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Title: Cytoskeletal Regulation in Cell Motility and Invasion

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Jeffrey A. Greenwood

Cell motility and invasion are important for development, immunity, wound healing, and tumor cell metastasis. Cells on two dimensional substrates migrate in three steps: protrusion of the front end, translocation of the cell body, and retraction of the rear end. For cells to migrate efficiently, these steps need to be well organized, and the actin cytoskeleton plays a critical role in each step. One of the actin structures important for cell motility is the stress fiber, a bundle of contractile actin filaments required for translocation of the cell body. Invasion or migration in three dimensional environments requires specialized actin rich membrane protrusions, called invadopodia, which regulate proteolytic activities that remodel the extracellular matrix for invasion. The purpose of this dissertation is to better understand the mechanisms regulating the actin cytoskeleton

during cell motility and invasion. The first specific aim of this study was to elucidate the mechanism of how cysteine-rich protein 1 (CRP1) binds or bundles actin, and is localized to actin stress fibers. Using CRP1 mutants in cosedimentation assays and fluorescence microscopy, we showed that LIM1 and glycine-rich region 1 (GR1) of CRP1 are required for actin binding/bundling and localization to actin stress fibers. This is the first report of the function of the glycine-rich region, unique to CRPs, in regulating the cytoskeleton, and LIM1/GR1 as a stand alone actin binding/bundling domain. The second specific aim was to determine the role of calpain 2 in glioblastoma cell invasion. Using a calpain 2 knockdown cell line and cell permeable calpain inhibitors in transwell assays or scratch assays, we demonstrated that calpain 2 activity is required for glioblastoma cell invasion, but not migration. Using gelatin zymography, calpain 2 was shown to be important for maintaining the level of extracellular pro-MMP2 and MMP2. These data clearly demonstrate that calpain 2 is required for glioblastoma cell invasion via regulation of invadopodia associated MMP2. Overall, the work in this thesis has advanced our understanding of actin cytoskeletal regulation in stress fibers and invadopodia important for the extracellular matrix remodeling and tumor cell invasion.

# Cytoskeletal Regulation in Cell Motility and Invasion

by

Hyo Sang Jang

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Hyo Sang Jang, Author

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# CONTRIBUTION OF AUTHORS

Hyo Sang Jang designed research under the supervision of Jeffrey A. Greenwood; Hyo Sang Jang performed research and analyzed data; Sangeet Lal was involved in designing, performing, and analyzing the transwell invasion assays with glutamate and preparing samples for gelatin zymography; Jeffrey A. Greenwood and Hyo Sang Jang wrote the papers in chapters 2 and 3.

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# Cytoskeletal Regulation in Cell Motility and Invasion

## Chapter 1

## General Introduction

## 1.1 Background and Significance

### 1.1.1 Cell Migration and Actin Cytoskeleton

Cell migration is essential for embryo development, inflammation, wound healing, immunity, and tumor cell invasion and metastasis [9,11,12,13,14]. For cells to migrate efficiently, three major steps need to be well organized: protrusion of leading edge, retraction of tailing edge, and movement of cell body [15]. In all of three steps, the regulation of the actin cytoskeleton plays critical roles. The actin cytoskeleton can be regulated at various levels; nucleation, polymerization, capping, severing, bundling, and crosslinking [16,17,18]. Disruption of the actin cytoskeleton regulation results in aberrant cell motility. For example, cell polarity and directed cell motility were shown to be impaired by interfering with actin polymerization [19]. The actin cytoskeleton consists of actin microfilaments and actin associated proteins. Filamentous actin or F-actin, which is a linear polymeric form of monomeric globular actin or G-actin, forms various structures including stress fibers and meshwork. A stress fiber is a bundle of F-actin, and is required for generating contractile force in cell body translocation (Fig. 1-1). The actin meshwork is an orthogonal network of F-actin, which is frequently found near the leading edge of a cell. Formation of these various actin structures is organized by actin associated proteins: actin bundling and crosslinking proteins as well as many accessory proteins. One of the major actin bundling proteins is α-actinin, which not only bundles F-actin to make stress fibers but also connects actin microfilaments to membrane bound integrins, which will in turn bind to extracellular matrix [20]. Cysteine-rich protein 1 (CRP1) is one of the α-actinin interacting proteins [21], and how CRP1 is localized to actin stress fibers is

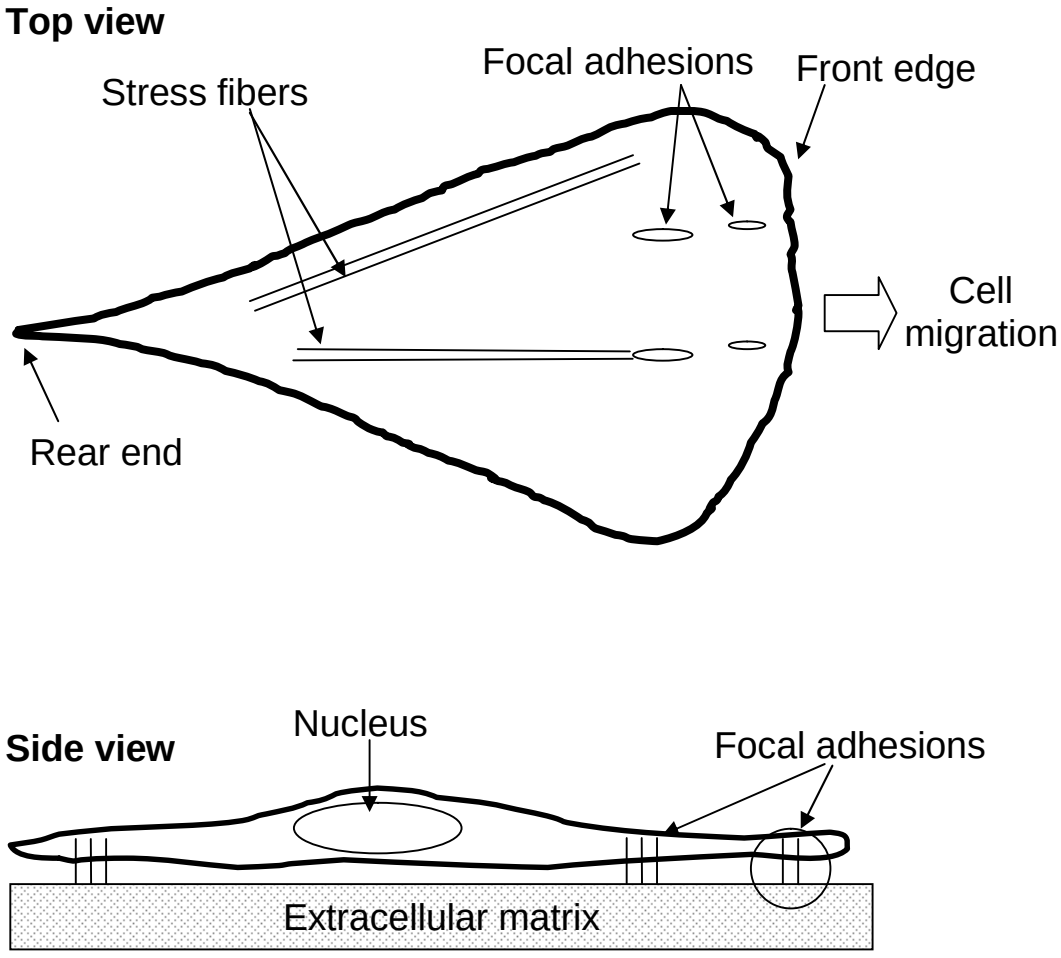


Figure 1-1. Diagram showing a migrating cell. In the top view, cytoskeletal structures that are important for migration on two dimensional surface are depicted: stress fibers and focal adhesions. Stress fibers are bundles of filamentous actin and have been shown to be critical for cell migration due to its contractility. Focal adhesions are integrin mediated structures that connect actin cytoskeleton to extracellular matrix and work as a signaling center. Focal adhesion dynamics are important for efficient cell migration; focal adhesions need to be formed in the front edge and disassembled in the rear end. In the side view, focal adhesions are illustrated as lines showing the physical contact between integrin receptors and the extracellular matrix.

unclear. Greenwood and colleagues showed that CRP1 can directly bind or bundle F-actin [22]. To further our understanding of how CRP1 is localized to stress fibers, we present data that shows the critical actin binding/bundling regions within CRP1 and its importance for the CRP1 localization to actin stress fibers.

### 1.1.2 Cysteine-Rich Protein 1 (CRP1) is an Actin Binding/Bundling Protein

CRP1 is a member of cysteine-rich protein family that has two LIM domains connected by a flexible linker region [23,24]. The LIM domain, named after three founding proteins lin-11, Isl-1, and mec-3, is comprised of two zinc fingers, which are tightly packed via hydrophobic interaction [25,26,27,28]. The LIM domain mediates protein-protein interaction, but two LIM domains in CRP1 have been shown to be independent units [26,29,30]. The CRP family is unique in that a glycine-rich region is located adjacent to each LIM domain [23,24]. Also, CRP members have two distinct locations in a cell: nucleus and actin cytoskeleton. Supposedly, CRPs have different functions depending on their locations. For example, nuclear CRP2 plays the role of a transcription factor. More specifically, CRP2 bridges two different transcription factors SRF and GATA, which are important for muscle cell differentiation [31]. CRP3, or muscle LIM protein, is important for the normal development and functioning of heart [32]. However, how CRP1 functions in the actin cytoskeleton remains to be clarified. Greenwood and colleagues showed that CRP1 is an independent actin binding and bundling protein as well as an α-actinin interacting protein [33]. As a first step to define the mechanism of CRP1 localization to the actin cytoskeleton, the actin binding/bundling

region was mapped using truncation forms and point mutants. To study the CRP1 population in stress fibers, rat embryonic fibroblast was used. Fibroblasts are the best model system for the study because they develop many strong stress fibers in the cell and stress fibers can be easily imaged by microscopy. In fibroblasts, yellow fluorescent protein (YFP)-tagged CRP1 was utilized to show that the glycine-rich region 1 is required for the CRP1 localization to actin microfilaments as presented in chapter 2.

### 1.1.3 Adhesion Formation in the Leading Edge of a Cell and De-Adhesion in the Tailing Edge

In mammals as well as other multicellular organisms, cells are in contact with other cells or surrounding matrices via molecular complexes. Also, *in vitro*, animal cells attach to the surface of culture dish by way of focal adhesions or focal adhesive complexes, which are integrin mediated linkages of the actin cytoskeleton and extracellular matrices [34]. In fact, almost 200 proteins have been proposed to comprise structural and functional networks centered on integrin mediated adhesions [35,36]. Some of the important adhesion proteins include talin, vinculin, and α-actinin to name a few. Talin [1,37], vinculin [38], and α-actinin [39,40] are connecting cytosolic microfilaments to membrane bound integrin molecules, and in turn, these integrin molecules interact with extracellular matrices (Fig. 1-2). Assembly and disassembly of focal adhesions are dependent on these focal adhesion protein interactions. Also, the focal adhesions change their size, locations, and molecular compositions according to the stage of adhesion formation [41]. For example, a nascent adhesion is smaller and located closer to the leading edge of a cell than a mature adhesion, and some proteins such as α-actinin are

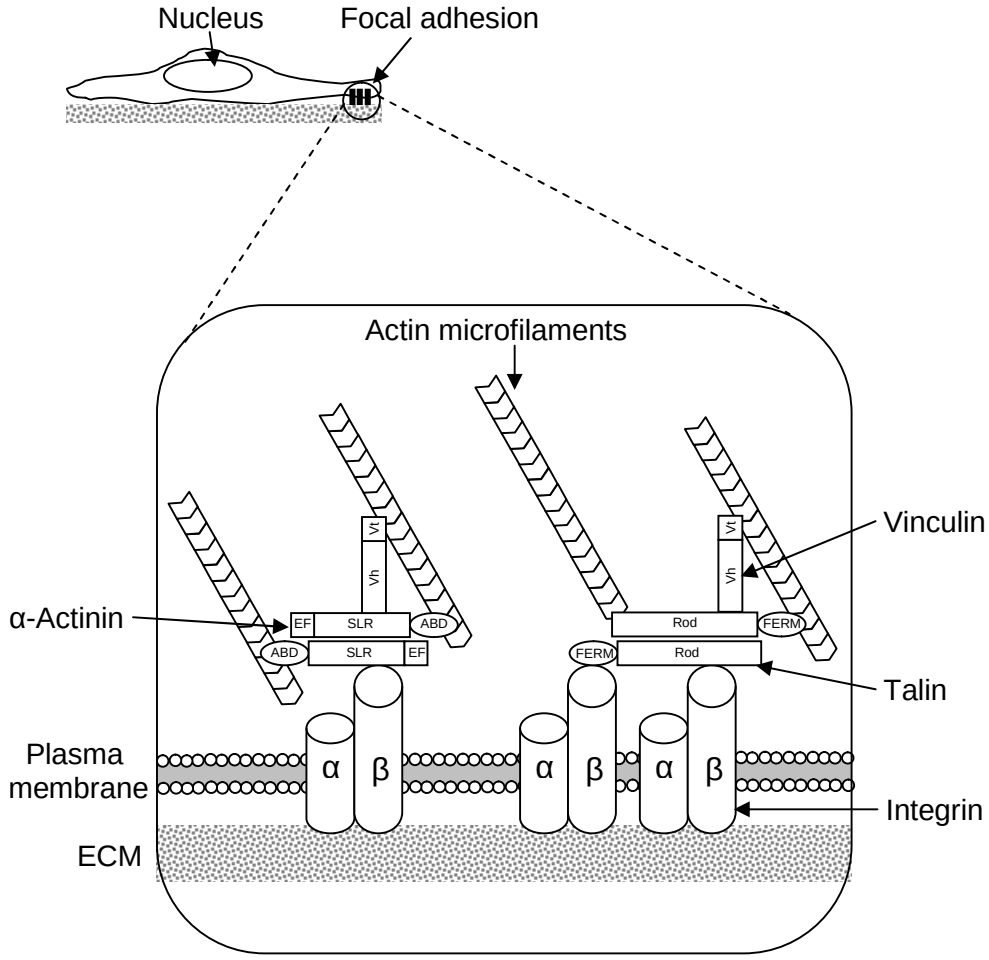


Figure 1-2. Diagram showing a focal adhesion. Focal adhesions are integrin mediated linkages of actin microfilaments and extracellular matrices. α-Actinin, an anti-parallel homodimer, binds to the β-integrin cytoplasmic domain via a spectrin-like repeat (SLR), and the N-terminal actin binding domain (ABD) binds to F-actin. The C-terminal EF hands domain (EF) binds to calcium ions. The N-terminal globular head domain, FERM, of talin binds to the β-integrin cytoplasmic domain [1]. The anti-parallel talin homodimer [2] also binds to F-actin via two distinct domains, FERM and the C-terminal rod domain (Rod). Vinculin binds to the SLR of α-actinin or the talin Rod domain via the N-terminal head domain (Vh). The C-terminal tail domain of vinculin (Vt) binds to F-actin.

acquired during the maturation of an adhesion. Focal adhesions of course are important for cell migration. In migrating cells, small membrane protrusions or lamellipodia are happening randomly, and only one of these is stabilized, thus giving directional persistence to the migrating cell [42]. Stable formation of focal adhesions will contribute to the stabilization of membrane protrusion. At the same time, focal adhesions in the tailing edge should be disassembled to allow cells to retract the tail and to translocate the cell body. Calpain, a family of calcium dependent proteases, has been shown to regulate cell adhesion and migration by regulating focal adhesion composition and disassembly of the focal adhesions [8,43]. For example, talin is focal adhesion protein cleaved by calpain, and if calpain resistant mutant talin is introduced into a cell, focal adhesion disassembly is inhibited in fibroblasts [43]. The effect of calpain 2 knockdown on talin proteolysis in glioblastoma cells will be examined in chapter 3. The third chapter of this thesis will further demonstrate the critical role of calpain 2 in cancer cell invasion.

### 1.1.4 Cells Require Invasion Specific Mechanisms for Migration in Three Dimensional Environment

When adherent cells are plated on a rigid surface, they migrate on a two dimensional surface probably going through the three major steps of cell migration as discussed before: protrusion, translocation, and retraction. However, most of cells *in vivo* are in three dimensional environments. In other words, they are surrounded by cells and also complex medium called the extracellular matrix (ECM). For cells to migrate efficiently in three dimensional space, which will be referred to as invasion throughout this thesis, remodeling of this cell-to-cell contact and the ECM would be required besides

the three steps of migration on two dimensional space [44]. The ECM consists of many different proteins and carbohydrates, and supports the cells physically. During invasion, the ECM may act as a barrier or provide guidance to invading cells depending on what type of migration strategy the cells are taking [45,46]. One of the ECM remodeling enzymes is matrix metalloproteinase (MMP), which is important for development and tumor cell invasion [47]. One of MMP's functions is the degradation of the ECM thereby making enough space for cells to migrate through. Consequently, MMP activity is more important for three dimensional migration or invasion than two dimensional migration. In tumor cells, MMP activities are concentrated at specialized membrane protrusions called invadopodia, thereby making the proteolysis occur more efficiently. Invadopodia diagram and some of major MMP isoforms found in glioblastoma cells are presented in Fig. 1-3. Invadopodia and MMP will be described in detail later in this chapter.

### 1.1.5 Invadopodia are Critical Structure for Tumor Cell Invasion

Invadopodia are specialized membrane extensions that are associated with extracellular or membrane-localized proteolytic activities and is frequently found in invading tumor cells [48,49]. Actually, invadopodia have been shown to be important for tumor cell invasion [50]. Invadopodia are dynamic structures and according to the size and molecular compositions, four distinct stages were proposed for invadopodia formation [51]; the initiation of invadopodia, pre-invadopodia, mature invadopodia, and late invadopodia. Briefly, actin and cortactin are enriched in the initiation step, and the level goes up slightly in the second and the third steps. As the membrane bound MT1-

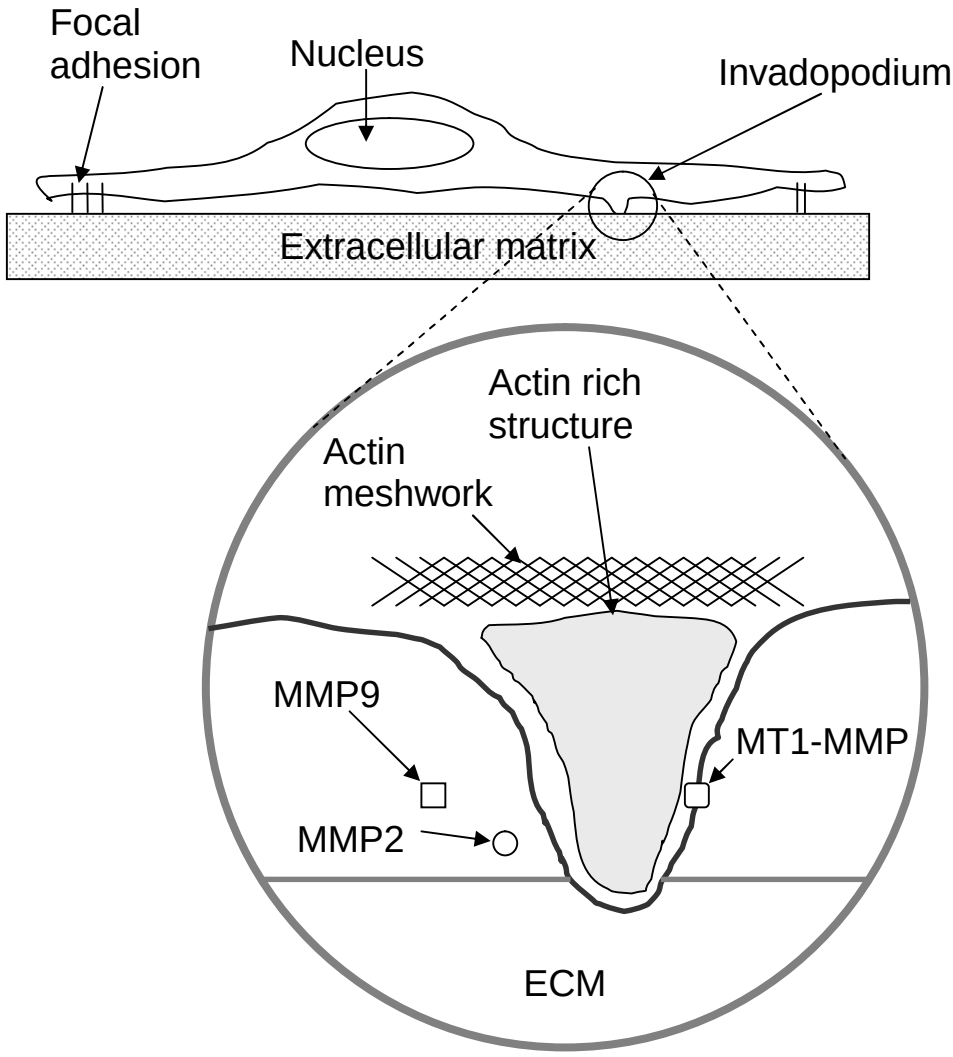


Figure 1-3. Diagram showing an invadopodium. Invadopodia are specialized membrane protrusions that are enriched with actin microfilaments. In the base of those actin rich structures, actin meshwork is supporting the structures. Invadopodia are also associated with proteolytic activities, which are important for migration through the three dimensional extracellular matrix. In glioblastoma cells, MMP2 has been shown to be one of major isoforms of secreted MMP along with MMP9. MMP2 is secreted as an inactive zymogen form (pro-MMP2) and activated by other activating proteases such as membrane-bound MT1-MMP, which cleaves the auto-inhibitory N-terminus of pro-MMP2.

MMP (or MMP14) level goes up in the second stage, proteolysis of the ECM begins, and the proteolytic activity becomes highest in the mature invadopodia, the third stage. In the last stage of late invadopodia, actin and cortactin are leaving the invadopodia leaving MT1-MMP in invadopodial membrane. As proposed in the four stage model, cortactin is one of the most important proteins for invadopodial structure and function. Cortactin, isolated as tyrosine phosphorylated proteins in v-src transformed fibroblasts, is a multi-domain protein (Fig. 1-4A). The N-terminal acidic domain (NTA) binds to Arp2/3 complex and initiates actin polymerization [52]. Also, cortactin directly binds to F-actin through its repeat. Cortactin has been shown to be required for invadopodia formation and ECM degradation in fibroblasts transfected with constitutively active form of Src [53]. Also, cortactin has been proposed to play an important role in exocytosis by targeting secretory vesicles from Golgi to invadopodia [54]. Endogenous cortactin can be visualized by immunostaining cells with anti-cortactin antibody (Fig. 1-4B, C). As mentioned earlier, cortactin is colocalized to actin rich invadopodia, and intense staining of cortactin is observed near nuclei of U87MG glioblastoma cells (Fig. 1-4B, C) as compared to several discrete cortactin staining in breast cancer cells [55]. Much effort has been put into figuring out the regulatory mechanism of invadopodia formation or maturation. Besides cortactin, another key regulator of invadopodia in glioblastoma is calpain 2, which was shown to regulate invadopodia dynamics and breast cancer cell invasion [55]. Interestingly, calpain 2 was proposed to carry out dual roles: invadopodia formation and disassembly. Calpain 2, activated by EGF signaling, turns on Src kinase activity, thereby activating cortactin and stimulating cortactin formation. On the other

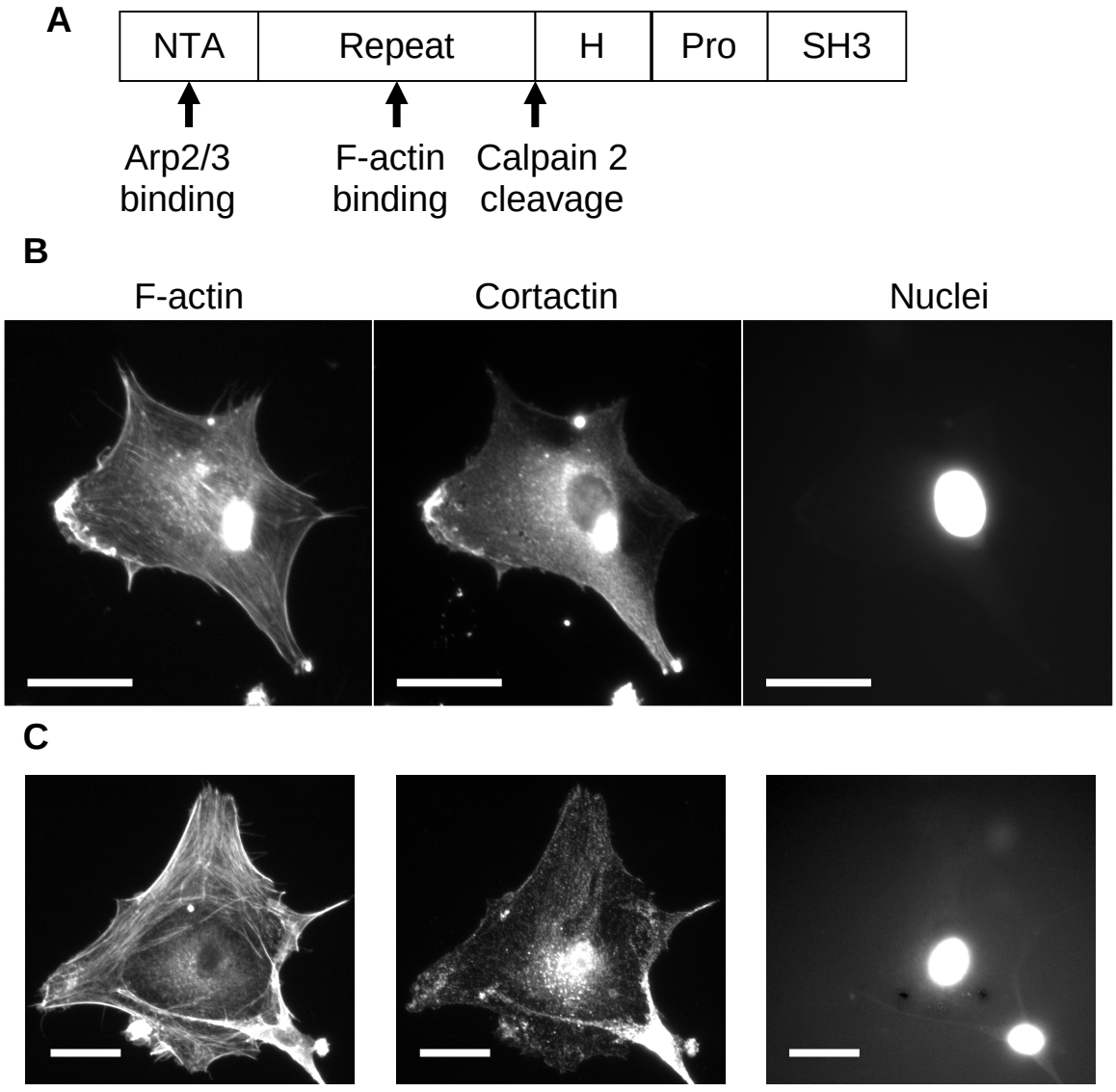


Figure 1-4. Cortactin is colocalized with actin rich membrane protrusions, invadopodia, in U87MG glioblastoma cells. (A) Domain structure of cortactin is presented. NTA is the N-terminal acidic domain. Repeat is the random repeat of six copies of 37 amino acids. H is the helix region. Pro is the proline rich domain. SH3 is src homology 3 domain. Cortactin was visualized by immunostaining of U87MG glioblastoma cells transfected with control shRNA plasmid (see Experimental Procedures in Chapter 3 for stable cell line generation). Briefly, cells grown on glass coverslips were fixed (B) or fixed and extracted with a detergent (C). Then, cells were treated with anti-cortactin antibody, followed by treatment with goat anti-mouse IgG FITC conjugate. F-actin was stained with rhodamine phalloidin, and nuclei were stained with DAPI. Bar = 30 μm.

hand, cortactin is the substrate of calpain 2 [56] (Fig. 1-4A). Thus, cleavage of cortactin by calpain 2 is thought to result in invadopodia disassembly in breast cancer cells. In chapter 3, the effect of calpain 2 knockdown on cortactin proteolysis will be discussed.

### 1.1.6 Matrix Metalloproteinases are Important for Tumor Cell Invasion

Matrix metalloproteinase is a family of proteolytic enzymes that requires zinc ion for the catalytic activity, and this enzyme family has more than 20 members, which differ in their substrate specificity, location, and structure [47]. MMPs are expressed in the inactive zymogen form, and activated by proteolysis of its N-terminus. Also, there are MMP inhibitors called tissue inhibitors of metalloproteinases (TIMPs), which comprise the MMP system in the cell.

When calpain 4, the small subunit of calpain, is disrupted in mice, neither calpain 1 or calpain 2 activity was detectable [57]. In embryonic fibroblasts of calpain 4 deficient mice that were immortalized by SV40 large T antigen, both MMP2 and uPA (urokinase type plasminogen activator) were not expressed at the level of mRNA and protein. Also, endogenous calpain inhibitor calpastatin derived peptide reduced the mRNA expression of both MMP2 and MMP9 in leukemic THP-1 cells [58]. In addition, there are many evidences showing the requirement of calpain activity or expression for MMP expression [59]. However, there are few reports showing isoform specific requirement of calpain for MMP expression. In chapter 3, we are going to show a calpain 2 requirement for MMP2 expression and activity in glioblastoma cells. We will focus on MMP2 in this thesis because MMP2 is one of the major secreted MMP isoforms expressed in glioblastoma

cell line U87MG [60], and MMP2 is required for three dimensional migration, but not two dimensional migration [61,62].

Since both MMP2 and MMP9 are two MMP isoforms that have gelatinolytic activity, their extracellular activities can be visualized by modified *in situ* gelatin zymography. Fig. 1-5 shows the pericellular gelatinolytic activities of U87MG glioblastoma cells. Most of the activity was observed near nuclei of cells.

### 1.1.7 Calpain is Calcium Activated Protease

Calpain is a family of calcium dependent cysteine proteases that have as many as 14 isoforms in mammals [63]. Calpain has been reported to be crucial for cell motility. Calpain is required for the regulation of adhesion complexes and for migration of a cell [64]. Furthermore, calpain has been shown to play a role in cell spreading, rear detachment, and membrane protrusion [65,66]. The requirement of calpain for tumor cell migration is also well documented for breast cancer [55], prostate cancer [67], and human fibrosarcoma [46]. Based on the above reports, we began to determine the connection of the autocrine glutamate signal and calpain 2 activation in glioblastoma cell migration and invasion.

The ubiquitous forms of calpain are calpain 1 and calpain 2. Both calpain isoforms are heterodimers of an 80 kDa catalytic subunit and a 27 kDa regulatory subunit, with the 27 kDa subunit, calpain 4, being common in both isoforms. When calpain 4 is disrupted in mice, the embryo was unable to develop normally and died during embryogenesis, even though calpain 4 deficiency was not affecting the proliferation of embryonic stem

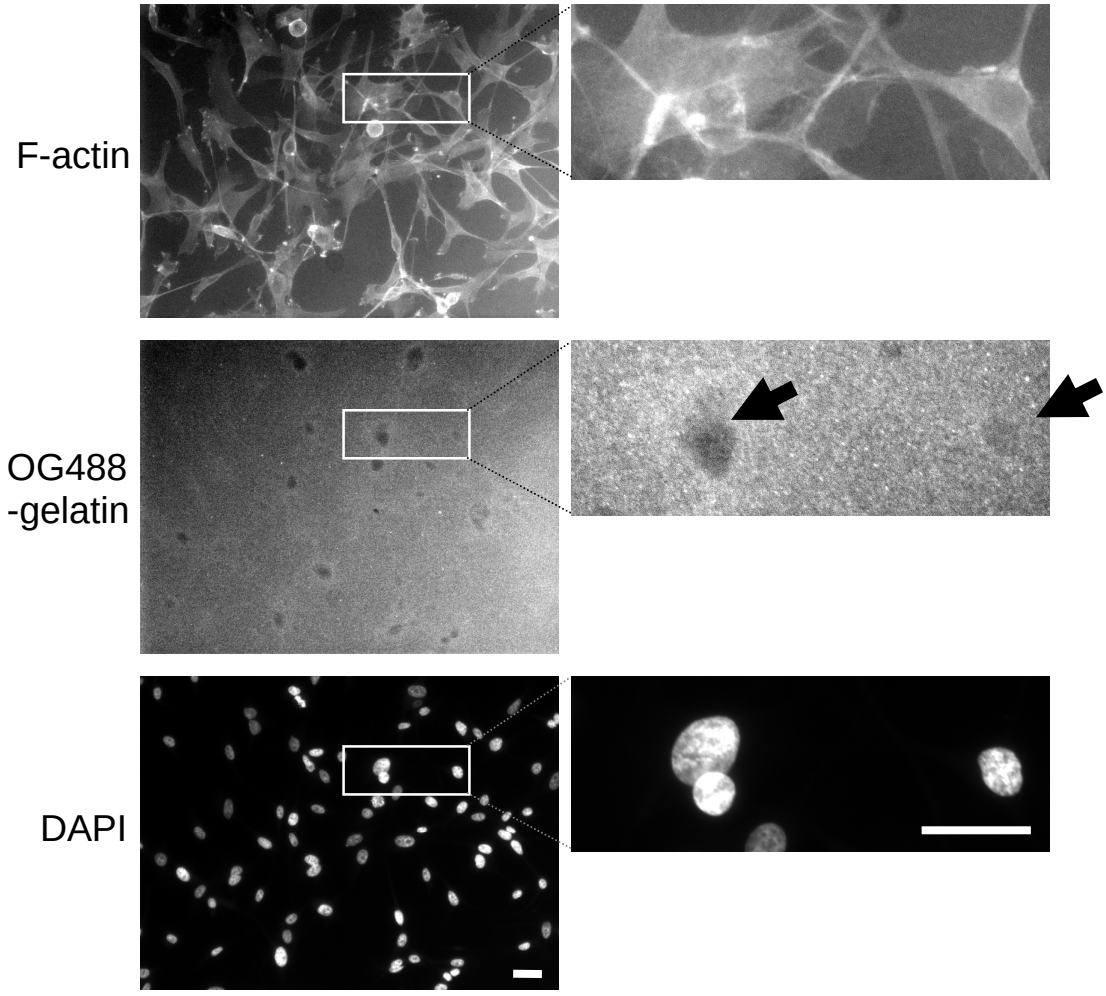


Figure 1-5. U87MG glioblastoma cells express the extracellular gelatinolytic activity. Pericellular proteolytic activities were visualized by gelatin zymography. Briefly, cells were plated onto Oregon Green 488-gelatin coated coverslips and incubated for 48 h. After fixing cells, the actin cytoskeleton was stained with rhodamine phalloidin, and the coverslips were mounted using DAPI containing antifade agent to locate nuclei. The images were captured using a cooled CCD camera (CoolSNAP-HQ, Photometrics, Tucson, AZ) connected to a fluorescence microscope Axiovert 100 (Carl Zeiss Thornwood, NY). Degradation of gelatin by extracellular proteases leaves dark regions (arrows) on a bright background. Bar = 30 μm.

cell or fibroblasts [57]. Homozygous disruption of calpain 2 led to preimplantation lethality suggesting the critical role of calpain 2 in embryogenesis [68]. Calpain 4 knockout cells were used to figure out the proteins whose expression has been changed compared to wild type cells, and some proteins were identified by two dimensional protein gel and mass spectrometry [69]. Even though calpain 4 is a regulatory subunit for both calpain 1 and calpain 2, it was also shown that calpain 4 has additional functions in regulating cell-substrate mechanical interaction [70].

### 1.1.8 Autocrine Glutamate is Important for Glioblastoma Cell Invasion

Glutamate is excitatory and toxic to neuronal cells. Thus, glutamate is quickly absorbed by glial cells. However, glioblastoma cells, which derived from glial cell population, are not absorbing glutamate but actually secreting glutamate using cystine glutamate exchanger system $Xc^-$ [5,71]. As briefly mentioned earlier, glutamate can kill neuronal cells and this was thought to be one function of secreted glutamate: killing the surrounding neuronal cells and making room for tumor cell expansion. Recently, Sontheimer and colleagues showed that glutamate can also work as an autocrine/paracrine signal for glioma cells [4]. More specifically, glutamate secreted from glioma cells is binding to AMPA receptor of its own or neighboring cells, inducing calcium influx (Fig. 1-6) [4]. Once glutamate export is blocked, glioblastoma cells can not migrate or invade efficiently. Another glutamate receptor NMDA receptor is not expressed in U87MG glioblastoma cell line, leaving AMPA receptor as a main glutamate

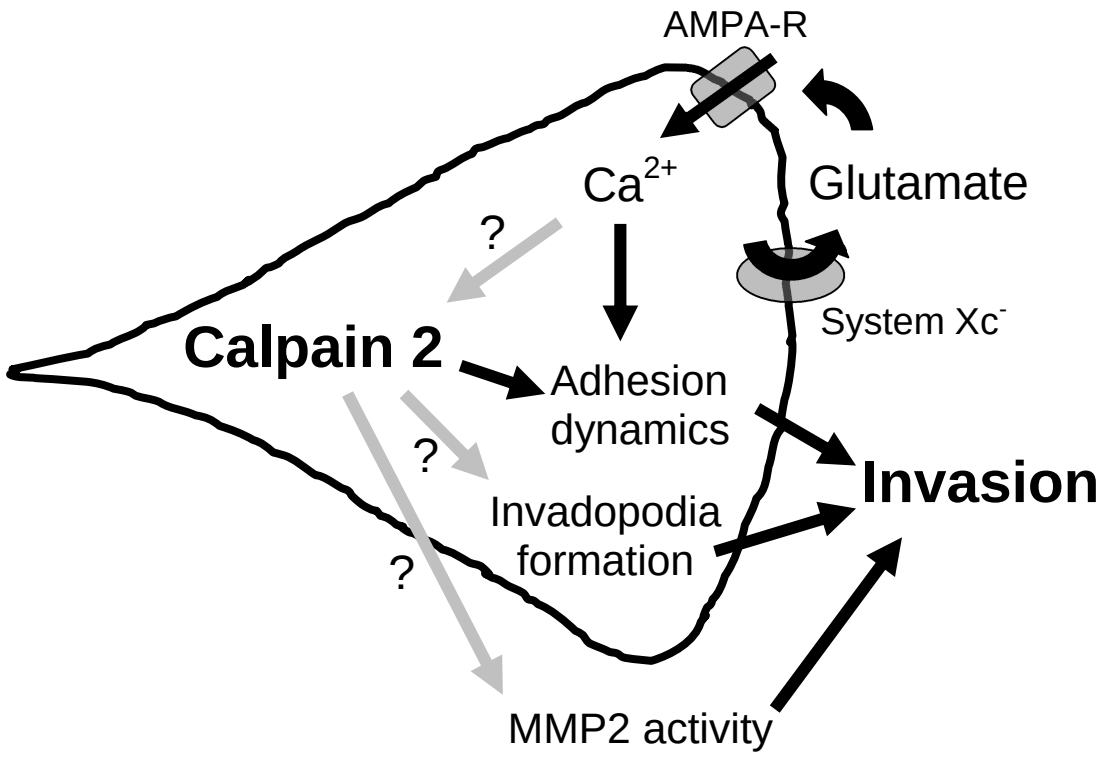


Figure 1-6. Autocrine glutamate is important for glioblastoma cell motility and invasion. Autocrine glutamate is secreted via system $Xc^-$ and binds to AMPA receptor (AMPA-R) stimulating calcium influx. Calcium has been shown to disrupt focal adhesions, but its downstream effectors have not been identified in glioblastoma cell motility. The calcium dependent protease calpain is one of the potential targets of calcium, and has been shown to regulate adhesion dynamics and cell motility. In this dissertation, we presented data that support the role of calpain 2 for glioblastoma cell invasion, but not migration, partly by regulating secreted MMP2 level.

receptor. AMPA receptor antagonist was also shown to be inhibitory to cell growth and motility [3].

Glioblastoma multiforme (GBM) is the most aggressive form of brain tumors [72,73]. The median survival time is no longer than 12 months, and only 5% of patients can survive for 5 years [74]. Once a patient is diagnosed with glioblastoma, as much tumor mass as possible is removed by surgical operation, and radiation therapy, chemotherapy, or the combination of the two are administered. But, usually, tumor is recurring near the resection cavity in about 12 months, and the patient dies shortly after the recurrence [74]. Among many characteristics of the tumor, invasiveness is a hallmark for malignant gliomas. Much of the effort has been put into defining the mechanism of invasion of brain tumor. But, the full understanding of the mechanism has yet to be obtained. Glutamate is known to be excitotoxic to neuronal cells and sequestered by astrocytes or microglia [75]. Surprisingly, however, GBM derived from glial cells secretes glutamate rather than absorbing it through system $Xc^-$, cystine-glutamate transporter present in glial cell [5]. Just by inhibiting the glutamate transporter, neurodegeneration and brain edema can be reduced [76]. Also, glutamate, an autocrine factor for glioblastoma, was shown to enhance glioblastoma cell migration and invasion via calcium oscillation [4,6]. Calcium oscillation generated through calcium permeable AMPA receptor has been shown to be critical for growth and migration of human glioblastoma cells [3]. One group also showed that calcium oscillation associated with integrin signaling led to focal adhesion disassembly in human U87 astrocytoma cells [7].

Taken together, these reports show the importance of glutamate signaling and calcium oscillation in the glioblastoma cell migration and invasion.

There are other important evidences that support the critical role of calpain 2 in glioblastoma other than calcium influx. First, EGF receptor expression is amplified in glioblastoma cells, and calpain 2 has been shown to be activated by direct phosphorylation by ERK in EGF signaling pathway [77,78,79]. Second, PI3 kinase pathway is unregulated in glioblastoma cells, because PTEN is mutated and PI3K is activated. As a result, PIP3 level is presumably increased. PIP3 can enhance the susceptibility of calpain substrates by modulating the conformation and exposing the hidden calpain cleavage sites in substrate proteins [80,81,82,83]. Thus, EGF receptor overexpression, activated PI3K signaling pathway, and constant calcium influx all point to the increased calpain activity in glioblastoma cells.

## 1.2 Dissertation Hypotheses and Aims

Actin cytoskeleton regulation is central to efficient cell migration, which is critical for many biological processes including development, wound healing, immunity, and tumor cell invasion and metastasis. Actin microfilaments are bundled together to form stress fibers which confer contractility to the migrating or invading cells. Also, located at the ends of stress fibers are focal adhesions, which connect the actin cytoskeleton to extracellular matrices via integrins, and need to be regulated for efficient cell migration. As described earlier in this chapter, cells require invasion specific mechanisms to efficiently migrate through three dimensional spaces, which is abundant in extracellular

matrices imposing a physical barrier to invading cells. To better understand how cells migrate in two dimensional and three dimensional spaces, we focused on the actin binding/bundling and cytoskeleton localization mechanism of CRP1 in the first specific aim. In the second specific aim, we focused on the role of calpain 2, calcium dependent protease, for glioblastoma cell invasion.

Specific aim 1. Determine the localization mechanism of CRP1 to actin stress fibers in fibroblast. – The working hypothesis is that glycine-rich region is required for CRP1 bundling of F-actin and localization to stress fibers. To test this hypothesis, we generated a series of truncation forms, domain swap mutant, and point mutant. Using these mutants in cosedimentation assays, we showed both LIM1 and glycine-rich region 1 are required for actin binding and bundling. Furthermore, using fibroblasts transiently transfected with YFP-tagged CRP1 in fluorescence microscopy, we demonstrated that the glycine-rich region 1 is also required, but not sufficient for CRP1 localization to stress fibers.

Specific aim 2. Determine the role of calpain 2 in glioblastoma cell migration and invasion. – The working hypothesis for this aim is that calpain 2 is required for glioblastoma cell invasion. To test the hypothesis, we first used calpain 2 targeting shRNA to knock down the expression in U87MG glioblastoma cell line, and examined invasion and migration by transwell assays and scratch assays. In these experiments, calpain 2 is shown to be required for glioblastoma cell invasion, but not migration. Cell permeable calpain inhibitors were also used to show that calpain activity is required for invasion. Calpain 2 was also shown to be important for proteolysis of adhesion proteins talin and filamin, but calpain 2 knockdown did not affect the morphology and the actin

cytoskeleton of glioblastoma cells. These results led us to hypothesize that calpain 2 is required for invasion specific processes. To test this hypothesis, we examined the proteolysis of cortactin, which is required for invadopodia formation and functions. As expected, calpain 2 is important for cortactin proteolysis. Furthermore, extracellular matrix metalloproteinase presented by invadopodia was visualized by gelatin zymography. Calpain 2 knockdown influences the level of extracellular MMP2 in invasive three dimensional environments.

# Chapter 2

## Glycine-Rich Region Regulates Cysteine-Rich Protein 1 Binding To Actin Cytoskeleton

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## 2.1 Abstract

Cysteine-rich protein 1 (CRP1) has a unique structure with two well separated LIM domains, each followed by a glycine-rich region. Although CRP1 has been shown to interact with actin-binding proteins and actin filaments, the mechanism regulating localization to the actin cytoskeleton in cells is not clear. Experiments using truncated forms showed that the first LIM domain and glycine-rich region are necessary for CRP1 bundling of actin filaments and localization to the actin cytoskeleton. Furthermore, domain swapping experiments replacing the first glycine-rich region with the second resulted in the loss of CRP1 bundling activity and localization to the actin cytoskeleton, identifying 7 critical amino acid residues. These results highlight the importance of the first glycine-rich region for CRP1 bundling activity and localization to the actin cytoskeleton. In addition, this work identifies the first LIM domain and glycine-rich region as a distinct actin filament bundling module.

Keywords:

Actin bundling

Cysteine-rich protein

Glycine-rich region

LIM domain

## 2.2 Introduction

Cysteine-rich proteins (CRPs) have been implicated in cell differentiation, cytoskeletal remodeling, and transcriptional regulation [23,29]. CRPs consist of two LIM domains (LIM1, LIM2) and are localized to the nucleus and the actin cytoskeleton. LIM domains are double zinc finger regions demonstrated to mediate protein-protein interactions. Whereas most LIM domains are linked by 8-10 amino acids, CRPs have a unique flexible linker consisting of more than 50 amino acids [23]. In addition, each LIM domain of the CRPs is uniquely followed by a glycine-rich region (GR1, GR2) (Fig. 2-1A). Although nuclear CRPs are important for transcriptional regulation involved in cell differentiation, the function of the cytoskeletal population of CRPs is not well defined. Previously, we demonstrated that CRP1 regulates the actin cytoskeleton by directly bundling actin microfilaments [33]. However, CRP1 has also been shown to interact with α-actinin and zyxin [21,84,85,86,87,88]. With the potential to interact with actin filaments and actin binding proteins, it is not clear if CRP1 localization to the actin cytoskeleton is dependent on interactions with α-actinin or zyxin. In order to understand the function of the cytoskeletal population, it is necessary to determine the mechanism by which CRP1 associates with the actin cytoskeleton in cells.

LIM domains have recently been reported to constitute a novel class of actin bundling modules [89]. In this study, we used truncation forms to determine which regions of CRP1 are required for actin bundling activity and localization to the actin cytoskeleton in cells. Our results limit the actin bundling activity of CRP1 to the LIM1 domain and extent the functional unit to include the glycine-rich region. Furthermore,

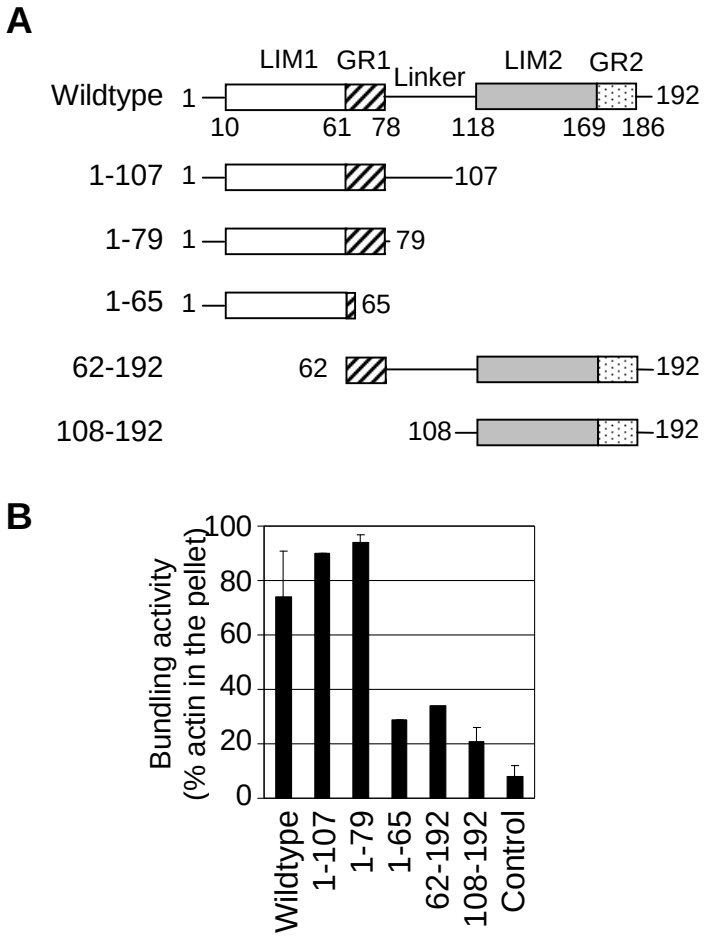


Figure 2-1. Residues 1-79 containing LIM1/GR1 are required for CRP1 bundling of F-actin. (A) Diagram of CRP1 structure illustrates the relative position of the LIM domains, glycine-rich regions (GR), and the flexible linker between the N- and C-terminal halves of the protein. Five truncation forms of CRP1 were generated to determine the regions involved in binding to actin filaments and localization to the actin cytoskeleton. CRP1 truncations forms, expressed in *E. coli* and purified by Ni-NTA agarose, were incubated with equimolar (10 µM) F-actin for 30 min at room temperature and centrifuged at 10,000 x g for bundling assay. The supernatant (S) and the pellet (P) were separated and analyzed by SDS-PAGE, and quantified by densitometry (B). n = 1-4 + SD.

we demonstrate that CRP1 associates with the actin cytoskeleton and focal adhesions in cells primarily as a result of direct interaction with actin filaments and independent of α-actinin and zyxin.

## 2.3 Materials and Methods

### 2.3.1 Proteins and DNA Constructs

Chicken CRP1 cDNA was a generous gift of Mary C. Beckerle (University of Utah, Salt Lake City, UT). 6xHis-tagged CRP1 and truncation mutants, domain swap mutants, and full-length mutant were generated using PCR, cloned into *E. coli* expression vector pPROEX HTc (Invitrogen, Carlsbad, CA), and confirmed by nucleotide sequencing. Proteins were purified using Ni-NTA agarose (QIAGEN, Valencia, CA). Purified proteins were dialyzed against 10 mM HEPES buffer, pH 7.0, 50 mM NaCl, and the concentration determined by measuring the absorbance at 280 nm. For the full-length CRP1 proteins and the truncation mutant 1-65 to be soluble, it was necessary to remove the 6xHis tag by cleavage with AcTEV protease (Invitrogen).

Each of the CRP1 proteins was also cloned into the animal expression vector pEYFP-C1 (Clontech, Mountain View, CA) and confirmed by nucleotide sequencing. Transfection of rat embryonic fibroblasts and fluorescence microscopy were carried out as previously described [33]. Briefly, rat embryonic fibroblasts were transfected using FuGENE6 (Roche, Indianapolis, IN) according to the manufacturer's protocol, and fixed in 3% formaldehyde containing 0.5% Triton X-100 for 30 min at room temperature. Actin was detected by staining cells with Alexa Fluor 350 phalloidin (Invitrogen). Images were captured using a CCD camera (CoolSNAP HQ, Photometrics, Tucson, AZ) attached to Carl Zeiss Axiovert 100S microscope.

### 2.3.2 Cosedimentation Assay

Cosedimentation assays were carried out as described previously [33,90]. Briefly, 10 μM F-actin (Cytoskeleton, Denver, CO) was incubated with 10 μM CRP1 at room temperature for 30 min followed by centrifugation at 10,000 x g for 30 min and the proteins from the supernatant and pellet separated by SDS-PAGE. Proteins were detected by staining with GelCode Blue Stain (Pierce, Rockford, IL), and quantified using a KODAK Image Station 440CF.

## 2.4 Results and Discussion

### 2.4.1 Direct Binding to Actin Filaments Mediates CRP1 Localization to the Cytoskeleton

CRP1 has been shown to localize to the nucleus, focal adhesions, actin stress fibers, and membrane ruffles [21,31,33,84,86]. A key step toward understanding the function of cytoskeletal-associated CRP1 is determining the mechanisms by which these populations are localized to actin filaments. *In vitro*, CRP1 has been demonstrated to bind directly to actin filaments and the actin-binding proteins zyxin and α-actinin [21,33,84,85,86,87,88]. However, it is not clear which of these interactions is responsible for the localization of CRP1 to the actin cytoskeleton and adhesion complexes in the cell. Previously, we found that CRP1 not only bound actin filaments directly, but had the potential to bundle actin filaments *in vitro* [33]. Based on these results, we proposed that CRP1 localization to the cellular cytoskeleton was mediated by direct interaction with actin filaments. To test this hypothesis, five truncation forms were constructed to determine the region of CRP1 required for the binding to and bundling of actin filaments (Fig. 2-1A). Filamentous actin binding and bundling was measured using an *in vitro* cosedimentation assay as previously described [33,90]. The cosedimentation assays were carried out with each of the truncation forms and the actin filament bundling activity of CRP1 was localized to residues 1-79 (Fig. 2-1B). Identical results were observed in actin filament binding assays in which cosedimentation was carried out at 100,000 x g (data not shown). Removal of just an additional 14 residues, comprising a majority of the GR1, resulted in the loss of binding and bundling (Fig. 2-1B). These results suggested that GR1 was mediating the interaction of CRP1 with actin filaments. However, when the

GR1 was expressed with the C-terminal portion of CRP1 (truncation form 62-192), little or no binding or bundling was observed (Fig. 2-1B). Therefore, both the LIM1 domain and GR1 are required for CRP1 to bind and bundle actin filaments *in vitro*. To determine the region regulating the localization of CRP1 to the actin cytoskeleton and focal adhesions in cells, the truncation forms were expressed in fibroblasts as YFP-tagged fusion proteins. To improve imaging of the actin cytoskeleton and associated proteins, cells were fixed in a buffer containing 0.5% Triton X-100 which extracts a majority of the cytosolic protein [33,91]. Consistent with previous reports, CRP1 was observed in the nucleus, along actin stress fibers, and in focal adhesions (Fig. 2-2). Also in agreement with prior studies [21], the fusion protein containing residues 1-107 of CRP1 was observed to have the same localization as the wildtype protein (data not shown). Most importantly, as observed in the actin filament binding and bundling assays, residues 1-79 were required for localization to the actin cytoskeleton (Fig. 2-2). The fusion protein containing residues 1-65 of CRP1 was almost entirely extracted with the Triton X-100 soluble cytosolic fraction leaving only the CRP1 population in the nucleus (Fig. 2-2). Images similar to residues 1-65 were observed for the fusion proteins containing the C-terminal regions of CRP1 with no cytoskeletal localization detected (data not shown). These results demonstrating that the LIM1 domain and GR1 are required for the direct binding of CRP1 to actin filaments and the localization of CRP1 to the actin cytoskeleton support the hypothesis that CRP1 localization to the actin cytoskeleton occurs through direct interaction with actin filaments.

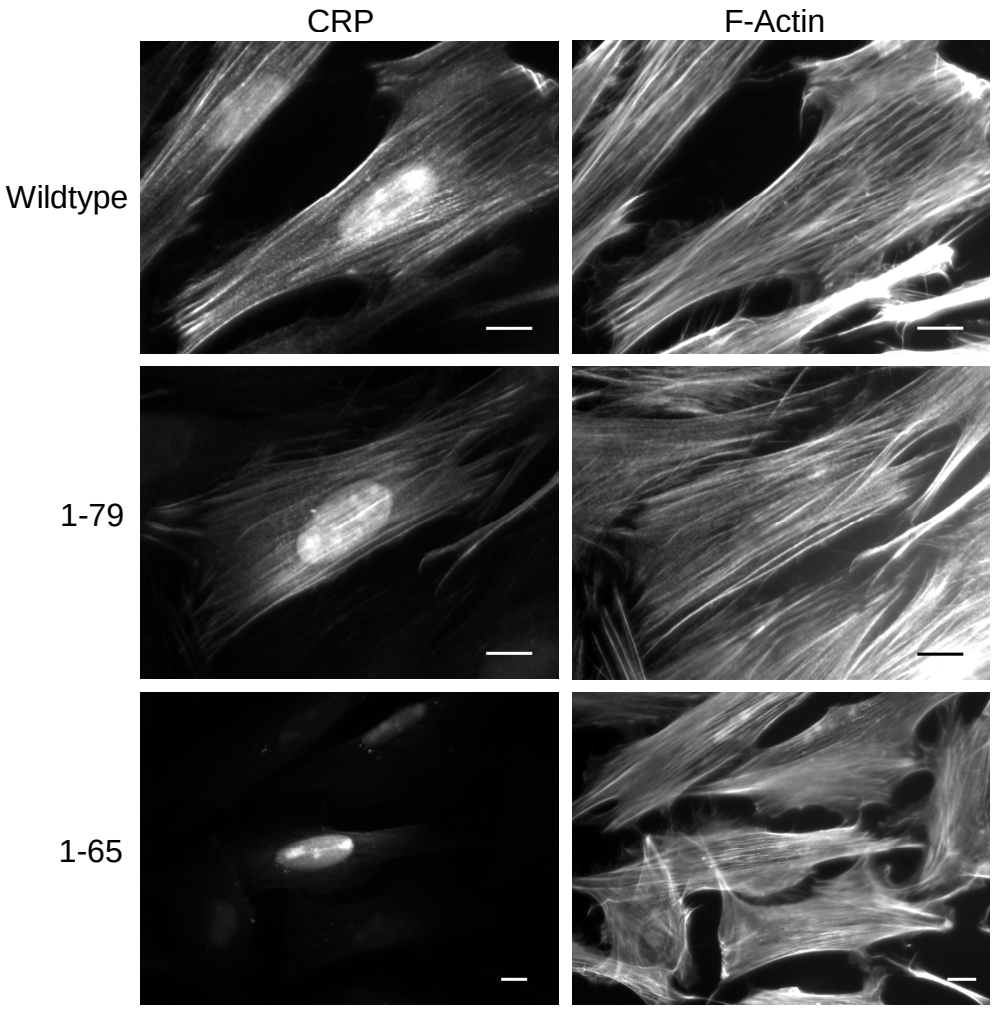


Figure 2-2. LIM1/GR1 is required for the localization of CRP1 to actin stress fibers. Rat embryonic fibroblasts expressing YFP-CRP1, YFP-CRP1 residues 1-79, and YFP-CRP1 residues 1-65, were fixed and stained with Alexa Fluor 350 phalloidin (F-actin) for fluorescence microscopy as described under Materials and Methods. Results are representative of 2-6 experiments. Bar = 10 μm.

Since CRP1 has also been shown to bind directly to α-actinin and zyxin *in vitro* [21,84,85,86,87,88], it was important to consider if CRP1 was associated with the actin cytoskeleton via one of these actin-binding proteins. CRP1 binding to zyxin requires the LIM2 domain [88]. Therefore, since the localization of CRP1 to the actin cytoskeleton is independent of the LIM2 domain, zyxin could not be responsible for the localization of CRP1 to the actin cytoskeleton. However, GR1 has been reported to mediate the interaction of CRP1 with the actin-binding domain of α-actinin *in vitro* [85]. Furthermore, mutation of lysine 65 to asparagine (K65N) was shown to reduce CRP1 binding to α-actinin to less than 10% of that observed for the wildtype protein [85]. To determine the role of α-actinin in the localization of CRP1 to the actin cytoskeleton, we expressed the CRP1 mutant tagged with CFP in fibroblasts. The K65N mutation had no effect on the localization of CRP1 to the actin cytoskeleton (data not shown). In addition, we were unable to detect any *in vitro* binding between CRP1 and the actin-binding domain of α-actinin in the presence of physiological salt concentrations (data not shown). Grubinger and Gimona [92] reported similar results showing that CRP2 binding to α-actinin was very weak under physiological salt concentrations. Collectively, the evidence presented above demonstrates that localization to the actin cytoskeleton and focal adhesions occurs primarily through direct interaction of CRP1 with filamentous actin.

### 2.4.2 CRP1 Localization to the Actin Cytoskeleton Requires the Non-Glycine Residues of GR1

Although, many LIM domain containing proteins have been shown to directly or indirectly associate with the actin cytoskeleton, it is not clear if a common mechanism is

involved in regulating this interaction [93]. The glycine-rich regions are a unique feature of the CRP family of LIM domain proteins [23]. Therefore, it is intriguing that GR1 is required for LIM1/GR1 binding to actin filaments. Understanding the role of GR1 in binding to actin filaments is important for elucidating the distinct function of CRP1 in regulating the actin cytoskeleton. To determine if the actin filament binding and bundling activity of LIM1/GR1 was specific for GR1, we substituted GR2 for GR1. LIM1/GR2 did not bind or bundle actin filaments *in vitro* (Fig. 2-3A) and did not localize to the actin cytoskeleton when expressed in fibroblasts (Fig. 2-3B). These results demonstrated that the specific amino acid sequence of GR1 was required for LIM/GR1 binding to actin filaments. Only 7 out of the 18 amino acid residues differ between GR1 and GR2 (Fig. 2-4A). Ironically, the domain swapping experiment demonstrated that the 7 non-glycine residues of the glycine-rich GR1 were required for LIM1/GR1 binding to actin filaments. When the 7 non-glycine residues were mutated in the full-length CRP1 protein, actin filament binding and bundling were reduced by 50% *in vitro* (data not shown). Furthermore, mutation of the 7 non-glycine residues within GR1 resulted in a significant decrease in the localization of the full-length CRP1 mutant to the actin cytoskeleton (Fig. 2-4B).

Since GR1 was not sufficient for binding to actin filaments, we propose that the GR1 plays a role in stabilizing the structure or regulating the orientation of LIM1 so that it can interact with filamentous actin. The LIM1/GR1 and LIM2/GR2 of CRP1 are

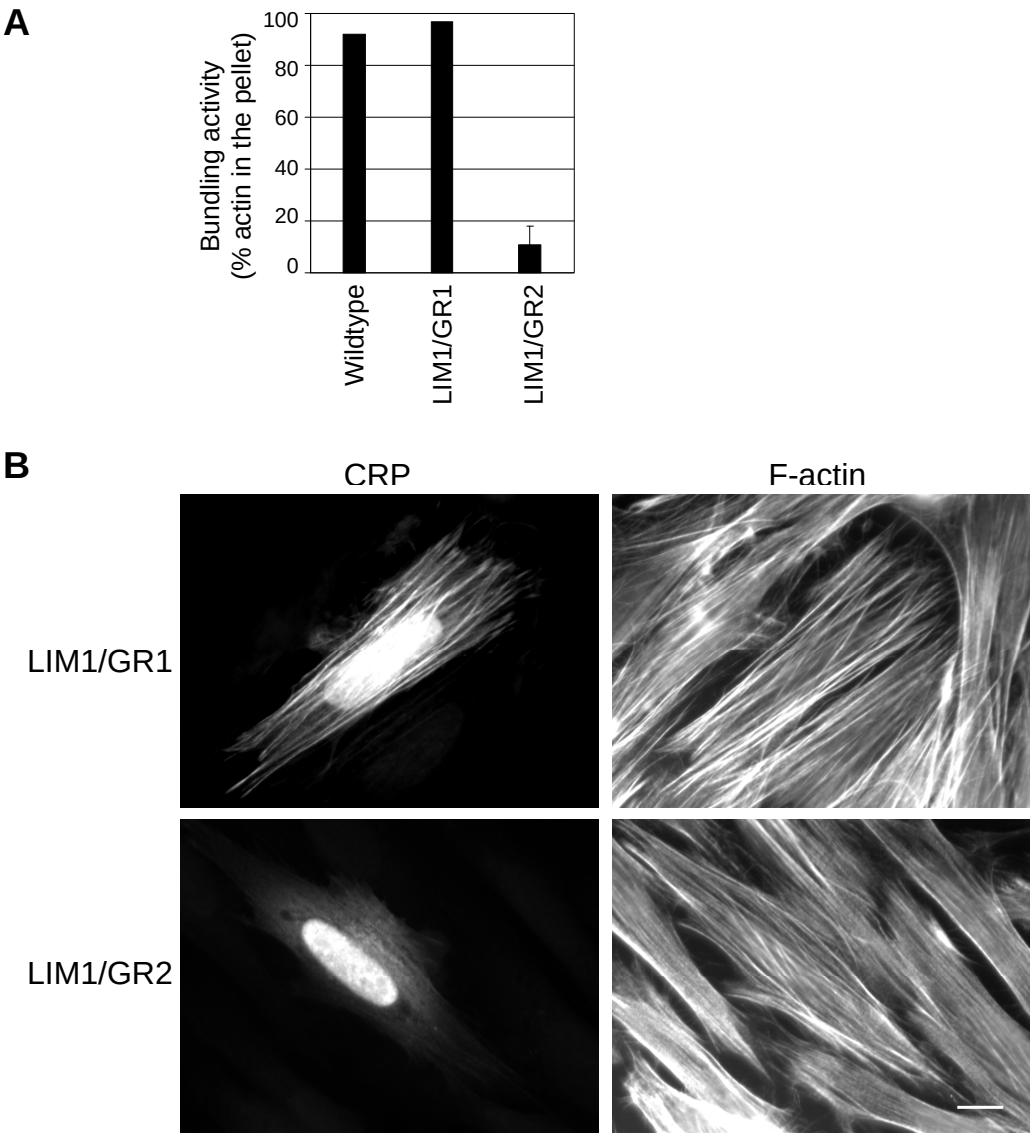


Figure 2-3. Glycine-rich region 1 (GR1) is required for the actin bundling activity of LIM1/GR1 and the localization to actin stress fibers. (A) To determine the requirement of GR1 for actin bundling and localization, GR1 of LIM1/GR1 was replaced by GR2 to make LIM1/GR2, which was purified with N-terminal His tag in *E. coli*, and F-actin bundling activity measured using the cosedimentation assay described under Materials and Methods. (B) Rat embryonic fibroblasts, transfected with either YFP-tagged LIM1/GR1 or LIM1/GR2, were fixed with 3% formaldehyde and 0.5% Triton X-100, and stained with Alexa Fluor 350 phalloidin (F-actin). Bar = 10 μm.

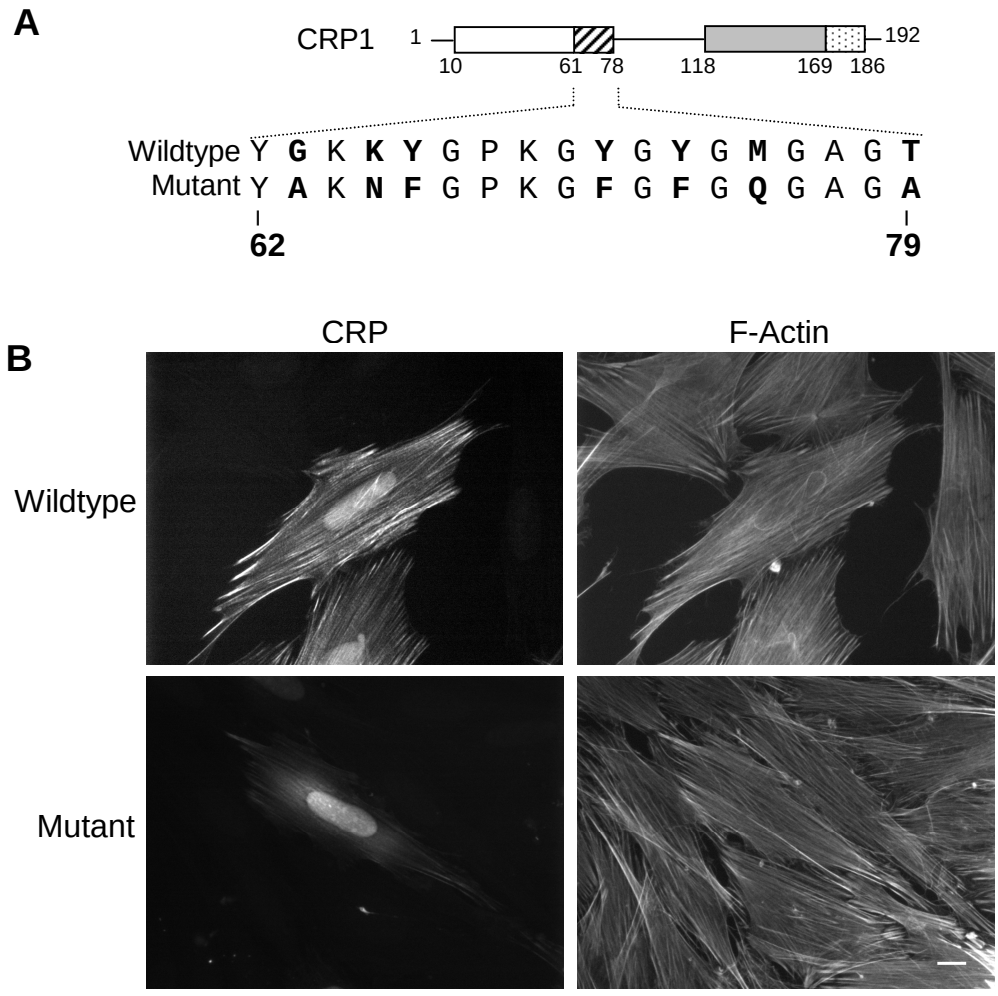


Figure 2-4. Non-glycine residues of GR1 are important for the localization of CRP1 to the actin cytoskeleton. (A) Based on the GR1/GR2 domain swapping experiments, the seven residues unique to GR1 were mutated as shown in bold. (B) Rat embryonic fibroblasts were transfected to express either YFP-tagged wildtype or mutant CRP1, fixed with 3% formaldehyde and 0.5% Triton X-100, and stained with Alexa Fluor 350 phalloidin (F-actin). Bar = 10 μm.

separated by a long flexible region of 40 amino acid residues. The GR1 may provide an important buffer between the flexible linker and the functional LIM1 domain. In summary, we have identified the LIM1/GR1 of CRP1 as a unique actin filament binding module which regulates the localization of CRP1 to the actin cytoskeleton.

The abbreviations used are: CRP, cysteine-rich protein; GR, glycine-rich region; Ni-NTA, nickel nitrilotriacetic acid; YFP, yellow fluorescence protein; CFP, cyan fluorescence protein; F-actin, filamentous actin; TEV, tobacco etch virus.

# Chapter 3

## Calpain 2 is Required for Glioblastoma Cell Invasion: Regulation of Matrix Metalloproteinase 2

Hyo Sang Jang, Sangeet Lal, and Jeffrey A. Greenwood

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## 3.1 Abstract

Glioblastoma is a rapidly progressing form of brain cancer with treatment options of limited success. The highly invasive nature of cells from glioblastoma tumors complicates surgical removal decreasing the potential impact of this therapeutic approach with recurrence of the tumor mass typically within months. The progression and likely outcome of this form of brain cancer highlights the importance of understanding the mechanisms of dispersal and characteristics of the invasive population of glioblastoma cells. The induction of calcium fluxes into glioblastoma cells by autocrine glutamate has been identified as a process critical for invasion. However, the intracellular target(s) by which calcium acts to stimulate the dispersal of glioblastoma cells is not clear. In this study, we tested the hypothesis that the calcium-activated protease calpain 2 is required for glioblastoma cell invasion. Using shRNA, calpain 2 was knocked down by greater than 80% in U87MG glioblastoma cells. In Matrigel coated transwell invasion assays, 90% less invasion was observed for the calpain 2 knockdown cells compared to controls. Interestingly, decreased expression of calpain 2 did not appear to have any influence on morphology or migration, suggesting regulation of an invasion specific mechanism. Consistent with this idea, 36% less extracellular MMP2 was measured in the media from the knockdown cells identifying the regulation of matrix metalloproteinases as one mechanism by which calpain 2 is involved in glioblastoma cell invasion. This is the first report to demonstrate that calpain 2 is required for glioblastoma cell invasion.

Keywords: Autocrine, Calcium influx, Calpain, Glioblastoma, Glutamate, Invasion, Matrix metalloproteinase

## 3.2 Introduction

Glioblastoma, which accounts for 20% of all primary brain tumors and is the most malignant form of brain cancer, is a devastating diagnosis with a 5-year survival rate of less than 4% [94]. Although the mechanisms responsible for the carcinogenesis and progression of glioblastoma tumors are unknown, several genes mutations have been identified in glioblastoma cells. Mutations resulting in inactivation of the PtdIns (3,4,5)-$P_3$ phosphatase PTEN have been detected in 31% of glioblastoma cells lines [95]. Mutations resulting in the activation of the p110α isoform of PI 3-kinase have been observed in 27% of glioblastomas [96]. In addition, Kubiatowski *et al.* [97] presented evidence correlating PI 3-kinase signaling with glioma cell invasion in rat brain implants. The results from these and other studies have identified a role for PtdIns (3,4,5)-$P_3$ in the migration and invasion of glioblastoma cells. We recently demonstrated that PtdIns (3,4,5)-$P_3$ binding regulates the susceptibility of the cytoskeletal adhesion protein α-actinin to proteolysis by calpain 2 [98], identifying a potential mechanism by which the PI 3-kinase pathway may influence cell adhesion and movement. However, the role of calpain 2 in glioblastoma cell migration and invasion has not been studied.

The first connection between calpain and cancer was identified by Shiba *et al.* [99], reporting increased levels of calpain activity in breast cancer tissues. A study by Kimura *et al.* [100] followed observing calpain-mediated proteolysis of the cytoskeletal protein merlin in nervous system tumors such as schwannomas and meningiomas. In addition, Braun *et al.* [101] showed a correlation between the expression of calpain 1 mRNA and metastasis of human renal cell carcinoma. More recent studies have further

established a role for calpain in both carcinogenesis and tumor progression [102,103,104,105,106,107]. In 2003, Mamoune *et al.* [67] published a study demonstrating that leupeptin inhibition of calpain activity or antisense down-regulation of calpain 2 decreased invasiveness of human prostate tumor cells by ~50% in an *in vivo* xenograft model. In addition, calpain 2 knockdown inhibited breast cancer cell invasion by ~50% regulating the invadopodial projections necessary for movement through the extracellular matrix [55]. These results are important because the data demonstrate that targeting calpain 2 during tumor invasion is a potential treatment for specific cancers.

Recent studies have identified unique factors involved in the regulation of glioblastoma cell migration and invasion that point to calpain 2 as a possible modulator required for glioblastoma cell migration and invasion. More specifically, autocrine glutamate was demonstrated to regulate the migration and invasion of glioblastoma cells [4,6]. These results are important because glutamate activates AMPA receptors on glioblastoma cells stimulating calcium influxes promoting migration [3]. In addition, Giannone *et al.* [7] have shown that calcium oscillations are required for focal adhesion disassembly in glioblastoma cells. The targets of calcium that are required for glioblastoma cell invasion in response to autocrine glutamate are not known. However, calpain 2 is a likely candidate.

In this study, we tested the hypothesis that the calcium-activated protease calpain 2 is required for glioblastoma cell invasion. Using calpain inhibitors and shRNA based knockdown of calpain 2, we demonstrate that calpain 2 expression and activity are required for glioblastoma cell invasion, but not migration. Furthermore, analysis of

matrix metalloproteinases (MMP) showed that calpain 2 is important for maintaining the level of extracellular MMP2 for glioblastoma cells. This is the first report demonstrating the requirement of calpain 2 for the invasion of glioblastoma cells.

## 3.3 Experimental Procedures

### 3.3.1 Antibodies and Reagents

Actin antibody (clone AC-40), talin antibody (clone 8d4), glutamate and gelatin were purchased from Sigma (St. Louis, MO); calpain 1 antibody (RP3) and calpain 2 antibody (RP1) were from Triple Point Biologics (Forest Grove, OR); cortactin antibody (clone 4F11) was from Millipore (Temecula, CA); filamin antibodies (E-3 and H-300) were from Santa Cruz Biotech (San Jose, CA); and peroxidase-conjugated goat anti-mouse IgG (H+L) antibody and peroxidase-conjugated goat anti-rabbit IgG (H+L) antibody were from Jackson ImmunoResearch Laboratories (West Grove, PA). Calpain inhibitor I (ALLN) and calpain inhibitor II (ALLM) were purchased from Calbiochem (La Jolla, CA). Matrigel, fibronectin, collagen type IV, and laminin were purchased from BD Bioscience (Bedford, MA). Transwell permeable supports were obtained from Corning (Corning, NY).

### 3.3.2 Cell Culture and Calpain 2 Knockdown Cell Line

U87MG glioblastoma cells were cultured and maintained as previously described [98]. Calpain 2 targeting short hairpin RNA (shRNA) plasmid was purchased from SABiosciences (Frederick, MD); the nucleotide sequence of calpain 2 shRNA was 5'-GGGCTGAAGGAGTTCTACATT-3' and the control shRNA was 5'-GGAATCTCATTCGATGCATAC-3'. Stable knockdown cell lines were generated according to the instruction of the manufacturer and maintained in the presence of 400

μg/ml Geneticin (Invitrogen, Carlsbad, CA). Transfections were carried out using FuGENE HD (Roche, Indianapolis, IN) according to the manufacturer's protocol.

### 3.3.3 Transwell Invasion Assay

To determine the effect of calpain 2 knockdown on glioblastoma cell invasion, an invasion assay was carried out using transwell permeable supports according to the manufacturer's instruction (Corning). Briefly, Matrigel was diluted to 2 mg/ml using ice-cold serum-free DMEM on ice, and 50 μl of diluted Matrigel were added to a transwell polycarbonate membrane of 6.5 mm diameter and 8.0 μm pore size, followed by 1 h incubation at 37°C. This membrane with a thin layer of Matrigel was transferred to 24 well plate containing 0.65 ml of DMEM plus 10% FBS per well or serum-free DMEM. For the experiment in figure 3-7, 100 μM glutamate was added to the lower well as a chemoattractant. The calpain2 knockdown and control cell lines were maintained in DMEM containing 10% FBS and 400 μg/ml Geneticin. Cells grown to confluence were trypsinized, rinsed twice with serum-free DMEM, and resuspended in serum-free DMEM at a density of $5\times10^5$ cells/ml. 50,000 cells were added to the upper well and incubated for 3 d at 37°C. After the incubation, cells and Matrigel in the upper well were wiped out using cotton swab, and cells on the bottom side of the membrane were fixed with methanol and mounted on slide glass using ProLong Gold antifade reagent with DAPI (Invitrogen) for counting the number of nuclei using a fluorescence microscope. Images were captured using a cooled CCD camera (CoolSNAP-HQ, Photometrics, Tucson, AZ) connected to the Axiovert 100 microscope (Carl Zeiss, Thornwood, NY). The captured

images were analyzed for the number of DAPI stained spots using MetaMorph 6.2 software (Molecular Devises, Downingtown, PA). The threshold was set by automatic thresholding function, and the DAPI stained nuclei were counted automatically by the count cell function of the software.

To determine the requirement of calpain activity for glioblastoma cell invasion, a transwell invasion assay was carried out using calpain inhibitors. U87MG glioblastoma cells were trypsinized, washed twice with serum free DMEM, pre-incubated for 30 min at 37°C with calpain inhibitor I or II and 50,000 cells were plated in Matrigel coated upper wells with appropriate concentration of calpain inhibitor I or II in both upper and lower wells. The following steps were done as described above.

### 3.3.4 Transwell Migration Assay

To determine the effect of calpain 2 knockdown on glioblastoma cell migration, transwell permeable supports were used for a migration assay according to the instruction of the manufacturer (Corning). Extracellular matrix proteins fibronectin, laminin, and collagen type IV were diluted to the concentration of 10 µg/ml with deionized water (for FN and LN) or 0.05 N HCl (for CL), and 50 µl were added onto the bottom surface of the transwell membrane, followed by incubation for 1 h at room temperature. The membrane was then washed twice with 0.1% BSA/serum free media and both the upper and lower surface were blocked by incubating the membrane in 0.1% BSA/serum free media for 30 min. This extracellular matrix protein-coated membrane was put into a 24 well plate containing 0.65 ml of DMEM plus 10% FBS per well. Then, 100,000 cells of the calpain

2 knockdown or control cell line, trypsinized and rinsed twice with serum-free DMEM, were added to the upper well and incubated for 3 h at 37°C. Following steps were done as for the transwell invasion assay.

### 3.3.5 Cell Migration Scratch Assays

Migration was measured using a scratch assay as described [108]. Monolayers of the U87MG cells were scratched using a 1.0 mm wide cell scraper, washed 3 times and incubated with 10% FBS-DMEM. Images of the scratch were recorded at 0 and 24 h as described above. Migration was determined by measuring the distance that the monolayer advanced from the edge of the scratch. Values from 3 regions were averaged to calculate the distance migrated. Distance was measured using MetaMorph 6.2 imaging software.

### 3.3.6 Gelatin Zymogram

MMP2 secreted into glioblastoma culture media was visualized by gelatin zymogram according to the standard protocol [109]. Briefly, 50,000 cells were plated in the Matrigel coated transwells in the absence of serum as in transwell invasion assays, and after 3 d, the culture media were centrifuged for 2 min at 12,000 rpm. The supernatant was mixed with equal volume of Laemmli buffer without reducing agent and separated by electrophoresis on 0.08% (w/v) gelatin containing SDS polyacrylamide gel. The gel was incubated for 1 h at room temperature with renaturing buffer [50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 μM $ZnCl_2$, 5 mM $CaCl_2$, 0.02% (w/v) $NaN_3$, 2.5% (v/v) Triton X-100] and overnight at 37°C with renaturing buffer without Triton X-100. The

gel was stained with Gelcode Blue Stain Reagent (Pierce, Rockford, IL) and destained with water according to the manufacturer's protocol. The clear bands on the blue background were imaged and quantified using a Kodak Image Station 440CF and Molecular Imaging Software version 4.0 (Eastman Kodak, Rochester, NY).

For time course assays, 200,000 cells were seeded in 3 ml of 10% FBS-DMEM containing 400 µg/ml Geneticin in 6 well plate. After incubating for 1 d, cells were rinsed twice with serum free DMEM, and 2.5 ml of serum free DMEM were added to each well. At each time point, 100 µl of the culture media were centrifuged for 2 min at 12,000 rpm, mixed with equal volume of Laemmli buffer without reducing agents, and analyzed by gelatin zymogram as described above.

### 3.3.7 Calcium Ionophore Treatment and Total Cell Lysate Preparation

To examine the calcium-activated proteolysis of calpain 2 substrates talin and filamin, 200,000 cells of control or calpain 2 knockdown cell line were plated in 6 well plate in 10% FBS-DMEM. After incubating cells for 2 d at 37°C in a humidified 5% $CO_2$ incubator, cells were rinsed twice with serum free DMEM and incubated for 1 h at 37°C in 2 ml of serum free DMEM. Culture media were aspirated and replaced with 2 ml of serum free DMEM containing either 10 µM A23187, 4-bromo (Calbiochem, San Diego, CA) or 0.1% (v/v) DMSO as a negative control, followed by 5 min incubation at 37°C. Again, the culture media were aspirated and 200 µl of SDS-containing lysis buffer [0.25 M Tris-HCl pH 6.8, 2% (w/v) SDS, 5 mM EGTA, 5 mM EDTA, 25 mM dithiothreitol,

10% (v/v) glycerol, 0.05% (w/v) bromophenol blue] were added. The lysate was sonicated for about 1~2 sec, boiled for 2 min, and analyzed by Western blot.

## 3.4 Results and Discussion

### 3.4.1 Calpain 2 protease activity is required for glioblastoma cell invasion

We have proposed that calpain 2 is a critical target for calcium signaling regulating autocrine glutamate dependent invasion of glioblastoma cells. To test this hypothesis, we used shRNA to knockdown expression of calpain 2 in U87MG glioblastoma cells. Stable calpain 2 knockdown cell lines were generated by transfecting U87MG cells with calpain 2 shRNA plasmids and selecting in the presence of Geneticin. Calpain 2 expression was decreased by greater than 80% compared to U87MG cells containing control shRNA (Fig. 3-1). Actin was also immunoblotted verifying that equal protein was loaded for each sample. In addition, immunoblotting for calpain 1 was carried out showing the isoform specificity of the shRNA knockdown procedure.

Previously published studies have shown that fibroblasts from calpain 4 knockout mice, the small subunit required for calpain 1 and 2 activity [63], have altered morphology with decreased cell spreading and an increase in process formation [64]. These morphological observations were extended to calpain 2 knockdown fibroblasts [66]. Based on these reports, we expected the morphology of the U87MG calpain 2 knockdown cells to differ from that of control cells. However, to our surprise, we did not observe any morphological differences between the control and calpain 2 knockdown U87MG cells using phase contrast microscopy (Fig. 3-2). In addition, no differences were observed for cells plated on fibronectin and stained for F-actin. These unexpected results led us to examine the proteolysis of the well established calpain 2 substrates talin and filamin in the U87MG cells. Calcium-activated proteolysis was determined by

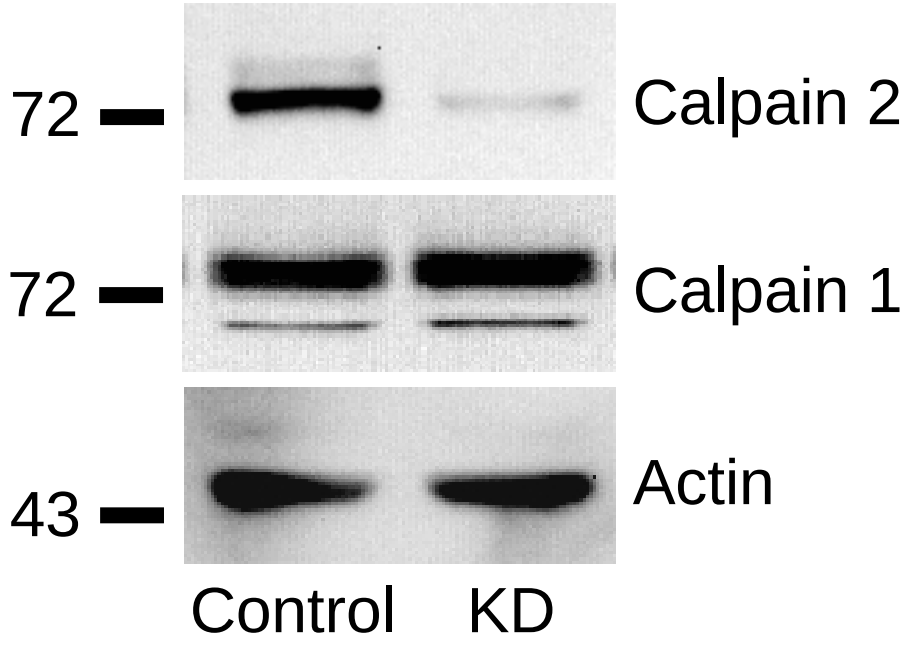


Figure 3-1. shRNA knockdown of calpain 2 in U87MG glioblastoma cells. Calpain 2 knockdown (KD) cells were generated by transfecting U87MG glioblastoma cells with calpain 2 targeting shRNA plasmid followed by selection for stable cells with Geneticin. Expression of calpain 2, calpain 1, and actin were measured by Western blotting of total cell lysates. Results are representative of three separate experiments.

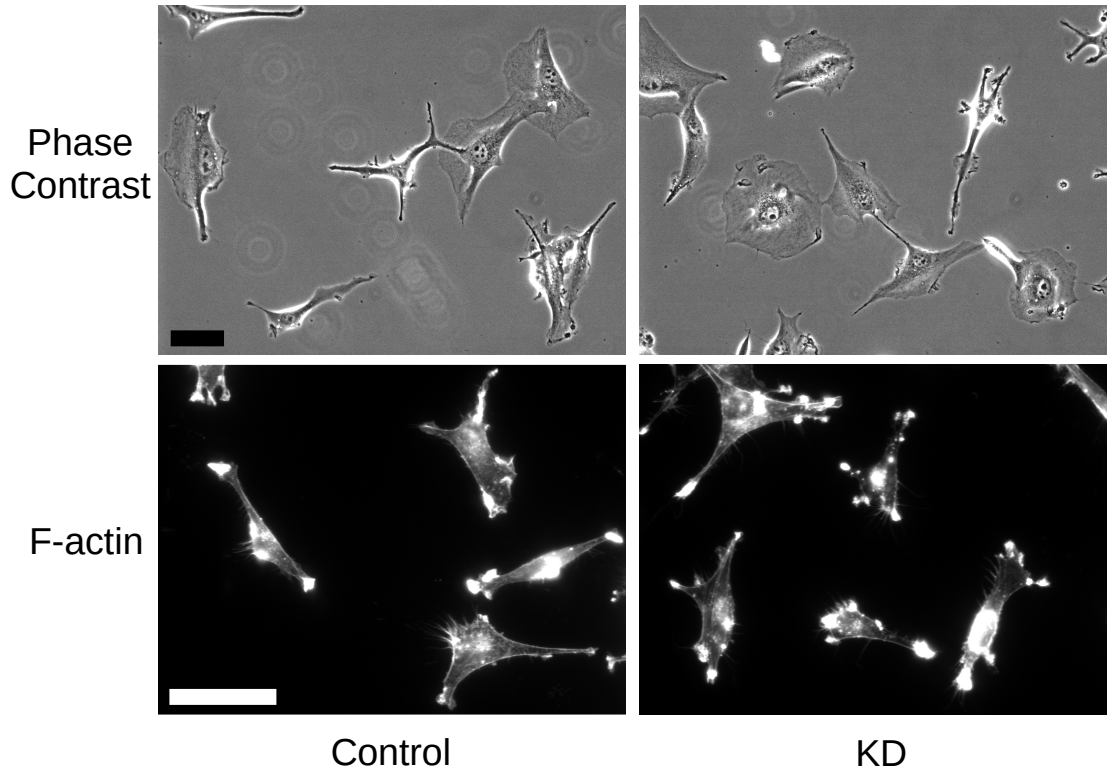


Figure 3-2. Knockdown of calpain 2 expression did not influence glioblastoma cell morphology or organization of the actin cytoskeleton. Control and calpain 2 knockdown (KD) cells were cultured on glass bottom dishes for an imaging by phase contrast microscopy or stained with rhodamine phalloidin to image filamentous actin (F-actin) by fluorescence microscopy. Bar = 50 μm.

immunoblotting total cell lysates from control and knockdown cells treated in the absence and presence of the calcium ionophore A23187 (Fig 3-3). The talin and filamin breakdown products which have previously been attributed to calpain proteolysis were observed in all samples [43,66,110]. The decrease in the intensity of the talin and filamin breakdown products in the calpain 2 knockdown cells compared to control cells demonstrate that both talin and filamin are cleaved by calpain 2 in the untreated cells. An even greater difference in the level of breakdown products was observed between the knockdown and control cells when treated with A23187, showing the calcium-activated calpain 2 proteolysis of talin and filamin. Therefore, knockdown of calpain 2 in the U87MG cells inhibited calpain 2 proteolytic activity even though no alterations in the morphology of the cells were observed.

To test the hypothesis that calpain 2 expression is required for glioblastoma cell invasion, movement was assayed for control and calpain 2 knockdown cells through an artificial extracellular matrix composed of Matrigel coated on transwell membranes as described in Experimental Procedures. Invasion was stimulated by 10% FBS-DMEM as a chemoattractant placed in the well containing the transwell insert (Fig. 3-4). Invasion of the calpain 2 knockdown cells was ~90% lower compared to control cells demonstrating the necessity of calpain 2 expression for glioblastoma cell invasion (Fig. 3-4 and 3-5). To determine if calpain activity was also required for glioblastoma cell invasion, movement of the parental U87MG cells was measured using the transwell assay in the presence of increasing concentrations of cell-permeable calpain inhibitors. Both calpain inhibitors 1 and 2 were observed to decrease invasion of U87MGs cells (Fig. 3-6)

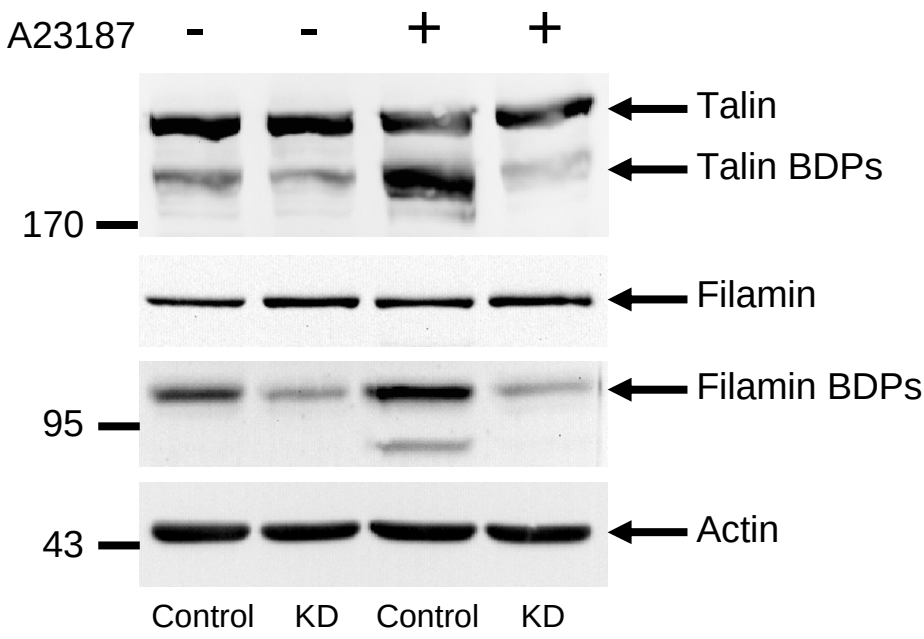


Figure 3-3. Calpain 2 mediates proteolysis of cytoskeletal proteins talin and filamin in glioblastoma cells. Total cell lysates from control and calpain 2 knockdown (KD) cells were immunoblotted to detect the full-length protein and calpain 2 breakdown products (BDP) of talin and filamin. Where indicated, the calcium ionophore A23187 (10µM) was added to the cells for 5 min to activate calpain 2. Immunoblotting for actin is included as a loading control. Results are representative of 3 experiments.

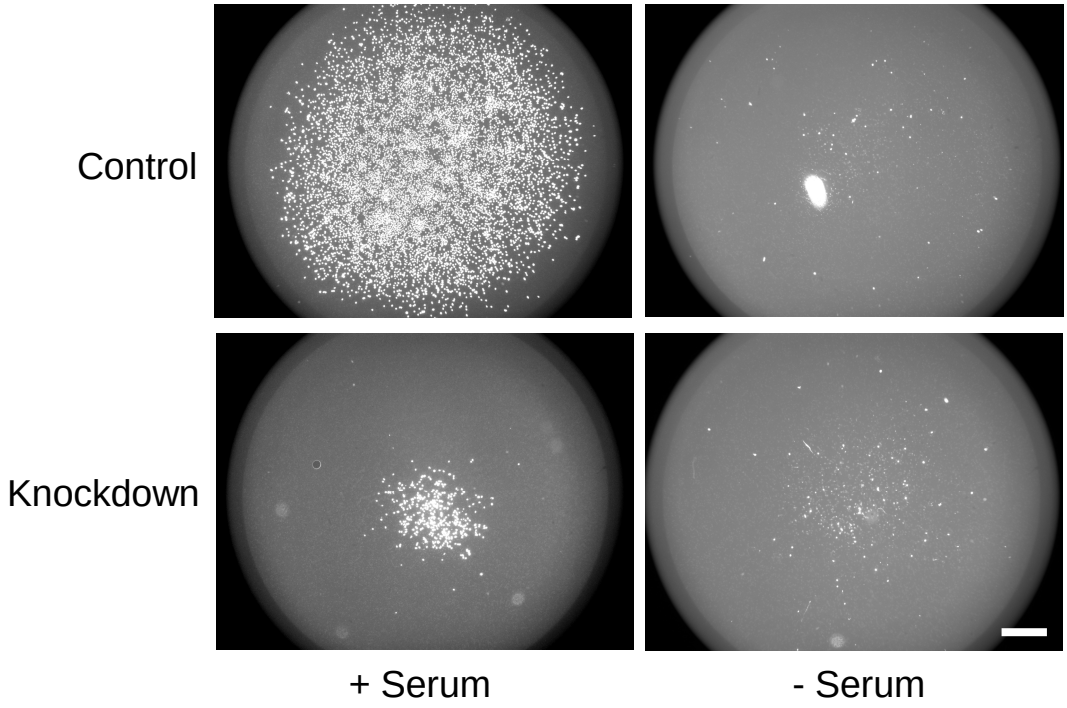


Figure 3-4. Calpain 2 expression is required for glioblastoma cell invasion. Glioblastoma cell invasion was measured by transwell invasion assays. Briefly, calpain 2 knockdown cells were plated in the Matrigel coated transwells with 10% FBS as a chemoattractant in the lower wells. After incubating for 3 d, cells on the bottom surface of the membrane were stained with DAPI, and the images were captured at 5x objective. 10% FBS-DMEM was replaced by serum free DMEM for negative control. The images are representative of three independent experiments. Bar = 0.5 mm.

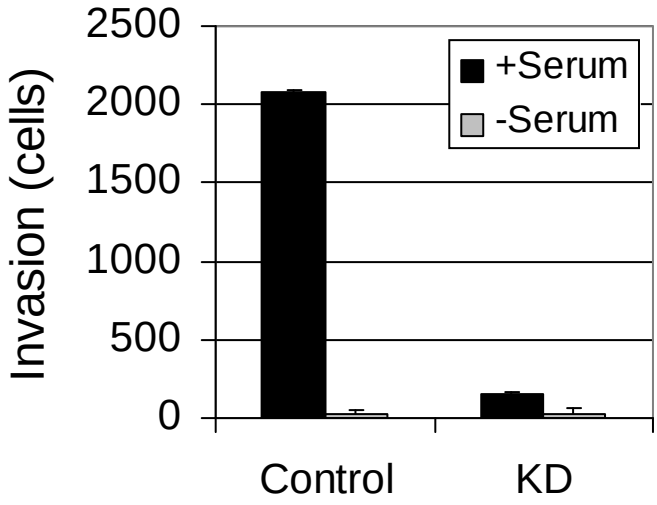


Figure 3-5. Invasion of calpain 2 knockdown cells is 90% less than control glioblastoma cells. To determine the requirement of calpain 2 for glioblastoma cell invasion, transwell invasion assay was carried out as in Fig. 3-4, and the number of DAPI stained nuclei was counted using MetaMorph ver 6.2 software. KD is calpain 2 knockdown cell line. n = 3 ± SEM.

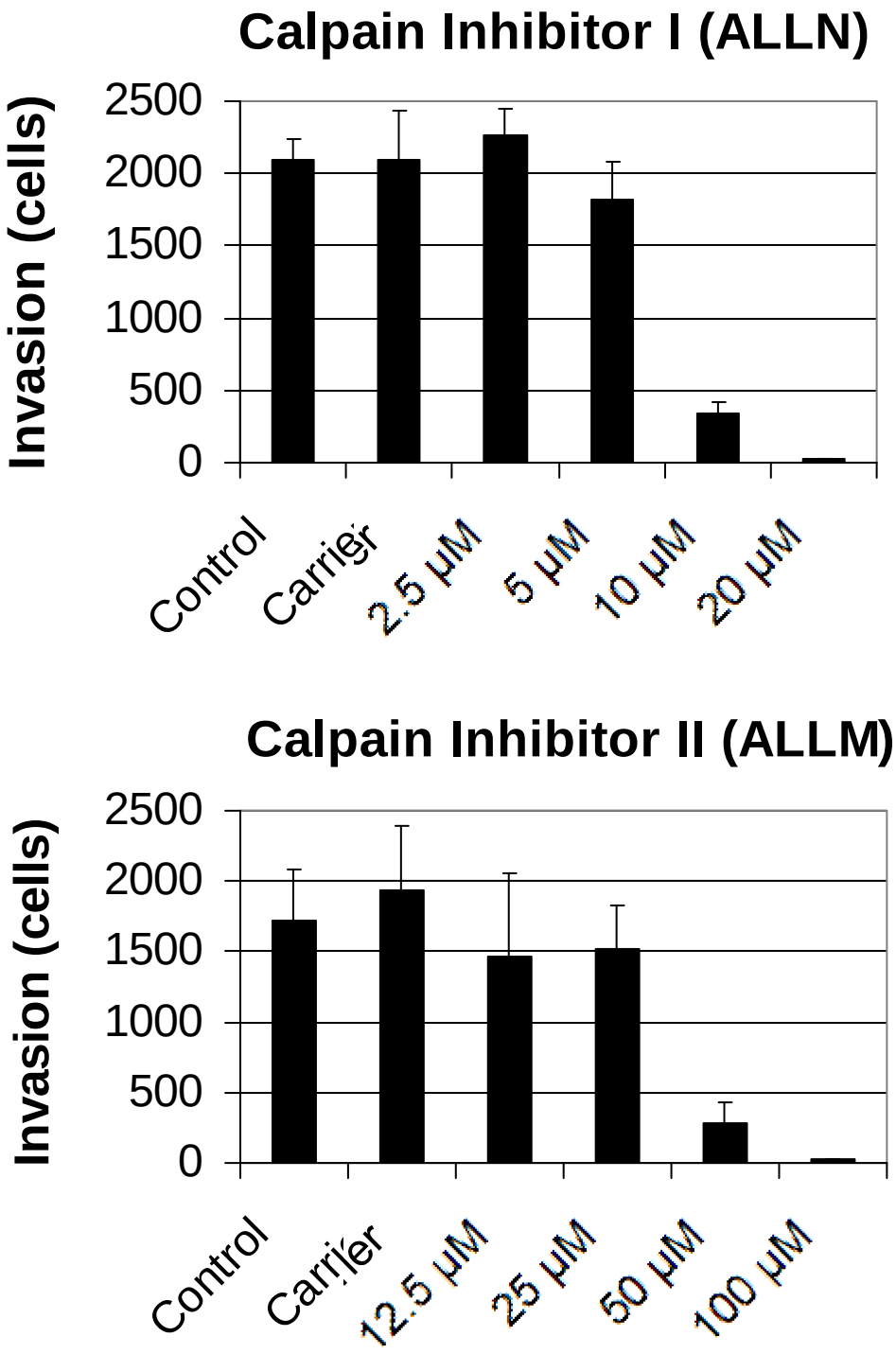


Figure 3-6. Calpain activity is required for glioblastoma cell invasion. Transwell invasion assays were carried out as in Fig. 3-4 with cell permeable calpain inhibitors I or II added to both upper and lower compartments. Ethanol was used as a carrier of the inhibitors and control contained neither inhibitors nor carrier. n = 3 ± SEM.

at concentrations previously reported to inhibit calpain 2 activity in cultured cells [8,79,104,111]. Therefore, data from transwell assays with calpain 2 knockdown cells and cell-permeable inhibitors demonstrate that calpain 2 activity is required for glioblastoma cell invasion.

We have proposed that calpain 2 is a key target for the previously identified calcium influxes induced by autocrine glutamate during glioblastoma cell invasion [4]. In addition, glutamate has been shown to induce chemotactic migration of brain microglial cells [112]. Therefore, it was important to determine if glutamate acted as a chemotactic factor during the invasion of glioblastoma cells. Transwell invasion assays were carried out with increasing concentrations of glutamate added in the lower wells. Even at concentrations as high as 800 μM, glutamate did not induce invasion of either the control or calpain 2 knockdown U87MG cells (Fig. 3-7). These results are consistent with the study published by Sontheimer and colleagues showing that exogenous glutamate did not increase glioblastoma cell migration [4] suggesting that glutamate is not a chemotactic factor for glioblastoma cell invasion. It is important to emphasize that autocrine glutamate was required for glioblastoma invasion in the study [4]. Therefore, the presence of autocrine glutamate in the microenvironment appears to enhance the ability of glioblastoma cells to invade. We propose that the glutamate induced calcium influxes activate calpain 2 to condition glioblastoma cells for invasion of the surrounding brain tissue.

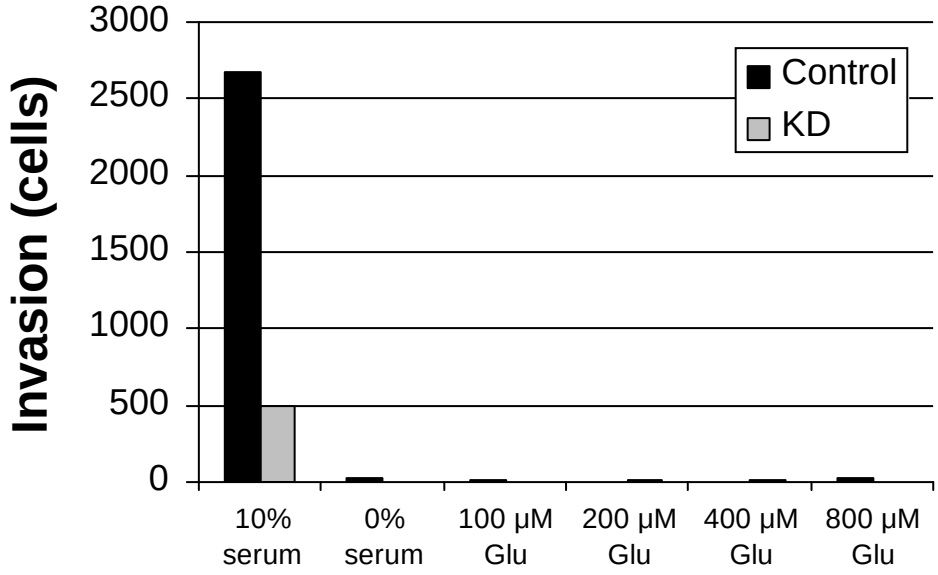


Figure 3-7. Exogenous glutamate is not a chemoattractant for glioblastoma cell invasion. To determine the requirement of glutamate in glioblastoma cell invasion, transwell invasion assays were carried out with increasing concentration of glutamate added to the lower wells. After incubating for 3 d, cells on the bottom surface of the membrane were stained with DAPI and counted. KD is calpain 2 knockdown cell line. n = 1~2.

3.4.2 Calpain 2 is not required for glioblastoma cell migration

Calpain 2 has been demonstrated to regulate many different processes important for the migration of a variety of cells types [43,56,66,113,114,115,116]. Therefore, we expected that the loss of these migratory mechanisms would account for the failure of the calpain 2 knockdown cells to invade the Matrigel in the transwell assays. However, the calpain 2 knockdown cells migrated at the same rate and extent as control cells (Fig. 3-8). Migration was measured using transwell assays coating the membrane with the purified extracellular matrix proteins found in Matrigel: laminin and collagen [117]. In addition, migration was assayed coating with fibronectin and FBS, which are commonly used to study the migration of glioblastoma cells [118,119]. For comparison with the invasion assays (Fig. 3-4), 10% FBS-DMEM was used as the chemoattractant. In the wells where no serum was added, haptotaxis was observed for membranes coated with fibronectin, collagen and FBS, but not laminin. In the wells where 10% FBS-DMEM was used as the chemoattractant, chemotaxis (migration above that observed with substrate alone) was observed for laminin, collagen, and FBS. However, no difference was observed in the migration of the calpain 2 knockdown cells compared to controls. To determine if the lack of a difference was a result of the assay method, the migration of calpain 2 knockdown cells compared to controls was examined using scratch assays as described in Experimental Procedures. As observed in the transwell migration assays, no difference in the rate and extent of migration was observed between the calpain 2 knockdown and control cells using the scratch assay (Fig. 3-9). Although these results differed from studies with fibroblasts and neutrophils showing a critical role for calpain 2 in the basic

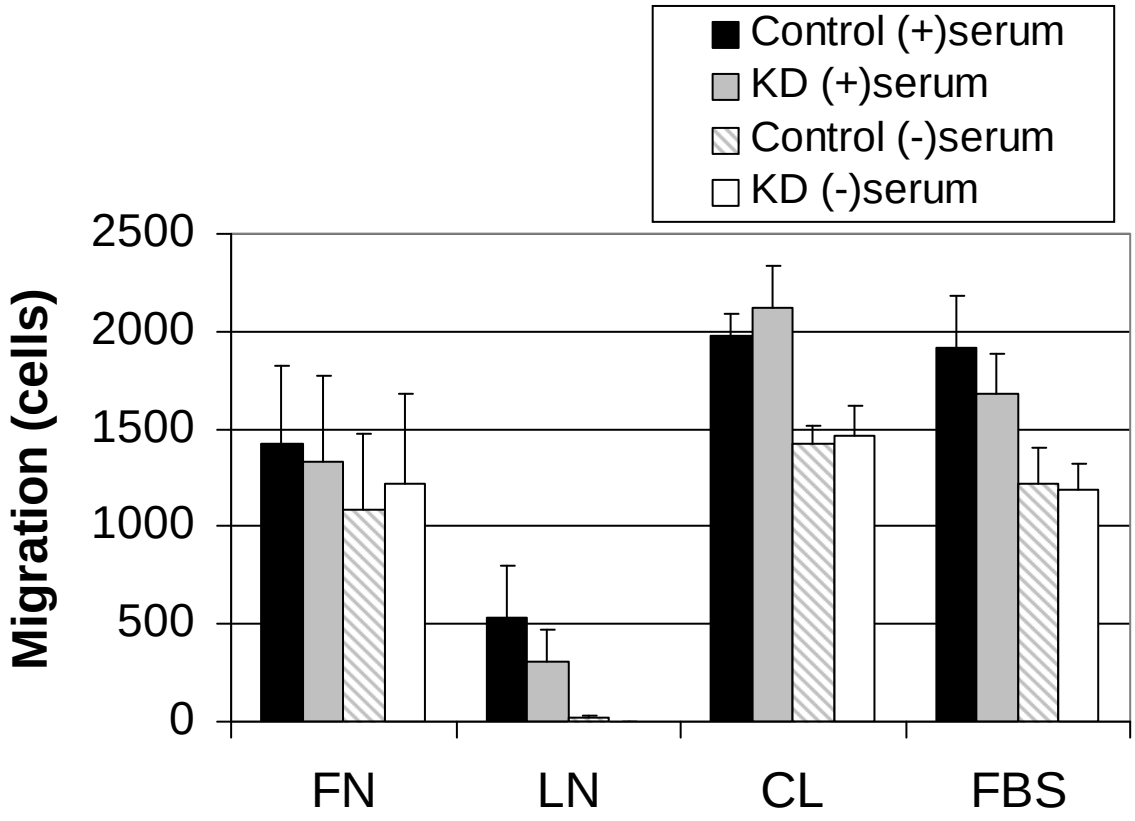


Figure 3-8. Calpain 2 expression is not required for glioblastoma cell migration on purified extracellular matrix proteins. Glioblastoma cell migration was measured by transwell migration assay in which cells were plated in the upper compartment and the bottom surface of the membrane was coated with ECM proteins. After cells migrated, cells on the bottom surface of the membrane were stained with DAPI and counted. Control is control cell line and KD is calpain 2 knockdown cell line. FN, fibronectin; LN, laminin; CL, collagen type IV; FBS, fetal bovine serum. n = 3~4 ± SEM.

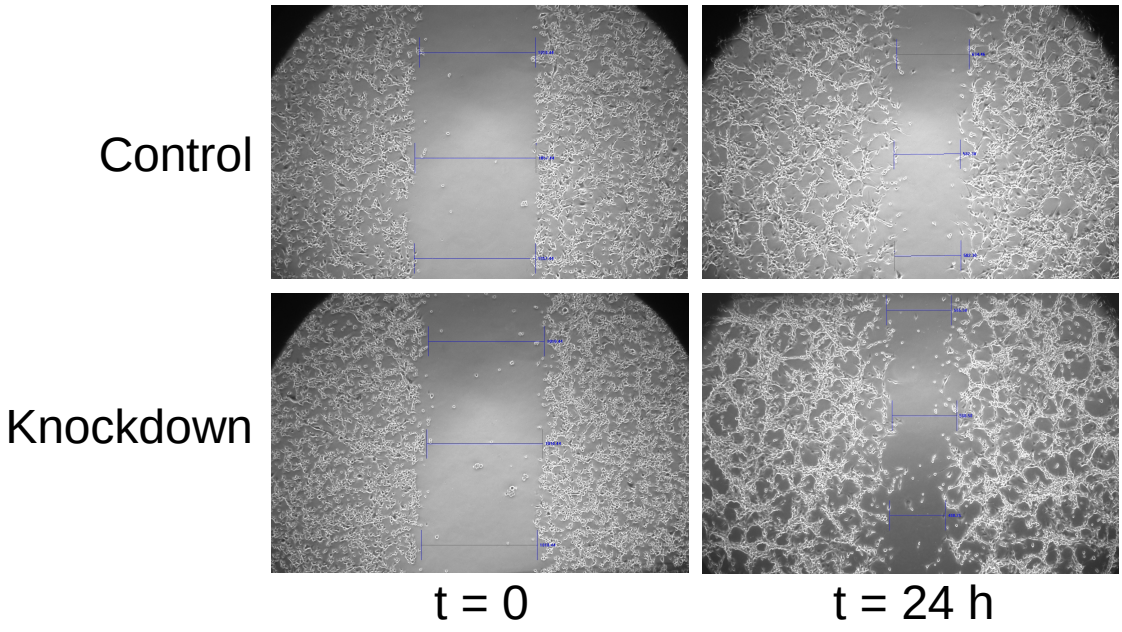


Figure 3-9. Calpain 2 expression is not required for glioblastoma cell migration in scratch assays. Two dimensional migration of calpain 2 knockdown cells or control cells was examined by measuring the advance of cell borders into denuded area.

processes required for cell migration, the data were consistent with the findings that knockdown of calpain 2 did not alter cell morphology or actin organization of the glioblastoma cells (Fig. 3-2). Furthermore, these results suggested that calpain 2 was not modulating glioblastoma cell invasion via control of migratory mechanisms, but through regulation of invasion specific processes.

### 3.4.3 Calpain 2 is required for matrix metalloproteinase-2 activity in glioblastoma cells

Invasion of cells through the extracellular matrix involves degradation of fibrous protein components allowing penetration and movement of tumor cells into adjacent regions of the diseased tissue. Tumor cell modification of the surrounding extracellular matrix is accomplished by specialized extracellular proteases controlled by cellular projections called invadopodia [120,121,122]. Based on our data indicating that calpain 2 was involved in the regulation of invasion specific processes in glioblastoma cells, we speculated that calpain 2 proteolysis was required for the modulation of invadopodial processes controlling extracellular matrix metalloproteinases (MMP). Recent studies have demonstrated that calpain 2 proteolysis of the cortactin is important for membrane protrusion and invadopodial dynamics and stability [55,56]. To determine if calpain 2 was involved in the regulation of the invadopodial protein, we examined the proteolysis of cortactin in the calpain 2 knockdown and control cells. As reported for breast cancer tumor cells [55], we observed an increase in full-length cortactin protein in the calpain 2 knockdown cells compared to controls (Fig. 3-10). Furthermore, the presence of cortactin breakdown products, which have previously been attributed to calpain

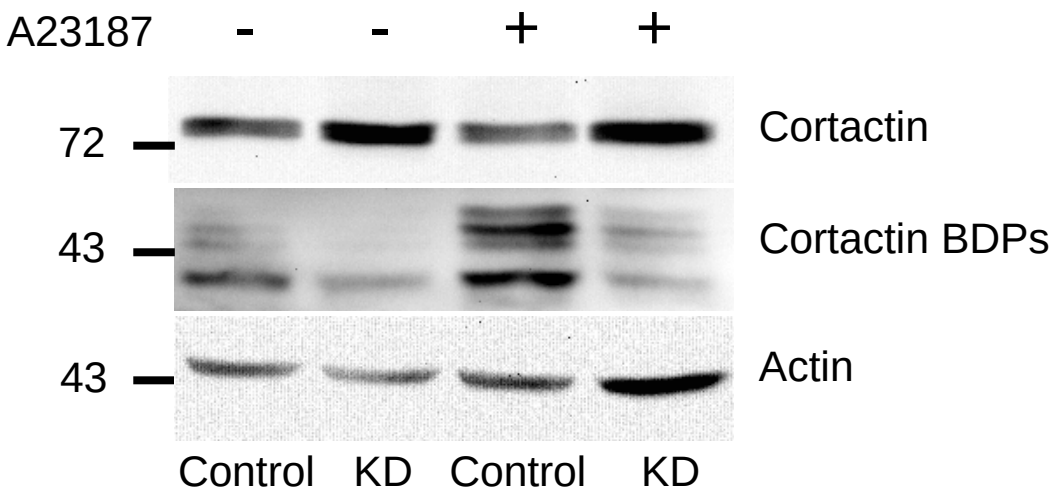


Figure 3-10. Calpain 2 mediates proteolysis of invadopodial protein cortactin in glioblastoma cells. As a first step to determine the calpain 2 requirement for formation of invasion specific structure invadopodia, proteolysis of cortactin, an invadopodial protein, was examined by Western blot. There were less cortactin breakdown products (BDPs) in calpain 2 knockdown cells than controls, and the difference in the amounts of BDPs was amplified by activating calpain activity by calcium ionophore A23187 treatment. Actin is a control. KD is calpain 2 knockdown cell line.

proteolysis, were reduced in the calpain 2 knockdown cells. Calpain 2 was clearly required for both basal and calcium-stimulated proteolysis of cortactin in glioblastoma cells. These results supported the hypothesis that calpain 2 was involved in glioblastoma invasion by regulating invadopodial presentation of MMPs. To determine if calpain 2 was also required for extracellular proteases, serum-free media from two-dimensional cultures of control and calpain 2 knockdown cells was sampled over time and assayed for MMP2 and MMP9 using gelatin zymography as described in Experimental Methods. A time-dependent increase in pro-MMP2 levels was observed in the media with less activity consistently observed in the samples from the calpain 2 knockdown cells compared to control (Fig. 3-11A). Under these conditions, a light band representing the active form of MMP2 was only detected at 36 hours and no MMP9 activity was observed. Serum containing media was also assayed verifying that the assay conditions were suitable for detecting both MMP2 and MMP9 activities. To measure MMP activity from cells within a three-dimensional environment, the experiments were reproduced sampling serum-free media from control and calpain 2 knockdown cells plated on Matrigel coated transwell inserts. Under these conditions, both pro-MMP2 and MMP2 were detected with 17% and 36% less activity, respectively, observed in the media from the calpain 2 knockdown cells compared to control (Fig. 3-11B). Similar results were observed in the media from the wells of the plate holding the inserts demonstrating the diffusion and widespread distribution of pro-MMP2 and MMP2 in the media. Consistent with the media from two-dimensional cultures, no MMP9 activity was detected suggesting that MMP9 may not play a major role in the invasion of U87MG glioblastoma cells. Together, these results

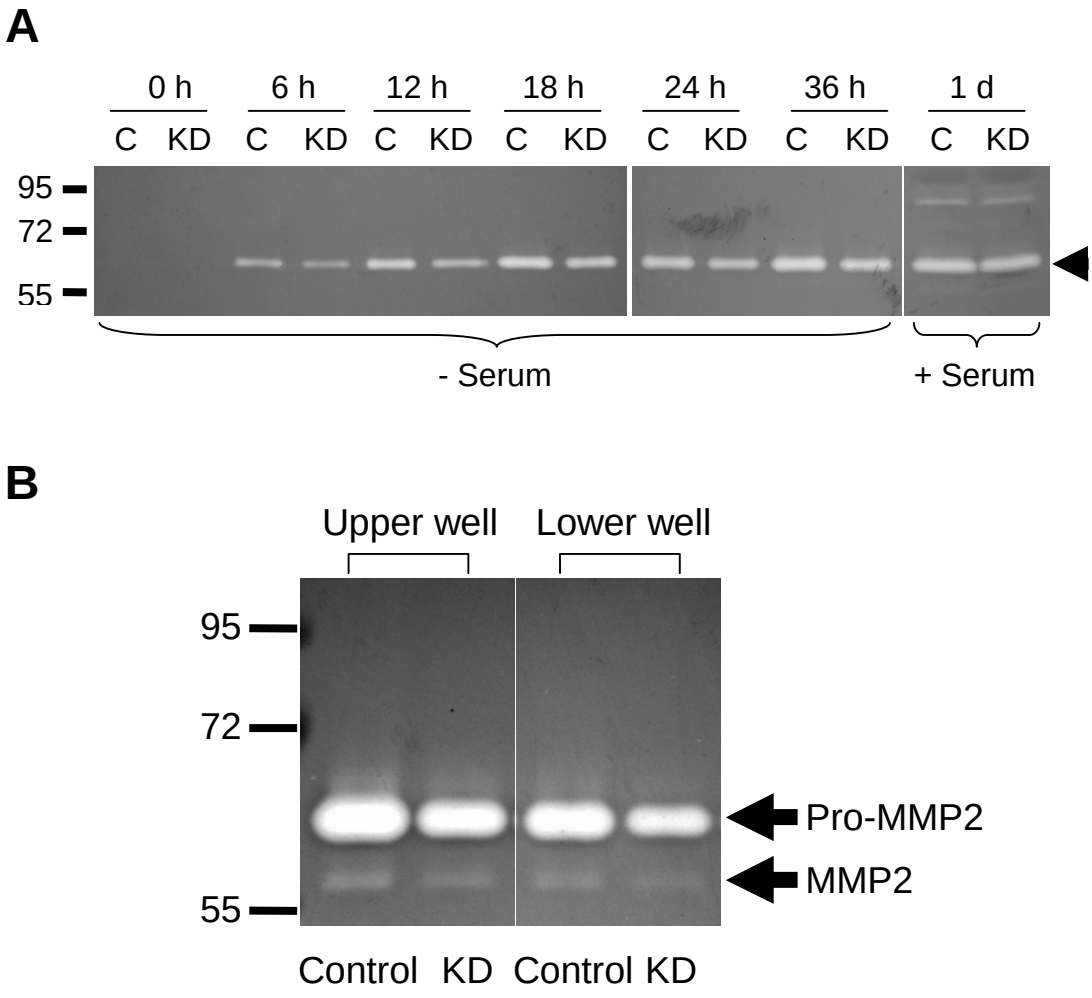


Figure 3-11. Calpain 2 expression is required for maintaining the level of secreted pro-MMP2 and MMP2 in glioblastoma cells. Pro-MMP2 or MMP2 was visualized by gelatin zymogram as described in Experimental Procedures. Briefly, culture media of glioblastoma cells grown in 6 well plate (A) or in Matrigel coated transwells as invasion assays (B) were separated by gelatin embedded polyacrylamide gel electrophoresis. After incubating the gel in the renaturing and developing buffers, the gel was stained with Coomassie blue, and the pro-MMP2 or MMP2 was visualized by clear bands on a blue background. (A) Extracellular pro-MMP2, indicated by the arrow head, was examined over 36 h period in the absence of serum in 6 well plate. The culture media taken 1 d after incubating in the presence of serum was loaded as a control. C is control cell line and KD is calpain 2 knockdown cell line. n = 1~2. (B) Extracellular pro-MMP2 or MMP2 in the upper and lower compartments of Matrigel coated transwells were examined at 3 d of incubation. n = 4.

suggest that calpain 2 is required for maintaining extracellular levels of MMP2 in an invasive microenvironment identifying one mechanism by which glutamate-dependent calcium-activated proteolysis mediates glioblastoma tumor cell invasion.

Based on the results presented in this study, we propose the following working model for the role of calpain 2 proteolysis in glioblastoma cell invasion (Fig. 3-12). It is well established that calcium influxes resulting from autocrine glutamate activation of AMPA receptors are required for glioblastoma cell invasion. Evidence has also been reported showing that these calcium influxes regulate turnover of adhesion contacts in U87MG glioblastoma cells. However, until this study, no direct target had been identified for calcium involved in mediating glioblastoma cell invasion. Our results show that the calcium-activated protease calpain 2 is required for glioblastoma cell invasion, in part, by regulating extracellular levels of MMP2. We propose that calpain 2 regulates MMP2 by influencing the function of invadopodia through proteolysis of invadopodial proteins such as cortactin. Further studies are necessary to identify substrates for calpain 2 and the mechanisms by which proteolysis are involved in glioblastoma cell invasion.

The abbreviations used are: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; CL, collagen type IV; DMEM, Dulbecco's modified Eagle media; FBS, fetal bovine serum; FN, fibronectin; LN, laminin.

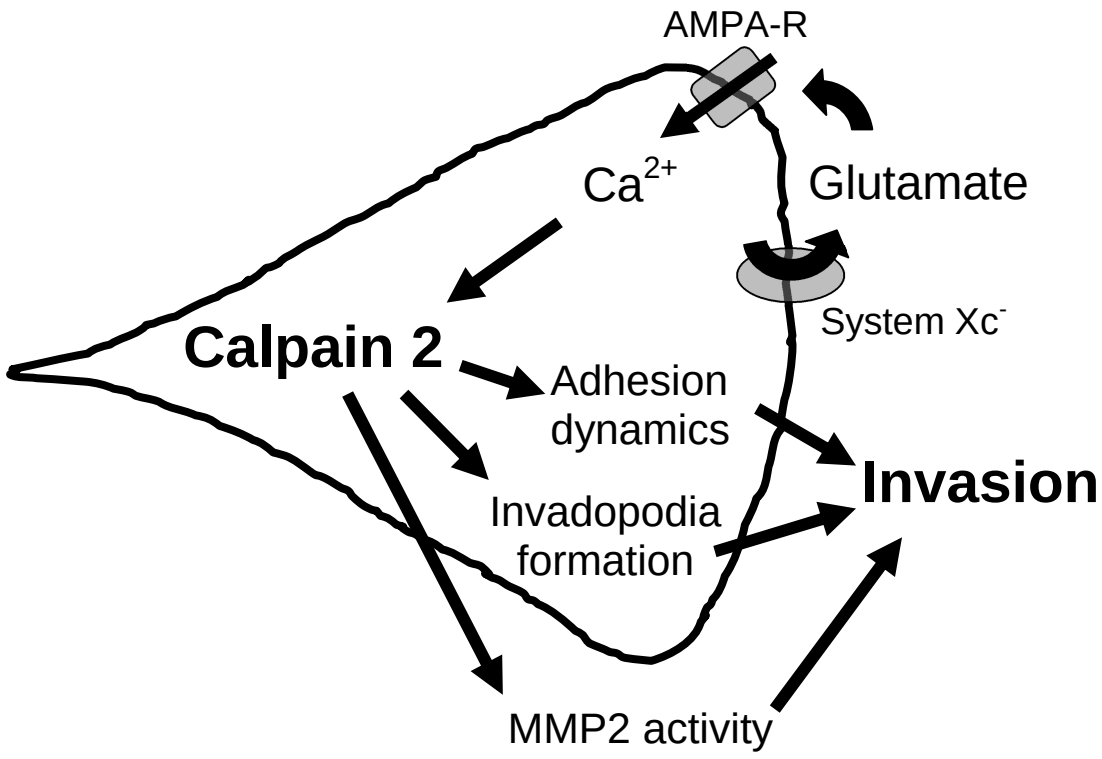


Figure 3-12. Working model. Diagram illustrates how glutamate stimulation of calcium influx via AMPA receptor (AMPA-R) is proposed to promote glioblastoma cell invasion. Previously published results have shown the importance of the autocrine glutamate secreted through system $Xc^-$, AMPA-R, calcium influx, and adhesion dynamics in glioblastoma cell migration and invasion [3,4,5,6,7,8,9,10,11,12]. However, the downstream targets of calcium have not been identified. One potential candidate is calpain 2, a calcium dependent protease, which is known to regulate adhesion dynamics and cell motility. In this study, we presented data that support the role of calpain 2 for glioblastoma cell invasion, but not migration. In addition, we showed that calpain 2 is important for maintaining the level of extracellular pro-MMP2 or MMP2 of glioblastoma cells in invasive three dimensional environments.

# Chapter 4

# General Conclusion

Cell migration is important for normal physiology and various processes of patho-biology. Migration on two dimensional substrates is a well organized process involving protrusion, translocation, and retraction. Migration in three dimensional environments or invasion follows a similar process with additional mechanisms required to overcome the physical barrier presented by the existing extracellular matrix. For cell motility in both two dimensional and three dimensional spaces, the actin cytoskeleton plays an important role in the regulation of adhesion, contractile stress fibers, and invasive membrane protrusions. Cell adhesion is integrin-mediated contact between the actin cytoskeleton and the extracellular matrix, and functions as a signaling center as well as a physical contact point. Contractile stress fibers are bundles of F-actin, connected to focal adhesions thereby providing contractile force to migrating cells. Migration in three dimensional space requires invasive membrane protrusions such as invadopodia or pseudopodia that are specialized in degrading surrounding matrices. In chapter 2, we studied the role of glycine-rich region 1 in CRP1 bundling of F-actin and localization to stress fibers. In chapter 3, we focused on the role of calpain 2 in glioblastoma invasion.

## 4.1 GR1 is Required for CRP1 Bundling of F-Actin and Localization to Stress Fibers

Besides filamentous actin, many actin binding or bundling proteins are found in the actin cytoskeleton regulating the structure. One of the major actin bundling proteins is α-actinin, which bundles filamentous actin and links the actin bundles to integrins in focal adhesions [40]. α-Actinin has been shown to be important for the regulation of adhesion and migration [91,123]. Interestingly, α-actinin was shown to interact with other

proteins, one of which is cysteine-rich protein 1 (CRP1) [21]. CRP1 is a double LIM domain protein, and originally identified as α-actinin binding protein. Greenwood and colleagues showed that CRP1 is directly binding or bundling actin filaments independent of α-actinin [22]. The data presented in chapter 2 support that not only LIM1 but also the adjacent glycine-rich region 1 (GR1) is required for actin binding/bundling activity and CRP1 localization to stress fibers. This work strengthens the idea of distinct functions for either LIM domain. This was shown to be true for nuclear CRP1 because LIM1 and LIM2 bind to different transcription factors [31]. Considering the fact that there is significant similarity of amino acid sequences between LIM1 and LIM2 (51% identity), and neither LIM1 nor LIM2 independently binds or bundles filamentous actin, the role of glycine-rich regions appears to be as important as that of the LIM domains. First, GR1 confers actin binding/bundling activity to CRP1 even though GR1 alone was not sufficient for that activity (Fig. 2-1). Second, the fact that only GR1, but not GR2, in CRP1 was able to confer actin binding/bundling activity as shown in domain swap (Fig. 2-3 A) and point mutant studies (Fig. A-3) is suggesting that GR1 may be partly responsible for distinct function of LIM1 and LIM2. It would be interesting to see if the glycine-rich regions play a similar role in nuclear population of CRP1. It is noticeable that only seven amino acid residues are different between GR1 and GR2 (Fig. 2-4 A). Further, to make actin bundles, actin bundling proteins need to have two actin binding domains in the protein or have to dimerize if it has only one actin binding domain. The example of the latter case is α-actinin, which is a homodimer with one actin binding domain on each subunit. The example of the former case is fascin, which bundles F-actin

as a monomer with two distinct actin binding domains in a single polypeptide [124]. Regarding the mechanisms of CRP1 bundling of F-actin, we examined both possibilities. The first possibility for CRP1 bundling is that LIM1/GR1 may have two actin binding domains, thus without the need to dimerize. If both binding domains are present in either LIM1 or GR1, each of them would bind actin, but that was not observed in cosedimentation assay (Fig. 2-1). In case LIM1 and GR1 have one binding domain respectively, this still does not explain the data because at least LIM1 or GR1 should be able to bind to actin even though each of them alone may not be able to bundle actin filaments. Therefore, the second possibility that LIM1/GR1 have a single actin binding domain and bundle actin filament via homo-dimerization appears to be more consistent with our data. Thus, we propose the model where LIM1 is a dimerizing domain as well as an actin binding domain. Since the LIM1 domain has two zinc finger domains, it may be possible that one of the zinc fingers is the dimerizing domain, and the other is the actin binding domain. Also, we propose that GR1 would be stabilizing LIM1 dimer and contributing partly to actin binding (Fig. 4-1).

## 4.2 Calpain 2 is Required for Glioblastoma Cell Invasion, Not Migration

As noted earlier, cell migration in three dimensional environments or invasion has critical role for normal physiology and patho-biology. The model system we used to study the actin cytoskeletal regulation in invasion was U87MG glioblastoma cell line. Glioblastoma has the activated PI3K pathway partly due to the EGFR overexpression [125], PI3K activation [96], and PTEN mutation [126], leading to increased PIP3. What

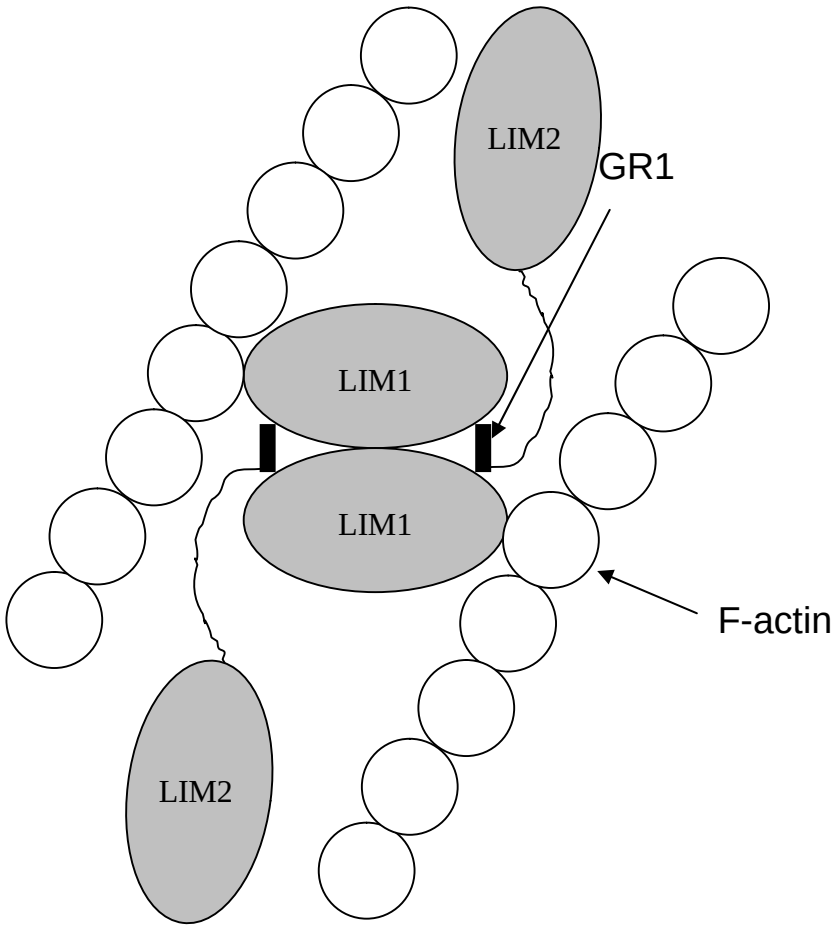


Figure 4-1. The model of CRP1 bundling of F-actin. Diagram illustrates how CRP1 can directly bind and bundle F-actin. LIM1 is dimerizing and GR1 is stabilizing the dimer. Also, LIM1 contains the actin binding region so that LIM1 dimer can make F-actin bundle. LIM2 is not binding F-actin.

would be the effect of higher PIP3 level on invasion? PIP3 has been shown to be important for initiating and maintaining cell polarity in neutrophils [19,127] or chemotaxing cells [128,129] by being involved in a positive feedback loop. Higher level of PIP3 may stabilize cell polarity fortifying the positive feedback loop. Also, the role of PI3K pathway is partly dependent on the type of a substrate on which cells are migrating [130]. Then, would PIP3 phosphatase affect chemotaxis? PTEN is a frequent mutation found in 44% of all glioblastoma [126]. Interestingly, PTEN did not affect chemotaxis in neutrophils, but another phosphatase SHIP1 was required for cell polarity and chemotaxis [131]. This suggests that glioblastoma still retains the responsiveness to chemoattractant, and SHIP1 may be a potential target for inhibiting tumor invasion.

One of the critical features of glioblastoma cells is that they are secreting glutamate, which is important for glioblastoma cell growth and invasion [4,6,132]. Glutamate secreted from glioblastoma is thought to have at least two distinct roles for glioma expansion and invasion. First is the excitotoxicity of glutamate; glutamate can kill neuronal cells by first increasing cytosolic calcium concentration [133] and inducing apoptosis [134]. Indeed, calpain inhibitor was able to reduce glutamate excitotoxicity [135]. Second is autocrine/paracrine signal. Autocrine glutamate binds the ionotrophic AMPA receptor and stimulates calcium influx. Calcium influx disrupts focal adhesion, contributing to cell migration [7]. Interestingly, extracellular calcium influx was shown to be a critical component of positive feedback loop that maintains cell polarity in macrophages [136]. If calcium is important for chemotaxis and cell polarity in glioma, AMPA receptor would be concentrated at the leading edge of a migrating cell. But the

downstream target of calcium influx has not been identified. One potential target of calcium is the calcium activated protease calpain. Here, we propose that calcium activated protease calpain is the downstream effector of calcium influx. To test this hypothesis, we first generated calpain 2 knockdown cell line by transfecting U87MG glioblastoma cells with calpain 2 targeting shRNA plasmid. Using calpain 2 knockdown cells in transwell invasion/migration assays and scratch assays, we clearly demonstrated that calpain 2 expression is required for glioblastoma cell invasion (Fig. 3-4 and 5), but not migration (Fig. 3-8 and 9). Also, cell permeable calpain inhibitors decreased the invasion in a dose dependent manner, suggesting that calpain 2 activity is required for glioblastoma cell invasion (Fig. 3-6). The effect of calpain 2 knockdown or inhibition on invasion is unlikely to result from cell toxicity. Based on the literature, we excluded the possibility of apoptosis induction by calpain knockdown or inhibition. First, the calpain inhibitor II did not induce apoptosis in a U373 glioblastoma cell line [137]. The concentration of the calpain inhibitor II used in their study was 100 μM, which inhibited invasion almost completely in our transwell assays. Second, mouse C6 glioma cells overexpressed calpain 1 protein, not calpain 2, and underwent apoptosis when exposed to various stimuli such as interferon-γ and oxidative stress. In fact, apoptosis of glioma cells was reduced by treating calpain specific inhibitor calpeptin [138]. Therefore, we concluded that the effect of calpain inhibition on glioblastoma cell invasion was largely due to the inhibition of invasion, not apoptosis induction.

It is of much interest to understand why and how glioblastoma cells preferentially invade along blood vessels or neurons, or toward subpial spaces [72]. Conceptually,

diffusible factors may be released from the blood stream or the extracellular matrix around the blood vessels or neurons may attract tumor cells. In the present work, we showed that fibronectin and type IV collagen are preferred substrates for glioblastoma cell migration through transwell membrane or haptotaxis (Fig. 3-8). Laminin 1 did not support migration. This may be isoform specific phenomenon because laminin 8 was shown to be one of the best substrates for glioblastoma cell migration on two dimensional surface [139]. Since ECM proteins bind to specific integrin receptors, by blocking the specific integrin receptor using antibody, glioma cell migration/invasion was inhibited [139]. It is also interesting that glioblastoma cells secrete their own extracellular matrices, and knockdown of laminin 8 expression resulted in inhibition of glioblastoma invasion [140].

Calpain has been clearly shown to regulate focal adhesion dynamics and compositions, thus affecting cell motility [8,64]. Much to our surprise, we could not detect any difference in motility of calpain 2 knockdown cells in transwell migration assays (Fig. 3-8) or scratch assays (Fig. 3-9). Besides the effect on cell motility, there are numerous reports of morphological changes caused by calpain down-regulation. In calpain 4 knockout mouse fibroblasts, membrane blebbing was significantly reduced partly due to down-regulation of RhoGDI-1 (Rho GDP-dissociation inhibitor) [69]. In another study, Huttenlocher and colleagues showed that calpain 4 knockout resulted in numerous thin membrane projections and increased transient membrane activity in immortalized fibroblasts [66]. Also, in the calpain 4 knockout fibroblasts, lamellipodial dynamics was deregulated leading to impaired net extension of lamellipodia. The same

group further showed that calpain 2, not calpain 1, is responsible for the altered membrane protrusion dynamics or less persistent protrusions. In contrast to these reports, surprisingly we did not observe any obvious morphological or actin cytoskeleton changes in the calpain 2 knockdown glioblastoma cell line (Fig. 3-2). It suggests that these effects may be cell type dependent, but further study is needed to address that question.

### 4.3 Calpain 2 is Critical for Invasion Specific Processes of Glioblastoma

One of the major differences between two dimensional migration and invasion is the presence of physical barrier imposed by extracellular matrices. One of the most studied mechanisms to overcome this barrier is degrading the matrices. The extracellular matrix is a very complex structure comprised of many proteins and carbohydrates. Consequently, many enzymes are present to remodel the extracellular matrix. One of them is matrix metalloproteinase, a family of zinc dependent peptidases. In tumor cells, MMP activity is closed associated with actin rich membrane protrusions, e.g. invadopodia, which have been shown to play an important role in tumor cell invasion. Hence, we investigated the role of calpain 2 in these invasion specific processes.

Consistent with fibroblasts [56], our results showed that cortactin is a substrate for calpain 2 in glioblastoma cells (Fig. 3-10). Since cortactin is required for invadopodia formation and matrix degradation [53], our result suggests that calpain 2 may be needed for invadopodia structure and function. To firmly establish the causal relationship, further study is needed.

Gelatin zymography showed that calpain 2 knockdown in glioblastoma led to about 30% decrease in extracellular MMP2 (Fig. 3-11). But, the other gelatinase MMP9 was not detected in glioblastoma cells. How calpain 2 affects the extracellular MMP2 is not clear yet. One of the most feasible mechanisms is interference with exocytosis or secretion of MMP2. Cortactin has been shown to be required for MMP secretion [54,141]. Cortactin binds to secretory vesicles from Golgi and directs them to invadopodia potentially by connecting them to the dynamic actin cytoskeleton. Cortactin is a substrate of calpain 2. Thus, calpain may affect the secretion of MMP. It would be interesting to see if calpain 2 affects the default pathway for protein secretion. Another possible way of calpain 2 affecting MMP level is affecting the invadopodia dynamics. Since invadopodia are the structures that present pro-MMP2, the decrease in the number of invadopodia may result in the decreased level of pro-MMP2.

Considering almost complete inhibition of invasion by calpain 2 knockdown, ~30% decrease in the extracellular pro-MMP2 or MMP2 does not appear to be convincing by itself. Then, is this really enough to cause such a dramatic decrease in invasion or are there any other mechanisms regulated by calpain 2?

First, there is tissue inhibitor of matrix metalloproteinase 2 (TIMP-2), which inhibits both pro-MMP2 and MMP2. It is thought that the balance between TIMP-2 and MMP2 is important for matrix degradation. Actually, the ratio of MMP2 to TIMP-2 has been shown to increase in the advanced stages of neuroblastoma [142] and in metastatic breast carcinomas [143]. If the balance is broken in the calpain 2 knockdown cell line,

that may lead to the lack of MMP2 activity while control cell line still retains the MMP2 activity.

Second, cells cannot migrate through the hole or space smaller than their nucleus. The exact size of permissible holes is dependent on cell types, but usually 3 μm are the limit. Matrigel was shown to have sub-micron sized pores which would not allow migration. Small decrease in the MMP2 activity may result in non-permissible size of holes, which would inhibit invasion.

Third, there may be other MMPs or proteases required for invasion. Considering the complexity of the ECM, it would not be surprising if multiple MMP isoforms, or other proteases, are required for timely and efficient matrix degradation. If MMP2 is activating some of those, small difference may result in large difference just like phosphorylation cascade.

Fourth, calpain 2 may be required for rear end retraction and pushing cell body/nucleus through the narrow intercellular spaces or the ECM. As Rosenfeld group demonstrated, myosin II is required for contractile force generation and cell body translocation [144]. In their study, myosin II knockdown [144] or inhibition [145] led to the defect in the ability of C6 glioma cells to migrate through 3 μm pores, but not 8 μm pores. Myosin II has regulatory light chains, which are phosphorylated by myosin light chain kinase (MLCK). MLCK phosphorylates and activates myosin II. There have been reports that shows the activation of MLCK by calpain [146,147]. Thus, it is very tempting to speculate that calpain 2 helps glioblastoma cells squeeze through the narrow intercellular spaces in normal brain by activating MLCK and myosin II.

Owing to the complex composition of serum, we did not include serum in our culture media when analyzing MMP2. But, it is hard to imagine growth factor free *in vivo* environments. Thus, it is worthwhile to review some of the important reports on the growth factor effect on MMP expression. First of all, EGF induced MT1-MMP expression at both transcriptional and translational level leading to the increase in active MMP2 [148]. This is particularly interesting because EGF receptors are overexpressed in glioblastoma cells [125]. EGF activates ERK, which is known to activate calpain 2. So, this further supports the importance of calpain 2 for MMP2 activity. Another interesting report is the MMP2 downregulation by IGF-1 receptor blockage leading to the inhibition of prostate cancer proliferation and invasion [149]. Also, the effect of extracellular growth factors on MMP secretion was shown by Rooprai *et al.* [150]. They showed that extracellular MMP2 and MMP9 of grade III astrocytoma and grade III oligoastrocytoma were increased by growth factor treatment in regular plastic culture flasks. These growth factors included EGF, bFGF, TGF-β1, TGF-β2, and VEGF. As Rooprai and colleagues pointed out, it is interesting to see both the positive or negative effects of one growth factor on another growth factor in terms of MMP expression or secretion. The discrepancy between *in vitro* and *in vivo* level of MMPs may be explained by the difference in microenvironments of tumor cells. Also, *in vivo*, it is likely that the MMP expression is regulated by actions of multiple growth factors. Interestingly, normal astrocytes have been shown to constitutively expresses MMP2 [151], MMP9 [152,153,154], both MMP2 and MMP9 [155], or MMP3 [156] upon stimulation. Moreover, MMP2 was shown to regulate normal astrocyte migration [157]. Thus, it

would be also interesting to compare the MMP expression profiles between normal astrocytes and glioblastoma cells and to investigate their implication in cell motility and invasion.

In conclusion, we showed for the first time that LIM1/GR1 is the unique actin bundling domain, and GR1 is required, but not sufficient by itself for CRP1 bundling of F-actin and localization to stress fibers. Also, to our knowledge, this is the first report of isoform specific function of calpain 2 in glioblastoma cell invasion. It is also interesting that calpain 2 expression was not required for migration. So far, there have been mounting evidences that calpain is important for cell migration. However, the glioblastoma cells did not require calpain 2 expression for migration on two dimensional substrates. Rather, invasion is of more interest, because glioblastoma is highly invasive, which makes it hard to treat the tumor. Thus, our data suggest that calpain 2 may be potential target for brain tumor treatment.

Overall, this dissertation has fulfilled the goal of better understanding the actin cytoskeletal regulation in stress fibers and invadopodia important for cell motility and invasion.

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# APPENDICES

Appendix A. CRP1 bundling of F-actin saturates at about 5 µM.

To determine the saturating concentration of CRP1 for F-actin bundling, cosedimentation assays were carried out with increasing concentration of CRP1 as described in the Materials and Methods of chapter 2. The percent of actin observed in the pellet in low speed cosedimentation assay increased with the concentration of CRP1 reaching almost to saturation at ~5 µM (Fig. A-1). This data suggest that CRP1 binds to F-actin roughly in a ratio of 1:2.

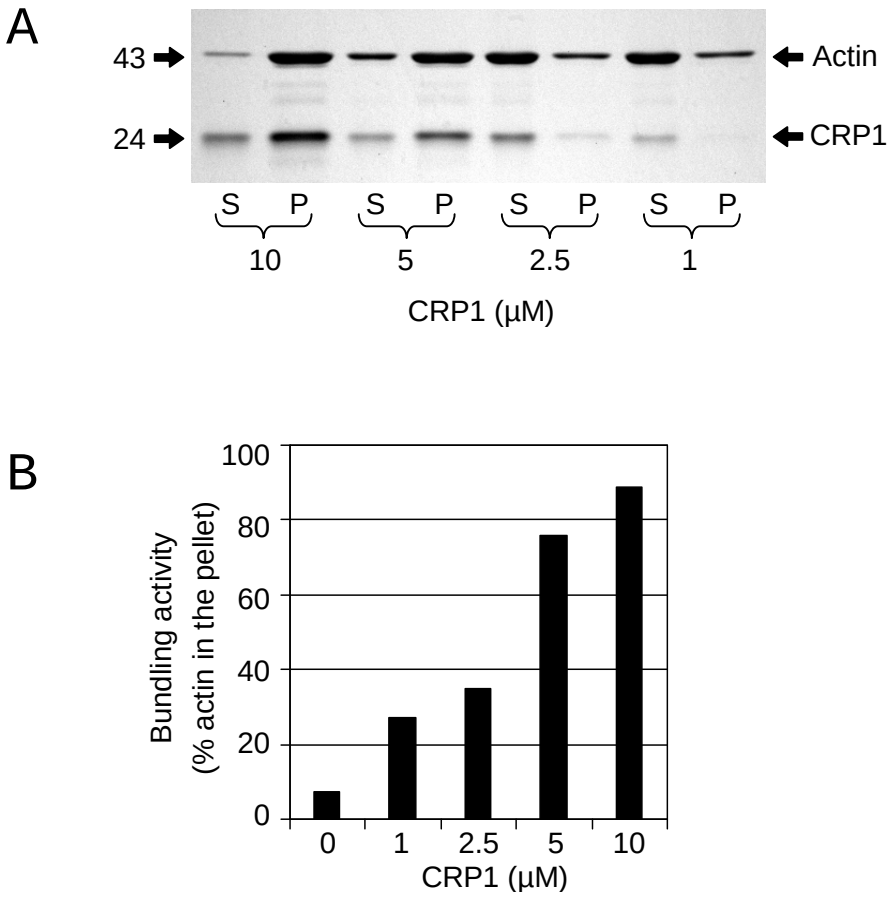


Figure A-1. CRP1 bundling of F-actin saturates at about 5 μM. Cosedimentation assay was carried out at 10,000 x g by incubating 10 μM F-actin with increasing concentrations of full-length wildtype CRP1 as described in Materials and Methods of chapter 2. (A) Proteins from the supernatant (S) and the pellet (P) were separated by SDS-PAGE and stained. (B) The percentage of total actin in the pellet was quantified as described in Materials and Methods of chapter 2.

Appendix B. LIM1/GR1 is required for CRP1 binding or bundling of F-actin.

To determine the F-actin binding/bundling domain in CRP1, five truncation forms were analyzed for their binding/bundling activities in cosedimentation assays as described in chapter 2. Cosedimentation assays were carried out at two centrifugal forces, 100,000 x g and 10,000 x g (Fig. A-2). At lower centrifugal force, truncation forms that can bundle F-actin can make an actin pellet. At lower centrifugal force, both binding and bundling proteins would be observed in the pellet. Data from both centrifugal forces were consistent with each other. These results suggest that binding and bundling in CRP1 are not separable character.

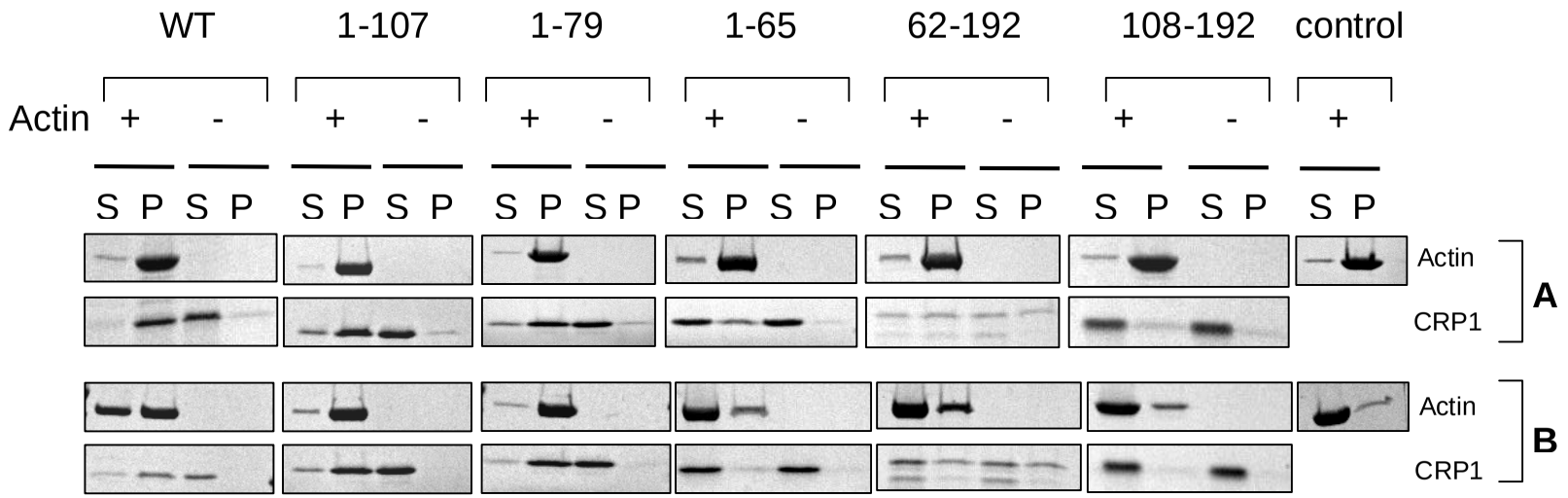


Figure A-2. LIM1/GR1 is required for CRP1 binding or bundling of F-actin. CRP1 truncation forms were subjected to cosedimentation assays at 100,000 x g (A) and 10,000 x g (B) as described in Materials and Methods of chapter 2.

Appendix C. Non-glycine residues in GR1 are important for full-length CRP1 bundling of F-actin.

To determine the effect of seven non-glycine residues identified in domain swap assay (Fig. 2-3) on the CRP1 bundling activity, cosedimentation assays were carried out with full-length CRP1 mutant. When the seven non-glycine residues in GR1 were mutated in the full-length CRP1 protein, actin filament bundling was reduced by ~50% *in vitro* (Fig. A-3 B, C). Cosedimentation assay data are also provided for domain swap experiments (Fig. A-3 A)

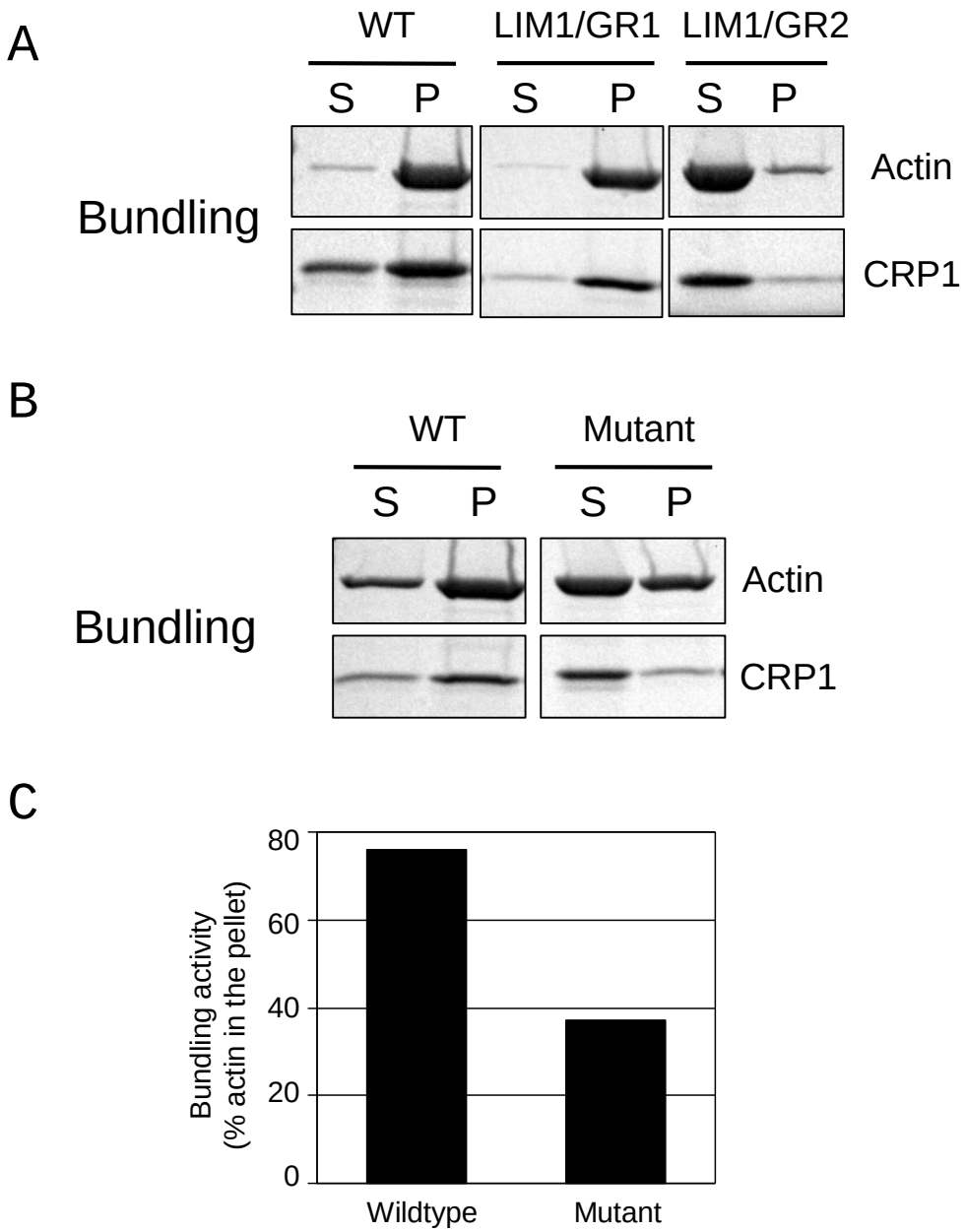


Figure A-3. Non-glycine residues of GR1 are important for actin bundling activity of full-length CRP1. (A) The bundling activity of domain swap mutant was analyzed by cosedimentation assay at 10,000 x g. Based on the GR1/GR2 domain swapping experiments, the seven residues unique to GR1 were mutated as shown in the Fig. 2-4, and its actin binding or bundling activities were determined using the cosedimentation assay described in Materials and Methods of chapter 2 (B) and quantified using densitometry (C).

Appendix D. Sequence of PCR primers.

PCR primer sequences used to make CRP1 truncation forms (Table A-1) and CRP1 domain swap mutants (Table A-2) in chapter 2 are provided.

Table A-1. Sequence of PCR primers for making 6xHis-tagged truncation forms of CRP1.

| Construct | Primer* | Primer sequence |
|---|---|---|
| Wildtype | F | 5′-ATAGAATTCCCCTCCGGGCCCCAACGCCG-3′ |
| | R | 5′-ACTTCTAGATCTGCGAGTGGATGAGCGCCC-3′ |
| 1-107 | F | 5′-ATAGAATTCCCCTCCGGGCCCCAACGCCG-3′ |
| | R | 5′-ACTTCTAGATTCTGGATGCGTTCGGGTTGG-3′ |
| 1-79 | F | 5′-ATAGAATTCCCCTCCGGGCCCCAACGCCG-3′ |
| | R | 5′-ACTTCTAGATGGTCCCGGCGCCCATCCCATAC-3′ |
| 1-65 | F | 5′-ATAGAATTCCCCTCCGGGCCCCAACGCCG-3′ |
| | R | 5′-ACTTCTAGATCTTCTTGCCATAGCAGGACTTG-3′ |
| 62-192 | F | 5′-ATAGAATTCCTATGGCAAGAAGTACGGCCC-3′ |
| | R | 5′-ACTTCTAGATCTGCGAGTGGATGAGCGCCC-3′ |
| 108-192 | F | 5′-ATAGAATTCCATGGCCCAGAAAGTCGGCGG -3′ |
| | R | 5′-ACTTCTAGATCTGCGAGTGGATGAGCGCCC-3′ |

* F, forward primer; R, reverse primer

Table A-2. Sequence of PCR primers for making GR-swapped construct.

| Construct | Fragment | Primer* | Primer sequence |
|---|---|---|---|
| LIM1/GR2 | 1-62 | F | 5′-ATAGAATTCCATGCCAAACTGGGGTGGAGG-3′ |
| | | R | 5′-GAAGTTCTTGGCATAGCAGGACTTGCAGTAG-3′ |
| | 171-187 | F | 5′-GCCAAGAACTTCGGGCCC-3′ |
| | | R | 5′-ACTTCTAGATCGCCCCGGCCCCCTGCCC-3′ |
| LIM2/GR1 | 115-170 | F | 5′-ATAGAATTCTGATGGGTGCCCGCGCTGCGG-3′ |
| | | R | 5′-GTACTTCTTGCCGTAGCAACCTTTGCAG-3′ |
| | 63-79 | F | 5′-GGCAAGAAGTACGGCCCC-3′ |
| | | R | 5′-ACTTCTAGATGGTCCCGGCGCCCATCCCATAC-3′ |

* F, forward primer; R, reverse primer

Appendix E. Gelatinolytic Activities on Zymograms are Dependent on Divalent Cations.

To show that clear bands on gelatin zymograms result from gelatinolytic activities of MMP2 or MMP9, we added 20 mM EDTA to the renaturing buffer and the developing buffer. Since the MMP activity is dependent on divalent cations calcium and zinc, the addition of EDTA would inhibit the MMP activity. As expected, the addition of 20 mM EDTA abolished clear bands on the gelatin zymograms (Fig. A-4).

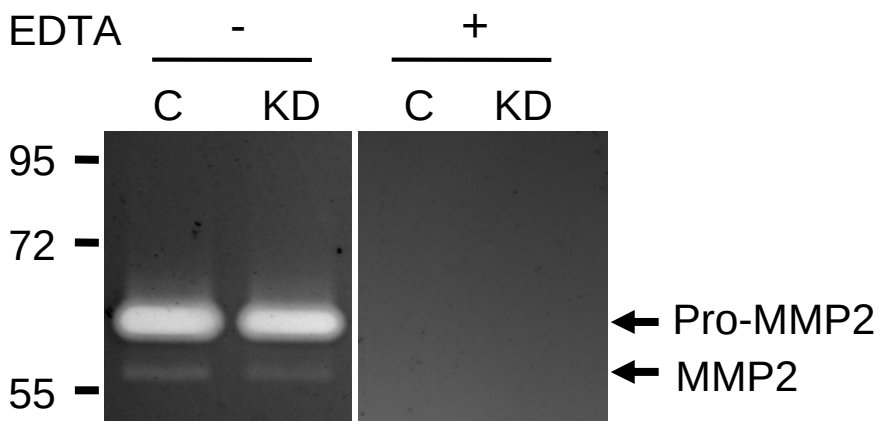


Figure A-4. Gelatinolytic activities on a gelatin zymogram visualize divalent cation dependent MMP2 proteins. To show that the clear band formation is due to the gelatinolytic activity of MMP2 or MMP9, gelatin zymogram was done as described in Experimental Procedures in chapter 3. EDTA was added to both the renaturing and the developing buffers at the final concentration of 20 mM.