb cDNA fragment encoding a portion of the MARK3 kinase domain (Figure 5.1).

5.3: MARK3 Interacts with SLK *in vitro*.

Full length MARK3 cDNA was then obtained through Open Biosystems and cloned into an expression vector in frame with the hemagglutinin-A (HA) epitope at the amino terminal. HA-MARK3 was *in vitro* translated (IVT) in the presence of ^{35}S-methionine, and tested for its ability to bind to recombinant GST-SLK *in vitro*. GST-SLK bound to glutathione conjugated agarose beads was incubated with IVT ^{35}S-MARK3 and resolved by SDS-PAGE. Binding was assessed by autoradiography of the Coomassie blue stained gel. We observed that MARK3 bound specifically to GST-SLK and not GST (Figure 5.2) suggesting that full length MARK3 may bind directly to SLK. Due to an in availability of a MARK3 antibody at the times these tests were conducted, we were not able to display endogenous association between SLK and MARK3 until later (see chapter 6).

5.4: MARK3 Phosphorylates Cdc25C on Serine 216.

To confirm that the MARK3 clone that was amplified had catalytic activity, its ability to phosphorylate Cdc25C was tested *in vitro*. Using an *in vitro* kinase assay it was confirmed that MARK3 was capable of phosphorylating Cdc25C *in vitro* but not Cdc25C^{S216A}, the phospho-site mutant (Figure 5.3). A review of the primary sequence of MARK3 suggests that K85 is the conserved lysine responsible for binding ATP and thus is required for enzyme activity (Hanks and Hunter, 1995). Through site directed mutagenesis, this lysine was substituted for arginine

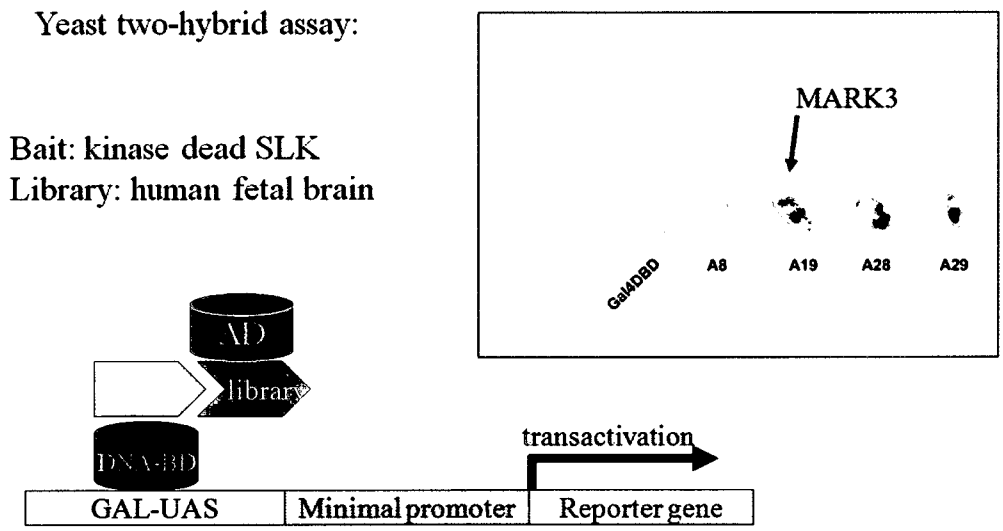

Figure 5.1. MARK3 is an SLK Interacting Protein: During the course of a yeast two-hybrid screen using SLK^{K63R} as bait we identified a 1.6kb cDNA clone whose translated product interacts with SLK. The clone was sequenced and identified as a fragment of the MARK3 kinase domain.

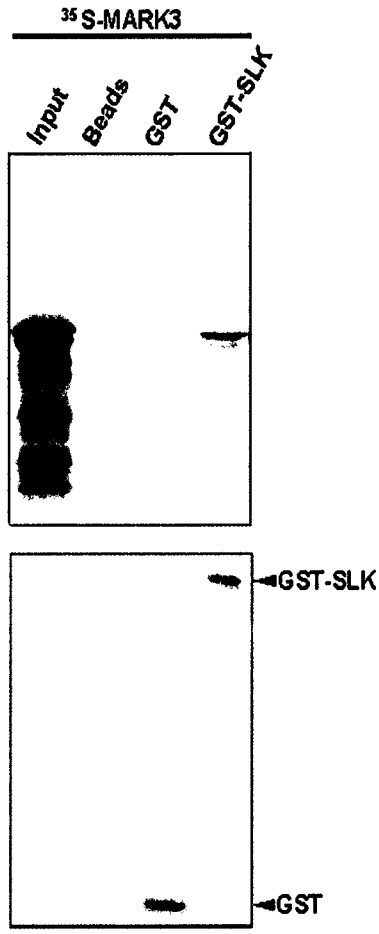

Figure 5.2. Full Length MARK3 binds SLK *in vitro*: Full length *in vitro* translated, ^{35}S-methionine labelled MARK3 was incubated with recombinant GST-SLK bound to glutathione conjugated agarose beads and resolved by SDS-PAGE. The Coomassie blue stained gel (lower panel) was exposed to X-ray film and MARK3 was observed to directly bind SLK and not GST (upper panel).

and tested for its ability to phosphorylate Cdc25C *in vitro*. When compared to MARK3, $MARK3^{K85R}$ displayed a large reduction in its ability to phosphorylate Cdc25C *in vitro* (Figure 5.3) indicating that this mutant results in a catalytically inactive form of MARK3.

5.5: The Activity of MARK3 and SLK is not Required for Their Interaction.

To further characterize the interaction between MARK3 and SLK, wild type constructs or their catalytically inactive counterparts were co-transfected in various combinations into HeLa cells. MARK3 was immunoprecipitated and SLK binding was monitored by Western blotting (Figure 5.4). When MARK3 or $MARK3^{K85R}$ was co-transfected in combination with SLK or SLK^{K63R} the binding affinities of MARK3 (or $MARK3^{K85R}$) to SLK (or SLK^{K63R}) did not show any marked differences indicating that the kinase activity of MARK3 and SLK is not required for these two proteins to form a complex.

5.6: MARK3 and SLK Interact with Each Other at Multiple Contact Points.

To study the role of the interaction between MARK3 and SLK, the binding needs to be disrupted which requires mutants that are incapable of binding each other. To construct these mutants the binding regions of SLK and MARK3 must first be indentified. Deletion mutants HA-$MARK3^{1-298}$ and HA-$MARK3^{299-729}$ were cloned and co-transfected into HeLa cells with domains of SLK that were previously described (Sabourin and Rudnicki, 1999; Sabourin et al., 2000). A 12CA5 monoclonal antibody was used to immunoprecipitate and detect the HA-tagged MARK3 fragments and the SLK fragments were detected with an αSLK polyclonal antibody (Sabourin and Rudnicki, 1999) or a 9E10 monoclonal antibody that recognizes Myc-tagged $SLK^{856-1202}$ (termed ATH). Whole cell lysate analysis indicated that $MARK3^{1-298}$ did not express

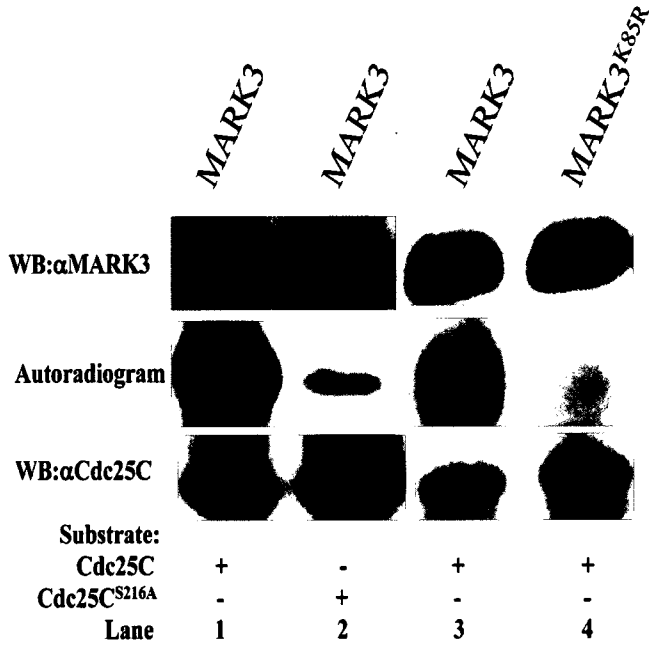

Figure 5.3. Cloned MARK3 has Kinase Activity: MARK3 cDNA was obtained and cloned into an expression vector and assessed for its ability to phosphorylate Cdc25C during *in vitro* kinase assays. MARK3 phosphorylated Cdc25C (lane 1and 3) but not $Cdc25C^{S216A}$ (lane 2). A review of the primary structure of MARK3 indicated that K85 is the candidate residue responsible for its kinase activity. A K85R mutant was created which is incapable of phosphorylating Cdc25C (lane 4) indicating that it is catalytically inactive. Cdc25C levels were normalized by Western blotting to confirm equal amounts of substrate were available in each reaction (bottom panel).

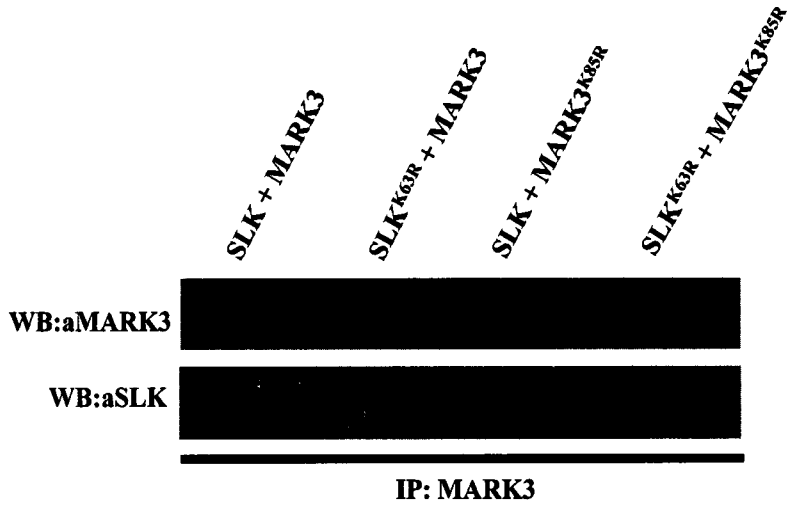

Figure 5.4. The Kinase Activity of SLK and MARK3 are not Required for Their Interaction: Combinations of wild type or catalytically inactive MARK3 and SLK and were co-transfected and tested for their ability to bind each other. MARK3 was immunoprecipitated out of co-transfected lysates and SLK binding was monitored by Western blotting. There was no change in binding affinities between any combination of wild type and catalytically inactive mutants.

as well as MARK3$^{299-729}$ (not shown) and as a result larger amounts of MARK3$^{299-729}$ were immunoprecipitated in comparison to MARK3$^{1-298}$ (Figure 5.5, bottom panel). MARK3$^{1-298}$ and MARK3$^{299-729}$ were both efficient in co-immunoprecipitating full length SLK (Figure 5.5, lanes 1 and 5) indicating that MARK3 has at least two points of contact with SLK. Notably, endogenous SLK was observed co-immunoprecipitating with MARK3$^{1-298}$ and MARK3$^{299-729}$ in samples in which Myc-SLK was not co-transfected (Figure 5.5, lanes 2-4 and 6-8) reaffirming the ability of MARK3$^{1-298}$ and MARK3$^{299-729}$ to bind SLK. Surprisingly, both MARK3$^{1-298}$ and MARK3$^{299-729}$ were efficient in binding SLK lacking the amino-terminal (ΔN, Figure 5.5 lanes 2 an 6), SLK lacking the carboxyl-terminal (Δ3', Figure 5.5 lanes 3 and 7), and both MARK3$^{1-298}$ and MARK3$^{299-729}$ bound the carboxyl terminal of SLK (ATH, Figure 5.5 lanes 4 and 8) indicating that the binding between MARK3 and SLK occurs at multiple contact sites. Alternatively, because the experiment was conducted *in vivo* there maybe other endogenous SLK or MARK3 binding proteins responsible for this binding pattern to take place and therefore the binding we observe using *in vivo* studies may be indirect and facilitated by other binding partners.

A disadvantage of using an *in vivo* system when characterizing protein-protein interactions is the possibility that, in this case, other MARK3 or SLK binding partners may be recruited (Figure 5.5). To rule out the possibility that proteins other than MARK3 and SLK are involved in the SLK-MARK3 interaction and to verify that the interaction is direct, an *in vitro* system was used in which SLK was translated and labelled with ^{35}S-methionine. Recombinant GST-MARK3 or recombinant MARK3 fragments of 250 amino acids in length (GST-MARK3$^{1-250}$, GST-MARK325$^{1-500}$, and GST-MARK3$^{501-729}$) were isolated from a bacterial expression system and binding assays were conducted using recombinant GST proteins that were bound to

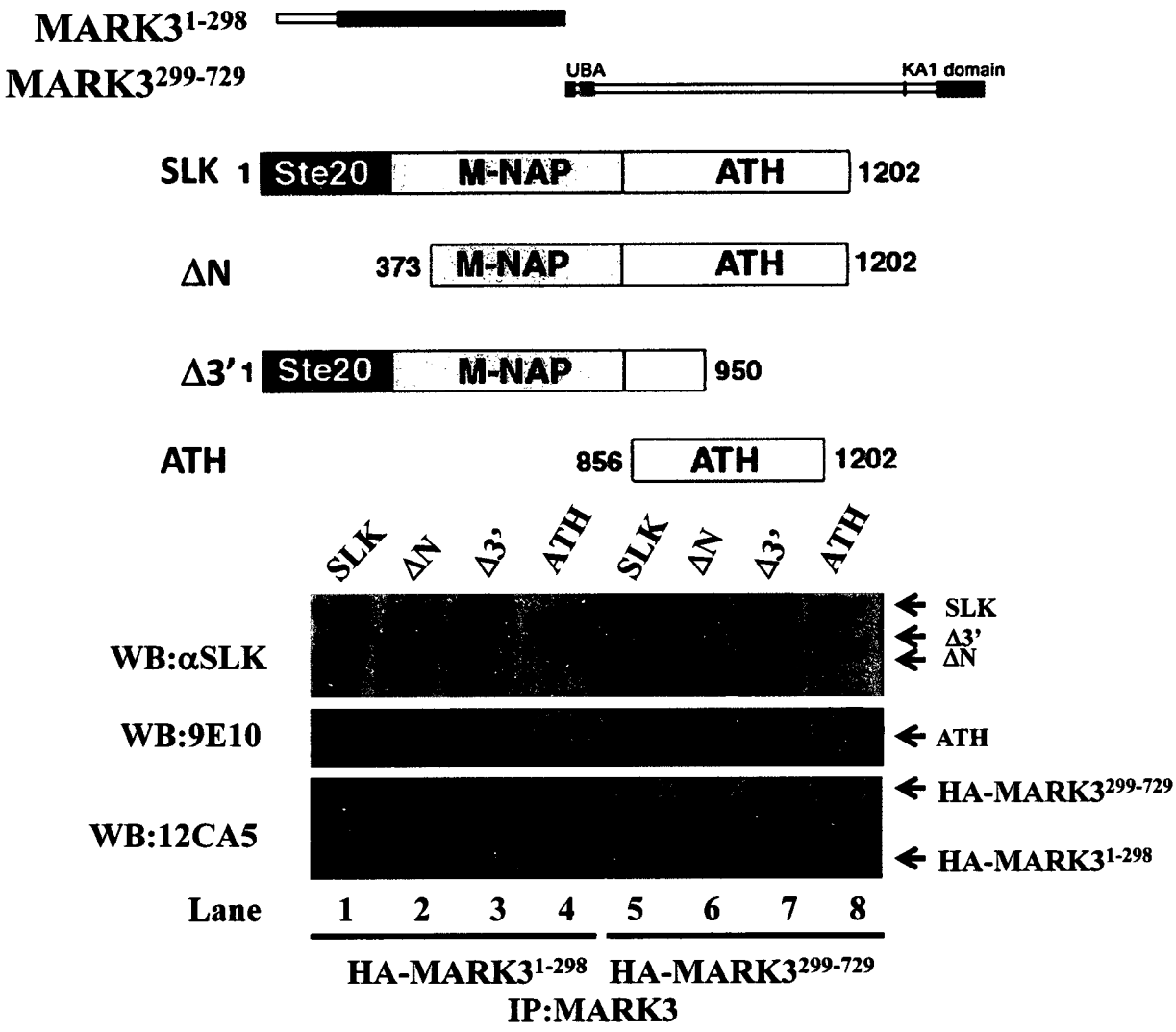

Figure 5.5. MARK3 Binds SLK at Multiple Points *in vivo*: HA-MARK3$^{1-298}$ and HA-MARK3$^{299-729}$ were transfected in combination with full length or various truncations of SLK. Both halves of MARK bound to full length SLK and the three other SLK fragments displayed here.

glutathione conjugated agarose beads. SLK efficiently bound full length MARK3 and, similar to the binding pattern obtained from the *in vivo* study, $MARK3^{1-250}$ and $MARK3^{501-729}$ also bound SLK efficiently (Figure 5.6). The central $MARK3^{251-500}$ domain bound SLK poorly with an efficiency similar to that of GST (Figure 5.6), suggesting that this may be background binding. One of the few recognizable domains present in MARK3 is its ubiquitination association domain (UAD)(Bachmann et al., 2004) which has been shown to bind monoubiquitin (Murphy et al., 2007). Since ubiquitylnation can facilitate protein-protein interactions, the UAD is a candidate domain for the SLK-MARK3 interaction. A requirement for this domain in the SLK-MARK3 interaction was tested using a MARK3 construct lacking its ubiquitination association domain (MARK3ΔUAD). This MARK3 mutant displayed an affinity for SLK similar to that of full length MARK3 (Figure 5.6). The UAD maps to amino acids 328-364 and is therefore a component of the $MARK3^{251-500}$ fragment which weakly binds SLK (Figure 5.6), supporting the observation that the UAD is not required for the SLK-MARK3 interaction to take place. These results indicate that the amino and carboxyl terminals of MARK3 bind SLK and the central UAD containing domain of MARK3 is not required for the SLK-MARK3 interaction.

These findings suggest that it may be difficult to define the regions of MARK3 responsible for binding SLK since there is at least two areas of contact and it may be more efficient to determine where $MARK3^{1-250}$ and $MARK3^{501-729}$ bind SLK. The $MARK3^{1-250}$ and $MARK3^{501-729}$ constructs, which are the fragments that bind SLK (Figure 5.6), were translated *in vitro* and labelled as described above and their ability to bind bacterially expressed recombinant YΔC, a central SLK fragment (termed XX1.2), and a carboxyl fragment (termed BG2631) was

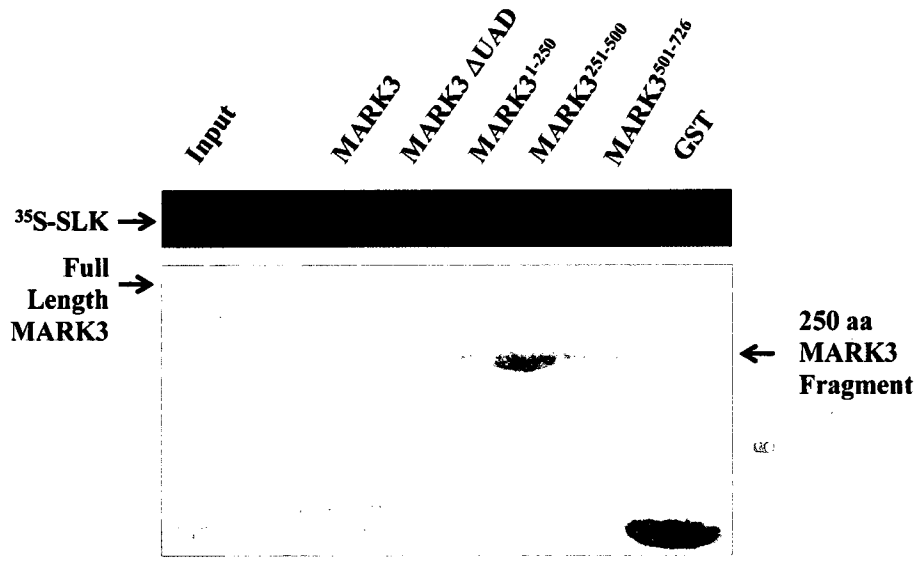

Figure 5.6. The Amino and Carboxyl Fragments of MARK3 Bind SLK: SLK was translated *in* vitro, labelled with ^{35}S-methionine, and tested for its ability to bind recombinant MARK3 constructs. SLK-MARK3 complexes were resolved by SDS-PAGE, stained with Coomassie blue (lower panel) and exposed to X-ray film (upper panel). The amino and carboxyl fragments of MARK3 were found to efficiently bind SLK and the central domain of MARK3 was observed to have an affinity to SLK similar to that of GST.

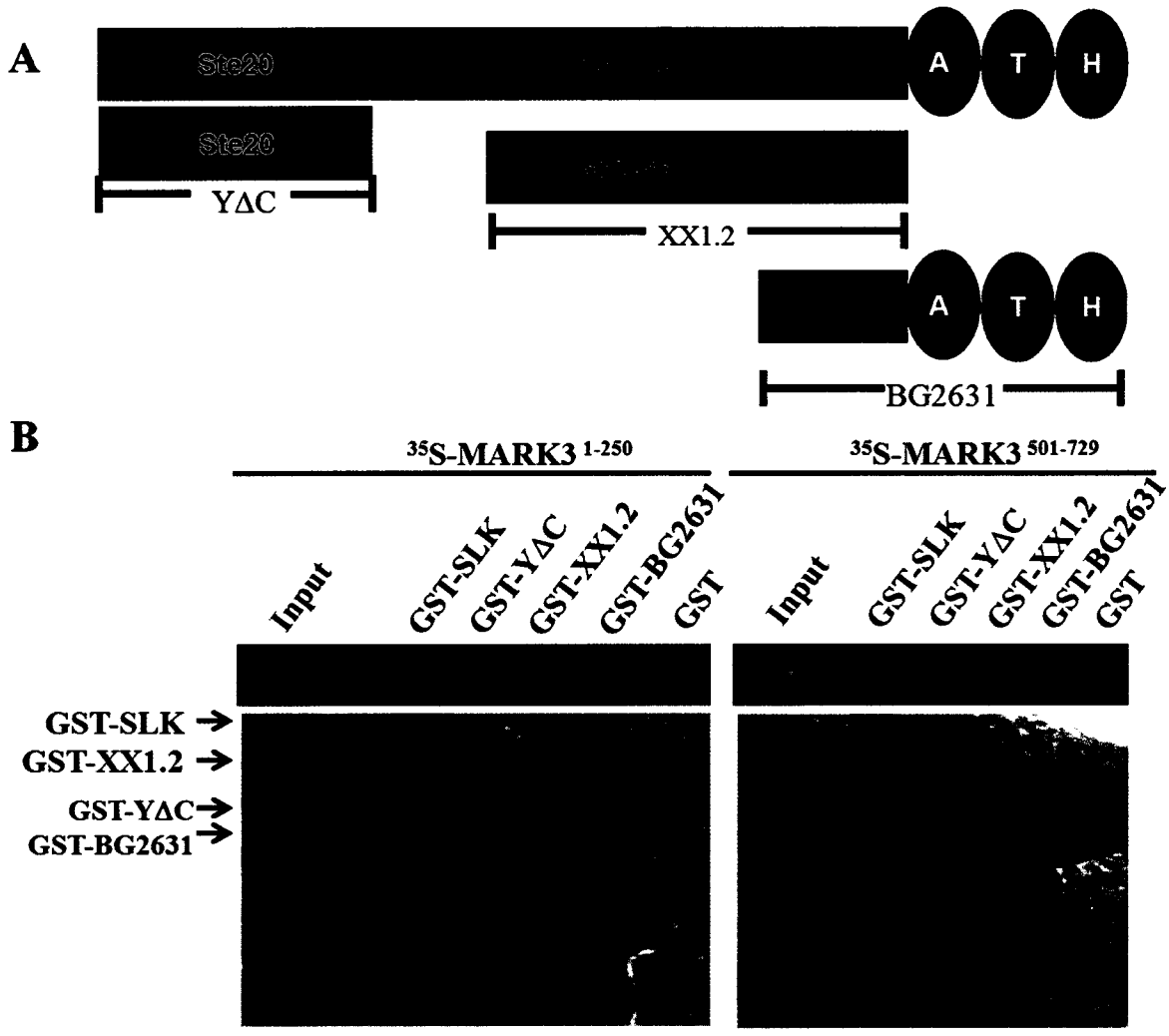

Figure 5.7. MARK3$^{1-250}$ and MARK3$^{501-729}$ Bind SLK at Multiple Points *in vitro*: MARK3$^{1-250}$ and MARK3$^{501-729}$ were *in vitro* translated and labelled with ^{35}S-methionine and tested for their ability to bind recombinant fragments of SLK. SLK-MARK3 complexes were resolved by SDS-PAGE and the gel was stained with Coomassie blue (lower panel) and exposed to X-ray film (upper panel). Both MARK3$^{1-250}$ and MARK3$^{501-729}$ were observed to bind full length SLK and the amino, central, and carboxyl domains of SLK.

assessed. Similar to the *in vivo* results displayed in Figure 5.5, both MARK3$^{1-250}$ and MARK3$^{501-729}$ can bind YΔC, XX1.2, and BG2631 but not GST (Figure 5.7). Collectively, the findings provided by the *in vivo* and *in vitro* binding assays indicate that the SLK-MARK3 interaction is extremely complex. Furthermore, the construction of a MARK3 or SLK mutant that does not bind the other may be problematic since more than one region of either protein must be deleted in order to negate their interaction.

5.7: SLK Phosphorylates MARK3 in its Central Domain.

Because we could demonstrate an *in vitro* and *in vivo* interaction between SLK and MARK3, we investigated if one is a substrate for the other. Although MARK3 efficiently phosphorylates Cdc25C *in vitro* (Figure 5.3), indicating that it is a functional kinase, phosphorylation of full length recombinant SLK^{K63R} or fragments of SLK by MARK3 was not detected following *in vitro* kinase assays (not shown). However, during reciprocal tests in which MARK3 was used as a substrate, SLK was shown to efficiently phosphorylate recombinant MARK3^{K85R} (Figure 5.8). To refine the region in MARK3 that is phosphorylated by SLK recombinant MARK3$^{1-250}$, MARK3$^{251-500}$, and MARK3$^{501-729}$ were tested as potential substrates for SLK in *in vitro* SLK kinase assays. Although SLK was incapable of phosphorylating the amino-terminal MARK3$^{1-250}$ and carboxyl-terminal MARK3$^{501-729}$ fragments, it efficiently phosphorylated the central UAD containing MARK3$^{251-500}$ fragment (Figure 5.8). SLK is also capable of phosphorylating recombinant MARK3^{K85R}ΔUAD further refining the site of phosphorylation between aa 251-327 and aa 365-500 since the UAD spans aa 328-364 of MARK3.

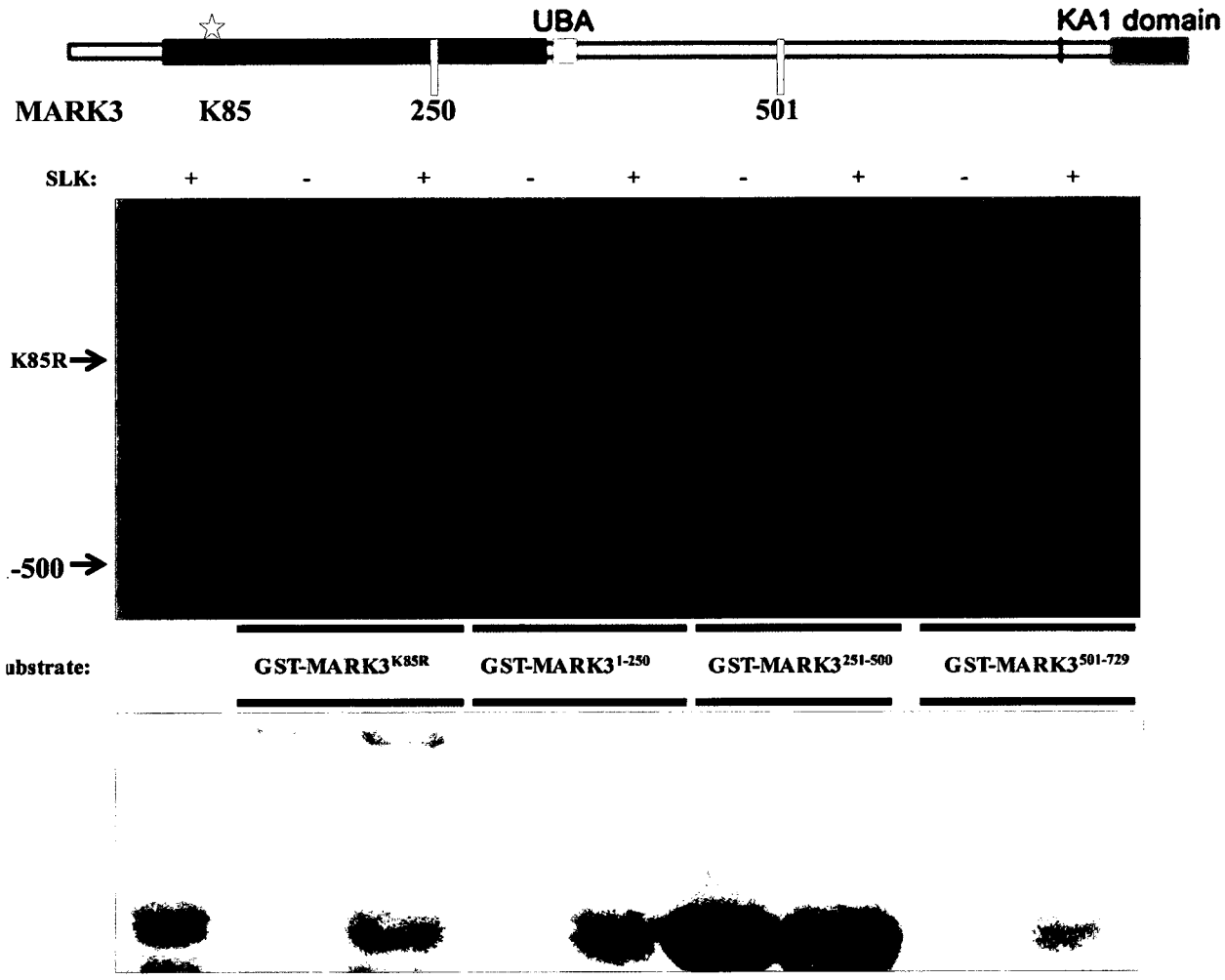

Figure 5.8. SLK Phosphorylates MARK3 Between Amino Acids 251-500: Recombinant MARK3 constructs were used as substrates for SLK during *in vitro* kinase assays. SLK was observed to phosphorylate full length MARK3^{K85R} and the MARK3$^{251-500}$ construct.

5.8: SLK and MARK3 do not Affect Each Others Catalytic Activity or Expression.

We have shown that SLK is required for G_2 progression upstream of cdc2 activation (Chapters 3 and 4). Furthermore, an absence of SLK interacting proteins has made it difficult to place it in a mechanistic pathway involved in G_2 progression. Here we show that SLK interacts with MARK3, a factor that has been linked to G_2 maintenance through phosphorylation of Cdc25C on its inhibitory residue (Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997). Studies involving the SLK-MARK3 interaction were therefore initiated to verify if SLK and MARK3 interact in a pathway that leads to cdc2 activation. If MARK3 is phosphorylating and holding Cdc25C in an inactive state, then at some point in G_2 MARK3 must receive a signal that negates this phosphorylation event. Therefore, we tested if SLK regulates MARK3 activity by expressing constitutively active SLK (YΔC) or catalytically inactive SLK (KΔC) and monitored the ability of MARK3 to phosphorylate Cdc25C *in vitro*. Kinase assays did not detect any change in MARK3 activity in cells expressing YΔC or KΔC (Figure 5.9). Alternatively, the signal that MARK3 must receive to prevent phosphorylation of Cdc25C may not act on MARK3's catalytic activity but rather on its level of expression. The presence of a ubiquitin binding domain in MARK3 and the role of ubiquitination in proteasomal degradation prompted us to test if SLK has an impact on MARK3 expression levels by transfecting YΔC and KΔC in HeLa cell followed by a an assessment of MARK3 levels by Western blotting. Cells expressing YΔC or KΔC did not show any marked differences in MARK3 protein levels (Figure 5.10) indicating that SLK interaction may not regulate MARK3 expression levels.

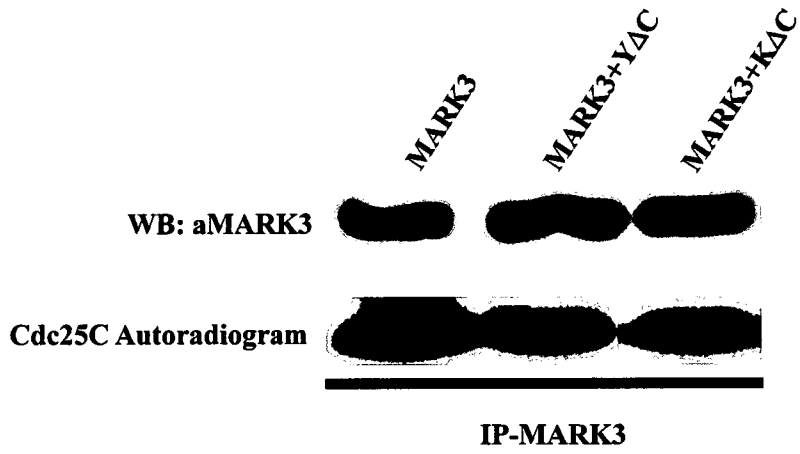

Figure 5.9. SLK does not Affect MARK3 Activity: HeLa cells were transfected with MARK3 or with MARK3 in combination with YΔC or KΔC and MARK3's ability to phosphorylate Cdc25C was assessed using *in vitro* kinase assays. YΔC and KΔC expression did not result in a marked difference in MARK3's catalytic activity.

Figure 5.10. SLK Does not Affect MARK3 Expression Levels: HeLa cells were transfected with MARK3 or MARK3 in combination with YΔC or KΔC. MARK3 levels did not display marked differences in the presence of YΔC or KΔC indicating that SLK does not have an impact on MARK3 expression levels.

5.9: Discussion and Interpretation.

Because SLK is required for G_2 progression and MARK3 has been linked to G_2 maintenance through Cdc25C phosphorylation (Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997), the observation that SLK and MARK3 interact provided a basis for expanding on this interaction. This prompted us to investigate the role of this interaction on G_2 progression.

In order to test if the SLK-MARK3 complex had a physiological role in G_2 progression the association was characterized. Identification of the interacting domains would provide the information required to construct SLK or MARK3 mutants that do not bind, allowing the disruption of the complex *in vivo*, providing a tool for studying the function of this complex. Both proteins are kinases and their catalytic activity is not required for the interaction to take place (Figure 5.4) indicating that they do not rely on phosphorylation by one another for this interaction. Transfection and co-immunoprecipitation studies using MARK3 constructs indicated, surprisingly, that both $MARK3^{1-298}$ and $MARK3^{299-729}$ efficiently bound amino and carboxyl-terminal SLK constructs *in vivo*, suggesting that the *in vivo* SLK-MARK3 interaction is intricate.

A disadvantage of an *in vivo* system for studying protein-protein interactions is the potential for other factors to tether the proteins of interest together which may lead to problems when attempting to interpret the binding results. To characterize the direct contact that takes place between SLK and MARK3 an *in vitro* system was used. During these studies, 250 amino acid fragments of MARK3 were tested for their ability to bind SLK. Similar to the *in vivo* studies, we showed that an amino-terminal $MARK3^{1-250}$ and a carboxyl-terminal $MARK3^{501-729}$ fragment were both capable of binding SLK (Figure 5.6) indicating that MARK3 contacts SLK

at two regions. Similar studies were conducted in which MARK3$^{1-250}$ and MARK3$^{501-729}$ were tested for their ability to bind regions of SLK and showed that each of these amino and carboxyl terminal fragments of MARK3 bind both amino and carboxyl fragments of SLK (Figure 5.7). These findings indicate that there are at least four regions in MARK3 that contact SLK since MARK3$^{1-250}$ can bind two distinct fragments of SLK. Since MARK3$^{501-729}$ displays a similar binding pattern, there must be at least four regions in MARK3 that interact with SLK. Further refinement of the interaction between SLK and MARK3 by these methods may prove to be tedious and may be better resolved by technologies such as X-ray crystallography of the protein complexes. However, some structural insight can be gained from these studies since other Ste20-kinases of the GCK family are capable of homo-dimerizing in a head to tail conformation (Ling et al., 2008). Our group has evidence supporting the hypothesis that SLK exits in a head to tail conformation thus rendering its amino and carboxyl terminals in close proximity to each other. For the amino and carboxyl terminals of MARK3 to be able to bind both the amino and carboxyl terminals of SLK they must be in close proximity to each other suggesting that MARK3 may also 4have a head to tail conformation.

Although we were incapable of showing MARK3 phosphorylation of SLK *in vitro*, we did show that SLK could phosphorylate MARK3 somewhere in the central MARK3$^{251-500}$ fragment, but outside of the UAD (Figure 5.8). Notably, this fragment does not bind SLK (Figure 5.6). The observation that SLK phosphorylates MARK3 *in vitro* suggests that this phosphorylation event may regulate MARK3 function such as its catalytic activity or expression levels. Since SLK is activated during G_2/M and is required for G_2 progression upstream of cdc2, our hypothesis is that SLK phosphorylation of MARK3 is required to inhibit the repressive signal to Cdc25C. The consequences of this phosphorylation event may either alter MARK3

activity or its expression levels. Our results also show that YΔC or KΔC expression did not affect the ability of MARK3 to phosphorylate Cdc25C (Figure 5.9), indicating that MARK3 phosphorylation by SLK does not affect its activity. Similar tests assessing if SLK affects MARK3 expression levels did not show any significant changes. However, one could speculate that this phosphorylation event may provide docking sites for other proteins or cause the dissociation of MARK3 bound proteins. Another possibility is that this in an *in vitro* artefact and until the phosphorylation site is identified we will be unable to assess the physiological role of this phosphorylation event.

Overall, we show here that SLK and MARK3 form an intricate complex and that SLK is capable of phosphorylating MARK3 *in vitro*.

Chapter 6

SLK, MARK3, and Paxillin Form a G_2/M Specific Complex

6.1: Introduction and Rationale.

Paxillin is a multi-domain adaptor protein that was initially identified as a tyrosine phosphorylated protein in v-src transformed cells (Glenney and Zokas, 1989). The cloning of paxillin has revealed that it comprises numerous discrete structural domains (Brown et al., 1996; Turner and Miller, 1994) including LIM domains and LD motifs. LIM domains are double zinc finger motifs that mediate protein-protein interactions (Perez-Alvarado et al., 1994; Schmeichel and Beckerle, 1994). The N-terminal half of paxillin contains a proline rich sequence that serves as a docking site for the SH3 domain of Src (Weng et al., 1993) and five leucine and aspartic acid rich motifs (termed LD1-5) that are responsible for most of the signalling that occurs through paxillin (Brown et al., 1996; Turner and Miller, 1994). Paxillin has several phosphorylation sites and its phosphorylation status regulates its binding affinity (Brown and Turner, 2004). Paxillin has also been demonstrated to be hyperphosphorylated on serines by a yet to be identified kinase and is degraded during mitosis (Yamaguchi et al., 1994; Yamaguchi et al., 1997) suggesting that it may receive post-translational modifications important for cell division.

SLK has been demonstrated to localize to paxillin containing leading edges during cell spreading (Wagner et al., 2002). The transcriptional co-factors Lbd1 and 2 are capable of binding to SLK (Storbeck et al., 2009) and to the LD domains of paxillin (Storbeck *et al.*, unpublished). There is also another link between SLK and paxillin in which MARK3 was identified as a paxillin interacting protein (Cell Migration Gateway, 2009). Our hypothesis is that MARK3 may recruit paxillin to SLK for phosphorylation. Although the majority of the literature pertaining to paxillin revolves around migration, one possibility is that phosphorylation

of adhesion proteins results in cell detachment from the matrix, a process that is required for cell division.

6.2: SLK Phosphorylates Paxillin.

SLK and paxillin have been found to co-localize at sites of adhesion during cell spreading (Wagner et al., 2002). Although SLK and paxillin have not been observed to interact directly, evidence from our laboratory suggests that SLK and paxillin associate weakly *in vivo* in exponentially growing and non-motile populations (Foucault *et al.*, unpublished). The potential interaction between paxillin and SLK is currently the focus of another study in our laboratory. Whether the interaction is direct or indirect does not have a major impact on what will be presented in this study. Since SLK localizes with paxillin at membrane ruffles during migration (Wagner et al., 2002) and motility is enhanced when paxillin is phosphorylated on serine residues (Bellis et al., 1997), we tested if SLK was capable of phosphorylating paxillin *in vitro*. SLK was isolated from exponentially growing fibroblast populations and subjected to *in vitro* kinase assays in which recombinant paxillin was used as a substrate. We observed that SLK can efficiently phosphorylate paxillin *in vitro* (Figure 6.1). Further refinement of the phosphorylation site was conducted by testing the ability of SLK to phosphorylate the amino-terminal LD domains (LD1-5) or the carboxyl LIM domains (LIM1-4) of recombinant paxillin. SLK was incapable of phosphorylating LIM1-4 but phosphorylated the LD1-5 subdomains with lesser efficiency than that observed for full length paxillin (Figure 6.1) which may be the result of a loss of conformation in the deletion mutant. Interestingly, the LD domains are responsible for the majority of the signalling that goes through paxillin (Brown et al., 1996; Turner and Miller, 1994).

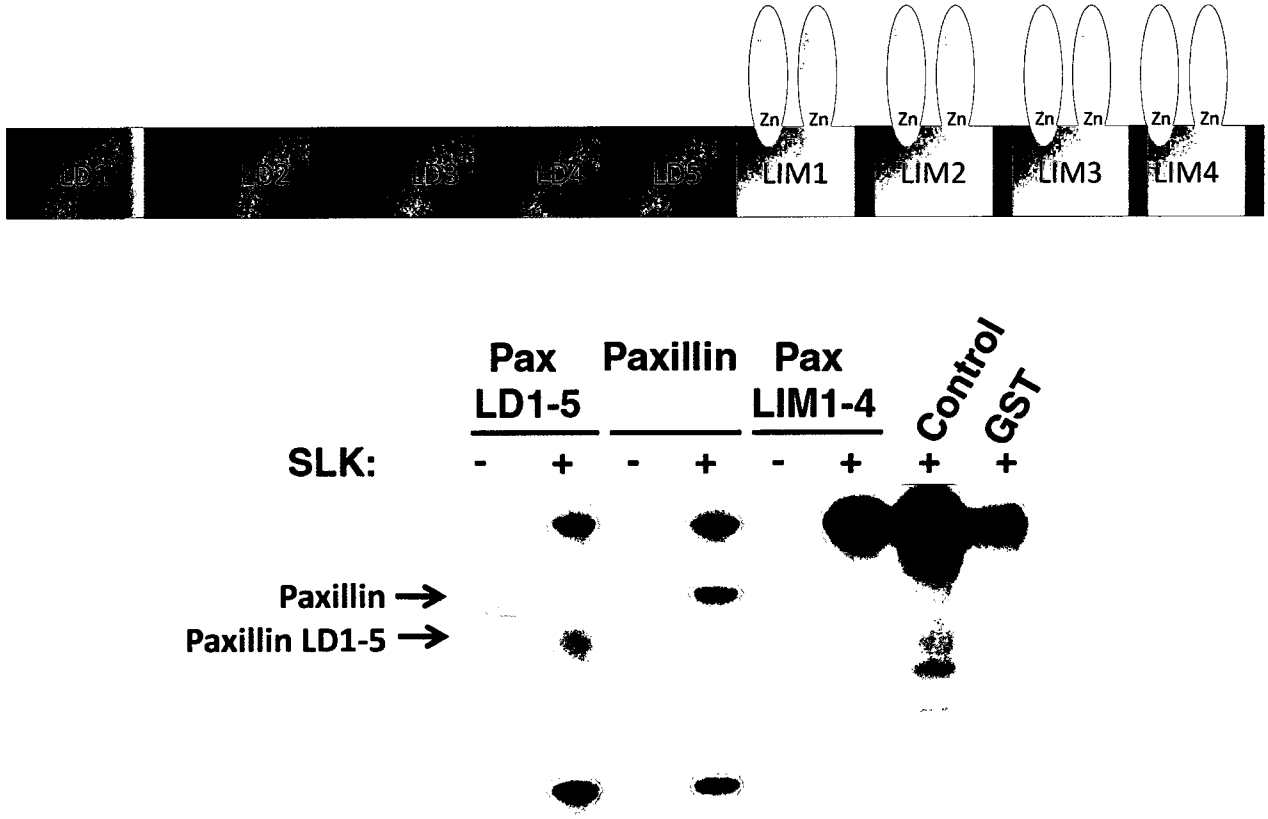

Figure 6.1. SLK Phosphorylates Paxillin *in vitro*: SLK was immunoprecipitated from exponentially growing, non-motile fibroblasts and used to test its ability to phosphorylate recombinant paxillin *in vitro*. SLK was observed to efficiently phosphorylate paxillin. To narrow down the region in paxillin that is phosphorylated by SLK recombinant LD1-5 or LIM1-4 constructs were tested as substrates and SLK was observed to phosphorylate the LD1-5 construct.

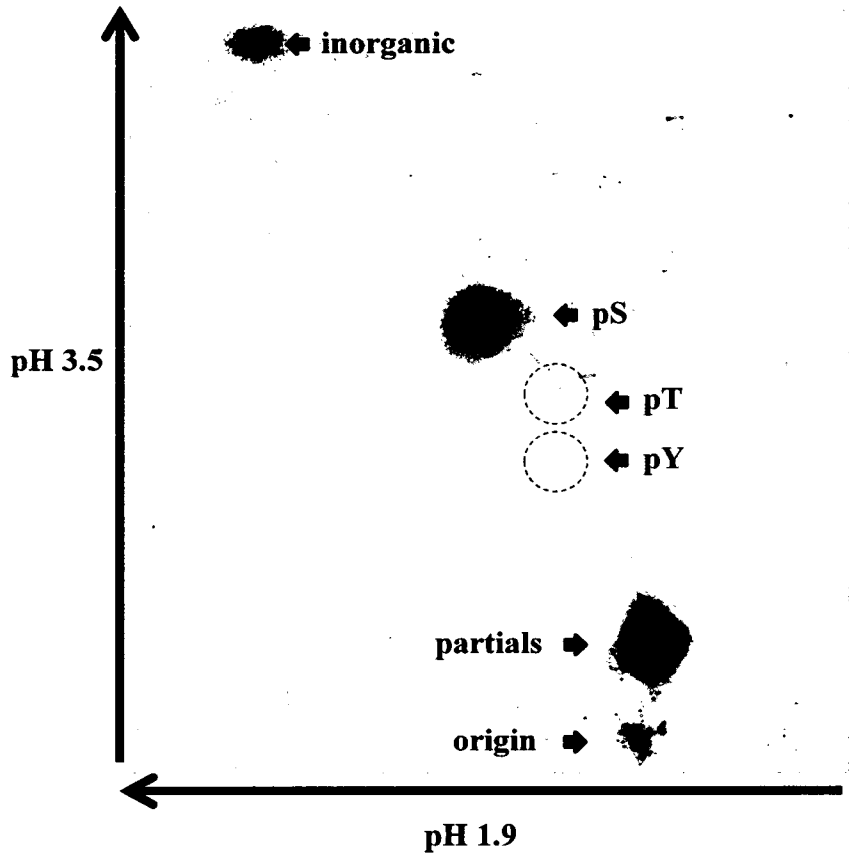

Figure 6.2. SLK Phosphorylates Paxillin on a Serine Residue: Paxillin was labelled with ^{32}P *in vitro* by SLK, purified, and hydrolyzed in hydrochloric acid. The resulting amino acids monomers were spotted on thin layer cellulose plates and resolved by electrophoresis. The amino acid that incorporated ^{32}P migrated with a phospho-serine standard.

Phosphoamino acid analysis was then conducted on paxillin that was *in vitro* phosphorylated by SLK. Following acid hydrolysis of paxillin, the resulting amino acids, along with phospho-amino acid standards were spotted on thin layer cellulose plates and resolved by 2D electrophoresis. The plate was then exposed to X-ray film and the amino acid that incorporated ^{32}P was found to migrate with the phospho-serine standard indicating that SLK phosphorylates paxillin on a serine residue. Notably, paxillin requires serine phosphorylation for efficient migration (Bellis et al., 1997) and is hyperphosphorylated on serine residues during mitosis (Yamaguchi et al., 1994; Yamaguchi et al., 1997).

6.3: Paxillin Binds MARK3.

MARK3 has been suggested to play a role in cytoskeletal dynamics due to its homology to other MARK family members and has recently been identified as a paxillin interacting protein during a mass spectrometry (Migration Gateway, 2009). During that study, several hundred other proteins were identified as paxillin interacting proteins raising questions regarding the stringency of the cut off limits for filtering *bona fide* interacting proteins and residual background. Also, it did not take into consideration if the interaction was direct or mediated by other binding partners. To validate this observation, paxillin was *in vitro* translated, labelled with ^{35}S-methionine, and its ability to bind recombinant MARK3 was tested as described above. Binding assays indicated that paxillin efficiently binds MARK3 *in vitro* (Figure 6.3). Similarly, paxillin also bound recombinant MARK3ΔUAD, $MARK3^{1-250}$, and $MARK3^{501-729}$ with efficiency similar to that of full length MARK3 indicating that MARK3 also has multiple points of contact with paxillin similar to SLK (Figures 5.6 and 5.7). Paxillin was also found to bind recombinant $MARK3^{251-500}$ with an affinity similar to that of GST suggesting that the observed binding of paxillin to $MARK3^{251-500}$ is background interaction.

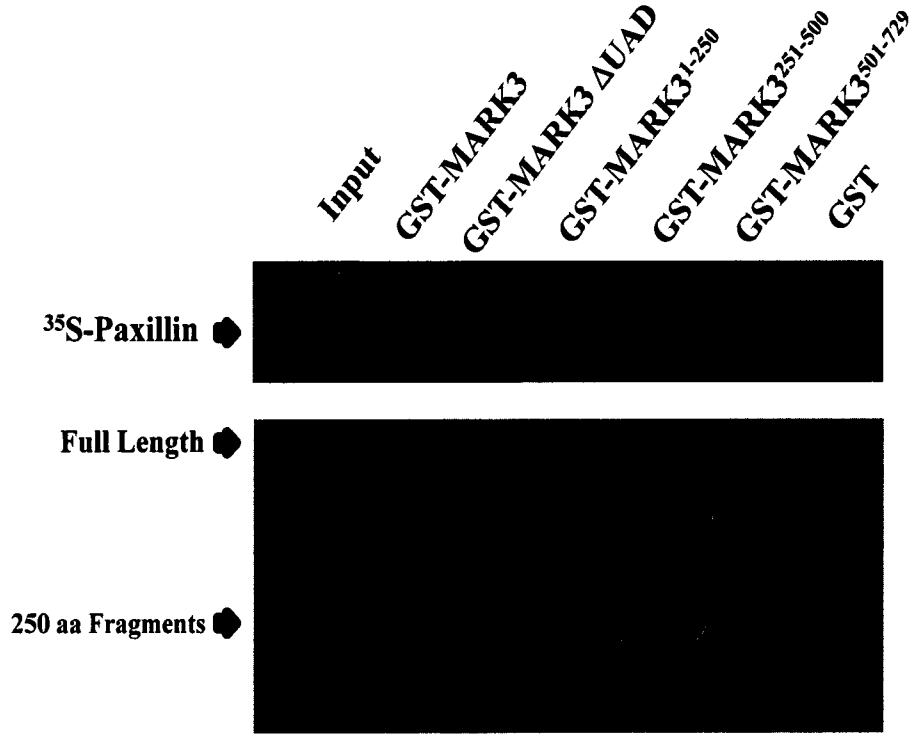

Figure 6.3. Paxillin Binds MARK3 *in vitro*: Paxillin was *in vitro* translated, labelled with ^{35}S-methionine, and tested for its ability to bind recombinant MARK3 or recombinant MARK3 fragments. MARK3-paxillin complexes were resolved by SDS-PAGE and the gel was stained with Coomassie blue (lower panel) and exposed to X-ray film (upper panel). Paxillin was found associated with full length MARK3, MARK3ΔUAD, MARK3$^{1-250}$, and MARK3$^{501-729}$.

To identify the regions in paxillin that bound MARK3$^{1-250}$ and MARK3$^{501-729}$, paxillin was divided into its LD1-5 domains and its LIM1-4 domains and these recombinant proteins were tested for their ability to bind *in vitro* translated MARK3$^{1-250}$ and MARK3$^{501-729}$. Consistent with the above observations, both MARK3$^{1-250}$ and MARK3$^{501-729}$ bound full length recombinant paxillin. Further deletion showed that MARK3$^{1-250}$ and MARK3$^{501-729}$ both bound recombinant LIM1-4 more efficiently than full length paxillin. MARK3$^{1-250}$ and MARK3$^{501-729}$ bound the recombinant LD1-5 construct with weak affinity, similar to that of GST (Figure 6.4) suggesting background binding. The interaction was further refined by dividing LIM1-4 into LIM1-2 and LIM3-4 and the ability of paxillin to bind these recombinant proteins was assessed. As displayed in Figure 6.4, both MARK3$^{1-250}$ and MARK3$^{501-729}$ bound LIM1-2 with efficiency similar to that of LIM1-4 indicating that MARK3$^{1-250}$ and MARK3$^{501-729}$ bind within LIM1-2 of paxillin.

6.4: SLK Activity Affects the MARK3-Paxillin Association.

We have demonstrated that SLK is capable of phosphorylating both MARK3 and paxillin *in vitro*. To address the physiological role of this observation the effects of KΔC expression on the MARK3-paxillin interaction was monitored by infecting exponentially growing fibroblasts with a KΔC or LacZ adenovirus expressing vector. The MARK3-paxillin interaction was assessed 24 hours post-infection by immunoprecipitating MARK3 and monitoring the binding of paxillin or SLK by Western blotting. Immunoprecipitation of MARK3 from LacZ control samples reproducibly displayed efficient co-immunoprecipitation of paxillin, suggesting that MARK3 and paxillin bind *in vivo*. MARK3 precipitates were also able to co-immunoprecipitate SLK and KΔC (Figure 6.5). Consistent with this observation, immunoprecipitation of MARK3

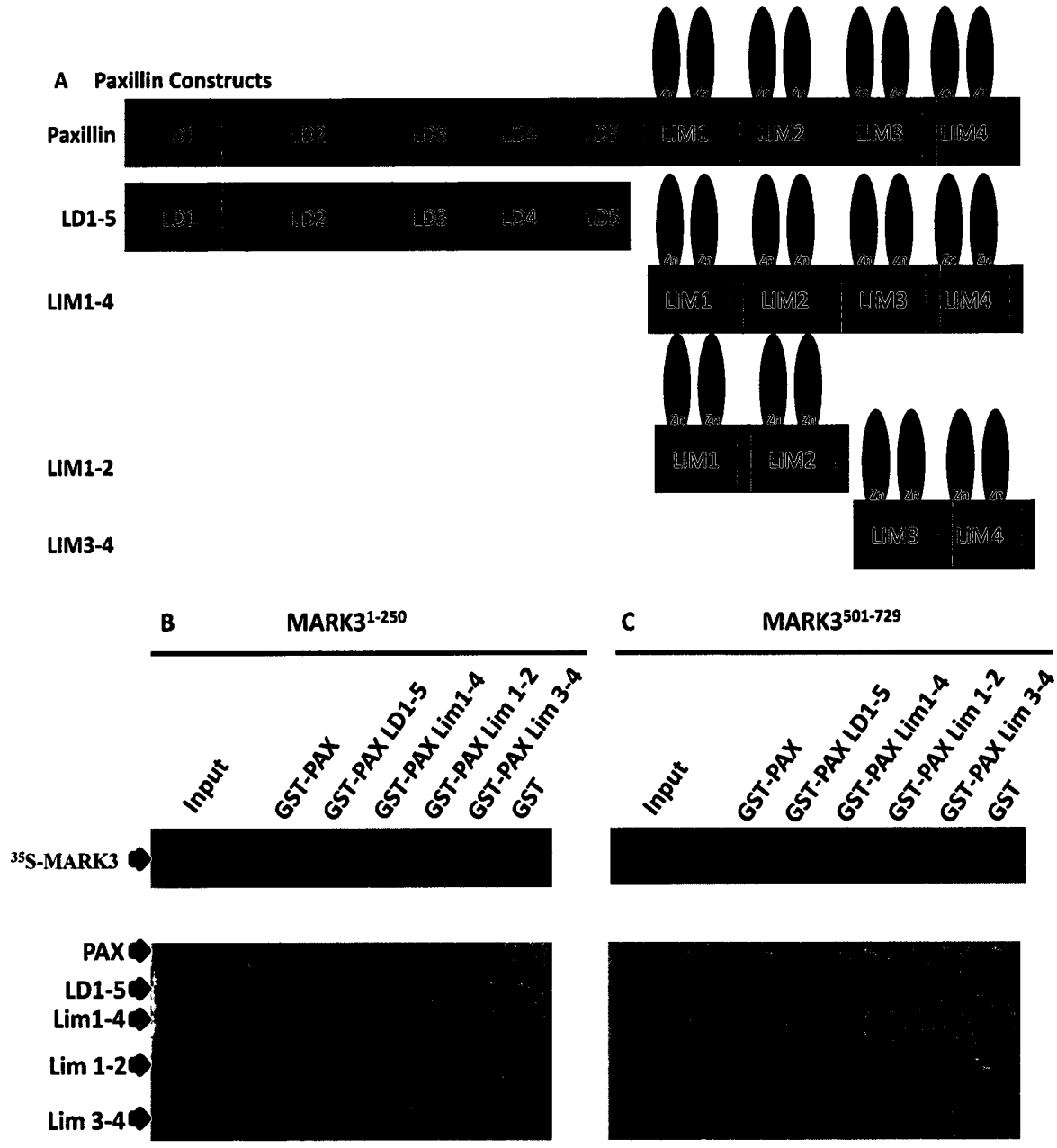

Figure 6.4. MARK3$^{1-250}$ and MARK3$^{501-729}$ Bind LIM1-2 of Paxillin: (A) Schematic representation of the paxillin constructs used during MARK3-paxillin binding assays. MARK3$^{1-250}$ (B) and MARK3$^{501-729}$ (C) constructs were *in vitro* translated, labelled with ^{35}S-methionine, and tested for their ability to bind recombinant paxillin or recombinant paxillin domains. MARK3-Paxillin complexes were resolved by SDS-PAGE and the gel was stained with Coomassie blue (lower panel) and exposed to X-ray film (upper panel). MARK3$^{1-250}$ and MARK3$^{501-729}$ efficiently bind LIM1-2 of paxillin.

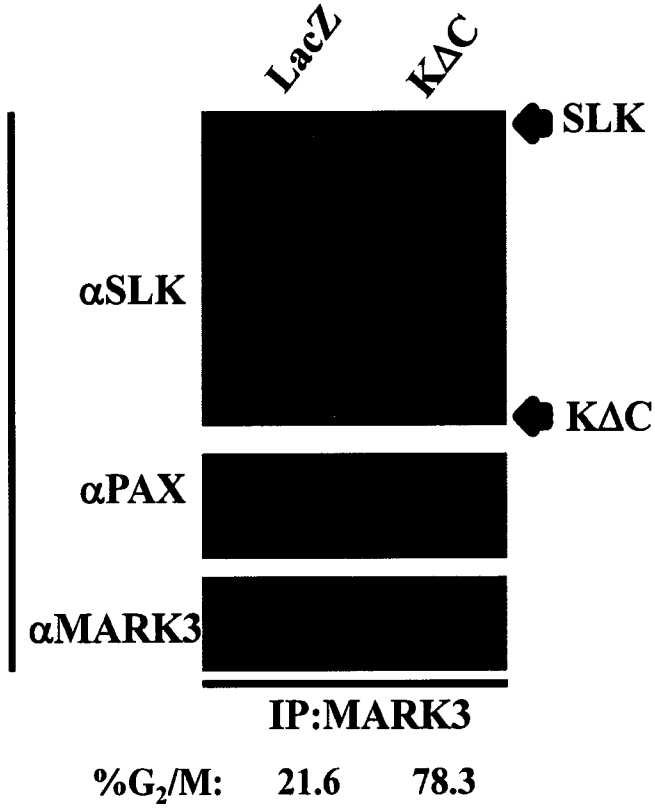

Figure 6.5. KΔC Expression Enhances the MARK3-Paxillin Interaction. MARK3 was immunoprecipitated from cultures expressing KΔC or LacZ 24 hours after being infected with their indicated adenoviral vectors. Normalization to MARK3 populations expressing KΔC displayed a stronger MARK3-paxillin interaction and also showed an increased G_2/M population when compared to LacZ control populations.

from KΔC infected populations co-immunoprecipitated paxillin but with greater efficiency than the LacZ control (Figure 6.5) suggesting that SLK activity may affect the MARK3-paxillin association.

6.5: MARK3 Binds SLK and Paxillin Differentially Throughout the Cell Cycle.

Our results have shown that KΔC expression results in delayed G_2 kinetics (Chapter 4). During the studies involving the monitoring of the MARK3-paxillin interaction in KΔC expressing populations, samples were collected in parallel and their cell cycle profile was assessed. MARK3 immunoprecipitated from KΔC expressing populations co-immunoprecipitates paxillin with greater efficiency than LacZ control populations. However, 78% of the KΔC population was is in the G_2/M compartment of the cell cycle compared to 22% of the LacZ population (Figure 6.5). This suggests that cell cycle phase could contribute to the enhanced MARK3-paxillin interaction observed in KΔC expressing populations rather than an absence of SLK activity.

To address if the SLK-MARK3-paxillin interaction is cell cycle phase specific, synchronized populations of C3H10T1/2 fibroblasts were collected at times when the majority of the population was in the G_2/M compartment of the cell cycle as described above. MARK3 was immunoprecipitated from these samples and complex formation with SLK and paxillin was monitored by Western blotting. The amount of MARK3 immunoprecipitated from each sample during the time course is similar at each time point. This suggests that changes in the affinity of any of MARK3's binding partners arise as a result of differential binding to MARK3 throughout the cell cycle and is not due to MARK3 immunoprecipitation efficiencies (Figure 6.6). At 20 hours post serum stimulation the population is entering the G_2/M compartment of the cell cycle

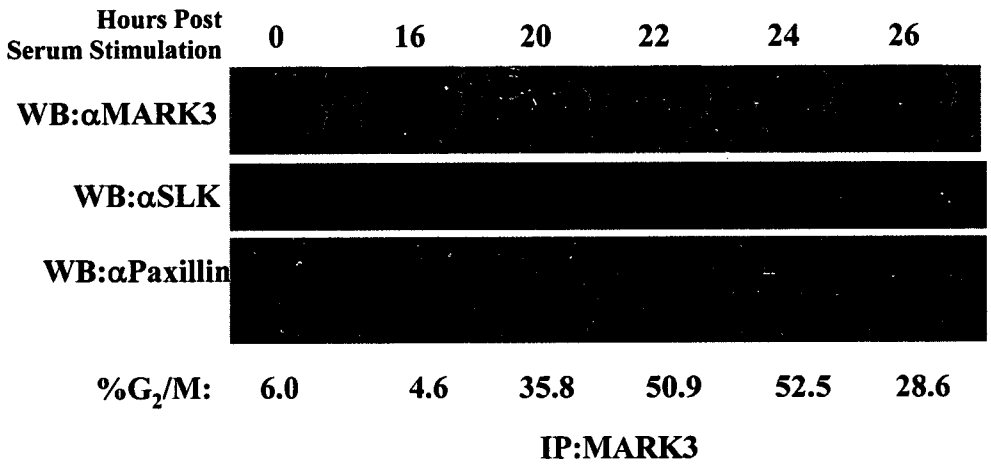

Figure 6.6. SLK, MARK3, and Paxillin Associate During G_2/M: Synchronized populations of C3H10T1/2 fibroblasts were collected at various times following serum stimulation and the ability of SLK and paxillin to bind immunoprecipitated MARK3 was assessed by Western blotting. SLK, MARK3, and paxillin were observed to interact with greater affinity in populations that are largely in the G_2/M compartment of the cell cycle suggesting that this is a G_2/M specific protein complex. One representative experiment is shown.

at which time paxillin can be efficiently co-immunoprecipitated with MARK3, which continues to increase and then stabilize by 22 hours post-serum stimulation. Similarly, SLK displays a similar co-immunoprecipitation pattern, which is maximal at 24 hours post-serum stimulation and begins to dissipate by 26 hours post-serum stimulation. Taken together, these findings indicate that SLK, MARK3, and paxillin can associate with a much greater affinity during G_2/M than other phases of the cell cycle. This suggests that the complex may have a cell cycle specific function.

6.6: Discussion and Interpretation.

SLK localizes with paxillin in membrane ruffles during migration at times when SLK activity is elevated (Wagner et al., 2002; Wagner et al., 2008) and paxillin is known to be phosphorylated on serine residues (Bellis et al., 1997; Turner, 2000; Yamaguchi et al., 1994; Yamaguchi et al., 1997). Based on these observations paxillin was tested for its ability to be phosphorylated by SLK. We observed that SLK phosphorylates paxillin on a serine residue in its LD1-5 domains *in vitro* (Figures 6.1 and 6.2). Interestingly, SLK phosphorylated full length paxillin with greater efficiency than its LD1-5 counterpart suggesting that conformational issues contribute to this phosphorylation event which is supported by the inability of SLK to phosphorylate LD1-2 or LD3-5 constructs (Foucault *et al.,* unpublished).

Following on a recent report showing that MARK3 interacts with paxillin (Cell Migration Gateway, 2009) we were able to establish a link between SLK, paxillin, and MARK3. We validated that MARK3 binds paxillin directly and showed that there are at least two points at which MARK3 interacts with paxillin since both $MARK3^{1-250}$ and $MARK3^{501-729}$ are capable of binding to LIM1-2 of paxillin (Figures 6.3 and 6.4). This binding pattern supports the head to

tail conformation of MARK3 proposed above (section 5.8). This would require that the amino and carboxyl terminals of MARK3 be in close proximity to be able to interact with LIM1-2 of paxillin.

The ability of SLK to phosphorylate paxillin and MARK3 and the observation that paxillin and MARK3 interact prompted us to investigate the effect of SLK activity on the MARK3-paxillin interaction. Our results show that KΔC expression resulted in an enhanced MARK3-paxillin interaction (Figure 6.5). This is the first observation showing that SLK and paxillin can form a complex *in vivo*. KΔC also leads to G_2/M accumulation in these populations suggesting that the increased MARK3-paxillin binding may be a G_2/M specific aggregation. However, studies using synchronized populations indicate that the SLK-MARK3-paxillin interaction is greatly enhanced when populations are in the G_2/M compartment of the cell cycle (Figure 6.6) indicating that the enhanced MARK3-paxillin interaction observed in cultures expressing KΔC is cell cycle phase specific rather than dependent on SLK activity. Previous studies have shown a requirement for SLK during migration (Roovers et al., 2009; Storbeck et al., 2009; Wagner et al., 2002; Wagner et al., 2008) and our group has hypothesized that SLK is recruited to sites of adhesion during migration where it phosphorylates paxillin. This results in focal adhesion disassembly and allows for efficient migration. In the absence of SLK, paxillin is not phosphorylated, focal adhesions do not break down, and migration is inhibited (Figure 1.6). Using this ideology and based on the differential binding of SLK, MARK3, and paxillin throughout the cell cycle we propose that during interphase, SLK and MARK3 do not interact and are not at sites of adhesion (Figures 6.6 and 6.7A). As cells enter the G_2/M compartment of the cell cycle, SLK and MARK3 receive signals that cause them to interact and MARK3 tethers

SLK to paxillin allowing SLK to phosphorylate paxillin, relaxing focal adhesions and allowing cells to become loosely adherent and capable of dividing (Figures 6.6 and 6.7B).

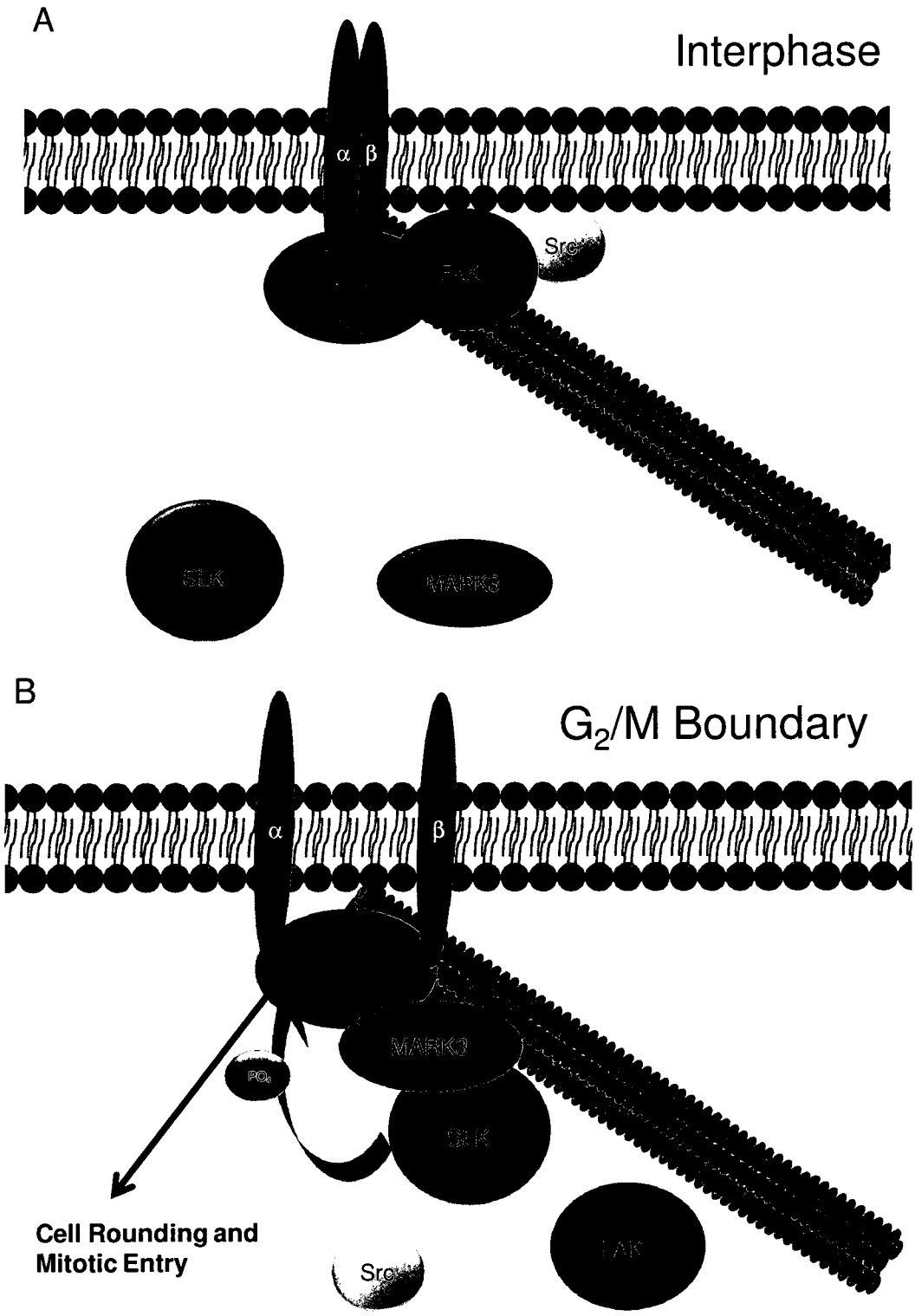

Figure 6.7. Working Model: **(A)-** During interphase focal adhesions are assembled and SLK and MARK3 do not associate with each other. **(B)-** at the G_2/M boundary MARK3 associates with SLK and tethers SLK to paxillin. SLK then phosphorylates paxillin which leads to focal adhesion disassembly, cell rounding, and mitotic entry.

Chapter 7

General Discussion

7.1: General Discussion.

Cell cycle progression is a tightly monitored process that is carried out by highly regulated intracellular signalling networks that evolved to ensure that cells do not proliferate uncontrollably. Here we show that SLK is required for G_2 progression and propose that it is required to relax adhesions of proliferating cells by phosphorylating paxillin allowing cells to round up and divide (Figure 6.7).

When this study was initiated there was very little literature available on SLK and what was available either detailed the cloning of SLK homologs in other species (Itoh et al., 1997; Pytowski et al., 1998; Sabourin and Rudnicki, 1999; Yamada et al., 2000), was conducted by our group (Sabourin and Rudnicki, 1999; Sabourin et al., 2000), or implicated SLK in an unknown G_2/M pathway (Ellinger-Ziegelbauer et al., 2000). Our group was well on its way to showing a role for SLK during migration when this study started. Although the majority of the literature that has been published regarding SLK function since this study commenced revolves around migration or cytoskeletal dynamics (Burakov et al., 2008; Chaar et al., 2006; Roovers et al., 2009; Storbeck et al., 2009; Wagner et al., 2002; Wagner et al., 2008), we chose to pursue a preliminary observation that indicated that SLK was required for proliferation. We are aware that migration and G_2/M progression are very divergent processes but we propose here that they may converge at the level of focal adhesion disassembly.

Another initial observation was that SLK localized to the mitotic spindle suggesting that SLK may be required for the mitotic spindle to function properly. Following biochemical characterization of the KΔC induced cell cycle block we were surprised to discover that KΔC resulted in a G_2 block rather than halt the cell cycle at mitosis. Although we were unable to

make a functional link between SLK and spindle function during this study, it would not be surprising to observe a mitotic block if cells were induced to express KΔC at the onset of M phase, following G_2.

YΔC expression has been shown to induce actin dissolution which is followed by apoptosis (Sabourin and Rudnicki, 1999; Sabourin et al., 2000; Wagner et al., 2002) demonstrating that studying SLK can be difficult. Indeed, increasing its activity results in death and down-regulation of its activity results in an absence of proliferation (O'Reilly et al., 2005). Notably, populations expressing KΔC for several days remain viable (Figure 3.4) indicating that KΔC is not toxic unlike its YΔC counterpart. We have shown that YΔC induces apoptosis by providing evidence that it is responsible for inducing a mitotic catastrophe (Figure 4.7) and the actin dissolution previously reported in cells expressing YΔC (Sabourin and Rudnicki, 1999; Sabourin et al., 2000) may not be a direct result of YΔC but rather a secondary consequence of mitotic entry (Sanger and Sanger, 1976; Schoroeder, 1976; Yamashiro et al., 1990).

Our laboratory has conducted several yeast two hybrid screens using SLK (or domains of SLK) as bait in order to identify interacting proteins, providing us with an overwhelming amount of putative SLK binding partners that require validation. To date, proteins that we have verified as SLK interacting proteins through yeast two hybrid screens include SLK itself, Lbd1/2 which have recently been shown to interact with SLK to regulate migration and SLK activity (Storbeck et al., 2009). MARK3 was the only protein we identified as an SLK interacting protein that has links to G_2.

Since MARK3 phosphorylates Cdc25C on its inhibitory site (Ogg et al., 1994; Peng et al., 1998; Peng et al., 1997) and since Cdc25C activation is required for mitotic entry (Dunphy

and Kumagai, 1991; Lee et al., 1992; Millar et al., 1991a) a cell cycle arrest similar to the one observed in populations expressing KΔC would be observed if MARK3 remained constitutively active. Since MARK3 phosphorylates Cdc25C on in its inhibitory site holding Cdc25C in an inactive state there must be a signal sent to MARK3 to inhibit it from phosphorylating and inhibiting the activity of Cdc25C in order for cdc2 activation.

Based on this reasoning and because SLK and MARK3 interact with each other, we tested if SLK was responsible for providing MARK3 with a cue that negates it from phosphorylating Cdc25C by expressing KΔC and YΔC. After numerous attempts using various strategies we were unable to show that SLK has an impact on MARK3s ability to phosphorylate Cdc25C. Alternatively, MARK3 may be degraded during G_2 which would negate its signalling to Cdc25C which is a reasonable hypothesis since the cell cycle is regulated by the expression and destruction of various proteins. We were unable to observe any regulation of MARK3 levels by SLK. Interestingly, MARK3ΔUAD expression levels were similar to that of wild type MARK3 suggesting that the consequence of MARK3 ubiquitination may facilitate its interaction with other proteins rather than target it for degradation.

Although we were unable to demonstrate a functional cell cycle link between MARK3 and Cdc25C, the inhibitory signal sent from MARK3 to Cdc25C may be negated due to re-localization in which during G_2/M, MARK3 localizes to paxillin (Figure 6.6) and is incapable of interacting with cytosolic Cdc25C. Alternatively, since we were unable to determine the effects of MARK3 phosphorylation by SLK, one possibility is that this phosphorylation event results in differential binding of MARK3 interacting proteins such as 14-3-3 which have been shown to bind MARK3. This binding is dependent on the phosphorylation of serine residues present in the $MARK3^{251-500}$ fragment that SLK phosphorylates (Goransson et al., 2006). Paxillin is also

phosphorylated on serine residues by several kinases (Deakin and Turner, 2008) and has also been shown to bind 14-3-3 proteins (Deakin et al., 2009) suggesting that its binding partners may bind differentially throughout the cell cycle (Yamaguchi et al., 1997).

Our results display that SLK, MARK3, and paxillin form a complex during G_2 and our binding studies suggest that MARK3 may act as a scaffold that is responsible for bridging SLK and paxillin. The SLK-MARK3 interaction appears to be intricate since MARK3 contacts SLK at a minimum of four regions. Evidence from our group indicates that SLK homo-dimerizes in a head to tail manner. (Figure 7.1) similar to the conformational structure displayed by other Ste-20 GCK family members (Ling et al., 2008). This would make the SLK-MARK3 complex even more perplexing when considering the likely possibility that this is a multimeric protein complex. Taking this into consideration and the fact that one MARK3 molecule has four regions of contact with SLK, there are four possible binding conformations in which one MARK3 molecule can bind an SLK dimer. For example, $MARK3^{1-250}$ may bind the amino and carboxyl portion of one SLK molecule (Figure 7.1A) or may bind the amino portion of one SLK molecule and the carboxyl portion of the other SLK molecule in the dimer (Figure 7.1B). Since the carboxyl region of MARK3 has a similar binding pattern there are four ways which one MARK3 molecule can bind an SLK dimer. Finally, our group has evidence to support that the head to tail conformation of the SLK dimer changes when SLK is activated in which the dimer goes into an open conformation similar to the mode of action of other Ste-20 GCK family members. This may lead to changes in the manner in which MARK3 binds SLK and one can only speculate on what those changes may be.

$MARK3^{1-250}$ and $MARK3^{501-729}$ both bind LIM1-2 of paxillin in addition to the amino and carboxyl domains of SLK. Together with the observation that these three proteins interact in a

complex during G_2/M (Figure 6.6), it suggests that MARK3$^{1-250}$ and MARK3$^{501-729}$ are important domains for creating the SLK-MARK3-paxillin complex. These domains are likely buried deep in the complex with little access to other proteins due to the steric hindrance imposed by SLK and paxillin. In this hypothetical model in which MARK3 is in a head to tail conformation, MARK3$^{1-250}$ and MARK3$^{501-729}$ are both bound to the amino and carboxyl terminals of SLK as well as LIM1-2 of paxillin which would leave the MARK3$^{251-500}$ domain relatively free for SLK phosphorylation (Figure 7.2). Until the identification of the phosphorylation sites within MARK3$^{251-500}$, the physiological consequences of this phosphorylation event remains elusive. However, it is likely that this event dictates MARK3 binding partners since SLK does not affect MARK3 kinase activity.

SLK phosphorylates paxillin *in vitro* somewhere in its LD1-5 domain and these domains are responsible for most of the signalling that goes through paxillin (Brown et al., 1996; Turner and Miller, 1994). In addition, KΔC expression results in reduced rates of migration (Storbeck et al., 2009; Wagner et al., 2002; Wagner et al., 2008) and motility is enhanced when paxillin is phosphorylated on serine residues (Bellis et al., 1997) by kinases that are yet to be identified (Deakin and Turner, 2008; Turner, 2000). The links that MARK3 has to G_2, in addition to Cdc25C, may be to recruit SLK to focal adhesions, inducing their turnover similar to its requirement during migration. Here we present additional evidence to support that SLK phosphorylation of paxillin may result in focal adhesion break down not only during migration but at times when cells must become less adherent in order to divide. When the sites in paxillin that are phosphorylated by SLK are validated we will be able to test that hypothesis.

We have recently identified S243, S244, and S250 of paxillin as candidate SLK phosphorylation sites (Foucault *et al.*, unpublished). Furthermore, we have obtained PAX$^{-/-}$

fibroblasts (Hagel et al., 2002) and initial studies indicate that they do not display delayed G_2 kinetics when expressing KΔC further supporting the hypothesis that paxillin is phosphorylated by SLK in G_2. Therefore, future experiments will consist of expressing paxillin mutants that can not be phosphorylated by SLK followed by an assessment of cell cycle kinetics. In addition, phospho mimics can be expressed to monitor for the presence of ectopic spindle formation. We expect that the expression of paxillin mutants that SLK can not phosphorylate will mirror the KΔC-induced delay in G_2 and this observation should support a role for SLK phosphorylation of paxillin in G_2 to induce focal adhesion disassembly. This would allow for the characteristic cell rounding that is observed during cell division.

In summary, we have shown that SLK activity is elevated during G_2/M and is required for efficient G_2 progression. Here we report two novel interactions that include the binding of MARK3 to both SLK and paxillin. The SLK-MARK3 interaction is intricate in which MARK3 makes at least four contacts with SLK and the MARK3-paxillin interaction could be mapped to the LIM1-2 region of paxillin. SLK, MARK3, and paxillin are capable of forming a complex *in vivo* and this complex is most evident during G_2/M. We find that SLK phosphorylates the LD domains of paxillin *in vitro* and propose that during G_2, SLK, MARK3, and paxillin form a complex in which MARK3 is responsible for recruiting SLK to paxillin. We propose that SLK then phosphorylates paxillin and the consequences of this phosphorylation event results in focal adhesion disassembly followed by the characteristic cell rounding that is observed during cell division.

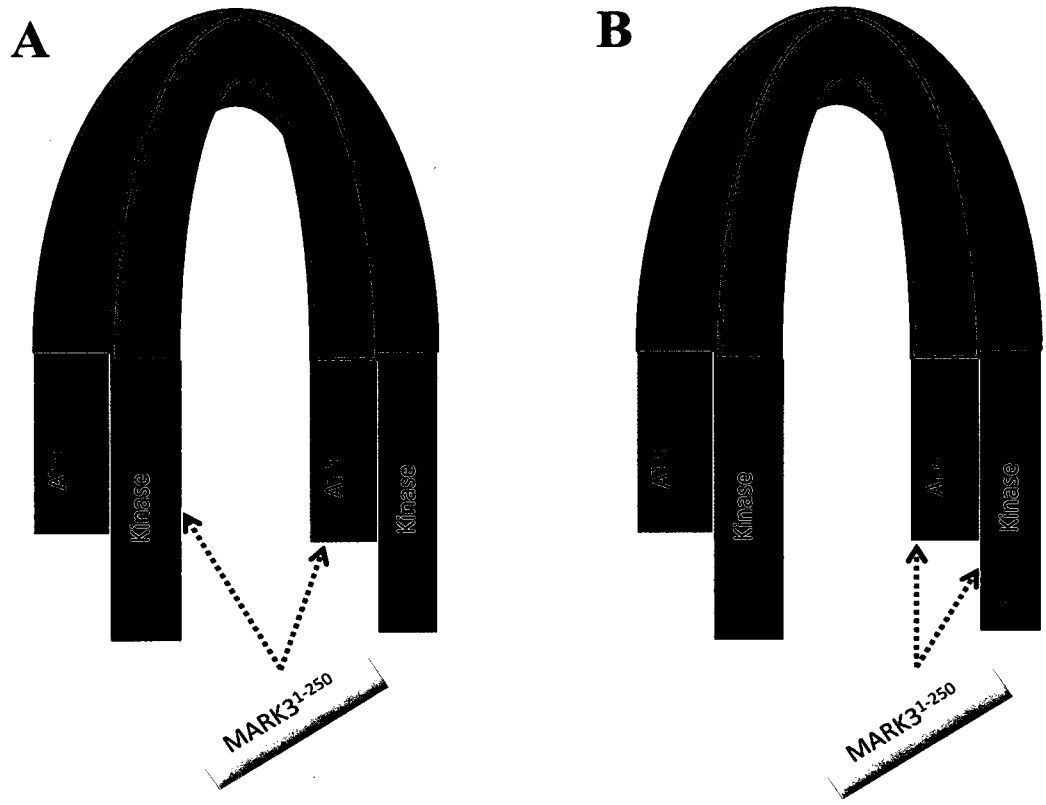

Figure 7.1. Possible MARK3 Interactions with SLK Dimers. MARK3$^{1-250}$ may bind the amino and carboxyl portion of one SLK molecule (A) or may bind the amino portion of one SLK molecule and the carboxyl portion of the other SLK molecule in the dimer (B).

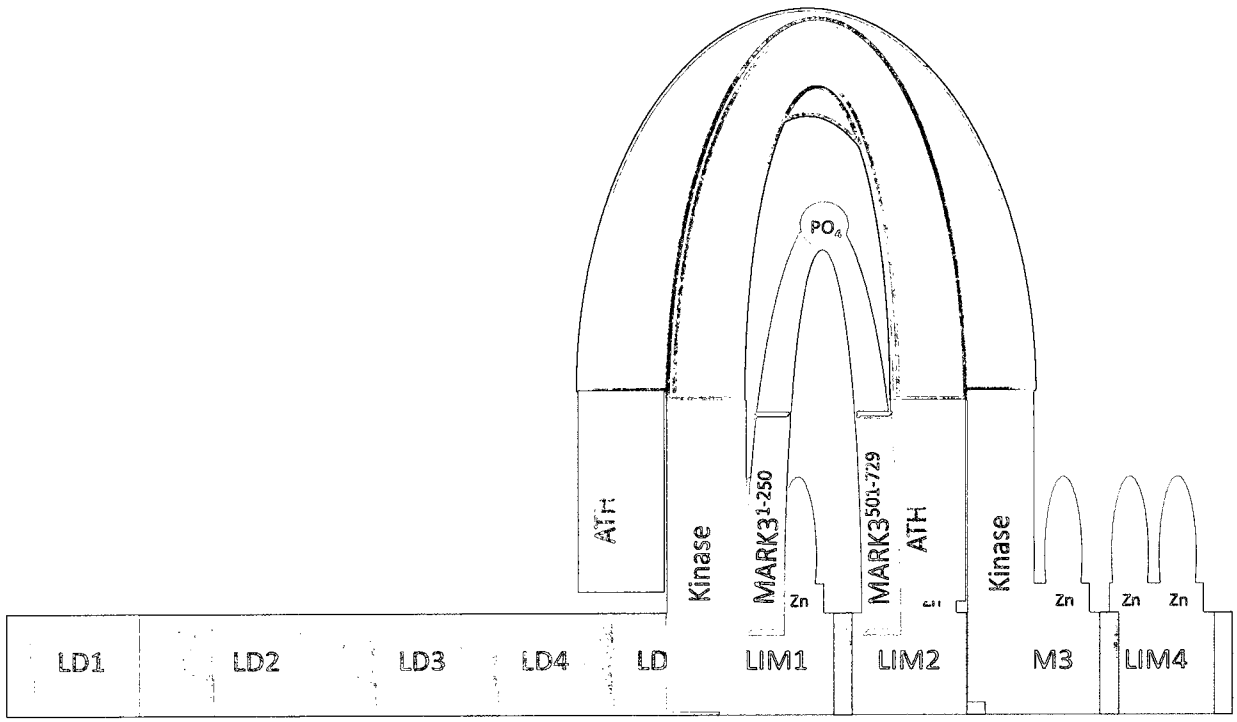

Figure 7.2. Proposed SLK-MARK3-Paxillin Complex. Our tests showed that MARK3$^{1\text{-}250}$ and MARK3$^{501\text{-}729}$ binds both the amino and carboxyl domains of SLK and both bind LIM1-2 of paxillin. Those observations were used to develop this model in which the MARK3$^{251\text{-}500}$ fragment that SLK phosphorylates is free of steric hindrance from the rest of the complex (note: the four points of interaction between MARK3 and SLK are not displayed here).

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