Aggravated Aggregates: The Role of a Retroviral Oncoprotein and Pseudophosphatase in Cellular Responses

Michelle R. Munyikwa

College of William and Mary

Follow this and additional works at: https://scholarworks.wm.edu/honorstheses

Recommended Citation
https://scholarworks.wm.edu/honorstheses/389

This Honors Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
Aggravated Aggregates: The Role of a Retroviral Oncoprotein and Pseudophosphatase in Cellular Responses

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Interdisciplinary Studies from The College of William and Mary

by

Michelle Munyikwa

Accepted for ____________________________
(Honors)

Dr. Shantà Hinton, Advisor

Dr. Lizabeth Allison

Dr. Mark Forsyth

Dr. Lisa Landino

Williamsburg, VA
April 28, 2011
Abstract

Proteins, the workhorses of the cell, are immensely important to biological processes in all forms of life. Cellular signaling is a vital component of cellular function in which proteins play a major role. This thesis research focuses on two distinct proteins, an oncoprotein (v-ErbA) and a pseudophosphatase (MK-STYX), and their role in responses to extracellular signals. The retroviral oncoprotein v-ErbA (p75\textsuperscript{gag-v-ErbA}) is a highly mutated variant of the thyroid hormone receptor \(\alpha1\) (TR\(\alpha1\)) that interferes with cellular response to thyroid hormone. v-ErbA has been observed to form punctate cytoplasmic foci reminiscent of aggresomes, perinuclear accumulations of misfolded proteins. Aggresomes have several key features; specifically, this research provides evidence that v-ErbA foci, like aggresomes, colocalize with proteasomes and mitochondria. Additionally, v-ErbA aggresome formation is dependent upon the viral Gag sequence. These studies provide evidence that v-ErbA is recruited to aggresomes, highlighting a complex mode of oncogenesis across several cellular compartments. The second protein, MK-STYX [MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein] is a pseudophosphatase member of the dual-specificity phosphatase subfamily of the PTPs (protein tyrosine phosphatases). MK-STYX lacks catalytic activity due to the absence of two key amino acids from the signature motif that are essential for phosphatase activity. MK-STYX is known to bind G3BP, an RNA-binding protein that is involved in stress granule assembly, and decreases stress granule formation. This thesis research provides evidence that MK-STYX inhibition of G3BP-induced stress granule formation is not phosphorylation-dependent; MK-STYX still decreased stress granule assembly in cells expressing a non-phosphorylatable G3BP mutant. Additionally, an active mutant of MK-STYX was able to induce stress granule assembly in cells expressing the phosphomimetic G3BP-S149E. Finally, MK-STYX is homologous to a class of phosphatases responsible for regulating MAP kinase pathways, which are involved in diverse processes of cellular growth and differentiation. In PC12 cells, a model of neuronal differentiation, MK-STYX appears to induce the formation of multiple small outgrowths in untreated cells and enhances the length of outgrowths in cells treated with NGF. This data indicates a potential role in regulation of cellular response to extracellular signals. In summary, this project elucidates the recruitment of v-ErbA to aggresomes, and the pseudophosphatase MK-STYX as a regulator of the cellular stress response and neuronal differentiation.
Table of Contents

List of Figures.................................................................5
Acknowledgements ............................................................6

General Introduction.........................................................7
Cellular signaling and communication......................................7
Part 1: The targeting of v-ErbA to aggresomes..............................8
  The thyroid hormone receptor..........................................8
  v-ErbA..........................................................................9
  Protein folding and misfolding: an introduction to the aggresome ....12
  Mechanism of aggresome formation....................................13
  Degradation and clearance mechanisms induced by the aggresome ....14
  Rearrangement of cell components in response to the aggresome ....14
  Naturally occurring aggresomes.........................................15
  Aggresome markers.......................................................15
Part 2: The involvement of MK-STYX in the stress response and neuronal
differentiation................................................................16
  Protein phosphorylation and cellular communication................16
  Protein tyrosine phosphatases..........................................18
  PTP signature motif and catalytic mechanism..........................18
  Dual specificity phosphatases..........................................19
  Receptor tyrosine kinases..............................................20
  Ras: a ubiquitous signaling protein...................................21
  Mitogen-activated protein kinases......................................22
  Classes of MAP kinases................................................24
  MAP kinase phosphatases and MAP kinase cascade regulation......25
  “Dead” phosphatases and their cellular significance................26
  MK-STYX....................................................................27
  G3BP........................................................................28
  Stress granule formation.................................................30
  G3BP and stress granule assembly......................................31
Specific aims........................................................................33

Methods.............................................................................34
Plasmids............................................................................34
Cell culture, transfections and treatments.................................34
Fixation, immunofluorescence, and organelle staining...............35
Cell scoring and statistical analysis......................................36

Results ...............................................................................37
Part 1: The Targeting of v-ErbA to Aggresomes..........................37
  v-ErbA aggresomes recruit proteasomes................................37
  v-ErbA aggresomes recruit mitochondria...............................38
  The AEV Gag sequence is essential for v-ErbA recruitment to aggresomes ....42
Part 2: The involvement of MK-STYX in the stress response and neuronal
differentiation................................................................45
  Inhibition of stress granule assembly by MK-STYX is not wholly G3BP
  phosphorylation dependent.............................................45
  MK-STYX increases outgrowth formation in PC12 cells.............51
List of Figures

Figure 1: Schematic Comparison of v-ErbA and TRα. ...........................................11
Figure 2: Classification of Protein Tyrosine Phosphatases. .................................17
Figure 3: MAPK and MK-STYX signaling cascades in neuronal cells...............23
Figure 4: Schematic Representations of MK-STYX and G3BP. .........................29
Figure 5: The recruitment of proteasomes to the site of the aggresome..........39
Figure 6: Mitochondria are recruited to v-ErbA aggresomes..........................41
Figure 7: The AEV Gag sequence plays a role in targeting of v-ErbA to aggresomes. ...........................................................................................................44
Figure 8: Phosphorylation independent inhibition of stress granule assembly by MK-STYX .................................................................47
Figure 9: Phosphorylation independent inhibition of stress granule assembly by MK-STYX .................................................................48
Figure 10: Induction of G3BP149E stress granule assembly by the active mutant .................................................................49
Figure 11: Induction of G3BP-S149E stress granule assembly by the active mutant .........................................................................................50
Figure 12: MK-STYX promotes outgrowth formation in PC12 cells.................54
Figure 13: MK-STYX causes a shift in the distribution of outgrowth lengths...55
Figure 14: MK-STYX causes the most significant shifts between 20 and 60 μm .................................................................................................................56
Figure 15: MK-STYX affects the morphology of untreated cells......................57
Figure 16: MK-STYX elongates outgrowths in cells treated with NGF ..........58
Acknowledgements

This thesis came together because of the efforts of several fantastic people. First, I would be nowhere without the mentorship of Drs. Lizabeth Allison and Shantà Hinton, who have talked me through these projects more times than I can count and offered me guidance throughout this process. I also can’t express my gratitude enough to Vinny, who has been a great help since I first entered the lab and without whom my figures would be a mess. Finally, I’d love to thank the members of the Awesome Allison Lab and the Happening Hinton lab for being such great friends during my time at William and Mary. I’ll never forget you all. Last, but not least, I’d like to thank my friends and family for putting up with me over the last few months and providing endless support.
General Introduction

Cellular signaling and communication

Cell signaling is a complex system of communication responsible for governing basic cellular activities and physiological functions. Critical to these processes is the ability of cells to perceive and respond to their environment. These communication systems depend heavily on extracellular signaling molecules, which are produced by cells to signal to their neighbors or other cells further away. These signal molecules interact with receptor proteins that transmit the signal to the necessary parts of the cell, initiating shifts in DNA expression, protein function, and progression in the cell cycle. Response to extracellular signals governs such diverse processes as growth and development, immunity, apoptosis, and homeostasis; misregulation of this cellular response can result in cancer, diabetes, and inflammation, among other diseases.

This thesis research encompasses two projects. The first portion of this research involved the characterization of a highly mutated variant of the thyroid hormone receptor, v-ErbA. This oncogenic variant, which is carried by the avian erythroblastosis virus, interferes with normal cellular response to the thyroid hormone. Notably, prior research has indicated that it has altered subcellular distribution with respect to the thyroid hormone receptor and forms large, perinuclear aggregates (Bonamy et al., 2005; DeLong et al., 2004).
These aggregates resemble aggresomes, sites of misfolded proteins that accumulate at the microtubule organizing center. This research aimed to determine whether v-ErbA foci are aggresomal in nature. Additionally, investigation of the AEV Gag sequence provides a potential rationale for the recruitment of v-ErbA to these foci.

The second component of this work was the characterization of a novel protein, the pseudophosphatase MK-STYX. MK-STYX, though it is catalytically inactive, has sequence homology to the MAP kinase phosphatases (MKPs), a class of proteins that is responsible for modulating the duration of MAPK signaling. MK-STYX has been shown to affect endogenous and stress granule assembly induced by G3BP (Hinton et al., 2010). This thesis research determined whether MK-STYX inhibition of stress granule formation is phosphorylation-dependent. MK-STYX is homologous to a class of proteins called MKPs, which are expressed in PC12 rat pheochromocytoma cells. Thus, we investigated the effect of MK-STYX on MAPK signaling in these cell lines.

**Part 1: The targeting of v-ErbA to aggresomes**

**The thyroid hormone receptor**

The thyroid hormone receptor (TR) is a nuclear hormone receptor that alters transcription of genes involved in homeostasis, development, and metabolism
in response to thyroid hormone (T₃). There are two major forms of thyroid hormone receptor, TRα1 and TRβ1; the focus of this thesis research will be TRα1 and its variants. TRα1 (hereafter referred to as TRα) binds to target genes both in the presence and absence of its ligand. When bound to T₃, depending on the response element to which TRα is bound, it can activate transcription of genes. When T₃ is absent, TRα represses these genes. Proper localization of the thyroid hormone receptor is integral in proper cellular response to thyroid hormone, particularly shuttling of the receptor between the cytoplasm and nucleus. Mutations in the nuclear localization sequence(s) of thyroid hormone receptor disrupt its transport into the nucleus, preventing it from interacting with thyroid hormone response elements and altering gene transcription. Evidence indicates that mislocalization of the receptor caused by mutations can lead to a variety of diseases, including cancer.

**v-ErbA**

The retroviral gag-v-Erb oncoprotein (p75\text{gag-v-ErbA}) is a mutated derivative of TRα that is unable to bind thyroid hormone. v-ErbA was first isolated as one of two oncogenes carried by the avian erythroblastosis virus (AEV); the other oncoprotein, v-ErbB, is a highly mutated form of the epidermal growth factor. AEV causes erythroleukemia and sarcomas in chickens (Braliou et al., 2001). Mutations in its amino acid sequence, including the fusion of AEV Gag to its
N-terminus, N- and C-terminal deletions, and 13 amino acid substitutions throughout the protein, lead to detrimental effects in infected cells (DeLong et al., 2004) (Figure 1).

v-ErbA has a dominant negative effect on TRα that was originally attributed to competition with TRα for T₃-responsive elements or other aspects of gene regulation. However, recent evidence has altered that understanding. It is now thought that oncogenic conversion of v-ErbA is linked to changes in DNA binding specificity and ligand binding properties, as well as altered nuclear export capabilities that result in a change in subcellular localization (Bonamy and Allison, 2006).

Nuclear export of v-ErbA occurs through a CRM1-mediated pathway, in direct contrast to the partially CRM1-independent transport of TRα (DeLong et al., 2004; Grespin et al., 2008). Prior studies have indicated that a nuclear export sequence resides within a 70-amino acid sequence in the C-terminal portion of the p10 region of the Gag sequence, which interacts directly with CRM1. Additional evidence suggests that, throughout its evolution, v-ErbA has lost the ability to follow the export pathway used by TRα (DeLong et al., 2004).

As opposed to the primarily nuclear localization of TRα, v-ErbA localizes primarily to the cytoplasm with punctate distribution in cytoplasmic and
Figure 1: Schematic Comparison of v-ErbA and TRα.

This figure illustrates v-ErbA homology with TRα. v-ErbA differs from TRα by fusion with a retroviral Gag sequence and by several amino acid substitutions along with deletions at both ends.
nuclear foci (Bonamy et al., 2005; DeLong et al., 2004). Evidence suggests that v-ErbA dimerizes with TRα and the retinoid X receptor, sequestering a significant fraction of these receptors in the cytoplasm, where they cannot affect gene expression (Bonamy et al., 2005). Preliminary evidence suggested that these v-ErbA foci were linked to aggresomes, a cellular response to accumulations of misfolded protein.

**Protein folding and misfolding: an introduction to the aggresome**

Correct folding and maintenance of a newly synthesized amino acid chain into its three-dimensional conformation is required for proper protein function. This does not always occur; however, misfolding of proteins can be caused by mutations in the amino acid sequence, errors in translation, or environmental stresses. Correctly folded proteins have their hydrophobic side chains embedded within the interior of the folded protein. Misfolded proteins often expose hydrophobic domains, accumulate in the cytoplasm, and associate with other misfolded proteins, resulting in aggregation (Garcia-Mata et al., 1999). Protein aggregation is linked to many diseases, including Alzheimer’s, Huntington’s, and Parkinson’s disease (Boeddrich et al., 2003; Tanaka et al., 2004; Waelter et al., 2001). Generally, misfolded and aggregated proteins are refolded with the aid of chaperones or degraded by proteasomes or the autophagic process. However, a third possibility exists—the formation of aggresomes (Garcia-Mata et al., 2002).
Mechanism of aggresome formation

Aggresomes are conglomerations of misfolded proteins that coalesce at the microtubule organizing center (MTOC) and are believed to be a mechanism for dealing with misfolded proteins in the cytoplasm. Large, mature aggresomes form when smaller aggregates are carried along the microtubule tracks by the dynein/dynactin motor complex towards the nucleus (Garcia-Mata et al., 2002).

Once the smaller aggregates reach the nucleus, they form a large aggregate of loosely associated particles surrounding the MTOC. This causes a disruption of the vimentin intermediate filament network, which collapses around the aggregated protein (Garcia-Mata et al., 1999). Mature aggresomes have been shown to recruit chaperones, proteasomes, ubiquitin, and mitochondria. Despite this recruitment, aggregates are only partially degraded (Garcia-Mata et al., 2002). However, the aggresome also induces autophagy, directing them to the lysosome for degradation (Fortun et al., 2003). Thus, the aggresomal pathway may act as a way to transport aggregated proteins from the cytoplasm into lysosomes.
Degradation and clearance mechanisms induced by the aggresome

The ubiquitin-proteasome system is a mechanism of protein turnover in which the protein of interest is linked to ubiquitin molecules that target the protein to the proteasome. The most common eukaryotic proteasome complex, the 26S proteasome, consists of a 20S core and two 19S regulatory caps. The 19S caps bind ubiquitinated proteins, remove ubiquitin, and unfold the substrate. At the 20S core, proteins are cleaved at peptide bonds to create short peptide fragments. It is reported that proteasomes are recruited to the aggresome, possibly to aid in degradation of accumulated misfolded protein (Garcia-Mata et al., 1999; Lee and Lee, 2002).

Rearrangement of cell components in response to the aggresome

The aggresome triggers disruption of intermediate filaments such as vimentin (Garcia-Mata et al., 1999; Johnston et al., 1998). However, it does not disrupt other components of the cytoskeleton, such as the microfilaments and microtubules (Garcia-Mata et al., 1999). The structure and function of the endoplasmic reticulum, Golgi, and lysosomes do not appear to be altered. While the mitochondria remain functional, they are reported to migrate to the aggresome and to similar sites of viral assembly (Bauer and Richter-Landsberg, 2006; Heath et al., 2001; Mittal et al., 2007; Nozawa et al., 2004; Wileman, 2007). This migration might be a mechanism to provide ATP to chaperones and proteasomes.
Naturally occurring aggresomes

Aggresomes were first identified in connection with a mutant protein characteristic of cystic fibrosis (Johnston et al., 1998). This protein, the cystic fibrosis transmembrane regulator (CFTR), functions as a chloride channel when properly folded; when misfolded, it cannot be transported to the cell membrane and is unable to carry out normal functions. This malfunction is associated with cystic fibrosis. Aggresomes are associated with a variety of other disorders, particularly neurodegenerative diseases, including Huntington’s disease, Parkinson’s disease, and Retinitis pigmentosa (Saliba et al., 2002; Waelter et al., 2001).

Aggresome markers

Aggresomes can be studied not only through naturally occurring proteins but also through synthetic protein constructs that mimic aggresome formation. These act as a model system to characterize aggresome composition, formation, and resulting cellular response. Additionally, these proteins are markers that act as reference points. The primary aggresome marker used in this thesis research is GFP-250, which is composed of green fluorescent protein (GFP) fused at its C-terminus to the first 250 amino acids of the protein p115 (Garcia-Mata et al., 1999). p115 is a 959 amino acid protein involved in the transport of cargo from the ER to the Golgi (Nelson et al.,
1998). This truncated GFP-250 protein coalesces into a single structure at the MTOC that displays aggresomal characteristics. GFP-250 aggresomes require microtubules for their formation, reorganize vimentin, and recruit chaperones and proteasomes (Garcia-Mata et al., 1999)

**Part 2: The involvement of MK-STYX in the stress response and neuronal differentiation**

**Protein phosphorylation and cellular communication**

Reversible protein phosphorylation is an important post-translational modification that has been shown to play a role in almost every cellular process. Phosphorylation states are controlled by kinases, which are responsible for adding phosphate groups to target proteins, and phosphatases, which cleave these groups from the protein residues. Because the first phosphatase was discovered a decade after the first kinase, research on phosphatases is less complete. However, it is now becoming clear that while kinases are responsible for setting the amplitude of a signal, phosphatases are key in determining the duration and rate of these cellular signals (Heinrich et al., 2002; Hornberg et al., 2005). Understanding the regulation of dephosphorylation is an integral part of elucidating cellular signaling and biological processes, especially given growing evidence that phosphatases are involved in a wide variety of pathologies, including cancer, neurodegeneration, diabetes, and inflammation (Tonks, 2006).
Figure 2: Classification of Protein Tyrosine Phosphatases.

PTPs can be classified into these subgroups based on sequence homology and presence of conserved functional or binding domains. Of note in this work are the MKPS, the active homologs of MK-STYX.
Protein tyrosine phosphatases

Unlike kinases, which are derived from a common ancestor, the phosphatases evolved in structurally and catalytically distinct families. The protein tyrosine phosphatases (PTPs) are a large, heterogeneous group of protein phosphatases (Neel and Tonks, 1997; Patterson et al., 2009; Tonks, 2006). Protein tyrosine phosphatases are defined by their ability to dephosphorylate phosphotyrosine residues within a substrate. These proteins can be divided into four basic subgroups (Figure 3). The first, the “type I cysteine based PTPs”, encompass both the classical, tyrosine-specific “PTPs” and the “DUSPs”, or dual specificity phosphatases. The second group contains just one PTP, “the low-molecular mass PTP (LMWPTP)”, a type II cysteine-based and tyrosine-specific phosphatase. The third group is the “type III cysteine-based, tyrosine- and threonine-specific phosphatases”; this class includes three cell cycle regulators, Cdc25A, Cdc25B, and Cdc25C, which dephosphorylate cyclin-dependent kinases, thus regulating the cell cycle. Finally, the fourth class is the “aspartic acid based PTPs”, which include the EyA and HAD phosphatases (Patterson et al., 2009).

PTP signature motif and catalytic mechanism

A hallmark of the protein tyrosine phosphatases is the signature motif, \( HCX_5R \), in which the cysteine and arginine residues are essential for catalysis. The catalytic mechanism shared by the PTPs is also characteristic
of several other structurally diverse phosphatases. Dephosphorylation requires two steps mediated both by active site residues and nearby residues that stabilize the intermediates. In the first step, the phosphate group on the substrate is transferred to the catalytic cysteine in the signature motif in an $S_N2$ nucleophilic attack; this is made possible by the neighboring histidine and arginine, which serve to lower the $pK_a$ of the cysteine and enhance its nucleophilic abilities at physiological pH. Additionally, the neighboring arginine is essential in stabilizing the transition state, resulting in a phosphocysteine intermediate. Surrounding residues stabilize the negative charge in the region, allowing a water molecule to position for nucleophilic attack on the phosphocysteine intermediate (Zhang, 1998; Zhang and Dixon, 1994).

**Dual specificity phosphatases**

As members of the PTP superfamily, a unique feature of dual specificity phosphatases is their ability to dephosphorylate both phosphotyrosine and phosphoserine/threonine residues within one substrate (Patterson et al., 2009; Tonks, 2006). This is made possible by a shallower active site cleft that allows both tyrosine and serine/threonine to fit into the groove (Hinton et al., 2010). DUSPs possess both the $\text{HCX}_5\text{R}$ consensus sequence and an aspartic acid upstream of this sequence that acts as a general acid/base catalyst and helps to stabilize the phosphoryl-intermediate (Patterson et al., 2009). DUSPs are classified into multiple subgroups that highlight their heterogeneous
nature (Figure 2). Of particular note are the MKPs (mitogen-activated protein kinase phosphatases), which are one of the best-characterized DUSP subgroups and the primary subject of this study. The MKPs include ten proteins that dephosphorylate MAP kinases (mitogen-activated protein kinases) at both phosphoserine/threonine and phosphotyrosine residues (Patterson et al., 2009). Once thought to act merely as antagonists of MAP kinase signaling cascades, there is growing evidence that MKPs are critical regulators of signaling, capable of positively or negatively impacting signal transduction.

**Receptor tyrosine kinases**

Receptor tyrosine kinases (RTKs) are a subclass of enzyme-linked receptors that phosphorylate specific tyrosine residues on intracellular signaling proteins. Of the 90 tyrosine kinases in the human genome, 58 are receptor tyrosine kinases (Robinson et al., 2000). They can be divided into 16 structural subfamilies. In mammalian cells, these include the epidermal growth factor (EGF) receptors, the insulin/insulin-like growth factor receptors, the nerve growth factor (NGF) receptors, the platelet-derived growth factor receptors/macrophage colony-stimulating factor receptors, the fibroblast growth factor (FGF) receptors, the vascular endothelial growth factor (VEGF) receptors, and the ephrin (Eph) receptors.
Binding of a signal protein to the ligand-binding domain on the exterior of the cell activates the intracellular tyrosine kinase domain. Rearrangement of the cytosolic domains enables autophosphorylation, in which the kinase domains of the receptors can cross-phosphorylate each other on multiple tyrosines. This process contributes to the activation of the molecule in two ways. The first is that phosphorylation of tyrosines within the kinase domain increases the kinase activity of the enzyme. Additionally, phosphorylation of tyrosines outside the kinase domain generates high affinity docking sites for the binding of intracellular signaling proteins in the cell, which triggers the formation of a large intracellular complex. The result of this process is that phosphate groups are added to selected tyrosine side chains on both the receptor protein itself and on intracellular signaling proteins that go on to engage in downstream signaling within the cell (Alberts, 2008; Robinson et al., 2000).

**Ras: a ubiquitous signaling protein**

The Ras proteins are a class of proteins that are part of the Ras superfamily of monomeric GTP binding proteins. GTPases are proteins that are able to bind and hydrolyze guanosine triphosphate (GTP). The Ras superfamily includes five subfamilies: Ras, Rho, Rab, Arf, and Ran (Colicelli, 2004; Wennerberg et al., 2005). Ras proteins generally contain a covalently attached lipid groups that help anchor them to the cytoplasmic face of the plasma membrane. There, they interact with other signaling molecules to
relay signals from the cell surface to other parts of the cell, aiding in processes such as growth and differentiation.

Ras is activated and inactivated by guanine nucleotide exchange factors (GEFs) and GTP-ase activating proteins (GAPs). GEFs promote the exchange of GDP for GTP, thereby activating Ras, while GAPs increase the rate of hydrolysis of bound GTP, inactivating Ras. Thus, the coupling of a receptor tyrosine kinase to a GEF drives Ras into its active state. It has been indicated that RasGAP (a GAP specific to Ras) may have an integral role in the transducing of signals by Ras. The N-terminal domain of RasGAP has an SH3 (SRC Homology 3) domain flanked by two SH2 (Src Homology 2) domains responsible for phosphotyrosine binding. The SH3 domain in RasGAP, it appears, is essential for transducing signals downstream of Ras in a MAPK-independent manner and is also essential for transformation by oncogenic Ras (Irvine et al., 2004).

**Mitogen-activated protein kinases**

Ras can activate a serine/threonine phosphorylation cascade that is highly conserved in eukaryotic cells: the mitogen-activated protein kinase (MAPK) pathways (Figure 3). MAPKs are activated by dual phosphorylation on TXY motifs (Robinson and Cobb, 1997). The prototypical MAP kinase cascade comprises a three kinase module, including a MAP kinase (or ERK) a
Growth factors such as neurotrophins (including NGF) induce RTKS to signal to ERK/MAPK via a signaling cascade that begins with the small G-protein Ras. The Ras-dependent pathway is ubiquitous in signaling processes of growth and development. MKPs deactivate MAPKs by dephosphorylating them. We propose that MK-STYX either binds a MAPK or binds to the MKP.

**Figure 3: MAPK and MK-STYX signaling cascades in neuronal cells.**
MAPK/ERK kinase (MEK), and a MEK kinase (MEKK) (Robinson and Cobb, 1997). In this cascade, a receptor tyrosine kinase or other enzyme-linked receptor would receive a signal from the exterior of the cell, generally in the form of a signaling molecule. Then, the RTK would activate Ras in the mechanism discussed above. From there, Ras activates a MEKK, Raf. Raf phosphorylates the MEK at two serines, thereby activating it (Alessi et al., 1994). Finally, the MEK, or MAP kinase kinase, catalyzes phosphorylation of both a threonine and a tyrosine on a MAP kinase. This MAP kinase, now activated, can phosphorylate target proteins in the cytosol, affecting protein activity, or enter the nuclear to phosphorylate one or more components of a gene regulatory process, altering gene expression (Robinson and Cobb, 1997).

Classes of MAP kinases
There are more than a dozen MAPK genes in the human genome. The most completely characterized are the extracellular signal regulated kinases ERK1/2, the c-Jun N-terminal kinases JNK1-3, ERK5, and the p38 kinases. The c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK) are activated in response to stresses, cytokines, and DNA/protein synthesis inhibition. The p38 kinases are activated in response to cell stressors and osmotic stress. This particular study focuses on the ERK1/2 family of MAP kinases. ERK1 and ERK2 are 42 and 41 kDa respectively, share 83%
identity, and are ubiquitously expressed throughout eukaryotic cell types. ERK1/2 is activated to varying extents by growth factors, serum, cytokines, and microtubule depolymerization. In response, these kinases are known to phosphorylate the p90 ribosomal S6 kinase, mitogen and stress activated kinase (MSK), and MAPK interacting kinase, among many other targets. These and many diverse targets have linked ERK1/2 to cell attachment and migration, growth and differentiation. Though ERK1/2 generally localize to throughout the cell, activation of ERK1/2 results in translocation to the nucleus, where they play a role in interactions with nuclear transcription factors. MEK1, which harbors a strong NES, binds to both non-phosphorylated ERK1 and ERK2, highlighting a mechanism for sequestering inactive ERK1/2 in the cytosol (Raman et al., 2007).

MAP kinase phosphatases and MAP kinase cascade regulation

The first MAP kinase phosphatase, MKP1, was originally cloned as an immediate early gene product (3CH134) expressed upon stimulation with growth factor. It was found to specifically dephosphorylate MAP kinases at the phosphothreonine and phosphotyrosine residues. This dephosphorylation was shown to inactivate the catalytic activity of MAP kinases and to suppress cellular signaling (Sun et al., 1993). After the discovery of MKP1, nine other MKPs were identified on the basis of sequence homology, including a highly conserved C terminal domain. Along with MKP1, MKP3 is extensively
studied, and shown to bind specifically to Erk2. Other DUSPs, such as JSP-1 and SKRP1, have been shown to modulate MAPK signaling through upstream regulators, indicating that there are significant, multi-faceted mechanisms for the control of MAP kinase pathways by phosphatases (Schwertassek et al., 2010).

“Dead” phosphatases and their cellular significance

The pseudophosphatase STYX is the prototypic pseudophosphatase. It is structurally related to the dual-specificity phosphatases but lacks the nucleophilic cysteine residue (Wishart and Dixon, 1998). There have been many other pseudophosphatases identified; this discovery in conjunction with the presence of naturally-occurring pseudokinases suggests that catalytically impaired proteins may influence phosphorylation and dephosphorylation in vivo (Tonks, 2009).

Aside from the 10 MAP kinase phosphatases with CH2 motifs, a protein called MK-STYX also contains the same motifs and is homologous to the MKPs. Interestingly, this protein is naturally catalytically inactive due to the substitution of serine and phenylalanine in place of the conserved cysteine and histidine residues. MK-STYX thus constitutes a naturally occurring “dead” phosphatase, or pseudophosphatase. Despite their catalytically inactive status, there is growing evidence that pseudophosphatases may perform
regulatory functions \textit{in vivo}. Mutation of the first discovered MKP, MKP1, at the active site led to sustained phosphorylation of MAPK (as opposed to the normally transient phosphorylation state of this protein) (Sun et al., 1993). Thus, it is possible that the mutant protein binds to its phosphorylated substrate, thus blocking catalytically active endogenous phosphatases from cleaving the phosphate group. Additionally, expression of the catalytically inactive MKP1 mutant was sufficient to generate an accumulation of phosphorylated MAP kinase (Sun et al., 1993). This potential for mutated, catalytically inactive phosphatases to bind tightly to their substrates has been exploited in the elucidation of targets for poorly characterized phosphatases. However, these experiments also shed light on possible mechanisms of action for pseudophosphatases in mammalian cells.

\textbf{MK-STYX}

MK-STYX [MAPK (mitogen-activated protein kinase) phosphoserine/threonine/tyrosine-binding protein] is a pseudophosphatase member of the dual-specificity phosphatase subfamily of the PTPs (protein tyrosine phosphatases) (Figure 4). MK-STYX is catalytically inactive due to the absence of two amino acids from the signature motif that are essential for phosphatase activity. The conserved nucleophilic cysteine residue and adjacent histidine residue are replaced by serine and phenylalanine residues, respectively, in MK-STYX. G3BP [Ras-GAP (GTPase-activating protein) SH3
(Src homology 3) domain-binding protein-1, a regulator of Ras signaling, has been identified as binding partner of native MK-STYX. MK-STYX is homologous to the MKPs, which are responsible for regulating diverse cellular signaling pathways.

G3BP
The isolation of G3BP (Ras-GTPase activating protein SH3 domain binding protein) was reported in 1996 (Parker et al., 1996) (Figure 4). Since then, G3BP has been implicated in a diverse array of cellular processes. Mammals have 3 G3BPs: G3BP 1, 2a, and 2b, which are the products of differential splicing of 2 genes. They are ubiquitously expressed. Structurally, the proteins contain an RNA Recognition Motif (RRM), an arginine-glycine rich box (RGG box), and a Nuclear Transport Factor 2 (NTF-2) - like domain. NTF2 has been involved in RanGTP-dependent nuclear import of proteins through the nuclear pore complex (Irvine et al., 2004). The distribution of G3BP is primarily cytoplasmic, but it can enter the nucleus (Parker et al., 1996). Functionally, all G3BPs bind specifically to the SH3 domain of RasGAP, perhaps modulating signaling events downstream of Ras. The RNase activity of G3BP is positively regulated by phosphorylation at several sites. Serine 149 (ser149), in particular, is dependent on RasGAP and
**Figure 4: Schematic Representations of MK-STYX and G3BP.**

MK-STYX (A) has dual specificity and rhodanese domains. MK-STYX is catalytically inactive due to the absence of two amino acids (shown) within the active site that are essential for phosphatases activity. G3BP (B) has an NTF2-like domain as well as an RNA recognition motif. Formation of stress granules is regulated by phosphorylation at site 149.
negatively regulated by Ras signaling (Parker et al., 1996; Tourriere et al., 2001).

**Stress granule formation**

Cells have evolved protective responses to stresses such as elevated temperature, oxidative conditions, or exposure to UV light. One response involves the induction of the heat-shock proteins, stress-induced transcription factors, and the formation of stress granules (Kedersha et al., 2000; Kedersha et al., 1999). Stress granules are large, cytoplasmic structures where untranslated mRNAs accumulate (Anderson and Kedersha, 2002; Kedersha and Anderson, 2002). Assembly of stress granules can be triggered by the phosphorylation of the translation initiation factor eIF2α, which prevents formation of the eIF2-GTP-Met-tRNA complex (Kedersha et al., 1999). This inhibits protein synthesis and leads to accumulation of the 48S preinitiation complex (Kedersha and Anderson, 2002; Kedersha et al., 1999). Stress granules contain many components of this complex as a result. Multiple RNA-binding proteins are associated with the stress granule complex, including TIA-1 and TIAR, poly(A)+ binding protein I (PABP-I), and the mRNA stabilizing protein HuR (Kedersha and Anderson, 2002; Kedersha et al., 1999).
**G3BP and stress granule assembly**

G3BP is diffusely distributed throughout the cytoplasm of untreated cells, but when exposed to environmental stresses, such as heat shock, UV irradiation, hypoxia, arsenite or high temperature, G3BP becomes localized in large cytoplasmic structures called stress granules (Tourriere et al., 2003). Stress granules are stalled sites of mRNA translation, where mRNA is triaged and sent to other sites of storage, initiation of translation, or degradation. The overall effect is to promote expression of proteins that allow the cell to adapt to the stress (Kedersha et al., 2005). Additionally, it appears G3BP recruitment to stress granules is influenced by Ras. Phosphorylation at site 149 seems to have an effect on stress granule formation as well; a phosphomimetic mutant of G3BP (S149E) was unable to form stress granules (Tourriere et al., 2003). This data indicates that G3BP-induced formation of stress granules is dependent on dephosphorylation of Ser149, particularly because phosphorylation at this site prevents oligomerization of G3BP (Tourriere et al., 2003).

Using co-immunoprecipitation studies, G3BP was determined to be a binding partner for MK-STYX. MK-STYX reduced stress granule formation in cells transfected with a GFP-tagged G3BP expression vector (Hinton et al., 2010). Additionally, expression of MK-STYX was observed to result in a dramatic
decrease in stress granule formation in arsenite treated cells (Hinton et al., 2010).
Specific aims

This research aims to answer several primary questions, elucidating the importance of protein structure, function, and localization in biological processes:

1. Do v-ErbA aggresomes colocalize with proteasomes?
2. Do v-ErbA aggresomes colocalize with mitochondria?
3. What is the effect of the retroviral Gag sequence on v-ErbA localization?
4. Is MK-STYX inhibition of stress granule formation affected by G3BP phosphorylation state?
5. Does MK-STYX have an effect on NGF-stimulated differentiation?
6. What is MK-STYX’s effect on the activation state of ERK 1/2?
Methods

Plasmids

The GFP-250 expression vectors were a generous gift from Elizabeth Sztul (University of Alabama) (Fu et al., 2005; Garcia-Mata et al., 1999). The aggresomal marker GFP-250 is composed of GFP fused at its C-terminus to the first 250 amino acids of p115, a protein involved in the transport of cargo from the endoplasmic reticulum to the Golgi (Nelson et al., 1998). GFP, GFP-TRα1, GFP-Δ Gag-v-ErbA, and YFP-Gag (1–70) expression vectors have been previously described (Bonamy et al., 2005; DeLong et al., 2004).

pMT2-FLAG-MK-STYX-FLAG and pMT2-FLAG-MK-STYX_{active}-FLAG were generated as described previously. (Hinton et al., 2010). The integrity of all constructs derived from PCR was confirmed by DNA sequencing. The G3BP-GFP, G3BP149A-GFP, and G3BP149E-GFP constructs were kindly provided by Jamai Tazi (Institut de Génétique Moléculaire, France).

Cell culture, transfections and treatments

HeLa cells (human cervix epithelioid carcinoma; ATCC CCL-2) were grown and maintained at 37°C and 5% CO₂ in Minimum Essential Medium (Gibco), supplemented with 10% fetal bovine serum (Invitrogen). PC12 cells (rat pheochromocytoma) were grown and maintained at 37°C and 5% CO₂ in DMEM high glucose Minimum Essential Medium (Gibco), supplemented with
10% fetal bovine serum (Invitrogen).

For transient transfection assays, 1–3 × 10^5 HeLa cells per well were added to each well of a 6-well plate with glass coverslips (Fisher) and incubated at 37°C for approximately 24 h. Alternatively, 1-7 × 10^5 PC12 cells were grown on 100-mm round dishes and incubated at 37°C for approximately 24 h. Various combinations of plasmid expression vectors were introduced using either Lipofectamine or Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cells were fixed 24–48 h post-transfection and analyzed by fluorescence microscopy.

For live cell imaging, PC12 cells were plated onto 100 mm plates (Fisher) and incubated at 37°C for approximately 24 hours. Various combinations of plasmid expression vectors were introduced using either Lipofectamine or Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. In some experiments, cells were treated with nerve growth factor. Cells were imaged 24-48 hours post transfection.

**Fixation, immunofluorescence, and organelle staining**

Approximately 24–48 h after transfection, cells were fixed in 3.7% formaldehyde and, in some experiments, permeabilized with 0.2% Triton-X-100 for antibody staining. Antibodies used were as follows: anti-20S
proteasome ‘core’ subunits, 1:500 (BioMol); Cy3-goat anti- mouse or Cy3-goat anti-rabbit, 1:500 (Zymed Laboratories). To visualize mitochondria, cells were treated with 10 nM MitoTracker Red (Molecular Probes) approximately 48 h post-transfection. Upon addition of MitoTracker Red, cells were incubated at 37°C for 30 min. After processing, coverslips were mounted onto microscope slides in Fluoro-Gel II (Electron Microscopy Sciences) containing the DNA counter stain DAPI (4,6-diamino-2-phenylindole dihydrochloride) (Sigma).

**Cell scoring and statistical analysis**

For some experiments, prepared slides were analyzed with an Olympus fluorescence microscope. Cells were photographed with a Cooke SensiCamQE digital camera. IP Lab software and Adobe Photoshop CS were used to pseudocolor, and layer the captured images. At least 2–3 replicate transfections were performed, with a minimum of 100 cells scored per replicate. For PC12 experiments, live cells were analyzed with a Nikon Eclipse Ti microscope. NIS Elements software was used to analyze outgrowth length.
Results

Part 1: The Targeting of v-ErbA to Aggresomes

v-ErbA aggresomes recruit proteasomes

Strong evidence suggests that v-ErbA foci are aggresomal in nature. First, v-ErbA aggresomes have been shown to associate with aggresome markers (Bondzi et al., 2011). Additionally, they have been shown to disrupt the vimentin intermediate filaments, depend on the microtubules and the dynein motor for formation, and recruit cellular components like HDAC6 (Bondzi et al., 2011).

Aggresomes have been reported to recruit chaperones, ubiquitination enzymes, and proteasomes (Garcia-Mata et al., 2002). In order to investigate the potential colocalization of GFP-v-ErbA inclusions with proteasomes, HeLa cells were transfected with expression vectors for either GFP-250 or GFP-v-ErbA. Subsequently, we immunostained with anti-20S proteasome subunit antibodies (the catalytic core of the 26S proteasome) and observed by fluorescence microscopy. In order to increase the amount of mature aggresome formation, some cells were treated with MG132. First, we investigated the colocalization of the aggresome marker GFP-250 with 20S proteasomes. The 20S proteasome subunits showed a diffuse distribution throughout the cytoplasm with 59% of cells displaying distinct colocalization of a subpopulation of the proteasomes with these aggregates. Of cells that
formed cytoplasmic inclusions of GFP-v-ErbA, 68% of cells showed colocalization with a subpopulation of 20S proteasome subunits.

**v-ErbA aggresomes recruit mitochondria**

The accumulation of proteins at the aggresome causes disorganization of the microtubule network. Because microtubules regulate the distribution of cellular organelles, this disruption leads to retraction of the mitochondria from the periphery to the cell and towards the area of the aggresome and of the viral replication factories (Bauer and Richter-Landsberg, 2006; Heath et al., 2001; Mittal et al., 2007; Nozawa et al., 2004; Wileman, 2007). It is hypothesized that this recruitment takes place to provide energy for the processes of protein folding and/or degradation (Waelter et al., 2001). Given that both the 20S proteasome subunits and chaperones have been shown to colocalize to the aggresome, and both structures require ATP to function, this is plausible (Bondzi et al., 2011; Garcia-Mata et al., 1999).

In order to investigate possible association between GFP-250 and GFP-v-ErbA aggresomes and mitochondria, HeLa cells were stained with MitoTracker Red, a mitochrondrion-selective dye. In untransfected cells, mitochondria were distributed throughout the cytosol. This was true of cells transfected with expression vectors for pEGFP and GFP-TRα1. In contrast, in
Figure 5: The recruitment of proteasomes to the site of the aggresome.

Colocalization of 20S proteasome subunits with GFP-250 and v-ErbA aggresomes. HeLa cells overexpressing GFP-250 or GFP-v-ErbA) were fixed 48 h post-transfection and immunostained with anti-20S proteasome antibodies (red). In the merged images, yellow indicates colocalization and blue indicates nuclei stained with DAPI.
61% of cells that formed GFP-250 aggresomes, the foci colocalized with mitochondria retracted from the cell periphery.

Prior work indicated that when v-ErbA was present in punctate foci throughout the cytoplasm, these pre-aggresomal inclusions did not colocalize with mitochondria (Bonamy et al., 2005). In this investigation, v-ErbA foci were allowed to mature into full aggresomes. Consequently, colocalization of v-ErbA with mitochondria was observed in 83% of cells that formed mature aggresomes.
Figure 6: Mitochondria are recruited to v-ErbA aggresomes.

In untransfected cells (A), and in cells transfected with pEGFP (B) and GFP-TRα1 (C), mitochondria are distributed throughout the cytoplasm. In cells transfected with expression vectors for GFP-250 (D) or GFP (E), mitochondria colocalized to aggresomes.
The AEV Gag sequence is essential for v-ErbA recruitment to aggresomes

Viral factors, sites of viral assembly, are utilized by many viruses to increase the efficiency of replication by isolating viral components. Similarly to aggresomes, viral factories rearrange vimentin, utilize the microtubule tracks, converge at the MTOC, and recruit chaperones, ubiquitin, and mitochondria. Because v-ErbA is a retroviral oncoprotein, studying its inclusions offers insight in the study of both aggresomes and viral factories. v-ErbA is expressed by AEV as a Gag-v-ErbA fusion protein. The Gag sequence appears to be essential for biological activity of v-ErbA and contains a strong nuclear export sequence (DeLong et al., 2004). The viral Gag sequence is involved in formation of the viral capsid; it is possible that it is recognized by the dynein motors, like other viral components, and it is carried along the microtubule tracks towards the nucleus.

In order to investigate the role of the Gag sequence in the formation of v-ErbA foci, HeLa cells were transfected with a GFP-ΔGag-v-ErbA (Gag sequence deleted) expression vector and viewed by fluorescence microscopy. Generally, over 50% of cells expressing GFP-v-ErbA contain large cytoplasmic inclusions. In this experiment, ΔGag-v-ErbA showed a significant nuclear population, more diffuse cytoplasmic distribution, and a drastic reduction in cytoplasmic foci (~ 1% of cells) (see Figure 7). Further
investigation into the role of the Gag sequence involved the overexpression of YFP-tagged Gag (1-70), which has been shown to localize entirely to the cytoplasm (DeLong et al., 2004). This protein formed multiple foci in the cytoplasm of a subset of the cells. It is possible that the 70 amino acids at the N-terminus play a role in promoting the formation of cytoplasmic inclusions of v-ErbA.
Figure 7: The AEV Gag sequence plays a role in targeting of v-ErbA to aggresomes.

HeLa cells were transfected with plasmids for GFP-ΔGag-v-ErbA (v-ErbA with deletion of Gag sequence). 24 h post-transfection, cells were fixed and analyzed by fluorescence microscopy. GFP-ΔGag-v-ErbA was more nuclear than GFP-v-ErbA and had few cytoplasmic aggregates compared with GFP-v-erbA.
Part 2: The involvement of MK-STYX in the stress response and neuronal differentiation

Inhibition of stress granule assembly by MK-STYX is not wholly G3BP phosphorylation dependent

Stress granule assembly is induced by a variety of environmental stressors, including heat shock and UV radiation, as well as overexpression of G3BP (Tourriere et al., 2003). It has also been shown that MK-STYX binds G3BP and decreases stress granule assembly (Hinton et al., 2010). Dephosphorylation of G3BP at serine 149 has been shown to induce stress granule assembly (Tourriere et al., 2003). To determine whether inhibition of stress granule assembly by MK-STYX is phosphorylation-dependent, we coexpressed MK-STYX with GFP-tagged G3BP-S149A or G3BP-S149E mutants in HeLa cells. This mutant cannot be phosphorylated, nor does it possess the charge that might mimic phosphorylation. Consistent with previous studies (Tourriere et al., 2003), our study found that G3BP-S149A induced stress granule assembly in ~60% of cells expressing the mutant. In our research, MK-STYX prevented stress granule assembly; only 35% of cells expressing MK-STYX and G3BP-S149A formed stress granules.

The non-phosphorylatable G3BP-S149A mutant mimics dephosphorylated G3BP, which is required for stress granule assembly (Tourriere et al., 2003). MK-STYX is able to regulate stress granule assembly in cells expressing
G3BP-S149E, suggesting that its mode of action is not wholly dependent on G3BP phosphorylation state. Additionally, the expression of MK-STYX had no effect on stress granule formation in cells expressing the phosphomimetic mutant G3BP-S149E.

Intriguingly, the presence of MK-STYX\textsubscript{(active mutant)} had a significant effect on cells expressing the non-phosphorylatable and phosphomimetic 149 mutants. Cells expressing G3BP-S149A in the presence of MK-STYX\textsubscript{(active mutant)} formed larger granules, with 55% forming aggregates. Additionally, cells expressing the active mutant and G3BP-S149E formed intermediate-sized granules that resemble prior studies with MK-STYX and wild type G3BP (Hinton et al., 2010). Approximately 53% of cells expressing the phosphomimetic G3BP-S149E and the active mutant were able to induce stress granule formation, an unexpected finding given the necessity of dephosphorylation at site 149 for stress granule assembly (Tourriere et al., 2003).
Figure 8: Phosphorylation independent inhibition of stress granule assembly by MK-STYX.

Representative examples of subcellular distribution of G3BP-S149A-GFP. HeLa cells cotransfected with expression constructs for G3BP-S149A-GFP and wild-type MK-STYX showed fewer cells with stress granules. The overexpression of G3BP-S149A alone resulted in stress granule assembly. Cells cotransfected with G3BP-S149A and the active MK-STYX mutant accumulated larger perinuclear granules. Green = G3BP-S149A. Blue = DAPI.
Figure 9: Phosphorylation independent inhibition of stress granule assembly by MK-STYX.

Cells were scored for the presence or absence of stress granules. Three replicate experiments were performed (n=100); the error bars indicate +/- SEM.
**Figure 10: Induction of G3BP149E stress granule assembly by the active mutant**

Representative examples of subcellular distribution of G3BP-S149E-GFP. Cells expressing G3BP-S149E mutant alone or in the presence of MK-STYX did not accumulate stress granules. However, cells expressing G3BP-S149E accumulated small granules throughout the cytoplasm. Green = G3BP-S149E. Blue = DAPI.
Figure 11: Induction of G3BP-S149E stress granule assembly by the active mutant

Cells were scored for the presence or absence of stress granules. Three replicate experiments were performed (n=100); the error bars indicate +/- SEM.
MK-STYX increases outgrowth formation in PC12 cells

It has been shown that the DUSP PTEN attenuates G3BP in neuronal cell lines (Huang et al., 2005). Thus, we sought to determine whether MK-STYX would have similar effects on signaling in neuronal cell lines. In order to do this, we selected the PC12 cell as a model. The PC12 cell line is derived from a pheochromocytoma of the rat adrenal medulla. After stimulation with nerve growth factor, the cell stops growing, forms processes, and becomes electrically excitable. After exposure for weeks, the cells can form processes up to 500-1000 μm (Greene & Tischler, 1976). In PC12 cells, binding of NGF and EGF to their respective cell surface receptor tyrosine kinases activates Ras-MAPK, phosphatidylinositol (PI) 3-kinase, and phospholipase C cascades (source). EGF transiently activates ERK1/2 and stimulates cell proliferation. NGF stimulation leads to sustained MAPK activity and nuclear accumulation of ERK, leading to cell cycle arrest, neurite outgrowth, and expression of neural markers (Qiu et al., 2004).

In addition to MAP kinases, PC12 cells also express phosphatases. For example, MKP3 mRNA is highly induced after treatment with NGF in PC12 (Camps et al., 1998). Dual specificity phosphatases may also play a regulatory role of the MAPK cascade in this cell line. Overexpression of PTEN caused resistance to NGF in PC12 cells, while PTEN knockdown caused elevated MAPK signaling (Jia et al., 2010). Even more intriguingly, a rat
homologue of G3BP, the only known binding partner of MK-STYX, is expressed endogenously in PC12 cells (Parker et al., 1996). Additionally, G3BP1 mRNA is induced downstream of human epidermal growth factor receptors (Irvine et al., 2004). Given this information, we hope to investigate the possibility that MK-STYX is affecting this process through either regulation of the MAPK or the principal MKPs (see Figure 3).

In order to test what effects MK-STYX might have on neuronal cells, we used PC12 cells as a model. We performed transient cotransfections using the expression vectors for pEGFP and pMT2-FLAG or pMT2-FLAG-MK-STYX-FLAG. In cells that expressed these constructs, 33% of cells expressing MK-STYX formed outgrowths, as compared to 2% of untransfected cells (Figure 12). Cells expressing MK-STYX exhibited multiple small outgrowths (Figure 15).

**MK-STYX increases outgrowth length in cells treated with NGF**

To determine MK-STYX effects on the normal cellular signaling in response to growth factors such as NGF, we performed transient cotransfections in PC12 using expression vectors for pEGFP and pMT2-FLAG or pMT2-FLAG-MK-STYX-FLAG. In cells that expressed these constructs, we observed a dramatic shift in the distribution of outgrowth length (Figure 13). The largest shift was in the categories of 20-40 μm and 40-60 μm (the diameter of a...
PC12 cell ranges from 6-14 μm). This data would suggest that MK-STYX is enhancing MAPK signaling in the PC12 cell. Interestingly, however, our preliminary Western blotting data suggests that ERK 1/2 phosphorylation, and thus activation, is actually lower in cells expressing MK-STYX. Thus, further explanation is needed to determine which MAPK MK-STYX is affecting to achieve this response.
Figure 12: MK-STYX promotes outgrowth formation in PC12 cells.

In order to perform a preliminary investigation of MK-STYX’s effects on outgrowth formation, PC12 cells were transfected with either pEGFP & pMT2 or EGFP & MK-STYX. Co-expression of pEGFP allowed visualization of successful transfection and cell morphology. Cells were fixed and analyzed 24 h post-transfection for neurite outgrowth. Cells were scored (n=100) for the presence or absence of outgrowths.
Figure 13: MK-STYX causes a shift in the distribution of outgrowth lengths.

PC12 cells were transfected with either pEGFP & pMT2 or pEGFP & MK-STYX. Co-expression of pEGFP allowed visualization of successful transfection. Cells were treated with 100 ng NGF 24 h post-transfection. 48 h post-transfection, cells were visualized using a combination of phase contrast and fluorescence microscopy. Cells were scored (n=200) for outgrowth length using NIS Elements software.
Figure 14: MK-STYX causes the most significant shifts between 20 and 60 μm.

PC12 cells were transfected with either EGFP & pMT2 or EGFP & MK-STYX. Co-expression of EGFP allowed visualization of successful transfection. Cells were treated with NGF 24 h post-transfection. 48 h post-transfection, cells were visualized using a combination of phase contrast and fluorescence microscopy. Cells were scored (n=200) for outgrowth length using NIS Elements software. Error bars indicate +/- SEM.
Figure 15: MK-STYX affects the morphology of untreated cells.

PC12 cells were transfected with either pEGFP & pMT2 or pEGFP & MK-STYX. 48 h post-transfection, cells were visualized using a combination of phase contrast and fluorescence microscopy. Cells expressing MK-STYX (B) display small outgrowths surrounding the cell in 33% of cases, unlike those expressing the empty vector (A).
A) pEGFP + pMT2

B) pEGFP + MK-STYX

Figure 16: MK-STYX elongates outgrowths in cells treated with NGF

PC12 cells were transfected with either pEGFP & pMT2 or pEGFP & MK-STYX. Co-expression of EGFP allowed visualization of successful transfection. Cells were treated with 100 ng NGF 24 h post-transfection. 48 h post-transfection, cells were visualized using a combination of phase contrast and fluorescence microscopy. A – pEGFP + pMT2, B – pEGFP + MK-STYX.
Discussion

Part 1: The Targeting of v-ErbA to aggresomes

Prior research in the Allison lab indicated that the v-ErbA oncoprotein forms cytoplasmic foci and that a significant portion of cellular TRα in these foci; this likely contributes to the protein’s oncogenic properties (Bonamy and Allison, 2006; Bonamy et al., 2005). Additionally, research in the Allison lab indicates that a subpopulation of v-ErbA aggregates colocalize with aggresomal markers (such as GFP-250), recruit HDAC6 and chaperones, disrupt vimentin intermediate filaments, and are enhanced in size upon treatment with proteasome inhibitors (Bondzi et al., 2011). Formation of v-ErbA foci is also microtubule- and dynein-dependent (Bondzi et al., 2011). This thesis research provides further support for the targeting of v-ErbA to aggresomes; both mitochondria and proteasomes are recruited to the site of v-ErbA foci. Additionally, aggresome formation appears to be dependent on the viral Gag sequence.

Significance of recruitment of mitochondria and proteasomes

v-ErbA is an oncoprotein carried by the avian erythroblastosis virus (AEV) that mislocalizes to cytoplasmic foci, sequestering the thyroid hormone in heterodimers within these foci; this property contributes to its oncogenic properties (Bonamy et al., 2005). This thesis research provides evidence that these cytoplasmic foci that are aggresomal in nature, recruit proteasomes and
mitochondria, and are reliant upon the Gag sequence for formation. These results indicate that v-ErbA foci are aggresomes, but also that they may represent a holdover from viral replication.

It is possible that the targeting of v-ErbA to aggresomes is a cellular response to the accumulation of the mutant protein in the cytoplasm. If so, the aggresome serves as a mechanism of sequestering the protein for disposal by the proteasome machinery. Our data indicates that proteasomes are recruited to the site of the aggresome, presumably to aid in the degradation process. The 20S proteasome subunit has been shown to localize to the aggresome (Garcia-Mata et al., 1999), despite evidence that proteasome-mediated degradation is not particularly efficient at the aggresome (Garcia-Mata et al., 2002).

Additionally, we found that mitochondria were recruited to the site of the v-ErbA aggresomes. It has been postulated that mitochondria are recruited to aggresomes to provide energy for protein folding and/or degradation (Waelter et al., 2001). It is also possible that because v-ErbA causes the disruption of the vimentin intermediate filaments (Bondzi et al., 2011), the reorganization of the mitochondria could be a side effect of the disturbance of the cytoskeleton. Mitochondria have also been shown to be involved in apoptotic pathways, so it is possible that mitochondria, once recruited to the aggresome, engage in
signaling pathways that promote apoptosis (Cereghetti and Scorrano, 2006; Debure et al., 2003; Soo et al., 2009).

**Significance of the viral Gag sequence in v-ErbA localization**

It is also plausible that targeting of v-ErbA to aggresomes is a remnant viral behavior for assembly at a viral factory. While the aggresomal response is likely adapted to reduce the toxicity of misfolded proteins, viruses may exploit it as a way to gather structural components necessary for viral assembly in a central location (Wileman, 2006). Viral factories have been shown to colocalize with the aggresome marker GFP-250, and like aggresomes they are dependent upon the microtubules for assembly and disrupt vimentin intermediate filaments (Arnaud et al., 2007; Heath et al., 2001; Liu et al., 2005). v-ErbA is a fusion protein containing a portion of the AEV Gag sequence, which encodes structural proteins that form the viral capsid. Prior studies indicate that the Gag portion of v-ErbA mediates CRM1-dependent nuclear export (DeLong et al., 2004). v-ErbA has also been shown to have a more nuclear localization when the Gag sequence is deleted (DeLong et al., 2004). This thesis research indicates that deletion of the Gag sequence results in aggresome formation in less than 1% of cells, partially because of the predominantly nuclear localization. This data supports the hypothesis that the formation of v-ErbA foci is mediated by the Gag sequence, and that this represents a remnant of viral assembly at viral factories.
Aggresomes: cytoprotective or pathogenic?

v-ErbA can bind to TRα and sequester the receptor in cytoplasmic foci, preventing the regulation of target genes in response to the thyroid hormone (Bonamy and Allison, 2006; Bonamy et al., 2005; Bondzi et al., 2011). Other dominant negative variants of TRα form cytoplasmic foci (Bunn et al., 2001; Lee and Mahdavi, 1993). It is thus possible that this mislocalization plays a role in their repressive action as well. Mutations in TR genes are associated with endocrine disorders and human cancers (Chan and Privalsky, 2009; Chan and Privalsky, 2010), and have been associated with a mouse model of thyroid cancer (Zhu et al., 2010). There is significant potential that mislocalization of thyroid hormone receptor variants is key in their pathogenesis.

Characterizing v-ErbA aggresomes helps in answering a key question in studies of protein aggregation: are these mechanisms cytoprotective or pathogenic? Given the association between protein aggregation and such disorders as cystic fibrosis, Alzheimer’s, and Parkinson’s (Johnston et al., 1998), it is tempting to hypothesize that aggresomes are detrimental to the cell. Alternatively, the recruitment of chaperones, proteasomes, mitochondria, and other cellular components suggests that this is a mechanism utilized by the cell in order to sequester the protein in order to degrade it.
Future directions

To further investigate the cytoprotective or pathogenic nature of v-ErbA aggresomes, it would be helpful to continue to investigate the role of the mitochondria at the site of the aggresome. One potential is to test for apoptotic markers at the site of the aggresome.

Given the association between thyroid hormone receptor mutations and disease, further characterization of the effects of other thyroid hormone receptor mutations on cellular localization could clarify the role of mislocalization in dominant negative activity.

Part 2: The involvement of MK-STYX in the stress response and neuronal differentiation

A role for pseudophosphatases in cell signaling

Several pseudophosphatases have been identified with a diverse range of functions (Azzedine et al., 2003; Conner et al., 2006; Tonks, 2009; Wishart and Dixon, 1998). While these proteins adopt a similar structure, they lack key residues that are essential for catalytic activity and thus will not be able to cleave phosphate groups from their target substrates. Despite their lack of
catalytic activity, however, these proteins have meaningful effects on signaling pathways. This highlights the importance of protein-protein interactions.

MK-STYX is a pseudophosphatase member of the MAP kinase phosphatase class of dual specificity phosphatases (Hinton et al., 2010). MK-STYX is catalytically inactive due to the absence of two key residues in the active site that are essential for phosphatase activity (Andersen et al., 2004; Hinton et al., 2010). Mutations made in these key residues restored catalytic activity (Hinton et al., 2010). It consists of a PTP domain, as well as a rhodanese domain homologous to the cdc25 (cell dependent cyclin phosphatase 25kDa) (Keyse and Ginsburg, 1993; Wishart and Dixon, 1998). The PTP domain carries out the catalytic activity, while the rhodanese domain is thought to act as a docking domain for MAPKs (Hofmann et al., 1998). MKPs are known to bind to MAPKs to dephosphorylate and deactivate them (Camps et al., 1998; Sun et al., 1993; Sun et al., 1994). Given MK-STYX's homology to MKPs, the most likely target for MK-STYX would be MAPKs, particularly ERK1/2. However, MK-STYX has diverse binding capabilities and functions, including acting as a regulator of G3BP in response to stress and as an effector in the apoptotic pathway (Hinton et al., 2010; Niemi et al., 2011). This thesis research has provided further evidence that MK-STYX is a dynamic regulator of cell signaling pathways.
Significance of MK-STYX’s effects on stress granule assembly

MK-STYX has been shown to bind G3BP (Hinton et al., 2010), an RNA-binding protein that is a regulator of Ras function and stress granule assembly (Tourriere et al., 2003; Tourriere et al., 2001). It has been shown that MK-STYX inhibits stress granule formation induced by G3BP, while the active mutant does not have this effect (Hinton et al., 2010). Dephosphorylation at Ser149 of G3BP is necessary for the formation of stress granules (Tourriere et al., 2003). The G3BP-S139A mutant is designed to mimic dephosphorylation at site 149. Interestingly, our results show that MK-STYX inhibits stress granule assembly induced by the non-phosphorylatable mutant G3BP-S149A. These results indicate that MK-STYX is capable of inhibiting stress granule assembly independent of G3BPphosphorylation state. Additionally, while MK-STYX does not have an effect on cells expressing the phosphomimetic mutant G3BP-S149E, the active mutant induces stress granule assembly in these cells. These results highlight MK-STYX as a potential regulator of G3BP and stress granule formation. Potentially, MK-STYX may regulate the disassembly of stress granules. Additionally, this study provides further evidence that pseudophosphatases such as MK-STYX are more than dominant negative proteins that block phosphatase function, but rather powerful signaling regulators.
Significance of MK-STYX effects on MAPK signaling in neurons

MAPK cascades regulate diverse cellular responses including growth, differentiation, apoptosis, and proliferation (Schaeffer and Weber, 1999). MAPKs are abundantly expressed throughout the nervous system, so it is no surprise that their misregulation should be particularly interesting with respect to neurological disorders. Both decreased and increased levels of MAPK activation have been linked to mental retardation, impaired learning, and memory loss (English and Sweatt, 1996; English and Sweatt, 1997; Peng et al., 2010; Sweatt, 2001). Neurological disorders have been linked to both hyperphosphorylation and the inhibition of MAPK phosphorylation.

The significance of MAPK signaling cascades in proper neurological function is becoming increasingly clear. However, not much is known about the regulation of the MAPK signaling pathways. There are several phosphatases that serve as regulators of MAPK signaling; a good example is the PTP SHP-2 (Easton et al., 2006; Shi et al., 1998; Wright et al., 1997). It would appear that MK-STYX may play a role in modulating response to cellular signaling molecules. Additionally, G3BP, a known binding partner of MK-STYX, has been implicated in neuronal outgrowth formation and interacts with axonal tau mRNA (Atlas et al., 2004).
Future Directions

The evidence we have gathered suggests that MK-STYX may be interacting with another MAPK or signaling protein in order to stimulate neuronal differentiation. Other MAPKs have been implicated in PC12 differentiation, including p38 (Iwasaki et al., 1999; Morooka and Nishida, 1998); it has even been suggested that activation of the p38 MAPK alone can induce neuronal differentiation of PC12 cells (Iwasaki et al., 1999). Further investigation of this connection would involve the use of PD98059, a small molecule that selectively blocks the activity of MEK, inhibiting phosphorylation and activation of ERK1/2 in vivo (Alessi et al., 1995; Pang et al., 1995). If MK-STYX stimulates outgrowth formation in the presence of this inhibitor, it would suggest involvement of other pathways, particularly p38. Particularly, though, this research indicates a dynamic role for MK-STYX and other pseudophosphatases in regulation of cellular signaling mechanisms. Far from being simple antagonists of their active homologues, pseudophosphatases are emerging as key players in a variety of physiological mechanisms.

Summary

The preceding thesis research has provided evidence for the importance of cellular signaling to normal cellular functions. v-ErbA, a mutated variant of the thyroid hormone receptor, interferes with the normal processes of the cell. This protein forms large, cytoplasmic foci that may act as a cellular
mechanism to deal with this misregulation of cellular response to the thyroid hormone receptor. MK-STYX, a pseudophosphatase, is also involved in the response to environmental cues. The first, stress granule formation, is a cellular response to high stress conditions. The second is the normal physiological process of differentiation in PC12 cells.

This work highlights aggregation as a cellular mechanism to deal with stress, whether it is viral infection or an overload of misfolded proteins (aggresomes), or stressors such as UV irradiation or heat shock (stress granules). Key in the formation of these aggregates are complex cell signaling mechanisms, the misregulation of which can be detrimental to the cell.
References


the Golgi and earlier secretory compartments and contains distinct
domains required for its localization and function. *J Cell Biol.* 143:319-
331.

Niemi, N.M., N.J. Lanning, J.A. Klomp, S.W. Tait, Y. Xu, K.J. Dykema, L.O.
Murphy, L.A. Gaither, H.E. Xu, K.A. Furge, D.R. Green, and J.P.
Mackeigan. 2011. MK-STYX, a catalytically inactive phosphatase
regulating mitochondrial dependent apoptosis. *Mol Cell Biol.*

2004. Formation of aggresome-like structures in herpes simplex virus
type 2-infected cells and a potential role in virus assembly. *Exp Cell
Res.* 299:486-497.

kinase kinase blocks the differentiation of PC-12 cells induced by nerve

Parker, F., F. Maurier, I. Delumeau, M. Duchesne, D. Faucher, L. Debussche,
A. Dugue, F. Schweighoffer, and B. Tocque. 1996. A Ras-GTPase-
extivating protein SH3-domain-binding prot

Patterson, K.I., T. Brummer, P.M. O'Brien, and R.J. Daly. 2009. Dual-
specificity phosphatases: critical regulators with diverse cellular

Peng, S., Y. Zhang, J. Zhang, H. Wang, and B. Ren. 2010. ERK in learning

is dependent on continual NGF receptor regeneration. *Dev Growth
Differ.* 46:393-403.

Raman, M., W. Chen, and M.H. Cobb. 2007. Differential regulation and


Saliba, R.S., P.M. Munro, P.J. Luthert, and M.E. Cheetham. 2002. The
cellular fate of mutant rhodopsin: quality control, degradation and


Schwertassek, U., D.A. Buckley, C.F. Xu, A.J. Lindsay, M.W. McCaffrey, T.A.
phosphatase c-JUN N-terminal kinase (JNK) stimulatory phosphatase
1 is necessary for its activation of JNK signaling and apoptosis. *Febs J.*
277:2463-2473.


