2009

Mechanisms of non-conventional cell death in brain tumor cells

Aparna Kaul
Medical University of Ohio

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
Mechanisms of Non-Conventional Cell Death in Brain Tumor Cells

Submitted by:
Aparna Kaul

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

<table>
<thead>
<tr>
<th>Examination Committee</th>
<th>Signature/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major Advisor:</strong> William Maltese, Ph.D.</td>
<td></td>
</tr>
<tr>
<td><strong>Academic Advisory Committee:</strong></td>
<td></td>
</tr>
<tr>
<td>Ronald Mellgren, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Ana Maria Oyarce, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Kam Yeung, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Jean Overmeyer, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Kathryn Eisenmann, Ph.D.</td>
<td></td>
</tr>
</tbody>
</table>

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

Date of Defense: April 21, 2009
Mechanisms of Non-Conventional Cell Death in Brain Tumor Cells

Aparna Kaul
University of Toledo
Health Science Campus

2009
DEDICATION

I dedicate this work to my parents, as a testament of my love and gratitude.
ACKNOWLEDGEMENTS

I am grateful to Dr. Maltese for giving me an opportunity to be a part of his lab, which was my second home for over four years. His patience, positive-outlook, and dedication for science are exemplary. My graduate training in his lab was very enriching in terms of scientific development and critical thinking. I do not have enough words to thank him for being such a wonderful mentor.

I want to express my gratitude to Dr. Jean Overmeyer for training me when I knew nothing about molecular biology, and making me feel comfortable even when I asked the most stupid questions. I thank her for lending a helping hand during those long and tedious experiments. It was fun working with her.

All members of the Maltese lab deserve special mention for making our lab so much fun. Kristen, Ashley, and Haymanti have been very helpful and considerate. I also want to thank the former lab members, Dr. Erin Johnson, Dr. Xuehuo Zeng, and Jane Ding for their help and support.

I would like to thank the members of my committee, Dr. Ronald Mellgren, Dr. Han-Fei Ding, Dr. Ana Maria Oyarce, Dr. Kam Yeung, and Dr. Kathryn Eisenmann for their time and valuable advice.

I want to extend my gratitude to all my friends who have helped me over the years, with special thanks to Dr. Ananya Banerjee for all the many ways she has helped me out. My family has been a constant source of encouragement and emotional support. Since the time I can remember, my big sister, Meenakshi, has been my inspiration, and I thank her for being there for me always. Finally, I want to acknowledge the person
without whom I could not have come this far, my husband, Gaurav. I thank him for his unconditional love and support.
# TABLE OF CONTENTS

Dedication ........................................................................................................................... ii

Acknowledgements ............................................................................................................. iii

Table of Contents ............................................................................................................... v

Introduction .........................................................................................................................1

Literature ............................................................................................................................. 7

Manuscript #1 .................................................................................................................... 50
Active Ras Triggers Death in Glioblastoma Cells through Hyperstimulation of Macropinocytosis.

Manuscript #2 .................................................................................................................. 104
Activated Ras Induces Cytoplasmic Vacuolation and Non-apoptotic Death in Glioblastoma Cells via Novel Effector Pathways

Manuscript #3 .................................................................................................................. 149
Killing of Cancer Cells by the Photoactivatable Protein Kinase C Inhibitor, Calphostin C, Involves Induction of Endoplasmic Reticulum Stress

Summary ............................................................................................................................ 191

Bibliography ....................................................................................................................... 195

Abstract .............................................................................................................................. 227
INTRODUCTION

Malignant gliomas are the most common form of primary brain tumors in adults, (Lefranc et al., 2005) making them the leading cause of central nervous system (CNS) tumor-related deaths (Ishii et al., 1999). Malignant gliomas comprise anaplastic astrocytomas, oligodendromas, ependymomas, and oligoastrocytomas. As per the World Health Organization (WHO) classification, grade IV astrocytomas are known as glioblastomas (Omuro et al., 2007). Glioblastomas are associated with a very poor prognosis and have been considered the most treatment refractory of all human tumors. Conventional chemotherapeutic agents do not offer much improvement in the overall clinical outcome for the patients diagnosed with glioblastomas. Temozolomide is the only agent that has been proven to be effective, when combined with surgery and radiotherapy, for adults with glioblastoma (Stupp et al., 2005b; Stupp et al., 2005a). Although promising, temozolomide extends the median survival only by a few months. Clearly, there is a pressing need for developing therapeutic agents that can improve the prognosis of patients diagnosed with glioblastomas.

One of the hallmarks of cancer cells is their acquired resistance toward apoptosis (Hanahan and Weinberg, 2000). This feature not only confers enhanced proliferative capability, but also helps them acquire chemoresistance (Plati et al., 2008). As with other cancers, malignant gliomas harbor genetic alterations which render them resistant to apoptosis. These include mutations in the \( p53 \) gene, over-expression of anti-apoptotic Bcl2 family members, and PTEN loss (Steinbach and Weller, 2004; Sathornsumetee and Rich, 2008).
Apoptosis is a type of programmed cell death, morphologically characterized by cytoplasmic shrinkage, membrane blebbing, and chromatin condensation. Cells are eventually fragmented into apoptotic bodies that are cleared, before their lysis, by phagocytes, which recognize the exposed phosphatidylserine (PS) on cells dying via apoptosis (Fadok et al., 1998; Fadok and Henson, 1998). Apoptosis is a fundamental biological process, crucial for the regulation of tissue homeostasis. Over-activation of the apoptotic machinery has been associated with degenerative disorders and under-activation with cancer and autoimmunity (Danial and Korsmeyer, 2004).

Apoptosis can be initiated by intracellular or extracellular cues, which ultimately converge on a family of cysteine proteases known as caspases. Caspases choreograph the “limited proteolytic events” (Fuentes-Prior and Salvesen, 2004) that constitute the morphological and biochemical changes characteristic of apoptosis. The two main pathways that lead to the activation of caspases are: the intrinsic pathway that results in the mitochondrial-dependent cytochrome-c release, and subsequent activation of caspase-9; and the extrinsic pathway that depends on ligand dependent activation of cell surface death receptors, followed by the activation of caspases-8 and 10. The activation of initiator caspases of either pathway results in proteolysis-dependent activation of the executioners, caspases-3, 6 and 7.

It is well recognized that there is an imbalance between pro-survival and pro-death signals in cancer cells, with the balance tilted toward the survival signals. An understanding of the molecular machinery of apoptosis has led to the identification of
targets that can be manipulated to tilt the balance in favor of death in cancer cells (Call et al., 2008).

Despite advances in understanding and manipulating apoptosis in cancer cells, resistance remains a problem, which has led researchers to re-evaluate the concept of death-induction in cancer cells. This has special relevance in the light of studies establishing that apoptosis is not the only type of death program hard-wired in a cell. It has now been established that death can occur via different mechanisms, resulting in distinct morphological characteristics (Kroemer et al., 2005). Some important non-apoptotic death pathways include autophagic death, necrosis, paraptosis, and necroptosis (Kroemer et al., 2008). It has been postulated that understanding the molecular machinery involved in the induction of different death pathways will be vital in developing effective treatment strategies in apoptosis-resistant tumors (Kolenko et al., 2000).

In part I of this thesis, I will describe the characterization of a novel form of non-apoptotic death, triggered by activated Ras in glioblastoma cells. This form of cell death is characterized by the accumulation of large phase-lucent vacuoles, derived from macropinosomes. Vacuolated cells expressing Ras ultimately detach from the substratum and lose viability. The broad spectrum caspase inhibitor, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk), is not able to inhibit cell death, indicating that cellular demise occurs in a caspase-independent manner. This is the first evidence of a form of cell death that can be initiated by dysregulation of macropinocytosis (cell drinking). Hence, this form of cell death has been named ‘methuosis’ (derived from the Greek word methuo, which means to drink to intoxication).
As shown in the incorporated manuscript (Overmeyer et al., 2008), ectopic expression of activated Ras GTPase (Ras G12V) elicited the same phenotype in a variety of glioblastoma cell lines, irrespective of their genetic makeup, indicating that glioblastomas are specifically sensitive to this mode of death. Also, Ras did not induce methuosis in other commonly used cell lines (HeLa, HEK293, and HEp2), indicating that there is a degree of cell-type specificity in the ability of Ras to induce methuosis. By understanding the signaling mechanisms that trigger this unique form of cell death, it may be possible to uncover specific molecular targets that can be manipulated to induce this form of death in glioblastomas, and perhaps other cancer cells. An understanding of the signaling mediators will also provide an insight into the basis for differential sensitivity of certain cell types toward the induction of methuosis, and thus be important in evaluating the therapeutic potential of this form of cell death.

As a first step toward defining the signaling pathway, part II of this thesis details a series of studies that were performed to determine which downstream signaling effector is activated by Ras to trigger methuosis. Farnesylation and membrane attachment of Ras was required for the induction of methuosis. However, activation of the most well studied Ras effectors (Raf, PI-3K and RalGDS) was not absolutely required for the induction of methuosis.

Macropinocytosis is an actin-dependent endocytic process utilized by the cells to internalize bulk extracellular fluid. Macropinocytic vesicles are derived from plasma membrane ruffles or lamellipodia and are different from other endocytic vesicles that are derived as a result of plasma membrane invagination. Molecular mechanisms regulating
the formation, maturation, and trafficking of this unique organelle remain poorly understood. A member of the Ras-superfamily, Rac1, has been shown to play an important role in the biogenesis of macropinosomes (Ridley et al., 1992). Also, Ras communicates with Rac1, via different signaling intermediates (Lambert et al., 2002). Therefore, we postulated that Rac1 might play a key role in methuosis. Indeed, expression of constitutively active Rac1 recapitulates the effect of Ras(G12V) in glioblastoma cells. Other Rho family members (RhoA and Cdc42) did not cause vacuolation when over-expressed in glioblastoma cells, indicating that induction of methuosis is specific to activated Ras and Rac. While these data suggest that Rac1 might be acting downstream of Ras to induce the accumulation of macropinosomes, further studies need to be performed before making this conclusion. Specifically, the downstream effector that mediates the activation of Rac1 by Ras in these cells needs to be identified.

The fact that glioblastoma cells are more susceptible to death induced by dysregulated macropinocytosis, as compared to other cell lines, indicates that these cells may have a defect in the intracellular trafficking of the macropinosomes. The studies done with dextran tracers showed that the Ras-induced vacuoles do not fuse with the lysosomes, pointing toward a defect in the trafficking of macropinosomes. Therefore, understanding the key molecular events involved in the biogenesis and accumulation of Ras-induced macropinosomes will be instrumental in uncovering a target that can be manipulated therapeutically in glioblastomas.
Genetic alterations, like loss of \( p53 \) and over-expression of Bcl2, are known mediators of resistance to DNA damaging drugs and radiation in glioma cells (Steinbach and Weller, 2004). Therefore, in addition to looking for non-apoptotic forms of cell death, it is also worthwhile to seek alternative approaches to initiate apoptosis, independent of DNA damage or death receptor pathways. To this end, the identification of the endoplasmic reticulum (ER) as a player in apoptotic cell death has been consequential. Endoplasmic reticulum stress-initiated apoptosis appears to be a ‘weak-point’ in cancer cells and can be exploited for therapy (Bakhshi et al., 2008; Chauhan et al., 2008b; Kardosh et al., 2008). The successful use of bortezomib (Velcade/PS-341) in multiple myeloma has provided evidence that ER stress triggered apoptosis is a viable therapeutic target. In part III of my thesis, I will discuss the mechanism of calphostin-C (cal-C) induced cytotoxicity in cancer cells. Calphostin-C is a protein kinase C inhibitor, whose activity is strictly dependent on light activation (Bruns et al., 1991). My data shows that cal-C induces massive ER stress due to an impairment of protein trafficking from ER to Golgi. There is a rapid accumulation of ER-derived cytoplasmic vacuoles and activation of ER stress mediators like JNK and PERK. This is followed by induction of apoptosis and death of cancer cells. These findings suggest that cal-C induces cytotoxicity, at least in part, by the induction of ER stress-initiated apoptosis. It is my hypothesis that using cal-C to initiate ER stress in cancer cells may prove effective in targeting cells that are otherwise resistant to conventional chemotherapeutic agents that work by causing DNA damage. This is important in the light of studies which indicate that ER stress inducers may be useful in potentiating the cytotoxicity of approved
chemotherapeutic agents, like temozolomide, 5-fluoro-uracil etc. (Amiri et al., 2004; Cusack, Jr. et al., 2006). Calphostin-C is an interesting candidate in this regard because its activity can be restricted specifically to tumors by photoactivation.
GLIOBLASTOMA MULTIFORME

Malignant gliomas are the most common tumors of the human brain, accounting for 40-50% of all primary CNS tumors (Lefranc et al., 2005; Lu and Shervington, 2008). The most aggressive subtype, glioblastoma multiforme (GBM), is associated with a very poor prognosis (Colman and Aldape, 2008). Median survival of patients diagnosed with GMB is only about 12-15 months, despite intensive treatment efforts (Sathornsumetee and Rich, 2008). The current treatment strategy for GBM includes a combination of surgery, radiation, and chemotherapy (da Rocha et al., 2002). However, even after surgical resection, the tumor most often recurs within 2-3 cm of the original tumor or the resection margin. This has been attributed to the high proliferative index and tremendous infiltrative capacity of the cells comprising this tumor (Nakada et al., 2007). Currently available chemotherapeutic drugs have a low specificity towards GBM, and temozolomide is the only available agent that has been reported to improve survival of patients diagnosed with GBM (Sathornsumetee and Rich, 2008).

The genetic alterations commonly observed in glioblastomas include epidermal growth factor receptor (EGFR) amplification, loss-of-function mutations in p53 and retinoblastoma (Rb) genes, deletion or mutation of cyclin-dependent kinase inhibitor p16\(^{\text{INK4A}}\), phosphatase and tensin (PTEN) deletion, and over-expression or hyperactivation of protein kinase C (PKC) (Maher et al., 2001; da Rocha et al., 2002; Rao and James, 2004; Sathornsumetee and Rich, 2008; Ohgaki, 2005). These genetic
alterations form the molecular basis for the aggressive phenotype, and resistance to available chemotherapeutic agents.

An understanding of the molecular abnormalities has led to the development of targeted therapies for glioblastomas. These include targeting the EGFR (e.g. the kinase inhibitors, erlotinib, gefitinib), inhibiting angiogenesis (e.g. bevacizumab), and inhibiting the phosphatidylinositol 3-kinase (PI-3K)-AKT pathway (e.g. AKT inhibitor, perifosine). These agents are currently being evaluated in clinical studies, alone or in combination with other chemotherapeutic agents, for their effect on patient survival and anti-tumor effects (Sathornsumetee and Rich, 2008). Glioblastomas are also characterized by an intrinsic resistance to apoptosis. Furthermore, migrating glioma cells up-regulate anti-apoptotic pathways, thus decreasing their susceptibility towards the cytotoxicity of chemotherapeutic agents (Joy et al., 2003). Since currently available therapeutic agents induce cytotoxicity via conventional apoptotic pathways, to which these cells are resistant, research efforts are being focused on identifying novel mechanisms by which cell death can be triggered in these highly intractable tumors.

**PROGRAMMED CELL DEATH**

Programmed cell death (PCD) is a vital part of embryogenesis, development, and the maintenance of tissue homeostasis. This process results in cell death via a regulated, energy dependent mechanism, and requires gene expression (Golstein and Kroemer, 2007; Sperandio et al., 2000). Historically, the terms PCD and apoptosis have been used synonymously. Morphological features of apoptosis (Greek: apo-from, ptosis-falling)
include cell membrane blebbing, cytoplasmic shrinkage, chromatin condensation (pyknosis), and nuclear fragmentation (karyorrhexis) (Fig. 1) (Galluzzi et al., 2008a; de Bruin and Medema, 2008). Finally, the cell breaks down into small membrane-bound fragments, known as apoptotic bodies, which are cleared by phagocytosis. Apoptotic cell death is thus considered a ‘clean’ way to die, as it does not elicit inflammatory responses (Reed, 2000).

![Figure 1. Electron micrograph depicting, a normal cell (A), and an apoptotic cell (B) (Edinger and Thompson, 2004)](image)

Even though apoptosis is the most common and relatively well characterized form of PCD, it is now a well accepted fact that apoptosis is not the only form. Cells can utilize numerous mechanisms to initiate programmed death, depending upon cellular context and signaling mediators. I will first describe, in brief, the mechanisms and mediators of apoptosis, and then provide a brief overview of non-apoptotic death pathways.
Apoptosis

Apoptosis is an evolutionarily conserved pathway, which involves the sequential activation of caspases, a family of cysteine proteases that cleaves sites following an aspartate residue in their substrates (Riedl and Shi, 2004; Degterev and Yuan, 2008). Functionally, caspases are divided into two categories. Apical or initiator caspases (caspases-2, 4, 8, 9 and 10) are responsible for initiating disassembly of the cell in response to apoptotic stimuli, while the executioner or effector caspases (caspases-3, 6 and 7) account for executing cellular disassembly (Thornberry and Lazebnik, 1998). This vital role in the apoptotic machinery requires a stringent regulation of caspase activity. Central to this regulation is the fact that all caspases are synthesized as inactive zymogens (pro-caspases) (Riedl and Shi, 2004). In response to appropriate stimuli, initiator caspases are activated via the proximity-induced dimerization mechanism (Boatright et al., 2003), and they in turn, cleave and activate effector caspases (Riedl and Salvesen, 2007; Okada and Mak, 2004).

In mammalian cells, induction of apoptosis is mediated either via the death receptor ('extrinsic') or the mitochondrial ('intrinsic') pathway (Fig. 2). The extrinsic pathway is activated when extracellular ligands (e.g. Fas ligand [FasL], tumor necrosis factor [TNF]) bind to cell surface death receptors, leading to the formation of the death-inducing signaling complex (DISC). This signaling complex initiates activation of caspase-8, which in turn activates the effector caspases to execute cell death (Boatright et al., 2003). Intracellular stress signals trigger the intrinsic pathway, wherein, mitochondria play a key role. In response to stress signals (e.g. DNA damage), mitochondria undergo
alterations in their transmembrane potential, which leads to mitochondrial outer membrane permeabilization (MOMP) or the production of reactive oxygen species (ROS) (Kim et al., 2006). The mitochondrial membrane permeabilization leads to the release of cytochrome-c, resulting in the activation of apoptotic protease activating factor-1 (Apaf-1), which in turn induces the assembly of a signaling complex, known as the apoptosome. Apoptosome assembly is the key event in the activation of caspase-9 and other downstream executioner caspases (Riedl and Salvesen, 2007).

This apparently simple description of the above pathways is, in reality, highly complex. There are a number of activators and inhibitors for each pathway with a substantial degree of cross talk at several levels (Reed, 2000). Some important regulators include, the B-cell lymphoma-2 (Bcl2) family of pro- and anti-apoptotic proteins, inhibitor of apoptosis proteins (IAPs) (Reed, 2000), and mitochondrial pro-apoptotic proteins, second mitochondria-derived activator of caspase (Smac, also known as direct IAP binding protein with low pI [DIABLO]) (Verhagen et al., 2000; Du et al., 2000) and high temperature requirement A2 (HtrA2, also known as OMI) (Hegde et al., 2002).
The Bcl2 family is an important modulator of the mitochondrial apoptotic pathway. Members of this family are characterized by the presence of at least one of the four conserved Bcl2 homology (BH) domains (Fig. 3) (Adams and Cory, 1998). The anti-apoptotic members (e.g. Bcl2, Bcl-XL) are involved in maintaining the integrity of mitochondrial outer membrane, endoplasmic reticulum, and the nuclear membrane. They prevent cytochrome-c release which is perhaps one of the factors involved in the...
induction of a conformational change in Apaf-1, required for caspase-9 activation. The pro-apoptotic members are further divided into two subfamilies, the Bax family and the BH3-only family. Bax and Bak are important for MOMP, however, the exact mechanism by which Bax and Bak cause MOMP remains controversial. It has been postulated that they get inserted as homo-oligomerized multimers to form channels within the mitochondrial outer membrane, resulting in the release of cytochrome-c (Adams and Cory, 1998; Danial and Korsmeyer, 2004). The resistance of Bax and Bak double knock-out cells to a variety of death stimuli, highlights their regulatory role (Cory and Adams, 2002). The function of BH3-only subfamily has been proposed to follow the “two-class” model (Letai et al., 2002). Amongst them, the Bad-like members interact with the anti-apoptotic members to antagonize their function, whereas, BID promotes death by activating Bax and Bak. In the absence of apoptotic stimuli, the BH3-only proteins are regulated by diverse mechanisms, such as transcriptional control, post-translational modifications, and sequestration by scaffolding proteins (Cory and Adams, 2002). Interestingly, caspase-8 is involved in the proteolytic cleavage-mediated activation of BID. Truncated-BID (tBID), in turn, triggers the mitochondrial pathway, acting as an important node of interaction between the intrinsic and the extrinsic pathways (Danial and Korsmeyer, 2004).
The IAP family of proteins (XIAP, cIAP1, and cIAP2) bind and inhibit the caspases, and are thus capable of inhibiting apoptosis initiated via the extrinsic and the intrinsic pathways (Reed, 2000). The repression of caspases, by IAPs, is de-repressed by the mitochondrial residents Smac/DIABLO (Verhagen et al., 2000; Du et al., 2000) and OMI/ HtrA2 (Hegde et al., 2002), which are released from the mitochondria during apoptosis.

ER Stress and Apoptosis

More recently, the endoplasmic reticulum (ER) has been implicated as the third intra-cellular compartment which can regulate apoptosis (Rao et al., 2004). Endoplasmic reticulum, being the principle site for protein synthesis, folding, and calcium homeostasis, is highly sensitive to alterations in its normal environment. Changes that compromise normal functioning of the ER are referred to as ER stress. In order to restore
normal conditions, the ER initiates a series of signaling events, known as the unfolded protein response (UPR) (Lai et al., 2007). In the event that cellular homeostasis is restored, the cell survives, else, severe ER stress can lead to activation of the apoptotic machinery (Rao et al., 2004). Cell death resulting from this form of stress has been implicated in various pathophysiological conditions like neurodegenerative disorders, diabetes, and ER storage disease (Zhao and Ackerman, 2006). Hypoxia, an inducer of ER stress, has frequently been observed in the tumor microenvironment. Induction of the UPR signaling pathway, in this case, confers growth advantage by up-regulation of the pro-survival arm of the UPR (Feldman et al., 2005).

While some pro-apoptotic ER stress mediators are known, the molecular mechanisms connecting ER stress response to apoptosis remain poorly understood. The known pro-apoptotic inducers include, C/EBP homologous protein (CHOP) (Oyadomari and Mori, 2004) and c-Jun N-terminal kinase (JNK) (Szegezdi et al., 2006). The anti-apoptotic molecules include, Bcl2 (Oakes et al., 2006) and the ER chaperone, glucose related protein 78 (GPR78), also known as immunoglobulin-binding protein (BiP) (Li and Lee, 2006).

As mentioned earlier, caspase activation is central to the apoptotic machinery. Several caspases are activated during ER stress initiated apoptosis. Literature carries reports of ER stress induced activation of caspases-12, 4, 9, 8, 7, 6 and 3. However, identity of the apical caspase, specific for ER stress remains controversial. Caspase-12, a rodent specific protein, which localizes to the ER membrane, has been proposed to be the caspase specific for ER stress induced apoptosis. It was shown to be activated specifically
by known ER stress inducers like brefeldin-A (BFA), tunicamycin, thapsigargin, and was not activated by other apoptotic inducers (Nakagawa et al., 2000). The human homologue of caspase-12, though identified, has several mutations rendering it non-functional (Szegezdi et al., 2006). Caspase-4, which shares 48% homology with caspase-12, has been proposed to be its functional substitute in humans (Hitomi et al., 2004). Caspase-4 also localizes to the ER and is specifically activated by ER stress initiated apoptosis (Hitomi et al., 2004). On the other hand, it has also been shown that caspase-4 and 12 activities are not always required for the induction of ER stress mediated apoptosis (Obeng and Boise, 2005).

Given the significance of Bcl2 family members in apoptosis, their role in regulating ER stress induced apoptosis is not surprising. Several studies have shown that ER localized Bcl2 proteins control the calcium (Ca) levels, thereby regulating apoptosis. Over-expression of Bcl2 and Bcl-XL protects against thapsigargin (an inhibitor of ER calcium ATPase) induced apoptosis (Srivastava et al., 1999). Phosphorylation of Bcl2 increases the discharge of calcium from the ER, thereby, modulating mitochondrial uptake and apoptosis (Bassik et al., 2004). These proteins can thus be involved in the cross-talk between ER and mitochondria-initiated apoptotic cascades. Mouse embryonic fibroblasts (MEFs) lacking both Bax and Bak are resistant to tunicamycin and thapsigargin induced cell death. The ER stress modulators, in turn, affect the functioning of Bcl2 family members. The pro-apoptotic protein CHOP can decrease Bcl2 protein expression and JNK can inhibit Bcl2 activity in a phosphorylation dependent manner (Lai et al., 2007).
Caspase-Independent Apoptosis

The fact that caspases are central to apoptosis is well established, however, evidence in the literature suggests that, under certain conditions, non-caspase proteases may be involved in death execution. Caspase-independent apoptosis, also known as apoptosis-like PCD, mimics the morphological features of apoptosis, e.g., plasma membrane blebbing, chromatin condensation, and externalization of PS before cell lysis (Leist and Jaattela, 2001). The non-caspase proteases involved in this process include cathepsins, calpains, and granzymes (Johnson, 2000). The lysosomal cathepsins implicated in this form of cell death include, cathepsins B, D, and L. (Boya and Kroemer, 2008). Death receptor induced apoptosis can proceed in the absence of caspases, via cathepsin B, in WEHI-S fibrosarcoma cells (Foghsgaard et al., 2001). Cathepsins D and B have also been implicated in camptothecin-induced death of liver cancer cells (Leist and Jaattela, 2001). Calpains, the calcium dependent neutral cysteine proteases, mediate cell death in retinal ganglia (McKernan et al., 2007). Calpains have also been placed upstream and downstream of caspase-3 in radiation-induced apoptosis (Waterhouse et al., 1998), indicating that there is a substantial degree of cross-talk between different protease sub-families (Johnson, 2000). Also, lysosomal membrane permeabilization (LMP) and release of cathepsins can lead to MOMP and activation of the intrinsic apoptotic pathway (Kroemer and Jaattela, 2005).

Apoptosis-inducing factor (AIF), a mitochondrial resident protein, is another important mediator of caspase-independent apoptosis. Following its release from the mitochondria, AIF translocates to the nucleus and mediates chromatin condensation and
DNA fragmentation. Classical features of apoptotic death are observed, without the activation of caspases (Hong et al., 2004). Apoptosis-inducing factor is important in the mediation of caspase-independent cell death caused by over-activation of poly-adenosine diphosphate (ADP) ribose polymerase-1 (PARP-1) (Yu et al., 2002; Cregan et al., 2004). The exact molecular mechanisms elucidating preferential activation of caspase-independent vs. caspase-dependent death pathways are under investigation. However, it is clear that cells are capable of activating various kinds of death mediators, depending on the intensity and the type of insult.

Non-Apoptotic Programmed Cell Death

For decades, a dichotomous classification of cell death existed, wherein, apoptosis was considered a programmed or controlled form of cell death and necrosis was considered an accidental/passive form of cellular demise. However, it is now well recognized that this classification is an oversimplified view of highly complex mechanisms that are essential for normal growth, development, and cellular homeostasis (Broker et al., 2005). Many studies have documented that a cell can complete programmed cell death in the absence of caspases and that apoptosis is not the only form of programmed cell death (Xiang et al., 1996; Johnson et al., 1998; Degterev and Yuan, 2008). Also, evasion of apoptotic cell death is a hallmark of cancer cells, therefore, there is a lot of interest in studying alternative mechanisms of PCD.

Autophagic or type II PCD (Fig. 4C) is characterized by an accumulation of double or multi-membrane structures known as autophagosomes. Cell death usually
occurs in a caspase-independent manner (Gozuacik and Kimchi, 2004). Macroautophagy (henceforth called autophagy), is a catabolic process utilized by the cell to degrade long-lived proteins and damaged organelles (Shintani and Klionsky, 2004). It is characterized by the sequestration of cytoplasmic constituents into double or multi-membraned autophagic vesicles (or autophagosomes), followed by their delivery to the lysosomes, and subsequent degradation by the lysosomal hydrolases (Broker et al., 2005; Shintani and Klionsky, 2004). Under basal conditions, eukaryotic cells utilize autophagy for maintaining cellular homeostasis (Kondo et al., 2005; Stromhaug and Klionsky, 2001). However, autophagy can be stimulated under conditions of cellular stress. Accumulation of autophagic vesicles has been observed in cells dying in response to cancer treatment or in certain pathological conditions, like neurodegeneration. However, it is still a matter of debate whether increased autphagic activity, in such cases, is the cause of death or a futile attempt to survive (Debnath et al., 2005). There is evidence in the literature, for and against autophagy playing a causative role in PCD. Autophagic cell death is observed during mammalian development; e.g., in the morphological remodeling of the male Mullerian duct and the female Wolffian duct, during genital development (Dyche, 1979), and the involution of mammary and prostate glands (Helminen and Ericsson, 1971; Helminen and Ericsson, 1968). Treatment of MCF-7, human mammary carcinoma cells, with tamoxifen has been reported to cause an accumulation of autophagic vacuoles and subsequent cell death (Bursch et al., 1996). Caspase-8 inhibition causes autophagic cell death in L929 fibrosarcoma cells. On the other hand, starvation conditions induce apoptosis in cervical cancer cells, when the autophagic machinery is inhibited (Galluzzi
et al., 2008b). Bafilomycin-A1, an inhibitor of autophagy, sensitizes glioma cells to radiation-induced apoptosis (Ito et al., 2005). Even though there are conflicting reports on the role of autophagy in PCD, it has emerged as an important target that can be manipulated in cancer cells, depending on the signaling mediators and the specific response elicited by the cells.

Necrosis, which in the past was referred to as a passive or uncontrolled form of cell death, has now been recognized to be a regulated event, categorized under PCD. The hallmark features of necrotic cell death include, bioenergetic failure and rapid loss of cellular membrane potentials (Zong and Thompson, 2006). Morphological changes commonly observed are, gain in cell volume (oncosis), swelling of cytoplasmic organelles, plasma membrane rupture, and subsequent loss of intracellular contents (Fig. 4D) (Kroemer et al., 2008). Studies indicate that necrotic death may act as a ‘fail-safe’ mechanism for a cell when caspase dependent pathways (and sometimes autophagic pathways) are inhibited (Festjens et al., 2006). It is also worth noting that apoptosis and necrosis can often be initiated by the same type of cellular insult, depending upon the intensity of the insult.

Necrotic cell death involves interplay between diverse signaling pathways. Several mediators of necrosis have been identified; however the exact correlation between the molecular events has not been well established. Receptor interacting protein-1(RIP-1), a death domain containing adapter protein, has been documented to be important in receptor-induced necrosis, providing evidence that necrosis can be initiated by a set of specific signaling events (Chan et al., 2003; Zheng et al., 2006). Main players
during the propagation and the execution phase include calcium and reactive oxygen species (ROS). Calcium is important for the activation of calpains, phospholipase A$_2$ (PLA$_2$), and lipoxygenases, which disrupt the organelles and cellular integrity (Festjens et al., 2006). The activation of PARP can also cause necrosis by depletion of the cytosolic nicotinamide adenine dinucleotide (NAD) pools (Zong and Thompson, 2006).

Recent studies have described some novel non-apoptotic death pathways with necrosis-like features. Paraptosis is induced by the human insulin-like growth factor-I receptor (IGFIR) and is characterized by cytoplasmic vacuolation, lack of apoptotic morphology, and the lack of inhibition by caspase inhibitors (Fig. 4F) (Sperandio et al., 2000; Sperandio et al., 2004). Necroptosis is induced by the stimulation of Fas/TNFR receptor family, in conditions when apoptosis is blocked. Necroptosis is accompanied with necrotic morphology (Fig. 4E) and induction of autophagy, which in this case is a cellular response and not a part of the death execution machinery (Degterev et al., 2005).

More recently our lab has described methuosis, a novel form of non-apoptotic cell death, characterized by the accumulation of large phase lucent vacuoles derived from macropinosomes (Fig. 4G). While caspase activation is observed, death is not inhibited by zVAD-fmk, a pan caspase inhibitor. Methuosis is specifically triggered by the ectopic expression of activated Ras or Rac1 GTPases, in glioblastoma cells. In the first two chapters of this thesis, I will describe the studies performed to characterize this unique death pathway and our current knowledge about the signaling pathway(s) involved in the induction of methuosis.
Figure 4. Morphological features of various modes of cell death. (A) A normal cell (B) An apoptotic cell (C) A cell undergoing autophagy (D) Cell dying via necrosis (E) Necroptosis (F) Paraptosis (G) Methuosis (Golstein and Kroemer, 2007)

Understanding Cell Death Pathways: Therapeutic Implications

Initiation of the death program is a basic response hardwired in a cell for normal tissue development and homeostasis. Aberrations in this fundamental process lead to various pathological conditions. While inappropriate activation leads to pathologies like neurodegeneration, diabetes, and tissue injury (myocardial infarction, stroke); defects in cell death activation lead to immortalization and cancer (Degterev and Yuan, 2008; Blank and Shiloh, 2007). The development of cytoprotective therapies for inappropriate activation of cell death pathways is a separate area of investigation. In the following
section, I will cite a few examples of how the current understanding of apoptotic and non-apoptotic death pathways has been translated toward designing targeted therapies for cancers.

Chemotherapeutic agents, like DNA damaging drugs, target the more ‘conventional’ pathways of apoptosis, which might be mutated in a tumor (Linder and Shoshan, 2005). However, not all apoptotic targets are mutated in cancer cells, therefore, armed with an understanding of the molecular machinery, researchers can now find targets to which cancer cells are still susceptible. For example, the extrinsic apoptotic pathway can be targeted specifically in tumor cells by using tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), belonging to the death ligand family. Unlike the TNF family members (e.g. FasL), TRAIL specifically targets a wide range of tumor cells (Kabore et al., 2004). Monoclonal antibodies that specifically activate TRAIL death receptors are currently in clinical trials (Plummer et al., 2007; Tolcher et al., 2007). This strategy also appears promising in preclinical models for gliomas. Intracerebral injections of TRAIL inhibited intracranial xenografts in nude mice and increased their survival rate (Roth et al., 1999). Studies have shown that combining TRAIL with other chemotherapeutic agents can enhance apoptotic death in glioblastoma cell lines (Rohn et al., 2001; Siegelin et al., 2008; Gaiser et al., 2008).

Inhibitor of apoptosis proteins are potential therapy targets because of their up-regulated expression in cancers. Targeting IAPs could sensitize cancer cells to undergo apoptosis by releasing their inhibition on caspases. Survivin is the smallest IAP and the most differentially expressed between tumors and normal tissues. This IAP has been
targeted using an antisense oligonucleotide (LY21813308) strategy and has shown promise in preclinical studies (Call et al., 2008).

The fact that balance between pro- and anti-apoptotic Bcl2 proteins is often altered in human tumors, has made this group of proteins an attractive target for therapy. Indeed, small molecule Bcl2 antagonists have been developed and some of them are now being evaluated in clinical trials. Obatoclax (GX-15-070) binds to the BH3 groove of the prosurvival Bcl2 members. It inhibits Mcl-1-Bak interaction, up-regulates Bim and induces apoptosis in multiple myeloma (Trudel et al., 2007). It also displayed synergy with bortezomib in mantle-cell lymphoma (Perez-Galan et al., 2007). ABT-737 is another small molecule inhibitor, which disrupts Bcl2-Bim and Bcl2-Bax complexes and has shown promise in the treatment of lymphoid malignancies and small cell lung cancer (Warr and Shore, 2008; Papadopoulos, 2006). Local administration of ABT-737 improved survival in a glioma xenograft tumor model and potentiated the cytotoxicity of other chemotherapeutic agents like vincristine, etoposide, and TRAIL (Tagscherer et al., 2008).

The endoplasmic reticulum is another target which has shown potential for therapeutic application. The high protein turnover in cancer cells can be targeted by disrupting normal functioning of the ER (Linder and Shoshan, 2005). The successful use of bortezomib (Velcade/PS-341) in multiple myeloma has provided evidence that ER stress-induced apoptosis is a viable therapeutic target. Bortezomib acts by inhibiting the proteasome, which leads to an accumulation of misfolded/unfolded proteins in the ER (Landis-Piwowar et al., 2006), resulting in ER stress, and sustained ER stress can result
in the induction of apoptosis. Furthermore, bortezomib, when combined with other ER stress inducing agents (e.g. celecoxib, geldanamycin, edelfosine), yields synergistic/additive therapeutic effects, thus increasing the overall efficacy and reducing toxicity (Chauhan et al., 2008b; Chauhan et al., 2008a; Kardosh et al., 2008; Nieto-Miguel et al., 2007). The success of bortezomib has paved the way for the development of second generation proteosome inhibitors and identification of other agents like curcumin (Bakhshi et al., 2008; Pae et al., 2007), edelfosine (Nieto-Miguel et al., 2007), and selenium (Wu et al., 2005) that can induce ER stress-initiated apoptosis in cancer cells.

Recent studies have revealed that the ER component of apoptosis can be successfully targeted in malignant gliomas. Knockdown of BiP enhanced the cytotoxicity of temozolomide and 5-flurouracil (5-FU) (Pyrko et al., 2007). Bortezomib has also shown promise in glioblastomas (Yin et al., 2005; Kardosh et al., 2008). In combination with celecoxib, bortezomib was effective in killing glioblastoma cells, both in vitro and in vivo (Kardosh et al., 2008). It is currently being evaluated in clinical trials to study its effectiveness in patients with recurrent or progressive gliomas (Reardon and Wen, 2006). In chapter three of this thesis, I will describe my findings on ER stress-induced apoptosis stimulated by calphostin-C in different cancer types. Among other cancer cells, glioblastoma cells were found to be very sensitive to calphostin-C induced ER stress and subsequent death. Thus, agents targeting ER stress, alone or in combination with other chemotherapeutic agents, might prove effective in eliminating resistant glioblastoma cell populations.
Autophagic cell death has received considerable attention in tumors that are resistant to apoptosis. While there is evidence both for and against autophagy being a pro-death pathway, certain agents have shown promise in the induction of autophagic cell death. Glioblastomas are susceptible to agents like rapamycin (Takeuchi et al., 2005), RAD001 (Alonso et al., 2008), ceramide (Daido et al., 2004), and arsenic trioxide (Kanzawa et al., 2003), that induce autophagic cell death. Some of these agents (rapamycin and arsenic trioxide) are currently being evaluated in clinical trials to establish their efficacy in patients with glioblastoma (Lefranc et al., 2007).

Photodynamic therapy (PTD) can activate necrosis-mediated cell death (Ricci and Zong, 2006). Photolon, a photosensitizer, induces ROS-mediated necrotic cell death, with no caspase activation, in cancer cells (Copley et al., 2008).

While molecular targets for non-apoptotic death pathways have been identified, they are still quite far from being evaluated in patients. A lot still needs to be done to discover viable drug candidates that can induce non-apoptotic death in tumors.

THE SMALL G-PROTEIN SUPERFAMILY

The small guanosine-5’-triphosphate (GTP) binding proteins (G-proteins) are low molecular weight proteins (20-40 kDa), that are critical signaling mediators of pathways that control cell division, apoptosis, cell-cell adhesion, cell-matrix adhesion, vesicle trafficking, and cell migration. The family members are further sub-divided, based on their structure, into subfamilies of Ras, Rho, Arf, Rab, and Ran GTPases (Takai et al., 2001; ten Klooster and Hordijk, 2007). In general, these proteins act as molecular
switches, shuttling between their inactive guanosine-diphosphate (GDP) bound form and active GTP bound form (Fig. 5). The active GTP bound protein is capable of interacting with its downstream target(s) and activating appropriate signaling pathway(s) (Bernards and Settleman, 2004). The intrinsic GTP hydrolysis (or GTPase) activity helps terminate the signal. There are several regulators of the GTPase cycle and they will be discussed along with the specific subfamily. In the following section, I will describe the Ras subfamily in detail and will provide a brief overview of the Rho GTPases.

**Figure 5. The GTPase switch** (Modified from Bernards and Settleman, 2004)

**THE SMALL GTPASE: RAS**

Ras proteins are critical components of signaling pathways, linking cell surface receptors to the transcriptional machinery involved in cell growth and differentiation. The acronym Ras is derived from *rat* sarcoma because Ras genes were identified based on their ability to contribute to the transforming abilities of the Harvey and Kirsten strains of
rat sarcoma virus (Barbacid, 1987). In mammalian cells, there are three Ras genes, $H$-$Ras$ (from Harvey), $K$-$Ras$ (from Kirsten) and $N$-$Ras$ (from Neuroblastoma). Ras genes encode four 21kDa proteins, H-Ras, N-Ras, K-Ras4A and K-Ras4B. The two K-Ras proteins are derived as a result of alternate exon utilization (Perez-Sala and Rebollo, 1999). The Ras proteins are homologous in their first 164 amino acids; they differ completely in the 25 carboxy-terminal (C-terminal) residues, except for the cysteine 186 residue. The C-terminal is therefore known as the hypervariable region (HVR) (Fig. 6). The HVR has two signal sequences that enable the Ras proteins to be targeted to the plasma membrane. The effector domain (amino acids 32-40) is the region where Ras proteins bind their downstream signaling partners. The effector domain is identical in all four proteins (Crespo and Leon, 2000).

![Figure 6. Domain structure and the hypervariable region of Ras proteins (Prior and Hancock, 2001)](image-url)
The Ras proteins undergo post-translational modification in their carboxy-terminal, enabling their membrane recruitment. Membrane localization is mandatory for their function. All Ras proteins have a C-terminal CAAX box (amino acids 186-189 in H-Ras), where ‘C’ is always cysteine, ‘AA’ aliphatic amino acids, and ‘X’ is any amino acid (Takai et al., 2001; Macaluso et al., 2002). The cysteine 186 residue is first farnesylated in the cytosol; this event targets the protein to the ER, where the downstream ‘AAX’ residues are cleaved, and then the now terminal cysteine residue is methylated. Subsequently, H-Ras, N-Ras and K-Ras4A are palmitoylated at the upstream C-terminal cysteine residues and are targeted to the plasma membrane by vesicular trafficking, via the classical exocytic pathway. Palmitoylation does not occur in K-Ras4B, which bypasses the exocytic pathway and anchors the plasma membrane by virtue of its positively charged lysine residues. However, the exact mechanism by which this occurs is still unclear (Prior and Hancock, 2001; Hancock, 2003; Karnoub and Weinberg, 2008).

Ras Signaling Pathways

Ras (henceforth used as a general term for Ras proteins) activation is induced by a variety of extracellular growth factor signals (e.g. epidermal growth factor [EGF], platelet derived growth factor [PDGF]), triggering the membrane recruitment of guanine nucleotide exchange factors (GEFs), which facilitate the exchange of GDP for GTP (Takai et al., 2001; Hancock, 2003). In the classical model for Ras activation, binding of EGF to its receptor leads to phosphorylation of the tyrosine residue and association of the adaptor protein, growth factor receptor-bound protein-2 (GRB2), which in turn recruits
Ras-GEF (e.g. son of sevenless [SOS], Ras guanine nucleotide releasing factor [GRF]) to the membrane. The exchange factor promotes GDP dissociation and GTP loading, leading to a conformational change in the protein, allowing Ras to bind to its downstream effectors. The signal is terminated by the hydrolysis of GTP to GDP. The intrinsic GTPase activity of Ras is relatively weak, therefore, another set of regulators known as GTPase-activating proteins (GAPs) mediate the signal termination by stimulating the GTPase activity of Ras. Point mutations in codons 12, 13, 59, 61 and 63 make the protein resistant to the GAP activity, thus rendering the Ras protein constitutively active (Crespo and Leon, 2000; Marshall, 1995).

Ras proteins regulate diverse biological processes by interacting with numerous downstream effectors (Fig. 7). The most well studied Ras effectors are: Raf kinase, PI 3kinase and Ras-related-GDP dissociation stimulator (RalGDS).

Figure 7. Ras signaling pathways
Raf-1 serine/threonine kinase was the first bonafide Ras effector to be identified. Ras mediated activation of Raf-1 is a multistep process in which Ras enables membrane association of Raf-1. It is now believed that once Raf-1 attaches to the membrane, Ras binds to a second site in the cysteine rich domain of Raf-1, and promotes subsequent events, leading to the complete activation of Raf-1 kinase (Campbell et al., 1998). Activation of Raf-1 initiates signaling via the mitogen activated protein (MAP) kinase pathway. Raf-1 phosphorylates MAP kinase extracellular signal-regulated kinase (MEK), which in turn phosphorylates extracellular signal-regulated kinase (ERK 1/2). Subsequently, ERK gets translocated to the nucleus where it activates several transcription factors involved in cell proliferation and differentiation (Crespo and Leon, 2000).

Another well characterized Ras effector, class I PI-3K, phosphorylates phosphatidylinositols (PtdIns) at the 3’ position of the inositol ring to generate phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3). The phosphorylated product acts as a second messenger to activate proteins with the pleckstrin homology (PH) domain (Lindmo and Stenmark, 2006). Ras directly interacts with the p110 catalytic subunit of PI-3K, leading to its activation (Crespo and Leon, 2000). The catalytic product then activates the PH domain containing proteins e.g., phosphoinositide dependent kinase-1 (PDK1), protein kinase B (PKB, also known as AKT), to generate cell survival signals. Ras- activated PI-3K can also activate Rac GEFs (e.g., Sos and Vav), thereby activating another small GTPase Rac, which is involved in actin reorganization, membrane ruffling, and increased cellular motility (Campbell and Der, 2004).
Ras binds to RalGDS in a GTP and effector domain dependent manner to activate RalA and RalB. Ral proteins transmit the signal downstream by binding to their effectors, Ral binding protein-1 (RalBP1, also called RLIP76), phospholipase D (PLD), and Cdc42, which are involved in membrane trafficking, tumor survival, and metastasis (Bodemann and White, 2008; van Dam and Robinson, 2006). The RalGDS pathway can also inhibit the forkhead transcription family members that are involved in promoting cell cycle arrest and apoptosis (Downward, 2003).

In addition to activating unique signaling pathways, Ras activates a number of other small GTPases, e.g. Ral, Rab5, Rac and Rap (Mitin et al., 2005). A recurrent theme in Ras-induced activation of proteins of its own kind is that a number of Ras effectors serve as GEFs for other GTPases (Fig. 8). Thus, the small GTPases have developed efficient mechanisms by which they cooperate amongst themselves to regulate various cellular processes.

Figure 8. Ras effectors as guanine nucleotide exchange factors
(Modified from Mitin et al., 2005)
Other Ras binding partners include, Ras interaction/interference protein-1 (Rin-1), novel Ras effector-1(Nore-1), Ras-interacting protein (RAIN), phospholipase C-ε (PLCε), and T-cell lymphoma invasion and metastasis-1 (TIAM1). Among these proteins, Rin1 and Nore-1 (a member of Ras-association domain family (RASSF) of proteins) possess tumor-suppressive properties (Karnoub and Weinberg, 2008; Vos et al., 2003). In breast cancer cell lines, Rin1 gene silencing has been reported and reactivation of Rin1 was able to inhibit anchorage independent growth of tumors. It has also been documented that Nore1 regulates apoptosis via selective interaction with the pro-apoptotic protein, mammalian sterile-20-like protein kinase-1 (MST-1) (Khokhlatchev et al., 2002).

Ras and Human Cancers

Approximately 30% of human tumors harbor point mutations (at residues 12, 13 or 61) in the Ras protein. As mentioned earlier, these mutations render the Ras protein constitutively activated and thus oncogenic. Persistent activation of Ras signaling also occurs as a result of over-expression of growth factor receptors like EGFR, human epidermal growth factor receptor-2 (HER2/neu), or breakpoint cluster region-abelson (Bcr-Abl) (Campbell and Der, 2004). Under normal physiological conditions, Ras regulates diverse biological processes. Its ability to interact with a wide variety of signaling effectors makes Ras an ‘efficient’ oncogene for cancer cells. Ras has been implicated in almost all aspects of malignant transformation, namely, increased proliferation, protection against apoptosis, invasion, and metastasis. It is now well
recognized that Ras utilizes a combination of downstream effectors to mediate malignant transformation (Shields et al., 2000). While the Raf pathway is important for stimulating proliferation, the PI-3K/AKT pathway facilitates cell survival and anti-apoptotic function of Ras (Feig and Buchsbaum, 2002). Activation of the PI-3K/AKT pathway downstream of Ras is also important in preventing anoikis or apoptosis induction due to matrix deprivation. Ras contributes to invasion and metastasis by stimulating PI-3K and/or TIAM-1 dependent activation of Rac, which facilitates cellular motility.

Association of Ras with Cell Death

Although, the anti-apoptotic role of Ras is well established, emerging evidence now indicates that Ras can also promote cell death (Rebollo and Martinez, 1999). The dual effect of Ras on survival and death depends on the cell type and regulatory inputs to the cell (Downward, 1998). In order to explain this paradoxical behavior of Ras, it was postulated that in untransformed cells, excessive stimulation of Ras-induced hyperproliferative signals may lead to a pro-apoptotic response to prevent oncogenesis. On the other hand, in cells that are already transformed, stimulation of Ras pathways would mediate pro-survival responses (Cox and Der, 2003). Also, the modulation of survival and death signals by Ras was believed to be mediated mainly through the regulation of apoptosis. As mentioned earlier, Ras utilizes PI3-K/AKT, Rac etc. to elicit anti-apoptotic responses, and Nore-1 for pro-apoptotic stimulation. The Raf stimulated mitogen activated protein (MAP) kinase pathway can control both pro- and anti-apoptotic pathways (Cox and Der, 2003). However, signaling pathways stimulated by Ras are
much more complex, and recent reports indicate that pro-death effects of Ras are not confined to untransformed cells. Also, the observation by Chi et al. (1999) that activated H-Ras can induce non-apoptotic death in glioblastoma and gastric cancer cells has added to the complexity. We have recently clarified that the latter form of non-apoptotic cell death stimulated by Ras results from dysregulated macropinocytosis (Chapter 1) and we have named this novel form of cell death, methuosis. Thus, it is evident that pro-death signaling of Ras can initiate both apoptotic and non-apoptotic death, depending on the genetic make-up and regulatory inputs to the cell.

**RHO FAMILY GTPASES**

The Rho subfamily proteins are involved in the regulation of cytoskeletal reorganization, membrane trafficking, cell cycle progression, and gene expression. More than twenty Rho GTPases have been identified, which have been subdivided as follows: the Rho proteins (RhoA, RhoB and RhoC), the Rac proteins (Rac1, Rac2, Rac3, RhoG), the Cdc42-like proteins (Cdc42, TC10, TLC, Chp), the Rnd proteins (Rnd1, Rnd2, Rnd3/RhoE), the RhoBTB and Miro proteins (Ellenbroek and Collard, 2007). Among them, RhoA, Rac1, and Cdc42 have been well characterized. Like the Ras proteins, Rho family members shuttle between activation and inactivation cycles, depending on their association with GTP or GDP, and interact with downstream targets in the GTP bound form, to induce biological responses. In addition to GEFs and GAPs regulating their activation and deactivation states, these proteins are also regulated by GDP dissociation
inhibitors (GDIs), which sequester the proteins into inactive cytoplasmic complexes (Takai et al., 2001; Ridley, 2001b).

The stepwise post-translational modification of Rho family proteins proceeds in a manner similar to the Ras proteins. The conserved cysteine residue of the CAAX box is in most cases geranylgeranylated and, less frequently, farnesylated. The upstream cysteine residues are palmitoylated in case of RhoB, whereas, Rac1, Rac2, and RhoG are targeted to their sub-cellular destination(s) by virtue of the upstream polybasic amino acid sequence (Bustelo et al., 2007).

A number of downstream effectors of the Rho family members have been identified. Some effectors, like, phospholipase-D1 (PLD1), protein kinase C alpha (PKC-A), and filamin A (FlnA) can bind to multiple family members (RhoA, Rac and Cdc42). Effectors specific for Rac1 include, CybA (NADPH oxidase complex subunit) implicated in superoxide production, synaptotagmin 2 (SynJ2), involved in the regulation of clathrin mediated endocytosis, arfaptin 2, a scaffold protein that mediates cytoskeletal regulation, and plexinB1, the semaphorin receptor, involved in axon guidance and cell migration. Proteins like p21 activated kinase (PAK), neutrophil cytosolic factor-1, 2 (Ncf1, 2), Stat3 can bind to both Rac and Cdc42. RhoA regulates the formation of stress fibers and focal adhesions by interacting with Rho-associated coiled-coil containing protein kinase (ROCK), and mammalian homolog of Drosophila diaphanous protein (mDia). Other downstream binding partners of RhoA include rhophilin and citron kinase. (Bishop and Hall, 2000; Karnoub et al., 2004; Bustelo et al., 2007).
Similar to Ras proteins, the effector region (amino acids 25-49 in Rac1) is critical for interaction with downstream effectors. However, in the case of the Rho family, another region, known as the ‘insert region’ (residues 123-135 in Rac1) has been shown to be involved in effector interactions and signaling. This alpha-helical insert region is present in all Rho proteins, but not other Ras superfamily members (Karnoub et al., 2004). Mutations in the insert region affected Rac-induced activation of NADPH oxidase but had no effect on PAK activation (Freeman et al., 1996). However, Karnoub et al. (2001) showed that the insert region was required for Rac1-induced membrane ruffling, but was dispensable for the production of reactive oxygen species. In another study it was shown that the insert region of RhoA was required for activation of Rho kinase but not for binding (Zong et al., 2001). These observations suggest that the insert region can regulate interaction of Rho proteins with certain effectors and may have a differential effect on the signaling outcome.

Biological Processes Regulated by Rho GTPases and Association with Cancer

The best characterized biological processes regulated by Rho proteins stem from their ability to modulate the actin cytoskeleton. Restructuring of the actin machinery is required for various processes like cell migration, shape change, cell adhesion, cytokinesis, and vesicle trafficking. Rho/Rac/Cdc42 coordinate the organization of actin into distinct structures. Rho proteins are involved in the stress fiber formation, Rac proteins coordinate the formation of ruffles and lamellipodia, and Cdc42 regulates filopodia formation (Fig. 9) (Takai et al., 2001).
Thus, the Rho family members are involved at various levels in cell migration. Rac coordinates lamellipodia extension at the leading edge of migrating cells by stimulating new actin polymerization. It is also involved in the assembly of focal adhesion complexes that allow the attachment of lamellipodia. The cell body then moves forward by actin-myosin filament contraction which in turn can be regulated by Rho. Remodeling of the extracellular environment to facilitate migration is influenced by Rac and Cdc42 via the transcriptional activation of matrix metalloproteases (MMPs) (Ridley, 2001a).

Regulation of the actin cytoskeleton is the most well studied function of Rho GTPases, however, they are also involved in the regulation of various transcription factors like E-twenty-six (ETS), activator protein-1 (AP-1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which in turn control growth and survival signals. The role of Rho proteins in the regulation of membrane trafficking and endocytic pathways will be discussed later.
Considering the important biological processes regulated by the Rho family members, it is not surprising that altered Rho GTPase signaling has been observed in tumor initiation, progression, and metastasis. It is interesting to note, while activating mutations in Ras proteins are commonly observed in tumors, mutations in Rho proteins are rare. However, alteration in the expression or activity of Rho proteins has been documented in various cancers (Vega and Ridley, 2008). Proteins of this family are implicated in the regulation of Ras-induced oncogenic transformation. Dominant negative mutants of the Rho subfamily can inhibit Ras induced transformation, whereas, activated mutants co-operate in the process (Shields et al., 2000). Cells derived from TIAM-1 null mice are resistant to Ras-induced tumor development (Malliri et al., 2002). Rho family proteins play a substantial role in tumor invasion and migration. For example, over-expression of RhoC in untransformed breast cells makes them highly motile and invasive (Wu et al., 2007), whereas a dominant negative RhoC mutant or its siRNA-mediated knockdown, reduces invasion and migration of cancer cells (Heasman and Ridley, 2008). Increased RhoC expression has been correlated with breast cancer progression (Kleer et al., 2005). Several studies have implicated Rac1 in the increased migration and invasiveness of cancer cells, especially, glioblastomas (Jarzynka et al., 2007; Salhia et al., 2008).

However, unlike its other family members, RhoB acts as a tumor suppressor, because it inhibits tumor growth, cell migration, and invasion (Vega and Ridley, 2008). In agreement with its role as a tumor suppressor, levels of RhoB have been reported to be down-regulated in several types of cancers (Adnane et al., 2002; Mazieres et al., 2004;
Couderc et al., 2008). The mechanism by which RhoB functions as a tumor suppressor is not clear. Its role in endosomal trafficking has been speculated to play a role in its ability to act as a tumor suppressor (Vega and Ridley, 2008).

**ENDOCYTIC PATHWAYS**

Endocytosis includes processes by which cells internalize solutes, macromolecules, and particles via vesicles derived from the plasma membrane (Conner and Schmid, 2003). Endocytosis is sub-divided into two broad categories based on the cargo internalized: phagocytosis (cell eating or internalization of particles) and pinocytosis (cell drinking or internalization of fluids). Phagocytosis typically occurs in specialized cells like macrophages, monocytes, and neutrophils. Pinocytosis occurs in all cells and is further subdivided into: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-and caveolae-independent endocytosis (Fig. 10) (Liu and Shapiro, 2003; Conner and Schmid, 2003). In the proceeding sections, I will focus on macropinocytosis, a poorly understood phenomenon, compared to other endocytic processes.
Macropinocytosis: Introduction

Macropinocytosis is a process by which cells internalize bulk extracellular fluid, via vesicles derived from plasma membrane ruffles or lamellipodia. As evident in figure 10, macropinocytic vesicles or macropinosomes are formed as a result of ‘evagination’ of the plasma membrane, and are thus different from other pinocytic vesicles, which are derived as a result of ‘invagination’ of a portion of the plasma membrane (Amyere et al., 2002). The lamellipodia then collapse and fuse with the plasma membrane, trapping a portion of the extracellular milieu (Conner and Schmid, 2003). The vesicles thus derived range anywhere from 0.5-5 µm in diameter (Swanson and Watts, 1995; Liu and Shapiro, 2003). The diagnostic features of macropinosomes that differentiate them from other endocytic vesicles, are: their formation is accompanied by membrane ruffling, they rapidly (within minutes) incorporate fluid phase tracers like fluorescent dextran or lucifer yellow, they do not concentrate transferrin receptor, and their formation can be inhibited.
by agents that disrupt the actin cytoskeleton, like cytochalasin A/D/E or agents that specifically block the Na\(^+/\)H\(^+\) exchanger, like amiloride (Racoosin and Swanson, 1992; Liu and Shapiro, 2003; Gong et al., 2008; Jones, 2007).

Most cells activate macropinocytosis in response to growth factors or mitogenic signals, however, constitutive macropinocytosis can be observed in tumors and specialized cells such as dendritic cells (Johannes and Lamaze, 2002). Macropinocytosis thus contributes to immune surveillance by dendritic cells which sample large volumes of extracellular fluid for antigen presentation (Conner and Schmid, 2003). However, macropinocytosis can also be utilized by pathogens, like \textit{Salmonella}, \textit{Shigella}, and \textit{Chlamydia}, to gain entry inside the cells, and evade being phagocytosed by macrophages (Swanson and Watts, 1995; Amyere et al., 2002). More recently, our lab has characterized a novel form of cell death induced by de-regulated macropinocytosis in glioblastoma cells expressing activated Ras or Rac GTPases (Chapter 1) (Overmeyer et al., 2008)

**Macropinocytosis: Molecular Components**

Macropinocytosis can be induced by growth factors, phorbol esters (Swanson and Watts, 1995), and oncogenes, like H-Ras (Bar-Sagi and Feramisco, 1986) and Src kinases (Veithen et al., 1996; Kasahara et al., 2007). While the molecular signaling mechanisms involved in biogenesis and turnover of macropinosomes remain poorly understood, certain key mediators have been identified. Macropinocytosis is closely linked to actin reorganization, which results in plasma membrane ruffling, and eventual closure of the
ruffling edge via membrane fusion, to generate a macropinocytic vesicle (Conner and Schmid, 2003). It can therefore be inhibited by agents, like cytochalasin D, that disrupt the actin cytoskeleton (Liu and Shapiro, 2003; Araki et al., 1996). Several studies have also documented the requirement of the enzyme PI-3K (Class I), by virtue of its inhibition by wortmanin and LY-294002 (Araki et al., 1996; West et al., 2000; Amyere et al., 2000; Falcone et al., 2006). In the macrophages stimulated with macrophage-colony stimulating factor (M-CSF), PI-3K was involved in the closure of ruffles to form vesicles (Araki et al., 1996), vesicle maintenance, and trafficking (Murray et al., 2000). In v-src or K-Ras transformed fibroblasts, PI-3K activation was required for signal transduction to initiate actin reorganization (Amyere et al., 2000). 3-methyladenine, an inhibitor of class III PI-3K, inhibited the homotypic fusion of macropinosomes in EGF-stimulated epidermoid A431 cells (Araki et al., 2006), suggesting that different PI-3K enzymes and their products may be involved at various stages of macropinosome biogenesis and maturation.

Small GTPases act as important modulators at distinct stages of the life cycle of a macropinocytic vesicle. Given the close association of Rac GTPases with actin reorganization, it comes as no surprise that Rac plays an important role in the biogenesis of macropinosomes. Active Rac1 (Rac1[G12V]) induced macropinocytosis in rat fibroblasts, whereas, the dominant negative mutant, Rac1(T17N), blocked macropinocytosis triggered in response to growth factors and oncogenic Ras, indicating that endogenous Rac is required for growth factor-stimulated macropinocytosis (Ridley et al., 1992). Rac1 is also required for constitutive macropinocytosis by immature dendritic
cells (West et al., 2000). Downstream of Rac, PAK1 is critical for the induction of macropinocytosis. Specifically, PAK1 co-localizes with F-actin in membrane ruffles and plays a regulatory role in the induction of macropinocytosis in Swiss 3T3 cells (Dharmawardhane et al., 2000; Dharmawardhane et al., 1997). More recently, alsin2 (ALS2), a novel Rac1 effector, was shown to be recruited on nascent macropinosomes by Rac. The exact function of ALS2 in macropinocytosis is not clear, although, it has been proposed to act as a Rab5GEF, thus, activating Rab5 and promoting macropinosome maturation (Kunita et al., 2007).

The small GTPase, Rab5, an important regulator of intracellular trafficking in receptor-mediated endocytosis (Woodman, 2000; Stenmark and Olkkonen, 2001), has been documented to play a role in macropinocytosis. Rab5 has emerged as a regulator of actin remodeling, downstream of receptor tyrosine kinases (RTKs) (Lanzetti et al., 2004). Lanzetti et al. (2004) reported that simultaneous signaling from Rab5, PI-3K, and Rac1 was required for the induction of ruffles in response to RTKs. Rab5 localized on the macropinosomes, induced actin cytoskeleton remodeling via its effector RN-tre and was indispensable for RTK-induced circular ruffling, leading to macropinocytosis. Another Rab5 effector, Rabankyrin, co-localizes on macropinosomes and may play a role in generation and trafficking of macropinosomes (Schnatwinkel et al., 2004). The above observation coupled with the fact that Rac1 activates a Rab5GEF, ALS2, which in turn activates Rab5 (Kunita et al., 2007), points toward an important regulatory role of Rab5 at various stages of macropinocytosis.
Stimulation of ADP-ribosylation factor 6 (ARF6), a GTPase of the ARF subfamily, also results in the activation of macropinocytosis. This GTPase appears to be vital for the biogenesis and trafficking of macropinosomes, by virtue of its ability to activate different signaling mediators. Activation of ARF6 by aluminum fluoride (AlF) (Radhakrishna et al., 1996) or over-expression of its GEF, EFA6 (Brown et al., 2001), leads to plasma membrane ruffling corresponding to macropinocytosis. Regulation of biogenesis by Arf6 involves activation of Rac1 via kalirin (a RacGEF) (Koo et al., 2007) or via the dedicator of cytokinesis/engulfment and cell motility (DOCK/Elmo) complex (Santy et al., 2005). By modulating the activity of its downstream effector, phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase), an enzyme that generates phosphatidylinositol 4, 5-bisphosphate (PIP2), Arf6 regulates the maturation and trafficking of macropinosomes. Proper trafficking of these vesicles requires Arf6 to be activated and de-activated, since the constitutively activated form of Arf6 causes an accumulation of macropinosomes (Brown et al., 2001; Porat-Shliom et al., 2008).

Other proteins known to be associated with macropinosomes include sorting nexin 5 (SNX5) (Kerr et al., 2006; Lim et al., 2008), SWAP-70 (Oberbanscheidt et al., 2007), and Rab34 (Sun et al., 2003).

While many questions regarding the signaling events still need to be elucidated in order to fully understand this unique endocytic pathway, it is evident that small GTPases play a pivotal role in the biogenesis, trafficking and overall regulation of macropinocytosis.
Fate of the Macropinosomes

Despite similarities in the initial steps leading to the biogenesis of macropinosomes in different cell types, their intracellular fate appears to be cell type specific. Macropinosomes generated as a result of M-CSF stimulation of macrophages, acquire markers of early endosomes, late endosomes, and finally fuse with the lysosomes (Racoosin and Swanson, 1993). These structures rarely fuse back with the plasma membrane (Swanson and Watts, 1995). The macropinosomes of dendritic cells fuse with one another and acquire various endocytic markers, like, early endosome antigen 1 (EEA1) and transferrin receptor (recycling endosomes). However, these dynamic vesicles also undergo exocytosis and regurgitate their contents back into the extracellular space (Falcone et al., 2006). On the other hand, macropinosomes in human A431 cells interact with one another but do not appear to interact with other endocytic compartments (Hewlett et al., 1994; Veithen et al., 1998). They do recycle their contents back to the plasma membrane in a cyclic-adenosine monophosphate (c-AMP) dependent manner (Veithen et al., 1998). Sorting nexin-5 (SNX5)-positive macropinosomes, generated in response to EGF in HEK293 cells, acquire early endosome markers EEA1 and Rab5, which is followed by the recruitment of Rab7, a marker for late endosomes. These maturation steps culminate in the fusion of macropinosomes with the lysosomes (Kerr et al., 2006). Finally, the macropinosomes induced by H-Ras or Arf-6 share similar phosphoinositide composition, acquire Rab5 but are EEA1 negative and the majority of macropinosomes recycle back to the plasma membrane (Donaldson et al., 2009; Porat-Shliom et al., 2008).
CALPHOSTIN-C: A PROTEIN KINASE C INHIBITOR

A recurrent theme in signal transduction is the reversible phosphorylation of proteins, in order to modulate their function. This reaction is catalyzed by a group of proteins known as ‘protein kinases’. The protein kinase C (PKC) family is comprised of several serine/threonine specific kinases, classified as follows: a) classical or conventional PKCs (cPKCs, α, βI, βII, γ), which depend on calcium, diacylglycerol (DAG) and phosphatidylserine (PS) for activation, b) novel PKCs (nPKCs, δ, ε, θ and η), which are calcium independent but require DAG and PS, and c) atypical PKCs (aPKCs, ζ and λ/ι), which do not require calcium or DAG, but require PS (da Rocha et al., 2002; Hofmann, 2004). These enzymes play a significant role in diverse biological functions, like synaptic transmission, secretion, cell cycle control, differentiation, and proliferation (Hofmann, 2004).

Isoform specific variation in the activity of PKCs has been noted in various types of cancers. The PKCη isoform, increases cell proliferation and mediates resistance to radiation in glioblastomas (Martin and Hussaini, 2005). Elevated expression of PKCβII has been observed as an early event in colon cancer development (Gokmen-Polar et al., 2001). However, under-expression and over-expression of PKCs has been noted in cancers, therefore, it is difficult to assign a specific role to a particular isoform (Martiny-Baron and Fabbro, 2007). For example, PKCα activity is elevated in breast cancer and malignant gliomas but repressed in colon carcinomas (da Rocha et al., 2002). It is also important to note that PKC is implicated in the regulation of multidrug resistance in various tumors (Martiny-Baron and Fabbro, 2007).
Protein kinase C inhibitors have been developed as therapeutic targets against cancers. Some of them, e.g., enzastaurin (PKCβ specific), ruboxistaurin (PKCβ specific), and lestaurtinib are currently being tested in clinical trials.

Staurosporine is one of the most powerful PKC inhibitors used in various experimental systems, however, its poor kinase selectivity has prevented its therapeutic development (da Rocha et al., 2002). Calphostin-C (cal-C) is a specific PKC inhibitor that modifies the DAG binding site of the enzyme. Calphostin-C, a polycyclic hydrocarbon, is a secondary metabolite of the fungus *Cladosporium cladosporoides*. Because the DAG binding site is unique to PKC, cal-C potently inhibits the enzyme (IC₅₀=50nM) without affecting other kinases (Kobayashi et al., 1989). Another unique feature of this compound is that its inhibition of PKC is strictly light dependent (Bruns et al., 1991), making it a good candidate for photodynamic therapy.

Calphostin-C has been shown to be cytotoxic against various tumor types e.g., gliomas (Ikemoto et al., 1995), acute lymphoblastic leukemia (Zhu et al., 1998), bladder cancer (Beck et al., 1999), and prostate cancer (Dubauskas et al., 1998). However, the exact mechanism by which cal-C exerts its cytotoxic effects remains poorly understood. In this thesis, I will document the role of cal-C, as an inducer of ER stress in cancer cells (Chapter 3). My work shows for the first time that cal-C can induce rapid apoptosis initiated by ER stress in a number of cancer cells, making it an interesting therapeutic target, especially in the case of photodynamic therapy.
Active Ras Triggers Death in Glioblastoma Cells Through Hyperstimulation of Macropinocytosis

Jean H. Overmeyer*, Aparna Kaul*, Erin E. Johnson, and William A. Maltese§


Department of Biochemistry and Cancer Biology, University of Toledo College of Medicine, Toledo, Ohio, USA

Running title: Macropinocytosis and Cell Death Induced by Ras

Keywords: Ras, Macropinocytosis, Necrosis, Autophagy, Methuosis, Glioblastoma

*These authors contributed equally to the work.

Grant Support: This work was supported by National Institutes of Health grants R01 CA34569 and R01 CA115495, and by a grant from the Charlotte Geyer Foundation.

Note: Current address for E.E. Johnson: Dept. of Genetics and Complex Diseases, Harvard School of Public Health, 665 Huntington Ave, Boston, MA 02115

§Correspondence: William A. Maltese Ph.D., Department of Biochemistry & Cancer Biology, Block Health Sciences Bldg, Mail Stop 1010, University of Toledo College of Medicine, 3035 Arlington Ave., Toledo, Ohio, 43614, E-mail: william.maltese@utoledo.edu
Abstract

Expression of activated Ras in glioblastoma cells induces accumulation of large phase-lucent cytoplasmic vacuoles, followed by cell death. This was previously described as autophagic cell death. However, unlike autophagosomes, the Ras-induced vacuoles are not bounded by a double membrane and do not sequester organelles or cytoplasm. Moreover, they are not acidic and do not contain the autophagosomal membrane protein, LC3-II. Here we show that the vacuoles are enlarged macropinosomes. They rapidly incorporate extracellular fluid-phase tracers, but do not sequester transferrin or the endosomal protein, EEA1. Ultimately, the cells expressing activated Ras detach from the substratum and rupture, coincident with the displacement of cytoplasm with huge macropinosome-derived vacuoles. These changes are accompanied by caspase activation, but the broad-spectrum caspase inhibitor, z-VAD, does not prevent cell death. Moreover, the majority of degenerating cells do not exhibit chromatin condensation typical of apoptosis. These observations provide evidence for a necrosis-like form of cell death initiated by dysregulation of macropinocytosis, which we have dubbed ‘methuosis’. An activated form of the Rac1 GTPase induces a similar form of cell death, suggesting that Ras acts through Rac-dependent signaling pathways to hyperstimulate macropinocytosis in glioblastoma. Further study of these signaling pathways may lead to the identification of other chemical and physiological triggers for this unusual form of cell death.
Introduction

Glioblastoma is one of the most aggressive human brain tumors (1,2). Despite efforts to improve surgical, radiological and chemotherapeutic treatment strategies, the prognosis for patients with glioblastoma remains poor. A major problem is that residual cells remaining after surgical resection of the primary tumor rapidly acquire resistance to chemotherapeutic drugs (3). In addition, glioblastomas often harbor mutations in genes that regulate programmed cell death (e.g., PTEN, RB, p53), rendering them resistant to conventional pro-apoptotic stimuli (4). These characteristics have stimulated interest in identifying alternative pathways for inducing cell death in glioblastoma.

Apoptosis is the best characterized form of programmed cell death. However, non-apoptotic forms of cell death are now recognized as playing significant roles during embryonic development, neurodegeneration, and cancer regression (5). In these cases loss of cell viability may occur in a manner that is independent of caspase activation. Autophagic cell death (type-II programmed cell death), is the most widely studied form of non-apoptotic cell death. Its diagnostic morphological feature is accumulation of autophagosomes and degradative autolysosomes (6). Autophagic death has been reported to occur in several types of cancer cells (7), but it has received particular attention in glioblastoma, where it can be induced by alkylating agents (8), arsenic trioxide (9), ionizing radiation (10), and rapamycin (11). Nevertheless, it remains controversial whether increased autophagic activity is actually a direct cause of cell death. Recent evidence supports the alternative view that accumulation of autophagosomes may signify
a survival response intended to rid cells of misfolded proteins or damaged organelles (12-14).

In 1999, Chi et al. (15) reported that ectopic expression of activated Ras GTPase, which normally serves to stimulate cell proliferation, can trigger non-apoptotic cell death in glioblastoma and gastric carcinoma. This was described as autophagic death because the cells developed numerous cytoplasmic vacuoles. However, there have been no follow-up studies to confirm that the vacuoles induced by Ras are autophagosomes. In the present study we have determined that the large vacuoles that accumulate in glioblastoma cells expressing activated H-Ras are in fact derived from macropinosomes. Cell rupture coincides with continued expansion of these macropinocytotic vacuoles. These findings provide evidence for a novel form of cell death characterized by hyperstimulation of vesicular fluid uptake and accumulation of swollen macropinosomes. We have termed this process methuosis (from the Greek word methuo, which means to drink to intoxication).

Materials and Methods

Cell culture. U251 glioblastoma cells were obtained from the DCT Tumor Repository (National Cancer Institute, Frederick, MD, USA). Other cell lines were obtained from the same source, or from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained at 37°C with 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Phase contrast images of the live cells were obtained using an Olympus IX70 microscope.
equipped with a digital camera and SPOT imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

**Generation of stable cell lines for inducible expression of active Ras and Rac.** To generate stable cell lines capable of conditional Ras expression, U251 cells were nucleofected with pTet-on (Clontech, Mountain View, CA), which encodes the reverse tet-responsive transcriptional activator. Cells were selected with 500 μg/ml G418 and clonal lines were tested in transient transfection assays to determine which ones gave the tightest doxycycline (Dox)-regulated gene expression using the tet-responsive pTRE vector (Clontech). One such clone was used for generation of permanent cell lines by nucleofection with pTRE(myc-H-RasG12V) together with pTK-Hyg. Clones were selected in medium containing 200 μg/ml hygromycin + 200 μg/ml G418 and screened to measure the expression of myc-H-Ras(G12V) in response to 1 μg/ml Dox. The clonal line, U251-C18, was selected for use in the present studies, although other clones exhibited essentially identical morphological phenotypes when Ras(G12V) expression was induced. For Rac1(G12V) expression, lentiviral particles containing the gene for myc-tagged Rac1(G12V) under the control of a tet-inducible promoter were generated by America Pharma Source, LLC (Gaithersburg, MD). These were mixed with lentiviral particles containing a gene for blasticidin resistance and used to co-infect U251 pTet-on cells. Clones were selected in medium containing 10 μg/ml blasticidin and 200 μg/ml G418. Expression of myc-Rac1(G12V) was induced by adding 1 μg/ml Dox to the medium.
**Retroviral expression.** Myc-tagged H-Ras constructs (G12V and S17N) were subcloned into the EcoR1-BamH1 sites of the retroviral expression vector, pFBneo (Stratagene, La Jolla, CA, USA). Procedures for retrovirus production and infection of glioma cells have been described previously (16).

**Nucleofection.** H-Ras(G12V) was subcloned into pCMV5 that had been modified to encode an in-frame myc epitope tag (MEQKLISEEDL). The expression vector was introduced into the U251 cells by nucleofection, using the Nucleofector II system from Amaxa Inc., (Gaithersburg, MD) with Solution T and program T-30. Other cell lines were nucleofected with different solutions and programs: HEK293 cells, Solution V with program Q-001; HeLa cells, Solution R with program I-013; and HEP2 cells, Solution V with program G-016.

**Western blot analysis.** Antibodies were obtained from the following sources: myc epitope (EMD Biosciences, San Diego, CA, USA), PARP (BD Pharmingen, San Jose, CA), laminA/C (Cell Signaling Technology, Danvers, MA, USA), lactate dehydrogenase (LDH) and α-tubulin (Sigma, St. Louis, MO), and LC3 (APG8b, N-Term; Abgent, San Diego, CA, USA). Protein was quantified in cell lysates by colorimetric assay using the Bio-Rad reagent (Bio-Rad, Inc., Hercules, CA, USA). SDS-PAGE and western blot analyses were performed as described previously (16), using enhanced chemiluminescent (ECL) detection (GE Healthcare, Piscataway, NJ, USA). Immunoblot signals were
quantified using a Kodak 440CF image station or an Alpha Innotech FluorChem HD2 imaging system.

In order to block the lysosomal turnover of endogenous LC3-II, cells were incubated in medium containing protease inhibitors, E64D (10 μg/ml) and pepstatin A (10 μg/ml) (Peptides International, Louisville, KY) for 48 h prior to western blot analysis of LC3. Cells treated with 500 nM staurosporine (Cayman Chemical Company, Ann Arbor, MI) for 18-24 hrs served as positive controls for induction of PARP and lamin cleavage associated with apoptosis.

Cell viability and cell cycle distribution. Viability of individual cells was assessed by fluorescence microscopy using the Viability/ Cytotoxicity Assay Kit (Biotium Inc, Hayward, CA), which measures hydrolysis of calcein acetoxy methylester (live) and uptake of ethidium homodimer III (dead). At least 50 cells per sample were counted to determine the percent dead population and all samples were analyzed in triplicate. Viability of cell populations in culture was quantified by metabolic activity assay, measuring the conversion of 3- [4,5-dimethylthiazol2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to the formazan derivative using the Cell Growth Determination kit from Sigma (St. Louis, MO). The formazan derivative was quantified by measuring its absorbance at 570 nm with a Spectra Max 384 Plus plate reader. Caspase-3 activity was detected by the NucView fluorescence-based assay (Biotium Inc.) according to the directions supplied by the manufacturer.
To compare relative levels of ATP in vacuolated versus non-vacuolated cells, the cells were collected by trypsinization and assayed using the CellTiter Glo kit from Promega (Madison, WI). The luminescence produced by the ATP-dependent mono-oxygenation of luciferin by luciferase was normalized to the number of cells added to the assay, determined with a Coulter counter.

For colony forming assays cells were seeded in 100 mm dishes at 1,400 cells/dish. The day after plating, H-Ras(G12V) expression was induced in half of the cultures by inclusion of 1 μg/ml dox in the medium. Medium was replaced on all of the cultures every 2-3 days. Colony formation was assessed after three weeks by washing the cultures with PBS, fixing the cells for 10 minutes in ice-cold methanol and staining for 10 minutes with 1% crystal violet in 35% methanol. Colonies containing at least 50 cells were counted using a dissecting microscope.

Cell cycle distribution was determined from DNA histograms generated by flow cytometric analysis of cells prepared as described (17), using a Beckman-Coulter EPIC XL MCL cytometer. Data were analyzed using Multicycle DNA cell cycle analysis software (Phoenix Flow Systems, San Diego, CA).

DNA fragments were isolated by the method of Herrmann et al. (18) and resolved by electrophoresis in a 1.2% agarose gel. Images were obtained using the FluorChem HD2 system after staining the gel with ethidium bromide.

**Immunofluorescence microscopy.** Cells were prepared for immunofluorescence as described previously (19). Myc-tagged proteins were detected with a monoclonal
antibody (EMD Biosciences, San Diego, CA, USA) followed by goat anti-mouse IgG conjugated with Alexa Fluor™ 568 (Invitrogen, Carlsbad, CA, USA). For detection of endogenous LC3, we used the purified rabbit polyclonal antibody, APG8b (MAP1LC3B, N-Term) from Abgent, San Diego, CA, USA, followed by goat anti-rabbit IgG conjugated with Alexa Fluor 488. Antibodies used for immunofluorescence localization EEA1 and LAMP1 were obtained from Abcam (Cambridge, MA) and the Developmental Studies Hybridoma Bank (Iowa City, IA) respectively. Cells were examined with a Nikon Eclipse 800 fluorescent microscope equipped with a digital camera and ImagePro software (Media Cybernetics, Silver Spring, MD, USA) or with a Leica TCS SP5 multiphoton confocal microscope, using 488 nm and 561 nm laser excitation lines.

Electron microscopy. Cell pellets were fixed, dehydrated, and infiltrated as described previously (19). Ultrathin sections were collected on copper 300-mesh support grids, stained with uranyl acetate and lead citrate, and examined using a Philips CM 10 transmission electron microscope.

Uptake of fluid-phase and organelle-specific tracers. Lucifer Yellow (LY) and dextran-Alexa Fluor (AF) 488 (10,000 MW) were purchased from Invitrogen/Molecular Probes (Eugene, Oregon). Cells were incubated with LY (1.5 mg/ml in Hanks balanced salt solution; HBSS) for 15 minutes in a 37°C, 5% CO₂ incubator. The LY was removed and the cells were washed once with HBSS. Phase contrast and fluorescent images were immediately taken of the live cells using an Olympus IX70 microscope equipped with a
digital camera and SPOT imaging software. Uptake of LY was quantified by flow
cytometry. Briefly, cells were grown in phenol-red-free medium, incubated with LY as
described above, harvested by trypsinization and suspended in HBSS. For each sample
10,000 cells were analyzed with a Beckman-Coulter EPICS Elite ESP cytometer, with
488 nm excitation laser and 505-545 nm emission. Mean fluorescence intensity of the
population was determined after subtraction of autofluorescence background obtained
from parallel control samples incubated without LY. In some studies the cells were
preincubated with Cytochalasin D (1 μM) (Sigma) for 30 min prior to the addition of LY.

For labeling with dextran-AF488, the cells were washed twice with phenol red-
free DMEM containing 10% FBS, then incubated with 0.5 mg/ml dextran-AF488 in the
same medium for the period of time indicated in the figure legends. The cells were
washed two times in the same medium without the tracer, then images of the live cells
were acquired as described for LY staining. In some cases, cells were co-labeled for 15
min with dextran-Af488 and human holo-transferrin conjugated to Alexa Fluor 594
(Invitrogen-Molecular Probes) added at 5 μg/ml in serum free DMEM.

Intracellular acidic compartments were labeled by incubating live cells at 37°C
with Lysotracker Red DND-99 (Invitrogen) added to phenol red-free DMEM at a final
concentration of 1 μM. Staining of intracellular compartments for cathepsin B activity
was performed by incubating live cells with Magic Red ™ RR (Immunochemistry
Technologies, Bloomington, MN) according to the directions supplied by the
manufacturer.
Results

Effects of ectopic expression of activated Ras in human glioblastoma cells. U251 cells harbor mutations in the pro-apoptotic genes, PTEN and p53, and are widely used as a model for human glioblastoma (20). To begin a detailed characterization of the cellular phenotype triggered by expression of activated Ras in glioblastoma cells, we generated a stable U251 glioblastoma cell line (U251-C18) that exhibits tightly controlled conditional expression of myc-tagged H-Ras(G12V) in response to the addition of Dox (Fig. 1A). Our initial observations of these cells generally agreed with those reported by Chi et al. (15). That is, coincident with expression of myc-tagged Ras(G12V), the cells became filled with lucent cytoplasmic vacuoles that are readily detected by phase contrast microscopy (Fig. 1A). By day-6 there was a decrease in the number of viable cells in the cultures expressing H-Ras(G12V) compared with the controls (Fig. 1B). This coincided with noticeable cell rounding and detachment from the dish, with abundant floating debris suggestive of cellular disintegration. In cultures harvested on day-6 after addition of Dox, detached cells accounted for approximately 10% of the total cell population (not shown). Nearly 50% of these detached cells were nonviable when tested in a live/dead assay that measures hydrolysis of calcein acetoxy methylester (live) and uptake of ethidium homodimer III (dead) (Fig. 1C). On the other hand, the attached cells in the cultures expressing active Ras scored positive for viability (Fig. 1C) and exhibited DNA histograms with S-phase populations similar to the control cells growing without Dox (Supplement, Fig. S1). Although they were extensively vacuolated, the attached cells collected on day-6 after addition of Dox showed no significant decline in ATP levels
compared to the cells maintained without Dox (Fig. 1D). However, consistent with their reduced viability, the detached cells exhibited a marked reduction in ATP concentration (Fig. 1D). Taken together, these findings imply that metabolic failure leading to cell death occurs abruptly at a late stage coincident with or after the detachment of the vacuolated cells. The long-term consequences of H-Ras(G12V) expression for cell survival are evident in colony-forming assays where addition of Dox resulted in an 85-90% reduction in the number of colonies (Fig. 1E).

The same pattern of vacuolization and cell degeneration was observed when Ras(G12V) was introduced into the U251 cells by retroviral infection (Supplement, Fig. S2) or by transient nucleofection (21). Seven additional independently-derived human glioma cell lines, some without mutations in PTEN (LN229) or p53 (U87MG, A172) (20), also exhibited a similar phenotype after expression of H-Ras(G12V) (Supplement Fig. S2 A). However, introduction of myc-H-Ras(G12V) into other commonly used cells (HeLa, HEp2, HEK293) did not cause vacuolization (Supplement Fig. S2 B). As previously reported, the inactive GDP-locked (15,21) or non-farnesylated (21) forms of Ras did not cause vacuolization, indicating that the cellular phenotype is directly related to the activation of specific Ras signaling pathways.

**Ras-induced vacuoles are distinct from autophagosomes.** Electron microscopy of glioblastoma cells expressing H-Ras(G12V) revealed numerous electron-lucent vacuoles ranging from 0.5-2 μm in diameter, with some larger vacuoles reaching 7-8 μm (Fig. 2A). The vacuoles were generally devoid of cytoplasmic components or organelles,
although some contained unidentified membranous inclusions or small quantities of amorphous electron-dense material (Fig. 2A). At high magnification the thickness of the membranes surrounding the vacuoles was estimated at 6-8 nm (Supplement Fig. S3), consistent with a single membrane. The large electron-lucent vacuoles were clearly distinct from smaller structures fitting the description of ‘classical’ autophagosomes (22) which have double membranes surrounding luminal cytoplasmic contents. (Fig. 2A, black arrows).

To further confirm that the Ras-induced vacuoles were not derived from autophagosomes, we performed immunofluorescence staining of the cells with an antibody against a well-established autophagosome marker, microtubule associated protein light-chain 3 (LC3). LC3 exists in a cytosolic form (LC3-I) and a form that is conjugated to phosphatidylethanolamine on autophagosome membranes (LC3-II) (23). The relative amount of LC3-II correlates with the number of autophagosomes induced by starvation and other stimuli (23). The LC3 antibody from Abgent, Inc. reacts predominantly with LC3-II on western blots. Therefore, we used this antibody to determine if LC3-II was localized to the vacuole membranes. As we have reported previously (21), myc-H-Ras(G12V) was localized in membranes surrounding the vacuoles (Fig. 2B). In contrast, LC3-II was detected in much smaller punctate structures (Fig. 2B). Confocal microscopy showed clearly that the large vacuoles circumscribed by myc-H-Ras(G12V) were separate from the LC3-II positive autophagosomes (Fig. 2C).

Because the preceding study was done with cells that were transiently nucleofected, we were able to compare the cells expressing myc-H-Ras(G12V) with the
adjacent non-transfected cells (Fig. 2B, asterisks). We noticed that the punctate LC3 fluorescence was more intense in the transfected cells, suggesting that autophagosomes might accumulate separately from the phase-lucent vacuoles. To explore this possibility, we determined the relative amount of LC3-II in the stable U251-C18 cells with or without the expression of myc-H-Ras(G12V). As shown in Fig. 2D (left panel), expression of myc-H-Ras(G12V) was associated with a 2.7-fold increase in the amount of LC3-II, normalized to LDH. However, this could reflect either an increase in autophagosome biogenesis (stimulation of cellular macroautophagy pathways) or a decreased lysosomal turnover of LC3-II. An established method that can be used to distinguish between these possibilities is to compare the levels of LC3-II in the presence and absence of lysosomal protease inhibitors (24). As shown in Fig. 2D (right panel), addition of protease inhibitors to uninduced cells (-Dox) caused a 3.2-fold increase in the basal level of LC3-II, consistent with the expected impairment of lysosomal LC3-II turnover. However, when myc-Ras(G12V) was induced by addition of Dox, there was an additional 2-fold increase in LC3-II above the level caused by addition of protease inhibitors alone. This indicates that much of the increase in LC3-II induced by expression of Ras(G12V) is related to an increase in autophagosome formation, rather than a block in LC3-II turnover. Similar results were obtained when the cells were treated with rapamycin, an inhibitor of mTOR and a well-established inducer of macroautophagy (25) (not shown).

As shown in Fig. 3, expression of Ras(G12V) has identical effects on cell morphology and viability when expressed in a stable U251 cell line that we previously
established as being resistant to pro-autophagic stimuli because of a knockdown of the autophagy protein, beclin-1 (16). This suggests that the increased autophagic activity detected in Fig. 2 is not required for the death of the glioblastoma cells expressing activated Ras(G12V), and that cellular degeneration is most likely related to the progressive accumulation of the non-autophagic phase-lucent vacuoles.

**Ras-induced vacuoles are enlarged macropinosomes.** In considering possible origins for the phase-lucent vacuoles, we noted a previous study in which activated Ras was shown to stimulate macropinocytosis in fibroblasts (26). Macropinocytosis is a process whereby cells internalize extracellular fluid trapped beneath projections of the plasma membrane termed ruffles or lamellipodia (27). Macropinosomes typically appear as phase-lucent vesicles ranging in diameter from 0.5 μm to 5 μm. Rapid incorporation of extracellular fluid phase tracers is a hallmark of macropinosomes. When we added Lucifer Yellow (LY) to the medium, cells expressing H-Ras(G12V) incorporated the tracer into many of the phase lucent vacuoles within 10 min (Fig. 4A). Quantification of LY internalization by flow cytometry demonstrated a 3-fold increase in uptake of the tracer into the +Dox cells expressing Ras(G12V), compared with the basal level of LY uptake in the –Dox controls (Fig. 4B). Preincubation with cytochalasin D (CytoD), which disrupts the actin cytoskeleton involved in the formation of lamellipodia, had no effect on LY uptake in the –Dox cells, suggesting that most of the LY uptake in these cells is due to basal activity of the endocytic pathway. In contrast, addition of CytoD
caused a 50% reduction of LY incorporation in the +Dox cells, after subtraction of the basal uptake attributed to endocytosis (-Dox, +CytoD) (Fig. 4B).

Electron micrographs revealed numerous lamellipodia closing around regions of extracellular fluid to form nascent macropinosomes in the cells expressing H-Ras(G12V) (Fig. 4C). Control cells that were not expressing Ras also contained some lamellipodia, but closure of these structures to form enlarged macropinosomes was not evident (not shown).

In addition to labeling macropinosomes, fluid phase tracers can enter early endosomes. Macropinosomes lack a clathrin coat and can be distinguished from endosomes by their comparative inability to concentrate receptors (28). Therefore, to confirm that the vacuoles were derived from macropinosomes, cells expressing Ras(G12V) were subjected to short-term incubation with a bulk fluid-phase tracer, dextran-AF488, together with a ligand for the transferrin receptor, transferrin-AF594. The larger vesicles containing fluorescent dextran were distinct from much smaller endosomes that sequestered transferrin (Fig. 4D). In accord with this finding, we observed that the phase-lucent vacuoles were separate from smaller punctate structures detected by immunofluorescence with an antibody against the well known early endosomal protein, EEA1 (Fig. 4E). These findings, coupled with the morphological evidence in Fig. 4C, support the identification of the Ras-induced vacuoles as macropinosomes.

The lucent vacuoles induced by activated Ras are morphologically distinct from lysosomes and autolysosomes, which typically contain electron-dense organelle remnants.
or degraded cytoplasmic components (29) (Figs. 2A and 4C). However, on the basis of morphology alone it was difficult to rule out the possibility that some of the Ras-induced vacuoles might be swollen late endosomes, similar to those observed in cells where morphogenesis of multivesicular endosomes is disrupted by inhibiting the class-III PI 3-kinase, Vps34 (19,30). Because the latter retain the acidic characteristic of late endosomes, they readily incorporate lysosomotropic agents (19). Therefore, to test the possibility that some of the Ras-induced vacuoles might be late endosomal compartments, we performed supravital staining with Lysotracker Red™. As shown in Fig. 5A, there was no clear overlap between the phase-lucent vacuoles and the compartments labeled with Lysotracker Red. Similar results were obtained when the cells were stained with acridine orange, which is sequestered in late endosomes, lysosomes, and autolysosomes (31,32) (data not shown). Additionally, there was no substantial overlap between the phase-lucent vacuoles and compartments labeled with Magic Red™RR, a cell permeable peptide substrate that fluoresces when cleaved by cathepsin B (Fig. 5B). Taken together, these results support the conclusion that the majority of the phase-lucent vacuoles are derived from macropinosomes rather than late endosomes or lysosomes.

Although the vacuoles did not stain with markers for acidic or cathepsin-positive compartments (Fig. 5A & B), we found that many of them contained LAMP1, a membrane protein typically associated with lysosomes and late endosomes (Fig. 5C). Two possible models could explain this. In the first model, macropinosomes may fuse with late endosomes or lysosomes, acquiring LAMP1 and simultaneously neutralizing the
interior of these compartments so that they cannot be detected with acidophilic agents or cathepsin substrates. An alternative model is suggested by reports that LAMP1 can traffic directly to non-lysosomal compartments like early endosomes (33) or nascent phagosomes (34). Thus, the macropinosomes may remain separate from lysosomes while recruiting LAMP1 directly to their membranes. To discriminate between these models, we pre-labeled the lysosomal compartments of vacuolated glioblastoma cells by incubating them with Lysotracker Red for 3 h. Then, after removing the Lysotracker from the medium, we added fluorescent dextran for 4 h to determine if the dextran-labeled compartments would merge with the pre-labeled lysosomes (Fig. 5D). After 4 h we detected merger of some of the smaller dextran-labeled structures with the Lysotracker-positive compartments, presumably representing the fusion of endosomes with lysosomes. However, even after this extended period, the larger dextran-labeled vacuoles appeared to remain separate from the pre-labeled lysosomal compartments (Fig. 5D). Similar results were observed when the cathepsin substrate, Magic Red™ RR, was used to pre-label the lysosomes (not shown). These observations are consistent with the concept of direct recruitment of LAMP1 to the membranes of macropinocytotic vacuoles, with minimal fusion between these compartments and lysosomes.

**Activated Rac1 induces cytopathology similar to that caused by activated Ras.** The mechanisms underlying macropinocytosis are poorly understood, but previous studies have implicated the Rac1 GTPase and its effector, PAK1, as key regulators of this process (35,36). Since downstream targets of Ras include guanine nucleotide exchange
factors that can stimulate activation of Rac1 (e.g., Tiam1) (37), we hypothesized that Rac1 might be positioned downstream from Ras in the pathway that triggers macropinosome accumulation in glioblastoma cells.

To test this possibility we asked if expression of a constitutively active form of Rac1 could mimic the effects of Ras(G12V) in U251 glioblastoma cells. As shown in Fig. 6A &B, conditional expression of activated myc-Rac1(G12V) in U251 cells triggered a vacuolar phenotype closely resembling that observed with Ras(G12V). As in the case of cells expressing Ras(G12V), the viability of the cells expressing Rac1(G12V) declined between the fourth and eighth days after addition of Dox, coincident with extreme vacuolation and cell detachment (Fig. 6C). Moreover, the cells expressing Rac1(G12V) exhibited a substantial increase in the uptake of LY into the vacuolar structures (Fig. 6D). We have previously reported that activated forms of other Rho-family GTPases (e.g., Cdc42 and RhoA) do not cause vacuolation of U251 cells (21). Thus, the hyperstimulation of macropinocytosis appears to be a specific effect of Ras(G12V) and Rac1(G12V).

The mechanism of cell death in glioblastoma cells expressing activated Ras is distinct from apoptosis. In their initial report describing Ras-induced death of glioblastoma cells, Chi et al. (15) found no evidence for caspase activation in the vacuolated cells. However, in light of our observation that loss of cell viability coincides with detachment from the substratum (Fig. 1C), we re-examined this question by evaluating the cleavage of caspase substrates in both the attached and detached cell
populations. As shown in Fig. 7A, there was no cleavage of PARP or lamin A/C in the attached, mostly viable, vacuolated cells. However, in the detached cells fragments of PARP and lamin A/C were detected at molecular weights consistent with caspase-3 cleavage. The sizes of these fragments were identical to those observed in cells treated with staurosporine, a known inducer of apoptosis (Fig. 7A). Examination of the portion of the PARP blot below 75 kDa did not reveal any 50 kDa fragments indicative of degradation by lysosomal proteases (38). In accord with the PARP cleavage, 49.6% of the detached cells stained positive for caspase-3 activity (Supplement, Fig. S4 A), mirroring the percentage of non-viable cells in the detached population (Fig. 1C). Agarose gel electrophoresis revealed no evidence of DNA fragmentation in the attached cells, but the DNA recovered from the detached cells was extensively degraded, with detectable laddering suggestive of nucleosomal DNA fragmentation (Supplement, Fig. S4 B).

To determine if death of the cells expressing Ras(G12V) was dependent on caspase activation, we added the broad-spectrum caspase inhibitor, zVAD-fmk, during the critical period (days 4-6) when loss of cell viability begins to occur. The PARP blots in Fig. 7B indicate that zVAD was highly effective in blocking caspase activation. However, this did not prevent vacuolization (not shown) or loss of cell viability (Fig. 7C). Thus, although activation of caspases occurs in conjunction with the demise of the glioblastoma cells, this is not an obligatory feature of the death mechanism. In separate studies we also tested the ability of cathepsin and calpain inhibitors to preserve the viability of the vacuolated cells (Supplement Fig. S5). Consistent with the absence of
alternative 50 kDa PARP cleavage products, the lysosomal protease inhibitors were ineffective in preventing cell death induced by expression of Ras(G12V).

In light of the foregoing observations, we examined the morphology of the detached glioblastoma cells to determine if these cells exhibit typical features of apoptosis (Fig. 7D). Electron microscopy revealed that at least 80% of the detached cells contained numerous large cytoplasmic vacuoles with morphology similar to the macropinosomes described earlier in the attached cells (Figs. 2A and 4C). The cells were generally swollen to 20-30 μm diameter, compared to the attached cells, which typically ranged 10-15 μm. In about half of the cell population the expansion of vacuoles was so extreme that these structures filled most of the cytoplasmic space (Fig. 7D). While some cells contained numerous vacuoles of various sizes (Fig 7D, panel i), others contained only a few very large vacuoles (panels ii & iii), suggestive of an end-stage coalescence of these structures. In addition to the distorted cells with intact peripheral membranes, there were numerous remnants of cells that had ruptured (Fig. 7D, panel iv). However, even in the severely vacuolated or ruptured cells, the nuclei were generally intact and contained diffuse chromatin and a prominent non-fragmented nucleolus. These observations indicate that the morphological features of the dying glioblastoma cells resemble necrosis-like forms of cell death rather than classical apoptosis.

Discussion

The resistance of many types of cancer cells to apoptosis has stimulated interest in identifying non-apoptotic cell death pathways that might be targeted to slow tumor
A number of distinct non-apoptotic forms of cell death have now been characterized. These include: type-II or autophagic cell death (5,7,12), paraptosis (39,40), oncosis (41-43), and necroptosis (44,45). Even the term necrosis, previously used to indicate ‘passive’ cell death or the post-mortem state of cells (41), has more recently been used to describe forms of programmed cell death that involve progressive lysosomal damage, leakage of lysosomal proteases, and early disruption of the cell membrane (46-49). In the present study we provide a detailed characterization of a novel form of non-apoptotic cell death observed in glioblastoma cells upon constitutive stimulation of Ras signaling pathways. The hallmark cytopathological feature of this form of cell death is the marked accumulation of large fluid filled vacuoles derived from macropinosomes. Electron microscopy demonstrates a correlation between cellular disintegration and a progressive increase in the number and size of the macropinocytic vacuoles. These observations are highly suggestive of a causal relationship between the dysregulation of macropinocytotic fluid uptake and the eventual metabolic collapse and rupture of the cells. Final proof of this interrelationship must await the development of new approaches for long-term inhibition of macropinocytosis, because the drugs currently used to block this process (e.g., amiloride, cytochalasins) are toxic when applied to cultured cells for more than a few hours (50).

In contrast with an earlier report (15), we discovered that by examining detached cells expressing Ras(G12V) we could in fact detect caspase activation and DNA fragmentation. However, similar to the situation in most other forms of non-apoptotic death, caspase activation does not seem to be an obligatory step in the Ras-induced death.
program. Furthermore, our studies of cellular morphology did not show typical cell shrinkage, blebbing, and nuclear chromatin condensation observed in apoptotic cells. Therefore, our results support the classification of Ras-induced macropinocytotic cell death as non-apoptotic. As discussed below, comparison with other known types of non-apoptotic death suggests that macropinocytotic cellular degeneration represents a unique form of cell death.

Autophagic death is now the most widely recognized type of non-apoptotic cell death. The diagnostic feature of this form of death is the proliferation of autophagosomes and autolysosomes that engulf cytoplasm and organelles and cannibalize the cell (5,12). In cells expressing activated Ras, the large macropinocytotic vacuoles that eventually fill the degenerating cells are morphologically distinct from autophagosomes. Although autophagosomes seem to accumulate in parallel with the macropinocytotic vacuoles, our studies with beclin-1 knockdown cells suggest that macropinocytotic vacuolization and cell death induced by Ras(G12V) can occur independent of the autophagy machinery. Thus, in this case, autophagy may reflect an attempt of the cells to survive under the adverse metabolic conditions created by rampant macropinosome accumulation, rather than a direct cause of cell death. This would be consistent with accumulating evidence that autophagy can function as a protective strategy against apoptosis or necrosis in cells subjected to metabolic stress (14).

The cytopathology induced by activated Ras or Rac is also distinct from several lesser known forms of cell death. Necroptosis can be triggered by stimulation of death receptors under conditions where caspases are inhibited (44,45). Cell swelling and
membrane rupture occur, but a massive increase in vesicular fluid uptake is not a diagnostic feature of necroptosis. Moreover, we have observed that necrostatin, a potent inhibitor of necroptosis, does not impede Ras-induced vacuolization or cell death (unpublished). Oncosis is a form of caspase-independent death typically caused by ischemia or disruption of ion pumps (43,51). As in the case of the Ras-induced cell death, oncosis can include cell swelling, vacuolization, and membrane rupture. Cytoplasmic vacuolization also occurs in another distinctive form of cell death termed paraptosis (39). However, in both oncosis and paraptosis the vacuoles are derived mainly from distended endoplasmic reticulum and/or mitochondria rather than macropinosomes. Finally, although cytoplasmic vacuolization is sometimes mentioned as a feature in various forms of necrosis-like cell death involving lysosomal damage, we have not detected alternative 50 kDa PARP cleavage products that would signal the leakage of lysosomal cathepsins associated with this form of necrosis (38). Nor have we observed any mitigation of Ras-induced cell degeneration by treating glioblastoma cells with cathepsin or calpain inhibitors. In light of these differences with other types of cell death, and the unique association of cellular degeneration with hyperstimulation of macropinocytosis (cell drinking), we propose that this form of cell death be named “methuosis” (methuo, from the Greek; to drink to intoxication). Table 1 summarizes the features of methuosis in comparison with other reported forms of cell death.

Activating mutations in Ras have long been regarded as oncogenic because they result in chronic stimulation of signaling pathways important for cell proliferation (52). Activated Ras may also contribute to tumor progression by protecting transformed cells
from apoptosis, although some reports have described opposite pro-apoptotic functions for Ras (53). The stimulation of a non-apoptotic death mechanism by activation of Ras highlights a relatively unexplored aspect of Ras signaling pathways. Our finding that activated Ras can induce vacuolization and cellular degeneration in a variety of human glioma cell lines, including those like U251 and T98G which harbor PTEN and p53 mutations that render them relatively resistant to apoptosis, suggests that the presence of Ras-responsive pathways capable of hyperstimulating macropinocytosis and/or inhibiting the clearance or recycling of macropinosomes, may be a general feature of human glioblastoma. If so, further delineation of the relevant signaling mechanisms could suggest ways to manipulate this pathway to trigger cell death in these intractable tumors.

In this regard, our previous studies have indicated that stimulation of vacuolization in glioblastoma cells does not depend on conventional Ras effectors such as Raf, PI 3-Kinase, and Ral-GDS (21). In considering alternative possibilities, it is noteworthy that the Rac1 GTPase has been implicated as a regulator of macropinocytosis (35). Indeed, as we have shown here, expression of activated Rac1(G12V) in glioblastoma cells can mimic the effects of Ras(G12V). Since downstream targets of Ras include guanine nucleotide exchange factors like Tiam1, which can stimulate activation of Rac1 (37), our working hypothesis is that these exchange factors may be the key Ras effectors involved in transmitting signals via Rac to the macropinocytotic machinery.

Our studies comparing glioblastoma cells to HeLa, HEK293 and HEp2 cells suggest that there is definite cell-type specificity in the ability of Ras to stimulate methuosis. Similar conclusions were drawn by Chi et al (15), who observed Ras-induced
vacuolization in glioblastoma and two gastric cancer cell lines, but not in bladder carcinoma cells. Thus, obtaining a better understanding of the basis for the particular sensitivity of certain cell types to methuosis will be important for evaluating the therapeutic potential of this form of cell death. Our results with dextran tracers (Fig. 5D) indicate that, unlike normal macropinosomes, the Ras-induced vacuoles do not dissipate or fuse with lysosomal compartments after they are internalized. This raises the possibility that the explanation for the differential sensitivity to methuosis could reside not only at the level of induction of macropinocytosis, but also at the level of intracellular trafficking or membrane channel function.

Another important question for future study is whether stimuli other than ectopic expression of Ras or Rac can provoke methuosis. In this regard it is worth noting that cytoplasmic vacuolization is often mentioned as a morphological feature of necrotic cell death caused by cytotoxic drugs or adverse environmental conditions, but there is seldom any indication as to the specific origin of the vacuoles. Thus, it is conceivable that dysregulation of macropinocytosis may be a common occurrence in forms of cell death labeled as necrosis, and it may therefore be more widespread than previously recognized.

**Acknowledgments:** We thank Thomas Sawyer and Karen Domenico for help with flow cytometry, and William Gunning, Ph.D and Michelle Lewandowski for assistance with electron microscopy.
References


death in malignant glioma cells by arsenic trioxide. Cancer Res 2003; 63:2103-
2108.

10. Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. Molecular
response of human glioblastoma multiforme cells to ionizing radiation: cell
cycle arrest, modulation of the expression of cyclin-dependent kinase

Synergistic augmentation of rapamycin-induced autophagy in malignant
glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors.

12. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor


H, Yoshida M, Chen W, Asai A, Himeno M, Yokoyama S, Kuchino Y.
Oncogenic Ras triggers cell suicide through the activation of a caspase-
independent cell death program in human cancer cells. Oncogene 1999;
18:2281-2290.


Figure Legends

Figure 1. Expression of activated H-Ras induces cytoplasmic vacuolization and death of U251 glioblastoma cells. (A) On day-0 (24 h after plating) U251-C18 glioblastoma cells were switched to medium containing 1 μg/ml doxycycline (+Dox) or maintained in medium without Dox. Western blots show the expression of myc-tagged H-Ras(G12V) in parallel cultures at intervals after addition of Dox. Phase contrast microscopy of the same cultures shows extensive accumulation of cytoplasmic vacuoles in the cultures expressing Ras(G12V). (B) MTT assays show a decline the number of viable cells in cultures expressing H-Ras(G12V). Each point represents the mean (± S.D.) of determinations performed on four separate cultures in a 96-well plate initially seeded at 5,000 cells/well. MTT was added directly to the medium to avoid losing detached cells. (C) Live versus dead fluorescence assays were performed on detached cells collected in the medium from 3 parallel cultures between day-4 and day-7 after addition of Dox. The attached cells from the same cultures were collected on day-7. For each determination 50 cells were scored. (D) ATP assays were performed on attached or detached cells after 6 days in medium with or without Dox. Results are means (± S.D.) from determinations performed on three separate cultures. (E) Cells were plated for colony-forming assays as described in the Materials and Methods. 24 h after plating, cultures were switched to medium with or without Dox. After 3 weeks colonies with >50 cells were counted. The results are the mean ± S.D. from 4 plates.
Figure 2. Vacuoles induced by activated Ras are distinct from autophagosomes.  (A) U251 cells were infected with myc-H-Ras(G12V) retrovirus to induce vacuoles and then examined by electron microscopy after two days. Vacuoles (V) are electron lucent and much larger than the typical autophagosomes with double membrane morphology (indicated with black arrows). The white arrow points to the nuclear membrane and N denotes the nucleus. The bar in each panel is 1 μm.  (B) Nucleofected U251 cells transiently expressing myc-H-Ras(G12V) were co-stained with antibodies recognizing endogenous LC3 (upper panel) and the myc-epitope (lower panel). The asterisks mark cells in the same field that did not express myc-tagged Ras.  (C) A confocal image of stable U251-C18 cells incubated with Dox for 4-days, showing distinct localization of myc-H-Ras(G12V) (red) and endogenous LC3 (green) (bar = 10 μm).  (D) Accumulation of the autophagosome marker, LC3-II, in cells expressing Ras(G12V). On the day after plating, U251-C18 cells were changed to medium with or without Dox as indicated. At the same time the cultures received E64D and Pepstatin A (protease inhibitors), or DMSO (no inhibitors). After 2 days immunoblot analysis was performed to determine levels of myc-Ras(G12V), LDH, and LC3-II as described in Materials and Methods. The upper panels show representative blots (scans from the Alpha Innotech imaging system) documenting expression of myc-H-Ras(G12V), LC3-II, and LDH. The graphs in the lower panel show the results obtained when the ratio of LC3-II to LDH was quantified in triplicate samples.
**Figure 3.** Suppression of the expression of the pro-autophagy protein, beclin-1, does not interfere with Ras-induced vacuolation and cell death. (A) U251 cells expressing non-specific (control) or beclin-specific RNAi (beclin KD) were generated as described (16). These cells were nucleofected with the pCMV5myc-H-Ras(G12V) expression vector or empty vector and examined 24 h later. (A) Western blots show that myc-Ras(G12V) was expressed equally in the control and beclin KD cells. Expression of beclin-1 was reduced by >90%. Equal amounts of protein were loaded in each lane. (B) Representative cells showing the immunofluorescence localization of myc-H-Ras(G12V) to prominent cytoplasmic vacuoles in the control and beclin KD cells. Cell rounding and detachment occurred in both the control and beclin KD cells. (C) The bar graph shows the percentage of vacuolated cells in the control and beclin KD cultures depicted in panel B. The values were determined by counting 100 cells in random photomicrographs, using a threshold of two or more vacuoles (> 0.5 μm diameter) per cell for a positive score. (D) The stable tet-inducible U251-C18 cell line was infected with lentivirus containing inverted repeat stem-loop RNAi sequences matching a unique region of the human beclin mRNA, or a “control” sequence that did not match any known GenBank entry. Beclin knockdown was verified to be >90% in the beclin KD cells compared to the controls. The control and beclin KD cell lines were then incubated with or without Dox as described in the legend to Fig. 1. On the sixth day after addition of Dox, the number of attached cells in the control and beclin KD cultures expressing H-Ras(G12V) (+Dox) was determined and the results were expressed as percent of the number of attached cells in the parallel cultures without Dox. Results are the mean ± S.D. of separate determinations from three
cultures. (E) Aliquots of cells from the U251-C18 control and beclin KD cell lines were plated for colony-forming assays with or without addition of Dox to induce Ras(G12V) expression. The number of colonies was counted in three cultures from each group.

**Figure 4.** Vacuoles induced by Ras(G12V) in U251 cells exhibit characteristics of macropinosomes. (A) Phase-lucent vacuoles incorporate the extracellular fluid phase tracer, Lucifer Yellow (LY). Live cells were incubated with LY for 15 min on the fourth day after they were induced to express myc-H-Ras(G12V) by addition of Dox. (B) U251-C18 cells maintained for 2-days with or without Dox were incubated with LY for 15 min and uptake of the tracer was quantified by flow cytometry. Where indicated, the cells were pre-incubated for 30 min with cytochalasin D (Cyto D) before adding the LY. (C) Electron microscopy of attached U251-C18 cells expressing myc-H-Ras(G12V) reveals the presence of numerous lamellipodia that close to form nascent macropinosomes, indicated by asterisks. The scale bars represent 1 μm. (D) Vacuoles that incorporate the fluid phase tracer, dextran-AF488, are separate from compartments that incorporate transferrin-AF594. Live vacuolated cells were incubated with the indicated fluorescent probes for 15 min on the fourth day after induction of myc-H-Ras(G12V) with Dox. The same field of cells is shown in the phase and fluorescence micrographs. (E) Vacuolated cells expressing myc-H-Ras(G12V) were fixed and subjected to immunofluorescence microscopy to localize the endosomal marker, EEA1.
**Figure 5.** Vacuoles induced by Ras(G12V) in U251 cells are distinct from late endosomes and lysosomes. Four days after inducing U251-C18 cells to express myc-H-Ras(G12V), live cells were (A) incubated with Lysotracker Red to label acidic compartments or (B) incubated with Magic Red-RR to label compartments containing cathepsin B activity. (C) Vacuolated cells expressing Ras(G12V) were fixed and subjected to immunofluorescence microscopy to localize LAMP1. (D) Vacuolated cells expressing Ras(G12V) were pre-incubated with Lysotracker Red (red) for 3 h, washed, and then incubated for 4 h with dextran-AF488 (green). The phase image in the top panel shows the same field of cells depicted in the fluorescent images.

**Figure 6.** Activated Rac1 mimics the effects of activated Ras in glioblastoma cells. A stable U251 cell line for conditional expression of myc-Rac1(G12V) was generated as described in Materials and Methods. (A) On day-4 and day-8 after addition of Dox the expression of myc-Rac1(G12V) was checked by immunoblot analysis with an antibody against the myc epitope. (B) Phase contrast microscopy of the same cultures shows extensive accumulation of cytoplasmic vacuoles and cell detachment in the cultures expressing Rac1(G12V). (C) MTT assays show a decline in the number of viable cells in the +Dox cultures compared to the −Dox controls. Each point represents the mean (± S.D.) of determinations performed on six separate cultures in a 96-well plate. (D) U251 cells expressing Rac1(G12V) incorporate LY into the phase-lucent vacuoles. Cells were incubated with LY for 15 min on the fourth day after addition of Dox. The micrograph show a merge of the phase contrast image with the fluorescence image of the live cells.
Figure 7. Progressive degeneration of glioblastoma cells expressing activated Ras involves macropinosome expansion and non-essential activation of caspases. (A) Expression of myc-H-Ras(G12V) was induced by incubating stable U251-C18 cells with Dox. The medium was replaced every two days. Detached cells were collected between days 4-7. Attached cells were collected on day-7. As a positive control for apoptotic cleavage of PARP and lamin A/C, cells maintained without Dox were treated with staurosporine for 24 h. Immunoblots showing PARP (upper panel) and lamin A/C (lower panel) reveal cleavage products indicative of caspase activation. (B) U251-C18 cells were seeded and maintained with or without Dox as described in Fig. 1. Starting on day-4, half of the +Dox cultures were changed to medium with 50μM zVAD-fmk in DMSO. The –Dox cultures (no Ras expression) received DMSO alone. On day-6 the attached and detached cells were harvested for immunoblot analysis of PARP. (C) MTT assays were performed on day-4 and day-6 comparing the viability of the cells in the +Dox cultures with –Dox controls. Each result is the mean ± S.D. of assays performed on four wells of a 96-well plate. (D) Electron micrographs of representative detached U251-C18 cells collected between days 4-6 after induction of Ras(G12V) expression. N = nucleus. Scale bar is 10 μ.
Supplementary Material

**Figure S1.** Expression of Ras(G12V) does not alter the cell cycle distribution of attached vacuolated cells. Stable U251-C18 cells were grown with or without Dox, as in Fig. 1. Attached cells from three parallel cultures were harvested on day-6 and cell cycle distribution was determined by flow cytometry as described in Materials and Methods. Results are means (± S.D.) of separate determinations performed on three parallel cultures.

**Figure S2.** Activated H-Ras induces a vacuolar phenotype and cellular degeneration in multiple human glioma cell lines. (A) The indicated cell lines were infected with retrovirus expressing myc-H-Ras(G12V) or myc-H-Ras(S17N) and cells were photographed at 300x magnification. After three days the cells expressing Ras(G12V), but not those expressing Ras(S17N), began to detach from the dish and undergo lysis. (B) myc-H-Ras(G12V) was introduced into U251, HEK293, HeLa or HEp2 cells by nucleofection and cells were examined by phase contrast or immunofluorescence microscopy 24 h later.

**Figure S3.** Electron microscopy shows that the Ras-induced vacuoles are delimited by a single membrane. (A) U251 cells were infected with myc-H-Ras(G12V) retrovirus to induce vacuoles and then examined by electron microscopy after two days. The scale bar represents 1 μm. (B) The area outlined in panel A, viewed at higher magnification. The bar represents 0.1 μm.
**Figure S4.** Detached cells collected after induction of H-Ras(G12V) contain activated caspase-3 and show signs of nucleosomal DNA degradation. (A) U251-C18 cells were collected between days 4-7 after Dox was added to stimulate expression of H-Ras(G12V). The cells were stained with a caspase substrate that fluoresces when cleaved by caspase-3. Counts performed on three separate cultures (80-90 cells per culture) indicated that 49.6 ± 7.5% of the detached cells were positive for caspase-3 activity. There were no caspase-3 positive cells in the attached population (not shown). (B) Cells were maintained with or without Dox as described in Fig. 1. Detached cells were collected between days 4-7. Attached cells were harvested on day-7. The agarose gel shows DNA isolated from the attached and detached cells under conditions that favor extraction of DNA fragments, as described in Materials and methods.

**Figure S5.** Calpain and cathepsin inhibitors do not protect U251 cells from Ras-induced death. Cells were seeded in 96-well plates at 5,000 cells/well and maintained with or without Dox as described in Fig. 1. Starting on day-5, the –Dox and +Dox cultures were changed to medium with either 25 μM ALLN, an inhibitor of calpains, cathepsins B and L, and neutral cysteine proteases (EMD Chemicals, San Diego, CA), or 100 μM PD150606, a selective calpain inhibitor (EMD Chemicals), reconstituted in DMSO. The remaining +Dox and –Dox cultures received DMSO alone. On Day-7 the viability of the cells in the +Dox cultures (with or without protease inhibitors) was compared with the corresponding –Dox controls using the MTT assay. Each result is the mean ± SD of assays performed on four separate cultures in a 96 well plate.
Table 1. Comparison of Methuosis with Other Common Forms of Cell Death

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Methuosis†</th>
<th>Autophagic</th>
<th>Paraptosis</th>
<th>Oncosis</th>
<th>Necroptosis</th>
<th>Necrosis</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic vacuolation (origin)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(macropinosomes)</td>
<td>(autophagosomes, autolysosomes)</td>
<td>(ER, mitochondria)</td>
<td>(ER, mitochondria)</td>
<td>(ER, mitochondria)</td>
<td>(uncertain)</td>
<td></td>
</tr>
<tr>
<td>Cell swelling</td>
<td>+</td>
<td>- / +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Plasma membrane rupture</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Membrane blebbing</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Nucleosomal DNA fragmentation (laddering)</td>
<td>+</td>
<td>- / +(late)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chromatin marginalization or condensation</td>
<td>-</td>
<td>- / +(late)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Caspase activation</td>
<td>+</td>
<td>- / +(late)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- / +</td>
<td>+</td>
</tr>
<tr>
<td>Mitochondrial membrane depolarization</td>
<td>?</td>
<td>- / +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ATP depletion</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Autophagic Activity increased</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>- / +</td>
</tr>
</tbody>
</table>

* Information complied from references cited in the Discussion.
† Based on observations with U251 glioblastoma and other glioma cell lines listed in Supplementary Fig 2. Morphological features consistent with methuosis have also been observed when Ras(G12V) is expressed in MKN-1 and TMK-1 gastric carcinoma cells (15) and MCF7 breast carcinoma cells (unpublished observations)
FIGURE 1.
FIGURE 2.
FIGURE 3.
FIGURE 4.
FIGURE 5.
FIGURE 6.

A

Day 4
Dox - +

Day 8
Dox - +

B

Day 4
-Dox +Dox

Day 8
-Dox +Dox

C

Cell Viability (% of Control)

Day 4 Day 8

D

image of cells
FIGURE 7.

A

B

C

D

i

ii

iii

iv

PARP

Cleaved PARP

Lamin A/C

Cleaved Lamin

+Dox (RasG12V)

+Stauroporine

PARP

Cleaved PARP

Dox

Dox (RasG12V)

+Dox (RasG12V) +zVAD

Cell Viability

% of Control

Day-4

Day-6

N
FIGURE S1.
FIGURE S2.

### A

<table>
<thead>
<tr>
<th></th>
<th>U251</th>
<th>A172</th>
<th>T98G</th>
<th>U87</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Ras (G12V)</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>H-Ras (S17N)</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>SF295</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>SNB75</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>SNB19</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>LN229</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th></th>
<th>U251</th>
<th>HEK293</th>
<th>HeLa</th>
<th>HEp2</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc-H-Ras (G12V)</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>myc-H-Ras (G12V)</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
</tbody>
</table>
FIGURE S4.
FIGURE S5.
Activated Ras Induces Cytoplasmic Vacuolation and Non-Apoptotic Death in Glioblastoma Cells via Novel Effector Pathways

Aparna Kaul§, Jean H. Overmeyer§ and William A. Maltese*


Department of Biochemistry and Cancer Biology, University of Toledo College of Medicine, Toledo, Ohio, USA

Running title: Non-Apoptotic Death Induced by Ras

Key Words: Ras, Non-Apoptotic Cell Death, Vacuoles, Glioblastoma, Raf, PI 3-Kinase, Farnesylation, Autophagy

§These authors contributed equally to the work.

*Correspondence: Dr. William A. Maltese, Department of Biochemistry & Cancer Biology, Block Health Sciences Bldg, University of Toledo College of Medicine, 3035 Arlington Ave., Toledo, Ohio, 43614
E-mail: william.maltese@utoledo.edu
Abstract

Expression of activated H-Ras induces a unique form of non-apoptotic cell death in human glioblastoma cells and other specific tumor cell lines. The major cytopathological features of this form of death are the accumulation of large phase-lucent, LAMP1-positive, cytoplasmic vacuoles and increased autophagic activity. In this study we sought to determine if induction of cytoplasmic vacuolation a) depends on Ras farnesylation, b) is specific to H-Ras, and c) is mediated by signaling through the major known Ras effector pathways. We find that the unusual effects of activated H-Ras depend on farnesylation and membrane association of the GTPase. Both H-Ras(G12V) and K-Ras4B(G12V) stimulate vacuolation, but activated forms of Cdc42 and RhoA do not. Amino acid substitutions in the Ras effector domain, which are known to selectively impair its interactions with Raf kinase, class-I phosphatidylinositide 3-kinase (PI-3K), or Ral nucleotide exchange factors, initially pointed to Raf as a possible mediator of cell vacuolation. However, the MEK inhibitor, PD98059, did not block the induction of vacuoles, and constitutively active Raf-Caax did not mimic the effects of Ras(G12V). Introduction of normal PTEN together with H-Ras(G12V) into U251 glioblastoma cells reduced the PI-3K-dependent activation of Akt, but had no effect on vacuolation. Finally, co-expression of H-Ras(G12V) with a dominant-negative form of RalA did not suppress vacuolation. Taken together, the observations indicate that Ras activates non-conventional and perhaps unique effector pathways to induce cytoplasmic vacuolation in glioblastoma cells. Identification of the relevant signaling pathways may uncover
specific molecular targets that can be manipulated to activate non-apoptotic cell death in this type of cancer.
1. Introduction

Oncogenic mutations in ras genes occur in approximately 30% of human malignancies [1]. In their active GTP-bound conformation, Ras proteins interact with a variety of downstream effectors that regulate cell proliferation, differentiation, and survival [2,3]. Signals are normally terminated when GTP is hydrolyzed and Ras reverts to the inactive GDP state. The mutant forms of Ras typically found in tumors harbor amino acid substitutions (e.g., G12→V) that reduce their GTPase activity [4,5]. This results in sustained stimulation of Ras pathways that promote cell proliferation and survival [6]. However, in some types of cells activated Ras can trigger cellular senescence [7], apoptosis [8] or non-apoptotic cell death [9]. The most notable example of the latter has been observed in human glioblastoma, gastric carcinoma, and neuroblastoma cells, where introduction of H-Ras(G12V) triggers accumulation of cytoplasmic vacuoles and cell death without caspase activation or DNA fragmentation [9-11]. This form of cell death was originally described as Type-II programmed cell death [10], a term often used synonymously with autophagic cell death [12,13]. Type-II cell death has received increasing attention as a significant alternative pathway for cell death in cancer [14-18]. However, to date there have been no follow-up studies aimed at defining the signaling pathways through which activated Ras can trigger the initial cytoplasmic vacuolation that ultimately leads to loss of cellular integrity and non-apoptotic death.

In the present study we set out to determine a) if the induction of vacuolation in glioblastoma cells requires H-Ras farnesylation and membrane association, b) if the
effect is specific to H-Ras, and c) if vacuolation is linked to activation of the major known Ras effector pathways. We find that the induction of vacuolation requires Ras farnesylation and is sensitive to effector domain mutations, but it does not depend on activation of the Raf, phosphatidylinositide 3-kinase (PI-3K), or RalGDS signaling pathways. These observations raise the prospect that Ras may be acting through an atypical or perhaps unique effector pathway to induce vacuolation and non-apoptotic death in glioblastoma cells.

2. Materials and methods

2.1 Cell culture

U251 glioblastoma cells were obtained from the DCT Tumor Repository (National Cancer Institute, Frederick, MD, USA). Cells were maintained at 37°C with 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Phase contrast images of the live cells were obtained using an Olympus IX70 inverted microscope equipped with a digital camera, using SPOT imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.2 Generation of expression constructs for nucleofection

H-Ras (G12V) cDNA was purchased from Clontech (Palo Alto, CA, USA). The cDNA was subcloned into a pCMV5 vector that had been modified by PCR to encode either an in-frame myc epitope tag (MEQKLISEEDDL) [19] or a FLAG epitope tag (DYKDDDDK) [20]. Amino acid substitutions (C186S, Y40C, D38E and E37G) were
created by PCR modification of the *myc-H-Ras(G12V)* cDNA using Pfu polymerase
(Stratagene, La Jolla, CA, USA) and appropriate oligonucleotide primers. pcDNA3.1
containing the G12V mutant form of 2x myc-tagged *K-Ras2* (*i.e.*, *K-Ras4B*) was
purchased from the UMR cDNA Resource Center (http://www.cdna.org  Rolla, MO,
USA). PRK5 vectors encoding myc-Cdc42(G12V) and myc-RhoA(G14V) were
provided by A.L. Wilson-Delfosse, Case Western University, Cleveland, OH. Human
*PTEN* in pCMV6-XL5 was purchased from Origene Technologies Inc., Rockville, MD,
USA. The pCMV-*RafCaax* construct was purchased from Clontech. The insert was
subcloned into the pCMV5 vector with the myc-tag sequence described above. Human
*RalA* (GenBank accession number NM_005402) was modified by overlap extension PCR
to generate the S28N point mutation. The resulting PCR product was subcloned into the
aforementioned pCMV5 vector with an in-frame 5’ myc-tag sequence. All of the
expression vectors were introduced into the U251 cells by nucleofection, using the
Nucleofector II system from Amaxa Biosystems (Koeln, Germany) following the
manufacturer’s protocol with Nucleofector Solution T in combination with program T-
30. For each reaction, 1-5 μg of plasmid DNA was introduced into 3 x 10^6 cells that had
been harvested in RPMI 1640 medium. Following nucleofection, the cells were plated in
60 mm dishes and analyzed 18-24 hours later.

2.3 *Generation of Ras constructs for retroviral expression*

Myc-tagged H-Ras constructs, (G12V and S17N) were subcloned into the EcoR1-BamH1
sites of the retroviral expression vector, pFBneo (Stratagene, La Jolla, CA, USA).
Retrovirus was produced in HEK293-GPG packaging cells and glioblastoma cells were infected as described previously [21]. Non-infected cells were eliminated by incubation for 2 days post-infection in medium containing 2.0 mg/ml G418.

2.4 Immunofluorescence microscopy

Cells were grown on laminin-coated glass coverslips, washed with Hanks balanced salt solution (HBSS), fixed in ice-cold methanol for 10 min, and blocked with 10% goat serum in PBS. For detection of myc-H-Ras(G12V,C186S) and 2x-myc-K-Ras2(G12V) the procedure was modified slightly to include a preliminary fixation with 3% paraformaldehyde prior to the methanol step. Myc-tagged proteins were detected by incubation for 1 h with an anti-myc monoclonal antibody (Calbiochem, San Diego, CA, USA) diluted in PBS + 10% goat serum, followed by a 1-h incubation with goat anti-mouse IgG conjugated with Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA). Antibodies and procedures used for immunofluorescence localization of LAMP1 have been described previously [22]. For experiments where myc-tagged RalA was co-expressed with FLAG-tagged Ras, we used an anti-FLAG monoclonal antibody (Sigma, St. Louis, MO, USA) followed by goat anti-mouse IgG-Alexa Fluor 568, combined with rabbit anti-myc IgG conjugated directly to FITC (ICL Labs, Newberg, OR, USA). Photomicrographs were taken with a Nikon Eclipse 800 fluorescent microscope equipped with a digital camera and ImagePro software (Media Cybernetics, Silver Spring, MD, USA).
2.5 Western blot analysis

Antibodies used for western blotting were obtained from the following sources: p44/42 MAP Kinase (ERK1/2), phospho-p44/42 MAP Kinase (ERK1/2) (Thr202/Tyr204), phospho-Akt (Ser473), and total Akt (Cell Signaling Technology, Danvers, MA, USA); myc epitope tag (EMD Biosciences, San Diego, CA, USA); Raf-1 and PTEN (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein determinations, SDS-PAGE, and western blot analyses were performed as described previously [19]. Chemiluminescent immunoblot signals were quantified using a Kodak 440CF Image Station.

3. Results

3.1 Induction of cytoplasmic vacuolation in human glioblastoma cells by activated H-Ras(G12V) requires Ras farnesylation

In their initial study Chi et al. [10] demonstrated that expression of oncogenic H-Ras(G12V), but not GDP-locked H-Ras(S17N), causes massive accumulation of phase-lucent cytoplasmic vacuoles in U251 glioblastoma cells and gastric carcinoma cells. The vacuolated cells round up, detach from the substrate and disintegrate. This appears to be a form of non-apoptotic programmed cell death, since it occurs without substantial mitochondrial swelling, caspase activation or chromosomal DNA fragmentation [10]. We have observed a similar effect of activated H-Ras in a broad spectrum of human glioblastoma cell lines, but not in other commonly studied transformed cell lines (Overmeyer et al., unpublished).
H-Ras normally undergoes a series of sequential modifications commencing with the attachment of a farnesyl moiety to cys-186, followed by proteolytic removal of the three distal amino acids, and finally, carboxyl-methylation of the terminal cysteine [23]. In H-Ras, palmitylation of two upstream cysteines also occurs. In the absence of farnesylation, H-Ras remains predominantly in the cytosol, and subsequent modifications by membrane-associated enzymes do not occur [24]. Therefore, to evaluate the importance of membrane localization of H-Ras(G12V) for induction of the vacuolar phenotype, we introduced an amino acid substitution that prevents farnesylation; *i.e.*, C186→S. The cysteine mutation eliminated the ability of activated H-Ras to induce phase lucent cytoplasmic vacuoles (Fig. 1a) and the cells continued to proliferate without detaching from the dish (not shown).

Many of the Ras-induced vacuoles contain the lysosomal membrane protein, LAMP1 (ref. 10 and Fig. 4 in this report), suggesting that they arise from lysosomal compartments. However, these structures have not yet been characterized in sufficient detail rule out other origins (e.g., late endosomes, autophagosomes, autolysosomes). Immunofluorescent localization of myc-tagged H-Ras(G12V) revealed a striking association of the GTPase in the membranes surrounding the cytoplasmic vacuoles (Fig. 1b). In contrast to cells expressing H-Ras(G12V), the cells expressing the non-farnesylated myc-H-Ras(G12V, C186S) did not form vacuoles and the Ras protein exhibited a diffuse cytoplasmic distribution (Fig. 1b).
3.2 Activated K-Ras, but not RhoA or Cdc42, can mimic the effects of activated H-Ras

The amino acid sequences of Ras proteins are highly similar throughout their core regions (amino acids 1-170), which contain the nucleotide binding and effector domains [25]. Most of the variation among the Ras proteins occurs in the last 20-24 amino acids, which comprise the COOH-terminal “hypervariable” domain and the farnesylation motif. In the cases of H-Ras and K-Ras4B, differences in the COOH-terminal sequences (Fig. 2a) dictate how the proteins will associate with membranes. For instance, H-Ras associates with membranes via farnesylation and palmitylation, whereas K-Ras4B relies on farnesylation combined with the upstream polylysine domain [26]. The different lipid modifications, combined with variations in amino acids between 167-172, also influence the targeting of H-Ras and K-Ras to separate membrane microdomains [26,27].

In light of these distinctions, we asked whether activated K-Ras4B was also capable of inducing the vacuolar phenotype in U251 cells. As shown in Fig. 2c, cells expressing myc-tagged K-Ras4B(G12V) formed phase-lucent vacuoles that were rimmed with the expressed protein, similar to the observations with H-Ras(G12V) (Fig. 2b). As in the case of H-Ras(S17N), the GDP-locked K-Ras4B(S17N) was ineffective at inducing vacuoles (not shown). Although activated forms of both H-Ras and K-Ras produced similar morphological effects, overexpression of other Ras-related GTPases with activating mutations, Cdc42(G12V) (Fig. 2d) and RhoA(G14V) (Fig. 2e), did not trigger the formation of cytoplasmic vacuoles.
3.3 **Effector domain mutations have differential effects on the ability of H-Ras(G12V) to induce vacuoles**

The preceding results suggested that the ability of Ras to activate pathways responsible for causing vacuolation depends on domains common to H-Ras and K-Ras rather than on unique COOH-terminal sequences or membrane localization signals. In light of these findings, we used mutagenesis of the Ras effector domain as an initial experimental strategy to evaluate the relative importance of different Ras effector pathways for induction of the vacuolar phenotype. The effector domain (amino acids 32-40) is one of two major “switch” regions that change conformation when the nucleotide binding pocket of Ras is occupied by GTP versus GDP \[28,29\]. Specific amino acid substitutions in the effector domain selectively prevent the interaction of activated Ras with its major targets: Raf-1, PI-3K p110\(\alpha\), or RalGDS \[30-33\] (summarized in Fig. 3a).

We first tested the D38E substitution, which is permissive for binding to Raf, but not to PI-3K or RalGDS. Cells expressing H-Ras(G12V, D38E) exhibited robust vacuolation, reduced only slightly when compared with G12V alone (Fig. 3c). This finding suggested that interactions with PI-3K and RalGDS are not critical for vacuolation, and pointed to the potential importance of Raf. Consistent with this idea, introduction of the Y40C substitution, which allows interaction with PI-3K, but blocks interaction with Raf and RalGDS, markedly reduced the ability of H-Ras(G12V) to induce vacuoles (Fig. 3c).

The final mutant tested was H-Ras(G12V, E37G). The E37G substitution is known to prevent Ras interaction with both Raf and PI-3K, while preserving the interaction with RalGDS and several other effectors. As shown in Fig. 3c, incorporation of this
substitution caused a substantial reduction in the percentage of vacuolated cells. Thus, in summary, the combined results with all three effector mutants were inconsistent with a key role for PI-3K or RalGDS and pointed to the Raf pathway as the most likely mediator of Ras-induced vacuolation.

3.4 Induction of cytoplasmic vacuolation by activated Ras does not depend on activation of Raf signaling pathways

In light of the preceding studies, we used alternative approaches to determine if the stimulation of cytoplasmic vacuoles in U251 cells by H-Ras(G12V) is directly related to its activation of the Raf→MEK→ERK1/2 signaling pathway. A specific MEK inhibitor (PD98059) was included in the medium for 42 h after infection of the cells with retrovirus expressing myc-H-Ras(G12V). The MEK inhibitor suppressed the H-Ras(G12V)-induced phosphorylation of ERK1/2 to a level approaching that detected in the negative control cells expressing myc-H-Ras(S17N) (Fig. 4a). However, the suppression of ERK phosphorylation did not prevent the accumulation of LAMP1-positive vacuoles in the cells expressing H-Ras(G12V) (Fig. 4b).

As a different strategy to specifically address the role of Raf activation in cellular vacuolation, we activated the Raf→MEK→ERK1/2 signaling pathway independently of Ras. This was done by attaching a Caax prenylation motif to the C-terminus of Raf, thereby generating a constitutively active form of Raf that remains associated with the cell membrane independent of Ras [34]. When this construct was expressed in U251 cells, it induced an increase in ERK phosphorylation that was even greater than that
induced by H-Ras(G12V) (Fig. 5b). However, the activation of ERK by Raf-Caax did not trigger vacuolation of the glioblastoma cells (Fig. 5c). These results demonstrate that the induction of vacuolation by activated Ras involves effector pathways distinct from the Raf→MEK→ERK1/2 cascade.

3.5 Class I PI-3K is not an essential mediator of vacuolation induced by H-Ras(G12V)

The GTP-bound form of Ras activates the p110 catalytic subunit of class I PI-3K [35], resulting in increased production of PI(3,4,5)P3 [36]. Although the results of the effector domain mutagenesis studies (Fig. 3) suggested that interaction with PI-3K is not essential for H-Ras(G12V) to trigger cellular vacuolation, we wanted to confirm this by a separate method. The use of PI-3K inhibitors like wortmannin was not desirable because they can perturb endocytic trafficking and cause the formation of vacuoles derived from multivesicular endosomes [37]. Instead, we took advantage of the fact that U251 cells, like many other human glioblastoma lines, carry a mutation in the PTEN gene [38]. PTEN normally dephosphorylates the 3’ position of PI(3,4,5)P3, facilitating turnover of the PI-3K product and downregulating signaling to the Akt pathway [39,40]. By introducing normal PTEN into U251 cells expressing H-Ras(G12V) (Fig. 6a), we were able to effectively down-regulate PI-3K signaling, measured by Akt phosphorylation (Fig. 6b). Although coexpression of PTEN with H-Ras(G12V) reduced phosphorylation of Akt by more than 60%, there was no detectable reduction of vacuolation (Fig. 6c).
3.6 Ras-induced vacuolation is not blocked by coexpression with dominant-negative RalA

In addition to PI-3K and Raf, activated Ras binds and stimulates RalGDS [41,42]. The latter promotes exchange of GTP for GDP on two Ras-related GTPases, RalA and RalB. The Ral GTPases have been implicated in many of the downstream effects of Ras, including cell transformation, activation of transcription factors, and intracellular trafficking [43-45].

Our earlier observation that H-Ras(G12V, D38E) was a strong inducer of vacuolation (Fig. 3) despite its reported inability to interact with RalGDS [46], suggests that activation of RalGDS is not required for the Ras-induced phenotype. To confirm this observation, we coexpressed FLAG-tagged H-Ras(G12V) with myc-tagged RalA (S28N) in U251 cells (Fig. 7). Similar to Ras(S17N), substitution of S28→N in Ral locks the protein in the GDP state and renders it a dominant-negative suppressor of endogenous Ral function [47]. As expected, cells expressing FLAG-Ras(G12V) exhibited typical vacuolation, with localization of the Ras protein to the vacuole membranes. In the cells expressing myc-RalA(S28N) alone, there was no evidence of vacuolation and the expressed protein exhibited a diffuse localization with concentration in the plasma membrane. When FLAG-Ras(G12V) was co-expressed with myc-RalA(S28N), the dominant-negative Ral did not inhibit vacuolation. Interestingly, although activation of the Ral pathway does not appear to be causally related to vacuolation, coexpression of activated H-Ras(G12V) with RalA(S28N) changed the distribution of the latter so that it completely co-localized with H-Ras(G12V) on the vacuole membranes (Fig. 7b).
Although we did not explore this further, it would be consistent with a model in which activated H-Ras on the vacuole membrane binds to endogenous RalGDS or other Ral exchange factors, thereby shifting the distribution of the associated GDP-locked RalA(S28N) to this compartment.

4. Discussion

In the present study we report the first detailed exploration of the molecular signaling pathways underlying a unique form of Type-II (caspase-independent) programmed cell death triggered by expression of activated Ras in glioblastoma [9-10]. The major cytopathological features of this form of cell death are accumulation of phase-lucent, LAMP1-positive, cytoplasmic vacuoles, followed by cell detachment and disintegration.

Our results demonstrate that farnesylation is required for activated H-Ras to stimulate signaling pathways that induce cytoplasmic vacuolation. Farnesylation initially targets Ras proteins to the endoplasmic reticulum, where the Caax motif is further processed by a protease that removes the final three amino acids and a carboxyl methyltransferase that modifies the farnesylated cysteine. H-Ras and K-Ras subsequently take different routes to the plasma membrane [24]. Differences in their hypervariable C-terminal sequences and posttranslational modifications (see Fig. 2a) direct H-Ras to lipid rafts and K-Ras to non-raft regions [26]. We now show that both H-Ras(G12V) and K-Ras4B(G12V) are capable of stimulating cellular vacuolation and localizing to the vacuole membrane. This suggests that the reported difference in H-Ras versus K-Ras
membrane subdomain distribution is not a major factor in the signaling mechanism that triggers vacuolation. One possible explanation for this finding is that H-Ras leaves the rafts and joins K-Ras in the bulk plasma membrane when it is activated by GTP [48]. Alternatively, the signals that stimulate vacuolation may emanate from the pool of activated Ras associated with endomembrane compartments. This would be entirely consistent with evidence that Ras signaling can occur in the endoplasmic reticulum, Golgi apparatus and endosomes [49-52].

The amino acid sequences of H-Ras and K-Ras are identical in the domains termed switch-1 (amino acids 32-40) and switch-2 (amino acids 60-76), which undergo GTP-dependent conformational changes [28,53] and determine the interaction of Ras with downstream effectors [54,55]. In contrast, the Rho family members (Cdc42 and RhoA), diverge from Ras in these regions and do not stimulate vacuolation when expressed in their constitutively active forms (Fig.2). Together with the inability of GDP-locked Ras(S17N) to cause vacuolation and cell death [10], these findings strongly support the conclusion that the vacuolar phenotype is induced through stimulation of specific Ras effector pathways.

In seeking to define the relevant Ras signaling pathways, we focused initially on the well-known Raf→MEK→ERK1/2 kinase cascade. The first step in the cascade involves the activation of Raf by Ras-GTP [56,57]. Our interest in this pathway was motivated by several reports linking ERK activation to the regulation of macroautophagy, a common feature in Type-II cell death. Specifically, Codogno and colleagues showed that ERK1/2 stimulates autophagosome formation by phosphorylating and activating
GAIP (Gα-interacting protein), a GTPase activating protein for Gαi3, [58,59]. Later studies showed that this pathway can be stimulated by Ras [60]. Finally, a separate study in Sertoli cells treated with the carcinogen, lindane, has shown that activation of ERK can cause accumulation of LAMP1-positive vacuoles by interfering with the maturation of autophagosomes into autolysosomes [61]. The results of our studies with the Ras effector domain mutations supported the possible involvement of Raf. For instance, the Y40C and E37G substitutions, which impede interaction with Raf, reduced vacuolation, whereas the D38E mutation, which is permissive for Raf binding, had almost no effect (Fig. 3). However, two follow-up studies indicated that the reduced vacuolation caused by the Y40C and E37G mutations is probably related to interference with effectors other than Raf. First, blocking Raf→ERK signaling with a MEK inhibitor had no effect on Ras-induced vacuolation (Fig 4). Second, overexpression of a constitutively active form of Raf (Raf-Caaax) did not induce vacuoles, despite the fact that it stimulated ERK phosphorylation to an even greater extent than H-Ras(G12V) (Fig. 5).

The ability of Ras-GTP to bind to the p110 catalytic subunit and promote activation of Class-I PI-3K is now well established [62]. We initially thought that activation of Class I PI-3K by ectopic expression of Ras(G12V) would be an unlikely mechanism to stimulate Type-II cell death in glioblastoma, because the basal activity of the PI-3K→Akt pathway is already increased by the absence of PTEN in these cells. In addition, several studies indicate that class-I PI-3K is a negative regulator of autophagic activity [63-65]. Our suspicions were borne out by the data from the Ras effector domain studies, where the D38E mutation had little influence on the extent of vacuolation,
despite its reported ability to interfere with PI-3K interaction. Conversely, introduction of the Y40C mutation into H-Ras(G12V) reduced vacuolation even though this construct is competent to interact with PI-3K. Most importantly, our conclusion that vacuolation is not mediated by stimulation of PI-3K was strongly supported by the studies in Fig. 6, where introduction of PTEN into the glioblastoma cells expressing H-Ras(G12V) reduced the activation of Akt by more than 60% without having any effect on vacuolation.

Having ruled out Raf and PI-3K as likely mediators of the vacuolar phenotype induced by H-Ras(G12V), we turned our attention to a third major Ras effector, RalGDS. Activation of Ral GTPases has been implicated in the control of endocytic trafficking via the Ral effector, RalBP (RLIP76) [43,44,47]. However, our finding that neither the introduction of the non-permissive D38E effector mutation nor the coexpression of Ras(G12V) with the dominant-negative RalA(S28N) interfered with vacuolation argues against an essential role for pathways regulated by Ral nucleotide exchange factors.

Despite the evidence against Raf, PI-3K, and RalGDS as key effectors, the reduced vacuolation observed with the Y40C and E37G mutations supports the premise that vacuolation requires activation of specific Ras signaling pathways. Many additional Ras effectors have now been identified [66]. These include the RIN1 nucleotide exchange factor for Rab5 [67], Tiam1 and other exchange factors for Rac [68], Rain, an endomembrane receptor for Ras [69], Nore1, a pro-apoptotic tumor suppressor [8], and AF-6, a mediator of membrane-cytoskeleton interactions [70,71]. Although we have not yet evaluated all of these Ras effector pathways in detail, it is worth noting that the E37G
mutation, which reduced vacuolation in our studies, is permissive for Ras binding to RIN1 [72], Nore1 [73] and Rain [69]. Conversely, the D38E mutation, which did not have a major effect on vacuolation, would diminish Ras binding to AF-6 [70]. These preliminary observations raise the intriguing possibility that the unusual cytoplasmic vacuolation induced by activated Ras may be related to interactions with novel effectors found specifically in glioblastoma cells and perhaps other types of cells (neuroblastoma, gastric carcinoma) that respond to Ras in this unique way.

The precise definition of the Ras signaling pathway(s) responsible for induction of the vacuolar phenotype and Type-II cell death could be quite challenging because of the growing list of Ras targets. Further complicating the task, the effects of activated Ras may vary depending on the cell type or species [3,74]. For example, in a mouse glioblastoma model, the introduction of oncogenic K-Ras stimulates gliomagenesis and is essential for tumor maintenance [75,76], contrasting with the death-promoting effects in human glioma cells. Despite these challenges, future studies aimed at pinpointing the Ras signaling pathways responsible for induction of Type-II death could have important clinical implications. For example, migrating glioblastoma cells are generally resistant to apoptosis, due to constitutive activation of one or more pro-survival signaling pathways [77]. By defining the molecular mechanisms for alternative non-apoptotic forms of cell death, it may be possible to identify new targets that can be activated to reduce survival of the malignant cells.
5. Conclusions

Since the original demonstration that ectopic expression of activated H-Ras can induce cytoplasmic vacuolation and Type-II death in glioblastoma cells [10], there have been no systematic studies aimed at defining the molecular mechanisms underlying this unique effect. The results presented in this report represent a significant step forward in narrowing the search for the relevant Ras effectors. We are now able to draw the following conclusions: (1) Cytoplasmic vacuolation can be induced by K-Ras as well as H-Ras, suggesting that common switch regions, rather than unique hypervariable domains or lipid modifications, are the critical factors for stimulation of the relevant signaling pathways. (2) The pathways responsible for triggering vacuolation are specific to Ras; i.e., activated forms of RhoA and Cdc42 do not duplicate this effect. (3) Stimulation of vacuolation depends on farnesylation of Ras, implying that the relevant effectors are associated with intracellular membranes. (4) The ability of Ras to induce cytoplasmic vacuolation does not depend on activation of the widely studied Raf→MEK→ERK kinase cascade, the PI-3K → Akt pathway, or the RalGDS → Ral pathway. The latter findings underscore the possibility that activated Ras may stimulate unconventional effector pathways to induce non-apoptotic cell death. Although this study does not pinpoint the key effector pathway, it eliminates the most obvious candidates, thereby providing important direction for future studies. In the long term these studies may uncover specific targets that can be manipulated in a therapeutic context to increase the susceptibility of glioblastoma to this alternative form of cell death.
Acknowledgements

This work was supported by grants to W.A.M. from the National Cancer Institute (RO1 CA34569) and the Charlotte Geyer Foundation. We are grateful to Amy Wilson-Delfosse, Ph.D. for providing the Cdc42 and RhoA expression vectors.
References


131


[61] E. Corcelle, M. Nebout, S. Bekri, N. Gauthier, P. Hofman, P. Poujeol, P. Fenichel, B. Mograbi, Disruption of autophagy at the maturation step by the carcinogen lindane is associated with the sustained mitogen-activated protein


Figure Legends

Figure 1. Stimulation of vacuole formation by activated Ras depends on farnesylation.
(a) U251 glioblastoma cells were nucleofected with expression vectors encoding the indicated myc-tagged constructs. After 24 hours equal amounts of cellular protein were subjected to western blot analysis using the myc antibody. At the same time, live cells in parallel cultures were examined by phase contrast microscopy (300 x magnification). (b) U251 cells were nucleofected with expression vectors encoding the indicated myc-tagged constructs. After 24 hours the expressed proteins were localized by immunofluorescence microscopy as described in Materials and methods. The left panel of each figure shows the same cells under phase contrast.

Figure 2. H- and K-Ras, but not Rho family GTPases, induce vacuolation.
(a) Sequence alignment of the COOH-terminal hypervariable domains of H-Ras and K-Ras4B, showing sites of posttranslational lipidation. (b-e) U251 glioblastoma cells were nucleofected with expression vectors encoding the indicated myc-tagged constructs. After 24 hours the expressed proteins were localized by immunofluorescence microscopy using an antibody against the myc epitope (right panel). The left panel of each figure shows the same field of cells under phase contrast.

Figure 3. Mutations in the effector domain of H-Ras(G12V) have differential effects on its ability to induce cytoplasmic vacuolation. (a) Summary of Ras effector interactions known to be inhibited (-) or permitted (+) by specific amino acid substitutions, based on
information compiled from refs 8,30-33,69,70,72. (b) U251 cells were nucleofected with expression vectors encoding the indicated myc-tagged H-Ras constructs. After 24h, equal aliquots of cell protein were subjected to western blot analysis using the myc antibody (representative blot shown). (c) Parallel cultures were processed for immunofluorescence localization of myc-tagged Ras proteins. Cells staining positive with the myc antibody were photographed and scored for vacuolation. Cells were scored as vacuolated if they contained at least 2 vacuoles \( \geq 0.5 \mu m \) in diameter and/or \( >10 \) smaller vacuoles. At least 65 myc-positive cells were scored to determine the percentage of transfected cells that were vacuolated in each culture. The results for each Ras construct shown in the bar graph are derived from three separate experiments (mean \( \pm \) S.D.). The decreases in vacuolation observed with G12V+Y40C and G12V+E37G were significant at \( p<0.01 \) compared with G12V alone (Student’s t-test).

**Figure 4.** Induction of vacuoles by H-Ras(G12V) does not depend on activation of the ERK pathway. To obtain uniform expression of exogenous Ras, U251 cells were infected with retrovirus encoding myc-H-Ras(G12V) or myc-H-Ras(S17N). At the time that the cells were plated in selection medium (see Materials and Methods), the MEK inhibitor, PD98059 (25 \( \mu M \)) or an equivalent volume of DMSO vehicle, was added to the culture medium. Cells were processed for western blot analysis or immunofluorescent detection of LAMP1-positive vacuoles after 42 h in the presence of the inhibitor. (a) Activation of ERK was monitored in equal aliquots of whole cell lysate (normalized for protein) using antibodies to detect phosphorylated ERK1/2 (phos ERK) and total
ERK1/2. (b) Immunofluorescence microscopy to detect LAMP1-positive vacuoles was performed as described in Materials and Methods. The panels are composites showing representative cells from the cultures incubated with or without PD98059.

Figure 5. Overexpression of constitutively active Raf-1 does not mimic the effect of activated H-Ras. U251 cells were nucleofected with empty vector or with expression vectors encoding myc-Raf-Caax or myc-H-Ras(G12V). The cells were processed for western blot analysis or immunofluorescence 24 h later. (a) Immunoblot analysis with an antibody against Raf confirms that myc-Raf-Caax (upper band) is overexpressed relative to endogenous Raf. (b) Phospho-ERK and total ERK were determined by immunoblot analysis (representative blot shown). The ratio of phospho-ERK to total ERK was determined by quantifying the immunoblot signals with a Kodak Image station. The results shown in the bar graph are the means (±SD) of separate determinations performed on three cultures. (c) Cells expressing myc-Raf-Caax or myc-H-Ras(G12V) were identified by immunofluorescence using antibody against the myc epitope. The same cells were examined under phase contrast to assess the presence of vacuoles. The nucleofection efficiencies in both sets of cultures were approximately 50%. There were no vacuolated myc-positive cells in the cultures expressing myc-Raf-Caax.

Figure 6. Reduction of the activity of the PI-3K signaling pathway by introduction of PTEN does not prevent the induction of vacuoles by H-Ras(G12V). (a) U251 cells were nucleofected with expression vectors encoding PTEN and/or myc-H-Ras(G12V) as indicated. After 24 h equal aliquots of cell protein were processed for western blot
analysis to verify the expression of PTEN and myc-tagged Ras. (b) Phospho-Akt and total Akt were determined by immunoblot analysis (representative blot shown). The bar graph depicts the ratio of phospho-Akt to total Akt (mean ± S.D.) determined by quantifying the immunoblot signals from three separate cultures. The decrease in Akt phosphorylation in PTEN + myc-H-Ras(G12V) was significant at p < 0.05 compared with myc-H-Ras(G12V) alone (Student’s t-test). (c) Phase contrast images of representative fields of cells from the cultures harvested for the blots shown in a & b (200x).

**Figure 7.** Dominant-negative RalA does not block the induction of vacuolation by activated H-Ras. (a) U251 cells were nucleofected with expression vectors encoding FLAG-H-Ras(G12V), myc-RalA(S28N) or a combination of both. Equal amounts of cell lysate were subjected to western blot analysis to verify expression of the tagged proteins. (b) The upper panels show representative immunofluorescent localization of FLAG-H-Ras(G12V) alone and myc-RalA(S28N) alone. The bottom panels show a typical cell expressing both proteins.
Fig. 2

a) COOH-Terminal Hypervariable Domains of Human H-Ras and K-Ras4B

<table>
<thead>
<tr>
<th></th>
<th>polyetylation</th>
<th>deamidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Ras</td>
<td>L R K L N P F E S O P O M S E K E V L S -</td>
<td></td>
</tr>
</tbody>
</table>

b) myc-H-Ras(G12Y)

c) myc-K-Ras4B(G12V)

d) myc-Cdc42(G12V)

e) myc-RhoA(G14V)
### Fig. 3

#### a

**H-Ras effector domain mutations**

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Effector</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>D38E</td>
<td>PI3K, p110</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Raf</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>RafGDS</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AF-6</td>
<td>-</td>
</tr>
<tr>
<td>Y40C</td>
<td>PI3K, p110</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Raf</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RafGDS</td>
<td>-</td>
</tr>
<tr>
<td>E37G</td>
<td>PI3K, p110</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Raf</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RafGDS</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Nore1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>RIN-1</td>
<td>+</td>
</tr>
</tbody>
</table>

#### b

![Western blot analysis](image)

#### c

![Bar graph](image)
Fig. 4

a

<table>
<thead>
<tr>
<th>myc-H-Ras</th>
<th>G12V</th>
<th>G12V</th>
<th>S17N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD98059</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

phos ERK

total ERK

myc

b

H-Ras(G12V)

H-Ras(G12V) + PD98059
Fig. 5

(a) Western blot analysis showing the expression levels of myc-Raf-Caax, Raf (endogenous), myc-H-Ras (G12V), myc-Raf-Caax, and myc-H-Ras(G12V).

(b) Western blot analysis showing the phosphorylation status of ERK (phos ERK) and total ERK levels. Graph showing pERK/total ERK ratios for myc-Raf-Caax, myc-H-Ras (G12V), and pCMV5.

(c) Immunofluorescence images of myc-Raf-Caax and myc-H-Ras(G12V) expressing cells.
Fig. 6

(a) PTEN

(b) Phos Akt

(c) myc-H-Ras(G12V)

(d) myc-H-Ras(G12V) + PTEN

(e) PTEN

(f) Phos-Akt/Total Akt

(g) PTEN

(h) myc-H-Ras(G12V)

(i) myc-H-Ras(G12V) + PTEN

(j) PTEN
Killing of cancer cells by the photoactivatable protein kinase C inhibitor, calphostin C, involves induction of endoplasmic reticulum stress

Aparna Kaul and William A. Maltese§

Department of Biochemistry and Cancer Biology, University of Toledo College of Medicine, Toledo, Ohio, USA

Running title: Calphostin C induces endoplasmic reticulum stress

Keywords: Calphostin C, Protein kinase C, Endoplasmic reticulum stress, Apoptosis, Breast cancer

Grant Support: This work was supported by National Institutes of Health grant R01 01 CA115495.

§Correspondence: William A. Maltese Ph.D., Department of Biochemistry & Cancer Biology, Block Health Sciences Bldg, Mail Stop 1010, University of Toledo College of Medicine, 3000 Arlington Ave., Toledo, Ohio, 43614
E-mail: william.maltese@utoledo.edu

Abbreviations: cal-C, calphostin C; PKC, protein kinase C; DAG, diacylglycerol; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; IRE1, Inositol-requiring protein 1, PERK, protein kinase R-like endoplasmic reticulum kinase; CHOP, CCAAT/enhancer binding protein homologous transcription factor; STS, staurosporine; DMEM, Dulbecco’s modified eagle medium; β-APP, β-amyloid precursor protein.
Abstract

Calphostin C (cal-C) is a potent photoactivatable inhibitor that binds to the regulatory domain of protein kinase C (PKC), as well as to other proteins that contain diacylglycerol/phorbol ester binding sites. Cal-C is cytotoxic against many types of cancer cells, yet the basis for this activity remains poorly understood. Here we show that one of the earliest effects of cal-C is an impairment of endogenous glycoprotein export from the endoplasmic reticulum (ER), accompanied by formation of ER-derived vacuoles. Vacuolization of the ER is closely correlated with induction of an ER stress response that includes activation of JNK and PERK, as well as increased expression of CHOP(GADD153). These rapid effects of cal-C are not mimicked by staurosporine, a potent inhibitor of PKC catalytic activity, indicating that ER stress is due to interaction of cal-C with targets other than PKC. In conjunction with the induction of ER stress, breast carcinoma cells undergo caspase-dependent cell death characterized by early activation of caspases 9 and 7, concurrent with PARP cleavage. These observations suggest that induction of apoptosis by cal-C is related, at least in part, to ER-stress triggered by disruption of ER morphology and transport function. Drugs that exhibit antineoplastic activity due to induction of ER stress have gained increasing attention in pre-clinical and clinical trials. Thus, the present findings raise the possibility that cal-C may be useful for devising a targeted photodynamic therapy based on induction of ER stress in some forms of cancer.
Introduction

Protein kinase C (PKC) isozymes have been implicated as regulators of signaling pathways that promote proliferation, survival, metastasis, and drug resistance in cancer cells [1,2]. Elevated levels of PKC expression or activity have been noted in human malignancies such as gliomas [3], breast tumors [4], and metastatic gastric carcinoma [5]. Many investigations have explored the antineoplastic activity of PKC inhibitors, with particular emphasis on the indocarbazoles [e.g., staurosporine], which target the ATP binding site of PKC, and the macrocyclic lactones [e.g., bryostatin 1], which antagonize PKC activation and stimulate ubiquitin-mediated degradation [1,6]. Despite encouraging pre-clinical results, the efficacy of these compounds in clinical trials has been disappointing, possibly due to the number of functionally distinct PKC isozymes and the lack of kinase specificity [7].

The fungal perylenequinone, calphostin C (cal-C), was initially described as a potent inhibitor of PKC that operates through a novel mechanism, binding to a Ca$^{2+}$-induced hydrophobic site on the PKC regulatory domain and preventing activation by diacylglycerol (DAG) and phorbol esters [8,9]. The inhibitory activity of cal-C is strictly dependent on photoexcitation, which causes irreversible site-specific oxidative modification of PKC [10,11]. This has raised the prospect that cal-C might be a useful agent for photodynamic cancer therapy [12]. Thus far the evaluation of cal-C has been limited to preclinical studies. The results have established that this inhibitor can induce apoptosis in a broad spectrum of human cancer cell lines, including glioma [13-15], cervical carcinoma [16], lymphoblastic leukemia [17], prostate cancer [18], and
nasopharyngeal carcinoma [12]. However, important questions about the molecular mechanisms underlying the cytotoxicity of cal-C remain to be answered. In particular, accumulating evidence suggests that the compound could operate through mechanisms that are independent of PKC. For example, cal-C can directly inhibit phospholipase D [19], and at concentrations that trigger apoptosis, it can cause oxidation and degradation of lamin B1 [16], disassembly of Golgi membranes [20], and mobilization of calcium from intracellular stores [17]. Further complicating matters, a study of the effects of cal-C in wild-type and drug resistant MCF-7 breast cancer cells indicated that the inhibitor can kill the cells by a novel mechanism involving the accumulation of cytoplasmic vacuoles of unknown origin [21]. In the present study we have clarified the latter mechanism of calphostin-induced cell death by showing that the cytoplasmic vacuoles arise from the endoplasmic reticulum (ER), and that cell death is preceded by disruption of ER to Golgi trafficking and induction of a robust ER-stress response. These findings suggest that cal-C may merit further evaluation as a potential photodynamic agent that could be used to inhibit tumor progression by stimulating ER stress.
Materials and Methods

Cell Culture and Transfection

MCF-7 breast carcinoma cells were a gift from A. Parissenti, Northeastern Ontario Regional Cancer Centre. U251 glioblastoma cells were from the DCT Tumor repository (National Cancer Institute, Bethesda, MD). 501 MEL cells were from the cell culture core facility at the Yale Skin Disease Research Center. All other cell lines were from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) with a 5% CO₂ atmosphere. The pDsRed2-ER mammalian expression vector was purchased from Clontech (Palo Alto, CA, USA). The vector encodes the ER targeting sequence of calreticulin and an ER retention signal (KDEL) fused to red fluorescent protein. MCF-7 cells were transfected using the FuGENE HD transfection reagent (Roche, Indianapolis, IN, USA), according to the manufacturer’s protocol. Phase contrast images and fluorescent images of DsRed-ER distribution in live cells were obtained using an Olympus IX70 inverted microscope equipped with a digital camera and SPOT imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Drug Treatments and Photoactivation of Calphostin-C

Calphostin-C (Tocris, Ellisville, MO, USA) was dissolved in dimethylsulfoxide (DMSO). Cells were incubated in medium containing cal-C in the dark at 37°C for 30 min, followed by exposure to a 30-watt fluorescent light at 3 inches from the source for
another 30 min to activate cal-C. Time points indicated in the figures start after photoactivation of cal-C. Control dishes were incubated with an equivalent volume of DMSO under the same conditions. During co-treatments, cal-C and z-VAD-fmk (Bachem Bioscience, Inc., King of Prussia, PA, USA) or z-LEVD-fmk (BioVision, Inc, Mountain View, CA, USA), were added simultaneously to the medium. The cultures were then incubated at 37°C for 1 h, followed by photoactivation of cal-C, as described above. In some experiments cells were treated with 500 nM staurosporine (STS) (Cayman Chemical Company, Ann Arbor, MI, USA) or 2.5 μM brefeldin A (BFA) (Sigma, St. Louis, MO, USA).

**Cell Viability**

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based cell growth determination kit from Sigma (St. Louis, MO, USA), following the manufacturer’s protocol. The MTT assay was performed in quadruplicate, 24 h after photoactivation of cal-C. The absorbance at 570 nm was measured with a Molecular Devices SpectraMax plus plate reader. Statistical significance was assessed by Student’s t-test. IC₅₀’s for cal-C in different cell lines were determined by performing MTT assays on cells exposed to drug concentrations of 0, 1, 5, 10, 25, 50,100 and 150 nM for 24 h. IC₅₀’s were calculated from linear regression analysis of sigmoidal dose-response curves plotted with GraphPad Prism software (La Jolla, CA).
Western Blot Analysis

Antibodies used for western blotting were purchased from the following sources: JNK, phospho-JNK, caspase 7 and caspase 9 (Cell Signaling Technology, Danvers, MA, USA); PERK, phospho-PERK and CHOP (Santa Cruz Biotechnology, Santa Cruz, CA, USA); PARP (BD Pharmingen, San Jose, CA, USA); caspase 4 (MBL International, Woburn, MA, USA); and α-tubulin (Sigma, St. Louis, MO, USA). Protein determination in cell lysates, SDS-PAGE and western blot analyses were performed as described previously [22]. Chemiluminescence signals were quantified using an Alpha Innotech FluorChem HD2 imaging system.

Annexin V Assay

Attached cells were harvested by trypsinization and pooled with any detached cells in the medium. The cells were then stained with the Guava Nexin reagent (Guava Technologies, Hayward, CA, USA), containing pre-mixed annexin V-PE and 7-amino-actinomycin D (7-AAD), following the manufacturer’s protocol. Annexin V positive cells were quantified by flow cytometry, using a Guava Personal Cytometer.

Metabolic Labeling and Immunoprecipitation of β-Amyloid Precursor Protein (β-APP)

MCF-7 cells were washed twice with methionine-free DMEM and incubated with either 50 nM cal-C or DMSO in methionine-free DMEM, first for 30 min at 37°C and then exposed to a 30-watt fluorescent light source for 30 min. For pulse labeling, 100 μCi/ml [35S] methionine/cysteine mix (Easy Tag™ EXPRESS, NEN/Perkin Elmer,
Waltham, MA, USA) was added to the cultures and cells were incubated at 37°C for 30 min. The ‘pulse’ samples were harvested immediately. Parallel cultures (the ‘chase’ samples) were washed with PBS and incubated in DMEM containing 10% FBS, 200 μM methionine and 200 μM cysteine, with or without cal-C. The cultures were exposed to light for 30 min after addition of the chase medium containing fresh cal-C, and then incubated at 37°C for 1.5 h (total 2 h chase period). Cells were harvested and lysed in PBS containing 1.0% Triton-X 100 and protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and radiolabeled β-APP was immunoprecipitated as described previously [23], using the Anti-BX6 polyclonal antibody [24]. Immune complexes were collected by incubation for 1 h at 4°C with protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) coupled to goat anti-rabbit IgG (MP Biomedicals, Inc., Solon, OH, USA). Immunoprecipitated proteins were subjected to SDS-PAGE and fluorography as described [23]. The relative amounts of mature and immature β-APP were determined by densitometry using a Kodak 440CF image station.

**Fluorescence Microscopy**

MCF-7 cells were grown on glass coverslips for 24 h and treated with 50 nM cal-C or DMSO as described above. Two hours after cal-C treatment, cells were washed with HBSS, fixed in ice-cold methanol for 10 min, and blocked with 10% goat serum in PBS. For visualization of the Golgi apparatus, cells were incubated for 1 h with the anti-GM130 antibody (BD Transduction Labs, San Jose, CA, USA) diluted in PBS+10% goat serum, followed by incubation with goat anti-mouse IgG conjugated with Alexa Fluor 488.
568 (Molecular Probes/Invitrogen, Eugene, OR, USA). Images were obtained using an Olympus IX70 fluorescence microscope equipped with a digital camera. Merger of phase contrast and fluorescent images was accomplished with the SPOT imaging software.

For visualization of acidic lysosomal or late endosomal compartments, cells were incubated with Lysotracker red DND-99 (Invitrogen Corp, Carlsbad, CA) as described previously [25], and live cell imaging was performed as described above for Ds-Red.
Results

Cytoplasmic Vacuolization Precedes Loss of Viability in Tumor Cell Lines Treated with Activated Calphostin-C

Exposure of MCF-7 breast carcinoma cells to photoactivated cal-C in the concentration range of 15-100 nM caused a significant reduction in cell viability within 24 h (Fig. 1A). Loss of viability was accompanied by increased cleavage of the caspase substrate, PARP, which was detectable as early as 2 h after activation of cal-C (Fig. 1B). The 85 kDa PARP fragment was identical in size to that generated in cells treated with staurosporine, a known PKC inhibitor and a potent inducer of apoptosis (Fig. 1C). As reported previously [21], death of the MCF-7 cells treated with cal-C was preceded by extensive cytoplasmic vacuolization (Fig. 2A). We were able to detect the accumulation of phase-lucent vacuoles within 1-3 h after activation of cal-C. Vacuoles did not form and there was no increase in cell death if the photoactivation of cal-C was omitted (Fig. 2B). Shortly after the onset of vacuolization, some of the cells began to detach from the surface of the culture dish, so that by 24 h more than half of the cells in the culture were floating. Examination of PARP cleavage in the detached versus attached cell populations (Fig. 1D) indicated that most of the floating cells were undergoing cell death. Unlike the cells treated with cal-C, MCF-7 cells treated with staurosporine rounded and detached from the dish, but did not undergo vacuolization (Fig. 2B).

To determine if early cytoplasmic vacuolization is a general characteristic of cal-C treatment in other types of cancer cells, we exposed U251 glioblastoma and PANC-1 pancreatic carcinoma cells to similar concentrations of the compound. In both cases,
vacuoles accumulated within 3 h (Fig. 2A), with substantial loss of cell viability (Fig. 1A) and increased cleavage of PARP (Fig. 1C&D) occurring by 24 h. A broader survey of established cancer cell lines indicated that cal-C generally induced substantial cytotoxicity within 24 h, with IC50’s ranging from 15-37 nM. Most of the adherent cell lines exhibited extensive vacuolization, but a few did not (e.g., SW480, SKOV3).

**Cytoplasmic Vacuoles are Derived from the Endoplasmic Reticulum**

The close temporal correlation between the onset of cytoplasmic vacuolization and PARP cleavage (Fig. 1B and Fig. 2A) prompted us to conduct further studies with MCF-7 cells to establish the origin of the vacuoles induced by cal-C. Phase-lucent vacuoles can arise from various subcellular compartments. For example, inhibition of the class-III phosphatidylinositol-3-kinase, Vps34, causes swelling and vacuolization of late endosomes [26,27], whereas accretion of misfolded proteins in cells treated with inhibitors of Hsp90 and the ubiquitin-proteasome pathway causes distension of the ER [28]. Most recently, we described a novel cell death pathway in which cells are disrupted by accumulation of large fluid-filled vacuoles derived from macropinosomes [25].

To test the hypothesis that the vacuoles induced by cal-C are derived from the ER, we transfected MCF-7 cells with a vector encoding a red fluorescent protein (DsRed) that contains specific ER targeting and retention sequences. In non-treated MCF-7 cells, the marker was localized diffusely in the perinuclear region, a pattern typical for the ER (Fig. 3A). However, within 3 h after treatment of the cells with cal-C, the DsRed-ER was concentrated in discrete globular structures that matched the lucent vacuoles observed by
phase contrast microscopy (Fig. 3A). In contrast to these results, the phase lucent vacuoles induced by cal-C did not label with the late endosome/lysosome tracer, Lysotracker red (Fig. 3B). Nor did these structures internalize detectable quantities of fluid-phase tracers such as Lucifer yellow or Dextran-Alexafluor488 (not shown). Essentially identical results were obtained when the studies were performed with U251 and PANC-1 cells. These findings support the conclusion that the cytoplasmic vacuoles induced by cal-C are formed by dilation of ER cisternae.

**Induction of Vacuoles by Calphostin-C is Accompanied by ER Stress**

In light of the known association between accumulation of unfolded proteins and ER swelling, we undertook a series of studies with MCF-7 cells to determine if cal-C induces a prototypical ER stress response concurrent with vacuolization. One of the earliest signposts of ER stress is the phosphorylation of the stress-activated kinase, JNK. JNK activation appears to be mediated through a signaling cascade triggered by IRE1, an ER resident kinase/endoribonuclease that senses accumulation of misfolded proteins [29,30]. As shown in Fig. 4A, treatment of MCF-7 cells with cal-C caused a spike in JNK phosphorylation that was maximal at 15 min -1 h. The level of JNK phosphorylation tapered off slightly after 1 h, consistent with a report that cells respond to unmitigated ER stress by first activating and then attenuating IRE1 and JNK [30].

A second major branch of the ER stress response involves the activation of PERK, a transmembrane protein kinase. PERK is phosphorylated in response to ER stress, resulting in the activation of its cytoplasmic kinase domain, which targets eukaryotic
initiation factor 2α and attenuates translation [31]. Activation of PERK is sustained with prolonged ER stress [30]. Fig. 4B shows that within 15 min after activation of cal-C, phosphorylation of PERK was increased to approximately twice the level seen under baseline culture conditions, and remained elevated for the duration of the 4 h experiment.

The preceding studies were extended by examining the expression of CHOP (GADD153), a bZIP transcription factor that is upregulated during the ER stress response [32] and is implicated in apoptosis caused by ER stress [33]. As shown in Fig. 4C, treatment of MCF-7 cells with cal-C caused a substantial increase in CHOP commencing around 2 h, coinciding with the timing of cytoplasmic vacuolization and the first detectable increase in PARP cleavage (Fig. 1B). In contrast to the robust increase in CHOP triggered by cal-C, staurosporine, which targets the catalytic domain of PKC, did not increase the expression of CHOP, even after 18 h (Fig. 4C).

**Death of Calphostin-Treated Cells is Caspase-Dependent**

The close temporal correlation between ER vacuolization, induction of ER stress markers, and onset of PARP cleavage in MCF-7 cells treated with cal-C suggests that, contrary to a previous report [21], cell death is mediated by a caspase-dependent apoptotic pathway. To directly test the importance of caspase activation in the death of cal-C-treated MCF-7 cells, we added the broad-spectrum caspase inhibitor, z-VAD-fmk together with 50 nM activated cal-C for 24 h. As shown in Fig. 5A & B, z-VAD effectively inhibited the cleavage of PARP and significantly increased cell survival.
Similar results were obtained with U251 and PANC-1 cells, and with a separate MCF-7 cell line obtained directly from the American Type Culture Collection (not shown).

Independent evidence that the cal-C-treated MCF-7 cells were undergoing apoptosis was obtained by staining them with the apoptosis marker, annexin V. Cal-C caused an increase in the percentage of annexin V-positive MCF-7 cells, comparable to what was observed in cultures treated with staurosporine (Fig. 5C).

Cal-C has been reported to induce rapid activation of mitochondrial death pathways and cleavage of procaspase 3 in several cell lines [34,35]. Separate studies have implicated the mitochondrial apoptosome and caspase 3 as important mediators of ER stress-induced apoptosis [36,37]. Consistent with these reports, we observed substantial activation of the mitochondrial initiator caspase, caspase 9, within 1 h after photoactivation of cal-C (Fig. 6A). This coincided with the initial appearance of vacuoles (Fig. 2A) and the early spike in JNK and PERK phosphorylation (Fig. 4A & B). By comparison, staurosporine, which targets the catalytic site of PKC but does not cause ER vacuolization or ER stress, did not begin to cause procaspase 9 cleavage until 2 h (Fig. 6A).

Since MCF-7 cells lack the executioner caspase, caspase 3, we examined the activation of caspase 7. The latter is widely viewed as an alternative executioner caspase that can act downstream from caspase 9 [38,39], but it has also been implicated as an initiator caspase in the ER [40]. As shown in Fig. 6B, the time course of procaspase 7 cleavage following treatment with cal-C closely matched that observed for procaspase 9 (Fig. 6A).
Finally, we examined the effects of cal-C on caspase 4. Some studies have pointed to a role for caspase 4 in ER stress-induced cell death [41,42], while others have argued that caspase 4 is not required [43]. We observed an increase in pro-caspase 4 cleavage by 24 h after addition of cal-C, although the increase was not as great as that seen with brefeldin A, a commonly used inducer of the ER stress response (Fig. 6C). However, we did not find a consistent increase in cleavage of pro-caspase 4 above baseline levels during the critical early period (1-4 h) when ER vacuolization and ER stress markers were induced (Fig. 6C). In agreement with these observations, the inhibitor, z-LEVD-fmk, used at a concentration reported to specifically block caspase 4 [44,45], failed to protect MCF-7 cells from cal-C-induced cell death (Fig. 6D).

Protein Trafficking Between the ER and Golgi Compartment is Blocked by Cal-C.

The observation that ER vacuolization and stress were caused by cal-C, but not staurosporine, suggested that the induction of ER stress could be due to PKC-independent effects of cal-C. In this regard, our attention was drawn to an early study showing that cal-C, used at concentrations similar to those employed in the present study, can block export of proteins from the ER in a PKC-independent manner [46]. At significantly higher concentrations (>500 nM), cal-C can also cause disassembly of the Golgi apparatus [20,46]. To determine whether the swelling of the ER observed within 1-2 h after adding cal-C might reflect an underlying inhibition of protein export from the ER, we took advantage of the fact that MCF-7 cells, like most other cultured cell lines, express measureable amounts of the Alzheimer’s β-amyloid precursor protein (β-APP).
The latter has been used as an endogenous tracer to detect perturbations in vesicular trafficking of proteins from the ER to the Golgi compartment [23,47].

β-APP is a type-1 transmembrane glycoprotein that exhibits an increase in its apparent molecular mass on SDS gels after it is exported from the ER and enters the Golgi apparatus. The mobility shift is due to O-glycosylation of the exodomain [24,48]. The modified protein is then further processed by endoproteases (α or β secretases) that cleave off the glycosylated exodomain, which is secreted as soluble APP. Subsequent proteolytic events catalyzed by γ-secretases can result in the formation of amyloid β peptides from the residual C-terminal membrane-anchored remnant of the protein [49]. To determine if cal-C causes an early block in export of β-APP from the ER, we performed a pulse-chase study depicted in Fig. 7. After a 30 min pulse with 35S-methionine, the radiolabeled β-APP immunoprecipitated from control cells migrated predominantly as the immature (approx. 115 kDa) ER-resident form, with a lesser amount of β-APP migrating as the mature Golgi form (approx. 135 kDa) (Fig. 7A). A similar distribution was observed for β-APP when cal-C was included during the 30 min pulse. However, when the cells were chased in the absence of 35S-methionine for an additional 2 h to allow time for the immature β-APP to be exported to the Golgi apparatus, a significant impairment of β-APP maturation was noted in cells treated with cal-C (Fig. 7A). In the control cells the movement of the labeled β-APP into the Golgi compartment was manifested as significant increase in the ratio of the mature form relative to the immature form. In contrast, the immature ER form of β-APP remained predominant in the cells exposed to cal-C. In fact, the ratio of the Golgi form to the ER
form at the end of the chase was essentially unchanged compared with the ratio at the end of the 30 min pulse (Fig. 7B). These findings are similar to what happens to β-APP processing when cells express a dominant-negative form of the Rab1b GTPase, which regulates vesicular trafficking between the ER and Golgi compartments [23]. Therefore, the results indicate that cal-C causes a rapid arrest of ER → Golgi protein trafficking in MCF-7 cells. Immunofluorescent localization of the Golgi marker, GM130, revealed a similar perinuclear Golgi staining pattern in both control and cal-C-treated cells (Fig. 7C), with some cells exhibiting a discrete juxtanuclear cluster and others showing a more dispersed staining pattern. The distended ER-derived vacuoles in the cal-C-treated cells were not stained with anti-GM130, suggesting that the Golgi stack did not collapse into the ER. This implies that the observed block in ER → Golgi trafficking of β-APP is most likely related to functional impairment of the vesicular transport mechanism, rather than complete disruption of the Golgi architecture, which has been reported to occur at much higher cal-C concentrations [20].
Discussion

The present study provides several new insights into the previously reported anti-cancer activity of the photoactivatable perylenequinone, calphostin-C. First, we show that the tendency of cal-C to cause rapid cytoplasmic vacuolization, initially reported only in breast cancer cells [21], extends to several other cancer cell lines, and thus represents a general characteristic of this compound. Second, we establish that the vacuoles induced by cal-C arise from dilated ER cisternae. Third, we show that early vacuolization of the ER is accompanied by a perturbation of ER → Golgi protein trafficking and a concurrent induction of the ER stress response. Finally, we demonstrate a very close temporal correlation between ER vacuolization, induction of the stress response, and the early onset of caspase-dependent apoptosis. Based on these observations, we suggest a mechanism to explain the potent cytotoxic effects of cal-C in neoplastic cells. Specifically, we postulate that cal-C initially inhibits proteins with phorbol ester/DAG binding sites that control vesicular trafficking out of the ER. The resulting inhibition of protein and membrane export leads to distention of the ER and accumulation of misfolded or aggregated proteins, which trigger the ER stress response. Unabated ER stress finally leads to activation of a caspase-mediated apoptotic death pathway.

In the original report describing cal-C mediated vacuolization and killing of parental and drug-resistant MCF-7 cell lines, PARP cleavage and mitochondrial cytochrome-c release were not observed, suggesting a non-apoptotic mechanism of cell death [21]. However, in our studies we clearly observed PARP cleavage, caspase
activation, increased staining with annexin V, and protection with z-VAD. One possible explanation for this discrepancy may lie in the methods used for harvesting the cells. In our studies all of the PARP and caspase assays were done with pooled attached and floating cells, to avoid losing detached cells that accounted for most of the apoptotic population (Fig. 1D). Our findings agree with numerous reports indicating that cal-C triggers caspase-dependent apoptosis in other types of cells [12,13,17,34].

Our observation that staurosporine, a potent inhibitor of PKC, did not stimulate ER vacuolization or induction of CHOP expression, strongly suggests that the initial effects of cal-C on ER function are related to interaction with targets other than PKC. One possibility, raised by the work of Sciorra et al. [19], is that the effects of cal-C on protein export are due to binding to the catalytic domain of phospholipase D1 (PLD1), a known regulator of ER → Golgi trafficking [50]. An alternative possibility is that the effects of cal-C could be mediated by disruption of calcium homeostasis. For example, in acute lymphoblastic leukemia cells the cytotoxic effect of cal-C correlates with rapid calcium mobilization from intracellular stores and appears to be independent from inhibition of PKC [17]. A third possibility, suggested by the early work of Fabbri et al. [46], is that cal-C disrupts protein export from the ER through interaction with novel DAG/phorbol ester binding proteins that have yet to be identified.

As noted above, staurosporine did not mimic the effects of cal-C on ER vacuolization and stress response. Nevertheless, it was clearly able to induce caspase 9 activation and apoptosis. Therefore, although the disruption of ER morphology and protein export by cal-C is probably due to inhibition of a non-PKC target, apoptosis could
be related to composite effects of cal-C mediated through direct inhibition of PKC as well as PKC-independent ER stress. We found that cleavage of procaspase 9 (Fig. 6A) occurs very soon after photoactivation of cal-C, implying early mitochondrial involvement in the apoptotic response. This could be triggered by rapid induction of the mitochondrial permeability transition, as noted in previous studies with staurosporine [51,52]. Alternatively, the close temporal correlation of caspase 9 activation and PARP cleavage with swelling of the ER and activation of JNK, PERK and CHOP could support a model wherein apoptosis induced by cal-C is driven mainly by unmitigated ER stress. These mechanisms need not be mutually exclusive.

The exact sequence of molecular events that occurs between the onset of ER stress and the activation of a mitochondrial apoptotic response remains incompletely understood [53-55]. One possibility is that apoptosis is mediated by downregulation of Bel-2 and Bcl-X\textsubscript{L} [56], with concomitant oligomerization of Bax or Bad on the ER or mitochondria [57]. This scenario would fit with a study in glioma cells showing that cal-C stimulates rapid integration of Bax into mitochondrial membranes, followed by cytochrome-c release [34]. The early spike in activation of JNK in cells treated with cal-C (Fig. 4A) raises a second possibility that mitochondrial injury might be caused by the ability of JNK to stimulate cleavage of Bid [58] or activation of other BH3-only Bel-2 family members like Bim or Bmf [59]. Finally, it is conceivable that ER stress caused by cal-C could trigger apoptosis via local activation of an initiator caspase in the ER. Procaspa 12 has been reported to localize in the ER and undergo cleavage in response to ER stress in rodent cells [60]. However, a specific requirement for caspase 12 for
subsequent apoptosis remains controversial [36,43,61]. Caspase 12 is truncated or inactive in human cells [62], and the related caspase 4 has been proposed to function as an alternate ER stress-sensitive initiator of apoptosis [41,42]. We found evidence of increased pro-caspase 4 cleavage after 24 h of cal-C treatment, but we did not observe a consistent increase in caspase 4 activation during the early period when caspase 9 activation and PARP cleavage were first noted. Thus, it seems unlikely that caspase 4 plays a key role as an initiator caspase in the apoptotic response induced by cal-C.

Caspase 7 is traditionally regarded as an executioner caspase that functions downstream from pro-apoptotic signals emanating from mitochondria [38]. However, it has also been proposed to function as an ER-localized initiator caspase that can respond to ER stress by promoting cleavage of pro-caspase 12 [40] and, by inference, caspase 4. We observed activation of caspase 7 together with activation of caspase 9 (Fig. 6) and cleavage of PARP (Fig. 1) within 1-2 h after photoactivation of cal-C, well before increased cleavage of pro-caspase 4. This timing would be most consistent with caspase 7 acting as an executioner caspase in concert with activation of caspase 9.

The findings of the present study could have important implications for the design of new cancer therapeutic strategies aimed at exploiting the ER stress response. Bortezomib, which causes accumulation of misfolded proteins in the ER by blocking the activity of the 26S proteasome, has already been approved for treatment of multiple myeloma and is being evaluated for efficacy against other malignancies [63-65]. Other ER stress-inducers have shown promise in preclinical trials. For example, 2,5-Dimethyl-celecoxib, which is inactive against cyclooxygenase-2 but causes ER stress by increasing
intracellular free calcium [66], has proven to be effective in reducing the growth and survival of several different types of cancer in cell culture and animal models [67,68]. In addition to their intrinsic antineoplastic activity, there is some evidence that ER stress inducers may be useful in potentiating the effects of established therapeutic agents that trigger cell death through other mechanisms (e.g., DNA damage) [69-72]. Most recently, several studies have suggested that combining drugs that induce ER stress through distinct mechanisms may yield synergistic anticancer activity [73-75]. The results of the present study raise the prospect that cal-C may be a useful addition to the arsenal of agents that can be used to promote death in tumor cells by inducing morphological and functional perturbation of the ER. In this respect, cal-C could offer a unique advantage over other ER stress-inducers because its activity depends on photoexcitation, allowing for the possibility of restricting cytotoxicity to localized tumor sites. We suggest that this concept merits further evaluation in preclinical studies.

**Acknowledgement:** The authors thank Dr. Jean Overmeyer for helpful advice and discussions throughout these studies.
References


Figure Legends

**Figure 1.** Calphostin-C reduces cell viability and induces PARP cleavage in multiple tumor cell lines. (A) Cells were treated with the indicated concentrations of cal-C (light-activated) and viability was assessed 24 h later by MTT assay. Control cells (0 nM) were incubated with a volume of DMSO equal to that added in the cultures that received 100 nM cal-C. Each bar represents the mean (± SD) obtained from quadruplicate wells of a 96-well plate. The decreases observed at all cal-C concentrations for all cell lines were significant at p < 0.01 relative to the corresponding controls. (B) Immunoblots showing cleavage of the caspase substrate, PARP, in MCF-7 cells treated with 50 nM cal-C and harvested at the indicated time points. Tubulin served as control to demonstrate comparable loading of protein in each lane. (C) Cultures of MCF-7, U251 and PANC-1 cells were treated with 50 nM cal-C for 18 h. Parallel cultures were treated with 500 nM staurosporine (STS) for 18 h. Attached and detached cells were pooled for immunoblot analysis of PARP. (D) Cells were treated with cal-C as in panel C, except that the attached (Att) and detached (Det) cells were collected separately prior to immunoblotting for PARP. (E) The indicated cell lines were treated with graded concentrations of cal-C for 24 h, and IC_{50}’s were calculated from the results of the MTT assays. Separate cultures of each cell line were treated with cal-C at concentrations of 30-50 nM and observed by phase contrast microscopy after 3 h to determine if cytoplasmic vacuolization occurred.

**Figure 2.** Calphostin induces early accumulation of cytoplasmic vacuoles in multiple tumor cell lines. (A) MCF-7 and PANC-1 cells were treated with 50 nM cal-C. U251
cells were treated with 30 nM cal-C. Parallel control dishes were incubated with vehicle (DMSO). (B) MCF-7 cells were incubated with 50 nM cal-C, 500 nM staurosporine (STS), or DMSO (control) for 3 h, with or without a 30 min light-activation step. Phase contrast images (400X) of live cells were obtained after 3 h.

**Figure 3.** Vacuoles induced by cal-C are derived from the endoplasmic reticulum. (A) MCF-7 cells were transfected with the pDsRed2-ER vector as described in Materials and Methods. 24 h after transfection, the cells were treated with 50 nM cal-C or DMSO (control). Live cell phase contrast images and fluorescent images showing the localization of the DsRed-ER protein (400X) were obtained 3 h after activation of cal-C. (B) Untransfected MCF-7 cells were treated with cal-C as in panel A, and cells were incubated with Lysotracker red for 1 h prior to phase contrast microscopy and fluorescence imaging.

**Figure 4.** Cal-C treatment induces an ER stress response concurrent with ER vacuolization. (A) MCF-7 cells were harvested at the indicated times after treatment with 50 nM cal-C and equal amounts of protein were subjected to immunoblot analysis for phospho-JNK (Thr183/Tyr185) or total JNK. Tubulin served as a loading control. The ratio of phospho to total proteins was determined by quantifying the immunoblot signals with an Alpha Innotech FluorChem HD2 imaging system. The upper panel depicts a representative immunoblot of phospho-JNK and total JNK. The bar graph shows the mean (± SD) of the ratio of phospho-JNK to total JNK, determined from three cultures.
Asterisks denote values that were significantly increased relative to the 0-h time point ($p \leq 0.01$). (B) Immunoblot showing the time course of PERK (Thr 981) phosphorylation after treatment with cal-C. The ratios of phospho-PERK to total PERK (mean ± SD) determined from three cultures are shown in the bar graph. Asterisks denote values that were significantly increased relative to the 0 h time point ($p \leq 0.02$). (C) Upper Panel, Immunoblot showing the time course for induction of CHOP after treatment with cal-C. Lower panel, Immunoblot showing that CHOP is not induced after treatment with 500 nM STS. Lysate from MCF-7 cells treated with cal-C for 4 h served as a positive control for CHOP in this immunoblot. α-Tubulin was used as the loading control.

**Figure 5.** Cell death caused by cal-C in MCF-7 cells is caspase-dependent. (A) MCF-7 cells were treated with 50 nM cal-C and/or 50 μM z-VAD-fmk for 18 h. Attached and detached cells were pooled for immunoblot analysis of PARP. (B) MCF-7 cells were treated with 50 nM cal-C and/or 50 μM z-VAD-fmk. Parallel cultures treated with DMSO served as controls. Viability assays were performed 24 h after addition of the compounds. Each bar represents the mean (± SD) of determinations performed on four separate cultures in a 96-well plate. The increase in viability of the cells treated with cal-C plus z-VAD compared with cal-C alone was significant at $p < 0.001$. (C) MCF-7 cells were incubated with 50 nM cal-C, 500 nM STS, or DMSO for 18 h. Attached and the detached cells were pooled and stained with the Guava Nexin reagent. Annexin V positive cells were analyzed using a Guava personal cytometer. Each bar represents the
mean (± SD) of determinations performed on three separate cultures. The increases in annexin positive cells with STS or cal-C were significant at p < 0.01.

**Figure 6.** Cal-C induces early activation of caspases 9 and 7, but not caspase 4. MCF-7 cells were harvested at the indicated times after activation of 50 nM cal-C. At each time point, attached and detached cells were pooled and equal amounts of protein were subjected to immunoblot analysis for the indicated caspases. (A) *Upper Panel*, Time course of procaspase 9 cleavage to active 37 and 35 kDa fragments in cal-C-treated MCF-7 cells. *Lower Panel*, Time course of procaspase 9 cleavage after treatment with STS. (B) Time course of procaspase 7 cleavage following cal-C activation. (C) Time course of procaspase 4 cleavage after treatment with cal-C. The separate lanes at the right show procaspase 4 cleavage in MCF-7 cells that were treated with 2.5 μM brefeldin A (BFA) for 24 h. (D) MCF-7 cells were treated with cal-C alone or in combination with 20 μM z-LEVD-fmk (caspase 4 inhibitor), as described in “Materials and Methods”. MTT assays were performed after 24 h. Each bar represents the mean (± SD) of determinations performed on four separate cultures in a 96-well plate.

**Figure 7.** Export of an endogenous type-I membrane glycoprotein (β-APP) from the ER is impaired in MCF-7 cells treated with cal-C. Control and cal-C treated MCF-7 cells were labeled with [35S] methionine for 30 min. One dish from each condition was harvested immediately (pulse), and the other set was chased for 2 h in medium containing excess unlabeled methionine. β-APP was immunoprecipitated as described in Materials
and Methods. (A) The pulse and chase samples were run on separate gels and subjected to fluorography. The images are representative of results obtained from three separate cultures. (B) The bar graph shows the densitometric ratio of mature (135 kDa) to immature (115 kDa) β-APP, determined in the pulse and chase samples. The results are expressed as mean (± SD) determined from three separate cultures. The asterisk denotes a significant increase at p< 0.01. (C) MCF-7 cells were incubated with activated cal-C or DMSO for 2 h and then processed for immunofluorescence detection of the Golgi apparatus using an antibody against GM130. The immunofluorescent images were merged with the phase contrast images of the fixed cells.
Fig. 1

A

![Graph showing viable cell count over concentration of cal-C (nM)]

B

![Western blot showing PARP, Cleaved PARP, and Tubulin over time after activation of cal-C (h)]

C

![Western blot comparing PARP, Cleaved PARP, and Tubulin across different cell lines (MCF-7, U251, PANC-1)]

D

![Western blot comparing PARP, Cleaved PARP, and Tubulin across different cell lines (MCF-7, U251, PANC-1) with cal-C treatment]

E

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Vacuolation</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 (Breast Cancer)</td>
<td>+</td>
<td>16.7</td>
</tr>
<tr>
<td>ZR75 (Breast Cancer)</td>
<td>+</td>
<td>31.9</td>
</tr>
<tr>
<td>U251 (Glioblastoma)</td>
<td>+</td>
<td>16.4</td>
</tr>
<tr>
<td>T98G (Glioblastoma)</td>
<td>+</td>
<td>26.6</td>
</tr>
<tr>
<td>501 Mel (Melanoma)</td>
<td>+</td>
<td>12.9</td>
</tr>
<tr>
<td>U22S (Osteosarcoma)</td>
<td>+</td>
<td>18.4</td>
</tr>
<tr>
<td>PANC-1 (Pancreatic Cancer)</td>
<td>+</td>
<td>24.9</td>
</tr>
<tr>
<td>SW480 (Colon Cancer)</td>
<td>-</td>
<td>14.7</td>
</tr>
<tr>
<td>SKOV3 (Ovarian Cancer)</td>
<td>-</td>
<td>37.1</td>
</tr>
</tbody>
</table>
Fig. 7

A

B

C

Phase

GM-130

Merge
SUMMARY

Initiation of programmed cell death is central during embryogenesis and for the maintenance of normal tissue homeostasis. Apoptosis or type I PCD is an important death pathway utilized by multicellular organisms to get rid of damaged/abnormal cells. However, molecular aberrations allow certain cells to suppress the death program, enabling abnormal growth and cancer formation. Also, apoptosis evasion not only contributes to increased growth but also to treatment resistance. Among the primary brain tumors, glioblastoma multiforme is considered most treatment refractory. The majority of currently available chemotherapeutic agents induce death by DNA damage-initiated apoptosis, to which the glioblastomas are known to be resistant. It is therefore imperative to find alternative strategies to initiate death in these highly intractable tumors.

With the growing understanding of the molecular mechanisms of PCD, it is now being appreciated that cell death can occur via apoptotic and non-apoptotic death programs, both of which have relevance in cancer therapy. In this dissertation I have described two different strategies that can be used to initiate effective cell killing in glioblastomas. The first being a novel form of non-apoptotic cell death induced by expression of the activated form of Ras GTPase. The second approach involves initiation of ER stress-induced apoptosis, using calphostin-C, a specific inhibitor of the regulatory subunit of protein kinase C. Calphostin-C, at nanomolar concentrations, effectively killed a wide variety of cancer cells, including glioblastomas.

The expression of H-Ras(G12V) in glioblastoma cells results in the accumulation of large cytoplasmic vacuoles, subsequently the cells detach from the substratum and die.
This observation was first made by Chi et al., in 1999, wherein they suggested that the cells were dying via type-II or autophagic cell death. However, our findings clearly show that the Ras-induced vacuoles are derived from macropinosomes, and not from autophagosomes or lysosomes. The vacuoles rapidly incorporate extracellular fluid phase tracers, like dextran-Alexa Fluor and Lucifer yellow, which is a characteristic feature of macropinosomes. Macropinocytosis is an actin dependent process. The uptake of these tracers was inhibited by pre-incubating the cells with cytochalasin-D, further confirming that Ras-induced vacuoles are derived from macropinosomes.

Even though we observed an induction of LC3-II, a protein that specifically associates with the autophagosomes, immunofluorescence studies revealed that LC3-II positive structures were distinct from Ras-induced vacuoles. Furthermore, knockdown of beclin-1, a well established autophagy protein, had no effect on the vacuolar morphology or induction of death. These studies clearly indicate that autophagy does not play a causative role in the death of glioblastoma cells, induced by activated Ras.

In order to evaluate the role of apoptosis, if any, in the death induced due to accumulation of macropinosome-derived vacuoles, we performed detailed morphological and biochemical evaluations. Electronmicrographs of dying cells did not reveal chromatin condensation, a characteristic feature of apoptosis. Interestingly, caspase activation was observed in the dying cells but the broad spectrum caspase inhibitor, zVAD-fmk, did not prevent cell death. These observations indicate that caspase activation is not essential for Ras-induced cell death. This work thus provides the first
evidence for a form of cell death induced by accumulation of vacuoles derived from macropinosomes, which we have named “methuosis”.

The constitutively active form of Ras is oncogenic in a variety of cancers, but it has also been shown to induce death or senescence in certain cell types (Cox and Der, 2003). This duality underscores the complexity of Ras signaling networks. Since Ras is known to bind to a variety of different downstream effectors, it is important to understand which signaling pathway is specifically activated by Ras to induce methuosis, in order to exploit this unique death pathway as a targeted therapy in glioblastomas. We have shown that the most well-studied Ras effectors, Raf kinase, RalGDS and PI-3K, are not required for induction of the vacuolar phenotype. Our studies also revealed that activated Rac1 recapitulates the effect of Ras in glioblastomas, and other Rho family members (RhoA and Cdc42) do not play a role in methuosis. However, further studies need to be performed to confirm that Rac1 is indeed downstream of Ras, and if so, which downstream Ras effector is responsible for the activation of Rac1.

The essential role played by the endoplasmic reticulum in protein synthesis, folding, and calcium homeostasis has long been known. However, the molecular connections between the ER and cell death have emerged more recently. There is a growing body of evidence demonstrating the susceptibility of tumors to ER stress-initiated apoptosis (Kim et al., 2008). Agents that induce cytotoxicity via this mechanism are being developed as chemotherapeutic agents. This work describes the mechanism by which calphostin-C, a PKC inhibitor, exerts its cytotoxic effect on cancer cells. The
unique feature of cal-C is that it requires light activation, making it an attractive agent for photodynamic therapy.

Calphostin-C induces efficient killing of a wide variety of cancer cells. The majority of cell lines, including those derived from glioblastomas, exhibit an early accumulation of ER-derived vacuoles when exposed to this drug. Swelling of the ER is linked with an accumulation of proteins destined for the Golgi compartment. Calphostin-C treated cells are positive for indicators of ER stress signaling, which include phosphorylated PERK and JNK, along with induction of the pro-apoptotic protein, CHOP. Activation of caspases-9 and 7 is followed by PARP cleavage. Finally, co-treatment with zVAD-fmk increases the percentage of viable cells, indicating that caspases are essential for cal-C induced cell death. These observations strongly indicate that cal-C is causing apoptosis via sustained and severe ER stress. At this point we cannot rule out the possible involvement of the mitochondrial pathway because of the activation of caspase-9 after cal-C treatment. However, our data strongly suggests that part of the mechanism of cal-C induced apoptosis involves ER stress. Interestingly, staurosporine, a classical PKC inhibitor, does not cause vacuolation or induction of the pro-apoptotic ER stress protein CHOP. We therefore postulate that cal-C may be binding to targets other than PKC to inhibit export of proteins out of the ER, consequently inducing ER stress. Our data raises the possibility that cal-C could be developed as an antineoplastic agent; it is particularly an attractive candidate for photodynamic therapy.
BIBLIOGRAPHY


combination with celecoxib or its non-coxib analogue, 2,5-dimethyl-celecoxib.

Cancer Res. 68, 843-851.


ABSTRACT

The concept of programmed cell death has evolved over the years to include both apoptotic and non-apoptotic death mechanisms. This study describes a novel form of non-apoptotic cell death induced as a result of dysregulated macropinocytosis. We have named this cell death “methuosis”. Methuosis is observed when the activated form of Ras GTPase is over-expressed in glioblastoma cells. It is accompanied by the accumulation of large phase-lucent cytoplasmic vacuoles, followed by rounding up, detachment, and disintegration of the cells. The vacuoles quickly take up extracellular fluid-phase tracers, a hallmark of macropinosomes. Our studies also show that the Ras-induced vacuoles are not acidic and are negative for LC3-II (a marker for autophagosomes), transferrin and EEA1 (endosomal markers). These observations rule out the vacuoles originating from autophagosomal, endosomal or lysosomal compartments. Even though caspase activation is observed in dying cells, death is not prevented by zVAD-fmk, a pan caspase inhibitor. Electron microscopy revealed that the dying cells did not show classical signs of apoptosis, like chromatin condensation. These findings indicate that caspase activation is not required for methuosis to occur. Studies performed to decipher the signaling pathway(s) stimulated by Ras revealed that methuosis does not depend on the activation of Raf kinase, PI-3K orRalGDS, the most well-studied Ras signaling intermediates. Interestingly, constitutively active Rac1 induces an identical vacuolar phenotype in glioblastoma cells. Rac1-induced vacuoles are also derived from macropinosomes. We postulate that activated Ras is stimulating Rac GTPase via a unique downstream effector to initiate methuosis in glioblastoma cells.
ER stress-initiated apoptosis has recently gained attention as an effective death inducer in cancer cells. This work shows for the first time that the mechanism by which calphostin-C, a photoactivatable inhibitor of protein kinase C, induces apoptosis in cancer cells involves ER stress. Calphostin-C potently reduces the viability of a number of cancer cell lines, including glioblastomas. The cell death induced by cal-C involves accumulation of vacuoles derived from the ER with a concomitant block in the protein trafficking from ER to Golgi. There is a rapid activation of ER stress markers, JNK, PERK, and the induction of pro-apoptotic protein CHOP. Activation of caspases-9 and 7, along with PARP cleavage, is observed following the activation of ER stress signaling. Our studies indicate that apoptosis induced by cal-C has a strong ER-stress component and that this compound has a potential of being exploited as a chemotherapeutic agent for photodynamic therapy.