EFFECT OF DESMIN PEPTIDES AND THE CHARACTERIZATION OF NEBULIN FAMILY PROTEINS IN GLIAL CELLS

An Undergraduate Research Scholars Thesis

by

LYUBOV DUNINA-
BARKOVSKAYA

Submitted to Honors and Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor   Gloria M. Conover, Ph.D

May 2014

Major: Food Science Technology

TABLE OF CONTENTS

ABSTRACT

Effect of desmin peptides and the characterization of nebulin family proteins in glial cells.
(May 2014)

Lyubov Dunina-Barkovskaya
Department of Nutrition and Food Science
Texas A&M University

Research advisor: Gloria M. Conover, Ph.D
Department of Biochemistry & Biophysics

A 3-dimensional highly integrated cytoskeletal network dynamically responds to numerous mechanical stresses faced by cells in various tissue types. Intermediate filament proteins are a major part of the cytoskeleton of cells specified in a tissue-dependent, cell-type dependent manner that play a central role in the maintenance of cellular integrity and adaptation to mechanical stress. Limited information is available in regards to the functional interplay between microtubules and intermediate filaments to maintain cellular homeostasis. Recently, it was reported that tubulin-binding intermediate filament (IF) (neurofilament NFL and vimentin) specifically killed glioma cells but not healthy astrocytes (Bouquet et. al, 2009; Berges et al. 2012). Because it is known that desmin IFs contains four binding sites predicted to bind tubulin, based on SPOT peptide arrays, that also share homology to desmin nebulin-binding peptides (Bocquet, 2009; Conover and Gregorio, 2011), we hypothesized that a conserved function of nebulin to bind IFs to stabilize actin found in myocytes could also be maintained in nerve cells. This project aims to first investigate the uptake ability of desmin peptides homologous to NFL peptides and next test their effect on the distribution of the cytoskeleton of glioma T98G cells and astrocytes. Our results show that most of the desmin peptides were internalized into glioma cells with similar efficiencies as the vimentin (58-81) peptide without causing significant apoptosis or necrosis. Surprisingly, we found that some of the desmin peptides promoted cell survival and appeared to enhance mitochondria activity. Addition of the desmin peptides did not significantly prevent tubulin polymerization assays *in vitro*. Moreover, immunofluorescence studies showed that the majority of the desmin peptides collapsed the microtubule networks but had little effect on the actin and vimentin cytoskeletons. Notably, we found a novel speckled nuclear staining for nebulin in glial cells, and a close association of tubulin to nebulette. Consistent with our results, the nebulin mRNA expression was recently reported in neurons and glia cells (Laitila et al, 2012), while in myocytes, nebulin and nebulette directly binds to desmin (Conover et al 2009, Dunina-Barkoskaya et al, 2014 under review). Future studies should address whether the actin-binding nebulin or nebulette proteins are capable of directly binding tubulin and further investigate their relationship to the IF cytoskeleton of nerve cells.

DEDICATION

This research is dedicated to my supporting and dedicated mother, who has aided and given me direction through all my endeavors.

ACKNOWLEDGEMENTS

NOMENCLATURE

IF	Intermediate Filament
NFL	Neurofilament - Long Protein
DMEM	Dubleco's Modified Eagle Media
FBS	Fetal Bovine Serum
FACS	Fluorescence-Activated Cell Sorting
F-actin	Filamentous actin
G-actin	Globular actin
RB	Retinoblastoma

CHAPTER I

INTRODUCTION

The cytoskeleton is the network of proteins by which cells are held together, and in eukaryotes it consists of three kinds: microfilaments, microtubules, and intermediate filaments (IFs). IFs provide structural support, shock absorbance, and also function to integrate the rest of the cytoskeleton. (Herrmann 2007). One type of intermediate filament is the neurofilament (NF), which is primarily thought to both regulate axon diameter and provide structural support in neurons and neuron-like cells. These filaments are ~10 nm long and consist of repeating subunits and keratin. There are three kinds of NF – NFH (heavy), NFM (medium), NFL (light).

Intermediate filaments are present all over the body, notably in central nervous system cells. The central nervous system is present in all bilaterally symmetrical animals and is composed of neurons which transmit signals through synapses, and neuroglia or glial cells, which function to insulate and provide structural support for the neurons. Glioma is a cancerous glial cell that has a poor prognosis, particularly in children. Recently, a NFL peptide (NFL 40-63) located with its head domain was shown entering and killing the glioma cells selectively. Most strikingly, this NFL peptide specifically killed the glioma cancer cells and not the normal glial cells, and diminished the viability of the cancer cells by disrupting their tubulin filament network. Unlike vimentin another IF proteins that undergo uptake by glial cells, the NFL peptide targets the cancer cells specifically, which leads to new therapeutic applications. For example, the possibility of using it as a specific vehicle to introduce cancer drugs co-incubated with the peptide offers an intriguing treatment option for glioma patients. To date the mechanisms behind the uptake process within glial cells are unknown but is thought to be related with vimentin.

Another type of intermediate filament protein is desmin. This medium size protein is most abundantly expressed in smooth and striated muscle cells. It is found to localize near the Z-discs electron dense structures that define the borders of sarcomeres, the basic multi-protein unit of muscle cells. Desmin aids in the stabilization of F-actin through its interactions with nebulin and nebulette. Actin filaments pull away from myosin filaments at the center of the sarcomere in order to produce cycles of relaxation and contraction allowing muscle cells to beat and move.

The nebulin family of actin binding proteins is composed of nebulin, nebulette, Lasp-1, and Lasp-2 (reviewed in Pappas et al, 2011). All nebulin proteins bind actin and share actin-binding modules by its motif. Nebulin and nebulette stabilize actin in striated muscles. Recently, it was shown that nebulin is also expressed in significant amounts in the brain as is lasp-2 (Laitila 2012). Our findings suggest that nebulette is also expressed in the brain. Mutations in nebulette have been linked to lymphoma (Cosér 2010) and mutations in Lasp-2 have been linked to breast cancer and mast tumor cells. These findings raise the interesting possibility that nebulin family of proteins participate in cancer. To test this possibility in this study we evaluated the distribution of nebulin in in order to examine how these affected the cytoskeleton of the cells.

Because it was reported that desmin had multiple binding peptides to nebulin (Conover and Gregorio, 2011) scattered throughout the protein in a similar fashion as that found in desmin for tubulin (Bocquet, 2009) we hypothesized that the peptides that overlapped in desmin may also preferentially penetrate muscle cells. To test our hypothesis, we investigated if these desmin peptides functioned in a similar way to NFL and vimentin head peptides which share significant

primary sequence homology. Therefore, we predict that these desmin peptides penetrate cells and perturb the tubulin cytoskeleton distribution in a similar way than vimentin. Different modules from the desmin peptide were tested separately to see the individual effects of each module on the cell, in order to determine what components of the total peptide would have an effect. In this study we tested the efficiency of uptake in cells as compared to that for NFL and vimentin. We also tested if the viability of the cells was affected, as reported for vimentin peptides (citation). Uptake was analyzed by by fluorescence microscopy and cell viability were analyzed using the mitochondrial-based MTT and FACS-Annexin V assays.

CHAPTER II

METHODS

The astrocytes that were tested in this assay were acquired from rat brain, while the U87 and the T98G were human glioblastoma cells (Balzeau 2012). Labeled peptides were manufactured by Century 21 Biochemicals. First, cells were unfrozen and proliferated in DMEM at 37 °C and 5% CO_2 with 10% FBS, 5% L-glutamine, and 5% penicillin/streptomycin. For the T98G cells it took 48 hours to proliferate, while the astrocytes took 7 days to be an acceptable size for experimentation. Cells were plated on coverslips 24 hours before the uptake assay, during which they were exposed to the peptides for either 30 minutes or 6 hours.

Immunocytochemistry of Astrocytes, U87, and T98G

For this experiment, cells were first plated overnight on coverslips. Then, cells were incubated with the peptides (the NFL, vimentin, tubulin, and various desmin modules that were tested for uptake) for either 30 minutes or 6 hours**.** They were then washed with 1X PBS for two minutes. Media was removed with vacuum line and cells were treated with PFA (2% or 4% at pH 7.4) for ten minutes, then washed three times with 1x PBS. They were subjected to MTSB (triton permeabalization) for 15 minutes, washed with PBS, and treated with 5% BSA for 15 minutes, and treated with antibody (anti-mouse α-tubulin, anti-GFP, or streptavidin anti-mouse antibodies, alongside anti-nebulin M160-164, anti-nebulette M1-5, Tmod1, or Tmod 4 antibodies for rabbit) overnight at 4 °C, washed with PBS, and treated with antibody in 5% BSA for 2 hours at room temperature. After one more wash with PBS, the cells were stained with DAPI for 5 minutes at room temperature, washed, and mounted on coverslip for viewing with the Leica DMR or the inverted fluorescence microscope Leica CTR 6000, and imaged with ImageJ or Metamoph.

Uptake & Viability Assay (FACS)

Cells were first seeded in 6 well dishes overnight. The next day, some of the cells were treated with the peptide for the uptake assay (30 min at 37°C), after which 20 μl of NFL2-FITC were added for 30 minutes. The cells were then washed in 1X PBS. Then 500 μl of trypsin were added per well and incubated for 10 minutes at 37°C, removed, and 1 mL of DMEM medium added. Cells were re-suspended in 300 μl of 1X PBS, and twice centrifuged for 5 minutes at 2000 rpm, resuspending each time. The cell suspension was then transferred to the FACS tube where 5 μl of propidium iodine were added, and then the cells were transferred to the FACS machine and analyzed by flow cytometry. In the viability assay, the cells were incubated for 3 days at 37 °C, and were exposed to the peptide for a total of 6 hours following their incubation. While using the Annexin V-FITC kit for the viability assay, for every million cells, 1 ml 1x Binding Buffer was added. Cells were centrifuged at 300xg for 10 minutes, then the supernatant was removed and pellet resuspended in 100 μl of 10x Binding Buffer per million cells counted. 10 μl of Annexin V-FITC per 10^6 cells were then added and the mixture incubated for 15 minutes in the dark at room temperature. Cells were then washed with Binding Buffer, centrifuged at 300 rpm for 10 minutes, and the pellet resuspended in binding buffer. PI solution was added immediately prior to analysis by flow cytometry for both the uptake and the viability assay. For astrocytes, the tested amounts were no treatment and vimentin 58-81. For T98G it was tested with no treatment, vimentin modules 58-81, and NFL.

Cell proliferation (MTT) Assay

This assay measured the proliferation and growth rate effects of the T98G, U87, and astrocyte cells post desmin incubation. 500 cells were seeded in 96-well plates, incubated at 37 °C, and treated with peptides after 72 hours. 120 µl of MTT mix, which included 10 µl of medium, 2 µl of MTS and 100 µl of PMS solutions. The dish was then incubated at 37 °C for 2 hours, and read in a spectrophotometer at 490 nm after 2, 3 and 4 hours. The dye turns darker with higher activity.

CHAPTER III

RESULTS

Desmin peptides are internalized in T98G glioma cells.

To determine if the neurofilament homologous desmin peptides were able to get internalized and affected viability of glioma cells (Bouquet et al. 2009), synthetic 21-13mer biotinylated desmin peptides, identified in a previous study as binding sites for nebulin, (Conover and Gregorio, 2011) were added to cells for 6h at 37°C (Figure 1).
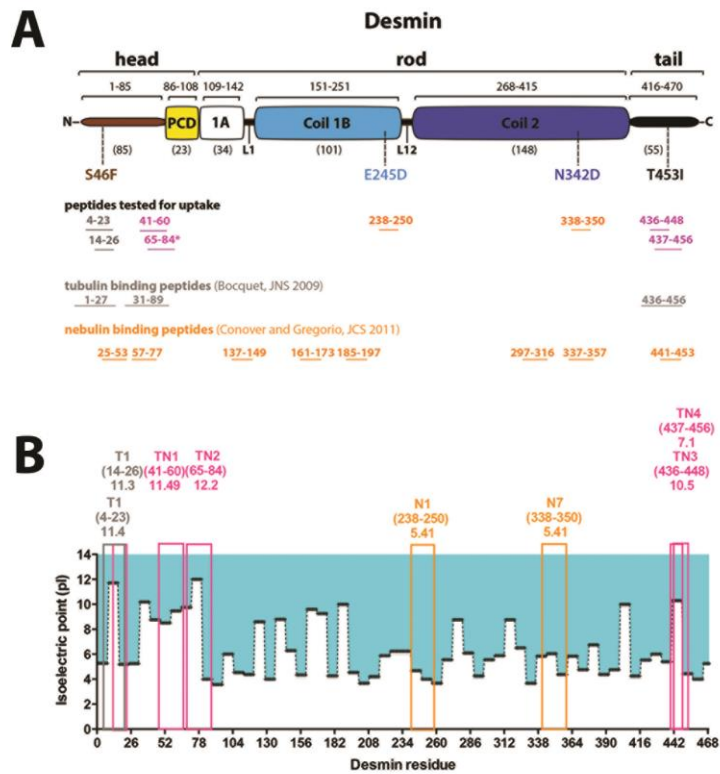

Figure 1. Desmin segments tested for uptake A) Desmin IFs have multiple binding sites for nebulin scattered throughout the molecule. Some binding sites in desmin for nebulin overlap with those found for tubulin. **B)** Map of the isoelectric point (pI) of desmin nebulin/tubulin binding peptides.

Immunofluorescence staining of these uptake assays resulted in the observation that all of the desmin peptides examined (both head, one from coil 1B, one from coil 2B, and one from the tail) were delivered inside cells in amounts approximately equal to that reported for vimentin 58-81 (Balzeau et al, 2011). Uptake occurred in slightly lower amounts than the NFL 40-63, but above the control dye being used by itself. The scrambled peptides served as positive control for viability MTT assay on the glial cells, and its uptake occurred in equal amounts as the uptake of native desmin peptide (Figures 2 and 3). The extent of peptide uptake in this assay is sometimes unclear because there are parts where the streptavidin appears as background, which made counting of the peptides difficult.
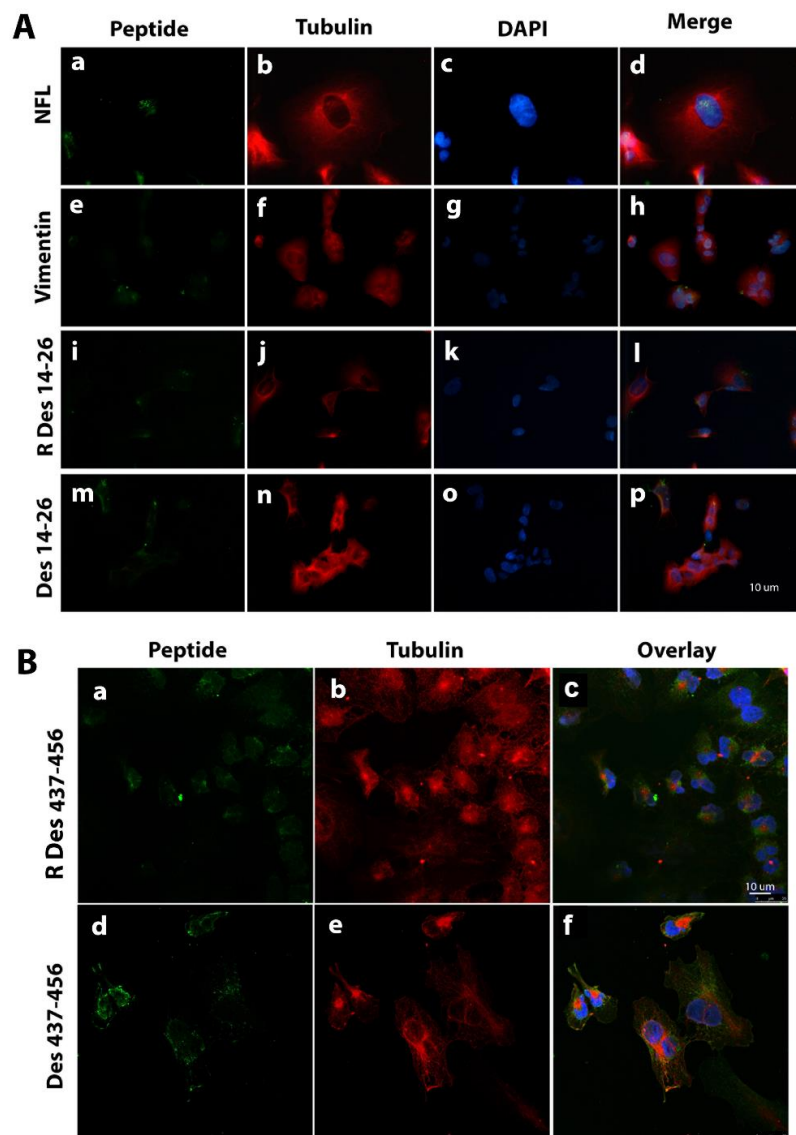
Figure 2. Uptake of desmin peptides (14-26; 437-456) into T98G glioma cells. Cells were incubated with 10µM of biotinylated desmin peptide for 6 hours at 37°C. The desmin peptides (random compared to native peptides) uptake were visualized using Streptavidin-Alexa 488. The effect on the microtubule cytoskeleton and nuclei was assessed by immunofluorescence light microscopy. At 10 µM, these cells internalized a moderate amount of desmin peptide with no significant difference found between random and native peptides.
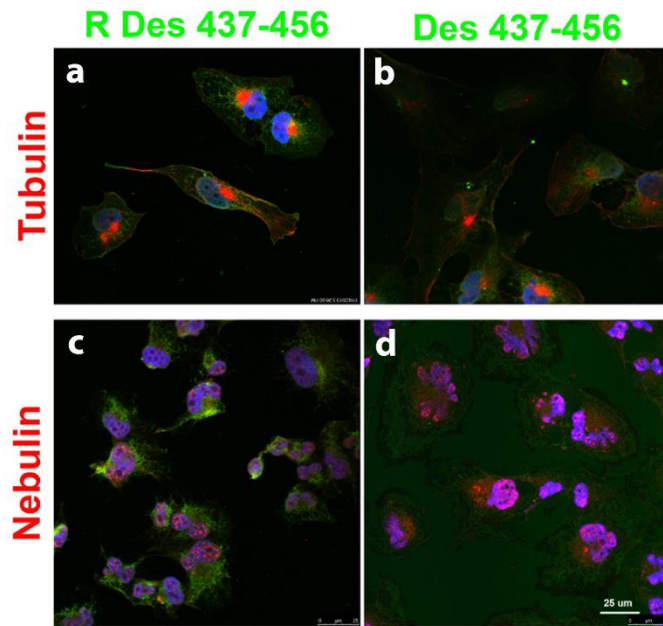
Figure 4. Uptake of tail desmin head and tail peptide into T98G glioma cells. Cells were incubated with 10µM of biotinylated desmin peptide for 6 hours at 37°C. The desmin peptides (random compared to native peptides) uptake were stained using Streptavidin-Alexa 488. The effect on tubulin, nebulin and nuclei shape was assessed by confocal microscopy. Under these conditions, a moderate amount of desmin peptide with no significant difference was found.

Increased survival of T98G glioma cells upon desmin internalization.

The mitochondrial activity, cell proliferation, and viability of T98G cells exposed to desmin peptides for 3 days were assessed using an MTT assay. This colorometric assay monitors the conversion of tetrazolium salt into formazan (Figure 3). Cytochalasin D, Paclitaxel, and NFL 40-63 were used as controls for having an inhibitory effect on viability, while incubation with vimentin peptide as well as treating the cells with scrambled peptides were used as positive controls, that is, minimal effect on cell viability. All of the desmin peptides were demonstrated to have no significant adverse effect on cell viability. In comparison, the vimentin 58-81 peptide more severely inhibited the glial cell mitochondrial activity than did any of the desmin peptides tested (Figure 4). If most of these current results are looked at closely, it appears that the cells

16

incubated with the desmin peptides had a higher amount of cells that were viable than the controls, suggesting enhancement of cell proliferation. This observation would indicate that the various desmin peptides could cause increase cell proliferation of glial cells. However, more tests involving other uptake trials would be needed to prove that this is statistically significant as well as if this observation could apply to other cell types.
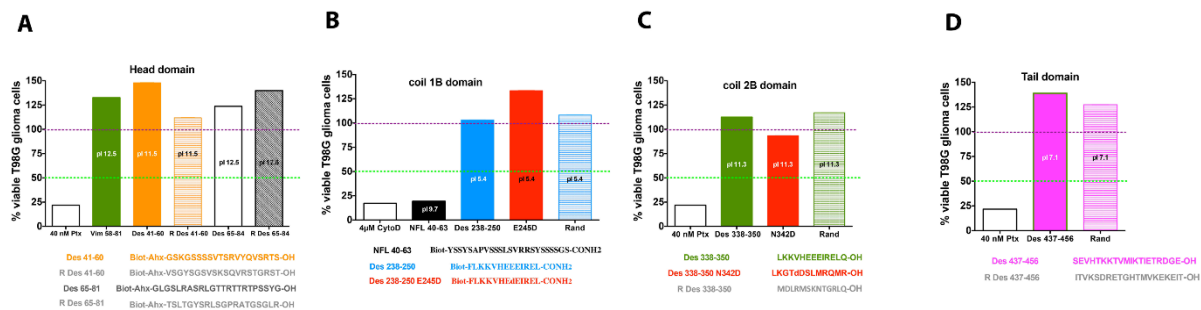

Figure 5. Normal viability after desmin peptide internalization. T98G cells were exposed to 80µM of the indicated desmin peptide every day for 3 days. 40 nM Placlitaxel (a MT depolymerizing agent) was used a negative control. Results for desmin peptides mapping in the **A)** head domain; **B)** the coil 1B domain **C**) the coil 2B and the **D)** tail domains of desmin are shown.

Another assay used a flow cytometer (FACS) to count the amount of necrosis as well as an early-stage apoptosis marker in the glial cells stained by Annexin-V FITC dyes. The results of this assay demonstrated that there was negligible effect on viability in cells exposed to the desmin peptides, expanding and validating the MTT results (data not shown). The results of the FACS assay suggest that the desmin peptides do *not* enhance apoptosis in these cells. Furthermore, it supports the results of the MTT assay that there was no effect on the viability of glial cells following uptake with the native desmin peptides.

The viability tests that have been done so far, including the MTT and FACS, demonstrate that there is no decreased viability due to the internalization of desmin segments, therefore it is highly unlikely that desmin destructively disrupts the cytoskeleton network. However, the distinct possibility of increased survival of the cancer cells incubated with peptide would suggest that the cytoskeleton of the cells is impacted in some way – perhaps growing faster, or perhaps some other

17

unexamined possibilities.

Effect of desmin peptide internalization in the cytoskeleton of T98G cells

To test the effect of desmin uptake in the cytoskeletal architecture of T98G cells, cells were exposed to peptide and either stained for tubulin or actin (Figure 2, 3), or tested for viability (Figure 4). Our results show a lack of a dramatic collapse or visible structural impact on all of the cytoskeleton networks examined in glial cells following the uptake of these desmin peptides compared to the controls. Nevertheless, we observed that some cells appeared to incorporate the tail desmin peptide into the cytoskeleton intermediate filament network, as determined from confocal optical slices (Figure 3). More analyses are needed examining how the cytoskeletal distribution is modulated in response to peptide uptake in order to definitively rule out that there is in fact no significant impact.

Nuclear speckled localization of nebulin module 164 in nervous system cells

Nebulin is a giant muscle protein located near the sarcomere Z-disc in muscle cells, and has had several hypothesized functions. It has been said to serve as a molecular ruler for actin at the z-disc, but more recently it evidence has been found to have more of an actin-stabilizing function (cite source). Nebulin module M164 (found in the C-terminal domain) has a bright punctate appearance inside the nucleus of glial cells and astrocytes (Figure 5). This is present across all glial cells that were stained – astrocytes as well as tumor cell lines which included T98G and U87.
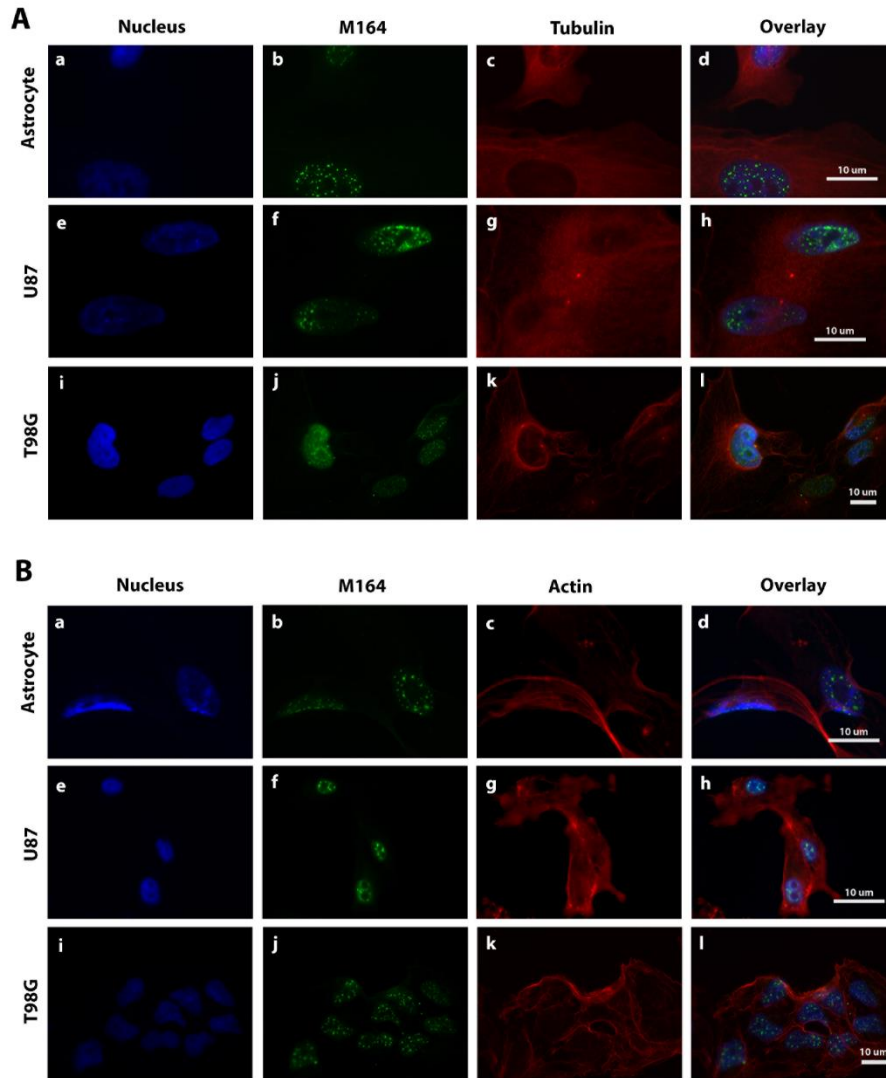
Figure 6. Nuclear speckled distribution for nebulin in astrocytes and glioma cells. A) The distribution of the C-terminal nebulin module M164 and tubulin or **B)** nebulin and actin was compared in astrocytes, U87 and T98G glioma cells. Immunofluorecence images revealed a novel speckled nuclear distribution for nebulin in neuronal cells that did not co-distribute with cytosolic actin or tubulin filaments.

However, this does not appear to be the case in other tumor cells, specifically in skeletal muscle tumors - retinoblastoma [RB] cells - wherein nebulin appears to be dispersed fairly evenly throughout the cell (Figure 7). Therefore, it appears that nebulin has this distinct speckled

appearance uniformly and solely at the nucleus of glial cells and astrocytes, or possibly across all

nerve cells including neurons. However, other nerve cells such as neurons have not been

immunostained for nebulin as of yet. Nebulin does not appear to co-localize nor associate with

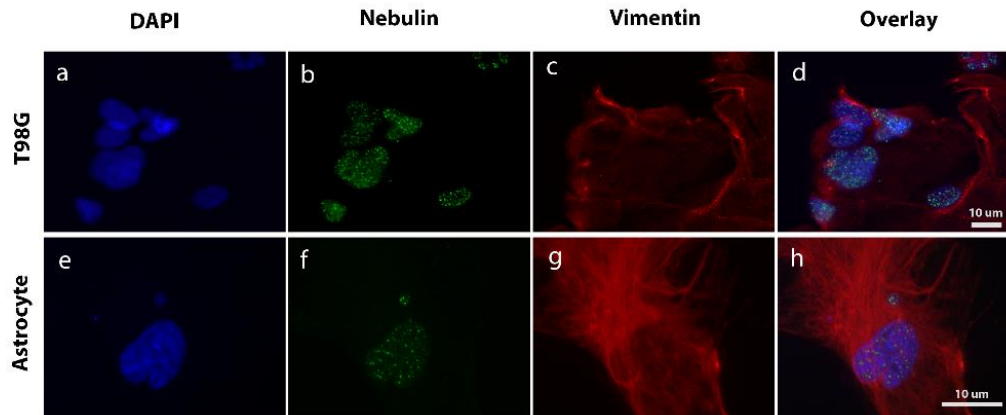tubulin or F-actin (Figure 5), nor with vimentin (Figure 6).

Figure 6. Nebulin shows no relationship to *vimentin* in tumor cells or astrocytes. There does
not appear to be a proximal relationship between nebulin and vimentin, just like F-actin and
tubulin, both in the tumor (T98G) and astrocyte cells. Nebulin and vimentin are located at a
distance away from each other, as nebulin stains solely in the nucleus, and vimentin appears to be
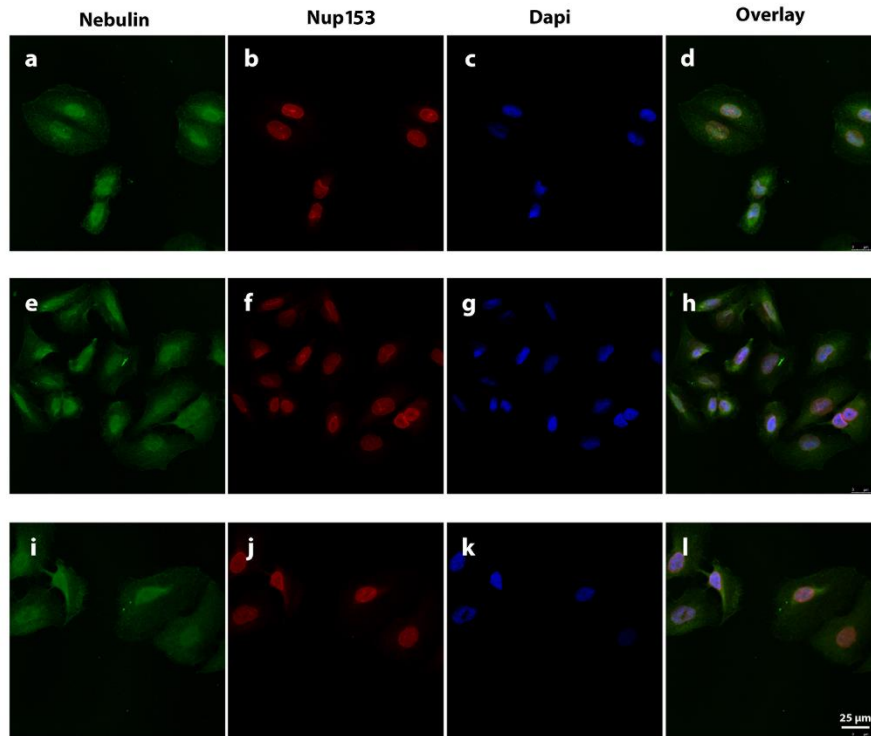exclusively in the cytosol.

Figure 7. Distribution of nebulin and nuclear pore complex protein Nup153 in U2-0S bone osteosarcoma cells. The N-terminal nebulin module M1 distribution pattern in U2-0S cells was disperse and dramatically different as compared to the punctate speckled pattern found for C-terminal nebulin in glial cells (Figure 5, 6). Moreover, it did not co-distribute with nuclear pore complex protein.

Association of nebulette with microtubules of glioma cells

Unlike nebulin, nebulette, another member of the nebulin family of proteins, has a disperse, yet fibrous appearance in the cytosol of the glial tumor cells T98G and U87 (Figure 8). Although co-localization was not visible with neither actin, vimentin, nor tubulin, it appears that nebulette associates with tubulin to a higher degree than with actin in the cytoskeleton of glioma cells (Figure 8-C). The actin was stained for by using phalloidin, thus, it is still unclear whether or not nebulette stabilizes actin in glial cells as it does in heart muscle cells (Moncman and Wang, 1999). However, it is clear that nebulette has a marked, distinct appearance from nebulin, which is mostly associated with the nucleus in glial cells as described above. There appears to be some nebulette in the nucleus with a diffuse appearance, but whether or not it is actually present or whether it is background from the stain is as of yet unclear. However, the fact that it appears as fibrous protein in the cytoplasm of glioma cells indicates that at least some of the stain is specific to this antibody.
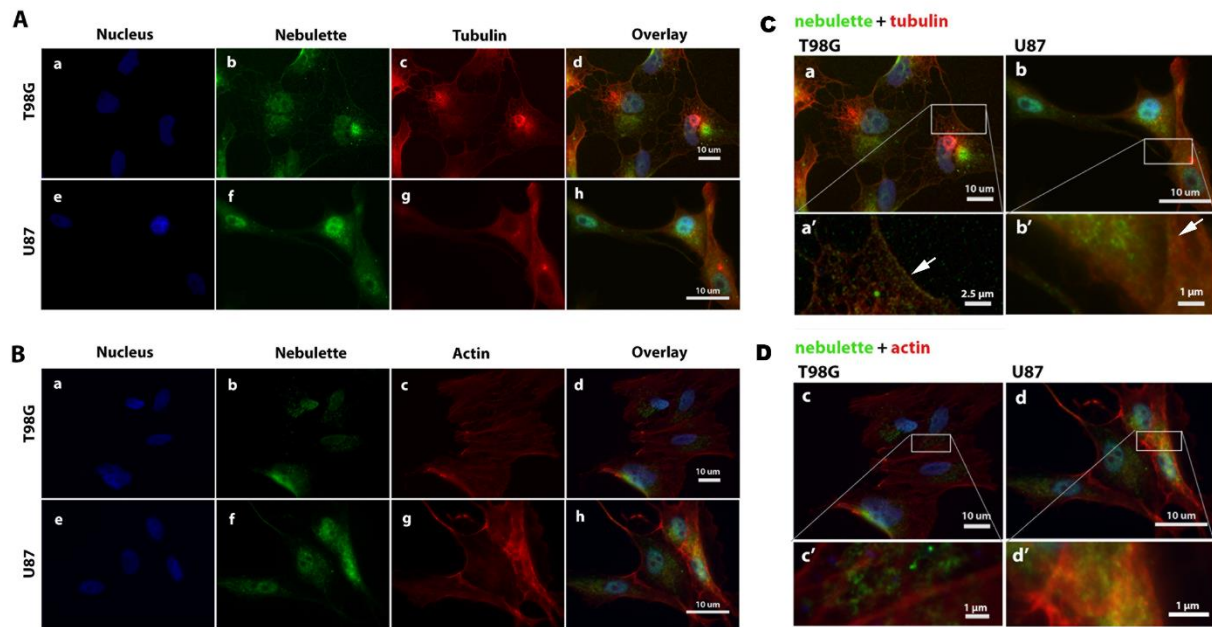
Figure 8. Distribution of nebulette, tubulin and actin in glioma T98G cells. A) Nebulette appears to be closely co-localized with microtubules. A pattern between nebulette and tubulin, possibly suggests that these proteins interact. In contrast, **B)** there seems to be no close distribution among nebulette and actin in T98G glioma cells. **C)** Magnified inset shows a close association of tubulin and nebulette. **D)** Magnified inset reveal no clear association between actin and nebulette in T98G or U87 glioma cells.

CHAPTER IV

DISCUSSION

Partial uptake of desmin peptides occurred in both T98G and U87 at 10 µM.

Uptake of selected desmin peptides known previously to bind to nebulin (Conover and Gregorio, 2011) or tubulin (Bouquet et al, 2009) by SPOTs blot overlay assays, was observed for the first time in the glial cells at a rate above the control (Figures 3, 4). The isoelectric point of these desmin peptides (amino-acids 14-26, 41-60, 65-84, 239-250, 338-250, and 436-448) used in peptide delivery experiments (Figure 1), ranged from basic to acidic. Specifically, we found that the head and coil 2 domain desmin peptides are basic, the coil 1B is acidic while the tail domain peptides is neutral. However, in our studies neither viability nor uptake appeared to depend dramatically on the isoelectric point of the peptide used.

Our finding that desmin peptides are internalized in glial cells is noteworthy because despite the visibility of the stain present in the control, there are darker, more obvious stains present in the desmin antibody immunostained fluorescent images, therefore supporting the cell uptake explanation. Despite the punctate appearance of peptide uptake, there was significant background present in these cells, which makes counting the number of cells with internalized peptides difficult, and thus, this experiment should be followed up with additional assays. As predicted, uptake occurred in scrambled desmin peptides, since those segments were utilized to assess if structural conformation of the peptide rather than only residue identity and charge or potentially other unknown features affects these peptide uptake ability or cell viability.

The delivery of the full-desmin protein in neurons or glial cells needs to be determined as well as other assays to define whether the mechanism of desmin peptide internalization observed here requires endocytosis or largely depends on the amount of membrane lipid oxidation. In addition to functioning as a potential drug delivery vehicle to correct for cytoskeletal defects caused by genetic mutations of IF proteins, the desmin peptide uptake reported here future studies should address the mechanisms at play to explain if IF-peptide delivery involves pockets of membrane lipids with negative charge or is invokes a general change in membrane rigidity that is unique to cells of the nervous system. The tubulin-binding peptides suggest that tubulin plays a role in the cytoskeleton of glial cells, while vimentin, also like desmin, a type III intermediate filament protein, is also suspected to be a key player (Granger 1979).

Limited effect on cell viability or proliferation upon desmin peptide uptake in glial cells.
The results obtained in the MTT and the FACS assay supports the idea that, while uptake of each of the desmin peptides used in this study occurs to some extent, there was no adverse effect on viability after 3 days in tissue culture. Notably, however, the MTT test results suggest that cancer glial cells had a slight increase in cell viability after incubation with peptides (Figure 4). However, this appears to be a global effect for all desmin peptides, since it was observed in native peptides, in some of the scrambled peptides, and in the mutant E245D peptide. Still, it is surprising that at 80 μM dosage nearly all of the peptides tested, the amount of viable glial cells is significantly higher for the cells incorporating desmin segments, mutant or otherwise, than they appear for the control. Follow-up experiments to better understand this observation should include a dose-response at different peptide dosages and evaluate the effect every 12 hours time points over 3 days rather than only a 72 hours final reading. Cell viability and proliferation studies should also be performed in

primary myocytes and fibroblast derived cell lines to test whether the effect is specific to T98G cells.

When comparing the desmin peptide color changes to the control wells treated with cytoskeletal inhibitors such as Cytochalasin D (inhibits actin polymerization), Paclitaxel (inhibits microtubule polymerization), and NFL 40-63 peptide (IF marker of Alzheimer/Parkinsonian diseases that kills glioma cells), desmin peptides delivery into T98G cells proved to be ineffective at inhibiting viability. This result was somewhat expected, as delivery of the vimentin 58-81 peptide we find killed about 20% of cells. Our result is consistent with previously published data (Balzeau et al, 2011). We predict that these desmin peptides have several possible mechanisms of entry to cross the membrane of glial cells. Although a full-desmin protein on its own has not been tested for uptake as of yet, it is likely that since the smaller peptides are taken into the cell, the large desmin molecule could be delivered in conjunction of other highly known cell penetrating peptides CPPs. Together, these tests demonstrate that desmin does not have the highly specific cancer-killing effects in glioma cells as NFL 40-63 peptides, and indicate that desmin peptides could be considered a new class of cytoskeleton-based cell penetrating peptide that may promote viability rather than killing. In the near future, it will be important to test these effects in other cell types.

A punctate nebulin distribution in the nucleus of glial cells
Immunofluorescence images of astrocytes and two kinds of glial tumors revealed that nebulin had a previously unrecognized distinct nuclear speckled distribution across all different types of glial neural cells, including both healthy cells and cancerous cells. As seen (Figures 5 and 6), nebulin has a speckled appearance that is distinctively marks the nucleus of the cells. This same

appearance is not visible in other kinds of cancer cells, such as bone cancer (Figure 7), nor in muscle cells where C-terminal nebulin distributes to the Z-discs (Gurgel-Gianetti 2001). Furthermore, this study is the first to show nebulin distribution in the nervous system cells, although previous reports demonstrated mRNA presence in brain using RT-PCR. Its function in the brain remains largely unknown, as there have not been any reports of nebulin nuclear function as of yet. However, based in part on its location in these cells, and in part by its inferred function in the muscle tissue, further areas for experimentation can be designed to understand the biological function of nebulin in nervous system cells.

This report showed that nebulin in T98G cells does not appear to stabilize F-actin as predicted from studies in muscle cells. Our results show that co-staining the nebulin C-terminus with the F-actin probe phalloidin did not appear to have any visible pattern correlating the F-actin to the neuronal nebulin. However, it is important to realize that the actin that was stained for in these experiments used phalloidin, a fungal toxin that only stains F-actin, a form of filamentous actin that is generally not found in the nucleus. Instead it is proposed that actin is mostly in a G-actin form (globular actin) that is inaccessible to phalloidin. Thus, it will be important to determine if the nebulin speckles co-distribute with globular actin (G-actin) by staining with DNAse I. If it does, similar assays can be carried out on these cells as have been carried out for to establish how neuronal nebulin might functional interact with nuclear actin.

Remarkably, nuclear titin, another giant protein in muscle tissue, has been identified in the nucleus of cells as a binding partner for lamin type A and B (Zastrow, 2006). There is a known functional relationship between nebulin and titin in muscle cells, so it is possible that all of these proteins work together in the nucleous as well. It is now predicted that the identification of nuclear nebulin

may aid in the understanding of the molecular mechanism responsible for inherited neuromuscular myopathies, since the two types of tissues both have nebulin in common.

Nebulette appears to associate with the microtubule cytoskeleton of glial cells

In cardiac muscle tissue, nebulette is presumed to stabilize actin in much the same way as nebulin does in the skeletal muscle. Similarly to their seemingly close relationship in muscle tissue, these two proteins did not appear to have identical functions in glial T98G cells. As seen by co-staining glial tumor cells with anti-nebulette module 5 with either anti-tubulin or phalloidin, nebulette appears in a fibrous distribution throughout the cytoplasm of glial cells. It associates loosely with the tubulin cytoskeleton but shows no obvious relationship with F-actin as it is completely delocalized from the filamentous actin. When observing the slides, nebulette appears to be present in the nucleus but only with a diffuse appearance. However, due to its known stabilizing function in cardiac tissues, and its lack of distinct distribution in the nucleous of glial cells, it is doubtful that nebulette would bind to globular actin.

REFERENCES

Bocquet, A; Berges, R.; Frank, R.; Robert, P.; Peterson, A.C; Eyer, J. 2009. Neurofilaments bind tubulin and modulate its polymerization. J. Neurosci 29, 11043-11054

Berges, R; Balzeau, J; Takahashi, M.; Prevost, C., Eyer, J. Structure-function analysis of the glioma targeting NFL-TBS.40-63 peptide corresponding to the tubulin-binding site on the light neurofilament subunit. PLoS ONE 7, e49436

Conover, GM1; Gregorio, CC. 2011. The desmin coil 1B mutation K190A impairs nebulin Z-disc assembly and destabilizes actin thin filaments. J Cell Sci. 1243464-76.

Laitila, J1; Hanif, M; Paetau, A; Hujanen, S; Keto, J; Somervuo P; Huovinen S; Udd, B; Wallgren-Pettersson, C; Auvinen P; Hackman P; Pelin, K. 2012. Expression of multiple nebulin isoforms in human skeletal muscle and brain. Muscle Nerve. 5:730-7

Conover, GM; Henderson, SN; Gregorio, CC. 2009. A myopathy-linked desmin mutation perturbs striated muscle actin filament architecture. Mol Biol Cell. 3:834-45.

Herrmann H; Bär H; Kreplak L; Strelkov SV; Aebi U. 2007. Intermediate filaments: from cell architecture to nanomechanics. Nat Rev Mol Cell Biol. 7:562-73

Pappas CT, Bliss KT, Zieseniss A, Gregorio CC. 2011. The Nebulin family: an actin support group.Trends Cell Biol. 1:29-37

Virginia M. Cóser, Claus Meyer, Rosania Basegio, Juliane Menezes, Rolf Marschalek, Maria S. Pombo-de-Oliveira. 2010. Nebulette is the second member of the nebulin family fused to the MLL gene in infant leukemia. Cancer Genetics and Cytogenetics, Volume 198, Issue 2, 2010, Pages 151-154

Balzeau, J.; Peterson, A.; Eyer, J. 2012. The vimentin-tubulin binding site peptide (Vim-TBS.58-81) crosses the plasma membrane and enters the nuclei of human glioma cells. International Journal of Pharmaceutics, Volume 423, Issue 1, Pages 77-83

Moncman CL1, Wang K. 1999. Functional dissection of nebulette demonstrates actin binding of nebulin-like repeats and Z-line targeting of SH3 and linker domains. Cell Motil Cytoskeleton. 44(1):1-22.

Granger BL, Lazarides E. 1979. Desmin and vimentin coexist at the periphery of the myofibril Z disc. Cell. (4):1053-63.

Gurgel-Giannetti J, Reed U, Bang ML, Pelin K, Donner K, Marie SK, Carvalho M, Fireman MA, Zanoteli E, Oliveira AS, Zatz M, Wallgren-Pettersson C, Labeit S; Vainzof M. 2001. Nebulin expression in patients with nemaline myopathy. Neuromuscul Disord. (2):154-62.

Zastrow MS1, Flaherty DB, Benian GM, Wilson KL. 2006. Nuclear titin interacts with A- and B-type lamins in vitro and in vivo. J Cell Sci. 119(Pt 2):239-49.