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Characterization of actin stress fibers: involvement of PDZ-LIM adapter proteins and the novel Clik1 kinase

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Academic Dissertation

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To Markku and Sara

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ABBREVIATIONS

aa	amino acid
ab	antibody
ABD	actin binding domain
ADF	actin depolymerizing factor
ALP	actinin-associated LIM protein
Arp2/3	actin related protein 2/3
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Bp	base pair
Ca^{2+}	calsium
CAK	CDK-activating kinase
CALI	chromophore-assisted laser inactivation
CDC2	cell division cycle 2
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CKI	CDK inhibitor
Clik1	CLP-36 interacting kinase 1
CLP-36	C-terminal LIM protein
CTD	carboxyl-terminal domain
C-terminus	carboxyl terminus
DHR	disc homology region
Dlg	discs large
DsRed	discosoma sp. red fluorescent protein
EGFP	enhanced green fluorescent protein
ENH	enigma homology protein
EST	expressed sequence tag
F-actin	filamentous actin
FAK	focal adhesion kinase
FRAP	fluorescence recovery after photobleaching
FSGS	focal and segmental glomerulosclerosis
G-actin	globular actin
GST	glutathione S-transferase
GTP	guanosine triphosphate
HMM	heavy meromyosin
INK4	inhibitors of CDK4
kb	kilobase
kDa	kilodalton
LIM	lin-11, isl-1, mec-3
LIMK	LIM-kinase
MAGUK	membrane-associated guanylate kinase
MAT1	Menage a trois
mDia	mammalian homologue of *Drosophila diaphanous*
MEKK1	MAPK/ERK kinase kinase 1
MLC	myosin light chain
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
NMDA	n-methyl-D-aspartate receptor

nNOS	neuronal nitric oxide synthase
N-terminus	amino terminus
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDZ	PSD-95, dlg, ZO-1
PIP2	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PKN	protein kinase N
pol II	RNA polymerase II
PSD-95	postsynaptic density 95
PTP-BL	protein tyrosine phosphatase
Ret	rearranged during transfection
Rho	Ras homology gene family
RIL	reversion-induced LIM protein
RNAi	RNA interference
SH3	Src homology region 3
STK35	serine/threonine kinase 35
TFIIH	transcription factor IIH
TGF-ß	tumor growth factor ß
Thr	threonine
TRIP-6	thyroid receptor interacting protein-6
Tyr	tyrosine
ZO-1	zonula occludens-1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I Vallenius, T., Luukko, K., and Mäkelä, T.P.: CLP-36 PDZ-LIM protein associates with nonmuscle α-actinin-1 and α-actinin-4. *J Biol Chem* 275, 11100-5, 2000.

II Vallenius, T. and Mäkelä, T.P.: Clik1: a novel kinase targeted to actin stress fibers by the CLP-36 PDZ-LIM protein. *J Cell Sci* 15,115, 2067-73, 2002.

III Vallenius, T., Scharm, B., Vesikansa, A., Luukko, K., Schäfer, R., and Mäkelä, T.P.: The PDZ-LIM protein RIL modulates actin stress fiber turnover and enhances the association of α-actinin with F-actin. *Exp Cell Res*, in press.

ABSTRACT

The actin cytoskeleton is best known for its role in muscle cells, where it forms a major part of the contractile machinery. In other cell types the actin cytoskeleton is involved in multiple processes including cell shape, motility, cell polarity, and cytokinesis. To perform all of these tasks, the nonmuscle actin cytoskeleton is composed of a number of specialized subtypes of actin networks that communicate with different signaling pathways. One of these is the actin stress fiber network that develops under cellular tension. Stress fibers in several ways resemble the contractile apparatus of muscle cells based both on their partially characterized molecular composition and their ability to contract. However, stress fibers are highly dynamic structures in terms of their rapid reorganization ability. Although actin stress fibers have an established role as the model system in which to study actin cytoskeleton dynamics in cells, the function of these fibers in nonmuscle cells is only partially characterized. This includes numerous unidentified proteins associating with the actin stress fibers and signaling events regulating their dynamics. This thesis focuses on characterization of three new actin stress fiber-associated proteins (Clik1 kinase; CLP-36 and RIL PDZ-LIM proteins), and their effects on actin stress fiber dynamics.

We identified a novel kinase termed Clik1 during our attempts to search for human homologues of yeast CDK-activating kinases. Soon after its identification Clik1 was found to associate with the previously identified but functionally uncharacterized CLP-36 PDZ-LIM protein. Clik1 association with CLP-36 led to relocalization of the otherwise nuclear Clik1 kinase to actin stress fibers. Interestingly, the localization of Clik1 to actin stress fibers resulted in partial disorganization of these structures suggesting that Clik1 may present a novel regulator of actin cytoskeleton. This prompted us to investigate the Clik1-CLP-36 interaction in more detail. During these studies we found that CLP-36 associated with Clik1 through its LIM domain, whereas the PDZ domain of CLP-36 bound to nonmuscle □-actinins. These findings led us to propose a model where CLP-36 PDZ-LIM protein functions as an adapter for Clik1 kinase targeting it to actin stress fiber.

To investigate the specificity of the noted interactions, we compared CLP-36 and the closely related RIL in their ability to interact with Clik1 and the cytoskeleton. While RIL did not associate with Clik1, it mimicked CLP-36 in several aspects including its localization to actin stress fibers, and its association with □-actinin in PDZ domain dependent fashion. However, live cell imaging studies demonstrated that RIL expression led to altered stress fiber dynamics, which was in stark contrast to stationary actin stress fibers detected in CLP-36 expressing cells.

The studies presented on three new actin stress fiber-associated proteins extend our understanding of the structure of these contractile elements of nonmuscle cells. Furthermore, the results demonstrate the dynamic nature of stress fibers, and how the associated proteins are critical in this regulation. Finally they point to active interplay between the actin cytoskeleton and other signaling processes in the cell - probably in part mediated by adapters such as CLP-36 and by signaling molecules such as the Clik1 kinase.

REVIEW OF THE LITERATURE

At the onset of this study, we identified Csk1 kinase as a CDK-activating kinase (CAK) in fission yeast (Hermand et al., 1998). Together with the prior identification of the related Cak1 kinase as a CDK-activating kinase in budding yeast (Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996), and rumors of the biochemical purification of a novel CDK-activating kinase in humans (later also published; Kaldis, 1999; Nagahara et al., 1999), this suggested that also metazoans might have a single subunit kinase related to the Csk1 and Cak1 kinases. Work described here was based on a hypothesis that such a kinase might be identified through a careful similarity search of mammalian databases, which at the time contained a vast amount of uncharacterized sequences due to high-throughput sequencing of expressed sequence tags (ESTs). The homology searches were planned to include identification of potential new CDK-like kinases as well. The present work describes characterization of a novel kinase Clik1 identified in this search. During the characterization of Clik1, we determined that this kinase does not appear to function as a CDK-activating kinase. However, a breakthrough in understanding its possible function came with the results indicating that Clik1 associates with the CLP-36 PDZ-LIM protein, and that these two proteins were likely to have role in actin stress fibers. Based on this sequence of events the following review includes a limited part on CDKs and cell cycle regulation followed by a more extensive review of actin stress fibers, α-actinin and PDZ-LIM proteins.

1. The cell division cycle and cyclin-dependent kinases

Cell division is essential for the propagation of unicellular organisms, and for the development and maintenance of tissue homeostasis in multicellular organisms. Cell division is controlled by a highly conserved molecular mechanism, namely the cell cycle. The key enzymes of the cell cycle are cyclin-dependent kinases (CDKs), a group of serine/threonine kinases, whose sequential activation initiate and coordinate cell cycle progression. Activated CDKs phosphorylate their target proteins resulting first in the duplication of genetic material and later segregation of chromosomes into identical daughter cells (reviewed in; Morgan, 1995).

To date, eleven different CDKs have been identified in human cells, of which five (CDK1, CDK2, CDK4, CDK6 and CDK7) have an established role in cell cycle regulation (reviewed in; Morgan, 1995). In addition, it has become evident that CDKs control other cellular processes including transcription (CDK7, CDK8, CDK9, CDK10) (Kasten and Giordano, 2001; Napolitano et al., 2002; Serizawa et al., 1995; Shiekhattar et al., 1995; Tassan et al., 1995b), mRNA splicing (CDK11) (Hu et al., 2003), and neuronal migration (CDK5) (Dhavan and Tsai, 2001).

The structure of CDK kinases is very simple, and consists of little more than a protein kinase domain with the twelve conserved subdomains found in almost all protein kinases

(Reviewed in; Hanks and Hunter, 1995). CDK family members have approximately 300 residues, and are 35-65% identical to the prototype CDK1 (also known as CDC2) (reviewed in; Morgan, 1997). Structural studies of CDK2 indicate that CDKs have an amino-terminal lobe rich in ß-sheets and a larger, mostly □-helical, carboxyl-terminal lobe (De Bondt et al., 1993). The cavity between the two lobes contains the active site, where substrate binding and catalysis occur (Fig. 1) (Jeffrey et al., 1995). Characteristic features for CDKs include a conserved cyclin binding sequence (the PSTAIRE-like region), two inhibitory phosphorylation sites in the middle of the glycine rich area located near the N-terminus of CDKs and a flexible T-loop, which contains an activating phosphorylation site (reviewed in; Morgan, 1996).

1.1 Cyclin-dependent kinases are highly regulated enzymes

The precise timing and coordination of cell cycle progression is ensured by tight regulation of CDKs. The primary regulators of CDK activity are the regulatory cyclin subunits, of which the first were identified as proteins that oscillate during the cell cycle (Evans et al., 1983). Subsequently a number of cyclins have been identified due to the presence of the "cyclin box" - a 100 amino acid domain responsible for CDK binding and activation (Kobayashi et al., 1992; Lees and Harlow, 1993). In mammalian cells, each CDK interacts with a specific subset of cyclins (CDK-cyclin pairs involved in cell cycle: CDK4-cyclin D, CDK6-cyclin D, CDK2-cyclin E, CDK2-cyclin A, CDK1-cyclin A, CDK1-cyclin B). Analysis of crystal structures of CDK2-cyclin A complexes indicate that cyclin binding induces conformational changes in the catalytic cleft of the CDK subunit. This change in conformation is required for correct ATP orientation and substrate binding (Fig. 1) (Jeffrey et al., 1995).

Two families of CDK inhibitors, the INK4 family ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$) and Cip/Kip family ($p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$), mediate negative regulation of CDKs. The INK4 family of inhibitors binds to CDK4 and CDK6, whereas Cip/Kip proteins inhibit cyclin E-and A-dependent kinases through their binding both to cyclin and CDK subunits (reviewed in; Sherr and Roberts, 1999). The Cip/Kip proteins can also act as positive regulators by stabilizing the CDK-cyclin interaction when associating with cyclin D-dependent kinases (LaBaer et al., 1997).

Phosphorylation provides another level of regulation of CDKs. CDKs contain two inhibitory phosphorylation sites within the ATP-binding pocket (Thr-14 and Tyr-15 in human CDK2), which are phosphorylated by Wee1 and Myt1 kinases, and dephosphorylated by the CDC25 family of phosphatases (reviewed in; Morgan, 1997). Additionally, CDKs contain an activating phosphorylation site within the T-loop also conserved in a wide variety of other Ser/Thr kinases (reviewed in; Hanks and Hunter, 1995). Phosphorylation of this conserved residue (Thr 160 in human CDK2) is mediated by the CDK-activating kinase (CAK; see below), and is required for full activity of CDK-cyclin complexes (reviewed in; Morgan, 1997). This phosphorylation leads to further

conformational changes (in addition to cyclin binding), making the catalytic cleft fully accessible to ATP and substrate binding (Fig. 1) (Russo et al., 1996).

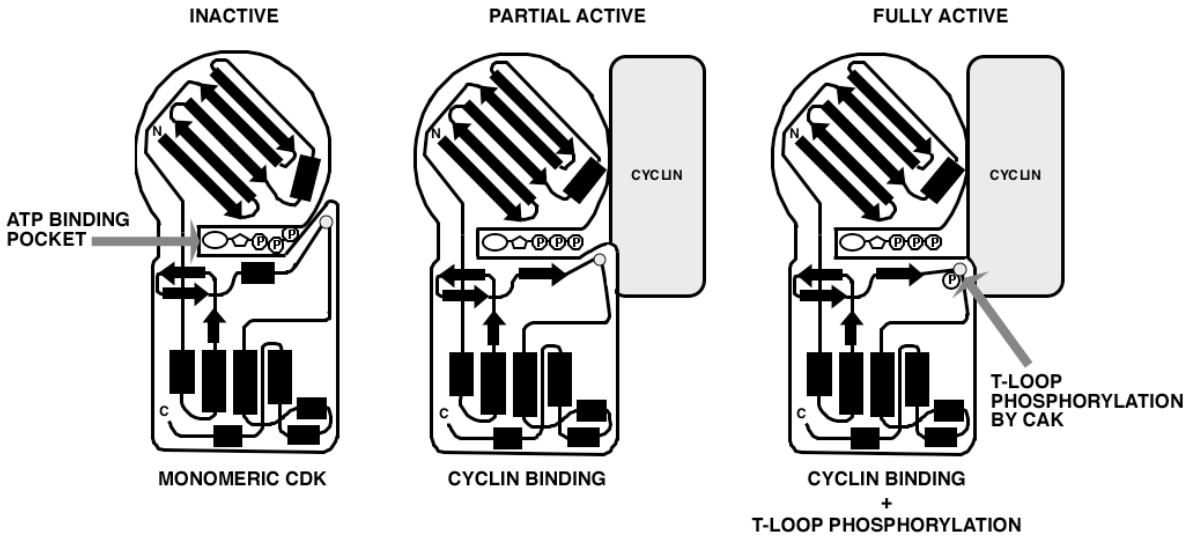

Figure 1. Cyclin-dependent kinase (CDK) activation.
A monomeric CDK subunit is catalytically inactive. Binding of the regulatory cyclin subunit and T-loop phosphorylation by the CDK-activating kinase (CAK) result in conformational changes that allow the phospho-transfer reaction from ATP to the substrate to happen. The schematic is modified from (Morgan, 1996), and based on structural studies of CDK2 and cyclin A (Russo et al., 1996).

2. The CDK-activating kinases (CAKs)

2.1 The CDK7 complex - regulating both cell cycle and transcription

CDK-activating kinase (CAK) was initially identified as an activity in Xenopus oocyte extract capable of phosphorylating the conserved threonine residue in the T-loop of CDK1 (Solomon et al., 1992). Subsequent purification and cloning of the subunits of CAK from different species identified it as a heterotrimeric complex composed of a catalytic subunit, $p40^{MO15}$, later renamed CDK7 (Fesquet et al., 1993; Poon et al., 1993; Shuttleworth et al., 1990; Solomon et al., 1993), a regulatory subunit, cyclin H (Fisher and Morgan, 1994; Mäkelä et al., 1994), and a third subunit MAT1 (Devault et al., 1995; Fisher et al., 1995; Tassan et al., 1995a). *In vitro* the mammalian CDK7-cyclin H-MAT1complex mediates the activating phosphorylation of CDK1, CDK2, CDK3, CDK4 and CDK6, thus suggesting that it is a potential candidate as a mammalian CAK (Kaldis, 1999). In support of this, CDK7 kinase activity has been shown to be required for the proper activation of the mitotic CDK1 *in vivo* in *Drosophila melanogaster* (Larochelle et al., 1998). Consistent with this the severe loss of CDK7 activity blocked cell division in *Caenorhabditis elegans* embryos (Wallenfang and Seydoux, 2002). Other *in vivo* support for CDK7-cyclin H-MAT1

complex function in cell cycle regulation comes from studies indicating that *Mat1* deficiency in the mitotic germ cell lineages resulted in rapid cell death, whereas the post-mitotic Schwann cells were viable upon ablation of *Mat1* (Korsisaari et al., 2002).

Shortly after the identification of CAK activity in the CDK7 complex, an entirely new function for this complex was identified through studies indicating that the CDK7-cyclin H-MAT1 trimer, along with its budding yeast homologue, Kin28-Ccl1-Tfb3/Rig2 is part of the transcription factor IIH (TFIIH)(Feaver et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). In TFIIH, CDK7 (and Kin28) phosphorylates the C-terminal domain of the large subunit of RNA polymerase II (CTD), which is thought to be a critical event for efficient transcription to occur. In budding yeast it is very clear the TFIIH kinase Kin28 has a critical role in regulating transcription of the majority of all RNA Pol II genes (Holstege et al., 1998). In mammalian cells it appears that CDK7 may be regulating transcription in a more focused way based on mouse knockout phenotypes (Korsisaari et al., 2002). In support of this notion, CDK7 within TFIIH has been demonstrated to regulate transcription through phosphorylation of specific transcription factors such as the retinoic acid receptor (Bastien et al., 2000; Keriel et al., 2002; Rochette-Egly et al., 1997).

2.2 New CAKs identified from *S. cerevisiae* and *S. pombe*

A peculiar difference has been identified in CDK-activating kinases between mammalian cells and budding yeast cells: although the budding yeast Kin28-Ccl1-Tfb3/Rig2 complex is the structural homologue of CDK7-cyclin H-MAT1 and forms part of TFIIH, it does not function as a CAK in budding yeast (Cismowski et al., 1995). Instead, budding yeast has a structurally unrelated, monomeric kinase termed Cak1 (or Civ1) that is the CAK for the CDK1 homologue Cdc28 as well as for other yeast CDKs - including Kin28 itself (Fig. 2) (Espinoza et al., 1996; Espinoza et al., 1998; Kaldis et al., 1996; Kimmelman et al., 1999; Thuret et al., 1996).

Like budding yeast, the fission yeast *Schizosaccharomyces pombe* also contains homologues for all three subunits of the mammalian kinase (Fig. 2). The kinase subunit is called Mcs6 (Damagnez et al., 1995), the cyclin subunit Mcs2 (Molz and Beach, 1993), and the MAT1 homolog Pmh1 (Hermand et al., 2003). However, unlike the budding yeast complex, the fission yeast Mcs6-Mcs2-Pmh1 complex is both a CAK and a CTD kinase, and activates the CDK1 homologue Cdc2 and phosphorylates the C-terminal domain of the large subunit of RNA polymerase II (Hermand et al., 2001). Thus fission yeast *S. pombe* is similar in this respect to the mammalian and fly mechanism, and budding yeast *S. cerevisiae* is - somewhat surprisingly - the exception (Fig. 2).

Interestingly, fission yeast is the only species in which two different CAKs have been found: in addition to the CDK7 homolog (Mcs6) activating the critical cell cycle CDK (Cdc2), fission yeast has a unrelated monomeric kinase called Csk1, which was identified at the onset of this study as kinase activating the Mcs6 CDK (Fig. 2) (Hermand et al.,

1998). The sequence comparison of fission yeast Csk1 and Cak1 revealed that these two kinases are somewhat related to each. This together with the rumors of the biochemical purification of an additional human CAK (later also published; Kaldis, 1999; Nagahara et al., 1999) prompted us to search for human homologue for Csk1 or Cak1 through a careful similarity search of mammalian databases.

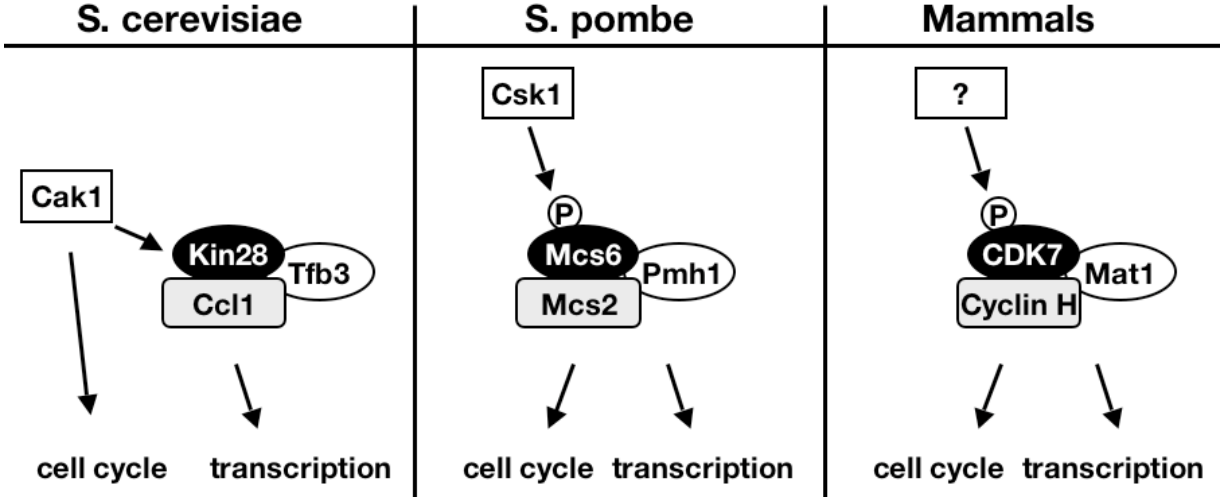

Figure 2. CDK-activating kinases (CAKs) in yeasts and our working hypothesis of mammalian CAKs, and their involvements in cell cycle and transcription.
On the left a single subunit CAK (Cak1) and the trimeric Kin28-Ccl1-Tfb3 complex in *S. cerevisiae*. In the middle a single subunit CAK (Csk1) and a trimeric CAK complex (Mcs6-Mcs2-Pmh1) in *S. pombe*. On the right a trimeric CAK (CDK7-Cyclin H-Mat1) and a question mark suggesting the existence of a single subunit CAK in mammals.

3. Actin stress fibers in nonmuscle cells

The cytoplasm of the cell is filled with protein polymers that maintain shape and transmit mechanical forces. Three types of cytoplasmic protein polymers have been identified, namely microtubules, intermediate filaments, and actin filaments, which together form the cytoskeleton of a cell. Actin filaments, also called microfilaments, are flexible filaments, whose major function in muscle cells is to act as part of the contractile machinery. In nonmuscle cells these filaments are involved in multiple dynamic processes including cell shape changes, cell spreading, motility, cytokinesis, polarity, and vesicle trafficking (reviewed in; Pollard et al., 2000).

Actin filaments (F-actin) are formed through polymerization of actin, a globular 42 kDa protein (G-actin or globular actin) (reviewed in; Oosawa, 2001). A total of six actin genes have been identified in *Homo sapiens,* four of which are expressed in muscle cells (□-skeletal actin, □□cardiac actin, □-vascular actin and □-enteric actin) whereas ß-actin and □-actin isoforms present nonmuscle or cytoplasmic actins (Herman, 1993; Mounier et al.,

1997). Although the different actin proteins vary by only a few amino acid residues, functionally they appear to have great diversity (Mounier et al., 1997).

In nonmuscle cells F-actin is arranged into a number of specialized actin networks depending on accessory proteins participating in network formation: i) beneath the plasma membrane actin filaments form a "cell cortex", which controls cell surface movements ii) in filopodia actin filaments form tight parallel bundles that maintain structure and expand surface area for e.g. for nutrient transport and sensory processes iii) migrating cells contain a "leading edge" or lamellipodia, where actin filaments are organized into a criss-crossing network, apparently facilitating forward movement of the leading edge iv) during the final moments of cell division two daughter cells are pinched apart through the contraction of a contractile ring, which is a specialized antiparallel actin filament structure, and v) in several nonmuscle cells actin filaments are organized into prominent and densely packed bundles, called actin stress fibers, which will be discussed in more detail in the following paragraphs (reviewed in; Welch and Mullins, 2002). The different actin networks are visualized in Figure 3.

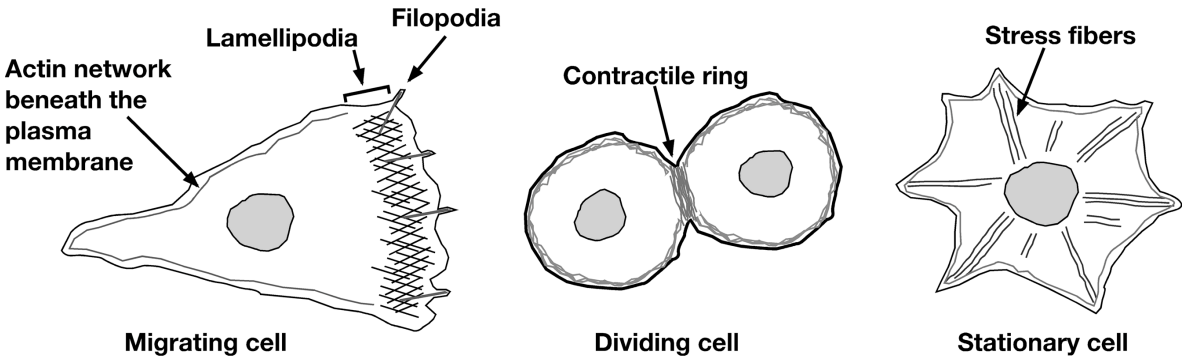

Figure 3. A schematic figure of different actin networks in nonmuscle cells.

3.1 Actin stress fibers are sarcomeric-like structures

Actin stress fibers were first described in 1924 as thin cytoplasmic striae with a dynamic nature, a study performed using live cell microscopy (Lewis and Lewis, 1924). The name "stress fiber" was introduced in 1967 by Buckley and Porter (Buckley and Porter, 1967), as these fibers were believed to be formed within lines of physical stress produced in the cytoplasm as the cells spread out and moved over a glass substratum. Interest in stress fibers was aroused in part because their organization resembled the structure of striated muscle myofibrils, first demonstrated by their ability to bind heavy meromyosin (HMM) (Ishikawa et al., 1969), a specific proteolytic fragment of myosin. This notion was further supported by immunofluorescence studies indicating the localization of actin (Lazarides and Weber, 1974), myosin (Weber and Groeschel-Stewart, 1974), tropomyosin (Lazarides, 1975), and □-actinin along the stress fibers (Lazarides and Burridge, 1975). Subsequent

studies have revealed that stress fibers are not only morphologically similar to myofibrils, but that they in fact - like myofibrils - are contractile elements. Initially this was suggested by the ability of stress fibers to shorten, when permeabilized cells were exposed to ATP (Isenberg et al., 1976; Kreis and Birchmeier, 1980), but this phenomenon could not be reproduced in living cells for technical reasons. Support for the contractility of stress fibers in living cells came from studies demonstrating that serum stimulation of starved fibroblasts resulted in stress fiber shortening and a more compact pattern of microinjected myosin (Giuliano and Taylor, 1990). Further evidence indicative of the contractility of stress fibers comes from studies where "wrinkling" of cells grown on flexible silicone rubber surfaces was only detected in cells having visible stress fibers (reviewed in; Burridge and Chrzanowska-Wodnicka, 1996).

Thus stress fibers are sarcomeric-like structures based on both their protein composition and ability to contract. Yet to date it is not entirely clear what the function of these fibers is. Clearly the formation of stress fibers is related to isometric tension, and for example the detachment of cells from their substratum induces a rapid vanishing of stress fibers. In line with this, actin stress fibers are absent from rapidly migrating cells (reviewed in; Burridge, 1981).

3.2 Actin stress fibers in tissue culture and *in vivo*

While actin stress fibers are a prominent and much-studied feature of many cultured cells stress fibers have been more difficult to demonstrate *in vivo*. This has prompted a hypothesis that these fibers might in fact represent an adaptation of cells to the two-dimensional tissue culture conditions and interaction with an artificial substratum (reviewed in; Burridge, 1981). Subsequently this notion has been put to rest.

One of the first attempts to address this question in mammalian tissues *in vivo* was a study where the authors analyzed numerous tissues by histochemical staining of filamentous actin using phallacidin, an acidic derivative of the widely used phalloidin (Wong et al., 1983). The only cells that exhibited clearly detectable stress fibers were a subset of endothelial cells under a high-velocity flow (cells lining the left ventricle, aortic valve and aorta), whereas similar cells lining the venous system lacked actin stress fibers. This suggested that stress fiber formation is linked to tension also *in vivo*. In agreement with these results stress fibers have been shown to develop during pathological conditions including atherosclerosis and hypertension (reviewed in; van Nieuw Amerongen and van Hinsbergh, 2001). It is proposed that the induction of actin bundles in these pathological conditions assist cells to maintain shape and to avoid detachment from underlying tissues.

A third situation in which actin stress fibers have been detected *in vivo* is platelet activation (Fig. 4). Under normal conditions platelets circulate in our blood stream as small, disk-shaped cells (left panel), but in the case of endothelial injury platelets contact with the subendothelial collagen and they become activated (middle panel). This activation leads to

the formation of actin bundles and reshaping of the platelet (right panel) (Tanaka and Itoh, 1998).

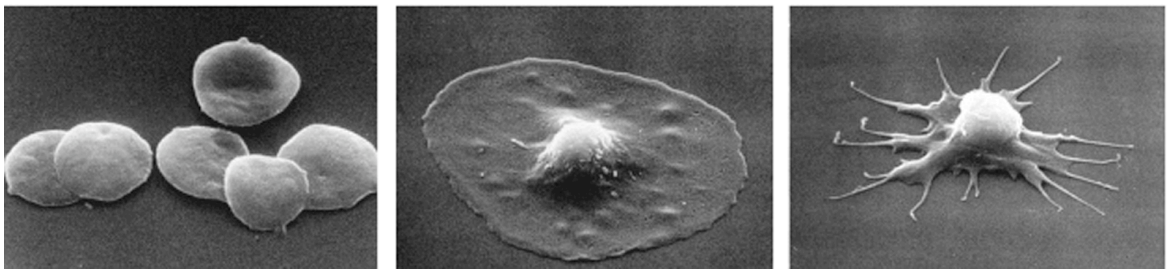

Figure 4. Platelet activation.
Left panel; disk-shaped platelets, middle panel; a spreading platelet, right panel; an activated platelet at a later stage. (Courtesy of Dr. James G. White).

3.3 The structure of actin stress fibers

Under the electron microscope actin stress fibers are detected as straight bundles, which vary in size and have broadened termini pointed to cell membrane (Porter and Buckley 1967). Actin filaments in stress fibers are ordered in opposite polarities, and are bundled together through actin filament cross-linking proteins. Stress fibers are linked to the plasma membrane via their association with focal adhesions (reviewed in; Small et al., 1998). Darker areas along the bundles are called "dense regions", and are likely to be analogues to dense bodies in smooth muscle cells and to the Z-discs in striated muscle cells (Spooner et al., 1971). Immunofluorescence studies have revealed that actin localizes as continuous lines along the fibers (Lazarides and Weber, 1974), whereas myosin and tropomyosin demonstrate an overlapping periodic staining pattern (Lazarides, 1975; Weber and Groeschel-Stewart, 1974). □-Actinin is also distributed in a similar periodic pattern but it does not overlap with tropomyosin and myosin, and instead colocalizes with the dense regions (Lazarides and Burridge, 1975).

A large and expanding list of proteins has been reported to localize to stress fibers (de Lanerolle et al., 1981; Geiger, 1979; Lazarides, 1975; Owada et al., 1984; Wang and Singer, 1977 and references therein), and several of these are also found in sarcomeres. However, the precise architecture of stress fibers with associated proteins is very unclear, and is in contrast to the intensively studied sarcomere architecture (reviewed e.g. in Clark et al., 2002). One of the few studies addressing the components and their stoichiometry in stress fibers suggested the presence of at least twenty proteins, of which several remain to be characterized (Katoh et al., 1998).

3.4 Dynamics of actin stress fibers

Stress fibers, as well as other specialized actin filaments in nonmuscle cells, are dynamic structures. Actin filaments are polar having a fast-growing barbed end (+ end) and a slow-

growing pointed end (- end). Actin filament elongation or polymerization occurs mostly at the barbed end, and shortening or depolymerization at the pointed end, and is tightly controlled by regulatory proteins (reviewed in; Welch and Mullins, 2002). Several proteins regulating polymerization-depolymerization processes have been identified, and at least partially classified according to their function such as monomer binding proteins profilin and thymosin (reviewed in; Pollard et al., 2000), and more recently identified monomer binding proteins twinfilin (Vartiainen et al., 2000) and MIM (Mattila et al., 2003), capping proteins such as gelsolin and CapZ, and severing proteins like ADF/cofilin (reviewed in; Pollard et al., 2000).

A key event for polymerization to occur is the existence of free barbed ends, which act as templates for incorporation of new actin monomers (reviewed in; Welch and Mullins, 2002). Free barbed ends can be created by different mechanisms; uncapping pre-existing filaments, severing filaments or de novo filament nucleation. One of the recent breakthroughs in understanding how new actin filaments form from actin monomers is the identification of the Arp2/3 complex, which is able to nucleate actin filaments especially in the leading edge of migrating cells (Reviewed in; Higgs and Pollard, 2001; Pollard et al., 2000; Welch and Mullins, 2002). However, genetic experiments in budding yeast *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* indicate, that the Arp2/3 complex is not the only nucleator of actin filaments (reviewed in; Evangelista et al., 2003). For example, in budding yeast the deletion of the Arp2/3 complex disrupts the assembly of cortical actin patches but actin cables remain unaffected (Winter et al., 1999), suggesting that another mechanisms for actin nucleation exists. Indeed, recent studies have implicated that formins are required for the formation of actin cables in budding yeast (Pruyne et al., 2002; Sagot et al., 2002). This together with other studies have led to a proposal that formins might have role in formation of actin cables such as actin stress fibers also in higher eukaryotes. This remains to be elucidated, and to date the knowledge of how stress fibers form in mammalian cells is very limited, and it is even uncertain whether this process involves the nucleation of new filaments, the reorganization of existing filaments or a combination of these (Reviewed in; Welch and Mullins, 2002).

In addition to the dynamic polymerization – depolymerization processes of actin filaments, there are numerous actin filament associated proteins, which are likely to participate in the regulation of stress fibers. As an example of such regulators is the actin filament cross-linking protein α-actinin, which dynamically moves in and out of actin stress fibers demonstrated elegantly by microinjection studies and photo-bleaching experiments (Edlund et al., 2001; Feramisco, 1979). However, it is not clear how α-actinin dynamics is regulated or more generally what is the recruitment order of α-actinin or other proteins that associate with actin stress fibers.

3.5 Rho and MLCK signaling pathways communicating with actin stress fibers

To summarize briefly the pathways communicating with actin stress fibers is extremely difficult because of the complexity of signaling molecules and cytoskeletal components

that are involved (e.g. part of those presented in Fig. 3 in Beck et al., 2001). Thus I concentrate on two well-characterized pathways Rho - Rho-kinase and MLCK.

During the past ten years it has become evident that the small GTP-binding protein Rho (more precisely in the majority cases RhoA) is a key regulator of actin stress fibers. A breakthrough was the discovery that microinjection of an active form of Rho GTPase induced formation of actin stress fibers and focal adhesions in fibroblasts (Ridley and Hall, 1992). Subsequent studies have revealed that the active GTP- bound form of Rho controls actin stress fibers by interacting with a set of effector proteins including Rho-kinases, PI 5-kinase, PKN and mDia proteins (reviewed in; Burridge and Chrzanowska-Wodnicka, 1996; Ridley, 2001; Watanabe et al., 1999). One of the well-characterized effectors of Rho is Rho-kinase (ROCK) (Matsui et al., 1996), which mediates the phosphorylation of several target proteins such as LIM-kinase (LIMK) (Maekawa et al., 1999; Ohashi et al., 2000), myosin regulatory light chain (MLC) (Amano et al., 1996; Kureishi et al., 1997) and MLC phosphatase (Kimura et al., 1996). The activated LIMK in turn phosphorylates the actin filament depolymerizing/severing factor cofilin, which leads to cofilin inactivation, and the accumulation of actin stress fibers (Maekawa et al., 1999). The phosphorylation of MLC by Rho-kinase results in increased contraction and assembly of stress fibers. Another way for Rho-kinase to promote actomyosin contraction is to phosphorylate MLC phosphatase. MLC phosphatase is composed of three subunits, and the phosphorylation of the myosin binding subunit inactivates its ability to dephosphorylate MLC (reviewed in; Burridge and Chrzanowska-Wodnicka, 1996). The activation of mDia by Rho promotes actin stress fibers assembly as well, but in this case the mechanisms is not fully characterized, but apparently it is a result of cooperative action of Rho-kinase and mDia (Watanabe et al., 1999).

In addition to Rho-kinase, the myosin light chain kinase (MLCK) regulates actin stress fibers. MLCK can also phosphorylate MLC, which leads to increased contractility of stress fibers. MLCK itself is regulated by $Ca2^{+}$/calmodulin binding in the way that elevated intracellular calsium results in calmodulin binding to MLCK leading to conformational changes and activation of MLCK (reviewed in; Burridge and Chrzanowska-Wodnicka, 1996). Even if both Rho-kinase and MLCK phosphorylate MLC, it is of interest to note that Rho-kinase and MLCK could differentially regulate distinct stress fiber networks termed "central stress fibers" and "peripheral stress fibers" (Totsukawa et al., 2000).

4. □-actinin in nonmuscle cells

4.1 The structure of □-actinin

□-actinin was first purified from skeletal muscle as a component that increased the superprecipitation of actin and myosin (Ebashi and Ebashi, 1964). In electron microscopy studies □-actinin appears as a long, narrow, rod-like molecule that is composed of two antiparallel 97-102 kDa □-actinin monomers (Podlubnaya et al 1975). With the completion □-actinin sequences in chicken (Baron et al., 1987) and Dictyostelium discoidecum (Noegel

et al., 1987), domain structure began to emerge indicating that a globular N-terminus of α-actinin contains the binding site for actin, and is accordingly called the actin-binding domain. This is followed by a rod-like domain, which consists of four spectrin-like repeats and is responsible for dimer formation. The C-terminal domain of α-actinin contains two EF hands, which are able to bind calcium in nonmuscle α-actinin isoforms (reviewed in; Blanchard et al., 1989).

The structural data of the spectrin-like repeats of α-actinin implicates that the rod domain is a twisted dimer, which may have an important structural role in stabilizing the rod, and preventing bending as well as it might serve two similar binding sites for proteins interacting with the rod-like domain (Ylänne et al., 2001). While the structural data of the entire α-actinin molecule remains to be resolved, the electron microscopy studies suggest that the N-terminal actin-binding domain can have several different conformations through the movements of a flexible neck region between the actin binding domain and the spectrin-repeats (Winkler et al., 1997). The domain structure of α-actinin is presented in Figure 5.

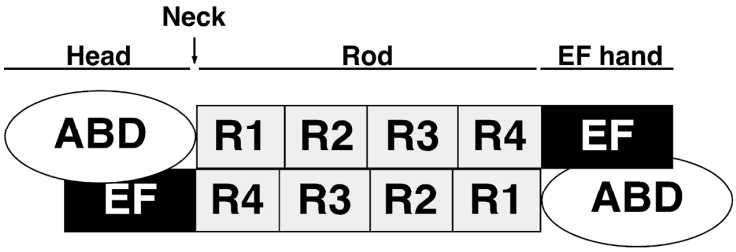

Figure 5. The schematic figure of α-actinin structure.
The actin binding domain (ABD), four spectrin-like repeats (R1-R4) and C-terminal EF hand (EF). The flexible region between the actin binding domain and the rod-like domain is indicated with an arrow (Neck).

4.2 α-actinins in nonmuscle cells

Although originally identified from muscle cells, it has subsequently become clear that α-actinin has important functions also in nonmuscle cells (reviewed in; Blanchard et al., 1989). In *H. sapiens* four α-actinin genes have been identified. α-actinin-2 and α-actinin-3 represent the muscle specific α-actinins forming a part of the contractile machinery anchoring actin filaments at the Z-discs in striated muscle and at the dense bodies in smooth muscle (Endo and Masaki, 1984). Two other α-actinins, α-actinin-1 and α-actinin-4 (Honda et al., 1998; Youssoufian et al., 1990), are expressed in a variety of nonmuscle cells, and subcellular localization studies demonstrate that they are localized to various, and at least partially distinct subcellular structures. α-actinin-1 localizes along actin stress fibers, to focal adhesion sites, to cell-cell contacts, and to the contractile ring during cytokinesis (Knudsen et al., 1995; Lazarides and Burridge, 1975; Pavalko et al., 1995), whereas the subcellular localization of α-actinin-4 is not as clear, but apparently more

concentrated on cell edges than in actin stress fibers (Gonzalez et al., 2001; Honda et al., 1998). Interestingly, while most studies suggest that α-actinins form homodimers, at least α-actinin-2 and α-actinin-3 apparently can heterodimerize as well (Chan et al., 1998). This suggests that also the nonmuscle α-actinins might form heterodimeric complexes when coexpressed in the same cells.

In addition to associating with various cytoskeletal structures, part of nonmuscle α-actinins are found in soluble form in the cytoplasm as demonstrated by fractionation and microinjection studies (Feramisco, 1979; Kreis and Birchmeier, 1980; Tanaka and Itoh, 1998), as well as more recently by photo-bleaching experiments demonstrating a rapid turnover rate (Edlund et al., 2001). Another characteristic feature of nonmuscle α-actinin is that the binding to actin is inhibited by calcium, whereas the binding of muscle specific α-actinins to actin is calcium-insensitive (Burridge and Feramisco, 1981).

4.3 α-actinin interactions in nonmuscle cells

A thorough search of the NCBI Pubmed database produced reports of over 40 different molecules associating with nonmuscle α-actinins. At the first glance this number appears to be surprisingly high. However, in the majority of cases it has not been determined whether the associated protein binds to both nonmuscle α-actinins or only with α-actinin-1 or α-actinin-4. In addition, some of α-actinin associated proteins exhibit restricted tissue distribution e.g. MAGI-1, LPP, L-selectin (Patrie et al.; 2002, Li et al., 2002; Pavalko et al., 1995), and therefore are likely to present α-actinin binding partners only in specific tissues. Considering the binding sites on α-actinin, which have been mapped approximately in the half of the cases, it is of interest to note that the spectrin-like repeats and the C-terminus of α-actinin serve as binding sites for a great part of the reported α-actinin-associated proteins whereas only few molecules bind to the N-terminal actin-binding domain of α-actinin.

Reviewing proteins that associate with α-actinin in different subcellular compartments, and their suggested function there clearly demonstrates that the function of α-actinin is not limited to cross-link actin filaments, and as examples some of those are discussed below in more detail. In focal adhesions one of the first α-actinin interacting proteins found was $ß_1$ integrin subunit (Otey et al., 1990). Since α-actinin was noted to interact both with integrin and actin, it was proposed that α-actinin might anchor the cytoplasmic actin filaments to the plasma membrane. The anchoring role of α-actinin is supported by more recent data indicating that an inactivation of EGFP-α-actinin by chromophore-assisted laser inactivation method (CALI) disrupts the α-actinin - integrin interaction in focal adhesions, resulting in detachment and retraction of actin stress fibers (Rajfur et al., 2002). Interestingly, in PDGF-treated cells PI 3-kinase activation leads to restructuring of focal adhesions. In these cells phosphatidylinositol 3,4,5-triphosphate binds directly to α-actinin, and *in vitro* is able to disrupt the association between α-actinin and the integrin $ß_1$ subunit suggesting a means of regulating α-actinin-integrin association in focal adhesions (Fraley et al., 2003; Greenwood et al., 2000).

In addition to integrins, α-actinin interacts with other membrane-associated molecules, such as the adenosine A_{2A} receptor and syndecan-4 (Burgueno et al., 2003; Greene et al., 2003). Syndecan-4 is a transmembrane proteoglycan, which communicates with extracellular matrix, and is involved in formation of focal adhesions and actin stress fibers. A recent study suggests that syndecan-4 is linked to the actin cytoskeleton through its association with α-actinin in integrin-independent fashion (Greene et al., 2003). Adenosine A_{2A} is a membrane receptor, and its association with α-actinin is proposed to anchor this receptor to the actin cytoskeleton, which is likely to be important for receptor internalization (Burgueno et al., 2003).

In cell-cell contact sites α-actinin associates both with adherens and tight junction proteins (Knudsen et al., 1995; Patrie et al., 2002) as well as with intercellular adhesion molecules, such as ICAM-1 (Carpen et al., 1992). More specifically, in adherens junctions α-actinin binds to β-catenin, and this interaction anchors the cadherin-catenin complex to cytoplasmic actin cytoskeleton (Knudsen et al., 1995). Recent data also provide evidence that in adherens junctions α-actinin could act as linker between two adherens junction systems (cadherin-catenin and nectin-afadin complexes) through its association with β-catenin and ADIP (Asada et al., 2003). In tight junctions α-actinin binds to MAG-1, but the significance of this association remains to be elucidated (Patrie et al., 2002).

In actin stress fibers the major function of α-actinin appears to be cross-linking actin filaments. Several proteins that associate with α-actinin also localize to stress fibers, such as Zyxin, Palladin and MEKK1 (Crawford et al., 1992; Parast and Otey, 2000; Christerson et al., 1999;). Although in several cases the function of α-actinin associated molecules in stress fibers is not known, it is likely that they facilitate α-actinin cross-linking role or regulate more generally stress fiber dynamics through α-actinin.

Finally, it is of interest to mention that in addition to MEKK1 kinase, α-actinin has been shown to associate with other signaling molecules including PKN (Mukai et al., 1997) and the p85 subunit of phosphatidylinositol 3-kinase (Shibasaki et al., 1994). The importance of these associations comes from the inferred possibility of α-actinin regulation since α-actinin itself is phosphorylated on both tyrosine and serine/threonine residues (Gronborg et al., 2002; Izaguirre et al., 2001; Izaguirre et al., 1999). In activated platelets α-actinin is tyrosine phosphorylated on the actin-binding domain by the focal adhesion kinase (FAK), and this phosphorylation reduced the associated of α-actinin with actin filaments (Izaguirre et al., 2001). PKN can also phosphorylate the N-terminal region of α-actinin *in vitro*, but the relevance of this phosphorylation is unknown (Mukai et al., 1997). Taken together, these studies indicate that α-actinin associates with a variety of distinct proteins in different subcellular structures, and α-actinin is regulated through several mechanisms, including association and dissociation of binding partners as well as phosphorylation, although these processed are only partially understood.

4.4 Genetic analyses of α-actinins

The requirement of α-actinin for the viability of cells and organisms has been addressed in several species using gene disruption approaches. In the amoeba *Dictyostelium discoideum*, with a single *α-actinin* gene, development proceeds normally and no apparent phenotypes are noted following disruption of the *α-actinin* gene (Witke et al., 1987). By contrast, null mutations of the single *α-actinin* gene in *Drosophila melanogaster* are lethal (Fyrberg et al., 1998) due to severe muscle dysfunction. The Drosophila *α-actinin* gene encodes three alternatively spliced isoforms of α-actinin; a 107 kDa protein expressed in larvae and adult supercontractile muscle, a 104 kDa broadly expressed muscle α-actinin, and a 104 kDa nonmuscle isoform. Interestingly, the exogenous expression of the adult muscle α-actinin isoform driven by a ubiquitous promoter was sufficient to rescue the larval lethality observed in Drosophila (Dubreuil and Wang, 2000). In *C. elegans* the null phenotype has not been characterized, but two RNAi-based knockdowns of the single *α-actinin* gene did not reveal abnormalities in the assays used (Kamath and Ahringer, 2003; Maeda et al., 2001).

In humans the nonmuscle α-actinin-4 was originally identified as protein closely associated with enhanced motility of cancer cells (Honda et al., 1998). However, an entirely new perspective for the function of α-actinin-4 was opened by results indicating that a point mutation in *α-actinin-4* gene caused a hereditary kidney disease called focal and segmental glomerulosclerosis (FSGS). A typical feature of this disease is abnormal protein secretion to the urine due to increased glomerular filtration (proteinuria) gradually causing renal insufficiency. Histological analysis revealed morphological abnormalities in podocytes, which are specialized epithelial cells with numerous foot processes involved in glomerular filtration. Interestingly, the authors also performed *in vitro* experiments indicating that the mutant α-actinin-4 proteins demonstrated increased binding to filamentous actin suggesting that actin cytoskeleton regulation may be altered in this group of patients. It is interesting to note that all identified point mutations were located between the actin-binding domain and the first spectrin-like repeat, in a region suggested to form a flexible neck region of α-actinin. After the identified link between kidney disease and α-actinin-4 a spur of activity can be noted in the field. Several interacting proteins have been reported including BERP, BP180, E3KARP, MAGI-1 and iNOS (Daniliuc et al., 2003; El-Husseini et al., 2000; Gonzalez et al., 2001; Kim et al., 2002; Patrie et al., 2002). Recent studies also provide two α-actinin-4 mouse models. Mice deficient in *α-actinin-4* were viable but showed progressive proteinuria and kidney disease resulting in death after several months of age (Kos et al., 2003). Interestingly, even though α-actinin-4 is ubiquitously expressed, the histological analysis of mice deficient in *α-actinin-4* indicated abnormalities only in the kidneys, where the morphology of podocytes was disrupted, and resembled the phenotype detected in FSGS patients. Another interesting result obtained from this study was that leukocytes from *α-actinin-4* $^{-/-}$ mice showed increased chemokinesis and chemotaxis. In another transgenic approach, podocyte specific expression of a α-actinin-4 mutant mimicking the FSGS mutation, caused proteinuria and abnormal podocytes architecture much like the FSGS disease (Michaud et al., 2003). Taken together these results indicate

that in kidney, where both α-actinin-1 and α-actinin-4 are expressed, α-actinin-4 is important for podocyte function, but apparently this protein is not absolutely essential in other cell types where its expression has been noted.

5. ALP and Enigma subfamilies of PDZ-LIM proteins

PDZ-LIM proteins form a family based on single PDZ and LIM domains. Enigma represents the founding member of PDZ-LIM proteins, and was initially identified in a yeast two-hybrid screening as a LIM-domain containing protein that associates with the human insulin receptor (Wu and Gill, 1994). Soon after the identification of Enigma several other related genes, including *Ril* (Kiess et al., 1995), *Clp-36* (Wang et al., 1995) and *Enh* (Kuroda et al., 1996) were identified through different approaches. However, it was the discovery of ALP that eventually revealed that these related genes encode proteins that not only have C-terminal LIM domains but also contain a highly similar N-terminal PDZ domain (Xia et al., 1997). The family now contains seven proteins, each of which contains one N-terminal PDZ domain followed either by one C-terminal LIM domain (ALP subfamily; ALP, RIL, CLP-36/hClim1/Elfin, Mystique) or three C-terminal LIM domains (Enigma subfamily; Enigma/LMP-1, ENH, ZASP/Cypher1) (Fig. 6) (Xia et al., 1997, and references therein). The PDZ and LIM domains of these family members are highly related (up to 70% sequence similarity), whereas the middle part between PDZ and LIM domain is more diverse.

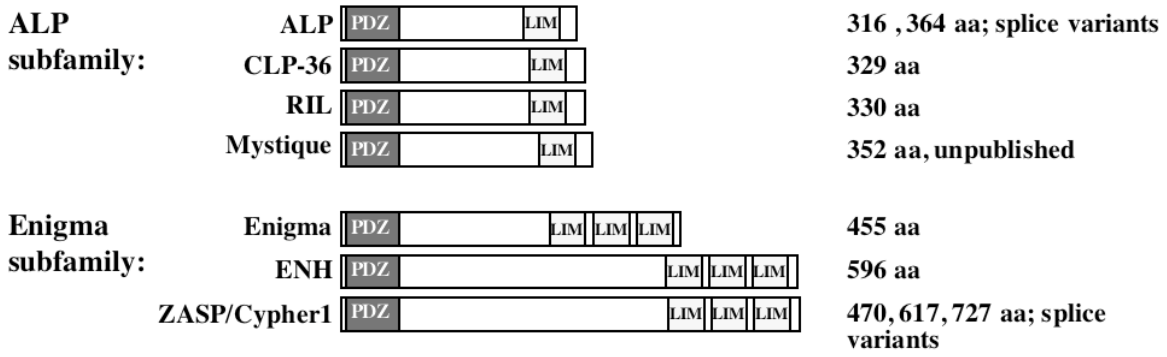

Figure 6. Human ALP and Enigma subfamilies of PDZ-LIM proteins.

In addition to ALP and Enigma subfamilies the human genome contains genes for three more distantly related proteins, namely LIMK-1 (Mizuno et al., 1994), LIMK-2 (Okano et al., 1995), and LMO7 (Semenova et al., 2003), that each contains both PDZ and LIM domains but also additional domains such as a kinase domain in LIMK-1 and LIMK-2. Due to the different domain organization and lower sequence similarity within the PDZ and LIM domains, these three proteins are not considered to belong to the PDZ-LIM family here.

By comparing the available sequences of several genomes it is evident that all human PDZ-LIM proteins have homologues in mice. *Drosophila melanogaster* genome encodes four putative PDZ-LIM proteins, and the *C. elegans* genome contains a single gene that encodes different isoforms through alternative splicing. Fission yeast and budding yeast do not have any obvious PDZ-LIM homologues.

5.1 PDZ domain

The PDZ (PSD-95, Dlg, ZO-1) domain was originally called a GLGF (Gly-Leu-Gly-Phe) repeat or a DHR region (discs large homology region), and is currently of the most abundant protein interaction domain found in organisms ranging from bacteria to man (reviewed in; Nourry et al., 2003). The human genome encodes for more than 400 PDZ domains in over 200 different proteins (reviewed in; Hung and Sheng, 2002). These numbers indicate that frequently a single protein possesses more than one PDZ domain - sometimes even up to 13 as is the case with MUPP1 (Mancini et al., 2000). Another typical feature is that PDZ domains are grouped into pairs or triplets, and it has been proposed that these tandem PDZ domains might have an influence on protein folding and ligand binding (reviewed in; van Ham and Hendriks, 2003). In several cases PDZ domains are found in combination with other domains, and depending on domain combinations PDZ domains have been classified into three main families; (1) proteins that contain only PDZ domains, (2) MAGUK PDZ proteins, which have one to three PDZ domains, a single SH3 domain, and a guanylate kinase domain, and (3) PDZ proteins that contain additional domains, such as LIM domains in the PDZ-LIM proteins (reviewed in; Nourry et al., 2003).

The three dimensional structures of numerous PDZ domains indicate that they consists of six ß-strands (ßA to ßF) and two □-helices (□A and □B) that are folded as a six stranded sandwich (reviewed in; Nourry et al., 2003). This structure forms a positively charged binding groove between the ßB-strand and the □B-helix, where a target sequence of interacting protein binds. The most firmly established binding partner for PDZ domains is the C-terminal tail of the interacting protein. This was initially demonstrated with the PDZ domain of PSD-95, which binds to the C-terminal T or S-X-V (T is threonine, S is serine and X is any amino acid residue and V is valine) motif of Shaker-type K^+ channels or the NMDA receptor (Kim et al., 1995; Kornau et al., 1995). Subsequently different types of specific C-terminal target sequences have been identified (reviewed in; Nourry et al., 2003).

However, an increasing number of reports demonstrate interactions of PDZ domains with other targets. For example, nNOS PDZ domain interacts with the PDZ domain of □-syntrophin demonstrating an interaction between two PDZ domains (Brenman et al., 1996). The second PDZ domain of PTP-BL associates with the LIM domain of RIL indicating an interaction between PDZ and LIM domains (Cuppen et al., 1998). The PDZ domain of ALP interacts with the spectrin repeats of □-actinin, and thus demonstrates an interaction of PDZ domain with internal sequence of interacting protein (Xia et al., 1997). Recent data

shows that PDZ domains can even bind with lipids, such as PIP2 (Zimmermann et al., 2002).

Numerous proteins containing PDZ domains localize to membrane structures, and are a part of bigger protein complexes such as membrane receptor complexes, ion channels, tight and adherens junctions (reviewed in; Nourry et al., 2003). Originally it was proposed that the function of PDZ domains in these structures is to act as clustering molecules for bigger protein complexes. However, the observation that PDZ domains interact with a number of non-membrane bound polypeptides suggests additional roles in protein targeting and signaling events. (reviewed in; Nourry et al., 2003).

5.2 LIM domain

The LIM domain is a 50-60 amino acid residue double zinc-finger motif, which was initially identified as a cysteine-rich domain in three different transcription factors (Lin-11, Isl-1, mec-3; reviewed in; Brown et al., 2001; Khurana et al., 2002). LIM domains can be found from a variety of proteins including transcription factors, cytoskeletal proteins and signaling molecules. LIM domain contains conserved cysteine, histidine, and aspartic acid residues, which are important for zinc binding whereas the rest of the amino acids in this domain are variable, and apparently involved in interaction with target protein. No consensus binding domain has been ascribed to LIM domains, and subcellular localization studies indicate that LIM proteins are found throughout the cell.

Considering functional aspects, it has been suggested that LIM domains might be involved in signaling and protein targeting. Consistent with this idea pinch, a protein containing five tandem LIM domains, has been suggested to link two signaling pathways - the integrin pathway and the tyrosine kinase growth factor receptor pathway - through its simultaneously association with integrin-linked kinase (ILK) and Nck-2 (Tu et al., 1999). The proposal that LIM domains regulate subcellular localization is supported by studies showing that phosphorylation of one of the three LIM domains of paxillin promotes its localization to focal adhesion sites (Brown et al., 1998). Another example is provided by a study demonstrating that phosphorylation of the LIM domain of ENH PDZ-LIM protein resulted in movement of ENH from the cell membrane to the cytosol (Kuroda et al., 1996). Additionally, it has been reported that Ret binding to the second LIM domain of Enigma is important for the mitogenic signaling of Ret (Durick et al., 1996).

5.3 PDZ-LIM proteins act as adapters

Interestingly, several mammalian PDZ-LIM proteins exhibit restricted expression patterns. For example ALP and ZASP/Cypher1 are exclusively expressed in muscle tissues (Faulkner et al., 1999; Xia et al., 1997; Zhou et al., 1999), whereas the expression of RIL and CLP-36 is prominent in epithelial tissues (Cuppen et al., 1998; Kiess et al., 1995; Vallenius et al., 2000). Despite these expression differences the majority of PDZ-LIM proteins have been shown to associate with the actin cytoskeleton, suggesting that their

functions are related (Guy et al., 1999, and references therein). This idea has gradually developed from several independent studies indicating that PDZ-LIM proteins associate on one hand with the actin cytoskeleton via their PDZ domain, and on the other hand with kinases via their LIM domain. The LIM – kinase interaction was initially identified through association of the third LIM domain of Enigma with the cytoplasmic tail of the insulin receptor in yeast two-hybrid assays (Wu and Gill, 1994). This interaction was apparently important for endocytosis of the receptor (Wu and Gill, 1994). Subsequently, mostly using yeast two hybrid screens, several other LIM – kinase interactions have been reported including interaction of the second LIM domain of Enigma with Ret/ptc2 - an interaction, which is required for the plasma membrane localization and mitogenic activity of Ret/ptc2 (Durick et al., 1998; Durick et al., 1996). The ENH PDZ-LIM protein was discovered through its binding to PKC (Kuroda et al., 1996), and soon after also Cypher1 was found to associate with PKC (Zhou et al., 1999). A different type of interaction for LIM domains was described in a yeast two-hybrid study where the LIM domain of RIL associated with the second and fourth PDZ domain of protein tyrosine phosphatase PTP-BL (Cuppen et al., 1998).

The actin cytoskeleton association with PDZ-LIM proteins was first demonstrated when ALP was found to associate with α-actinin-2 via its PDZ domain, and also by the ability of ALP to localize to the Z line of striated muscle (Xia et al., 1997). Soon after this other studies indicated that ZASP/Cypher1 and Enigma also localize to Z lines of striated muscle, and associate with α-actinin-2 and ß-tropomyosin via their PDZ domains, respectively (Faulkner et al., 1999; Guy et al., 1999; Zhou et al., 1999). However, in this regard it is notable to mention a recent study, which provides evidence that the PDZ domain may not be essential for actin cytoskeleton targeting. In this work deletion constructs of Cypher1 lacking the PDZ domain still localized to Z-discs in cardiomyocytes (Zhou et al., 2001). Several initial observations indicating that PDZ-LIM proteins associate with actin cytoskeleton were obtained from muscle specific PDZ-LIM proteins. Subsequently these results have been extended to nonmuscle cells, and as those results represent a part of this work, they will be discussed on results and discussion section.

5.4 Genetic models involving PDZ-LIM proteins

Thus far mouse models have been generated only for the muscle specific PDZ-LIM proteins ALP and Cypher1. Results obtained from mice deficient in *Alp* or *Cypher1* genes implicate these genes in normal sarcomere function. *Cypher1*$^{-/-}$ mice die postnatally with a severe form of congenital myopathy (Zhou et al., 2001). Interestingly, ultrastructural analysis of *Cypher1*$^{-/-}$ muscle tissues indicates that the initial formation of the Z-disc is normal but soon after contraction Z-discs structures become disorganized and disrupted, suggesting that Cypher1 is required specifically for maintenance of the Z-disc (Zhou et al., 2001). The *Alp* gene has been targeted by two independent research groups, and in the first study authors concluded that *Alp*$^{-/-}$ mice were indistinguishable from wild type counterparts (Beck et al., 2001), whereas in the second study *Alp*$^{-/-}$ mice gradually developed

cardiomyopathy (Pashmforoush et al., 2001). The cardiomyopathy was suggested to be due to the instability of □-actinin in Z-discs as *in vitro* assays demonstrated that ALP directly enhanced the capacity of □-actinin to cross-link actin filaments (Pashmforoush et al., 2001).

Another model organism, *C. elegans* has a single PDZ-LIM encoding gene, namely *eat-1*. *Eat-1* gene was originally identified as a screen for pharyngeal feeding defects in worms (Avery, 1993). In this screen two different mutations isolated in the *eat-1* gene caused slow and irregular pumping of the pharynx. Interestingly, in addition to pharyngeal phenotype these worms had problems in locomotion, and the body shape was slightly longer and thinner (Avery, 1993). Two recent global knock-down screens in worms did not reveal any notable phenotypes for Eat-1 (Kamath and Ahringer, 2003; Maeda et al., 2001). In another large-scale analysis for protein-protein interactions, the EAT-1 protein was found to associate with Mek-2 and LIN-15 proteins, both of which are required for normal vulval development (Walhout et al., 2000). In the light of these results, it was of interest to note, a recent meeting abstract reporting cloning of the *eat-1* gene, which was noticed to encode a combination of one ALP and three Enigma proteins from one gene by alternative splicing. The germline introduction of ALP/Enigma DNA was reported to be able to rescue *eat-1* mutants. Moreover, the abstract indicates that Enigma proteins localize to the dense bodies and to the sites of cell-cell contact in body wall muscle, whereas ALP exhibited a more broad expression pattern, and localized to dense bodies but was absent from cell-cell adhesions (McKeown C. and Beckerle M.; International Worm Meeting, 2003). It remains to be seen what is the final outcome of these interesting studies, but they may assist us in elucidating the role of PDZ-LIM proteins human cells.

AIMS OF THE STUDY

This study was undertaken to identify human kinases that might activate the CDK-activating kinase (CAK; CDK7-CyclinH-MAT1) or to identify additional CDK-like kinases. We hypothesized that identification of this kinase would reveal signaling pathways regulating the activity of human CDK7-CyclinH-MAT1 complex.

PRIMARY AIMS:

1. Identification and cloning of novel mammalian serine/threonine kinases resembling the fission yeast Csk1 or the budding yeast Cak1 by sequence comparison analysis
2. Functional characterization of such candidate kinases including identification of interacting proteins, and identification of substrates including testing the ability of these candidates to function as CAKs.

Using this strategy a novel nuclear kinase was identified and dubbed Clik1. Subsequently a strong interaction was identified between Clik1 and an uncharacterized PDZ-LIM protein CLP-36. This interesting and unexpected finding redirected the aims of this study.

SECONDARY AIMS:

3. Functional characterization of CLP-36 in order to understand why the Clik1 kinase interacts with a PDZ-LIM protein.
4. Comparison of mRNA and protein expression of Clik1 and CLP-36 in mammalian tissues
5. Investigation of the specificity of the Clik1-CLP36 interaction using other PDZ-LIM family members.
6. Characterization of the functional significance of the Clik1-CLP-36 interaction.

MATERIALS AND METHODS

The materials and methods used in this study are listed below, and described more in detail in the original publications, which are here referred to using Roman numerals.

The list of antibodies used in this study.

Antigen/name	Description	Reference or source	Used in
Myc (9E10)	Mouse monoclonal ab against Myc-tag	Babco Inc.	I, II, III
Myc (PRB-150C)	Rabbit polyclonal antiserum against Myc-tag	Babco Inc.	I
□-actinin-1 (A5044)	Mouse monoclonal ab against human □-actinin-1	Sigma	I, III
□-actinin-4 (NCC-Lu-632)	Mouse monoclonal ab against human □-actinin-4	(Honda et al., 1998)	I, III
CLP-36	Rabbit polyclonal antiserum against human GST-CLP-36	This study	II
ALP (K55)	Rabbit polyclonal antiserum against chicken ALP	(Pomies et al., 1999)	II
EGFP (RDI-GRNFP4abr)	Rabbit polyclonal antiserum against EGFP	Research Diagnostics Inc.	II, III

The list of cell lines used in this study.

Cell lines	Description	Reference or source	Used in
U2OS (HTB-96)	Human osteosarcoma cells	ATCC	I, II, III
COS-7 (CRL-1651)	Monkey kidney cells	ATCC	I, III
A549	Human lung epithelial cells	ATCC	III
REF52	Rat embryo fibroblasts	(Kiess et al., 1995)	III

The list of different expression vectors used in this study.

Plasmids	Description	Reference or source	Used in
Mammalian expression vectors			
CLP-36/pAMC	N-terminal Myc-tag, full-length CLP-36	This study	I, III
CLPΔ1-24/pAMC	N-terminal Myc-tag, aa 25-329	This study	I
CLP-36/pAHC	N-terminal HA-tag, full-length	This study	II
CLP-36/EGFP-C2	N-terminal EGFP-tag, full-length	This study	II, III
CLP1-231/EGFP-C2	N-terminal EGFP-tag, aa 1-231 of CLP-36	This study	II
Clik1/pAMC	N-terminal Myc-tag, full-length	This study	II

K98M/pAMC	N-terminal Myc-tag, full-length, point mutation: K98 -> M	This study	II
ALP/pAHC	N-terminal HA-tag, full-length	ALP/pBS, gift from P.Pomies	II
RIL/pcDNA3	Untagged, full-length	R. Schäfer	III
RIL/pAMC	N-terminal Myc-tag, full-length	This study	III
RIL/EGFP-C2	N-terminal EGFP-tag, full-length	This study	III
Myc-CDK7/pcDNA3	N-terminal Myc-tag, full-length	(Mäkelä et al., 1994)	II
***E. coli* expression vectors**			
ABD/R1-R2/pGEX	N-terminal GST-tag, human □-actinin-1, containing ABD and spectrin-like repeats 1-2	Dr. D. Critchley, (Salmikangas et al., 1999)	I, III
R1-R4/pGEX	N-terminal GST-tag, human □-actinin-1, containing spectrin-like repeats 1-4	Dr. D. Critchley, (Salmikangas et al., 1999)	I, III
R3-R4/EF/pGEX	N-terminal GST-tag, human □-actinin-1, containing spectrin-like repeats 3-4 and EF hand	Dr. D. Critchley, (Salmikangas et al., 1999)	I, III
RIL-PDZ1-100/pGEX2T	N-terminal GST-tag, aa 1-100 of RIL	B. Scharm	III
RIL-LIM 249-330/pGEX2T	N-terminal GST-tag, aa 249-330 of RIL	B. Scharm	III
RIL-82-253/pGEX2T	N-terminal GST-tag, aa 82-253	B. Scharm	III
RIL 1-330/pGEX2T	N-terminal GST-tag, full-length	B. Scharm	III
Insect expression vectors			
CLP-36/pAcGHLT-A	N-terminal GST-tag, full-length	This study	I, II
47/ pAcGHLT-A	N-terminal GST-tag, aa 158-329 of CLP-36	This study	II
Yeast 2-hybrid expression vectors			
38/pJG4-5	Full-length CLP-36	This study	II
51/pJG4-5	aa 24-329 of CLP-36	This study	II
47/ pJG4-5	aa 158-329 of CLP-36	This study	II
Clik1/EG202	aa 40-401 of Clik1	This study	II

Different methods used in this study are listed below, and detailed descriptions or references are provided in original publications.

Methods	**Used in**
Cell culturing	I, II, III
Cell transfection	I, II, III
Immunofluorescence analysis	I, II, III
Immunoprecipitation assay	I, II, III

Western blot analysis	I, II, III
Generation of recombinant proteins	I, III
Solution binding assay	I, II
In situ hybridization	I, III
Cytochalasin B treatment	I, III
Detergent extraction	I, III
Mass spectrometry	I
In vitro kinase assay	II
Yeast two-hybrid screen	II
Northern blot analysis	II
Live cell imaging	III
Blot overlay assay	III
F-actin cosedimentation assay	III

RESULTS AND DISCUSSION

1. Identification of Clik1 (II, unpublished)

1.1 Cloning of *CLIK1*

This study was initiated in an attempt to identify human homologues of the single subunit CDK-activating kinases from fission yeast (Csk1; Molz and Beach, 1993) and budding yeast (Cak1; Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996) through database searches. Three candidate partial human genes (ESTs) that were related to yeast CDK-activating kinases were initially identified in human EST databases. To isolate a full-length cDNA of the most promising EST (H85389), a 654 bp PCR fragment was generated based on a contig sequence of the original H85389, and a subsequently identified H29877 ESTs from a human fibroblast cDNA library. Subsequently, this PCR fragment was used to screen a human fetal liver cDNA library in lambda DR2 resulting in the isolation of a single 3.8 kb cDNA clone. Sequencing of this clone revealed an open reading frame of 1203 bp predicted to encode a protein of 401 amino acids. Subsequently the gene encoding for the cDNA was named *CLIK1* (CLP-36 interacting kinase), also termed STK35 (serine/threonine kinase 35) by sequence databases (GenBank accession numbers NP_543026 and Q8TDR2).

The predicted 44.6 kDa Clik1 protein contained a putative serine/threonine kinase domain between amino acids 69 and 390 (underlined in Fig. 7), with the serine/threonine active site signature between amino acids 223-235. Other possible functional regions of Clik1 included four potential NLS signals (dashed boxes in Fig. 7). In addition Clik1 sequence has several potential phosphorylation sites (black dots in Fig. 7) including two potential inhibitory phosphorylation sites (S79, Y80) just N-terminal of the kinase domain, a CDK1/Cyclin B phosphorylation consensus site (S14; S/T-P-X-K/R) (Moreno and Nurse, 1990) close to the N-terminus, and a possible activating phosphorylation sites in the hypothetical T-loop (S280, S281).

1.2 *CLIK1* gene encodes a putative kinase

In contrast to the noted similarity between EST H85389 and the yeast single subunit kinases, cloning of full-length Clik1 revealed only limited overall homology with any known serine/threonine kinases - including Csk1 and Cak1. Clik1 was most closely related to a group of uncharacterized CaM-kinase related kinases (e.g. GenBank CAC29064), with several CDKs, and with the PAK family of kinases. With the sequencing of the human genome it has later become evident that Clik1 does have a close relative located on chromosome 1 (GenBank accession number AAN03661, Fig. 7). This predicted gene encodes a protein that shares 58% identity with Clik1. In addition, rapidly expanding EST databases and available full genome sequences demonstrate that Clik1 is likely to have

homologues in mouse, rat, cow, zebra fish, and frog, whereas the genomes of *Drosophila melanogaster, C. elegans, S. cerevisiae* and *S. pombe* do not encode any obvious homologues for Clik1.

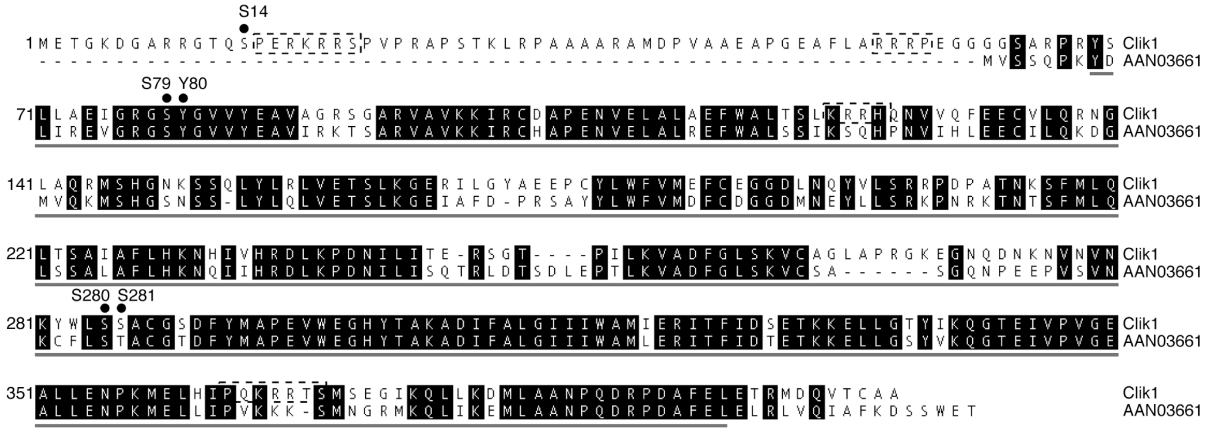

Figure 7. The alignment of human Clik1 and AAN03661 proteins.
The black boxes indicate identities. The kinase domain is underlined. Potential phosphorylation sites are marked with black dots (numbering from Clik1). The dashed boxes indicate possible nuclear localization signals.

2. Clik1 undergoes autophosphorylation, but apparently is not a CAK (II, unpublished)

2.1 Autophosphorylation of Clik1

In order to test whether the Clik1 cDNA encoded a kinase, the open reading frame of Clik1 was subcloned into a mammalian expression vector with a N-terminal Myc-epitope tag. Anti-Myc immunoprecipitates from U2OS or COS-7 cells transiently expressing Myc-Clik1 or controls were subjected to *in vitro* kinase reactions with a variety of substrates including GST-CDK2, Histone H1, CTD and myelin basic protein. Subsequent SDS-PAGE and autoradiography analysis indicated that Clik1 did not phosphorylate any of the tested substrates, suggesting it does not function as a CAK of CDK2 in the conditions previously used to test CAK activity, and where CDK7 is active. Instead, phosphorylation of a 46-48 kDa doublet was noted, and this doublet co-migrated with Clik1 as determined by Western blotting analysis. This result suggested that Clik1 might undergo autophosphorylation. To study this possibility, a mutant Clik1 construct was generated, where the conserved ATP-binding lysine was substituted to methionine (Myc-Clik1-K98M). The comparison of the wild type and mutant Clik1 immunoprecipitates indicated the presence of a broad radiolabeled band only in the wild-type lane, indicating that Clik1 undergoes autophosphorylation *in vitro*. Moreover, Western blot analysis showed that the Myc-Clik1-K98M protein co-migrated with the faster migrating band when compared to wild type Clik1 doublet, proposing that the slower migrating band in wild-type Clik1 represents a

phosphorylated form of Clik1. Despite an increasing repertoire of tested substrates (e.g. actin, □-actinin, CLP-36), to date we do not know of any substrates for Clik1 kinase besides its ability to autophosphorylate.

2.2 Is there any single subunit CDK-activating kinase in mammals?

As the original goal of my thesis was to try to identify human single subunit CDK-activating kinases, I would like to briefly discuss possible reasons why this subject is still unresolved. At the onset of the study, despite the rapidly expanding EST numbers, the significant fraction of human genome was not sequenced, leaving a possibility that the uncovered human genome might contain human CDK-activating kinase. However, looking back at the completed sequences of man and other species no clear homologues of the single subunit of CDK-activating kinases are identifiable using either full-length sequences or shorter domains. In the lack of primary sequence relatives, one could also look at related structural characteristics - but this would require structural data to be available for the one or both of the single subunit CAKs, which unfortunately are not yet available.

Attempts to identify the single subunit CAKs in mammalian cells have been tried by other strategies as well. Our own laboratory has utilized a genetic screen in fission yeast, to search for mammalian cDNAs capable of rescuing a temperature-sensitive strain with a disruption of *csk1* and a weak allele of the CDK7 homologue *mcs6* (*mcs6-13)*. Unfortunately this screen was not thorough as the transformation efficiency of this strain was suboptimal (Westerling T. et al., unpublished data).

Two studies from others have suggested the existence of the single subunit CAKs in mammalian cells: biochemical fractionation of HeLa cell lysates led to the identification of an approximately 40 kDa band, which showed reactivity with a budding yeast Cak1 antibody (Kaldis and Solomon, 2000). Additionally, a similar band in HepG2 cells has been reported, which cross-reacted with the same budding yeast anti-Cak1 antibody in TGF-ß dependent manner (Nagahara et al., 1999). However, these initial observations have not been corroborated either by the original groups or others.

Further complications to the unambiguous identification of critical CDK2 activating kinases were provided by the recent observations that mice deficient in *Cdk2* or *Cyclin E1*or *Cyclin E2* (genes that are thought to be essential for cell cycle progression), are viable, and fibroblasts isolated from these mice undergo normal mitotic cycles (Berthet et al., 2003; Geng et al., 2003; Ortega et al., 2003). The shattering of the dogma that Cdk2 activity would be required for cell cycle progression of course extends to the presumed required activation of Cdk2 by CAK. Therefore one no longer can presume that a candidate CAK kinase should be an essential gene.

In summary, while some evidence has been obtained for the existence of a single subunit CDK-activating kinase in mammalian cells, the recent convincing genetic results on the role of Cdk7 as a CAK in worms and flies have established Cdk7 as the only well demonstrated CAK in multicellular organisms to date.

3. Clik1 associates with the LIM domain of CLP-36 resulting in Clik1 localization to stress fibers (II)

3.1 Clik1 associates with CLP-36

Our kinase results in which Clik1 did not phosphorylate GST-CDK2 in vitro (see above) could have been due to the lack of an activating binding partner of Clik1 (like cyclin in the case CDK activation). Thus one of the strategies to try to generate an active kinase was to identify its partners. To this end we performed yeast-two hybrid screen. Using LexA-Clik1 as bait we identified 37 positive clones, of which 36 represented a poorly characterized CLP-36 gene (also called hClim1 (Kotaka et al., 1999) and Elfin (Kotaka et al., 2001)). The longest clones spanned the entire open reading frame of CLP-36 and the shortest clones were approximately half of the protein. All clones contained the C-terminal LIM domain, suggesting that the association between CLP-36 and Clik1 is mediated via this protein-protein interaction domain. Similar results were obtained from two independent yeast two-hybrid screens and from two different libraries. To study whether CLP-36 also interacts with cellular Clik1, the GST-CLP-36 pull-down experiments were performed from cells overexpressing Myc-Clik1 or control. Those results indicated that Clik1 associates with CLP-36, and also that the shortest C-terminal clone identified in the yeast two-hybrid assay was able to associate with Clik1, supporting the notion that the LIM domain of CLP-36 is responsible for interaction.

3.2 CLP-36 relocalizes the nuclear Clik1 kinase to stress fibers in a LIM-domain dependent fashion

The Clik1-CLP-36 association was further investigated with subcellular localization studies demonstrating that when Myc-Clik1 was transfected alone it localized mainly to the nucleus, whereas CLP-36 was detected in the cytoplasm and in actin stress fibers. Strikingly, in cells expressing both Clik1 and CLP-36, a prominent fraction of Clik1 was relocalized to the cytoplasm and colocalized with CLP-36 including actin stress fibers. This redistribution of Clik1 was specific as CDK7 - used as unrelated control kinase - remained in nucleus in cells coexpressing CLP-36 and CDK7. The interaction was highly specific also in regard to CLP-36, as neither of the closely related PDZ-LIM proteins ALP or RIL was able to relocalize Clik1. The redistribution of Clik1 was not kinase dependent, as it was also observed with the Clik-K98M mutant. The deletion construct of CLP-36 lacking the LIM domain was not able to direct Clik1 to actin stress fibers, thus we concluded that the LIM domain of CLP-36 is responsible for the Clik1 interaction as was suggested by the yeast two-hybrid analysis and the GST-binding assay. This observed LIM-kinase interaction was similar to studies done with other PDZ-LIM proteins, which also demonstrate that LIM domains mediate associations with kinases (Durick et al., 1996; Kuroda et al., 1996; Wu et al., 1996; Zhou et al., 1999).

4. α-actinin-1 and α-actinin-4 associate with the PDZ domain of CLP-36 (I)

4.1 CLP-36 localizes to the cytoplasm and to stress fibers, and associates with α-actinin via its PDZ domain

The Clik1-CLP-36 association directed us to study the role of CLP-36 PDZ-LIM protein further with the motivation that it might elucidate the function of Clik1 in cells. Immunofluorescence analysis indicated that CLP-36 localized both in the cytoplasm and periodically along actin stress fibers in cells overexpressing CLP-36. Interestingly, in immunoprecipitation studies we noted an approximately 100 kDa band that immunopurified with Myc-CLP-36, and by Western blotting analysis it was identified to represent α-actinin. This association was likely to be mediated via the PDZ domain of CLP-36, as a construct lacking the N-terminal part of CLP-36 was not able to associate with α-actinin. Additionally, this same mutant CLP-36 protein failed to localize to actin stress fibers. The prominent localization of CLP-36 in stress fibers in a PDZ domain-dependent fashion together with the results indicating that the association of CLP-36 with α-actinin occurs via the PDZ domain, suggested a model that the CLP-36 localization to stress fibers is mediated through α-actinin. Consistent with this CLP-36 and α-actinin colocalized in stress fibers. In line with these results it has been demonstrated that both ALP and Cypher1 associate with α-actinin-2 via their PDZ domains, and localize to Z-discs in muscle cells (Xia et al., 1997; Zhou et al., 1999). However, our results differ from those obtained by Bauer and coauthors reporting that the part between the PDZ and the LIM domain of CLP-36 was responsible for α-actinin binding in yeast two-hybrid analysis (Bauer et al., 2000). Furthermore Kotaka and coauthors reported that in muscle cells the LIM domain of CLP-36 was required for α-actinin-2 association (Kotaka et al., 2000). One possible cause of the apparent discrepancy could be that CLP-36 might homodimerize through LIM-PDZ interactions as has been reported for RIL (Cuppen et al., 1998). However, despite several attempts we have not been able to detect interactions between independently expressed CLP-36 domains or differentially tagged CLP-36 proteins (Vallenius et al., unpublished data).

The colocalization of CLP-36 and α-actinin in actin stress fibers together with results showing that CLP-36 can be immunopurified from low detergent cell extracts together with α-actinin, indicate that CLP-36 and α-actinin interact both in soluble and insoluble fractions in cells. These results raise an interesting possibility that soluble α-actinin may represent a free pool of α-actinin that is used for dynamic reorganization of actin stress fibers, and the associated CLP-36 could be involved in this function. This notion was one of the motivations for live cell imaging studies (see below).

4.2 CLP-36 associates with both α-actinin-1 and α-actinin-4

The abundance of cellular α-actinin that copurified with CLP-36 enabled us to address the stoichiometry of α-actinin binding, and also allowed us to perform mass spectrometry analysis to ask which isoform of nonmuscle α-actinin associated with CLP-36. To this end different amounts of the GST-CLP-36 protein were incubated with cellular lysates, and subsequently purified using glutathione-Sepharose beads, and analyzed by Coomassie stain. An approximate mass ratio 2:3 for GST-CLP-36 and α-actinin was linear until α-actinin became limiting in cell lysates, which led us to conclude that two CLP-36 molecules associated with a α-actinin dimer.

Interestingly, mass spectrometry analysis of the copurified α-actinin band revealed that both nonmuscle α-actinins (α-actinin-1 and -4) were found in the complex with CLP-36. The discovery of the α-actinin-4 interaction was particularly of interest due to a concomitant study indicating that α-actinin-4 mutations could cause an inheritable kidney disease (Kaplan et al., 2000). However, based on our expression studies it appears that CLP-36 is not highly expressed in glomeruli in kidney, and thus may not be the significant binding partner of α-actinin-4 in the glomeruli.

In this regard, it is also worthwhile to mention that although CLP-36 is found in complex with both α-actinin-1 and α-actinin-4, we do not know whether this interaction is mediated via both of these nonmuscle α-actinins or could they alternatively form heterodimers as has been shown with α-actinin-2 and β-actinin-3 (Chan et al., 1998).

5. CLP-36 as an adapter: α-actinin – CLP-36 – Clik1 (I, II)

Based on the results discussed above we have proposed a model where the CLP-36 PDZ-LIM protein targets the Clik1 kinase to actin stress fibers, and this is mediated through a bridge formed by CLP-36 and α-actinin. The results also provide direct evidence that CLP-36 acts as an adapter, as indirectly suggested previously for other PDZ-LIM proteins (Fig. 8) (Guy et al., 1999, and references therein). In cells expressing both Clik1 and CLP-36 the periodic and continuous staining pattern of CLP-36 was partly disrupted, and actin stress fibers appeared somewhat disorganized when detected with phalloidin. This result suggests that Clik1 may represent a novel regulator of actin stress fiber dynamics - possibly through phosphorylation of an as of yet unidentified substrate.

The relocalization of Clik1 to stress fibers also raises a question of the mechanism involved, and thus it was of interest to observe that some CLP-36 staining was detected in the nucleus, suggesting that CLP-36 might be able to shuttle between the cytoplasm and the nucleus as has been reported for LIMK-1 (Yang et al., 1998). In this case, Clik1 localization to stress fibers could be caused by nuclear export following the association with CLP-36. However, to date we have not address this question further, and this issue remains to be studied.

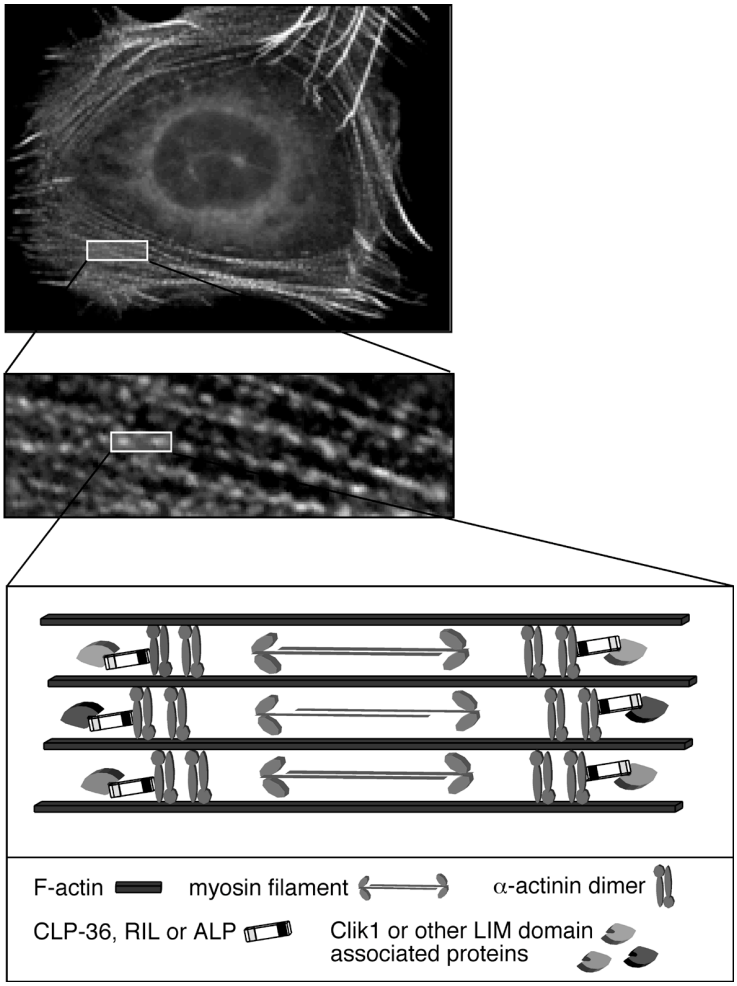

Figure 8. CLP-36 acts as an adapter.
The immunofluorescence picture in the top indicates the localization of CLP-36 in the cytoplasm and in actin stress fibers.
The middle panel represents a zoom-in picture, which demonstrates the periodic, dotted-like staining pattern of CLP-36 along actin stress fibers.
The schematic picture in the bottom illustrates the simplified architecture of actin stress fibers and how our proteins of interest might associate with this structure.

6. *CLIK1* and *CLP-36* are widely expressed genes (I, II, unpublished)

6.1 High expression of *CLIK1* in testis and ovaries

To study the possible physiological role of the association between Clik1 and CLP-36, the expression patterns of these genes were studied. Northern blotting analysis of *CLIK1* indicated that it is expressed at low levels in all tissues examined except for the testis, where significantly higher signals were noted. In order to study the observed testis expression further, we recently performed *in situ* analysis with an antisense *CLIK1* probe, which strikingly indicated a strong expression of *CLIK1* in spermatogenic cells and in oocytes (Vallenius et al., unpublished), suggesting that Clik1 might be involved in gametogenesis.

6.2 *Clp-36* is expressed in several nonmuscle tissues

Initial identification of CLP-36 by Wang et al. indicated that rat *Clp-36* mRNA was highly expressed in several nonmuscle tissues and in the heart (Wang et al., 1995), whereas Kotaka and coauthors reported strong signals in heart and in skeletal muscle using human *Clp-36* probe (Kotaka et al., 1999). To determine the expression pattern more accurately we performed *in situ* hybridization analysis from adult and embryonic tissues. This analysis was done both with human and mouse antisense *Clp-36* probes, and it clearly indicated that *Clp-36* was present in several epithelial tissues and in the heart, but absent in skeletal muscle. The difference observed in skeletal muscle expression in Northern blotting analysis (Kotaka et al., 1999) and *in situ* analysis could be due to the species-specific differences or the presence of a closely related gene. However, one more possibility is that the mRNA isolated from tissues contains a mixture of cells, for example skeletal muscle sample might contain cells from small vessels. In this regard it is of interest to mention that Bauer and coauthors measured that CLP-36 represents 0.3% of the total protein content in platelets and is also expressed in endothelial cells (Bauer et al., 2000). It should also be noted that, analysis of human ESTs does not support the notion that *Clp-36* would be strongly expressed in skeletal muscle.

In regard to analyzing protein expression, we have unfortunately not been able to raise Clik1 antibodies that would detect endogenous Clik1, and the generation of this reagent requires further work. However, we have been able to generate a good rabbit polyclonal antibody against CLP-36, which can immunopurify endogenous CLP-36 protein from a variety of tissues. As Clik1 showed high expression in testis, we performed immunofluorescence analysis of mouse testis sections indicating that the CLP-36 antibody stained the peritubular contractile cells surrounding the seminiferous tubuli and elongating spermatids. It will be interesting to investigate whether this noted overlap between Clik1 mRNA and CLP-36 protein will reflect in a shared function in testes.

7. Regulation actin stress fiber dynamics by CLP-36 and RIL (III)

7.1 Nonoverlapping expression patterns and subcellular localizations of CLP-36 and RIL

During the studies of CLP-36 we got interested in the closely related PDZ-LIM protein RIL. *Ril* was initially identified as a gene downregulated in H-*Ras* transformed cells (Kiess et al., 1995), and RIL was shown to associate with the PTP-BL phosphatase via its LIM domain (Cuppen et al., 1998). RIL is also interesting because of its expression pattern in several nonmuscle tissues, and especially in epithelial cells (Cuppen et al., 1998; Kiess et al., 1995). These results suggested that RIL would serve as a good control for our own studies with CLP-36, □-actinin and Clik1. To initiate these studies we compared the expression patterns of ALP family members by performing side-by-side *in situ* analysis with mouse antisense *Ril, Clp-36* and *Alp* probes. The comparison of *Clp-36* and *Ril* expression patterns revealed that although they both were expressed in several epithelial

tissues, the expression patterns did not overlap considerably, suggesting that they might have separate functions in cells. The following subcellular localization studies indicated that although RIL was localized along actin stress fibers and diffusely in the cytoplasm, several RIL expressing cells also exhibited irregular cables and clusters, which both colocalized with phalloidin staining. From these results we concluded that unlike to CLP-36, RIL overexpression lead to the formation of abnormal actin stress fibers, which further (together with finding that RIL did not change the nuclear localization of Clik1) proposed that these two nonmuscle PDZ-LIM proteins have distinct roles in cells.

7.2 RIL associates with α-actinin via its PDZ domain

RIL localization to actin stress fibers prompted us to study whether RIL could also associate with α-actinin. The immunoprecipitation analysis from cells overexpressing either RIL alone or together with EGFP-α-actinin indicated that both EGFP-α-actinin and endogenous α-actinin associated with RIL. However, the amount of α-actinin in the complex with RIL was remarkably lower than with CLP-36, demonstrating additional differences between these two PDZ-LIM proteins - specifically in their association with the soluble pool of α-actinin. To investigate the potential association of RIL and α-actinin in insoluble actin stress fibers, immunofluorescence studies were performed. These demonstrated the colocalization of RIL and α-actinin. The subsequent blot overlay assay using different fragments of purified GST-RIL protein as probes indicated that RIL could directly associate with α-actinin, and that this interaction was mediated via the PDZ domain of RIL. In addition, the PDZ domain of RIL associated with an unknown protein of approximately 55 kDa in this assay.

The difference in the ability of CLP-36 and RIL to associate with soluble α-actinin could have several alternative explanations. The association of RIL with α-actinin in general could be weaker than the association between CLP-36 and α-actinin. Alternatively RIL might prefer the insoluble α-actinin, which might also explain the detected abnormal actin cables and clusters in cells overexpressing RIL. Thirdly, RIL has been reported to form homodimers, which could affect binding to α-actinin. However, regarding the last alternative we have not detected dimerization in any of our assays. Finally, it was of interest to note a study indicating that RIL associated with Zyxin-related protein TRIP-6 (Cuppen et al., 2000), which could well represent the unidentified 55 kDa protein seen in the blot overlay assay described above. This interaction could also affect the ability of RIL binding to α-actinin.

7.3 RIL and CLP-36 dynamics in nonmuscle cells – distinct actin stress fiber turnover

As actin stress fibers are dynamic structures in nonmuscle cells, it was of interest to use live cell imaging to explore the stress fiber associated Clik1, CLP-36 and RIL dynamics in cells. To this end a time-lapse confocal microscopy system was setup that enabled detection of cells for several hours. Unfortunately, both EGFP-Clik1 and DsRed-Clik1 constructs

formed aggregates in cells (Vallenius et.al., unpublished), thus cells transiently expressing either EGFP-RIL or EGFP-CLP-36 were analyzed. Monitoring of these cells by collecting still frames revealed that EGFP-CLP-36 localized along the stress fibers in a periodic fashion, and in some cells a prominent centripetal movement of EGFP-CLP-36 was detected on stress fibers, much like the treadmilling movement of actin. Overall, CLP-36 expressing cells appeared stationary, which was in stark contrast to EGFP-RIL expressing cells, which undergo rapid changes in cell shape. In addition, EGFP-RIL expressing cells again demonstrated highly aberrant disorientated stress fibers. Strikingly these abnormal thick and irregular fibers exhibited rapid dynamics with continuous assembly and collapse. The observed dramatic alterations in actin stress fibers in RIL expressing cells was also in contrast to that reported for ALP; whose expression in rat myocytes enhanced the cytoarchitecture of sarcomeres (Pashmforoush et al., 2001). The abnormal behavior of actin stress fibers in cells expressing RIL suggests that RIL modulates actin stress fiber dynamics, and raises the question of mechanisms involved. Interestingly, our cosedimentation assays revealed that RIL enhanced the ability of □-actinin to cosediment with filamentous actin, suggesting that RIL might either recruit □-actinin into filaments or stabilize existing □-actinin in filaments. The live cell imaging studies indicated that once localized to actin stress fibers RIL overexpression caused irregular cables, which gradually became thicker prior to sudden breakdown, suggesting defective disassembly of stress fibers.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The identification of the Clik1 kinase formed the basis of this work, and the most interesting outcomes are its association with the CLP-36 PDZ-LIM protein, and its possible involvement in actin stress fiber dynamics. However, as frequently occurs in science a new discovery provides more questions than answers, and also in this case the results represent rather a start than an end. Several results presented in this work are based on overexpression models, and as such might not always reflect the *in vivo* role of the gene of interest. Thus one of the next important steps is to verify our results with endogenous Clik1. To address this, high-quality antibodies are required. Our recent *in situ* analysis of Clik1 has directed us to concentrate initial *in vivo* studies to the testes.

Continuing the future journey of Clik1, I would like to point out that although in this study we have concentrated on the role of Clik1 in actin stress fibers, the nuclear localization of Clik1 is a result, which should not be forgotten. It would be of great interest to study the nature of the bright clusters of Clik1, which are detected in the nucleus following detergent extraction. Considering functional studies of Clik1, one of the key issues is also to find its substrate(s). Finally, knockdown, knockout, or transgenic animal models would be a nice way to try to elucidate the *in vivo* function Clik1.

Other noticeable result of this study is the CLP-36 - □-actinin association. It is not that frequent that you immunopurify cellular proteins in amounts that are detectable in a Ponceau stain as is the case with the CLP-36 - □□actinin interaction. In addition CLP-36-□-actinin interaction was seen in actin stress fibers. These results together with the prominent CLP-36 expression in epithelial cells are of interest as in epithelial cells there is a constant actin network reorganization when cells divide, migrate, form cell-cell contacts and are under different stretch stimuli. Considering the *in vivo* significance of this association, it would be interesting to study the localization of CLP-36 and □-actinin in different epithelia, and try to clarify which type of actin networks they favor.

It would be also of interest to study the mechanism involved in CLP-36 and □-actinin (and also RIL and □-actinin) turnover in actin stress fibers. Comparison of CLP-36 and □-actinin turnover rates and the recruitment order in actin stress fibers by the means of fluorescence recovery after photobleaching technique (FRAP) would give insight into the dynamic nature of these processes. Elucidating signaling pathways involved in these processes could be initiated by testing available inhibitors known to affect actin stress fiber dynamics, such as Rho-kinase and MLCK inhibitors. In this regard the well-established platelet activation model (Fig. 4) (Tanaka and Itoh, 1998) might also assist us to study the CLP-36 and □-actinin dynamics, for example in the combination with RNAi technology. Indeed, as there are no CLP-36 animal models available it would be valuable to test the knockdown phenotype of CLP-36 with RNAi technology.

In summary, I hope the results presented and the tools generated during this thesis work provide a good basis for future studies on Clik1, CLP-36, □-actinin, and RIL, especially their role in regulating cellular processes through the cytoskeleton.

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