AN ABSTRACT OF THE THESIS OF

Justin Zhang for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on May 19, 2014. Title: Calpain 2 Cleavage of Akt in Glioblastoma Multiforme

Abstract approved: _____

Julie Greenwood

Glioblastoma is a malignant brain tumor that has extremely poor prognoses with a 5 year survival rate of less than 5%. The low survival rates of glioblastoma patients is attributed to glioblastoma cells being extremely resistant to radiotherapy and chemotherapy as well as being very invasive. We previously identified calpain 2, a calcium activated cysteine protease, to be required for invasion of glioblastoma. Here we identify a novel pathway in which calpain 2 directly cleaves Akt. Akt is a serine/threonine kinase that plays roles in numerous cellular processes including apoptosis, proliferation, and metabolism amongst others. We also identified through western blot analysis that calpain 2 is regulating cleavage of the isoforms Akt 1 and Akt 2. In addition we identified through immunofluorescence that calpain 2 appeared to be localized to the membrane ruffle region as well as the peri-nuclear region which is concomitant with Akt 2 and Akt 3 staining. We also identified that knockdown of calpain 2 in U87 glioblastoma cells caused a buildup of phosphorylated akt (ser473) as well as invadopodia at the membrane ruffle region indicating that calpain 2 may be regulating the turnover of invadopodia that are necessary for cell invasion.

Key Words: Glioblastoma, calpain 2, Akt, invadopodia

Corresponding e-mail address: zhangju@onid.oregonstate.edu

Calpain 2 Cleavage of Akt

By

Justin Xuan Zhang

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APPROVED:

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Mentor, representing Biochemistry and Biophysics

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Committee Member, representing the Linus Pauling Institute

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Committee Member, representing the Linus Pauling Institute

_____

Chair, Department of Biochemistry and Biophysics

_____

Dean, University Honors College

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

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Justin Zhang, Author

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Introduction

Glioma is the most common primary brain tumor, accounting for 2.5% of all cancer deaths in the United States with an estimated 12,500 deaths arising from primary malignant brain tumors (1). Gliomas make up about 30% of all brain and central nervous system tumors and about 80% of all malignant brain tumors, occurring at an annual incidence of 5 in every 100,000 individuals (2, 3). Gliomas arise from glial cells, non-neuronal cells that provide support and protection in the brain, and include glioblastoma multiforme, a highly malignant form of glioma. Approximately 66,290 primary brain tumors are diagnosed in the United States each year, 16% of which are glioblastomas (1). Despite the decades of research in cancer biology and specifically on glioblastomas, the prognosis of diagnosed patients remains poor (1,3). The median survival time after diagnosis is 3 months without treatment and 12 - 18 months with treatment, and the 5 year survival rate is less than 5% indicating a need for increased understanding of glioblastomas (4, 5).

The current standard of treatment involves surgical removal of as much of the tumor as physically possible followed with a combination of radiotherapy and chemotherapy using temozolomide, an oral chemotherapeutic that acts as an alkylating agent (6, 7). However, in recent years other chemotherapeutics have also been approved for use in the treatment of glioblastoma including the use of the humanized monoclonal antibody, Avastin (8). However, the effectiveness of current chemotherapeutics such as temozolomide is marginal as treatment with temozolomide increases median survival rates by 2 months from 12.1 months with radiotherapy to 14.6 months with radiotherapy and temozolomide

(53). Given such low survival rates, it is clear that the current treatments are still open for significant improvement. One of the main mechanisms by which glioblastoma cells are resistant to the standard treatments is due to its highly invasive nature. The first step of treatment, surgery, has the main purpose of removal of the bulk of the physical tumor. Studies have shown significant improvements in survival rate with removal of 98% or more of the tumor (9). However, even with surgical removal of most cells, residual tumor cells always remain. These residual cells are exceptionally invasive and spread throughout the brain after surgery (10). These residual cells pose the main problem with surgical techniques to treat glioblastoma as they are able to form new tumors either near the original tumor site or at distant satellite locations. Formation of new tumors and invasion of residual glioblastoma cells is often deadly as further removal of brain tissue may lead to neurodegeneration. Additionally, these residual glioblastoma cells are highly resistant to chemotherapy and radiotherapy (11) making patients especially vulnerable to glioblastoma cell invasion given the lack of therapeutics targeting invasion. Because of these low survival rates and lack of novel therapies for the treatment of glioblastoma, it is important to understand the mechanisms of glioblastoma cell invasion that leads to these poor outcomes.

Previous studies have shown that glioblastoma cell invasion and migration are regulated by autocrine glutamate signaling (12-14). Secretion of large amounts of glutamate by the glioblastoma cells trigger AMPA type glutamate receptors (AMPARs) which results in calcium permeability causing excitotoxic cell death of the surrounding neurons (15). However, secretion of glutamate also activates AMPA receptors on the glioblastoma cells

themselves which stimulate calcium influxes and promotes cell migration (16). Because AMPA receptors induce calcium oscillations it has also been found that calcium oscillations are required for focal adhesion disassembly in glioblastoma cells allowing for cellular migration (17). While the targets of calcium required for glioblastoma cell invasion are unknown, calpain 2 is a likely candidate given its calcium dependence.

The calpain proteins belong to a family of calcium dependent cysteine proteases (18). calpain 2 is a ubiquitously expressed isoform of the calpain family that regulates adhesion turnover and disassembly which aids in cell migration (19). Previous studies have linked calpain and cancer, reporting that there existed increased levels of calpain activity in breast cancer tissues (20). More recent studies have further established a role for calpain in both carcinogenesis and tumor progression (21-23). Studies have demonstrated that inhibition or down-regulation of calpain 2 decreased invasiveness of human prostate tumor cells by 50% in a rodent xenograft model (24). Calpain 2 has also been shown to regulate invadapodial projections affecting cell migration in breast cancer cells (25). These results point to calpain 2 as a potentially important mediator of glioblastoma cell invasion.

Our lab has previously examined the role of calpain 2 in glioblastoma cell invasion. In the study, both chemical calpain 2 inhibitors and shRNA based knockdown cell lines of calpain 2 were used. The results indicated that knockdown of calpain 2 causes a 90% reduction in glioblastoma cell invasion (26) suggesting that calpain 2 activity is required for the invasion of glioblastoma cells. The study also found that calpain 2 decreased the

amount of matrix metalloproteinase 2 (MMP2), a zinc dependent endopeptidase, in the extracellular matrix. These results indicate that calpain 2 may be affecting invasion processes through regulation of degradative extracellular enzymes. In addition to *in-vitro* studies, the lab also examined the effects of calpain 2 on invasion *in-vivo* by using a zebrafish xenograft model (27). In this model, human glioblastoma cells and calpain 2 knockdown cells were injected into zebrafish brains and observed for migration of the cancer cells. The results showed that there was a 2.9 fold decrease in human glioblastoma cell invasion when calpain 2 was knocked down compared to control human glioblastoma cells. These data together suggest that active calpain 2 is required for successful invasion of glioblastoma cells both *in-vitro* and *in-vivo*. Additionally, this study established the zebrafish model as an effective model for the study of brain tumor invasion making it extremely useful for further studies. Our lab has currently identified a novel protein, calpain 2, which is required for the invasion of glioblastoma cells. However, the mechanism and downstream effectors of calpain 2 involved in the regulation of invasion are currently unknown.

Because of the effect of calpain 2 on invasion and MMP2 levels, Akt has been identified as a potential target of calpain 2 due to its common role in regulation of invadapodia and MMP2 as well as other cellular processes such as cellular proliferation, glucose metabolism, apoptosis, and cell migration. Akt also known as protein kinase B (PKB) is a serine/threonine kinase which in mammals comprises three highly homologous members known as Akt1 (PKB alpha), Akt2, (PKB Beta), and Akt3 (PKB Gamma) (28). Akt is most well-known as part of the PI3K/Akt/mTOR pathway that is important in apoptosis

making it an important pathway in human cancers. Activation of Akt is phosphorylation dependent with two sites of phosphorylation, at threonine 308 or serine 473. Activation of the phosphoinositide-3-kinase (PI3K) leads to generation of phosphatidylinositol(3,4,5)-trisphosphate (PIP3) from phosphorylation of phosphatidylinositol(4,5)-bisphosphate (PIP2). Akt binds PIP3 through its pleckstrin homology (PH) domain (fig. A), resulting in translocation of Akt to the cell membrane. Akt is then activated through a dual phosphorylation mechanism in which phosphoinositide-dependent kinase-1 (PDK1) is brought to the membrane by the PH domain of Akt and phosphorylates Akt within its activation loop at threonine 308. A second phosphorylation at serine 473 within the carboxyl terminus is also required for activity and is carried out by the mTOR-rictor complex, mTORC2 (29, 30). Also involved in this pathway is a lipid phosphatase, phosphatase and tension homolog (PTEN), that catalyzes the dephosphorylation of PIP3 leading to inactivation. This dephosphorylation mechanism makes PTEN a major negative regulator of Akt signaling. Thus when PTEN is not expressed, the phosphorylation and dephosphorylation equilibrium of the PIP3 pathway is disrupted causing a buildup of PIP3 leading to hyper activation of Akt. Glioblastoma is known to have mutations in PTEN genes causing deactivation of the PTEN protein leading to hyper activation of Akt (31). Overactivation of Akt in cancer cells often leads to increased proliferation as well as resistace to apoptosis. PTEN mutations and deficiencies are also prevalent in many types of human cancers and have been associated with late stage tumors as well as therapeutic resistance potentially causing glioblastoma resistance to chemotherapy drugs (30).

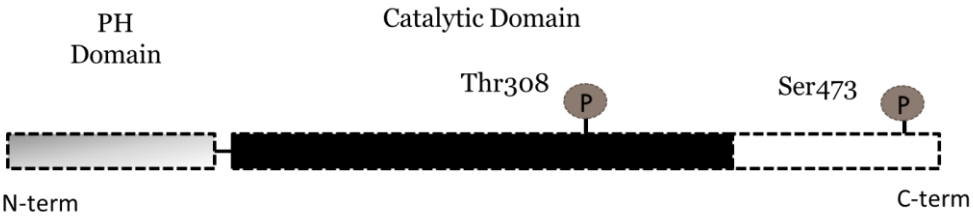


Figure A: Schematic of Akt protein domains highlights the pleckstrin homology (PH) domain and the catalytic domain. Connecting the PH domain and the catalytic domain is a linker region. The two phosphorylation sites, Thr308 and Ser473, are found in the catalytic domain and the C-terminal extension respectively.

Activated Akt phosphorylates a number of downstream targets to regulate their activity. One of the primary functions of Akt is to promote cell growth and protein synthesis through regulation of the mTOR complex 1 (mTORC1) signaling pathway. Akt can directly phosphorylate and activate mTOR, but can also inhibit the mTOR inhibitor proteins PRAS40 and tuberin (TSC2) leading to mTOR activation (32). Another major target of Akt is glycogen synthase kinase 3 (GSK-3). GSK-3 has two active forms, GSK-3alpha (Ser21) or GSK-3Beta (Ser9), and acts to promote glycogen metabolism, cell cycle progression, and regulation of wnt signaling. Phosphorylation of GSK-3 by Akt acts to inactivate GSK-3, which leads to activation of glycogen synthase among other downstream targets of GSK-3 (33).

Hyperactivation of Akt often leads to malignant cancers in large part due to Akt's role in regulation of cell death and apoptotic processes. Akt acts to attenuate the activation of the intrinsic apoptosis pathway. One of the most studied paths of regulation is through inhibition of Bax and Bak which are pro-apoptotic proteins (34). Akt also phosphorylates the Bcl-2 family pro-apoptotic BH3 only protein BAD. Phosphorylation of BAD causes its inability to block Bcl-2 protective function (35). Akt has also been shown to

phosphorylate the three members of the FoxO subfamily of Forkhead transcription factors (FoxO1, FoxO3, and foxO4). Phosphorylation of these FoxO proteins results in their retention in the cytosol which causes an inactivation in their transcriptional function. Blocking the functionality of the FoxO proteins is thought to promote cell survival due to the activation of certain FoxO target genes such as the ligand for the death receptor Fas, FasL (36, 37, 38).

Recently significant evidence has also garnered attention to Akt's role in cell migration which may be a significant contributing factor in the development of various cancers especially in regards to cell invasion as in glioblastoma. Previously Akt has been found to be localized in the leading edge membrane area of migrating HT1080 cells (39). In this particular study it was found that Akt potently promoted the invasion of HT1080 cells by increasing cell motility and matrix metalloproteinase-9 (MMP9) production. Both these increases were dependent on the kinase activity and membrane translocating ability of Akt.

One of the fundamental requirements of cell migration is the ability of cells to reorganize cytoskeletal related proteins. Previous studies have found that Akt is a major regulator of some of these proteins. Akt has been found to be important in F-actin polymerization and cytoskeletal contraction (40). This is in addition to findings that supported the claim that increased filopodia production occurs in an active Akt dependent manner in breast cancer cells (41). This evidence has supported the idea that Akt may influence cell motility through direct regulation of actin.

In addition to cell motility and migration, cell invasion requires the ability to break down surrounding extracellular matrix to allow for progression of invading cells. Akt has been linked to increased matrix metalloproteinase 2 (MMP2) and MMP9 secretion which are both responsible for proteolytic processing of extracellular matrix structural proteins (42). Akt has also been implicated in the regulation of invadopodia formation. Invadopodia are extracellular matrix degrading protrusions that are often formed by invasive cancer cells (43). Formation of invadopodia is also often associated with increased MMP protein secretion at the edges of the protrusions. Akt regulation of both MMP protein secretion and invadopodia formation make it an important regulator of cellular invasion.

Akt is known to regulate a number of cellular processes from cell proliferation to cell migration and invasion making the study of the regulation of Akt extremely important in understanding signaling pathways important for basic cellular function. Here we examine calpain 2 proteolysis of Akt as a novel mechanism for regulating the activity of Akt. Further understanding of regulation of Akt gives great insight into new ways to control the activity of a key promoter of cancer progression

Materials and Methods

Cell Culture – Three cell lines were used, U87MG paternal cells from the American Type Culture Collection (ATCC), 2pC cells and 2pR cells. The 2pC and 2pR cells represent stably transfected U87MG cells. The 2pC cell line is transfected with a control shRNA whereas the 2pR cell line is transfected with shRNA targeting calpain 2. U87MG Glioblastoma cells were cultured in DMEM containing 10% fetal bovine serum with an addition of a 2mM concentration of L-glutamine. Cells were plated on 100mm cell culture plates or T-75 flasks. 500,000 or 1,000,000 cells were plated at a time and checked every day for confluency and health of cells. All cells were maintained at 37^{o} C, 100% humidity, 95% air, and 5% CO_2.

Immunoprecipitation – $2x10^6$ cells were washed with 1X PBS and 400 ul of cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na3VO3, 1 ug/ml leupeptin. Cell Signaling Technology, Danvers, Massachusetts). 100 mM PMSF was added to the cells and incubated on ice for 5 minutes. Cells were then scraped into tubes and microcentrifuged for 10 minutes at 14,000 X g at 4^{o}C and supernatant was transferred to a new tube microcentrifuge tube. 5 ul of phospho-Akt (Ser473) sepharose bead conjugate (Cell Signalling Technology, Danvers, Massachusetts). was added to the lysate and incubated overnight at 4^{o}C with mixing. Beads were then washed with cell lysis buffer and resuspended with 2X SDS buffer and heated at 95^{o}C for two minutes.

Western Blot – Cells were harvested when 100mm plates or T-75 flasks were at 80% confluency. Adherent cell samples were harvested by washing with 1x PBS and then lysed with 2x SDS buffer. Cells were sonicated and then boiled for 2 minutes at 95^{o}C. Cells were suspended using 0.05% trypsin (Life Technologies, Carlsbad, California) and then pelleted. Trypsin was then aspirated and suspended cells were lysed with 2x SDS buffer then sonicated and boiled for 2 minutes at 95^{o}C. Protein was separated by PAGE using a 12.5% gel and standard procedures and then transferred for one hour at 100V to nitrocellulose membranes. Membranes were then blocked in 5% milk made in Tris buffered saline with tween 20 (TBST). Membranes were then incubated in primary antibody at 1:1000 concentrations in 5% milk at 4^{o}C overnight. Antibodies including anti-pSer473 Akt, Pan Akt, Akt1, Akt2, Akt3 (Cell Signaling Technology, Danvers, Massachusetts). Membranes were washed in TBST and incubated overnight at 4^{o}C overnight in secondary antibody conjugated to horse radish peroxidase to be imaged. Secondary antibodies were purchased from Cell Signal and were made at a concentration of 1:2000 in 5% milk. Blots were imaged using a Kodak imager with chemiluminescent substrates purchased from Thermo Scientific.

Immunofluorescence Microscopy –Immunostaining was done by placing one glass coverslip into each well of a 12 well plate. 30,000 cells were seeded onto each glass coverslip and fixed with 3% formaldehyde diluted in phosphate buffered saline (PBS) for 30 minutes at room temperature. Cell membranes were then permeabilized using 0.5% triton X-100 in PBS. The cells were then blocked using 1% bovine serum albumin in PBS for 8 minutes at room temperature. Cells were then tagged with anti-Akt1, Akt2, Akt3, p-

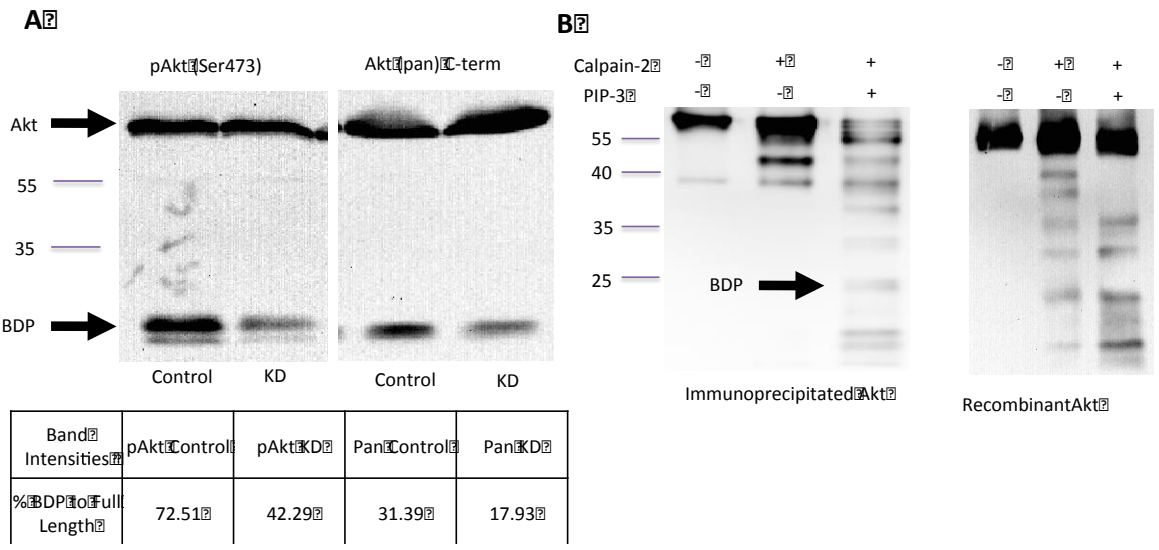
Akt(serine 473), Akt (Pan) and calpain 2 each at 1:50 dilutions in BSA for 30 minutes at 37^{o}C. Cells were then co-stained with either F-Actin at a dilution of 1:500 in BSA for 30 minutes at 37^{o}C. The cells were then incubated in their appropriate secondary antibodies and mounted onto glass slides with Prolong Gold antifade with DAPI reagent. Images of the cell were captured using a Zeiss Axiovert 100x microscope and uploaded using Metamorph imaging software.

Invadopodia Assay – Glass coverslips in a 12 well plate were coated with gelatin using a solution composed of 10 µl of 1mg/ml gelatin/PBS and 1ml of 2.5% sucrose/PBS solution. 100 µl of the gelatin solution was placed onto each coverslip and then most was aspirated off. 1 ml of 0.5% glutaraldehyde/PBS was added to each well and incubated on ice for 15 minutes then at room temperature for 30 min. Coverslips were then incubated in 1ml of 26.4 mM of sodium borohydride for 3 minutes at room temperature. The coverslips were then incubated in growth media before cells were plated onto the coverslips as done in immunofluorescence protocol. Cells were co-stained for cortactin at a 1:50 dilution.

Results and Discussion

Our lab has previously reported the role of calpain 2 as an important regulator of cellular invasion. Both Akt and calpain 2 have common downstream effectors making their potential interaction of interest to study. Using both a control and calpain 2 knockdown cell line made from U87 MG glioblastoma cells (26), it was found that Akt forms a breakdown product in the control cells at higher amounts compared to the calpain 2 knockdown cells indicating that calpain 2 may be required for the cleavage of Akt (fig. 1A). Here band intensities were measured and indicated by arbitrary units, and breakdown product band intensity was compared to full length band intensity as a percentage of full length band intensity. It is shown that there is a 42% decrease in the ratio of breakdown product to full length protein in the control cells compared to knockdown cells when blotting for phospho-Akt (fig. 1A). This result suggests that cleavage of Akt is regulated by calpain 2. The finding that calpain 2 mediates Akt cleavage is found using both antibodies targeting phosphorylated Akt (pSer473) and pan C-term Akt indicating that calpain 2 cleavage of Akt may be independent of the phosphorylation state of Akt (fig. 1A). Again when blotting for pan Akt there is a 43% decrease in the ratio of breakdown product to full length protein in the control cells compared to knockdown cells (fig. 1A). This result further supports the idea that cleavage of Akt is regulated by calpain 2. Additionally, the finding of a breakdown product that is detectable on the blot indicates that these fragments may be stable. Stable fragments have often been shown to retain activity and act as important regulators of cellular processes (44, 45, 46). The formation of a stable breakdown product of Akt may lead to further study of these breakdown products to determine if the fragments retain activity and what

their potential function is. To verify that calpain 2 was directly cleaving Akt and not merely upstream of this cleavage event, *in vitro* proteolysis reactions were carried out. Immunoprecipitated Akt from U87 MG glioblastoma cells was mixed with different combinations of buffer, calpain 2, and PIP-3. Akt alone in buffer showed little to no signs of proteolysis (fig. 1B lane 1). However, as calpain 2 is added to the reaction, there is seen an increase in breakdown of Akt indicating that calpain 2 is directly cleaving Akt and forming breakdown products (fig. 1B lane 2). Addition of both calpain 2 and PIP-3 show greatly increased cleavage of the Akt as seen in (fig. 1B lane 3). The breakdown product size of the proteolyzed Akt matches the size of the breakdown product in the control and knockdown cell lines. Addition of PIP-3 also increases cleavage above calpain 2 alone levels and is further confirmed by loss of intensity in the full length Akt band. These results are also consistent with previous findings that PIP-3 binding to substrate regulates the susceptibility of substrates to calpain proteolysis (47). Use of recombinant Akt instead of immunoprecipitated Akt mixed with combinations of calpain 2 and PIP-3 show similar proteolysis patterns (fig. 1C). However, with recombinant Akt, addition of calpain 2 alone shows more breakdown product bands than in the immunoprecipitated Akt. Addition of PIP-3 and calpain 2 do not show similar increases in proteolysis compared to immunoprecipitated Akt (fig. 1C lane 3). These *in-vitro* assays give further evidence that calpain 2 is directly acting on and cleaving Akt suggesting a novel mechanism of regulation of Akt.



Band Intensities	pAkt Control	pAkt KD	Pan Control	Pan KD
% BDP to Full Length	72.51	42.29	31.39	17.93

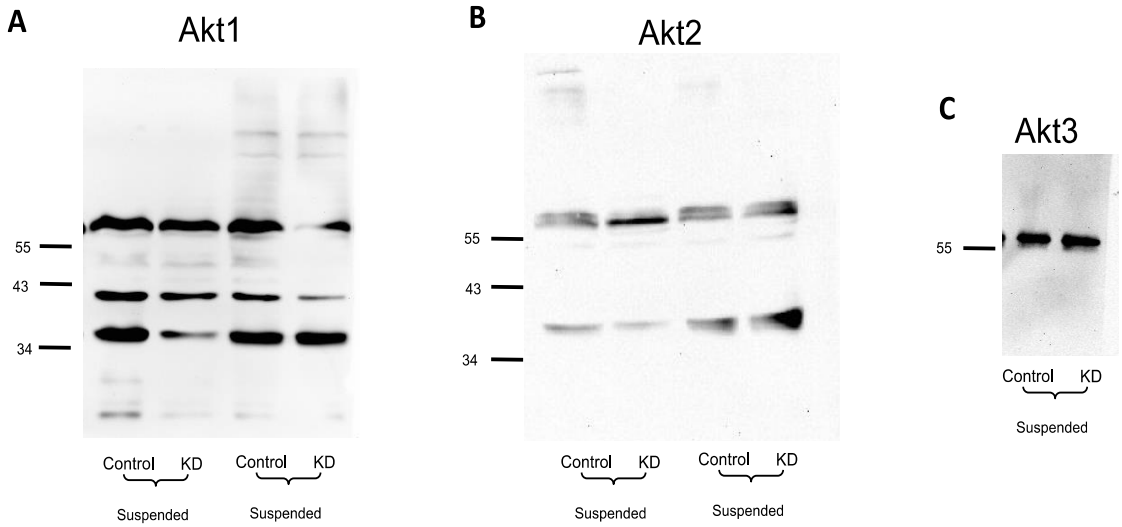
Figure 1: Calpain 2 regulates the proteolysis of Akt. (A) *In-vivo*, calpain-2 knockdown cells exhibit less break down product (BDP) than in control cells both in phosphorylated Akt and in pan Akt suggesting that calpain-2 is required for breakdown of Akt. (B) Immunoprecipitated Akt was mixed with calpain 2 and PIP-3 *in-vitro* and blotted for phosphorylated Akt at serine 473. Greater breakdown product formation is seen with addition of calpain 2 and PIP-3. (C) Recombinant Akt was mixed with calpain 2 and PIP-3 as in part B and again more breakdown product formation of phosphorylated Akt is observed with addition of calpain 2 and PIP-3.

Further characterization of the action of calpain 2 on Akt was done to determine potential cleavage of specific isoforms of Akt. As mentioned previously Akt has three isoforms: Akt1, Akt2, and Akt3 (alpha, beta, gamma respectively). These three isoforms have been shown to have varying functions that may give rise to the wide diversity of Akt signaling. Akt1 has been implicated in cell survival with Akt1 knockout mice being smaller than wild-type counterparts and display higher rates of apoptosis (48). Akt2 is involved in glucose homeostasis with Akt2 knockout mice displaying type 2 diabetes like phenotypes (49). Akt3 on the other hand has been implicated in regulating brain size (50, 51). The structure of Akt has been shown to contain a pleckstrin homology (PH) domain followed by a linker domain followed by a kinase catalytic domain and ending with a C-terminal extension containing a regulatory hydrophobic motif. Amongst the Akt isoforms the PH

domain is about 80% identical to each other, and the isoforms share 90% identity amongst each other in the catalytic domain. The C-terminal extension also has about 70% identity to each other. However, the linker region has low identity amongst the isoforms sharing only about 30% identity (52). To determine calpain 2 cleavage of Akt isoforms, calpain 2 knockdown and control cells were examined using antibodies targeting each specific isoform to probe for potential breakdown products. Antibodies obtained from Cell Signaling were specific to each antibody. Akt1 monoclonal antibodies were targeting Leu110 of Akt1, Akt2 monoclonal antibodies were targeted to residues of Akt2 (residue # not given), and Akt3 was targeted to Arg116 on Akt3. Targets for Akt 1 and 3 are both located in the linker region of Akt (Fig. A). Here both suspended and adherent cells were sampled. Sampling both suspended and adherent cells gives indication to potential changes in protein expression in the suspended and adherent states. As seen in Fig. 2A there appear to be significantly more breakdown product of Akt1 in control cells compared to calpain 2 knockdown cells. We observe a measured decrease of 66% of 40 kDa breakdown product compared to full length Akt1 from control cells to knockdown in suspended cells suggesting that calpain 2 regulates cleavage of Akt1 and its formation of a 40 kDa breakdown product (fig. 2A). For the 36 kDa breakdown product there is an 87% decrease in the ratio of breakdown product to full length in control and knockdown suspended cells suggesting again that calpain 2 regulates cleavage of Akt1 and its formation of a 36 kDa breakdown product (fig. 2A). However, the opposite effect is seen in adherent cell samples. For adherent cells there is a 320% increase in the ratio of knockdown to full length Akt1 for the 40 kDa breakdown product, and there is greater than a tenfold increase in the ratio of knockdown to full length for the 36 kDa breakdown

product (fig. 2A). This result suggests that adherent cells and suspended cells may regulate the cleavage of Akt1 differently. Interestingly, when probing for Akt1 breakdown products, there appears to be two major breakdown products at approximately 36 kDa and 40 kDa. This gives rise to evidence that calpain 2 proteolysis of Akt isoforms may produce multiple fragments that may give rise to different functionalities for the stable products. When probing for Akt2 it was measured that there was an 85% decrease in the ratio of breakdown product to full length in the control cells compared to knockdown suspended cells (fig. 2B). This suggests that calpain 2 also regulates the cleavage of Akt2. However, again in the adherent cells we observe a measured increase of 208% in the ratio of breakdown product to full length in the control cells compared to knockdown (fig. 2B). that in suspended cells there is more breakdown product in suspended control cells compared to suspended knockdown cells (fig. 2B lane 2). This result again suggests that the adherent and suspended cell populations regulate the cleavage of Akt differently. Interestingly the Akt2 breakdown product only forms one band around the 36 kDa mark with no second band at 40 kDa as seen in Akt1. This result suggests that calpain 2 may be differentially cleaving isoforms of Akt, which indicates that calpain 2 could be cleaving Akt in an isoform specific manner. To support this idea, it is seen in Akt3 that there is almost no formation of breakdown products at either previously observed 36 or 40 kDA bands suggesting that calpain 2 is not involved in cleavage of the Akt 3 isoform. Additionally, endogenous levels of Akt3 appeared to be almost undetectable from control and knockdown cell lysates leading to the use of immunoprecipitation to obtain detectable levels of Akt3. Even immunoprecipitating Akt3 there appears to be no formation of breakdown products which was also seen in blots for

endogenous Akt3. The lack of detectable levels of Akt3 in these cells is interesting as Akt3 is known to be significantly involved in brain size and function. However, previous studies have suggested Akt3 plays a major role mainly in neuronal cells (50, 51). These results potentially suggest that Akt3 is not as involved in the regulation of glial cells as it is in neuronal cells in regulating brain function and size.



Band Intensity	Control Suspended	KD Suspended	Control Adherent	KD Adherent
Full Length	1636.58	1481.55	2264.2	148.45
BDP 40	2007.62	614.61	630.21	173.37
BDP 36	1620.07	189.89	986.91	956.31

Band Intensity	Control Suspended	KD Suspended	Control Adherent	KD Adherent
Full Length	9.8222	30.26	8.7581	9.0996
Breakdown	7.3362	3.2814	7.9574	25.5
% Full Length	74.69	10.84	90.85	280.23

Figure 2: Calpain-2 is limited to specific isoforms of Akt. (A) Cleavage of Akt1 is more prominent in 2pC cells for both suspended and adherent cells. (B) Similar to Akt1, Akt2 has increased break down products in 2pC cells compared to 2pR cells for both suspended and adherent cells. (C) Akt3 levels were low in cell lysates leading us to immunoprecipitate Akt3. No breakdown products are seen in either 2pC cells or 2pR cells.

Immunofluorescence experiments were done next in order to determine localization of Akt isoforms in U87 MG glioblastoma cells. Cells were fixed and antibodies targeting

specific Akt isoforms were used to determine localization. Co-staining with filamentous actin (F-actin) was done as well in order to visualize cell shape. As seen in figure 3A. Akt1 is found largely localized in the nuclear region as well as generally dispersed in the cytoplasm. Co-staining with F-actin reveals no significant areas of overlap. However Akt2 and Akt3 exhibit similar localization patterns with intense signal in the peri nuclear region as well as at the specific sites of the membrane. Staining of F-actin reveals that membrane staining of Akt2 and Akt3 are at sites of membrane ruffles as indicated by more intense F-actin staining at those sites. Membrane ruffles are a characteristic feature of many actively migrating cells suggesting that Akt is involved in cell motility which confirms previous results.

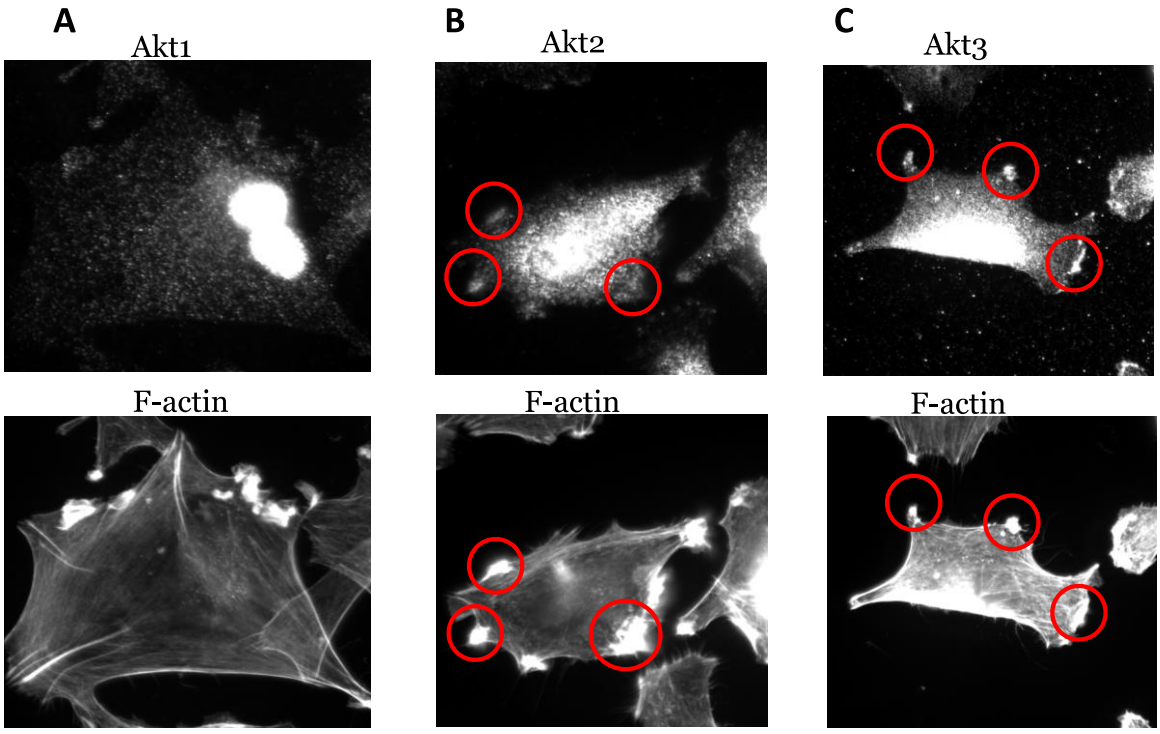


Figure 3: localization of Akt isoforms. (A). Akt1 is largely localized to the nuclear region (B) Akt2 has intense staining in the peri-nuclear region as well as along membrane ruffles (C) Akt3 also stains peri-nuclear region as well as along membrane ruffles

To further study the cleavage of Akt by calpain 2, cells were also stained for calpain 2 and F-actin in order to determine if calpain 2 and the Akt isoforms are co-localized. calpain 2 was found to be localized to the peri nuclear region as well as to membrane ruffle regions as indicated by colocalization with F-actin (fig4.). The localization to similar areas of the cell support the hypothesis that calpain 2 is directly cleaving Akt. The result indicating that calpain 2 is localized to membrane ruffle regions is also support for the fact that calpain 2 is involved in the invasion of glioblastoma cells as membrane ruffling is an important feature of cell motility.

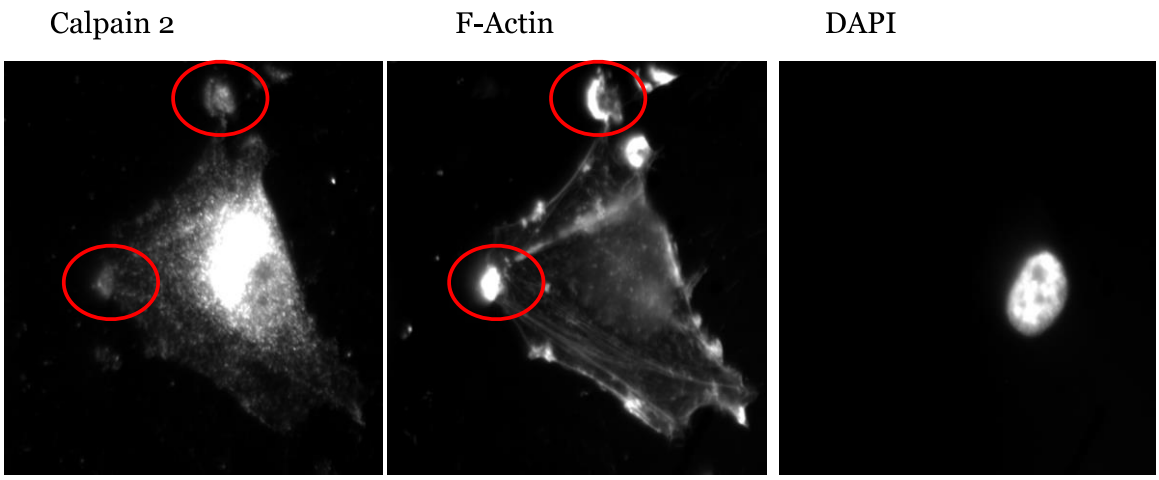


Figure 4: staining of calpain 2 indicates localization again in the peri-nuclear region as well as to the membrane ruffles

Akt was further studied to determine how cleavage by calpain 2 may regulate mechanisms leading to glioblastoma cell invasion. Phosphorylated Akt (Ser473) which is indicative of active Akt was co-stained with F-actin in U87 MG glioblastoma cells in order to determine the localization of active Akt. Here phosphorylated Akt (Ser473) was found to be localized largely to membrane ruffle regions as well as dispersed in the

cytoplasm (fig. 5). These data are consistent with previous findings done in HT1080 cells (40) and also further support that Akt is involved in cell migration and invasion of glioblastoma cells. The presence of active phosphorylated Akt at the membrane ruffles supports the idea that the Akt2 and Akt3 isoforms at the membrane ruffles were in active form as antibodies used to detect Akt isoforms were not phosphorylation dependent.

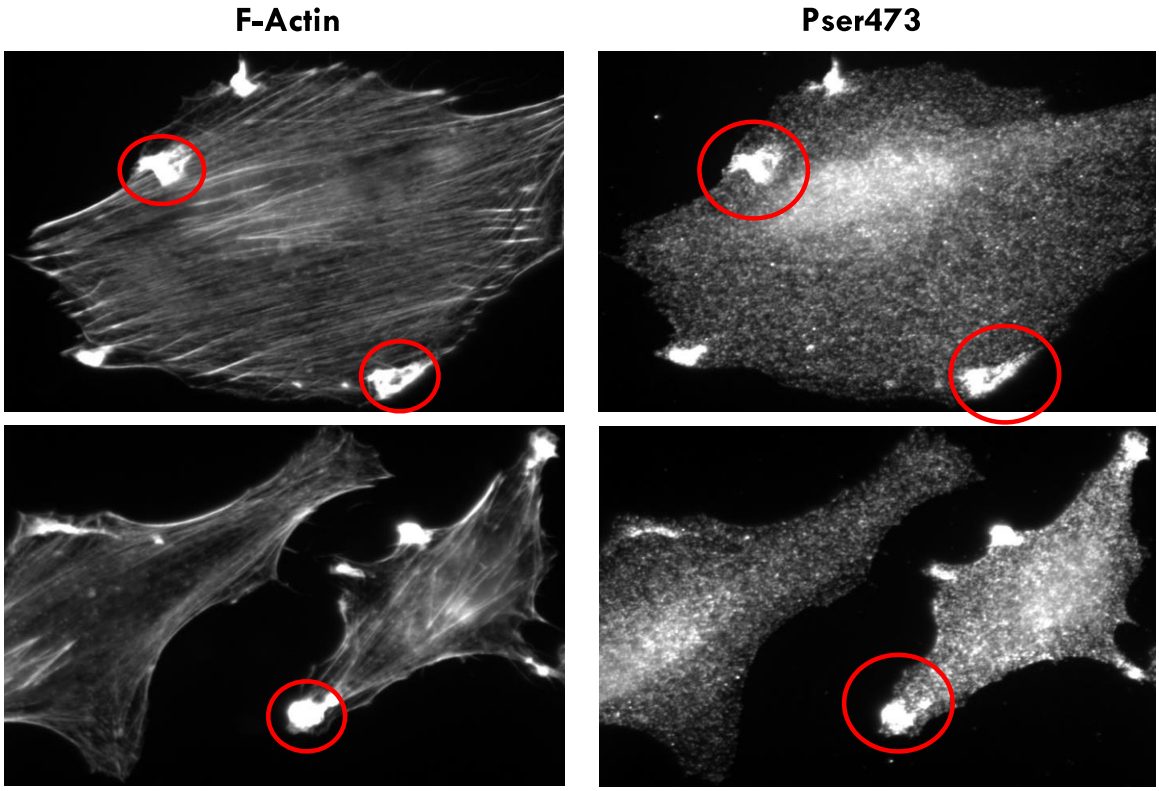


Figure 5: Immunofluorescence experiments indicated that Akt phosphorylated at residue 473 localizes to membrane ruffles consistent with F-actin staining

The finding that phosphorylated Akt (Ser473) is found at membrane ruffles is evidence that Akt is involved in cell invasion as changes in actin cytoskeleton at the ruffles is an important step in cellular invasion. Further experiments were done to show the link between calpain 2 cleavage of Akt and glioblastoma cell invasion. Both control and calpain 2 knockdown cells were stained with phosphorylated Akt (Ser473) and cortactin

and visualized using fluorescent microscopy. Cortactin is a monomeric protein that can be activated by external stimuli to promote actin cytoskeletal polymerization and rearrangement. Rearrangement of the actin cytoskeleton by cortactin is especially pronounced in the cell periphery. Cortactin is known to be a major regulator of invadopodia formation as well as MMP secretion. Knockdown of cortactin has been shown to significantly reduce the number of invadopodia, and has also been shown to be a substrate for Calpain 2 (53, 26). Cortactin has thus become a good indicator of invadopodia formation. As can be seen in fig. 6A, control cells stained with phosphorylated Akt (Ser473) and cortactin show little active phosphorylated Akt and little evidence of invadopodia formation as indicated by the lack of cortactin staining and puncta. However, in the calpain 2 knockdown cells there appears to be intense staining of phosphorylated active Akt (Ser473) at the cell membrane region (fig. 6B). This coincides with intense staining of cortactin in the same region as well as in the cell center. The intense staining of cortactin and phosphorylated Akt (Ser473) suggests that Calpain 2 knockdown may inhibit the turnover of invadopodia. Inhibiting turnover of invadopodia would give rise to the decrease in invasion of glioblastoma seen in the Calpain 2 knockdown cells as glioblastoma cells are unable to move into cleared out extracellular matrix space. The lack of intense staining of phosphorylated Akt (Ser473) and cortactin in the control cells indicates that there is successful turnover of invadopodia as there is no buildup of invadopodia. Additionally, the increased intensity of phosphorylated Akt (Ser473) at the cell membrane in knockdown cells suggests that calpain 2 cleavage of Akt may be causing this defect in invadopodia turnover.

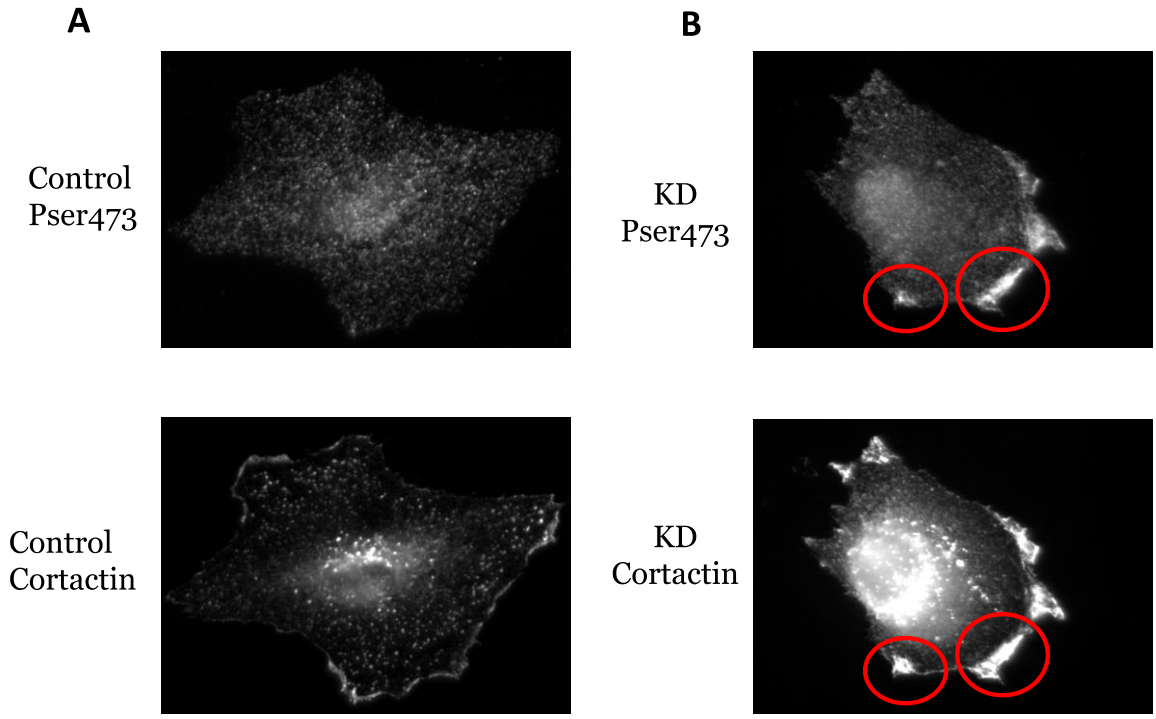


Figure 6: Localization of Pser473 and Cortactin in calpain 2 control cells and calpain 2 knockdown cells .(A) control cells indicate no intense staining of Pser473 where cortactin staining is seen. (B) In calpain 2 knockdown cells, there is intense staining of both cortactin and Pser473 on the membrane ruffles.

Our lab has previously identified calpain 2 as a necessary protein for glioblastoma cell invasion and release of matrix metalloproteinases. Akt has additionally been identified as a major signaling protein involved in a number of cellular processes including cellular migration and invasion as well as also being involved in MMP secretion. This study identifies a novel mechanism of Akt regulation in which calpain 2 directly cleaves Akt to form stable breakdown products. It was also discovered that calpain 2 regulation of glioblastoma cell invasion may be caused by controlling the formation of invadopodia through Akt regulation. Together these data identify a novel mechanism of Akt regulation

as well as identifying potential causes of calpain 2 regulation of glioblastoma cell invasion. Calpain 2 cleavage of Akt results in formation various breakdown products. Future studies will attempt to further characterize these breakdown products to assess their activity as well as potential roles in cellular processes.

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