A NOVEL ROLE FOR CALPAIN 4 IN PODOSOME ASSEMBLY

by

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Abstract

Podosomes are adhesive and invasive structures which may play an important role in numerous physiological and pathological conditions including angiogenesis, atherosclerosis, and cancer metastasis. Recently, the cysteine protease m-calpain (m-Capn) has been shown to cleave cortactin, an integral component of the podosomal F-actin core, as well as various proteins found in the peripheral adhesive region leading to the disassembly of these dynamic structures. In this study, I investigated whether Capn plays a role in the formation of podosomes downstream of c-Src. I show that: 1) phorbol-12, 13-dibutyrate (PDBu) as well as c-Src-Y527F expression induces podosome formation in mouse embryonic fibroblasts; 2) PDBu- and constitutively active c-Src-induced podosome formation is inhibited by the knockout of the m- and µ-Capn small regulatory subunit Capn4 in mouse embryonic fibroblasts (Capn4−/−), but is partially restored by re-expression of Capn4; 3) Capn4 localizes to podosomes; and 4) Inhibition of m- and µ-Capn proteolytic activity by the cell permeable calpain inhibitors has little effect on the formation of podosomes downstream of active c-Src. I conclude that Capn4 may play a role in the assembly phase of podosomes independent of calpain proteolytic activity. Work done in collaboration to determine a possible mechanism of action for the role of Capn4 in podosome assembly indicates that a possible binding partner of Capn4, β-PIX, co-localizes with, and shows in vivo association with Capn4. Furthermore, β-PIX and Capn4 bind directly in vitro in the presence of Ca^{2+}. We conclude that Capn4 plays a role in podosome assembly, and this role may be through direct interaction with β-PIX in a calcium-dependent manner.
Co-Authorship

Dr. Vinay Singh is a co-author of this thesis.

**Chapter 2**: This paper has been prepared for submission to *Biochemical Journal*. The authors are Thomas R. Dowler, Vinnay Singh, Madhav Jagannathan, Stephane Angers, Peter A. Greer, Zongchao Jia, and Alan S. Mak. I performed all of the experiments for the manuscript except for those noted below. Stephane Angers performed the pull-down of TAP-tagged β-PIX (Table 1). Fluorescence, circular dichroism, and *in vitro* GST-β-PIX pull-down experiments were performed by Dr. Vinay Singh (Figures 8, 9, 10).
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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>ALLM</td>
<td>N-acetyl-Lue-Lue-Met-CHO, Calpain inhibitor II</td>
</tr>
<tr>
<td>ALLN</td>
<td>N-acetyl-Lue-Lue-Nle-CHO, Calpain inhibitor I</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin related protein 2/3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>Capn</td>
<td>Calpain</td>
</tr>
<tr>
<td>Cast</td>
<td>Calpastatin</td>
</tr>
<tr>
<td>CAT</td>
<td>Cool-associated tyrosine phosphorylated</td>
</tr>
<tr>
<td>CH</td>
<td>Calponin homology</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cool</td>
<td>Cloned out of library</td>
</tr>
<tr>
<td>Src</td>
<td>Rous sarcoma</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology domain</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>GBD</td>
<td>GIT-binding domain</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIT</td>
<td>G-protein-coupled receptor kinase-interacting target</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neuronal-Wiskott Aldrich syndrom protein</td>
</tr>
<tr>
<td>NPF</td>
<td>Nucleation promoting factor</td>
</tr>
<tr>
<td>P95PKL</td>
<td>Paxillin kinase linker</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDBu</td>
<td>Phorbol-12,13-dibutyrate</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PIX</td>
<td>PAK-interacting exchange factor</td>
</tr>
<tr>
<td>PKCα</td>
<td>Protein Kinase C α</td>
</tr>
<tr>
<td>PTP1B</td>
<td>Protein tyrosine phosphatase-1B</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin Ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>WIP</td>
<td>WASP interacting protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
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</table>
Chapter 1

General Introduction

1.1 Cell Migration and Invasion

Cell migration and invasion are required for a number of physiological and pathological conditions including angiogenesis, wound healing, atherosclerosis, and cancer metastasis (1-3). Cell migration in a 2-dimensional system is a repetition of a four-step process that requires highly synchronized and dynamic changes in the cytoskeleton. This process consists of 1) protrusion of the leading edge of the cell, 2) adhesion of this leading edge through the formation of focal contacts with the extracellular matrix (ECM), 3) contraction of the cell body and 4) deadhesion of the trailing, which edge propels the cell forward. However, in a tissue environment the cell is surrounded by a basement membrane, which acts as a physical and biochemical barrier to cell motility. To move, the cell must either revert to amoeboid-type motility, squeezing through gaps in the ECM, or degrade the ECM in a focal, directed manner to allow for net migration to occur. Cells form specialized structures called podosomes and invadopodia that are responsible for the focal degradation of the ECM (2;4;5). Podosomes and invadopodia are formed in a variety of cell types and appear to be a common property of all cells that migrate in vivo (2;3;6). Indeed, podosomes and invadopodia have been shown to play a role in a number of normal and pathological processes such as angiogenesis, atherosclerosis, and cancer metastasis (2-4;7).
1.2 Migration and Invasion in Physiological and Pathological Conditions

1.2.1 Angiogenesis

Blood vessels are required to supply cells with oxygen and nutrients and remove waste products. Cell migration and invasion are important in the complex process of formation of new blood vessels, angiogenesis. Angiogenesis-stimulating growth factors, including platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), initiate angiogenesis by signalling to endothelial cells of pre-existing blood vessels (8;9). Upon stimulation, endothelial cells release proteases that degrade the basement membrane of the pre-existing vessel (10). Degradation of the tissue barrier allows the endothelial cells to migrate to the interstitial space. The lumen is the inner portion of a blood vessel through which blood flows and is lined with endothelial cells. The lumen of the new blood vessel forms followed by generation of a new basement membrane. To complete the angiogenesis process, the new vessel fuses with the pre-existing vessel, and blood flow is initiated. Cell migration and invasion is integral to the digestion and migration by endothelial cells required for the initial stages of angiogenesis(8).

1.2.2 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease in which plaque builds up on the lumen side of an arteriole wall. Progression of this pathological condition can lead to ischaemic heart disease and stroke, cerebrovascular disease or cardiac arrest. Initiation of atherosclerosis occurs when low density lipoprotein (LDL) accumulates on the inner wall
of the artery, termed the intima. Production of leukocyte adhesion molecules and chemokines leads to the recruitment of T cells, and monocytes, which differentiate into macrophages (11). The production and release of multiple growth factors and cytokines promote the recruitment and proliferation of vascular smooth muscle cells (VSMC). The recruitment of VSMC from the medial layer of the artery to the intima requires the crossing of tissue barriers, a process that is thought to require podosome formation (4;12). Plaque growth through the accumulation of cholesterol, immune infiltrates, macrophages, connective tissue, and smooth muscle cells can cause a partial blockage of blood flow. This blockage leads to ischaemic heart disease and cerebrovascular disease. Rupture of the plaque can induce formation of a thrombus blocking blood flow and lead to ischaemic stroke or cardiac arrest (13).

1.2.3 Cancer Metastasis

Cancer is a pathological condition in which uncontrollable proliferation occurs in a group of cells, called a tumor. Benign tumors often do not lead to complications as they stay localized to one discrete site. However, malignant tumors contain metastatic cancer cells with the ability to invade through tissue barriers and form secondary tumors in other areas and organs of the body. This is a major cause for mortality. When a cancer cell metastasizes, breaking away from the primary tumor, it must invade through the ECM to reach adjacent tissues, the bloodstream, or lymphatic system. Macrophages can act as metastasis promoters, as they produce molecules, such as growth factors, which can signal to cancer cells leading to an increase in their invasive abilities (14). While
tumor cells can use different mechanisms for invasion and metastasis, recently implicated cellular structures allowing for interaction of the cell with the ECM are podosomes and invadopodia (15). These specialized structures degrade the surrounding ECM and allow for the cell to migrate across tissue barriers to lymphatic and blood vessels, thus spreading the cancer (16). The metastatic cells form secondary tumors at new locations within the body leading to damage in organs and tissues.

1.3 The Actin Cytoskeleton

The actin cytoskeleton composes the cellular structures integral to migration and invasion. Actin monomers (G-actin) polymerize into double helical filaments (F-actin) and depolymerize, all at a rapid rate, leading to the formation of numerous dynamic cytoskeletal structures. Bundling of F-actin occurs to form the stress fibres and filipodia, while F-actin branching forms lamellapodia, dorsal ruffles, podosomes, and invadapodia.

1.3.1 Stress Fibres and Focal Adhesions

A cell pulls its body forward during migration by contraction along tensile cables. Stress fibres are the cables, bundles of F-actin along which actomyosin contraction occurs (Figure 1.1). The bundles are held together by crosslinking proteins such as α-actinin and fascin, and intercalated with non-muscle myosin and tropomyosin (17;18). Contraction along stress fibres occurs through the pulling of an actin filament towards or along another actin filament by the associated protein myosin (17). Found at the ends of stress fibres are focal adhesions. Focal adhesions form mechanical attachments to, and
Figure 1-1 The Actin Cytoskeleton. Stress fibres are bundles of actin fibres along which actomyosin contraction occurs. Focal adhesions adhere the cell to the ECM and are found at the ends of stress fibres. Lamellapodia protrude the cell membrane forward through the mechanical force of F-actin branching.
interact with the ECM through the transmembrane proteins, integrins (Figure 1.1) (19). The cytoplasmic portion of focal adhesions is composed of the structural proteins vinculin, talin, and paxillin and signaling proteins such as P21-activated kinase (PAK) and PAK interacting exchange factor (PIX) (20;21). Focal adhesions act as the cell ‘feet’, contacting the surface on which they lie, while stress fibres could be compared to the legs and muscles. Contraction along stress fibres attached to focal adhesions pulls the cell body forward, an integral step in the cell migration process.

1.3.2 F-actin Branching

Branching of F-actin forms the basis for the cytoskeletal structures lamellapodia, dorsal ruffles, podosomes, and invadapodia (22;23). It is a highly dynamic process which leads to deformation of the cell membrane through the mechanical force it creates (24;25). The basic proteins required for this process are actin, actin-related protein complex (Arp2/3), a nucleation promoting factor (NPF), barbed end capping proteins, cofilin, and profilin (22).

F-actin branching occurs through the nucleation of a daughter actin filament at a 70° angle to the side of a mother actin filament. Arp2/3 nucleates the actin polymerization of the daughter strand by mimicking the 2nd and 3rd G-actin molecules forming the new F-actin branch when activated by the NPF, neural Wiskott-Aldrich syndrome protein (N-WASP), increasing the affinity of Arp2/3 for ATP. N-WASP, in turn, is activated by binding of active Cdc42 which alleviates autoinhibition (26). WASP-interacting protein (WIP) binding to N-WASP is also required for regulation of
this complex in Arp2/3 mediated actin branching (22;27). The N-WASP/WIP activated Arp2/3 complex is localized to sites of F-actin branching by cortactin (28;29). Once localized to F-actin, cortactin acts as a scaffold, tethering the Arp2/3 complex to F-actin, further stabilizing the interaction (29;30). F-actin branching is a highly dynamic and complex process involving a variety of other actin-binding proteins including cofilin, profilin, thymosins, Scar family proteins, and capping proteins (22). Tightly regulated branching provides the mechanical force to protrude the membrane of the cell in the direction of branch elongation. Membrane protrusion is required for formation of sheet-like protrusions from the leading edge of the cell, termed lamellapodia. Protrusion of lamellapodia marks the first step in the cell migration process. F-actin branching also forms the basis of the cytoskeletal structures podosomes, invadopodia, and dorsal ruffles(23).

1.3.3 Podosomes and Invadopodia: Structure and Function

Podosomes and invadopodia are vertical branched F-actin columns protruding from the ventral surface of a cell. Both of these structures contact the substratum, are involved in focal matrix degradation, and have similar general protein compositions. In recent years, the distinctions between podosomes and invadopodia have been hotly debated. Recent review articles (2;3) report that while podosomes and invadopodia are actin-rich membrane structures which contact and degrade the ECM, podosomes are found in normal cells such as monocyctic cells, dendritic cells, and upon phorbol ester or cytokine stimulation, in endothelial cells and smooth muscle cells. Invadopodia are found
in cancer cells. Some key differences between these structures are the increased
membrane extension (2;3), matrix degradation (2), and lifetime (1;23) of invadopodia
compared to podosomes. While these views are generally accepted, there are
discrepancies within the literature regarding the proper term for Src-transformed
fibroblasts (1-3). Transformation of fibroblasts with active Src yields
podosomes/invadopodia which form into rosettes, circular arrangements of adhesive and
degradative structures, resembling podosomes produced in endothelial cells. These
structures, however, have lifetimes (≥2hr) and increased matrix degradation more similar
to invadopodia. As the Src-transformed fibroblasts appear as a podosome/invadopodia
hybrid, their proper definition has not yet been agreed upon.

My study focuses on phorbol ester-induced podosomes and constitutively active
c-Src-induced podosomes/invadopodia in fibroblasts (Fig. 1.1A). Despite a lack of
clarity in the literature, the vertical actin columns in fibroblasts with adhesive and
degradative properties brought about by these pathways will be referred to throughout
this document solely as podosomes.

Podosomes have been found in several cell types including dendritic cells,
osteoclasts, monocytic cells, phorbol ester-induced vascular smooth muscle cells, and
Src-transformed fibroblasts (2;3;5;23). These vertical actin columns can form as punctate
dots or as ring-like clusters of podosomes called rosettes. The structural basis of
**Figure 1-2 Podosome Structure.** (A) Epifluorescent microscopy images of phorbol ester- (Left) and Src-Y527F-induced (Right) podosomes in mouse embryonic fibroblasts. (B) Cross sections of podosomes (yellow lines in (A)) interaction with the ECM through integrins. They consist of a peripheral ring of adhesion proteins surrounding a branched F-actin core. Scale bars = 20 µm.
podosomes and invadapodia are a branched F-actin core surrounded by a ring of adhesion and regulatory proteins (Figure 1.1B).

The F-actin core is surrounded by a peripheral ring of focal adhesion proteins, which directly interact with the ECM through the signalling proteins integrins. The peripheral adhesive ring shares many common structural and signaling proteins with focal adhesions such as vinculin, paxillin, talin, PIX and PAK providing podosomes with their adhesive abilities (23;31). Podosomes differ from focal adhesions by not only their ability to protrude the membrane through actin branching, but also their reduced lifetimes, and ability to perform ECM remodeling (2;5;23).

For ECM remodelling, podosomes release proteases at discrete sites leading to focal matrix degradation (2;23). The proteases released from podosome sites have been found to be predominantly Zn$^{2+}$ and Ni$^{2+}$ matrix metalloproteases (MMPs) and ADAMS (a disintegrin and metalloproteinase). These proteases allow for degradation of matrix proteins such as fibronectin, collagen, fibrin and laminin (2;32-34). Degradation of the ECM through the release of these proteases and invasion through the degraded ECM may allow for the cell to move itself through tightly restricted areas.

1.3.4 Dorsal Ruffles

Less studied, but similar in composition to podosomes are dorsal ruffles sometimes termed circular dorsal ruffles or waves. They appear as circular waves of actin moving dynamically towards the leading edge of the cell. These structures compare
to podosomes in their ability to protrude the membrane of the cell, however, as their name implies, dorsal ruffles protrude the dorsal membrane (23;35). They are stimulated by the receptor-tyrosine-kinase growth factors platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), as well as phorbol esters (23;36). Within minutes of stimulation, dorsal ruffles often form, moving towards the leading edge of the cell with lifetimes between 5 and 20 minutes (23;37).

Dorsal ruffles have a similar composition to podosomes, the basis of these structures being branched F-actin. As F-actin branching occurs within these structures to modify the dorsal membrane, Arp2/3, N-WASP, WIP, and cortactin are all present. Also present in dorsal ruffles are the focal adhesion proteins found in podosomes including talin, paxillin, vinculin, and integrins (23). Localization of MMPs to these structures has also been reported (38).

Conclusive evidence as to the function of dorsal ruffles has not been presented to this date, however, due to their structural similarities to podosomes it has been hypothesized that dorsal ruffles have adhesive and ECM remodeling abilities (38). Their appearance on the dorsal surface of the cell may be required for ECM degradation and migration in a 3-dimensional setting. Other existing theories are that dorsal ruffles occur to transition the cell from a static to a motile state and that they are involved in rapid cell receptor internalization (23;35;39). These waves of F-actin may translocate actin and related proteins to the leading edge of the cell leading to polarization and cytoskeletal remodeling (23).
1.4 Regulation of Podosome Dynamics

Podosomes are dynamic structures with short lifetimes between 5-20 min. Due to their dynamic nature, undertaking the study of podosomes requires attention to both their assembly and disassembly phases. Studies so far have included PKCα and c-Src activity(40;41), as well as the actions of the PIX-PAK-GIT complex as regulators of podosome assembly(31;42). Recently, attention has been brought to calpain as a regulator of podosome disassembly(43). The duration of this section will detail the above requirements for podosome dynamics.

1.4.1 PKCα and c-Src

Activation of protein kinase C α (PKCα) leads to drastic remodelling of the cytoskeleton, cell migration and invasion. This cytoskeletal remodelling yields stress fibre disassembly, protrusion of lamellapodia at the leading edge of the cell, focal adhesion turnover, and podosome formation. Phorbol esters are commonly used to stimulate activation of PKCα in cultured smooth muscle cells. Phorbol esters mimic diacyl-glycerol (DAG), a physiological activator of PKC (44). Upon phorbol ester stimulation, PKCα indirectly activates non receptor tyrosine kinase c-Src through actin filament associated protein (AFAP-110) (41;45). Src activation leads to activation of Cdc42 and subsequent podosome formation (40). Furthermore, c-Src phosphorylates a number of proteins, including cortactin and N-WASP leading to activation of Arp2/3 required for F-
actin branching. Activation of PKCα and c-Src are common manners of podosome induction in cultured smooth muscle cells and fibroblasts (2;4;46;47).

1.4.2 β-PIX

PIX is a guanine nucleotide exchange factor (GEF), stimulating exchange of GDP for GTP on the Rho-family of GTPases Rac1 and Cdc42. The PIX family consists of the highly homologous α- and β-PIX. These proteins are made up of identical domains, barring the N-terminal calponin homology (CH) domain found in α-PIX, although a novel β-PIX isoform has been found to carry this domain (48). From N-terminal to C-terminal, PIX consists of an SH3 domain followed by dbl homology (DH) and pleckstrin homology (PH) domains (21;49). Following these tandem domains are a G protein-coupled receptor kinase-interacting target- (GIT) binding domain (GBD) and a coiled coil domain at the C-terminal end (21;50) (Fig. 1.2A).

The tandem DH and PH domains are thought to be responsible for the GEF activity of these proteins towards Cdc42 and Rac, while the SH3 domain binds PAK, a downstream effector of both Cdc42 and Rac (21;31;51). PAK activation occurs through the aforementioned activation of Cdc42 and Rac1 by PIX, leading to cytoskeletal remodeling and cell motility (31;42;52).

The PIX-PAK-GIT complex localizes to a number of cytoskeletal structures, including focal adhesions and podosomes (21;31). The localization of PAK to focal adhesions is dependent upon PIX and GIT interaction and further phosphorylation of focal adhesion proteins by PAK affects the dynamics of these structures (53). As well,
**Figure 1-3 Domain Structures of β-PIX and m-Calpain.** (A) From N-terminal to C-terminal, β-PIX consists of an SH3 domain, tandem dbl homology (DH) and pleckstrin homology (PH) domains, responsible for guanine nucleotide exchange factor (GEF) activity, a GIT-binding domain (GBD), and a coiled coil region. (B) m-Calpain consists of the large catalytic subunit Capn2 and the small regulatory subunit Capn4. Domains IIa and IIb harbor the active site. Domains IV and VI are penta-EF-hand domains. They heterodimerize through each of their 5th EF-hands. Domain V is a glycine rich domain. Residue numbers are shown in *italics*. 
the PIX-PAK-GIT complex localizes to the outer ring of podosomes and is required for podosome assembly and dynamics. Over-expression of PAK is sufficient to produce podosomes in aortic smooth muscle cells (31). Furthermore, PAK induction of podosome assembly occurs in a PIX dependent manner, as PIX-PAK binding is required for podosome formation downstream of activated PKCα (31). Although PAK kinase activity does not alter podosome assembly, phosphorylation of cortactin by PAK significantly reduces its ability to bind F-actin leading to podosome disassembly (54;55). The requirement for the PIX-PAK binding in podosome assembly and the role of PAK phosphorylation in podosome disassembly show the importance of this the PIX-PAK-GIT complex in podosome dynamics (31;42;54).

1.4.3 Calpain: Structure and Function

Calpains are calcium-dependent cysteine proteases with the ability to cleave proteins at limited sites. The calpain family consists of 13 members, several of which, including the ubiquitous m- and µ-calpain (Capn), have been widely studied. Both of these proteases consist of an 80 kDa catalytic subunit, sharing 60-65% sequence identity, bound to a common 28 kDa small regulatory subunit (56).

In 1999, X-ray crystallography yielded the structure of m-Capn (57). Capn2 is comprised of domains I, II, III, and IV, while Capn4 consists of domains V and VI. Domain I is a small 19-residue sequence on the NH₂-terminal end. The Cys, His, and Asn which make up the catalytic triad, the active site of this protease, are found in domain IIa and IIb. Domain IV is a penta EF-hand domain which shares close homology
to domain VI. On each of these domains, 4 of the 5 EF-hands bind Ca\(^{2+}\) while the 5\(^{th}\) EF-hands are responsible for the heterodimerization of Capn2 and Capn4 to form m-Capn. Domain V of Capn4 is glycine-rich leaving an unordered structure which could serve to tether the protease to discrete locations such as the phospholipid membrane (Fig. 1.2B) (56;58;59). The close homology between \(\mu\)- and m-Capn indicates a similar domain makeup and interaction between the large and small subunits. Heterodimerization of Capn4 and Capn1 or 2 to form \(\mu\)- and m-Capn, respectively, stabilizes the large subunit allowing for subsequent Ca\(^{2+}\) mediated alignment of the protease active site. Not only does Capn4 form a complex with the large subunit, but it can also form a homodimer through association of the 5\(^{th}\) EF-hands in the absence of the large subunit (60).

While the precise role of calpain \textit{in vivo} is not well understood, recent research has begun to elucidate its function. Calpain is thought to play a role in a number of diverse cellular events, including cell cycle progression (61), the regulation of gene expression (62), and the remodeling of cytoskeletal structures during cell migration and invasion (63-65). Numerous cytoskeletally linked \textit{in situ} substrates of \(\mu\)- and m-Capn have been discovered including cortactin, talin, N-Wasp, and various others (43;65-69). m-Capn regulates the turnover of focal adhesions at least in part through the cleavage of the actin binding protein talin (66). More recently, it was determined that \(\mu\)- and m-Capn regulate the disassembly of podosomes through the direct cleavage of cortactin (68), while leading to an accumulation of F-actin, N-WASP, \(\beta_2\)-integrins, talin, paxillin and vinculin at these sites (43).
1.4.4 Role of Calpain in Podosome Dynamics

While the structures of m-Capn and through homology comparisons, µ-Capn, are now well understood, work must still be done to determine the role of these proteases in various physiological and pathological processes such as angiogenesis and cancer metastasis. One such role recently studied is the proteolytic effects of m- and µ-Capn in podosome dynamics. Recent studies have shown that µ- and m-Capn are involved in the disassembly but not the assembly phase of podosomes (43;47). The proteolytic role of calpain in podosome disassembly occurs through the Capn-mediated cleavage of the podosome components cortactin, N-Wasp, talin, Pyk2 and possibly others (43;47;68). In this model, Capn is present in podosomes. Its proteolytic activity is integral to the disassembly phase of podosomes in that pharmacological inhibition of both m- and µ-Capn, as well as inhibition with the natural calpain inhibitor calpastatin (Cast), and siRNA knockdown of m-Capn leads to increased podosome size (43;47;68). In agreement with this model, calpain resistant cortactin leads to impairment of podosome disassembly and increased lifetime of these structures (68).

Recently, it has been shown that m-Capn proteolytic activity is required for podosome formation in breast cancer cells upstream of c-Src (68). Calpain cleavage of protein tyrosine phosphatase 1B (PTP1B) leaves an active PTP1B fragment which in turn activates c-Src through dephosphorylation of the inhibitory Y529 (68). This disagreement in the literature may be due to differential regulation of podosome dynamics between cell types. The reported role of m-Capn in c-Src activation, taken with
the role of µ- and m-Capn in podosome disassembly, places the proteolytic activity of calpain as a regulator of podosome dynamics.

1.5 Calpain 4 Interaction with β-PIX

In a study by Rosenberger et al. (70), the µ- and m-Capn small subunit, Capn4, was co-immunoprecipitated with the guanine nucleotide exchange factors, α- and β-PIX in CHO-K1 cells. In vitro examination of the Capn4-α-PIX interaction showed the minimal binding domains to be SH3-DH-PH of α-PIX interacting with domain VI of Capn4. Furthermore, association of GEF-deficient α-PIX with Capn4 was shown to be required for cell spreading, as α-PIX may actively recruit Capn4 to early integrin clusters resulting in µ- and m-Capn regulated actin cytoskeleton reorganization (21;70).

As of yet, no work has been done to determine the possible function of an interaction between Capn4 and β-PIX. As both PIX and Capn4 have proven to regulate podosome dynamics, an intriguing possibility is the requirement of this protein-protein interaction in podosome assembly or disassembly, and thus a novel role in cell migration and invasion.

1.6 Objectives

Podosomes are actin-based cytoskeletal structures with adhesive and degradative properties involved in cell migration and invasion. Understanding of these dynamic structures demands the study of both the assembly and disassembly phases. Evidence suggests that µ- and m-Capn proteolytic activity is involved in the disassembly phase of
podosomes. However, it has recently been determined that the small subunit Capn4 binds PIX, a guanine nucleotide exchange factor required for the assembly phase of podosomes. The objective of my research was to determine whether Capn4 has a function in podosome assembly independent of its protease regulatory role (Fig.1.3).

1.7 Questions to be Answered

**Does knockout of calpain 4 affect podosome assembly?**

As the small subunit of µ- and m-Capn is essential for the activity of these cysteine proteases and µ- and m-Capn are required for the regulation of podosome dynamics, we hypothesized that Capn4 was required for the efficient regulation of podosome dynamics. The aim of this study was to test this hypothesis and to assess which phase of podosome regulation, the assembly or disassembly, Capn4 regulates.

**Is the effect of calpain 4 on podosome assembly independent of its protease regulatory role?**

Upon determining the requirement of Capn4 in podosome assembly, it was essential to elucidate the mechanism by which Capn4 regulates podosome assembly. The aim of this study was to determine if the proteolytic activity of µ- and m-Capn are involved in podosome assembly or if a novel role for Capn4 independent of its protease regulatory role was required.
Figure 1-4 Role of Calpain 4 in Podosome Dynamics. µ- and m-Capn activity regulates the disassembly phase (k') of podosomes (right pathway). I hypothesize that Capn4 may play a role in the assembly phase (k+) of podosomes (left pathway).
Is an interaction between Calpain 4 and β-PIX involved in podosome dynamics?

Capn4 has been shown to interact with β-PIX in vitro and in situ. The aim of this study was to determine if the interaction between β-PIX and Capn4, both of which have been individually implicated in the regulation of podosome assembly, is required for the efficient regulation of podosome dynamics.
Chapter 2

Calcium-Dependent Association of Calpain 4 with β-PIX and its Implications in Podosome Assembly

2.1 INTRODUCTION

The calpain family of Ca\(^{2+}\)-dependent cysteine proteases has been shown to play a crucial role in the regulation of the dynamics of cell migration and invasion, which are vital processes in health and disease (56;65). The ubiquitously expressed μ- and m-calpain isoforms (or calpain 1 and 2) are heterodimeric proteases, each consisting of a large catalytic subunit encoded by the *Capn1* and *Capn2* genes, respectively, and a common small regulatory subunit, known as calpain 4, encoded by *Capn4/Capns1*. The large subunit comprises domains I to IV, and the regulatory subunit contains domains V and VI. Binding of Ca\(^{2+}\) to EF-hand motifs in domains IV and VI, as well as to non-EF-hand sites in catalytic domains confer Ca\(^{2+}\) sensitivity to proteolytic activities of calpain (71;72). While structural data have provided valuable information about the mechanisms by which enzymatic activities of m- and μ-calpain may be regulated, physiological functions of calpain have not been clearly established (56;58;65). There is accumulating evidence to suggest that calpain is involved in the turn-over of cytoskeletal structures by proteolytic degradation of their components such as talin, paxillin, vinculin and β-integrins in focal adhesions (56;63;65), and cortactin and N-Wasp in membrane protrusions (69). It has been shown recently that calpain also plays a role in cell invasion
by promoting the turnover of podosomes (43;47;68). Inhibition of µ- and m-Capn proteolytic activity impaired podosome disassembly, while having no effect on podosome assembly in dendritic cells, osteoclasts, and BHK-RSV cells (43;47;73). However, cleavage of protein tyrosine phosphatase 1B (PTP1B) by m-Capn was recently shown to be required for activation of c-Src in breast cancer cells, leading to podosome assembly (68). More work must be done to resolve this clear discrepancy in the literature which may arise from cell type dependent differences in the regulation of podosome dynamics.

Recent studies have demonstrated the association of Capn4 with α-PIX (PAK-interacting exchange factor, Cool2 or ARHGEF6) further implicating a direct link between calpain and the signaling pathway regulating integrin-mediated cell spreading and focal adhesion dynamics(70). α-PIX acts as a guanine nucleotide exchange factor (GEF) for Rac and interacts with its major effector, PAK (p21 activated kinase), which is a key player in cytoskeleton dynamics (74;75). α-PIX also interacts with GIT (G-protein coupled receptor kinase interacting target ) or paxillin-kinase linker (p95PKL), and CAT (Cool-Associated, Tyrosine phosphorylated), all of which are localized to focal adhesions (2;21;50;75;76). Overexpression of wildtype α-PIX and an SH3 mutant lead to coalescence of podosomes. Furthermore, the interaction between α-PIX and PAK4 is proposed to affect the actin dynamics of podosomes in human macrophages (42).

We have previously demonstrated that overexpression of PAK1 or β-PIX, a close homologue of α-PIX, promotes stress fiber disassembly with concomitant podosome formation in smooth muscle cells (31). This occurs even in the absence of phorbol 12,13
dibutyrate (PDBu) stimulation, which alone is capable of inducing podosome formation in smooth muscle cells(6).

Published data have shown that calpain proteolytic activities are involved in the disassembly, but not the formation, of podosomes. In this study, we ask if Capn4, in addition to activating μ- and m-Capn, may also be involved in the assembly phase of podosome formation by directly interacting with β-PIX.

2.2 MATERIALS and METHODS

2.2.1 Detection of μ- and m-Capn Activities by Casein Zymography

Both m- and μ-calpain activities were detected by casein zymography similar to the procedure performed by Croall et al., 2002 (77). This procedure called for a HEPES-Imidazole system in substitution for the standard Tris-glycine system (78;79). Mouse embryonic fibroblasts (MEF) were grown in 10 cm culture dishes. Cell lysates were prepared as previously described (77). The lysates (80 μg of total protein) were analyzed by casein zymography using 8% polyacrylamide gel. Relative μ- and m-Capn degradation of casein was quantified using ImagePro 6.0 software.

2.2.2 Induction of Podosome and Dorsal Ruffle Formation

MEF cells were stimulated with 1 μM phorbol 12,13-dibutyrate (PDBu) (Sigma-Aldrich, Oakville, ON) for 30 minutes or transfected with constitutively active c-Src for 24-48 hrs while incubating at 37°C in 5% CO₂ (80). The percentages of cells containing podosomes and the number of podosomes per cell containing podosomes in both
stimulated and non-stimulated cells were counted. Counts were performed on cells in the presence and absence of Capn proteolytic activity inhibitors. Criteria for podosome-containing cells consisted of the appearance of 3 or more vertical F-actin-based columns containing cortactin. Similar counts were performed in NIH-3T3-c-Src-YF cells in the presence and absence of Capn inhibitors.

The percentage of cells containing dorsal ruffles was counted in both stimulated and non-stimulated cells. Cells considered to contain dorsal ruffles included 1 or more dorsal ruffles, which were deemed circular waves of concentrated F-actin containing cortactin.

2.2.3 TAP-Tag Purification and Mass Spectrometry

Tandem affinity purification of TAP-tagged protein from HEK293T cells and subsequent LC-MS/MS was performed as described previously (81). Briefly, HEK293T cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing high glucose, 10% FBS and penicillin streptomycin mixture (Invitrogen Canada Inc., Burlington, ON) on 15 cm plates. These cells were transfected with pIRESpuro-Glue β-PIX and a polyclonal stable cell line was derived(81). The lysates were prepared from 1.5E8 cells and the β-PIX protein complexes isolated using sequential streptavidin and calmodulin affinity-purification steps. Eluted protein complexes were digested with trypsin and directly analyzed using LC-MS/MS using data dependent acquisition on a LTQ-XL ion trap mass spectrometer. Peptide and protein identifications were performed on the Sorcerer2 IDA system (SageNResearch) using the SEQUEST algorithm (81).
2.2.4 FLAG-Calpain 4 Co-Immunoprecipitation

NIH-3T3-Src cells were plated in 10 cm tissue culture plates (1 × 10^6 cells) and incubated for 24 hrs at 37°C 5% CO₂. Cells were transfected with 6 µg pFLAG-CMV-4-Capn4 or pFLAG-CMV-4-CassetteA, gifts from Dr. K. Kutsche (Universitätsklinikum Hamburg-Eppendorf) (70), with Lipofectamin Plus according to manufacturer’s protocol and incubated for 24 hrs (Invitrogen, Burlington, ON). A second transfection was performed the next day followed by another 24 hr incubation period. Cells were lysed with 1 mL lysis buffer, scraped, and spun down at 10000 × g for 10 min as previously described (70). Supernatant was added to M2 FLAG agarose (Sigma-Aldrich, Oakville, ON). After overnight incubation shaking at 4°C, beads were rinsed with wash buffer and eluted with 2 × SDS sample buffer (70). Western blotting was performed and blots were incubated with 1:10000 M2 anti-FLAG IgG (Sigma-Aldrich, Oakville, ON) or 1:1000 anti-β-PIX IgG (Millipore, Temecula, CA). Secondary anti-mouse or anti-rabbit antibodies (BioShop, Burlington, ON) were applied at 1:15000 dilutions. Blots were incubated in Immobilon Western chemiluminescent HRP substrate (Millipore, Temecula, CA) before exposure.

2.2.5 Cell Culture and Fluorescent Imaging

MEFs were cultured in DMEM containing low glucose (1 g/L), 10% FBS, and 1% penicillin streptomycin (Invitrogen, Burlington, ON) (79). Wild-type MEF with loxP sites inserted within exon 8 and downstream of the Capn4 coding sequence in exon 11 were used for our experimental studies as reported previously (79). Three cell lines were
generated: control cells (\(Capn4^{PZ/PZ}\)), loxP/Cre Capn4-knockout (\(Capn4^-\)), and Capn4-knockouts re-expressing the 28 kDa \(Capn4\) in a lentiviral-rescue system (\(Capn4^{res}\)). Prior to cell seeding, coverslips (12 mm) were incubated for 2 hrs at 37°C with 5 \(\mu\)g/ml human fibronectin (Roche Applied Science, Laval, QC). \(Capn4^{PZ/PZ}\) cells were seeded at 1 \(\times\) 10^4 cells/cover slip, while \(Capn4^-\) and \(Capn4^{res}\) cells were seeded at 1.5 \(\times\) 10^4 cells/cover slip. Cells were incubated on fibronectin containing coverslips for 24 hrs at 37°C in 5% CO_2.

At 24 hrs post-seeding, cells were stimulated with 1 \(\mu\)M PDBu (Sigma-Aldrich, Oakville, ON) for 30 minutes while incubating at 37°C in 5% CO_2. NIH-3T3-Src cells were cultured in DMEM containing low glucose (1 g/L), 10% BGS, and 1% penicillin streptomycin and seeded at 6 \(\times\) 10^4 cells/cover slip. After a 24hr incubation, cells were untreated or treated with 50 \(\mu\)M DMSO, ALLN, ALLM, 27mer peptide encoding exon 1B of calpastatin (Cast peptide), calpastatin negative control 27mer peptide (NC peptide) (Calbiochem, San Diego, CA) for 4 hr before fixing and staining.

Cell-processing and cell imaging using epi-fluorescence and confocal microscopy were performed as previously described (31). Primary antibodies were applied in 3% BSA-PBS in the following dilutions: 1:100 rabbit anti-calpain 4 and mouse anti-calpain 4 (Abcam Inc., Cambridge, MA), 1:100 rabbit anti-\(\beta\)PIX (Chemicon International, Temecula, CA), 1:200 mouse anti-vinculin (hVin-1; Sigma-Aldrich, Oakville, ON), 1:200 mouse anti-cortactin (4f11; Upstate, Temecula, CA), 1:200 rabbit anti-GFP. After rinsing 3 times for 10 minutes with PBS, secondary antibody was added to the cells in dilutions of 1:500. Goat anti-rabbit Alexa 488 conjugated secondary antibody (Molecular
Probes, Eugene, OR) allowed visualization of both Capn4 and β-PIX, while goat anti-mouse Alexa 488 allowed visualization of cortactin and vinculin. Actin filaments were visualized using 1:500 tetramethyl rhodamine isothyocyanate (TRITC) conjugated phalloidin (Sigma-Aldrich, Oakville, ON).

2.2.6 Lipofectamine Plus Transfections

Capn4^{PZ/PZ}, Capn4^{-/-}, and Capn4^{res} MEF cells were plated on coverslips coated with 5 µg/mL human fibronectin at 1 × 10^{4}, 1.5 × 10^{4}, and 1.5 × 10^{4} cells per well, respectively. After 24 hr incubation, cells were transfected with 0.2 µg of pBabe-Src-Y527F (82), a gift from Dr. B. Elliot (Queen’s University), and 0.2 µg pEGFP-β-actin (BD Biosciences, Palo Alto CA) as a transfection marker with Lipofectamine Plus reagent following the manufacturer’s protocol (Invitrogen). Control cells were transfected with 0.2 µg pEGFP-β-actin. After an incubation period of 24-48 hrs, cells were fixed, permeabilized and stained as described earlier.

2.2.7 Retroviral Infections

Pheonix ampho retroviral packaging cells were grown in DMEM 10% FBS 1% P/S to 60% confluency in 10 cm tissue culture plates. These cells were subjected to CaPO₄ transfection with 20 µg DNA (pWZL-Src-YF or empty pWZL) (83). 20 hr post-transfection, 10 mM sodium butyrate was added to cells for 12 hr followed by a change of media to fresh DMEM 10% FBS 1% P/S. NIH-3T3 were plated 24 hr pre-infection in T25 cell culture dishes (Corning) at 3.5 × 10⁵ cells/dish. 48 hr post-transfection of Pheonix ampho cells, viral supernatant was collected and an infection solution was mixed
(1 mL FBS, 2 mL DMEM 10% FBS 1% P/S, 7 mL viral supernatant, 4 µg/mL polybrene) (Aldrich). Infection mix (5 mL) was added to each T25. Supernatant was harvested, mixed, and added to cells 3 times with 6 hr intervals between additions of the infection mix. At 24 hr post-infection of NIH-3T3 cells media was changed to DMEM 10% BGS 1% P/S. Selection was performed 20 hr later with 6 µg/mL puromycin (Fisher).

2.2.8 InnoZyme Calpain Activity Assay

MEF Capn4Pz/Pz and NIH-3T3-Src cells were plated on 10 cm plates at 8 × 10⁵. After 48 hrs incubating at 37°C, 5% CO₂ cells were lysed with 500 µL Cytobuster Protein Extraction Reagent (Novagen). Protein concentrations of cell lysates were measured by a Bradford assay (BioRad, Mississauga, ON). InnoZyme™ Calpain1/2 Activity Assay Kit (Calbiochem, San Diego, CA) was used as per the manufacturers protocol with 100 µg protein lysates added to each reaction. Assays were performed in the presence and absence of the µ- and m-Capn chemical inhibitors ALLN, ALLM, Cast peptide, and NC peptide all at a concentration of 50 µM. Fluorescence was quantified using the SpectraMax M2e Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA., USA).

2.2.9 Statistical Analyses

The average percentage of cells containing podosomes and dorsal ruffles, the average number of podosomes per cell, and the average relative calpain activity were subjected to statistical analysis using a two-tailed student T-test with equal variance.
Results are presented at a significance level of $P < 0.05$ from a triplicate of experiment representative of three independent experiments.

2.2.10 Expression and Purification of Recombinant Proteins

The N-terminal His- or GST-tagged $\beta$-PIX constructs were expressed in *E. coli* and purified for *in vitro* binding studies and pull-down assays. The fusion constructs containing SH3-DH-PH $\beta$-PIX (residues 166-559), DH-PH $\beta$-PIX (residues 254-559, DH $\beta$-PIX (residues 254-429) and PH $\beta$-PIX (residues 429-554) were generated from the pCMV6 plasmid containing $\beta$-PIX variant 1 obtained from Dr. R. T. Premont (Duke University, Durham, NC) (76). The primers used for PCR amplification are described in Table 2.2 (Supplementary). In brief, recombinant clones were generated by ligating PCR products into pCR 2.1-TOPO vector (Invitrogen) and further subcloning as a *BamH*1-*Not*1 fragment into the *BamH*1 and *Not*1 sites of modified pGEX-4T-3 (Amersham Biosciences, GE Health Care Corp., Piscataway, NJ) and pET16b (Novagen, EMD Biosciences Inc., San Diego, CA). The clones for GST-DH and GST-PH single domains were constructed using In-Fusion™ 2.0 Dry-Down PCR Cloning Kit as per manufacturer’s instructions (Clontech, Mountain View, CA).

The His tagged Capn4 construct, which lacked the glycine-rich flexible region (Capn4ΔG), has been described previously (84). Expression of His-tagged proteins involved growth of freshly transformed *Escherichia coli* BL21 (DE3) in 1L of Terrific Broth medium (BioShop Inc., Burlington, ON) supplemented with 100 $\mu$g/L ampicillin at $37^\circ$C with shaking. The cells were grown to an OD$_{595}$ of approximately 0.7 when
isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to a final concentration of 0.25 mM, and growth was continued for an additional 10 hours at 22°C. The cells were harvested by centrifugation, re-suspended in 50 ml of buffer A (50 mM Hepes, pH 7.0, 300 mM NaCl, 10 mM Imidazole) containing 0.5 mM phenylmethane sulfonyl fluoride (PMSF), 10 mM dithiotheritol (DTT) and 1% Triton X-100, and disrupted by sonication (10 X 10 s cycles with 120 s pause time) at 4°C. Cell debris was removed by centrifugation for 1 hour at 16,000 rpm in a Beckmann JA 25.5 centrifuge rotor (Beckman Coulter, Inc. Fullerton, CA). The cleared supernatant was applied to a column packed with 5 ml of pre-equilibrated Nickel-Nitrilotriacetic (Ni-NTA) resins (QIAGEN Inc., Mississauga, ON), and His-tagged protein was purified according to the manufacturer’s instructions. The resins were first washed thoroughly with buffer A followed by a wash with 100 ml of buffer A containing 100 mM imidazole. The bound protein was eluted in buffer A containing 500 mM imidazole.

2.2.11 In Vitro Affinity Pull-Down Assays

GST-fusion proteins for affinity pull-down experiment were purified as per manufacturer’s instructions (Amersham Biosciences, GE Health Care Corp., Piscataway, NJ). In a typical in vitro pull-down assay, GST-tagged β-PIX constructs immobilized on Glutathione Sepharose-4B beads (Amersham Biosciences, GE Health Care Corp., Piscataway, NJ) were incubated with purified His-tagged Capn4ΔG for 1 hour at 4°C. For controls, Capn4 was incubated with Glutathione Sepharose-4B beads alone. The resins were washed 5 times using PBS followed by boiling in SDS-PAGE sample buffer.
Proteins were separated on a 15% SDS-PAGE gel and visualized after staining with Coomassie brilliant blue dye.

2.2.12 Circular Dichroism Spectrometry

Circular Dichroism (CD) experiments were performed at 25°C on a RSM 1000 spectropolarimeter (Olis Inc., Bogart GA) at the Protein Function Discovery facility (Queen’s University, Kingston ON). The instrument was purged with N₂ during the course of entire experiment. The protein concentrations and the path length used for far UV CD experiments were 25 µM and 0.1 mm, respectively. Each CD spectrum was an average of 8 accumulated scans of the protein in buffer containing 20 mM Tris-HCl (pH 8.0) and either 5 mM EGTA or 2 mM CaCl₂. The baseline was corrected by subtracting the spectra of the buffers with respective additives collected under identical conditions. The molar ellipticity [θ] was calculated from the observed ellipticity θ, using the equation [θ] = θ × 100/ncl, where n represents the number of amino acids in the protein, c is the molar protein concentration and l is the path length of the cell in centimeters.

2.2.13 Fluorescence Spectrometry

Intrinsic tryptophan fluorescence spectroscopy was used for in vitro binding studies using a lifetime fluorimeter (Fluorolog-Tau, Horiba Jobin Yvon Inc., NJ). The protein samples were excited at 285 nm and fluorescence emission was measured at 337.5 nm. Capn4ΔG was kept constant at 4 µM in a buffer containing 20 mM Tris-HCl (pH 8.0) and either 2 mM EGTA or 2 mM CaCl₂, to which aliquots of SH3-DH-PH β-PIX or DH-PH β-PIX stock solution was added. The emission intensities were averaged over 180 s/titration
point and normalized to the zero titration point. The baseline was corrected by subtracting the corrected spectra of the buffers with respective additives collected under identical conditions. The spectra were corrected for internal filter effect using the equation

$$I_{em(true)} = I_{em(measured)} \times 10^{(f \times c)}$$

for titration of SH3-DH-PH $\beta$-PIX or DH-PH $\beta$-PIX into buffer and

$$I_{em(true)} = I_{em(measured)} \times 10^{(f \times (1.477 + c))}$$

for titration of SH3-DH-PH $\beta$-PIX or DH-PH $\beta$-PIX into a buffer containing 2.57 $\mu$M Capn4.

With an assumption that total fluorescence $F = f_p(PIX) + f_c(Capn4) + f_{cp}(Capn4.PIX)$ where $f_p$, $f_c$ and $f_{cp}$ are the fluorescent coefficients of the respective proteins, an apparent $K_d$ estimation for association of Capn4 and SH3-DH-PH $\beta$-PIX or DH-PH $\beta$-PIX was obtained by fitting the data to the following quadratic equation:

$$\Delta F - F_0 = 0.5 F_C [P+C+K_d - ((P+C+K_d)^2 - 4.P.C)]^{1/2}$$

Where, $F_C$ is the fluorescent coefficient of the $\beta$-PIX fragment and Capn4 complex minus fluorescent coefficient of Capn4 and $\beta$-PIX fragment, i.e. $F_C = f_{cp} - f_p - f_c$. $\Delta F$ is the net fluorescence intensity obtained by subtraction of fluorescence intensities obtained by titration of SH3-DH-PH $\beta$-PIX or DH-PH $\beta$-PIX in Calpn4 solution from buffer, $F_0$ is the fluorescence intensity of Capn4 in the absence of SH3-DH-PH $\beta$-PIX or DH-PH $\beta$-PIX. $P$ and $C$ are total concentration of $\beta$-PIX fragments and Capn4, respectively.
2.3 RESULTS

2.3.1 Calpain 4-Knockout Causes Cytoskeletal Remodeling in Fibroblasts

As measured by casein zymography, and shown in Figure 2.1A, m- and µ-calpain activities were observed in control Capn4<sup>PZ/PZ</sup> MEF. While neither activity was detected in Capn4<sup>+/−</sup> cells, both m- and µ-calpain activities were restored in Capn4<sup>res</sup> cells to 2-fold and 3-fold the control levels, respectively (Fig. 2.1A). These data are in agreement with previous reports that knockout of Capn4 in fibroblasts destabilized the catalytic subunits of m- and µ-calpain, resulting in a loss of associated proteolytic activities (78;79).

Capn4<sup>PZ/PZ</sup> and Capn4<sup>+/−</sup> cells were of similar size, while Capn4<sup>res</sup> cells were consistently a smaller phenotype (Fig. 2.1B). Capn4 was distributed diffusely in the cytoplasm of Capn4<sup>PZ/PZ</sup> and Capn4<sup>res</sup> cells but was absent in Capn4<sup>+/−</sup> cells (Fig. 2.1B). A background perinuclear stain was seen in all 3 cell types. To assess the role of Capn4 in general cytoskeletal organization, we compared the stress fibres and focal adhesions (Fig. 2.2A) in Capn4<sup>PZ/PZ</sup>, Capn4<sup>+/−</sup> and Capn4<sup>res</sup> cells. Knockout of Capn4 has little effect on the overall structure of stress fibers in untreated MEF cells (Fig. 2.2A). While control cells produce many small vinculin-containing focal adhesions, Capn4<sup>+/−</sup> cells often develop elongated vinculin-staining focal adhesions at the end of stress fibers (Fig. 2.2A, arrows). Capn4<sup>PZ/PZ</sup>, and Capn4<sup>res</sup> cells had average focal adhesion lengths of 2.56 and 2.68 µm, while Capn4<sup>+/−</sup> cells had significantly elongated focal adhesions at an average of 4.42 µm (Fig. 2.2B). Our results thus corroborate with previous reports showing that
Figure 2-1 Disruption of *Capn4* Gene Knocks out m- and µ-Capn Activity. (A) Casein zymography shows that both m- and µ-calpain activity are absent in *Capn4*<sup>+/−</sup> cells (lane 2), while present in *Capn4<sup>PZ/PZ</sup>* (lane 1) and *Capn4<sup>−/−</sup>* cells (lane 3). Lower panel shows casein digestion by each cell type relative to *Capn4<sup>PZ/PZ</sup>* cells. (B) Images show F-actin staining TRITC phalloidin and anti-Capn4 immunofluorescence staining in (from top to bottom) *Capn4<sup>PZ/PZ</sup>* , *Capn4*<sup>+/−</sup> cells, and *Capn4<sup>−/−</sup>* cells. Capn4 is shown to be diffuse throughout the cytoplasm of *Capn4<sup>PZ/PZ</sup>* and *Capn4<sup>−/−</sup>* cells, while it is absent from *Capn4*<sup>+/−</sup> cells. Images were taken at 40× using epifluorescent microscopy. Scale bars represent 20 µm.
Figure 2-2 Knockout of Capn4 Results in Increased Focal Adhesion Length.

(A) Capn4^{PZ/PZ}, Capn4^{-/-}, and Capn4^{res} cells are shown using 40× epifluorescent microscopy stained with TRITC phalloidin and anti-vinculin. An increase in the size of peripheral focal adhesions is seen in Capn4^{-/-} cells relative to Capn4^{PZ/PZ} and Capn4^{res} cells. Actin based stress fibres remain similar between all three cell types. The scale bars represent 20 µm. (B) Graph of average focal adhesion lengths in MEF cells. Capn4^{-/-} cells have significantly increased focal adhesion length. Statistical * represents a P value < 0.05.
deletion of Capn4 is associated with a loss of m- and µ-calpain proteolytic activities and causes cytoskeleton remodeling in fibroblasts (78;85).

2.3.2 PDBu Induces Formation of Podosomes and Dorsal Ruffles in Fibroblasts

To study the role of Capn4 in podosome formation, we compared phorbol ester-induced podosome formation in control and Capn4<sup>-/-</sup> cells. Under non-stimulated conditions, Capn<sup>PZ/PZ</sup>, Capn<sup>-/-</sup> and Capn<sup>res</sup> cells did not develop podosomes. PDBu stimulation for 30 min, however, induced stress fibre disassembly in 82% of Capn<sup>PZ/PZ</sup> cells, with concomitant appearance of podosomes in 23.1% of the cells (Fig. 2.3A, B). On the other hand, less than 1% of the Capn4<sup>-/-</sup> cells produced podosomes in response to PDBu-treatment, while only 13% showed stress fibre disassembly. Re-introduction of Capn4 to Capn4<sup>-/-</sup> cells restored the ability of PDBu to induce stress fiber disassembly in 77% of the Capn<sup>res</sup> cells, and about 11.2% of these cells were able to develop podosomes (Fig. 2.3A, B).

Images of individual Capn4<sup>PZ/PZ</sup> control cells show that in the absence of PDBu-stimulation, cortactin is diffusely stained in the cytoplasm with occasional punctate spots, and is not localized to well-defined actin stress fibers (Fig. 2.3C). Treatment of Capn<sup>PZ/PZ</sup> cells with PDBu for 30 min induced disassembly of stress fibers and recruitment of actin and cortactin to form punctate podosomes that often aggregate to form belts close to the leading edge of the cells (Fig. 2.3D). Cross-sections of the podosomes clearly display the characteristic actin- and cortactin-columns arising from the ventral cell surface (Fig. 2.3D, insets). PDBu-induced podosomes in Capn<sup>res</sup> cells have
Figure 2.3. Capn4 is Required for PDBu-Induced Podosome Assembly. (A) Graph shows the percentage of cells showing loss of stress fibres. Upon PDBu stimulation (black bars), stress fibre disassembly occurred in 82.1% $\text{Capn4}^{PZ/PZ}$ and 77.6% $\text{Capn4}^{res}$ cells. The white bars represent unstimulated cells. Significantly less $\text{Capn4}^{-/-}$ cells showed loss of stress fibres (13.4%). Statistical * represents a P value < 0.05. (B) Graph shows the percentage of MEF cells forming podosomes upon treatment with PDBu. No $\text{Capn4}^{-/-}$ cells formed podosomes, while 23.1% of $\text{Capn4}^{PZ/PZ}$ cells and 11.2% of $\text{Capn4}^{res}$ cells formed podosomes. Statistical * represents a P value < 0.05. Statistical ** represents a P value < 0.05 to both $\text{Capn4}^{PZ/PZ}$ and $\text{Capn4}^{res}$ cells. (C) Unstimulated $\text{Capn4}^{PZ/PZ}$ cells stained with TRITC phalloidin and immunostained for cortactin, β-PIX, or Capn4 with Alexa 488. The scale bars represent 10 µm. (D) $\text{Capn4}^{PZ/PZ}$ cells were stimulated with PDBu. Cells were stained with TRITC phalloidin and immunostained for cortactin, β-PIX, or Capn4 with Alexa 488. The insets are the Z-X planes of regions containing podosomes (as indicated with arrows) and show localization of each protein to the vertical actin columns. These insets are (height × width) 3.6 × 5.1 µm, 2.6 × 6.7 µm, and 2.6 × 18.2 µm, respectively, while the scale bars represent 19.35 µm.
Figure 2-3
similar structures (Supplementary Fig. 2.11) These data indicate the requirement of Capn4 in PDBu-induced podosome formation in fibroblasts.

Next, we asked whether Capn4-dependent, PDBu-induced formation of podosomes require the association between Capn4 and β-PIX. As shown in Figure 2.3C, β-PIX and Capn4 are diffusely stained in the cytoplasm of Capn4<sup>PZ/PZ</sup> cells. Upon PDBu stimulation, both β-PIX and Capn4 were enriched in the actin columns of podosomes (Fig. 2.3D), suggesting the translocation of Capn4 and α-PIX from the cytoplasm to actin/cortactin columns in podosomes.

In addition to promotion of podosome formation in fibroblasts, we have observed that PDBu also induced the formation of circular dorsal ruffles, which are dynamic, actin-based structures arising from the dorsal cell surface (23). As shown in Fig. 2.4A, while almost none of the control, Capn4<sup>/−</sup>, and Capn4<sup>res</sup> cells produce dorsal ruffles, PDBu-stimulation for 30 min induces 16% of control Capn4<sup>PZ/PZ</sup> cells to form dorsal ruffles. However, only 3% and 6%, respectively, of the Capn4<sup>/−</sup> and Capn4<sup>res</sup> cells produce dorsal ruffles in response to PDBu-treatment (Fig. 2.4A). Although dorsal ruffles contain some of the same protein components found in podosomes, such as F-actin, cortactin, N-WASP and Arp2/3, dorsal ruffles are distinct, dynamic cytoskeletal structures with a life time of about 5-10 min that are formed at the dorsal cell surface (23;36). As shown in Figure 2.4B, actin-rich circular dorsal ruffles also contain cortactin, β-PIX and Capn4. To distinguish dorsal ruffles from podosomes, cross-sections of these structures are compared and shown in Figure 2.4C. The dorsal ruffles appear as large rings of actin that
**Figure 2.4. Capn4 is Required for PDBu-Induced Dorsal Ruffle Formation.** (A) Graph shows the percentage of cells containing dorsal ruffles. Upon PDBu stimulation (black bars), dorsal ruffle formation occurred in 15.8% of \(Capn4^{PZ/PZ}\) cells, which was significantly higher than \(Capn4^{-/-}\) cells forming these structures (3.7%). \(Capn4^{res}\) cells showed partial rescue of dorsal ruffle formation (5.1%). The white bars represent unstimulated cells. Statistical * represents a P value < 0.05 between \(Capn4^{PZ/PZ}\) and \(Capn4^{-/-}\) cells. Statistical ** represents a P value < 0.05 between \(Capn4^{PZ/PZ}\) and \(Capn4^{res}\) cells. There was no significant difference in dorsal ruffle formation between \(Capn4^{-/-}\) and \(Capn4^{res}\) cells. (B) \(Capn4^{PZ/PZ}\) cells stimulated with PDBu. Cells were stained with TRITC phalloidin and immunostained for cortactin, β-PIX, or Capn4 with Alexa 488. The insets represent 15 µm² and show each protein localizing with F-actin in circular dorsal ruffles. The scale bars represent 10 µm. (C) Confocal microscopy images of PDBu stimulated \(Capn4^{PZ/PZ}\) cells stained with TRITC phalloidin and immunostained for cortactin with Alexa 488. The left-hand images represent the X-Y (top) and Z-X planes (bottom) of a cell containing a circular dorsal ruffle, while the right-hand images represent a cell containing podosomes. The Z-X images for the circular dorsal ruffle and podosome containing cells represent (height × width) 2.9 × 44 µm and 2.9 × 44.6 µm, respectively. The Z-X planes show podosomes as actin columns developed from the dorsal to ventral surface of the cell, while the circular dorsal ruffles show actin structures beginning above the ventral surface and protruding above the dorsal surface of the cell. The white lines in the top panels indicate the sections from which the Z-X planes are taken. The scale bar represents 19.35 µm.
Figure 2-4
arise from the dorsal surface and extend downwards towards the cytoplasm but never protrude beyond the ventral cell surface. Podosomes, on the other hand, appear as vertical columns that span the entire cell body at the relatively flat cell periphery, and protrude out of the ventral surface.

2.3.3 Calpain 4 is Required for Src-Induced Podosome Assembly

To determine whether the requirement for Capn4 in podosome formation is upstream or downstream of c-Src, the MEF cells were transiently co-transfected with Scr-YF and GFP-β-actin as a transfection marker. Upon Src transfection, 21.8% of MEF Capn4<sup>PZ/PZ</sup> cells produced podosomes. Similar to the phorbol ester treatment, there was a significant decrease in the percentage of Capn4<sup>−/−</sup> cells transfected with Src-YF producing podosomes (1.2%). A partial, but insignificant rescue of podosome formation was seen in the Capn4<sup>res</sup> cells (5.3%) (Fig. 2.5A). These results indicate Capn4 regulates podosome assembly downstream of active Src.

Src-induced podosomes are punctate actin columns which appear as dots, as well as dots forming into more concentrated rings called rosettes. Src-induced podosomes were found throughout the cells rather than primarily at the leading edge as we reported for phorbol ester-induced podosomes. Confocal microscopy shows that Capn4 localizes to cortactin and actin containing Src-YF induced podosomes (Fig. 2.5B).
Figure 2-5 Capn4 Regulates Podosome Assembly Downstream of Src. (A) Graph of the percentage of cells forming podosomes in MEF control Capn4/−, and Capn4es− cells upon transfection with active Src. The percentage of Capn4PZ/PZ cells forming podosomes (21.8%) was significantly higher than Capn4es− (1.2%) and Capn4es− cells (5.3%). Statistical * represents a P value < 0.05. (B) Capn4PZ/PZ cells were transfected with constitutively active c-Src. Cells were stained with TRITC phalloidin and immunostained for cortactin or Capn4 with Alexa 488. The insets are the Z-X planes of regions containing podosomes (as indicated with arrows) and show localization of each protein to the vertical actin columns. These insets are (height × width) 3.6 × 5.1 μm and 2.6 × 18.2 μm, respectively, while the scale bars represent 19.35 μm.
2.3.4 Calpain proteolytic activity is not required for Capn4 dependent podosome assembly

Next, we investigated whether inhibiting calpain proteolytic activities may have an effect on phorbol ester- and Src-induced podosome assembly in fibroblasts. As shown in Figure 2.6A and B, addition of 50 µM ALLN, a cell permeable peptide aldehyde inhibitor of μ- and m-Capn activity, had little effect on the percentage of podosome-producing cells in phorbol ester-treated and Src-transfected MEFs, nor the steady state number of podosome per cell in phorbol ester treated MEFs. Addition of the inhibitor decreased the activity of μ- and m-Capn to 55% of the control levels in MEF Capn4PZ/PZ cells (Fig. 2.6C). This data suggests that calpain activity did not affect the assembly phase of podosomes, in agreement with previous reports (43;47;73).

To further demonstrate that calpain activity does not affect podosome assembly in fibroblasts, NIH-3T3-Src cells were treated with cell permeable calpain inhibitors. As shown in Figure 2.6D, treatment with either 50 µM ALLN or ALLM did not lead to significant changes in the percentage of cells forming podosomes and the steady state number of podosomes per cell relative to the DMSO treated control cells. As well, NIH-3T3-Src cells were treated with a 27mer peptide an inhibitory portion of calpastatin, the natural μ- and m-Capn inhibitor. Again no significant change was seen in the percentage of cells forming podosomes nor the number of podosomes per cell relative to the negative control calpastatin peptide (NC-peptide) and the DMSO-treated control cells (Fig. 2.6D).
Figure 2.6 Capn4 Regulates Podosome Assembly Andependent of its Proteolytic Regulatory Role. (A) Graph shows the percentage of Capn4^{PZ/PZ} cells producing podosomes upon treatment with PDBu or transfection with active Src in the presence (white bars) and absence (black bars) of the calpain inhibitor ALLN. No significant difference is seen in the percentage of cells producing podosomes upon treatment with 50 µM ALLN. (B) Graph shows the number of podosomes per cell found in Capn4^{PZ/PZ} cells stimulated with PDBu in the presence and absence of 50 µM ALLN. Control cells produced 12.9 podosomes per cell on average, while ALLN treated cells produced 12.2 podosomes per cell. (C) Graph of the relative fluorescent intensities representing μ- and m-Capn activity of Capn4^{PZ/PZ} cells untreated and treated with 50 µM ALLN. Statistical * represents a P value < 0.05. (D) A graph of the percentage of NIH-3T3-Src cells forming podosomes and the number of podosomes per cell in untreated cells and cells treated with 50 µM ALLN, ALLM, Cast peptide, or NC peptide relative to the counts in DMSO treated cells. (E) Graph of the relative fluorescent intensities representing μ- and m-Capn of NIH-3T3-Src cells treated as described in (D). (F) Western blot shows the inhibition of cortactin degradation upon treatment of cells with 50 µM ALLN and ALLM.
Figure 2-6
Incubation of cell lysates with ALLN, ALLM, and Cast peptide resulted in 56.8%, 52.2%, and 24.4% µ- and m-Capn activity levels, respectively, relative to control levels (Fig. 2.6E). The decrease in Capn proteolytic activity is reflected in the absence of cortactin degradation bands in ALLN and ALLM treated cells (Fig. 2.6F). InnoZyme calpain activity assay showed NC peptide-treated cells to have only 48.6% the µ- and m-Capn activity of control cells. As the NC peptide is the negative control for the Cast peptide, comparison of calpain activity between cells treated with these cell permeable peptides reports the Cast peptide as inhibiting about 50% µ- and m-Capn activity in NIH-3T3-Src cells. In light of this data and previously published work, we conclude that µ- and m-Capn activity does not affect podosome assembly in fibroblasts.

2.3.4 In Vivo Association of Calpain 4 and β-PIX

To determine whether the association of Capn4 and β-PIX plays a role in the effect of Capn4 on podosome and dorsal ruffle formation, we carried out a series of in vivo and in vitro protein interaction experiments. First, we confirmed that Capn4 and β-PIX do associate in vivo by carrying out pull-down assays using TAP- tag fused β-PIX expressed in HEK293T cells. As identified by mass spectrometry, the list of proteins associated with β-PIX includes Capn4 as well as a number of known β-PIX binding proteins such as GIT1, GIT2, PAK1, PAK2, βGIT, and βPAK (see Table 1 for details). Interestingly, neither m- nor µ-calpain was pulled down by the TAP-tagged β-PIX. From these results,
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Table 2-1 Representative Mass Spectrometry Results for the β-PIX Protein Complex
it can be concluded that β-PIX associates with Capn4 at a cellular level in HEK293T cells.

NH₂-terminal FLAG-tagged Capn4 was transiently transfected into NIH-3T3-Src cells followed by a FLAG pull down assay to determine whether Capn4 interacts with β-PIX in podosome forming cells. In this experiment, FLAG-Capn4 failed to pull-down β-PIX (Fig. 2.7). Interestingly, FLAG-Capn4 failed to pull down the m-Capn large subunit, Capn2. Due to the lack of a positive control, we cannot conclude that β-PIX does not interact with Capn4 in NIH-3T3-Src cells, however, this result may show that the placement of additional residues on the NH₂-terminal of Capn4 disrupts the protein conformation and thus binding with the large subunit, Capn2.

2.3.5 In Vitro Association of Calpain 4 and β-PIX

Our findings that Capn4 and β-PIX co-localize in podosomes and TAP-tagged β-PIX pulls down Capn4 indicate a direct or indirect association of β-PIX and Capn4. To distinguish these two possibilities, we carried out in vitro binding studies to: 1) determine if Capn4 and β-PIX interact directly, 2) assign the sites of interaction, and 3) provide binding parameters.

Identification of Capn4-binding sites on β-PIX. β-PIX and α-PIX contain similar functional domains with the exception of the N-terminal calponin-homologous (CH) domain being present in α-PIX but not in β-PIX (Fig. 2.8A) (21). It has been shown by Rosenberger et al. (70) that the SH3-DH-PH triple domains of α-PIX is sufficient and
Figure 2-7 NH$_2$-Terminal FLAG-Tagged Capn4 Lacks the Ability to Pull Down Capn2 or β-PIX. Pulldown of FLAG-Capn4 from NIH-3T3-Src cells. Exogenous NH$_2$-terminal FLAG-tagged Capn4 was expressed (left panel, bottom) and pulled down (right panel, bottom). While endogenous m-Capn and β-PIX was seen in cell lysates (right panel, top and middle), neither protein was pulled down by FLAG-Capn4 (left panel, top and middle).
Figure 2-8 *In vitro* affinity GST-β-PIX pull-down of Capn4. GST-β-PIX affinity pull-down of Capn4. Upper panel represents the β-PIX constructs used for the affinity pull-down experiments. Lower panel: Lane 1, purified Capn4 (100 μM); lane 2, pelleted GST beads incubated with Capn4; lane 3, pelleted GST SH3-DH-PH β-PIX beads; lane 4, pelleted GST SH3-DH-PH β-PIX beads incubated with Capn4; lane 5, pelleted GST DH-PH β-PIX beads; lane 6, pelleted GST DH-PH β-PIX beads incubated with Capn4; lane 7, purified Capn4; lane 8, pelleted GST DH β-PIX beads; lane 9, pelleted GST DH β-PIX beads incubated with Capn4; lane 10, pelleted GST PH β-PIX beads; lane 11, pelleted GST PH β-PIX beads incubated with Capn4; lane 12, supernatant of GST DH β-PIX incubated with Capn4 (shown in lane 9) and lane 13, supernatant of GST PH β-PIX incubated with Capn4 (shown in lane 11).
required for interaction with Capn4 using the Cyto-Trap pull-down protocol. To
determine if the Capn4 and β-PIX interaction requires similar binding sites, we used
GST-fused SH3-DH-PH domains of β-PIX in a Glutathione Sepharose bead pull-down
assay. His-tagged Capn4 devoid of the N-terminal Gly-rich region (Capn4ΔG) was
expressed in E. coli and purified for binding experiments. This construct was used instead
of the full length Capn4 because of its stability in the bacterial expression system (84)
and the Gly-rich region has no known protein-protein interacting properties (Fig.8A)
(84). As shown in Fig. 2.8B, upper panel, we demonstrated unequivocal binding of
Capn4ΔG with SH3-DH-PH and DH-PH domains of β-PIX (lane 4 and 6). Control (lane
2) shows no association of Capn4ΔG with Glutathione Sepharose resins. We further
observed that neither the DH nor PH domain binds to Capn4ΔG (lower panel, Fig.
2.8B). This data shows that Capn4 and β-PIX interact directly, and the DH-PH domains
of β-PIX appear to be sufficient for the binding (Lane 4 and Lane 6, respectively) but the
binding affinity may be further strengthened by SH3 (see below).

Fluorescence Spectroscopy. Using intrinsic tryptophan fluorescence
spectroscopy we assessed the association of Capn4ΔG with His-tagged SH3-DH-PH β-
PIX and DH-PH β-PIX in solution and compared their binding affinities. Binding curves
in Figure 2.9A and B show that Capn4 binds SH3-DH-PH β-PIX and DH-PH β-PIX in
the presence of Ca^{2+} with binding affinities, K_d = 16.20 ± 4.10 µM and 51.47 ± 3.67 µM,
respectively. Binding is essentially abolished in the absence of Ca^{2+} (data not shown).
This finding is consistent with the observation from the GST affinity pull-down
Figure 2-9 Fluorescence spectrometry of Capn4-β-PIX interaction. Fluorescence spectroscopy experiment for measuring an apparent K\textsubscript{d} of SH3-DH-PH β-PIX and DH-PH β-PIX with Capn4. Data was collected using a fluorescence spectrometer (Fluorolog-Tau, Horiba Jobin Yvon Inc., NJ) where samples were excited at 285 nm and emission was collected 337.5 nm with excitation and emission slit width of 5 nm. 2.57 μM solution of Capn4 in a buffer containing 10 mM Tris pH 8.0 and 2 mM CaCl\textsubscript{2} was titrated with additions of incremental quantities of SH3-DH-PH β-PIX (panel A) or DH-PH β-PIX (panel B). The experiment was performed in the presence of 2 mM calcium chloride or 5 mM EGTA. Measurements were performed in triplicate and the emission intensities were averaged, normalized to the zero titration point, and plotted against ligand concentration. Curve fitting was performed as described in the Materials and Methods section. Solid line represents the least-squared fitted curve.
experiment where GST-fused SH3-DH-PH β-PIX pulled down higher amount of Capn4ΔG compared to GST-fused DH-PH β-PIX.

Circular Dichroism Spectroscopy. To further examine the change in global secondary structure in β-PIX and Capn4 induced by complex formation, CD spectroscopy was employed. The secondary structures of His-tagged SH3-DH-PH β-PIX, DH-PH β-PIX and Capn4ΔG were analyzed individually or in complex, in the presence of calcium (Fig. 2.10A,B). The observed spectra of Capn4ΔG in combination with either SH3-DH-PH β-PIX (Fig. 2.10A) or DH-PH β-PIX (Fig. 2.10B) were significantly different than the calculated sum of the individual spectra, suggesting there were interactions between the proteins which affected their secondary structures. Furthermore, as demonstrated by the difference-CD plots in the presence or absence of calcium (Fig. 2.10 E,F) (Δ[θ] versus wavelength, as described in the Materials and Methods), association between Capn4 and either SH3-DH-PH β-PIX or DH-PH β-PIX clearly occurs in the presence of 5 mM Ca²⁺. In the absence of Ca²⁺, however, insignificant Δ[θ] is observed. It is noteworthy that the Δ[θ] plots differ significantly between the Capn4/SH3-DH-PH β-PIX and Capn4/ DH-PH β-PIX complexes, indicating major differences in secondary structures of the two complexes caused by the presence or absence of the SH3 domain. Since there is appreciable difference in affinity of SH3-DH-PH β-PIX and DH-PH β-PIX for Capn4 as demonstrated by fluorescence spectrometry data, these results suggest that primary Capn4-binding sites are located in the DH-PH domains of β-PIX because these two domains can bind Capn4 in the absence of SH3. However our data
Figure 2-10 Circular dichroism spectrometry of Capn4-β-PIX interaction. Circular Dichroism (CD) measurements of 25 μM calpain 4 and 25 μM each of SH3-DH-PH β-PIX or DH-PH β-PIX in the presence or absence of calpain 4 (25 μM). Spectra were collected in a buffer containing 10 mM Tris pH 8.0 with an addition of 2 mM calcium chloride (A,B) or without calcium (C,D). CD spectra were recorded on an OLIS RSM CD Spectrophotometer (OLIS Inc., Bogart, GA) at 25°C. An average of 12 scans was collected for each spectrum corrected against buffer blanks. The differential CD spectra, Δ[θ] of SH3-DH-PH β-PIX (E) and DH-PH β-PIX (F) in association with Capn4 evaluated in the presence (open circle) and absence of calcium (closed circle). The Δ[θ] plotted on the Y-axis was calculated by subtracting observed CD spectra of β-PIX and Capn4 from the theoretical CD spectra.
clearly indicates that SH3 also contributes to final binding affinity, suggesting that either there is a synergistic effect amongst the three domains or SH3 may also make contact with Capn4 which may further affect its interaction with Pro-rich sites of binding partners.

It is not clear how Ca\(^{2+}\) may affect the binding of Capn4 to the \(\beta\)-PIX fragments. However, it is conceivable that conformational changes in Capn4 induced by binding of Ca\(^{2+}\) to domain VI may present a favorable binding surface for the DH-PH domains of \(\beta\)-PIX. It is interesting to note that Ca\(^{2+}\) causes a significant change in secondary structure of the SH3-DH-PH \(\beta\)-PIX, as demonstrated by a 60% increase in \([\theta]\) at 195nm (Fig.10). Similar Ca\(^{2+}\) effects are not observed in DH-PH \(\beta\)-PIX fragment (Fig. 2.10C, D).

**2.4 DISCUSSION**

**2.4.1 Calpain 4 is Required for Induced Podosome Assembly in Fibroblasts**

Here we present novel data showing that Capn4 plays a crucial role in the phorbol ester- and constitutively active c-Src induced assembly of podosomes in fibroblasts, and this may involve direct interaction between Capn4 and \(\beta\)-PIX in a Ca\(^{2+}\)-dependent manner.

To validate the use of a Capn4 knockout embryonic cell line, \(\textit{Capn4}^{-/-}\), in our studies, we have corroborated and confirmed previous reports (78;79) that knockout of Capn4 has a significant effect on the cytoskeleton organization in fibroblasts and causes a down-regulation of the m- and \(\mu\)-calpain activities. The \(\textit{Capn4}^{-/-}\) cells showed a significant increase in focal adhesion length, as central focal adhesions were replaced by many elongated, vinculin-containing focal complexes at the cell periphery. This data is in
line with results reported previously by Dourdin et al. (78). We have demonstrated previously that disassembly of focal adhesions often occurs at the site of podosome biogenesis followed by actin polymerization requiring the actin-branching regulators, cortactin, Arp2/3 and N-WASP in smooth muscle cells (55). Since Capn4 plays a critical role in the regulation of focal adhesion formation, we asked whether Capn4 plays a similar role in the regulation of podosome formation induced by phorbol ester in fibroblasts. Phorbol ester is a potent inducer of cytoskeleton remodeling in a number of cell types generally involving disassembly of actin stress fibres and focal adhesions (55;80). Only recently, however, phorbol ester has been shown to induce podosome formation in smooth muscle (4;6;31;55;80) and endothelial cells (40). We found that within 30 min, PDBu induced the disassembly of actin stress fibers in control MEF and the appearance of podosomes at the leading edge of 23.1 % of the cells. However, Capn4−/− cells do not produce PDBu-induced podosomes, and the ability to produce podosomes was partially restored by re-expression of Capn4. This data clearly indicates that Capn4 is required for podosome formation in fibroblasts.

Calle et al. (43) studied the effects of calpain proteolytic activity on podosome disassembly in dendritic cells. Calpain was shown to be necessary for the regulation of the turnover of podosomes, as pharmacological inhibition of m- and µ-calpain activity led to a buildup of podosome size due an accumulation of F-actin, talin, N-WASP, β2-integrins, paxilin and vinculin (43). This data is consistent with our finding that the ability of PDBu to induce podosome formation was only partially rescued in the Capn4−/−
cells (Fig. 2.3B) which expressed significantly higher m- and μ-calpain activities than the control cells (Fig. 2.1A). The increase in calpain proteolytic activity may have led to premature cleavage of proteins necessary for podosome maturation resulting in a decreased podosome half-life or smaller, less detectable podosomes.

It is not clear whether general disassembly of actin stress fibers is a pre-requisite for podosome formation or, as suggested by recent data that showed localized dissolution of actin fibers, usually near the ends of stress fibers and focal adhesions, occurring at sites of podosome biogenesis in smooth muscle cells (55). The latter is consistent with data by Tatin et al. (40) showing that phorbol ester can induce podosome formation in endothelial cells in the presence of actin stress fibers (40). These results indicate a possible cell type-dependent mechanism for the regulation of podosome biogenesis which warrants further investigation.

It has previously been reported that activation of PKCα leads to activation of c-Src and subsequent podosome assembly (40;41;45). To determine whether Capn4-mediated regulation of podosome assembly lies upstream or downstream of c-Src we exogenously expressed active Src in MEF cells. Upon quantifying the percentage of cells producing podosomes in control, Capn4+/−, and Capn4res cells, we found that knockout of Capn4, again, significantly impaired the ability of the cells to produce podosomes. Only a partial but insignificant rescue of podosome formation was seen in Capn4res cells which may be due to the increase in μ- and m-Capn activity. This impairment of podosome assembly in Capn4−/− cells upon exogeneous expression of
active Src is consistent with our data concerning phorbol ester induction of podosomes in MEF cells. Furthermore, this evidence clearly states that Capn4 is required for podosome assembly downstream of Src.

In light of this involvement of μ- and m-Capn activity in podosome dynamics, it was important to determine whether it was the suppression of this proteolytic activity due to knockout of the small subunit or suppression of an unknown role of Capn4 which resulted in inhibition of podosome formation. Upon stimulation of podosomes with both phorbol ester and constitutively active c-Src in fibroblasts, followed by treatment with the chemical μ- and m-Capn inhibitors, ALLN and ALLM, as well as a 27mer peptide encoding exon 1B of the natural Capn inhibitor, calpastatin, suppression of calpain activity was shown to have little effect on podosome assembly. These results were in agreement with previous studies investigating podosome formation upon inhibition of Capn activity in dendritic, BHK-RSV, MTLn3 breast cancer cells and osteoclasts upon treatment with m-Capn siRNA, Cast peptide, ALLN, and ALLM (43;47;68;73).

In our study, a role novel role for Capn4 in podosome assembly downstream of active Src has been demonstrated. Furthermore, we have shown that this role is independent of its regulation of μ- and m-Capn activity.

2.4.2 Calpain 4 Interacts Directly with β-PIX

At present we are not clear how Capn4 may contribute to the regulation of podosome assembly. Our data provides evidence that Capn4 regulation of podosome assembly is independent of its ability to regulate proteolytic activity. However, our data
also indicates that direct interaction of Capn4 with β-PIX may contribute to the regulation of podosome dynamics. Their association *in vivo* is suggested by the finding that both proteins are recruited to and co-localize in the actin core of phorbol ester-induced podosomes. This is confirmed by the demonstration of direct interaction between Capn4 and β-PIX *in vitro* using pull-down assays, fluorescence and CD spectroscopy. Our data is also in agreement with previous results showing an association of Capn4 and α-PIX, a close homologue of β-PIX, in affinity pull-down assays(70). Moreover, TAP-tag fused β-PIX was purified from HEK293T cells, yielding further proof of an *in vivo* interaction of β-PIX with Capn4 in a multiprotein complex. The absence of the m- or µ-calpain catalytic subunits from this complex could be due to weaker binding, or a more intriguing possibility is that the β-PIX-Capn4 complex exists in the absence of m- or µ-calpain.

Interestingly, when a pull-down of NH2-terminal FLAG-tagged full-length Capn4 was attempted, interactions of Capn4 with both β-PIX and Capn2 were disrupted. Disruption of Capn4 binding to Capn2 may indicate that the NH2-terminal of Capn4 is required for the stability of interactions between Capn4 and specific binding partners *in vivo*. *In vitro* studies have focused on binding of an NH2-terminal truncated 21 kDa Capn4 binding to Capn2. Our results indicate a requirement for the unmodified amino end of domain V of Capn4 to interact with Capn2 *in situ*. Alternatively, cleavage of the amino end of Capn4 may be required to initiate binding with the m-Capn large subunit.
The minimal Capn4-binding sites on α-PIX were identified in the SH3-DH-PH domains by Rosenberger et al (70). In this study, we have shown that β-PIX fragments containing either the SH3-DH-PH or the DH-PH-domains have similar affinity for Capn4, suggesting that the major Capn4-binding sites are located in the DH-PH domains of β-PIX. However, the SH3 domain may also play a part in the interaction between the two proteins. As shown by CD data, binding of Capn4 to the SH3-DH-PH or DH-PH fragments elicits dramatic differences in conformational changes in the binding partners. This data suggests that although the actual contact sites between Capn4 and β-PIX are located at the DH-PH domains, this interaction may cause conformational changes in the SH3 domain, which in turn may affect its interaction with Pro-rich sites of binding partners. Alternatively and in addition, the SH3 domain may also be involved in the binding between Capn4 and β-PIX, and contribute to the overall affinity of the two proteins. Further analyses are required to distinguish these possibilities.

2.4.3 Calpain 4 and β-PIX Interaction Requires Calcium

It is clear from our binding studies that direct interaction between Capn4 and β-PIX requires Ca^{2+}. It is not clear, however, how Ca^{2+} may affect the binding of Capn4 to β-PIX. It is conceivable that conformational changes, albeit small, in Capn4 induced by binding of Ca^{2+} may present a favorable binding surface for the DH-PH domains of β-PIX. It is interesting to note that Ca^{2+} causes a significant change in the secondary structure of the SH3-DH-PH in the presence of Capn4, as demonstrated by a 60% increase in [θ] at 195nm (Fig. 5). Similar Ca^{2+}-effect is not observed in DH-PH
fragment mixed with Capn4. These findings suggest that Ca\(^{2+}\) may facilitate association of SH3-DH-PH and Capn4 through possible structural changes in the binding surface, which would not be as prominent in the case of the DH-PH fragment alone.

### 2.4.4 Implications of Calpain 4 and β-PIX Interactions In Podosome Assembly

The mechanism by which Capn4 regulates cytoskeletal organization has yet to be determined, although accumulating evidence suggests that Capn4 acts by activating the proteolytic activities of m- and μ-calpain, which have been shown to be involved in the turn-over of actin-binding proteins such as cortactin, and focal adhesion proteins, talin and paxillin. Consistent with this hypothesis, and in agreement with previous reports (78;79), is our data showing that knockout of Capn4 in MEF down-regulates the proteolytic activities of both m- and μ-calpain. However, our data indicates a novel role for Capn4, independent of activation of m- and μ-Capn proteolytic activities, in regulating podosome assembly. Our finding that Capn4 and β-PIX interact directly in a Ca\(^{2+}\)-dependent manner allows us to suggest additional or alternative regulatory mechanisms to what has been previously reported:

1) Treatment of fibroblasts with PDBu induces the recruitment of Capn4 to podosomes via its association with β-PIX in a Ca\(^{2+}\)-dependent manner. This association allows translocation of Capn4 via β-PIX to these cytoskeletal structures where activation of m- and μ-Calpain by Capn4 results in the turn-over of their protein components. However, it is not clear whether the short life time (2-20 min) of these
dynamic structures is compatible with the rate of calpain-dependent protein degradation during the disassembly phase.

2) An interesting, alternative possibility is that Capn4 may directly regulate β-PIX functions by acting as a Ca^{2+}-sensor, not unlike calmodulin, for Ca^{2+}-dependent regulation of β-PIX. Thus, binding of Ca^{2+}/Capn4 to β-PIX at the DH-PH domains might induce conformational changes near the binding sites and the adjacent SH3 domain, modulating interactions between β-PIX and other binding partners. Indeed, our previous structural characterization of calpain demonstrated a second protein-binding site in Capn4, which is distinct from the site that interacts with m- and μ-calpain; although the significance of this observation was not fully appreciated at the time of the structure determination (58). Furthermore, we have shown here that knockout of Capn4 abolishes the ability of the cells to form podosomes, suggesting that Capn4 is also required for the assembly phase of podosome formation.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>RE site</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH3-Start-F</td>
<td>CCGGATCCGTAAGACAAAGTTTAACTTCC</td>
<td>BamHI</td>
</tr>
<tr>
<td>DH-Start-F</td>
<td>CCGGATCTTATTACAATGGTTGCTACAG</td>
<td>BamHI</td>
</tr>
<tr>
<td>PH-Start-F</td>
<td>CCGGATCAAACGGAGGGCGATGAC</td>
<td>BamHI</td>
</tr>
<tr>
<td>DH-Stop-R</td>
<td>CCGCGGCGCTTTAGACCTTGGGCTGAAAGG</td>
<td>NotI</td>
</tr>
<tr>
<td>PH-Stop-R</td>
<td>CCGCGGCGCTTTAGACCTTGGGCTGAAAGG</td>
<td>NotI</td>
</tr>
<tr>
<td>DH-F-Fus</td>
<td>GGTTCGGCTGGATGCCGGATCCCTATTACAATGGTGCTACAG</td>
<td>BamHI</td>
</tr>
<tr>
<td>DH-R-Fus</td>
<td>AGTCAGATGCGCCGCCGCGCCGCTTTAGACCTTGGGCTGAAAGG</td>
<td>NotI</td>
</tr>
<tr>
<td>PH-F-Fus</td>
<td>GGTTCGGCTGGATGCCGGATCCCAACTGGGAGGGCGATGAC</td>
<td>BamHI</td>
</tr>
<tr>
<td>PH-R-Fus</td>
<td>AGTCAGATGCGCCGCCGCGCCGCTTAGACCTTGGGCTGCTG</td>
<td>NotI</td>
</tr>
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Table 2-2 Supplemental: List of β-PIX primers.
Figure 2-11 Supplemental: MEF Capn4<sup>ex</sup> cells form podosomes. Epifluorescent microscopy shows MEF Capn4<sup>ex</sup> cells form podosomes similar to those seen in Capn4<sup>PZ/PZ</sup> cells upon PDBu treatment. Cortactin, β-PIX and Capn4 all localize to the F-actin columns (Top, middle, and bottom rows, respectively). Insets represent 7.5 µm<sup>2</sup> X-Y section of podosomes. Scale bars represent 10 µm.
Chapter 3
General Discussion

3.1 Summary of Findings

Migration and invasion of cells across tissue barriers plays an important role in physiological and pathological conditions such as angiogenesis, atherosclerosis, and cancer metastasis. Migration involves the coordination of highly regulated cytoskeletal structures, allowing the cell to exhibit forward motility. In order for a cell to progress through the surrounding ECM and transmigrate through tissues, it must form specialized cytoskeletal structures with the ability to degrade barriers, termed podosomes. Podosomes are F-actin based structures with both adhesive and degradative properties, shown to secrete proteases at discrete sites leading to ECM degradation. Podosomes have been reported in numerous cell types including osteoclasts, macrophages, endothelial cells, carcinoma cells, and, upon stimulation with phorbol esters or Src, smooth muscle cells and fibroblasts. In this study, I focused on podosome formation in fibroblasts upon phorbol ester and Src stimulation.

As highly dynamic structures with lifetimes between 2-20 minutes upon phorbol ester stimulation and up to 2 hours upon exogeneous expression of active Src, podosomes use various methods of regulation during both their assembly and disassembly phases. Recently, the Ca^{2+}-dependent cysteine proteases µ- and m-Capn have been implicated as important regulators of podosome disassembly, but not assembly, in dendritic and BHK-RSV cells through their ability to cleave various structural and signalling proteins found
in these structures (43;47). Not only has this Capn proteolytic activity been highlighted in
the disassembly phase of podosomes, but as well, m-Capn proteolytic cleavage of PTP1B
was shown to be important in activating this phosphatase, leading to c-Src activation and
subsequent podosome formation in breast cancer cells (68). The contradiction of these
studies as to the role of Capn in the podosome assembly phase indicates regulation of
podosome dynamics may, in part, be cell type specific. In light of recent findings
regarding the role of Capn in podosome dynamics I pursued a novel question to further
elucidate the role of these proteins; is the µ- and m-Capn small regulatory subunit,
Capn4, required for podosome assembly in fibroblasts?

To answer this question, I first characterized MEF Capn4PZ/PZ, Capn4−/−, and
Capn4res cell lines. Our findings agreed with previous studies reporting that disruption of
the Capn4 gene results in loss of µ- and m-Capn activity and impairment focal adhesion
turnover. Upon reaffirming the effects of Capn4 knockout in untreated fibroblasts, our
efforts moved to answering the aforementioned question.

I have discovered a novel role for Capn4 in podosome assembly in fibroblasts.
Podosome stimulation was performed by both phorbol ester treatment and transient Src
transfection. Treatment of control cells with phorbol esters resulted in the disassembly of
actin stress fibres with concomitant formation of podosomes in 23.1% of cells. Capn4−/−
cells showed a significant decrease in stress fibre disassembly while less than 1%
produced podosomes upon phorbol ester treatment. A rescue of stress fibre disassembly
and partial rescue of podosome formation was seen in Capn4res cells. This data indicates
that Capn4 expression is required for podosome assembly downstream of phorbol ester stimulation of PKCα.

Only a 50% rescue of the percentage of cells forming podosomes was seen in Capn4<sup>res</sup> cells. This may be due to a significant increase in μ- and m-Capn activity relative to control cells, as shown by casein zymography. Proteolytic activity of μ- and m-Capn has been shown to regulate focal adhesion disassembly through the cleavage of talin (66), a component of the podosome adhesive ring (2). Furthermore, a number of other podosome components are known in vivo or in vitro substrates of μ- and m-Capn (65). The increase of calpain proteolytic activity within Capn4<sup>res</sup> cells may lead to depletion of various podosome components within the cell. In turn, a decreased concentration of intact podosome components may impair the assembly rate of podosomes.

Not only was Capn4 expression important in podosome assembly, but Capn4-knockout also decreased the formation of dorsal ruffles. As well, the rescue of Capn4, resulting in an overexpression of μ- and m-Capn activity lead to only a partial rescue of dorsal ruffle formation. Based on the similarities of structural components in both podosomes and dorsal ruffles, the comparable affects of Capn4 on the dynamics of the 2 F-actin-based cytoskeletal structures is not surprising. Addition of Capn4 regulation of the assembly phases of each of these structures provides further evidence supporting the theory presenting dorsal ruffles and podosomes as functionally similar structures (23).
Alternatively, regulation of dorsal ruffle assembly represents yet another role for Capn4 in cell migration and invasion.

My next goal was to place Capn4 in the PKCα-podosome assembly pathway. Not only is m-Capn shown to be required for c-Src activation and subsequent podosome assembly in breast cancer cells, but activation of c-Src lies downstream of PKCα (41;68). Upon exogeneous expression of active Src, 21.8% of Capn4<sup>PZ/PZ</sup> cells produced podosomes, while only 1.2% of Capn4<sup>-/-</sup> showed podosome assembly. A previous study placed m-Capn activity upstream of Src in breast cancer cell podosome assembly (68), however, my results consistently indicate that Capn4 plays a novel role in podosome assembly downstream of active Src.

The loxP/Cre Capn4 knockout system utilized in this study provides viable cells with a deletion of a gene which would, under normal conditions, lead to midgestation embryonic lethality (79). While the advantages of this system are the ability to determine the various processes and signalling pathways within the cell which are affected by the absence of Capn4, there are several limitations to this system. Lentiviral infection of MEFs with Cre-recombinase, required for excision of the Capn4 gene at loxP sites, is inserted into random points in the genome. These random insertion points may lead to disruption of important genes resulting in cell phenotypes falsely attributed to the knockout of Capn4. Another potential limitation is due to the possible redundancy of the Capn protease family. Capn is a family which consists of 13 proteins. The variety of Capns within the cell provides the potential for redundant functions within the family.
Thus, the functions of a knockout of \( \mu \)- and m-Capn activity may be partially rescued by other endogenous Capns.

Interaction of Capn4, with large subunits Capn1 and Capn2 of \( u \)- and m-Capn, respectively, is required for the stability of these proteases and, thus, their proteolytic activity within the cell. As it is the proteolytic activity of \( \mu \)- and m-Capn that regulates podosome disassembly, I tested the requirement of Capn proteolytic activity as a potential mechanism to explain the role of Capn4 downstream of Src in podosome assembly. Upon treatment of active Src expressing fibroblasts with pharmacological peptide aldehyde inhibitors and a peptide encoding an inhibitory domain of calpastatin we found no significant difference in podosome assembly relative to control levels. These results indicate the requirement for Capn4 in podosome assembly downstream of Src is independent of the role of Capn4 in \( \mu \)- and m-Capn proteolytic activity.

All previous literature regarding m- and \( \mu \)-Capn and their regulation of cytoskeletal structures focuses on the proteolytic activities of these proteins. The findings presented in this study however, contradict the dogma that Capn4 is solely responsible for regulation of proteolysis and suggest that Capn4 may also be involved in interactions with yet to be determined binding partners in the regulation of the assembly phase of podosome dynamics. Although I have not positively identified a binding partner of Capn4, data from my work and others have pointed to a possible candidate in the guanine nucleotide exchange factor \( \beta \)-PIX, which has been shown to mediate podosome assembly.
Capn4 binds PIX in situ in an interaction hypothesized to localize m-Capn to focal adhesions and initiate the turnover of these structures. Previous reports from our lab (31) and from Gringel et al. (42) indicate PIX in complex with PAK and GIT is required for podosome assembly and further regulation of podosome dynamics. As Capn4 and β-PIX interact in situ, and both are required for podosome assembly, we hypothesize the interaction of these 2 proteins regulates the assembly phase of podosomes. Our results indicate Capn4 and β-PIX interact either directly or indirectly in situ, however, modification of the NH2-terminal end of Capn4 abolishes the ability of Capn4 to interact with both β-PIX and the m-Capn catalytic subunit. No previous studies have reported pull-down of the catalytic subunit by affinity-tagged full length Capn4, while in vitro studies primarily focus on a 21 kDa Capn4 mutant lacking domain V due to the instability of the full-length protein upon purification (86). It is still unclear as to the function of domain V in vivo, however, our results indicate that its integrity may be important in protein-protein interactions.

I have shown that Capn4 and β-PIX co-localize at podosomes and dorsal ruffles in MEF cells. Furthermore, in vitro characterization of the β-PIX Capn4 interaction show direct binding of these proteins dependent upon domain VI of Capn4, the DH-PH domains of β-PIX, and the presence of Ca$^{2+}$. Due to Capn4 homodimerization in both the presence and absence of Ca$^{2+}$ (60) it is unclear whether the stoichiometry of the β-PIX Capn4 interaction involves 1-1 binding, or whether β-PIX interacts with Capn4 homodimers. The possibility exists that small conformational changes, primarily around
EF-hand 1 (60) in the homodimer may expose a β-PIX binding site. The significance of Capn4 homodimerization and of small Ca$^{2+}$ induced changes in structure in the β-PIX Capn4 interaction is currently unclear. While further work must be done to characterize the nature of this interaction in situ, the reported data indicates Capn4 may be involved in a complex with PIX whose action is required for and at sites of podosome assembly (Fig. 3.1).

3.2 Podosome Dynamics Model: Proposed Role for Capn4

My results indicate that Capn4 regulates podosome assembly downstream of active c-Src. Furthermore, Capn4 regulation of assembly is independent of its μ- and m-Capn regulatory role and may occur through calcium-dependent binding to β-PIX. Taken with previous findings, Capn4 is required in both the assembly and disassembly phases of podosomes. I propose a model to explain the actions of Capn4 in podosome dynamics downstream of active Src (Fig. 3.2). An influx of calcium at focal adhesion sites may initiate a conformational change of β-PIX and local Capn4. Due to this conformational change, a binding site on Domain VI of full-length Capn4 is exposed, allowing it to bind the DH-PH double domain of PIX at focal adhesion sites. As PIX is bound to PAK and GIT in a heterotrimeric complex, this binding causes the complex to undergo a conformational change, abrogating an interaction at the focal adhesion site and causing translocation of the newly formed Capn4-PIX-PAK-GIT complex to the peripheral ring of an early podosome structure. Alternatively, this binding of Capn4 may activate the complex, leading to onsite cytoskeletal manipulation and podosome assembly. Results in
Figure 3-1 Novel Pathway for Capn4 in Podosome Dynamics. \( \mu \)- and m-Capn activity regulates the disassembly phase (k\(^{-} \)) of podosomes (right pathway). Capn4 plays a novel role in podosome assembly (k\(^{+} \)) which may be through a calcium-dependent interaction with \( \beta \)-PIX (left pathway).
Figure 3-2 Proposed Model for Capn4 Regulation of Podosome Dynamics. (A) Upon a local influx of calcium at a focal adhesion site, Capn4 binds $\beta$-PIX in complex with PAK and GIT. (B) Once bound, these proteins translocate to an early podosome increasing the assembly rate or, alternatively, initiation of podosome assembly occurs at the focal adhesion site. (C) Podosome maturation results in localization of the catalytic subunit, Capn1 or 2, its binding to Capn4, and destabilizing the Capn4-$\beta$-PIX interaction. Proteolytic activities of $\mu$- and m-Capn regulate podosome disassembly.
Chapter 2 indicate the large Capn subunits, Capn1 and 2, are not involved in a PIX-Capn4 complex. Thus, I hypothesize that upon maturation of the podosome, Capn1 or 2 binds Capn4, destabilizing the Capn4-PIX interaction. Binding of the large and small subunits activates Capn proteolytic activity at podosomes, resulting in cleavage of various substrates including cortactin, N-Wasp, and talin. Cleavage of these calpain substrates increases the disassembly rate, as F-actin branching is destabilized.

This model highlights the requirement of Capn4 in the podosome assembly phase and disassembly phase, indicating the importance of Capn in podosome dynamics and, thus, cell migration and invasion. In light of this model which is in part based on known calcium-requirements for Capn4 interactions \textit{in vitro}, research must be undertaken to determine the affects of calcium concentrations on focal adhesion and podosome dynamics.

3.3 Future Directions

3.3.1 Is Capn4-β-PIX Binding Required for Podosome Assembly

Capn4 is required for podosome assembly independent of its protease regulatory role. Colocalization of Capn4 and β-PIX at discrete podosome sites, \textit{in situ} interaction between these proteins, and proof of direct binding \textit{in vitro} implicate this novel interaction as a potential regulator of podosome assembly. To determine the validity of this hypothesis, nuclear magnetic resonance could be used to elucidate the minimal binding region of the β-PIX-Capn4 interaction. Mutation of required residues could be performed on either
protein followed by exogeneous *in situ* expression to resolve the function of this interaction.

**3.3.2 Proteins involved in Capn4-β-PIX complex**

β-PIX mediated PAK and GIT localization to podosome sites is required for the assembly of these cytoskeletal structures and further modulation of their dynamics. Capn4 pulls down from cell lysate with TAP-tagged β-PIX in an interaction of, as of yet, unknown function. To determine whether Capn4 interacts with β-PIX while in complex with its binding partners PAK and GIT it will be essential to pull down Capn4 and clarify the other non-Capn proteins with which it interacts. Upon comparison with β-PIX associated proteins, a potential binding complex can be implicated.

**3.3.3 Is Capn4 Regulation of Podosome Dynamics Calcium-Dependent**

Capn4 contains 6 Ca$^{2+}$ binding sites to which the binding of Ca$^{2+}$ results in structural changes found to modulate μ- and m-Capn activity. Results from this study indicate the β-PIX-Capn4 interaction is also dependent upon Ca$^{2+}$ binding. As interactions with these Capn4 binding partners may play regulatory roles in the disassembly and assembly phases of podosomes, respectively, it will be important to test the effects on podosome dynamics of Ca$^{2+}$ binding to Capn4. Addition of calcium ionophores, as well as exogeneous expression of Capn4 EF-hand mutants in Src-transformed fibroblasts will help to determine these effects.
3.3.4 Roles of Capn1 and Capn2 in Podosome Dynamics

While µ- and m-Capn have similar structure and function in vitro differences in their in situ and in vivo functions still remain undetermined. Previous studies reporting calpain proteolytic activity to be involved in podosome disassembly have been undertaken through the use of pharmacological peptide aldehyde inhibitors and a 27mer calpastatin peptide, both of which inhibit µ- and m-Capn activity. However, no work has been performed to elucidate the individual roles of these proteases in regulation of podosome disassembly. To shed light on the individual roles of µ- and m-Capn on Src-induced podosome dynamics, I have created NIH-3T3-Src cell lines stably expressing shRNA directed to Capn1, Capn2, Capn1 and 2, and Capn4. Characterization of these cell lines will be required to determine the redundancies and disparities between µ- and m-Capn function in regulation of podosome disassembly.

3.3.5 Functional Significance of Capn4 Domain V

No previous studies have reported an in situ or in vivo pull-down of Capn2 with full length Capn4. However, my research shows disruption of Capn2 binding upon addition of a FLAG-tag to the NH2-terminal end of full length Capn4. Sequential truncations of Capn4 at increasing residue numbers followed by pull-down and immuno-fluorescent localization experiments will assist in determining the functional significance of domain V in vivo.
3.4 Conclusions
Proteolytic activity of μ- and m-Capn regulates the disassembly phase of podosomes, thus influencing cell migration and invasion. Our findings implicate the μ- and m-Capn small regulatory subunit Capn4 plays a role in the assembly phase of podosomes. Furthermore, this role is independent of its protease regulatory role, indicating Capn4 is involved in a novel interaction required for podosome assembly. I conclude that Capn4 may interact with β-PIX at sites of podosome assembly to regulate podosome dynamics in a Ca^{2+} dependent manner.
Appendix

Figure A1 pWZL-Src-Y527F. This image is taken from Addgene website (www.addgene.org).
**Figure A2** pEGFP-Actin. This image is taken from the Clontech website (www.clontech.com).
Figure A3 pFLAG-CMV-4-Capn4.
Reference List


Ref Type: Generic


