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

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Abstract

Invasion of glioblastoma cells significantly reduces the effectiveness of current treatments, highlighting the importance of understanding dispersal mechanisms and characteristics of the invasive population. Induction of calcium fluxes into glioblastoma cells by autocrine glutamate is critical for invasion. However, the 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. Knockdown of calpain 2 expression using shRNA or chemical inhibition of calpain activity reduced glioblastoma cell invasion by 90%. Interestingly, decreased expression of calpain 2 did not influence morphology or migration, suggesting regulation of invasion specific mechanisms. Consistent with this idea, 39% less extracellular MMP2 was measured from knockdown cells identifying one mechanism by which calpain 2 mediates glioblastoma cell invasion. This is the first report demonstrating that calpain 2 is required for glioblastoma cell invasion.

Keywords

Calcium; Calpain; Glioblastoma; Glutamate; Invasion; Matrix metalloproteinase

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% [5]. Although the mechanisms responsible for the carcinogenesis and progression of glioblastoma tumors are unknown, mutations in several genes have been identified in glioblastoma cells. Mutations resulting in inactivation of the PtdIns (3,4,5)-P₃ phosphatase PTEN have been detected in 31% of glioblastoma cell lines [24]. Mutations resulting in the activation of the p110 α isoform of PI 3-kinase have been observed in 27% of glioblastomas [36]. In addition, Kubiakowski et al. [22] 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)-P₃ in the migration and invasion of glioblastoma cells. We recently demonstrated that PtdIns (3,4,5)-P₃ binding regulates the susceptibility of the cytoskeletal adhesion protein α -actinin to proteolysis by calpain 2 [40],

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. [37], reporting increased levels of calpain activity in breast cancer tissues. A study by Kimura et al. [20] followed observing calpain-mediated proteolysis of the cytoskeletal protein merlin in nervous system tumors such as schwannomas and meningiomas. In addition, Braun et al. [2] 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 [4,17,23,25,34,35]. In 2003, Mamoune et al. [28] 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 [6]. 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 [27,39]. These results are important because glutamate activates AMPA receptors on glioblastoma cells stimulating calcium influxes promoting migration [19]. In addition, Giannone et al. [10] 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 is 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.

Experimental Procedures

Antibodies and Reagents

Anti-actin (clone AC-40), anti-talin (clone 8d4), glutamate and gelatin were purchased from Sigma (St. Louis, MO); anti-calpain 1 (RP3) and anti-calpain 2 (RP1) were from Triple Point Biologics (Forest Grove, OR); anti-cortactin (clone 4F11) was from Millipore (Temecula, CA); anti-filamin (H-300) was from Santa Cruz Biotech (San Jose, CA); and peroxidase-conjugated goat anti-mouse IgG (H+L) and peroxidase-conjugated goat anti-rabbit IgG (H+L) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Calpain inhibitor 1 (ALLN) and calpain inhibitor 2 (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).

Cell Culture and Knockdown of Calpain 2

Human U87MG glioblastoma cells, referred to as the parental U87MG cells, were obtained from American Type Culture Collection (Manassas, VA) and cultured as previously described [40]. Calpain 2 targeting and control short hairpin RNA (shRNA) plasmids were purchased from SABiosciences (Frederick, MD); the nucleotide sequence of calpain 2 shRNA was 5'-GGGCTGAAGGAGTTCTA CATT-3' and control shRNA was 5'-GGAATCTCATTTCG ATGCATAC-3'. Calpain 2 knockdown and control cells were generated by transfecting the parental U87MG cells with calpain 2 targeting or control shRNA plasmid followed by selection for stable cells with 400 µg/ml Geneticin (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol (SABiosciences). Stable cells were maintained in media containing 400 µg/ml Geneticin. Transfection was carried out using FuGENE HD (Roche, Indianapolis, IN) following instructions from the manufacturer. For all experiments examining the calpain 2 knockdown U87MG cells, comparison is made with U87MG cells transfected with control shRNA.

Transwell Invasion Assay

Invasion assays were carried out using transwell permeable supports (6.5 mm diameter and 8.0 µm pore size) according to the manufacturer's instructions (Corning, NY). Briefly, Matrigel was diluted to 2 mg/ml using ice-cold serum-free DMEM on ice and 50 µl added to the upper compartment of the transwell insert, followed by 1 h incubation at 37°C. Matrigel coated transwells were transferred to a 24 well plate containing 0.65 ml of media in each well. Fifty thousand cells in 100 µl of media were added to the upper compartment. Following 3 days incubation at 37°C, the Matrigel and cells remaining in the upper compartment were removed using a cotton swab and the cells on the bottom side of the membrane fixed with methanol and mounted on a glass slide using ProLong Gold antifade reagent with DAPI (Invitrogen). Images were captured using a cooled CCD camera (CoolSNAP-HQ, Photometrics, Tucson, AZ) connected to the Axiovert 100 microscope (Carl Zeiss, Thornwood, NY) and the number of DAPI stained cells quantified using MetaMorph 6.2 software (Molecular Devices, Downingtown, PA).

Transwell Migration Assay

Transwell permeable supports (6.5 mm diameter and 8.0 µm pore size) were used for migration assays following the manufacturer's instructions (Corning, NY). Extracellular matrix proteins fibronectin (FN), laminin (LN), and collagen type IV (CL) were diluted to 10 µg/ml with water (for FN and LN) or 0.05 N HCl (for CL) and 50 µl incubated on the bottom surface of the transwell membrane for 1 h at room temperature. The membrane was then washed and blocked with 0.1% BSA/serum free media for 30 min. The coated transwells were transferred to a 24 well plate containing 0.65 ml of media in each well. One hundred thousand cells in 100 µl of media were added to the upper compartment. After 3 h incubation at 37°C, cells remaining in the upper compartment were removed using a cotton swab and the cells on the bottom side of the membrane were fixed with methanol, mounted on a glass slide using ProLong Gold antifade reagent with DAPI, and counted as described above.

Cell Migration Scratch Assays

Migration was measured using a scratch assay as described [13]. 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, using the Calipers function under the Measure menu of MetaMorph 6.2, the monolayer advanced from the edge of the scratch. Values from 3 regions were averaged to calculate the distance migrated.

Matrix Metalloproteinase Assay

The levels of MMPs in the glioblastoma culture media was visualized by gelatin zymography following published protocols [43]. Conditioned media from U87MG cell cultures were centrifuged for 2 min at 12,000 rpm and the supernatant prepared for electrophoresis in the absence of reducing agent. Proteins were separated by SDS-PAGE on gels containing 0.08% gelatin. Gels were incubated for 1 h at room temperature with renaturing buffer [50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 μ M ZnCl₂, 5 mM CaCl₂, 0.02% NaN₃, 2.5% Triton X-100] and overnight at 37°C with developing buffer [50 mM Tris-HCl at pH7.5, 200 mM NaCl, 5 μ M ZnCl₂, 5 mM CaCl₂, 0.02% NaN₃]. Gels were then stained with Gelcode Blue Stain Reagent (Pierce, Rockford, IL) and the clear bands representing MMPs were quantified using a Kodak Image Station 440CF and Molecular Imaging Software version 4.0 (Eastman Kodak, Rochester, NY). Data were analyzed with Student's *t*-test and the level of significance was set at $P < 0.05$.

Results and Discussion

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. 1). Actin was also immunoblotted verifying that equal protein was loaded for each sample. In addition, immunoblotting for calpain 1 was carried out showing 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 [14], have altered morphology with decreased cell spreading and an increase in process formation [7]. These morphological observations were extended to calpain 2 knockdown fibroblasts [8]. Based on these reports, we expected the morphology of the U87MG calpain 2 knockdown cells to differ from that of control cells. However, we did not observe any morphological differences in terms of cell spreading, shape, and process formation, between the control and calpain 2 knockdown U87MG cells using phase contrast microscopy (Fig. 2). In addition, no differences were observed for cells plated on fibronectin and stained for *F*-actin (Fig. 2). 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 immunoblotting total cell lysates from control and knockdown cells treated in the absence or presence of the calcium ionophore A23187 (Fig. 3). The talin and filamin breakdown products which have previously been attributed to calpain proteolysis were observed in all samples [8,9,15]. 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. 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. 4). Cells were examined daily by light microscopy to verify that the decrease in invasion was not the result of differences in proliferation between the control and knockdown cells. Furthermore, cell counting and microscopic monitoring of the continuous cultures for the control and knockdown cells showed that there was no difference in the rate of proliferation or any evidence of apoptosis. 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 inhibitor 1 and 2 were observed to decrease invasion of U87MG cells (Fig. 5) at concentrations previously reported to inhibit calpain 2 activity in cultured cells [1,4,12,18]. 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 [27]. In addition, glutamate has been shown to induce chemotactic migration of brain microglial cells [26]. 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 μ M, glutamate did not induce invasion of either the control or calpain 2 knockdown U87MG cells (data not shown). These results are consistent with the study published by Sontheimer and colleagues showing that exogenous glutamate did not increase glioblastoma cell migration [27] 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 [27]. 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.

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 [8,9,29–31,33,38]. 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. 6). Migration was measured using transwell assays coating the membrane with the purified extracellular matrix proteins found in Matrigel: laminin and collagen [21]. In addition, migration was assayed coating with fibronectin and FBS, which are commonly used to study the migration of glioblastoma cells [16,42]. For comparison with the invasion assays (Fig. 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, under all conditions tested 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 method (Fig. 7).

Although these results differed from studies with fibroblasts and neutrophils showing a critical role for calpain 2 in the basic 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. 2). These results emphasize that calpain 2 may differentially regulate morphology and migration depending on the cell type and environmental conditions. More specifically, these results suggested that calpain 2 was not modulating glioblastoma cell invasion via control of migratory mechanisms, but through regulation of invasion specific processes.

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 [3,11,41,44]. 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 [6,31]. 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 cells [6], we observed an increase in full-length cortactin protein in the calpain 2 knockdown cells compared to controls (Fig. 8). Furthermore, the presence of cortactin breakdown products, which have previously been attributed to calpain proteolysis, was 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 Procedures. 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. 9a). Under these conditions, a light band representing the active form of MMP2 was only detected at 36 h and no MMP9 activity was observed. Fetal bovine serum (FBS) 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. Quantification by densitometry showed that there was 24% less pro-MMP2 and 39% less MMP2 in the media from the calpain 2 knockdown cells compared to control (Fig. 9c). Similar results were observed in the media from the lower chamber demonstrating the diffusion and widespread distribution of pro-MMP2 and MMP2 in the media. Consistent with the results from two-dimensional cultures, no MMP9 activity was detected in these assays suggesting that MMP9 may not play a major role in the invasion of U87MG glioblastoma cells. These results are consistent with observations reported by Postovit et al. [32] correlating expression of calpain 4, the small subunit common to calpain 1 and 2, with expression of MMP2 and invasion of fibroblasts from calpain 4 knockout embryos. Together, these results 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. 10). 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.

Abbreviations

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

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Fig. 1.

shRNA knockdown of calpain 2 in U87MG glioblastoma cells. Calpain 2 knockdown (KD) and control cells were generated by transfecting U87MG glioblastoma cells with calpain 2 targeting or control shRNA plasmid followed by selection for stable cells with 400 μ g/ml Geneticin as described in Experimental Procedures. a Expression of calpain 2, calpain 1, and actin were examined by Western blotting of total cell lysates (50,000 cells per lane). Anti-calpain 1 recognizes domain IV and anti-calpain 2 recognizes the amino-terminal end of domain III. b Bands representing calpain 2 protein were quantified using a Kodak Image Station 440CF and Molecular Imaging Software version 4.0 (Eastman Kodak, Rochester, NY). $n = 3 \pm \text{SEM}$. * Indicates $P = 0.001$

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

Fig. 3.

Calpain 2 mediates proteolysis of cytoskeletal proteins talin and filamin in glioblastoma cells. Total cell lysates (50,000 cells per lane) from control and knockdown (KD) cells were immunoblotted to detect the full-length protein and calpain 2 breakdown products (BDPs) of talin and filamin. Where indicated, the calcium ionophore A23187 (10 μ M) was added to the cells for 5 min to activate calpain 2. Anti-talin recognizes the full length protein and the 190 kDa carboxy-terminal rod domain. Anti-filamin recognizes residues 2,348–2,647 within the carboxy-terminus. Immunoblotting for actin is included as a loading control. Results are representative of three separate experiments

Fig. 4.

Calpain 2 expression is required for glioblastoma cell invasion. **a** Invasion of control and knockdown (KD) cells was measured using Matrigel coated transwells with or without 10% FBS-DMEM (serum) as a chemoattractant in the lower wells. After incubating 3 days, cells on the bottom surface of the transwell membrane were fixed, stained with DAPI, and images captured as described in Experimental Procedures. **b** DAPI stained cells were quantified using MetaMorph 6.2. $n = 3 \pm \text{SEM}$. Bar = 0.5 mm. * Indicates $P < 0.001$

Fig. 5.

Calpain activity is required for glioblastoma cell invasion. Invasion of the parental U87MG cells was measured using Matrigel coated transwells as in Fig. 4 in the presence of calpain inhibitor 1 or calpain inhibitor 2. Cells were pre-incubated with varying concentrations of calpain inhibitors for 30 min at 37°C before adding to the upper compartment of the transwells with the same concentration of the inhibitor in the lower compartment. After incubating 3 days, cells on the bottom surface of the transwell membranes were fixed and DAPI stained cells quantified as described in Experimental Procedures. Calpain inhibitors were neither washed off nor replenished during the assay. Ethanol was used as the carrier. $n = 3 \pm \text{SEM}$. * Indicates $P < 0.05$

Fig. 6. Calpain 2 expression is not required for glioblastoma cell migration. Migration of control and knockdown (KD) cells was measured using transwell chambers with the bottom surface of the membrane coated with fibronectin, laminin, collagen IV, or fetal bovine serum (FBS). After incubating 3 h, cells on the bottom surface of the transwell membrane were fixed and DAPI stained cells quantified as described in Experimental Procedures. $n = 3-4 \pm \text{SEM}$

Fig. 7. Calpain 2 expression is not required for glioblastoma cell migration in scratch assays. Migration of control and knockdown cells was measured using a scratch assay as described in Experimental Procedures. The width of the scratch was measured at three regions using MetaMorph 6.2 as shown in the images to calculate the distance migrated. $n = 4 \pm \text{SEM}$; Bar = 1.0 mm

Fig. 8.

Calpain 2 mediates proteolysis of the invadopodial protein cortactin in glioblastoma cells. Total cell lysates (50,000 cells per lane) from control and knockdown (KD) cells were immunoblotted to detect the full-length protein and calpain 2 breakdown products (BDP) of cortactin. Where indicated, the calcium ionophore A23187 (10 μ M) was added to the cells for 5 min to activate calpain 2. Immunoblotting for actin is included as a loading control

Fig. 9.

Calpain 2 expression is required for extracellular MMP2 in glioblastoma cells. **a** MMP levels in the media from control (C) and knockdown (KD) cells cultured in serum-free DMEM were measured over time using a gelatin zymogram assay as described in Experimental Procedures. Fetal bovine serum (FBS) was assayed along side as a positive control for identifying pro-MMP9 (pMMP9), pro-MMP2 (pMMP2), and MMP2. Results are representative of two separate experiments. **b** MMP levels were measured in the media from control (C) and knockdown (KD) cells cultured in Matrigel coated transwells for three days as for the invasion assays. Media was sampled from compartment above and below the transwell membrane. **c** The levels of pro-MMP2 and MMP2 were quantified by densitometry. $n = 3-5$. * Indicates $P = 0.027$. ** Indicates $P = 0.012$

Fig. 10.

Working model for the role of calpain 2 in glioblastoma cell invasion. Diagram illustrates how glutamate stimulation of calcium influxes via AMPA-R is proposed to promote glioblastoma cell invasion. Previously published results have shown that autocrine glutamate secreted through system Xc^- is required for glioblastoma cell invasion [1,10,19,27,39,45]. In this study, we show that calpain 2 expression and activity are also required for glioblastoma cell invasion identifying calpain 2 as a key target for glutamate-induced calcium influxes. We propose that calpain 2 regulates glioblastoma cell invasion by mediating adhesion and cytoskeletal plasticity, invadopodial dynamics, and extracellular MMP2