Exploitation and Regulation of Apoptotic Caspases

Scott Eron

Follow this and additional works at: https://scholarworks.umass.edu/dissertations_2

Part of the Biochemistry Commons, and the Structural Biology Commons

Recommended Citation

https://scholarworks.umass.edu/dissertations_2/873

This Open Access Dissertation is brought to you for free and open access by the Dissertations and Theses at ScholarWorks@UMass Amherst. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.
EXPLOITATION AND REGULATION OF APOPTOTIC CASPASES

A Dissertation Presented

by

SCOTT J. ERON

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
Of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2017

Chemistry Department
EXPLOITATION AND REGULATION OF APOPTOTIC CASPASES

A Dissertation Presented

by

SCOTT J. ERON

Approved as to style and content by:

Jeanne A. Hardy, Chair

Scott C. Garman, Member

Sankaran Thayumanavan, Member

Michael J. Knapp, Member

Craig T. Martin, Department Head
Chemistry Department
DEDICATION

To Thomas, Nancy, and Leigh for their endless support, patience, and love.

To my Grandma Eron, for always putting a smile on my face.

To my Uncle Tim, for showing me how to work hard,

my Aunt Mary, for teaching me faith and compassion,

and to my Grandma Bush, for pushing me to follow by ambitions.
ACKNOWLEDGEMENTS

First and foremost I would like to thank Jeanne, my advisor and mentor for the past six years. Your enthusiasm and passion for science had me hooked from the very first day of graduate school. I had essentially no biological background, yet you took a chance on me and are the reason I was able to succeed. You taught me how to think like a scientist, be curious, ask the right questions, and respect the hard work of others. You taught me how to present my research to virtually any audience and how to leave an impression.

I would like to thank Scott Garman for continuing to make me a better student and researcher every time we spoke. You always took the time to think about my projects and offer insightful advice and suggestions, no matter what setting. You also knew how and when to challenge me, which was never boring. It was also great to talk sports and share stories, not everyone can jump as high as Dr. Scott Garman.

I would also like to thank Mike Knapp and Thai. You both have a thirst for knowledge that has undoubtedly had a positive affect on my curiosity. I want to thank you for letting me collaborate with your groups and for showing me a different lens to view the same problem. I also want to thank Richard Vachet for all his help and guidance during my first year of graduate school, and for showing me professors aren’t all scary.

I absolutely have to thank my undergraduate research advisor, Jeff Peterson. You had incredible patience teaching me at Geneseo. Even after I broke the expensive Schlenk line on the very first day, you gave me a chance and I am forever grateful.

I cannot express enough my gratitude toward my Hardy Lab family. From the very first day I felt welcomed by all of the senior students. Sam, Kristen, Elih, Muslim, and Witold taught me how to work hard and how to accept failure. Because throughout your PhD you fail, a lot, and they were always supporting me through those times. And after their departure, a new family came together. Kevin, Bay, Derek, and Maureen, you are the foundation from which I gather strength. I absolutely could not be where I am without all of you, and I am sad for the day when my bench no longer borders yours. You have taught me so much, and not just in the lab, but in life. I will never forget our attempts at lab lunch, all the delirious late nights, a certain someone’s love for tequila, and the constant bothering of each other while we tried to do experiments. Thank you for making every day in the lab an enjoyable one, I will always treasure our time as a family.

To my friendship family back in Syracuse, thank you for always supporting me no matter what the circumstances. Mark, Catherine, Dan, Pat, and Matt, I wouldn’t know how to have fun without you guys. Without you there would be no old time hockey, no Eddie Shore.

To Matt and Mike, thank you for your constant advice and support. I would not be here without the two of you. To Tim, Steve and to my UMass friends; the boys of West street, the girls of High street, thank you for all the fun. We worked hard to play hard, and
play hard we did. You guys were a tremendous support system and I can never thank you enough.

And last, I would like to thank my family and relatives. I am blessed to have such a large and supportive family. From my Godmother, Aunt Sandy, to all of my cousins, aunts, uncles, and grandparents, I have felt overwhelming support and I thank you all.

But most importantly, I would like to thank my parents, Thomas and Nancy, and my younger sister Leigh. You taught me everything; how to live, love, learn, grow, succeed, and fail. You supported me in my darkest hours and commended me during my finest moments. You encouraged my every endeavor and inspired me to pursue my passions. It is because of you that I am the man I am today, and it is because of you that I have found success in what I enjoy the most, being a scientist. Thus, I dedicate this work to you.

“We keep moving forward, opening new doors, and doing new things, because we are curious... and curiosity keeps leading us down new paths.”

-Walt Disney
Caspases are the cysteine proteases that govern apoptotic cell death. The regulation of these enzymes is critical in order to restrain their death-inducing capabilities until the appropriate moment. Infidelity of caspase regulation and activation underlies a plethora of human diseases ranging from cancer to neurodegeneration. This establishes a pressing need for comprehensive studies of the apoptotic caspases in order to understand all aspects of their regulation, activation, substrate preferences, structure, and function. A detailed structural view of caspase regulation would have lasting implications for future therapeutic avenues targeting caspase function or apoptosis. This dissertation chronicles caspase regulation by phosphorylation as well as zinc. A mechanistic approach uncovering the precise means by which caspases and kinases co-regulate one another offers multiple avenues for therapeutic intervention. In addition, the influence of zinc on caspase activity at biologically relevant concentrations alters the perspective on zinc regulation of apoptosis. Lastly, this work utilizes the knowledge derived from these mechanistic studies for an application in which the cell death inducing potential of an executioner caspase is harnessed by delivering the caspase to a population of cancer cells.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>xvii</td>
</tr>
</tbody>
</table>

## CHAPTER

I. INTRODUCTION........................................................................................................1

*Apoptosis Depends on Caspases.................................................................1*

*The Apoptotic Pathway and the Activation of Caspases.............................2*

*Characteristics of Caspases.................................................................5*

*Regulation...............................................................................................9*

*Natural Regulation of the Apoptotic Caspases......................................11*

*The Caspase Potential.............................................................................17*

*References.........................................................................................18*

II. DUAL SITE PHOSPHORYLATION OF CASPASE-7 BY PAK2 BLOCKS APOPTOTIC ACTIVITY BY TWO DISTINCT MECHANISMS........24

*Abstract...............................................................................................24*

*Introduction.........................................................................................25*

*Results..................................................................................................28*

*The S239E Phosphomimetic Inactivates Caspase-7.................................28*

*Caspase-7 is Phosphorylated by PAK2 at S239 on the Small Subunit........31*
S239E Phosphomimetic Structure Suggests the Mechanism of Inhibition by Phosphorylation..........................35

Caspase-7 Phosphorylation Occurs on the Large Subunit Primarily at S30...........................................38

Phosphorylation of Caspase-7 Diminishes Processing by Initiator Caspases........................................39

Phosphorylation at S30 Slows Processing of Caspase-7 at the Intersubunit Linker.....................................42

Phosphorylation at S30 Interrupts the Binding Interaction Between Caspase-7 and Caspase-9..................43

Non-phosphorylatable Caspase-7 is Cleaved Faster by Caspase-9 Intracellularly.................................44

Discussion............................................................................................................................................47

Materials and Methods.........................................................................................................................54

DNA Expression Constructs and Antibodies..........................................................................................54

Generation of Caspase-7 Variants........................................................................................................54

Generation of the Caspase-9 Constitutive Dimer.................................................................................54

Caspase-7 Expression and Purification..................................................................................................55

Caspase-8 Expression and Purification..................................................................................................56

Caspase-9 Expression and Purification..................................................................................................56

PAK2 T402E Expression and Purification..............................................................................................57

Activity Assays......................................................................................................................................58

In vitro Phosphorylation Assays...........................................................................................................59

Caspase-7 S239E Crystallization and Data Collection.........................................................................60

Structure Determination.......................................................................................................................60
Materials and Methods.................................................................137

Materials.......................................................................................137

Synthesis of p(PEGMA-co-PDSMA) (P1).................................138

Synthesis of Nanogel-Caspase Conjugates.........................139

Activity Assay...............................................................................140

References......................................................................................141

V. HARNESSING THE APOPTOTIC POTENTIAL: INTERROGATING
CASPASE STRUCTURE TO CONTROL FUNCTION.........................145

Investigating Regulation of the Apoptotic Caspases............145

Caspase-7 and PAK2 Co-Regulation.........................................146

Apoptotic Caspases: Inhibition by Zinc..............................150

Exploiting Caspase Cell Death Potential via Nanogel Delivery.......154

The Killing Blow..............................................................................157

References......................................................................................157

BIBLIOGRAPHY..............................................................................160
LIST OF TABLES

Table

1. Crystallographic Data Collection and Statistics for the Caspase-7 S239E Variant ................................................................. 36

2. Parameters for Zinc Inhibition of Caspases under Metal Buffering Conditions and Unbuffered Conditions ........................................... 78

3. Thermal Stability Tm Measurements ........................................................................ 84

4. Thermal Stability Measurements for Caspase-7 ......................................................... 91
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The Apoptotic Pathway</td>
<td>3</td>
</tr>
<tr>
<td>2. Caspase Architecture</td>
<td>6</td>
</tr>
<tr>
<td>3. Caspase-7 Conformational Equilibrium Between States</td>
<td>8</td>
</tr>
<tr>
<td>4. Phosphorylation of the Apoptotic Caspases</td>
<td>13</td>
</tr>
<tr>
<td>5. Sites of Caspase-7 Phosphorylation by PAK2 and Kinetics of Phosphomimetic Variants</td>
<td>29</td>
</tr>
<tr>
<td>6. PAK2 Phosphorylates the Caspase-7 Small Subunit at S239</td>
<td>32</td>
</tr>
<tr>
<td>7. Caspase-7 cleaves PAK2 at D212</td>
<td>33</td>
</tr>
<tr>
<td>8. Caspase-7 WT can Recover Full Activity After Silencing with PG</td>
<td>34</td>
</tr>
<tr>
<td>9. Caspase-7 Wild-Type and C186A are both Phosphorylated on the Small Subunit in the Presence of Protecting Group</td>
<td>35</td>
</tr>
<tr>
<td>10. Phosphorylation at Caspase-7 S239 Sterically Blocks Substrate Binding</td>
<td>37</td>
</tr>
<tr>
<td>11. Caspase-7 Wild-Type Activity is Not Affected by PAK2 Phosphorylation</td>
<td>38</td>
</tr>
<tr>
<td>12. Phosphorylation on Caspase-7 Slows Processing by Upstream Initiator Caspases</td>
<td>40</td>
</tr>
<tr>
<td>13. Caspase-8 and Caspase-9 Activity is Unaffected by PAK2</td>
<td>41</td>
</tr>
<tr>
<td>14. Phosphorylation at S30 on Caspase-7 Slows Processing by Caspase-9</td>
<td>42</td>
</tr>
<tr>
<td>15. The Interaction Between Caspase-7 and Caspase-9 is Disrupted by Phosphorylation at S30</td>
<td>43</td>
</tr>
<tr>
<td>16. Fluorescence Polarization Controls</td>
<td>44</td>
</tr>
<tr>
<td>17. S30 Phosphorylation regulates Caspase-7 Processing Intracellularly</td>
<td>46</td>
</tr>
</tbody>
</table>
18. PAK2 uses Distinct Mechanisms at Two Sites of Phosphorylation: S30 Blocks Caspase-7 Zymogen Activation while S239 Directly Disrupts Substrate Cleavage

19. Known Zinc Binding Sites in Caspases

20. Zinc Inhibits Caspase-3, -7, and -8 from Cleaving Peptide & Protein Substrates.

21. Buffered Zinc Conditions Reveal Biologically Relevant Inhibition

22. Kinetic Fits to Determine Inhibition Constants

23. Stoichiometry of Zinc Binding

24. Zinc Binds to the Active Site of Caspase-8 and Influences the Oligomeric State

25. Zinc Destabilizes Caspase-8

26. Caspase-8 C360A is Only Destabilized by Zinc

27. Substrate-Mimic Occupying the Active Site Disrupts Zinc Binding to Caspase-7 but Not Caspase-3

28. Comparison of Raw Data for Caspase-8 and Caspase-7 Differential Scanning Fluorimetry

29. Thermal Shift Assays for Full-Length Caspase-7 and Active Site Variants

30. Caspase-3 is Stabilized by Zinc

31. The Influence of Zinc on Caspases and Apoptosis

32. Recovery of Caspase-3 Activity After Treatment with Various Polymerization Agents

33. Surface Exposed Cysteine Residues in Caspase-3
34. NMR Spectra of p(PEGMA-co-PDSMA) .................................................. 119
35. Absorption Spectra of Nanogel-Caspase Conjugates.......................... 121
36. Nanogel-Caspase Conjugates Characterization................................. 123
37. SDS-PAGE Gel Validating the Nanogel-Caspase Conjugation through
   Reducible Disulfide Linkages.............................................................. 124
38. Mass Specrometry of Nanogel-Caspase Conjugates............................ 126
39. Protein Enzymatic Activity............................................................... 129
40. Caspase-3 Activity Recovered after an Early Protocol for the Construction
   of Caspase-Containing Nanogels........................................................ 130
41. SDS-PAGE Gel Validating the Nanogel-Caspase Conjugation through
   Reducible Disulfide Linkages.............................................................. 131
42. Cellular Internalization of Nanogel-Caspase Conjugates with FITC Labeled
   Caspase-3............................................................................................. 132
43. No Observed Cellular Internalization of FITC-Caspase-3 Alone after 4 hours.. 133
44. Cell Viability after 24 hr Exposure of HeLa Cells with the Conjugates......... 135
### LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Preparation of Nanogel-Caspase-3 Conjugates</td>
<td>120</td>
</tr>
</tbody>
</table>

xvii
CHAPTER I

INTRODUCTION

Apoptosis Depends on Caspases

All multicellular eukaryotes depend on one critical biological pathway that dictates cell life and cell death. A programmed cell death cascade known as apoptosis has proven to be imperative to all metazoans, influencing a myriad of processes including organismal development, differentiation, maintaining homeostasis of cell populations, and elimination of damaged cells. This gene directed execution is so advantageous because it circumvents inflammation, leakage of cell contents, and does not damage the surrounding cells. This clean and methodical removal of cells begins with an energy dependent set of biochemical mechanisms that result in distinct morphological changes, including blebbing of the cell membrane, overall cell shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation and global mRNA decay. Ultimately, internal fragments of cell debris are packaged into apoptotic bodies and excreted, where they are quickly phagocytosed and degraded.

At the heart of this favorable cell death pathway are the caspases, a family of influential cysteine proteases who effectively manage this potentially lethal process. These caspases derive their name from their enzymatic function: using a cysteine for thiol chemistry in the active site to preferentially cleave after aspartate residues contained within a particular recognition motif. The proper regulation of the caspases is absolutely paramount to ensure that apoptosis occurs at the appropriate moment only under deliberate stimulation. Inappropriate apoptosis plays a major role in a plethora of diseases at both ends of the spectrum. Consequently, increased apoptosis is a factor in classic
neurodegenerative diseases such as Alzheimer’s\textsuperscript{5–7} and Huntington’s\textsuperscript{8,9} disease, as well as ischemic injury\textsuperscript{10} and AIDS.\textsuperscript{11} Meanwhile, a suppression of apoptosis is observed in many types of cancer\textsuperscript{12–15} and in autoimmune disorders.\textsuperscript{16}

**The Apoptotic Pathway and the Activation of Caspases**

Apoptosis comes from the Greek word meaning “falling off,” referring to the falling of leaves from a tree. Scientists have studied the concept of programmed cell death for (arguably) centuries, with examples ranging from observations of dying bark on oak trees to insect metamorphosis to neuronal death during embryonic development. However, it was not until 1972 that Kerr et al.\textsuperscript{17} coined the term apoptosis while examining morphological changes in hepatocyte development. The idea of an encoded program for cell suicide was brought to light.

The investigation of just 131 cells in a small nematode opened the window through which we now look at the mechanism of apoptosis. While observing the development of the nematode *C. elegans*, Horvitz and colleagues found that of the 1090 somatic cells of this worm, 131 of these cells die in a controlled fashion at specific stages during development.\textsuperscript{1} Amazingly, this was a consistent observation for each worm as it matured. The enzymes responsible for this programmed cell death (e.g. ced9 in *C. elegans*) were found to be homologous to human cysteine proteases, which are the caspases we know today. The study of this process behind gene-directed elimination of cells has since surged with researchers all over the globe who recognize the tremendous therapeutic potential of being able to dictate cell life or death.

This thesis aims to interrogate the regulation of the apoptotic caspases and exploit the knowledge gained for eventual therapeutic purposes. This begins by understanding
the activation mechanisms that trigger apoptosis. Apoptosis can be induced by multiple signals resulting in activating the initiation phase of cell death (Figure 1). This typically follows two main pathways: (1) the extrinsic pathway dependent on death receptors, or (2) the mitochondrially triggered intrinsic pathway. There is a third granzyme dependent cascade, which will not be discussed in detail here. However, an important observation is that all three initiation events converge on the activation of executioner caspases, emphasizing the critical role caspases play during apoptotic cell death.

**Figure 1: The Apoptotic Pathway**
The extrinsic and intrinsic apoptotic pathways are dependent on caspase activity. They begin by activating the upstream initiator caspases (procaspase-8 and -9 are activated by the DISC and apoptosome complexes respectively), which in turn cleave their substrates, the downstream executioner caspases. Both pathways converge on the activation of procaspase-3, -6, and -7, which are cleaved to generate caspase-3, -6 and -7, and which proceed to dismantle the cell in an orderly fashion through the cleavage of a myriad of specific substrates, ultimately terminating the cell.
The family of apoptotic caspases is broken down into initiator caspases -2, -8, -9, and -10, as well as executioner caspases -3, -6, and -7. The initiators act upstream, dependent on a trigger (dimerization or binding to an activating platform like the DISC or apoptosome) that enhances their ability to cleave and activate the downstream executioner caspases. Both the extrinsic and intrinsic apoptotic pathways rely on this cascade of caspase cleavage and activation events (Figure 1). The extrinsic pathway utilizes death receptors of the tumor necrosis factor (TNF) family, wherein stimulation of the receptors forces them to congregate at the cell surface and transmit the death signal through binding an intracellular ligand. This transfers information to the cytoplasmic region where formation of a death inducing signaling complex (DISC) can recruit caspase-8 and caspase-10 via their death effector domains (DED). Following recruitment, these initiator caspases dimerize and then activate via a cleavage event. This allows them to now cleave and activate the executioner caspases who then proceed to dismantle a myriad of downstream apoptotic targets.

The intrinsic apoptotic pathway is stimulated by various stress responses such as hypoxia, radiation, or viral infection. This program of cell death is contingent on permeabilizing the mitochondria, which relies on the action of the Bcl-2 family of proteins. This family consists of both pro-apoptotic and anti-apoptotic regulators (for review see Gross et al.). Under normal conditions, the anti-apoptotic Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 (among others) act in concert to inhibit the pro-apoptotic proteins Bax and Bak. When upstream signals activate this cascade, BH3 only proteins block Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, thus sequestering their anti-apoptotic activity. This allows Bax and Bad to form complexes at the mitochondrial outer membrane, which results in pore
formation and release of cytochrome c.\textsuperscript{22} Apoptotic protease activating factor-1 (Apaf-1) with the help of dATP, bind cytochrome c and form a heptameric complex known as the apoptosome.\textsuperscript{23} This enormous complex can recruit monomeric caspase-9 via its caspase activation and recruitment domain (CARD) and significantly enhance its activity.\textsuperscript{24} Caspase-9 then cleaves and activates executioner caspases -3 and -7\textsuperscript{25} for them to carry out the execution phase of apoptosis.

Both the extrinsic and intrinsic apoptotic cascades converge on the executioner caspases, highlighting the pivotal role they play in this powerful biological process. Controlling the activity of these enzymes would dictate life and death within cell populations, underscoring their therapeutic potential. It is with this in mind that we and others have pursued the exploration of understanding these proteases on a molecular level, with a particular focus on their regulation and activation.

**Characteristics of Caspases**

Ultimately, it is the caspases that drive the apoptotic machine during programmed cell death, making it imperative that we understand this powerful class of enzymes. This appreciation begins with activation. Due to their cell death inducing potential, apoptotic caspases are held in an inactive (uncleaved) zymogen form, also referred to as procaspases. An activating event must first occur in order to convert these proteins to highly functioning proteases. An extremely diverse set of stimuli can set this into motion, but it begins with the upstream initiator caspases. These are typically found in their monomeric zymogen (pro) form, and activation occurs via an induced proximity model. Whether it be caspase-8 associating with the DISC complex, or caspase-9 being recruited to the apoptosome (see above), the initiator caspases typically require the assistance of
other proteins for activation. Meanwhile, the executioner caspases naturally exist as dimers and their activation occurs upon cleavage by initiator caspases at their intersubunit linker.

Despite differences in activation, the caspase architecture is consistent across the apoptotic family. Executioner caspases are functional as homodimers, with each monomer comprised of a prodomain at their N-terminus, followed by a large and small subunit, which is connected by an intersubunit linker (Figure 2A). The length of the N-terminal prodomain is quite variable, with initiators typically having lengthy recruitment regions (ranging from 138 to 216 amino acids) and executioners having short regions closer to 20 amino acids. To reach maximum activity, caspases require cleavage of their intersubunit linker in order to properly form the active site.

Interestingly, executioner caspases are obligate dimers, with processing of the linker necessary for full catalytic activity. This linker rests across the dimer interface and only after cleavage

Figure 2: Caspase Architecture
(A) Domain architecture of an executioner caspase displaying the obligate homodimer. Each monomer is comprised of a prodomain, a large subunit, an intersubunit linker, and a small subunit. Arrows indicate processing sites where upstream initiator caspases cleave and activate the executioner. (B) Cartoon representation of a canonical caspase. Gray coloring indicates the N-terminal region, which is modeled in because it cannot be seen crystallographically. The large and small subunits are shaded light blue and dark blue respectively, along with a tetrapeptide substrate DEVD in each active site. The four critical and mobile loops are also labeled in one active site, including L2’ from the opposite monomer.
can it be liberated, where it proceeds to act as a loop assisting in formation of the active site on the opposite monomer.

There are two active sites per caspase homodimer and each is comprised of four critical mobile loops: L2, L3, L4, from one monomer and L2’ from the opposite monomer (Figure 2B). The orientation of these loops is absolutely essential for proper binding and cleavage of substrate. These flexible loops can sample different conformational states throughout the life of the protein, and a dynamic equilibrium exists between these states (Figure 3). Loop positioning has been thoroughly investigated with the executioner caspase-7 by x-ray crystallography. The pro-caspase-7 zymogen, mature (cleaved) unliganded (apo) caspase-7, and substrate-bound caspase-7 crystal structures have all been solved and combine to create a detailed understanding of loop positioning. In the uncleaved zymogen caspase-7 the active site loops are mobile and are not observed to be locked down to form the catalytic cleft. L2, which includes the catalytic cysteine C186, is held in the “down” conformation across the dimer interface. In addition, L3 and L4 are disordered. After cleavage of the intersubunit linker the mature caspase-7 is generated, and L2 is free to swing upward and make critical contacts with other active site loops including L4 and L2’. These interactions help form the proper active site loop bundle and allow the catalytic cysteine to rotate into a position amenable for substrate hydrolysis. Finally, several structures have been solved with an active site inhibitor bound, displaying all four loops in the proper orientation for binding and cleaving substrate. Notably, L2’ acts as a buttress for the L2 loop in the active site of the opposite monomer. It is important to note that a reversal of the active site loops back to the zymogen-like state have been observed by the addition of allosterically inhibiting
small molecules such as FICA and DICA (Figure 3). This will trap L2’ in the “down” conformation across the dimer interface and expel loop 3 from the active site. These observations underscore the mobility of these loops and the importance of proper active site geometry in order to recognize and process substrates.

Within each active site exists a catalytic dyad consisting of a cysteine and histidine to carry out the chemistry of catalysis. The caspase active sites have a strong specificity for acidic residues at the P1 cleavage site, typically cleaving after an aspartate residue on the protein substrate. However, glutamate and phosphoserine can occasionally be recognized by some caspases. The active site is also relatively conserved among all the apoptotic caspases, which makes targeting a specific caspase orthostERICally an extremely difficult task. Turning to allosteric regulation could provide a means to manipulate the activity of individual caspases in a selective fashion.

Figure 3: Caspase-7 Conformational Equilibrium between States
In the absence of any inhibitor caspase-7 primarily exists in the zymogen state (top). When bound to a small molecule in the active site the loops become organized and the equilibrium is shifted toward a conformation with a stable loop bundle (middle). Reversal to now favoring the zymogen state when bound to an allosteric inhibitor such as FICA or DICA (bottom).
The generation of a map of sensitive allosteric sites across the caspase family would provide new avenues to exploit individual caspases therapeutically.

**Regulation**

After the initiation of apoptosis, cells destined to die do so quickly and cooperatively. The cell death machinery is extremely efficient, primarily due to caspase activity. The executioner caspases have been documented to have hundreds of downstream substrates that are specifically cleaved, and in many cases the products have new functions based on this permanent posttranslational modification. Due to its staggering death potential, the apoptotic pathway and the caspases in particular require strict regulation intracellularly. Dysregulation can have dramatic effects. The majority of cancers have eluded the apoptotic response and therefore promote tumorigenesis or resist chemotherapies. Conversely, excess cell death and unwanted caspase activity has promoted the phenotypic impacts of neurodegenerative diseases such as Alzheimer’s and Huntington’s disease.

Numerous studies targeting caspases have aimed to either enhance or suppress apoptosis, although it is important to note that none have successfully completed clinical trials yet. Several molecules have been developed to activate caspases, particularly caspase-3, for use in cancer therapy. In particular, a high-throughput screen identified procaspase activating compound 1 (PAC-1).\(^3^3\) It was determined that the mechanism of procaspase-3 activation by PAC-1 was indirect, and was in fact due to PAC-1 chelating labile zinc bound to procaspase-3. It is well established that zinc inhibits procaspase-3\(^3^4\) and caspase-3,\(^3^5\) and PAC-1 was able to relieve that inhibition and sensitize procaspase-3 to activation. Clinical trials began on this compound in early 2015.\(^3^6\) Other compounds
currently under pursuit for apoptosis activation target other junctures in the apoptotic cascade that aim to activate caspases indirectly. One compound of interest is ABT-263\textsuperscript{37} (navitoclax), currently in clinical trials.\textsuperscript{38} This compound binds to three anti-apoptotic proteins Bcl-2, Bcl-xL, and Bcl-w,\textsuperscript{39} thus blocking their function and allowing pro-apoptotic Bcl proteins to permeabilize the mitochondrial outer membrane. This is a method to activate the intrinsic apoptotic pathway. However, some particular cancers overexpress other anti-apoptotic proteins, including Mcl-1, which can compensate for the loss of function of its inhibited Bcl-2 cousins after treatment with ABT-263 (for review see Williams et al.\textsuperscript{40}).\textsuperscript{41-43} Therefore, combination therapies are also currently under pursuit.\textsuperscript{44,45}

Another exciting application for activating the apoptotic pathway is the delivery of a caspase directly to any unwanted cell or population of cells. Protein therapeutics offer a variety of advantages over small molecules, including a more nuanced function with less off-target potential as well as catalytic opportunity that small molecules do not afford. The idea of caspase delivery is beginning to be explored, but the vehicle for such a delivery faces a variety of challenges including: reversibly encapsulating the caspase cargo, a timed intracellular release, and maintaining the native/active form of the protease. The development of nanogels to form a protein-polymer conjugates has been shown to meet these strict requirements and has successfully delivered active caspases which dose dependently eliminated a population of cancer cells.\textsuperscript{46} Expanding this approach has enormous potential for both caspase delivery and, in theory, delivery for any hydrophilic protein cargo.
Contrasting this school of thought, there has also been an effort to block caspase activation. Certain neurological diseases, such as Alzheimer’s disease, have been associated with enhanced irregular apoptosis. In addition, caspase-3 and -6 have been associated with disease onset, with studies suggesting they directly cleave amyloid precursor protein and tau.$^{6,47}$ As a result, many labs have been investigating the controlled silencing of this caspase activity specifically related to these neurological disorders. In addition, caspase inhibitors have been investigated for diseases or injuries that result in detrimental apoptosis. One example includes chronic hepatitis virus C infection that results in unwanted programmed cell death of hepatocytes.$^{48}$ Others involve battling excess apoptosis after ischemic injury, wherein a caspase inhibitor may provide benefit after stroke, brain injury, or an organ transplant.$^{49–51}$

Taken together, it is evident that there is a pressing need to study the variety of mechanisms of caspase regulation, activation, and suppression. The dysregulation of these enzymes has dramatic consequences and is associated with a plethora of diseases. By learning how to manipulate caspase function we may be able to make key therapeutic advances that take advantage of new, more specific mechanisms of regulation. In addition, with a strong focus on allostery we can uncover functionally sensitive sites on each individual caspase as a means of specific control.

**Natural Regulation of the Apoptotic Caspases**

The activity of apoptotic caspases is kept in check by layers of regulation that specifically modulate their biological roles. This begins with the fact that they are initially translated as inactive zymogens, but cellular control over proteolytic function becomes infinitely more complicated as the labyrinth of biological pathways engage and
intersect. It has become clear that natural regulation for the caspases has evolved at allosteric sites due to the unique role and regulation of each caspase. The entire family of enzymes catalyze the same reaction yet they display different biological roles and are regulated quite differently. Nature has embedded a record for this within the primary and tertiary structure of each caspase, with two notable recurring themes (1) posttranslational modifications, particularly phosphorylation and (2) inhibition via zinc binding. Although every apoptotic caspase is subject to these same two regulatory signals, each enzyme responds differently with a nuanced regulatory mechanism. Regulation is not only important to modulate apoptotic function, but also pivotal in non-apoptotic roles. Caspases have been increasingly implicated in a variety of non-apoptotic pathways such as immunity and cell behavior (for review see Kuranaga, 2012\textsuperscript{52}), for which responsibilities must be monitored.

Understanding any allosteric regulation could be extremely useful from a therapeutic perspective, and evidence for this continues to emerge. For example, a unique exosite on the N-terminal region of caspase-7 has been shown to enhance its recognition of particular apoptotic protein substrates.\textsuperscript{53} This enhancement is observed despite the fact that the close homolog caspase-3 shares the same sequence specificity as caspase-7 and displays a greater intrinsic activity compared to caspase-7.\textsuperscript{54} Further investigation into this sensitive N-terminal region could create an opportunity to specifically control caspase-7 activity. Implications for this grow in significance with continued discoveries that the similar executioners caspase-7 and caspase-3 actually have functionally distinct roles in apoptosis.\textsuperscript{55}
Posttranslational modifications also play an enormous role in caspase regulation. Caspases can be proteolytically modified, nitrosylated, ubiquitinated, and glutathionylated. But the most prevalent posttranslational modification is phosphorylation (for review see Dagbay et al.60). Every apoptotic caspase can be phosphorylated (Figure 4), and this typically leads to inactivation. What is so intriguing is the fact that this same signal, the addition of a phosphate group, has such a mechanistically differential impact on function for each independent caspase. In some cases, multiple phosphorylation events on the same caspase result in extremely different modes of regulation. In addition, phosphorylation can have differential effects on substrate processing. For example, caspase-6 is phosphorylated at S257, leading to

![Figure 4: Phosphorylation of the Apoptotic Caspases](image)

The apoptotic caspases are extensively phosphorylated, often leading to a decrease in catalytic activity.
inactivation. The phosphomimic S257D showed no self-activation potential and could not turnover a tetrapeptide substrate, however, it could cleave its own prodomain. The fact that caspase-6 recognizes different substrates uniquely suggests that exosites exist and that control over certain substrates is possible. This has enormous potential due to the influence caspase-6 has on neurodegenerative diseases; it would be incredibly powerful if one could block cleavage of harmful targets such as amyloid precursor protein, but retain caspase-6 activity for favorable biological substrates.

Phosphorylation by a variety of kinases has emerged as one of the most significant and influential forms of posttranslational modification on the caspases. This phosphorylation typically leads to inactivation. However, caspases have been found to cleave kinases and alter their function, and in a number of cases the substrate kinase is the same enzyme that phosphorylates that particular caspase (for review see Kurokawa et al.). This creates a dynamic interplay between these two enzymes, with each attempting to modulate the other with cell life and death hanging in the balance. Phosphorylation can act directly on the apoptotic caspases themselves, but kinases can also phosphorylate caspase substrates and alter the ability of the caspase to act on the newly modified substrate. For example, phosphorylation of phospholipase C-γ1 on a tyrosine residue sits adjacent to its caspase cleavage site. This addition of a phosphate blocks proteolytic processing by caspase-3 and -7. Conversely, some phosphorylation sites promote caspase cleavage. In PKCδ for example, a tyrosine phosphorylation event by Src kinase stimulates cleavage by caspase-3. Meanwhile, caspase cleavage of kinases results in altered forms of the respective kinases, which can have interesting downstream effects. Caspase processing can (1) separate inhibitory domains and possibly activate the kinase
for further downstream apoptotic applications\textsuperscript{70} (2) alter localization within the cell, such as exposing a nuclear localization signal,\textsuperscript{71} and (3) transform substrate specificities of the kinase itself.\textsuperscript{72} These events have dramatic implications for the morphological changes that occur during cell death, and they certainly open possibilities for positive feedback loops during the apoptotic cascade.

The interplay between caspases and the p21-activated kinase (PAK2) is an important example, which is the basis for one chapter in this thesis. PAK2 is a ubiquitously expressed kinase with implications in a variety of biological pathways including cell motility, mitosis, survival, and apoptosis. In its full-length form PAK2 stimulates cell survival,\textsuperscript{73,74} however, when cleaved by caspases it adopts a pro-apoptotic function. Caspase cleavage removes the autoinhibitory domain\textsuperscript{75} and allows the kinase domain to be transported to the nucleus\textsuperscript{76} where it can phosphorylate a new set of protein substrates that amplify the apoptotic response. Many forms of cancer have taken advantage of this duality by hyperactivating the full-length PAK2 and hijacking its anti-apoptotic activity.\textsuperscript{77} In fact, PAK2 has been found to phosphorylate and inactivate the executioner caspase-7,\textsuperscript{64} which would alter the ability of the cell to undergo apoptosis. As a result, PAK2 has been linked to resistance to chemotherapeutic agents. This example highlights the battle between caspases and kinases. In cancer, many caspases are losing this battle to phosphorylation by overexpressed kinases. Understanding the mechanism of inhibition by phosphorylation could inform scientists about (1) ways in which cancer is outlasting treatment and resisting chemotherapies and (2) continue to compile regions across the caspase family that are sensitive and able to modulate enzyme activity.
Regulation by metals is another powerful means to exert control over enzymatic activity. Notably, zinc has been found to be a vital cell regulator with recent discoveries putting more and more emphasis on zinc as a crucial biological regulator (for review see Fukada et al.\textsuperscript{78}). In fact, recent investigations confirmed nearly 3,000 proteins bind zinc, which is approximately 10\% of the human proteome.\textsuperscript{79} The influence of zinc extends to numerous biological processes, which is further emphasized by the fact that zinc affects a variety of proteins including transcription factors, enzymes, growth factors, receptors, and adapters (to name a few). Recently, the concept of cellular zinc buffering\textsuperscript{80} has surfaced as an important regulation technique for maintaining appropriate zinc concentrations while managing this array of biological processes. The discovery of zinc importers and exporters have uncovered relationships between zinc and numerous cellular functions.\textsuperscript{81} In addition, the importance of metallothioneins as zinc storage proteins adds a layer of complexity while analyzing the pool of available intracellular zinc. Maintaining zinc homeostasis is therefore a critical regulatory endeavor. Even a small disturbance in balancing the zinc concentration can have dramatic biological effects, including the activation or suppression of apoptosis.\textsuperscript{82}

The apoptotic caspases are inhibited by zinc,\textsuperscript{83–85} and in fact the entire apoptotic pathway has susceptibility to zinc regulation (for review see Truong-Tran et al.\textsuperscript{86}). Even small fluctuations in zinc concentration can disrupt cellular homeostasis and stimulate survival or force apoptosis. While caspase inhibition by zinc is well established, determining the mechanism of inhibition is still in its infancy. It was assumed that zinc bound to the caspase active site because of the cysteine-histidine dyad and the nucleophilicity of the catalytic cysteine. However, recent studies of caspase-9 demonstrate
that in addition to binding at the active site, the zinc also binds to an allosteric site. But even more exciting, investigation of caspase-6 revealed that zinc did not bind at the active site at all, but only to an allosteric region. This scrutiny at the molecular level suggests that zinc binding could be nuanced to each caspase. This again highlights how the same signal, the binding of zinc, has differential means of regulation in regards to the caspases. In addition, understanding each zinc binding site and its mechanism of altered protein function will only help build a map of allostery from which we can learn to control caspase function.

**The Caspase Potential**

The power and potential of the apoptotic caspases is staggering, requiring a tight regulatory network. Dysregulation has disastrous consequences for the cell, with many disease implications. The focus of this thesis centers on caspase regulation by phosphorylation and zinc, as well as exploiting the cell death potential of caspase-3 via targeted nanogel delivery. By understanding how these enzymes are regulated we can build an understanding of their structure and function that can aid in design efforts for therapeutic prospects. Specifically, chapter two will focus on how phosphorylation regulates caspase-7 function, with an application to chemotherapeutic resistance in cancers. Chapter three discusses a more global theme of zinc inhibition amongst the apoptotic caspases. Chapter four will conclude with delivering the executioner caspase-3 to cancer cells utilizing a redox-responsive nanogel vehicle. Ultimately, this thesis aims to expand the knowledge of caspase regulation with a strong focus on structural biology in order to intensify caspase therapeutic potential.


**References**


CHAPTER II

DUAL SITE PHOSPHORYLATION OF CASPASE-7 BY PAK2 BLOCKS APOPTOTIC ACTIVITY BY TWO DISTINCT MECHANISMS

The majority of this chapter has been published: Eron, S.J., Raghupathi, K., Hardy, J.A.

Dual site phosphorylation of caspase-7 by PAK2 blocks apoptotic activity by two distinct mechanisms. Structure. (2016).

Abstract

Caspases, the cysteine proteases that execute apoptosis, are tightly regulated via phosphorylation by a series of kinases. Although all apoptotic caspases work in concert to promote apoptosis, different kinases regulate individual caspases. Several sites of caspase-7 phosphorylation have been reported, but without knowing the molecular details, it has been impossible to exploit or control these complex interactions, which normally prevent unwanted proliferation. During dysregulation, PAK2 kinase plays an alternative anti-apoptotic role, phosphorylating caspase-7 and promoting unfettered cell growth and chemotherapeutic resistance. PAK2 phosphorylates caspase-7 at two sites, inhibiting activity using two different molecular mechanisms, before and during apoptosis. Phosphorylation of caspase-7 S30 allosterically obstructs its interaction with caspase-9, preventing intersubunit linker processing, slowing or preventing caspase-7 activation. S239 phosphorylation renders active caspase-7 incapable of binding substrate, blocking later events in apoptosis. Each of these mechanisms is novel, representing new opportunities for synergistic control of caspases and their counterpart kinases.
Introduction

Tens of billions of cells die each day by the controlled cell death pathways of apoptosis. Apoptotic programmed cell death is fundamental for all multicellular eukaryotes and is critical for organismal development, differentiation, eliminating damaged cells, and maintaining homeostasis. Dysregulation of apoptosis has been linked to diseases including cancer,\textsuperscript{1–4} autoimmune disorders,\textsuperscript{5} and neurodegeneration.\textsuperscript{6,7} In particular, transformation to a cancerous phenotype occurs when cells establish barriers to apoptosis and develop resistance to signals designed to eliminate malignant abnormalities via apoptosis.

The apoptotic cascade is ultimately dependent on the activation of the caspase family of cysteine proteases. These enzymes dismantle the cell by cleaving a diverse set of protein substrates\textsuperscript{8} with a specificity for acidic residues, typically aspartate, at P1\textsuperscript{9} although glutamate\textsuperscript{10} and phosphoserine can also be recognized.\textsuperscript{11} Apoptotic caspases are divided into two classes: the initiator caspases (caspase-8, -9, and -10) and their substrates, the executioner caspases (caspase-3, -6, and -7), which cleave specific cellular targets to invoke cell death. Caspases are functional as homodimers, with each monomer made up of an N-terminal prodomain, one large, and one small subunit (Figure 1A). As a means of inherent regulation, executioner caspases are translated as inactive procaspsase zymogens, with distinct cleavage events required for zymogen activation. Initiator caspases propagate the apoptotic signal by cleaving downstream executioner caspases at their intersubunit linker. Prior to cleavage, this linker rests across the dimer interface.\textsuperscript{12} Cleavage of the intersubunit linker generates loops 2 and 2’ (L2 and L2’), which, in the active, substrate-bound conformation, lock together the substrate-binding groove on the
opposite monomer (Figure 1B). This results in a dramatic conformational change in the dynamic active-site loop bundle from a zymogen state to an active state. Cleaved executioners can also experience a reversal to the zymogen-like state by small molecules, which forces a loop rearrangement that traps L2’ over the dimer interface and results in the expulsion of loop 3 from the active site pocket. Following activation in the cell, each executioner caspase cleaves over a hundred substrates to propagate the well-ordered termination of the dying cell.

Due to their cell death inducing potential, caspase activation and downstream activity is tightly regulated on multiple levels, with phosphorylation as one of the primary means to manage apoptotic caspase activity. In fact, caspases and kinases co-regulate each other, resulting in an intricate interplay of dramatic post-translational modifications that ultimately impact cell death and survival. Together apoptotic caspases are recognized to initiate and execute cell death, but their individual roles continue to come to light. Even highly related caspases play non-overlapping biological roles and are independently regulated by inhibitor proteins, zinc, and posttranslational modifications. Interactions with kinases are a major contribution to the unique regulation of individual caspases. The delicate nature of this balance between caspases and kinases has been exploited by a number of cancers, which have developed resistance to apoptosis, even after stimulation by chemotherapeutic agents.

Many cancers have capitalized on the vast signaling capacity of various kinases to manipulate cell processes. One particular example is the dysregulation of p21 activated kinase 2 (PAK2, γ-PAK). PAK2, the only ubiquitously expressed PAK, plays a role in a variety of biological pathways including cell motility, mitosis, survival, and
apoptosis. Interestingly, PAK2 plays a dual role in the context of apoptosis: active full-length PAK2 stimulates cell survival\textsuperscript{30,34} while cleavage of the autoinhibitory domain results in propagation of the pro-apoptotic response.\textsuperscript{35,36} This duality is dictated by the relationship of PAK2 with caspase cleavage\textsuperscript{37} PAK2 is cleaved by the executioner caspase-3 at D212, which separates the PAK2 kinase domain and autoinhibitory domains.\textsuperscript{38} The phosphorylated kinase domain, PAK2p34, is then translocated to the nucleus\textsuperscript{39} where it phosphorylates a new set of substrates, which in turn promote programmed cell death. A number of breast cancer cell lines have shown hyperactivity of PAK2,\textsuperscript{32} which tips the scale to favor full-length PAK2 and results in reduced levels of apoptosis. Full-length, active PAK2 stimulates cell survival through multiple mechanisms, including caspase phosphorylation.\textsuperscript{17} This overactivity has further been linked to resistance to chemotherapeutic agents.

The complexity of the caspase-kinase crosstalk has been intensified with the discovery that a pathway involving PAK2 is able to phosphorylate the executioner caspase-7 at three distinct residues: S30, T173, and S239.\textsuperscript{17} Although PAK2 and caspase-7 co-localize and co-immunoprecipitate, whether this phosphorylation of caspase-7 is mediated by PAK2 directly or by another kinase in that pathway was not fully elucidated by prior work. Phosphorylation of all three residues S30, T173, and S239 is reported to silence caspase-7 enzymatic activity, but the contribution from each individual site has not been studied nor has the mechanism of inhibition been determined. As a result of a total loss in caspase-7 activity, phosphorylation by PAK2 leads to a reduction in apoptosis. In addition, knockdowns of PAK2 in various breast cancer cell lines lead to increased apoptosis levels when stimulated with chemotherapeutics including
staurosporine or doxorubicin\textsuperscript{17} underscoring that PAK2 is critical in curtailing caspase-7 activity.

Here, we elucidate the molecular mechanism by which PAK2 phosphorylation inhibits caspase-7 function. Understanding the regulation of this executioner caspase has implications in cancer resistance and apoptotic activation. Our results show that PAK2 inhibits caspase-7 by two divergent mechanisms prior to and following caspase activation: initial phosphorylation allosterically slows activation by upstream initiator caspases by impeding cleavage at the intersubunit linker and a second phosphorylation site directly blocks substrate binding. Identifying and resolving the molecular mechanism behind both regulatory phosphorylation events provides valuable insight into therapeutically relevant means to control programmed cell death.

**Results**

The S239E Phosphomimetic Inactivates Caspase-7

Phosphorylation of caspase-7 by a PAK2-dependent pathway inactivates caspase-7 and this appears to attenuate apoptosis in several breast cancers after stimulation by chemotherapeutic agents\textsuperscript{17}. Caspase-7 phosphorylation by PAK2 occurs at three sites: S30, T173, and S239 (Figure 5A), but the impact of phosphorylation at these individual sites on caspase-7 function or their mechanisms of regulating caspase-7 have not been investigated. Prior to activation, caspase-7 exists as a zymogen. Proteolytic cleavage by upstream initiator caspases at the prodomain (D23) and the intersubunit linker (D198/D206) generates the active form of caspase-7 comprising large and small subunits of caspase-7 (Figure 5B). In the small subunit, S239 sits below the substrate-binding groove on loop 3, which is part of the active-site loop bundle. T173 is located at the
Figure 5: Sites of caspase-7 phosphorylation by PAK2 and kinetics of phosphomimetic variants

(A) Domain structure of caspase-7 highlighting the three reported sites of phosphorylation by PAK2 as ℗. Caspase-7 is cleaved and thereby activated by upstream initiator caspases (cleavage sites indicated by arrows). The full-length procaspase-7 dimer is processed to remove the prodomain, and at the intersubunit linker to form the large (light blue) and small (dark blue) subunits making active, cleaved caspase-7.

(B) Three phosphorylation sites (orange sticks) are present in the caspase-7 dimer consisting of two large (light blue) and small (dark blue) subunits. The N-terminus of active caspase-7, which is dynamic and unstructured, has not been resolved crystallographically, so a model of the S30 region has been added (gray). The prodomain (residues 1-23) is not shown. S239 sits below the active site in loop 3 and T173 rests at the bottom of the 160s helix. The active site of caspase-7 is composed of four loops from one half of the dimer (L1, L2, L3, L4) and one loop from the opposite half of the dimer (L2').

(C) Kinetic data of phosphomimetic variants. Substitution at S239, but at no other reported phosphorylation site, has a dramatic effect on caspase-7 activity.
bottom of the 160s helix, whereas S30, in the highly mobile N-terminal region, has not been resolved by any caspase-7 crystal structures.

To explore the impacts of phosphorylation, each of the three reported phosphorylation sites were replaced by glutamate to generate phosphomimetic variants, mirroring the negative charge and steric bulk of phospho-serine or -threonine. To ensure that activity was monitored in a cleaved (active) form, each phosphomimetic was expressed from a constitutively two-chain construct in which the large and small subunits are independently expressed. Neither S30E nor T173E had a significant effect on caspase-7 kinetics (Figure 5C). However, S239E had a dramatic effect on caspase-7 activity, dropping the catalytic efficiency by nearly three orders of magnitude as compared to the wild-type enzyme.

The introduction of negative charges in combination with steric bulk are two attributes that enable phosphorylation to have such significant effects on protein function. We next aimed to determine whether size, charge, or both, were responsible for the S239E loss of activity. Substituting S239 with Gln, retains the size of the Glu but removes the contribution of a negative charge. Conversely, the S239D variant retains the negative charge but removes the bulk. Both S239Q and S239D resulted in a significant drop in catalytic efficiency, but neither as dramatic as S239E, suggesting that both size and charge play a large role in inhibiting caspase-7 activity at S239. Notably, all three mutations (S239E, S239D, and S239Q) had a significant effect on $K_M$, highly suggesting that phosphorylation at S239 interferes directly in a steric manner with substrate binding. In addition, there was an effect on $k_{cat}$ for the S239E mutation, suggesting a disruption to the catalytic machinery. However, this $k_{cat}$ effect was much less pronounced with the Gln
and Asp mutations, further suggesting that both size and charge together are responsible for inhibition.

**Caspase-7 is Phosphorylated by PAK2 at S239 on the Small Subunit**

To investigate PAK2 phosphorylation of the caspase-7 small subunit, which contains S239, we performed an *in vitro* phosphorylation assay. PAK2 T402E, a constitutively active, auto-activating variant was pre-activated with ATP then incubated with $[^\gamma-3^2P]$ ATP and wild-type caspase-7 or the S239A variant, which cannot be phosphorylated at residue 239 (Figure 6A). Based on the low activity of S239E, we anticipated phosphorylation on the small subunit. We observed active caspase-7 completely cleaved PAK2 at D212 (Figure 7) to generate the kinase and autoinhibitory domains. In addition, in both the wild-type caspase-7 and S239A reactions, a phosphorylated band similar in molecular weight to the small subunit of caspase-7 appeared. To determine whether this 14 kDa band was a cleavage product of PAK2 or the small subunit of caspase-7, we repeated the *in vitro* phosphorylation assay on catalytically inactive caspase-7 (C186A, Figure 6B), which was expressed using the constitutively two chain construct. PAK2 cannot be cleaved by inactive caspase-7 C186A. The lack of the 14 kDa band with inactive caspase-7 confirms that the band is indeed a cleavage product of PAK2. Contrary to our expectations, we did not observe small subunit phosphorylation under these conditions.

These results demonstrate the difficulty in dissecting the competitive interplay between caspase-7 and PAK2. If active caspase-7 predominates, it cleaves PAK2 before PAK2 can phosphorylate the caspase-7 small subunit, which inactivates caspase-7. We sought a reagent that could block caspase-7 activity thereby preventing cleavage of
Figure 6: PAK2 phosphorylates the caspase-7 small subunit at S239

(A) PAK2 phosphorylates caspase-7 at multiple sites as assessed by autoradiography by \([\gamma^32P]\) ATP and compared to coomassie stained gels. The caspase-7 large subunit is phosphorylated rapidly, with phosphorylation observed even before the zero time point could be processed. PAK2 autophosphorylation indicates that PAK2 is active. After three hours, PAK2 is cleaved by caspase-7 resulting in multiple bands of cleaved PAK2.

(B) PAK2 phosphorylates catalytically inactive caspase-7 (C186A) at the large subunit. Because caspase-7 is not active, no cleaved PAK2 is observed.

(C) After incubation with a cysteiny1-2-pyridyl disulfide protecting group (PG), which blocks the catalytic cysteine, caspase-7 can be phosphorylated by PAK2 on both the large and small subunits. Substituting S239 (small subunit) with alanine results in the complete loss of caspase-7 phosphorylation on the small subunit, confirming PAK2 phosphorylates the caspase-7 small subunit at S239.

(D) After incubation with the PG both the wild-type caspase-7 and C290S were phosphorylated by PAK2 on the large subunit. The C290S variant prevents PG binding to this C290, which has known allosteric implications. Serine substitution at C290 results in a dramatic loss in small subunit phosphorylation at S239 by PAK2. The band intensity for small subunit phosphorylation is quantified in the bar graph at right, which shows the mean ± error represented as SD from three independent experiments.

(E) The dynamic loop bundle at the active site can adopt different conformations. Alignment of three crystal structures reveals that unliganded caspase-7 (blue, PDB ID 3IBF) is distinct from the full-length procaspase-7 (green, PDB ID 1GQF) and from caspase-7 bound to the allosteric inhibitor DICA (tan, PDB ID 1SHJ). In all three conformations C290 sits on \(\beta\)-strand 6, however, the base of loop 3 is in a dramatically different conformation. The unliganded structure loses \(\beta\)-character on \(\beta\)5 while both the full-length procaspase-7 zymogen and the DICA-bound structures extend \(\beta\)5 and expel loop 3 from this allosteric pocket at the dimer interface. Binding PG at C290 provides considerable steric bulk that is expected to force loop 3 of the unliganded caspase-7 (blue loop 3) to adopt a loop-accessible conformation (tan loop 3) as seen when DICA binds at this cysteine, C290.

In all panels of this figure, all caspase-7 variants were expressed from the constitutively two-chain construct, which produces the large (1-198) and small (199-303) subunits of caspase-7 as two independent polypeptides.
PAK2. Due to the location of S239, it was important that the reagent not fill the substrate-binding groove and thereby limit accessibility of S239. To meet these criteria we developed a cysteine protecting group (PG, L-cysteinyl-2-pyridyl disulfide, Figure 8A). PG (Figure 8B) modifies and inactivates the catalytic cysteine via reversible thiol chemistry, but because of its small size, does not fill the full substrate-binding groove. In the presence of PG, PAK2 clearly phosphorylated the small subunit of wild-type caspase-7 at S239, since no phosphorylation of S239A was observed (Figure 6C). In addition, the PAK2 remains uncleaved and the large subunit of caspase-7 is phosphorylated at the same levels as with unprotected caspase-7 demonstrating that S239 can indeed be phosphorylated by PAK2.

In addition to silencing the caspase-7 activity, the PG plays an orthogonal role at a second site, altering the equilibrium ensemble of the active-site loops by binding to a known allosterically acting cysteine (C290). Upon activation by cleavage at the intersubunit linker, caspase-7 experiences a rearrangement of its loops, which sample multiple conformations. For example, caspase-7 bound to the small molecule DICA (at C290) forces loop 3 to adopt a more accessible conformation, with an increased exposure
to solvent. We realized that binding of the PG at C290 should increase the accessibility of this region by forcing loop 3 into a more accessible conformation. To test this hypothesis, we mutated C290 to Ser, blocking the ability of the PG to bind at the allosteric Cys. We observed a decrease in phosphorylation on the small subunit of caspase-7 C290S compared to wild-type (Figure 6D). We did not observe a similar decrease when C186 was mutated to Ala (Figure 9) suggesting that PG binding to C290, but not C186, was responsible for loop movement and availability of S239 to phosphorylation by PAK2.

Thus, it appears that PG binding to C290 acts analogously to binding of other small molecules at C290, resulting in a structural change to loop 3 (Figure 6E). In unliganded caspase-7 (Figure 6E, blue) loop 3 is structured and protrudes inwards toward C290. When a small molecule, like DICA or PG binds at C290, loop 3 is forced upward in a loop accessible conformation that may resemble the zymogen full-length procaspase-7 (Figure 6E, green) or caspase-7 bound to DICA (Figure 6E, tan). This conformational shift increases the solvent accessibility of loop 3, including S239. The PG thus served two purposes (1) to silence caspase-7 activity allowing PAK2 to remain uncleaved so that
phosphorylation could proceed and (2) to alter the equilibrium of the caspase-7 active-site loop bundle to favor the solvent accessible conformation of loop 3, which includes the phosphorylation site S239. It is clear that phosphorylation of S239 occurs exclusively when loop 3 is in an exposed, loop-accessible conformation. This suggests either that S239 is only available for phosphorylation under certain conditions, or that PAK2 can distinguish these two distinct conformations of caspase-7.

**S239E Phosphomimetic Structure Suggests the Mechanism of Inhibition by Phosphorylation**

To determine the molecular mechanism of inhibition by phosphorylation at S239 we solved the crystal structure of the unliganded (apo) cleaved form of the caspase-7 S239E phosphomimetic at 2.2 Å resolution (Table 1). This caspase-7 phosphomimetic shares the same overall fold with all previously crystallized caspase-7 structures, including those unbound and bound to substrate. The heterotetrameric core consists of 12 β strands flanked by 10 α helices, and two symmetrical active sites made up a dynamic active-site loop bundle. Loop 3 sits just below the active site and contains R233, a critical
residue for binding substrate (Figure 10A). In both the unliganded mature structure (3IBF) and the substrate-bound structure (1F1J), this arginine is positioned identically (Figure 10B). R233 acts as an anchor poised to make four pivotal hydrogen bonds with the preferred DEVD tetrapeptide substrate. R233 interacts with both the Glu in the P3 position of the peptide substrate as well as the essential Asp side chain in the P1 position. Meanwhile, two additional hydrogen bonds are made with the carbonyl and nitrogen stemming from the R233 backbone. This behavior of R233 is in contrast to its behavior in the S239E structure.
The structure of unliganded caspase-7 S239E suggests a two-factor mechanism by which phosphorylation of S239 inhibits caspase-7. First, the position of loop 3 has been perturbed by the phosphomimetic (Figure 10C). The bulk from the introduced Glu side-chain at position 239, which mimics the phosphoserine in length and negative charge, forces the entire loop 3 to shift outwards toward a solvent channel. This reorients R233 away from the active site pocket so it can no longer make critical bonds with the substrate (Figure 10C). In addition, the phosphomimetic introduces a negative charge in the precise region where an aspartate would bind in the S1 pocket. This inhibitory mechanism is consistent with the kinetic data (Figure 5C), which suggested both size (S239Q) and charge (S239D) worked in combination to inhibit caspase-7 activity after modification at S239. Thus, the introduction of a negative charge in the P1 pocket would electrostatically repel an aspartate residue from the substrate.

Figure 10: Phosphorylation at caspase-7 S239 sterically blocks substrate binding
(A) Global alignment of the unliganded caspase-7 S239E phosphomimetic crystal structure (light blue) and the unliganded wild-type caspase-7 (dark gray, PDB ID 3IBF). Loop 3, which contains the S239E mutation, undergoes a clear shift in conformation.
(B) R233 interactions are essential for substrate binding and specificity. In both active-site liganded (gray, PDB ID 1F1J) and unliganded (dark gray, PDB ID 3IBF) caspase-7 structures, R233 is positioned to form two essential side-chain hydrogen bonds (black dots) that are critical for binding substrate (DEVD, orange sticks), anchoring it in place for catalysis.
(C) The unliganded S239E phosphomimetic crystal structure (blue) shows a dramatic shift in loop 3, with the introduced glutamate forcing R233 out of the position compatible for binding substrate. In addition, the introduction of a negative charge in the P1 pocket would electrostatically repel an aspartate residue from the substrate.
phosphorylation at S239 is effective at silencing activity of an already active caspase-7 once apoptosis has been initiated.

**Caspase-7 Phosphorylation Occurs on the Large Subunit Primarily at S30**

Phosphorylation of caspase-7 by PAK2 was also observed on the large subunit (Figure 6). This phosphorylation event was so fast that it occurred before the zero timepoint sample could be processed. In addition, the large subunit was phosphorylated regardless of the cleavage state of PAK2 (Figure 6A-C). Despite the overwhelmingly fast phosphorylation by PAK2 in the large subunit, no effect on the catalytic rate of cleaved caspase-7 was observed (Figure 11).

To determine if this phosphorylation occurred at the reported sites on the large subunit, S30 and T173 were individually replaced by alanine, a non-phosphorylatable residue. These substitutions were made in the background of caspase-7 catalytic site inactivation (C186A), which was used to prevent PAK2 from being cleaved. PAK2 did not phosphorylate S30A/C186A but did phosphorylate C186A and T173A/C186A. This was true for both full-length and cleaved caspase-7, indicating that S30 is the primary phosphorylation site on the caspase-7 large subunit. 

---

**Figure 11: Caspase-7 wild-type activity is not affected by PAK2 phosphorylation.**

Casp-7 wild-type CTC was allowed to incubate in PAK2 activity buffer in the presence and absence of autoactivated PAK2 and 1mM ATP. After a 60 minute incubation at 30°C the activity of the control caspase and the phosphorylated caspase was assessed by monitoring cleavage of the casp-7 fluorogenic peptide substrate DEVD-AMC. Assays were carried out in casp-7 activity assay buffer at 100 nM enzyme concentration. A student’s t test resulted in a P value of >0.05 indicating the control and the phosphorylated casp-7 are statistically identical. Data is represented as mean ± S.D.
subunit (Figure 12A). The ratios of the band intensities for the $^{32}$P:comassie stained bands were similar for FL C186A (2.14) and cleaved C186A (2.17) as well as for FL T173A/C186A (2.17) and cleaved T173A/C186A (2.11) suggesting that phosphorylation at S30A occurs similarly on the zymogen and the cleaved forms of caspase-7.

**Phosphorylation of Caspase-7 Diminishes Processing by Initiator Caspases**

Given that S239 phosphorylation directly impacts catalytic activity, it was surprising that the predominant site of phosphorylation, S30, had no direct effect on caspase-7 catalytic function. This suggested that S30 phosphorylation might play an orthogonal functional role. Executioner caspases including caspase-7 are dependent on activation by upstream initiator caspases including caspase-8 and -9, which cleave the intersubunit linker of full-length pro-caspase-7 at D198 or D206, generating active caspase-7. To determine if phosphorylation of the full-length caspase-7 impacted processing by initiator caspases, full-length caspase-7 C186A was phosphorylated by autoactivated PAK2 in the presence of [$\gamma^{32}$P] ATP or not phosphorylated in the no ATP condition. Phosphorylation was confirmed by autoradiography. Phosphorylated or unphosphorylated caspase-7 was then subject to cleavage by either caspase-9 or caspase-8. Unphosphorylated caspase-7 was cleaved by caspase-9 completely at the intersubunit linker after 3 hrs (Figure 12B). In contrast, phosphorylated caspase-7 was cleaved more slowly by caspase-9. Remaining full-length caspase-7 was quantified at each time point. We observed a significant decrease in the rate of cleavage when caspase-7 was phosphorylated (Figure 12C). Phosphorylated caspase-7 was also cleaved more slowly by caspase-8 (Figure 12D-E). For both initiator caspases, 30% less full-length caspase-7 was cleaved in the timecourse of the experiment. Notably, PAK2 has no effect on the function
Figure 12: Phosphorylation on caspase-7 slows processing by upstream initiator caspases
(A) PAK2 phosphorylates the large subunit in both full-length (FL) and in the cleaved state of caspase-7. The two reported phosphorylation sites in the large subunit were individually mutated to alanine. Lack of \([\gamma-^{32}\text{P}]\) ATP labeling of S30A suggests that phosphorylation primarily occurs at S30 on the caspase-7 large subunit and not at T173. All caspase-7 constructs contain the C186A mutation of the catalytic cysteine to prevent self-cleavage. Cleavage was monitored by coomassie stained SDS-PAGE and \(^{32}\text{P}\) autoradiography was used to confirm phosphorylation.
(B,D) Cleavage of full-length (FL) caspase-7 by upstream initiators caspase-9 (B) or caspase-8 (D) at the intersubunit linker. The addition of ATP activates PAK2 and stimulates caspase-7 phosphorylation, which slows the ability of caspase-9 or caspase-8 to cleave FL caspase-7 at the intersubunit linker.
(C, E) Quantification of FL caspase-7 remaining in (B,D) upon cleavage by caspase-9 (C) or caspase-8 (E). Values and error bars represent the mean ± SEM for measurements from three independent experiments.
of either caspase-8 or caspase-9 under these assay conditions (Figure 13A-B) and does not phosphorylate caspase-9 at all (Figure 13C), indicating that the decrease in processing is the result of phosphorylation of caspase-7.

Figure 13: Caspase-8 and caspase-9 activity is unaffected by PAK2
(A) Casp-8 and (B) casp-9 activity was assayed over a ten minute time course monitoring cleavage of the fluorogenic substrate LEHD-AFC. Enzymes were incubated with autoactivated PAK2 T402E (black bars, +ATP) or PAK2 T402E alone (white bars, No ATP) and activity was assessed at three time points. This experiment was performed in identical fashion to the caspase cleavage assays of full-length casp-7 (Figure 4B and 4D). Data is represented as mean ± S.D.
(C) Autoactivated PAK2 was allowed to incubate with casp-9 for up to 18 hours with [γ-32P] ATP. No bands for casp-9 appeared via autoradiography, suggesting PAK2 is unable to phosphorylate casp-9 under these assay conditions. In addition, full length casp-7 was incubated with casp-9 and autoactivated PAK2 for 18 hours as a positive control to demonstrate (i) PAK2 is active because it phosphorylates the large subunit of casp-7 and (ii) that casp-9 is active because it is able to cleave full length casp-7.
Phosphorylation at S30 Slows Processing of Caspase-7 at the Intersubunit Linker

By substituting alanine, a non-phosphorylatable residue, at each of the reported PAK2 sites in the caspase-7 large subunit (T173 and S30), we interrogated the contribution of phosphorylation at these sites to cleavage by caspase-9 (Figure 14A). Caspase-7 T173A/C186A was incubated with PAK2 in the presence or absence of [$\gamma$-32P]ATP and subsequently treated with active caspase-9 (Figure 14A-B). The cleavage pattern was nearly identical to caspase-7 C186A (Figure 12B), suggesting phosphorylation at T173 plays no role in slowing cleavage by caspase-9 at the intersubunit linker. In contrast, the cleavage pattern for full-length caspase-7

![Figure 14: Phosphorylation at S30 on caspase-7 slows processing by caspase-9](image)

(A) Caspase-7 phosphorylation sites T173 and S30 were replaced by alanine in the background of the C186A mutation of the catalytic cysteine to prevent self-cleavage. Caspase-7 T173A and S30A were treated with PAK2 with or without [$\gamma$-32P]ATP, then subjected to cleavage by caspase-9. Caspase-7 T173A/C186A was cleaved faster in the absence of ATP, with rates resembling caspase-7 FL C186A. Caspase-7 S30A/C186A was cleaved at a similar rate in the presence or absence of ATP.

(B, C) Quantification of cleavage in (A) of caspase-7 variants by caspase-9: T173A/C186A (B) or S30A/C186A (C). The nearly identical rate of cleavage of S30A with or with out ATP suggests that S30 phosphorylation is responsible for slowing cleavage of caspase-7 FL by caspase-9. Values and error bars represent the mean ± SEM of triplicates and are representative of three independent experiments. In all panels of this figure, all caspase-7 variants were expressed from a full-length construct which produces residues 1-303 as a single polypeptide chain.
S30A/C186A was identical in the presence or absence of phosphorylation (Figure 14A,C). This data strongly suggests that phosphorylation by PAK2 at S30 has considerable impact on the ability of caspase-9 to cleave caspase-7 at the intersubunit linker (residues 198-206).

**Phosphorylation at S30 Interrupts the Binding Interaction between Caspase-7 and Caspase-9**

S30 and the cleavage sites in the intersubunit linker (D198/D206) are distal in sequence, and due to the disorder in all structures of the caspase-7 prodomain, it is difficult to draw specific conclusions about the spatial proximity of these two regions. Nevertheless, phosphorylation at caspase-7 S30 clearly impacts the ability of caspase-9 to cleave caspase-7 at the intersubunit linker. Fluorescence polarization of FITC-labeled caspase-7 in the presence of a caspase-9, which forms constitutive dimer, showed a strong (1.8 μM) interaction (Figure 15A, 16A-C). In these experiments catalytically inactive caspase-7 and -9 were used to prevent cleavage if either caspase during the

**Figure 15: The interaction between caspase-7 and caspase-9 is disrupted by phosphorylation at S30**

(A) Fluorescently labeled uncleaved caspase-7 FL C186A interacts by fluorescence polarization with caspase-9 constitutive dimer (cDimer) C287A. (B) Phosphorylating caspase-7 FL C186A prior to a caspase-9 titration dramatically impacts the K_D. (C) Replacing S30 with a non-phosphorylatable alanine abrogates phosphorylation by PAK2, enabling a caspase-7: caspase-9 interaction.
reaction. When FITC-caspase-7 C186A was incubated with autoactivated PAK2 to allow phosphorylation prior to the caspase-9 titration, the $K_D$ weakened substantially, to greater than 27 $\mu$M (Figure 15B). This suggests that phosphorylation at S30 blocks the caspase-7:caspase-9 interaction. Consistent with that interpretation, repeating the experiment with caspase-7 S30A/C186A, which is non-phosphorylatable at position 30, shows a similarly strong interaction ($K_D = 1.7$ $\mu$M, Figure 15C) even in the presence of active PAK2. These data confirm the observation (Figure 12C and 14C) that phosphorylation at S30 has a profound effect on how the initiator caspase-9 interacts with the executioner caspase-7 at the initiation stage of apoptosis and may suggest that the N-terminal region of caspase-7 is critical for direct binding to caspase-9.

![Figure 16: Fluorescence polarization controls](image)

(A) Casp-7 C186A was fluorescently labeled with FITC and fluorescence anisotropy was measured over a casp-9 titration. Casp-7 C186A alone (filled circles), casp-7 C186A incubated in the presence of PAK2 and the absence of ATP (empty squares), and casp-7 C186A with ATP (triangles) all showed overlapping curves indicating addition of these molecules has no effect on casp-7 interacting with casp-9.

(B) Free lysine labeled with FITC was incubated with casp-9 at various concentrations and the fluorescence anisotropy was measured. A constant tumbling was observed even at high casp-9 concentrations, suggesting FITC itself does not interact with casp-9.

(C) Casp-7 C186A was labeled with FITC as before and fluorescence anisotropy was measured over a casp-3 titration. Only a small change in anisotropy was observed, demonstrating that fluorescently labeled casp-7 C186A does not behave in a similar manner with every caspase and has a unique curve with casp-9.

**Non-phosphorylatable Caspase-7 is Cleaved Faster by Caspase-9 Intracellularly**

It is clear that *in vitro*, phosphorylation at S30 leads to slower cleavage of caspase-7 by caspase-9 at the intersubunit linker. To observe the impact of S30
phosphorylation in a biological context we used MCF7 cells, a widely used breast cancer model. MCF7 cells are intrinsically deficient for caspase-3, putting the burden of executioner apoptotic activity on caspase-7. In addition, MCF7 cells overexpress PAK2, which phosphorylates caspase-7 intracellularly. MCF7 cells were transiently transfected with either phosphorylatable or non-phosphorylatable (S30A) caspase-7. Both transfected variants carried the catalytically inactivating C186S substitution to prevent caspase-7 self-processing. As predicted, following transfection of MCF7 cells, non-phosphorylatable caspase-7 (S30A/C186S) was processed by added caspase-9 significantly faster than was phosphorylatable caspase-7 (C186S) (Figure 17 A-B).

After observing the inhibitory effects of phosphorylation of caspase-7 at S30 in the MCF7 cellular milieu, we hypothesized that we would observe a similar effect in growing cells. MCF7 cells transiently transfected with caspase-7 C186S or S30A/C186S were treated with both ABT-263, a Bcl-2 inhibitor, and A1210477, an Mcl2 antagonist, which together stimulate the intrinsic apoptotic pathway and activate caspase-9 \(^43\). Monitoring the appearance of the cleaved and activated caspase-7 (Figure 17 C-D) revealed that caspase-7 S30A/C186S was processed from the full-length form to the cleaved form faster than the C186S variant. This suggests that, as was the case \textit{in vitro}, when S30 is available for phosphorylation by PAK2, this site becomes phosphorylated, decreasing binding, recognition, and cleavage by caspase-9. Thus it becomes clear that PAK2 regulates caspase-7 activity by inhibiting active (cleaved) caspase-7 through phosphorylation at S239, as well as preventing or dramatically slowing caspase-7 activation by caspase-9 due to phosphorylation at S30.
Figure 17: S30 phosphorylation regulates caspase-7 processing intracellularly

(A) Caspase-7 FL C186S or S30A/C186S were transiently transfected into MCF7 cells. Activated caspase-9 cleaved caspase-7 FL S30A/C186S faster than C186S in MCF7 lysates. Caspase-7 and histone deacetylase-1 (HDAC-1; a loading control) were observed by immunoblotting.

(B) The band intensity of the remaining FL caspase-7 transfected variants was quantified and normalized to the HDAC-1 loading control. The non-phosphorylatable caspase-7 variant S30A was cleaved faster by caspase-9 than caspase-7 containing native phosphorylatable S30. Data are shown as means ± S.D. from experiments performed on three separate days; the asterisks indicate a statistically significant increase in cleavage (** p<0.01) as determined by the Student’s t test.

(C) Caspase-7 FL C186S or caspase-7 FL S30A/C186S were transiently transfected into MCF7 cells and treated with two compounds known to activate caspase-9 via the intrinsic apoptotic pathway (ABT-263 and A1210477). Cells undergoing apoptosis cleaved caspase-7 FL S30A/C186S faster than C186S. Caspase-7 and histone deacetylase-1 (HDAC-1; a loading control) were monitored by immunoblotting.

(D) The band intensity of the appearance of cleaved caspase-7 was quantified and normalized to the HDAC-1 loading control. The non-phosphorylatable S30A/C186S variant was cleaved approximately 1.4 fold faster than the C186S counterpart. Data are shown as means ± S.D. from experiments performed on three separate days; the asterisks indicate a statistically significant increase in cleavage (* p<0.05) as determined by the Student’s t test.
Discussion

The regulation of caspases is paramount for determining whether cells live or die. Global regulation of apoptotic caspases has proven to be heavily modulated by phosphorylation, with extensive phosphorylation on both initiator and executioner caspases typically leading to inactivation (for review see Kurokawa and Kornbluth, López-Otin and Hunter). Prior to this work, the molecular details of these inactivating events have been sparse. Emerging regulatory themes have demonstrated an intricate complexity, with caspases as kinase substrates and kinases as caspase substrates. Biases in this interplay that favor cell survival influence diseases of proliferation, like cancer. In the caspase-7:kinase co-regulation, PAK2 hyperactivity is observed to contribute to a cancerous phenotype, with enhanced cell survival and chemotherapeutic resistance.

Our results mechanistically highlight the functional relevance of PAK2 directly phosphorylating the apoptotic target caspase-7 and demonstrate how PAK2 impacts caspase-7 activity independently prior to (S30 phosphorylation) or after (S239 phosphorylation) caspase-7 activation in apoptosis. The first mechanism slows initial activation by allosterically disrupting the ability of initiator caspases to activate full-length procaspase-7 prior to apoptosis, while the second mechanism involves direct blocking of substrate from binding to active caspase-7 during apoptosis (Figure 18).

Phosphorylation at S30 interrupts the binding interaction of caspase-7 with the initiator, caspase-9, and thereby slows caspase-7 cleavage at the distal intersubunit linker. Phosphorylation has been observed to directly block cleavage at the intersubunit linker of several caspases, including caspase-2, caspase-3, caspase-8, and murine caspase-9. A recent study identified a phosphorylation site distal from the intersubunit linker.
Figure 18: PAK2 uses distinct mechanisms at two sites of phosphorylation: S30 blocks caspase-7 zymogen activation while S239 directly disrupts substrate cleavage

The intrinsic apoptotic cascade proceeds when intracellular stresses promote release of cytochrome c from the mitochondria. Cytochrome c and Apaf-1 constitute the apotosome, which recruits and activates initiator caspase-9. Activated caspase-9 binds the full-length procaspase-7 zymogen, likely through interactions with the N-terminal region containing S30. Caspase-9 activates caspase-7 through cleavage at the intersubunit linker. Active caspase-7 continues the execution of apoptosis by processing over a hundred downstream substrates including p23, PARP, and other caspases. In addition, cleavage of PAK2 by caspases results in a release of the autoinhibitory domain (AID) and signals the kinase domain (KD) to be transported to the nucleus where phosphorylation of new targets propagates the apoptotic response. When active, full-length PAK2 can supersede cell death by performing either of two distinct phosphorylation events on caspase-7. Phosphorylation (⇒) at S30 interrupts the interaction between caspase-9 with the caspase-7 zymogen (repelling red lines), thus slowing zymogen activation at the early stages of apoptosis. PAK2 phosphorylation on active caspase-7 at S239 occurs most rapidly when the active-site loops adopt a zymogen like conformation but abolishes activity in all forms of caspase-7 by directly blocking substrate binding.
cleavage site that decreases caspase-8 processing through the sequential action of two kinases via an as yet unknown mechanism.\textsuperscript{49} Phosphorylation of S30 of caspase-7 is the first report of direct allosteric regulation of zymogen activation in caspases, where phosphorylation at a distal site impacted intersubunit linker cleavage. In many previous cases the phosphorylation sites that impact the rate of activation were located either on the linker itself or directly adjacent in the immediate flanking sequence, suggesting that phosphorylation at those linker sites directly prevents binding of the activating caspase by introduction of a charged residue. Inhibition by phosphorylation at S30 is the first example revealing details of a one-step allosteric mechanism where a distant site is utilized to control caspase maturation from the zymogen to the active form. We know of no other proteases for which this mechanism of phosphorylation regulating zymogen activation by a single kinase has been reported, but one might speculate that zymogen activation of other caspases or other proteases may likewise be allosterically modulated by relevant kinases. This identification of a vulnerable allosteric site on caspase-7 opens the door for future modulation of caspase-7 activation by both caspase and kinase modulators.

S30 sits in the N-terminal region of caspase-7. Our data suggest that this region may be critical for directly mediating the interaction of caspase-7 with caspase-9, potentially through an exosite interaction, that leads to zymogen activation. This N-terminal region has never been structurally characterized as it has been disordered in the many structures of caspase-7. Nevertheless, this region has proven to be critical for the participation of caspase-7 in apoptosis using allosteric mechanisms. An exosite at a basic lysine patch (K\textsuperscript{38}KKK) assists in the recognition of specific caspase-7 substrates.\textsuperscript{50} While
little is known about the region, it appears to be critical for caspase-7 specific activities. For example, the K$_{38}^{38}$KKK is not present in caspase-3 and is one example of how caspase-7 is able to fulfill its non-redundant apoptotic roles during the demolition phase. The PAK2 motif containing S30 is not present in caspase-3. We have also observed that PAK2 does not phosphorylate caspase-3 in this N-terminal region in vitro (data not shown), which further suggests that this region of caspase-7 is a region that is critical for regulation of caspase-7 specifically.

While phosphorylation of caspase substrates has been observed to block their cleavage by caspases, our work is the first to show that phosphorylation of an active-site loop in a caspase directly blocks substrate binding, thus inactivating active caspase-7 and later during apoptosis. The crystal structure of caspase-7 phosphomimetic S239E suggests that steric bulk and charge from phosphorylation results in a misalignment of the active-site loop 3. This mechanism is similar to that observed for caspase-6 phosphorylation of S257 by Ark5 kinase, which leads to misalignment of the loops in the substrate binding groove and inactivation of caspase-6. Many lines of evidence have suggested that active-site loop conformations are critical for caspase function. Cleavage of the intersubunit linkers liberates two loops which can reorganize to facilitate substrate binding. Mechanisms like those utilized by PAK2 and Ark5 take advantage of the requirement for precise positioning of the loops to enable substrate binding and cleavage. Small molecules and mutations that organize or disorganize the active site loops have proven to be effective activators or inhibitors. The discovery of the dimer-interface allosteric site in caspases led to speculation that a naturally-occurring regulatory small-molecule might bind and inactivate caspase-7. Our observation that S239 becomes
accessible upon binding small molecules at this allosteric site (containing C290) may suggest that such a molecule would be synergistic with PAK2, enabling phosphorylation at S239, thereby inhibiting caspase-7 activity in a covalent, and perhaps more stable, manner. Future caspase therapeutics may fruitfully focus on this adaptable regulatory mechanism exploited by PAK2.

Together, phosphorylation of PAK2 at both S30 and S239 combine to exert powerful control over the activation and subsequent activity of caspase-7. The effects of phosphorylation are maximized by taking advantage of a dual mechanism: an initial phosphorylation at S30 is responsible for slowing the critical activation event of intersubunit linker cleavage followed by a second modification on S239 to disrupt the active site loops and directly block substrate binding (Figure 18). This phenomenon, wherein one kinase engages two different molecular mechanisms that each lead to the same overall cellular outcome, has been observed for other kinase-substrate pairs. Ribosomal protein S6 kinase β-1 (S6K1) phosphorylation of insulin receptor substrate 1 (IRS-1) at S1101 blocks its interaction with PI3K, leading to insulin resistance.\textsuperscript{54} When alternative IRS-1 sites, S307/S312 are phosphorylated by S6K1, it enhances proteasomal degradation, also leading to insulin resistance.\textsuperscript{55,56} This pattern of regulation can also be observed for transcriptional regulation by the forkhead box proteins (FOXO). FOXO1 is phosphorylated by protein kinase B (PKB/Akt) at three sites, T24; S256, and S319,\textsuperscript{57} which results in a loss of transcriptional activity. Similar to PAK2 regulation of caspase-7, PKB phosphorylation utilizes different mechanisms of inhibition to achieve the same overall biological goal. Phosphorylation at S256 limits FOXO1 DNA binding.\textsuperscript{58} Meanwhile, phosphorylation at S319 creates a consensus sequence for CK1 to
phosphorylate additional serine residues at positions 322 and 325. This tight grouping of modified serines promotes the nuclear export of FOXO1, decreasing FOXO1 activity by a different mechanism. In these patterns of regulation, one kinase modifies a single substrate at multiple sites, which each exert mechanistically independent but biologically synergistic effects. This synergy in impact is perhaps expected, as uncorrelated outcomes would be non-productive. Thus, it is likely that full-length PAK2 would only play anti-apoptotic roles that have biological synergy to caspase-7 phosphorylation under related conditions.

In addition to S30 and S239, T173 has also been reported to be phosphorylated by PAK2. We have not observed phosphorylation of T173 under any conditions nor have we observed any functional effect of phosphomimetics at this residue. This could be because i) we have not explored the requisite conditions for phosphorylation, or ii) T173 is not a bonafide modification site but can only be recognized in a peptide, such as those used to identify this site, not in an intact protein, or iii) T173 is a bystander residue, which has no functional effect upon phosphorylation. The concept of bystander residues is replete in the kinase literature. Our work on S30 demonstrates that even for functional phosphorylation events, it can be difficult to decipher the role phosphorylation at a given site might play. It is possible that we may not have found the right conditions for T173 phosphorylation by PAK2, but if T173 is a target of PAK2 under conditions where S30 and S239 are phosphorylated it should likewise have an anti-apoptotic effect. We may likewise predict that if phosphorylation of T173 by PAK2 has a different (e.g. anti-apoptotic) functional effect, we would expect to observe it only under very different cellular conditions. Additionally, it is possible that each of the three sites of
phosphorylation are utilized under different biological conditions, providing nuanced control of caspase-7 activity by PAK2.

These findings illuminate the molecular details of a kinase inhibiting an apoptotic caspase. The implications extend to cancer resistance, suggesting a hyperactive PAK2 is able to limit apoptotic cell death and resist apoptotic stimulation by chemotherapeutic agents. In addition, our results pinpoint an allosteric region on a key apoptotic contributor, where alterations result in a slowed maturation and potential control over an executioner caspase. The significance of this regulation could extend beyond cancer resistance and apoptotic activation. Recent discoveries have implicated caspase-7 in inflammation, defense against L. pneumophila infection, osteogenesis, and tooth development, all of which show caspase-7 has distinct roles from caspase-3. These non-apoptotic roles could share regulation by phosphorylation and the mechanistic details uncovered here.

Finally, our increased mechanistic understanding of details of this caspase:kinase interplay may guide precision therapeutic approaches that exploit synergies between caspase and kinase directed modulators. Likewise, identification of the S30 site as critical for interaction with caspase-9 may implicate this S30 region in playing a role in the recognition of other caspase-7 substrates. If this is the case, synthetic inhibitors that block the S30 region, mimicking S30 phosphorylation, may prevent cleavage of the subset of substrates that utilize the S30 region for binding. This is contrast to active-site directed inhibitors, which like S239 phosphorylation, block cleavage of all caspase-7 substrates. S30-mimicking inhibitors that offered precision control of substrate selection would be unprecedented in the realm of synthetic caspase regulation.
Materials and Methods

DNA expression constructs and Antibodies

Human caspase-7 cDNA in pET 23b was a gift from Guy Salvesen. Human caspase-9 cDNA in pET 23b was purchased from Addgene. Human caspase-8 cDNA in pET 15b was a gift from JB Denault. Human p21 activated kinase 2 (PAK2) cDNA in pET 28b was a gift from John Kuriyan’s lab. The protecting group (PG, cysteinyl-2-pyridyl disulfide) was synthesized as previously described. Dulbecco’s modified Eagle’s medium (DMEM) and all other cell culture supplements were purchased from Gibco (Life Technologies). The pLX304 vector containing the caspase-7 cDNA for transient transfections was obtained from Harvard Plasmid. The caspase-7 antibody for detecting the large subunit and the HDAC-1 antibody were purchased from Cell Signaling Technologies.

Generation of Caspase-7 Variants

All wild-type and mutant versions of caspase-7 were expressed from a pET23b vector with a carboxy-terminal His6-tag. The full-length caspase-7 construct encodes residues 1-303, while a constitutively-two-chain corrected (CTC) construct was designed to produce the large (1-198) and small (199-303) subunits of caspase-7 as two independent polypeptides. All variants that were generated in either construct were created using QuikChange (Stratagene) site-directed mutagenesis. All DNA sequences were verified by DNA sequencing (Genewiz).

Generation of Caspase-9 Constitutive Dimer

Caspase-9 exists primarily as a monomer in solution, as seen by SEC. A constitutively dimeric caspase-9 has been reported, which decreases the distribution of caspase-9
monomers in solution and increases the more biologically relevant dimer. In this dimeric caspase-9, 5 residues at the dimer interface of caspase-9 were replaced with the residues from caspase-3. We followed this strategy, and replaced the codons for residues 402-406 to the caspase-3 equivalent C-I-V-S-M on the C-terminus of caspase-9 C287A using Phusion site directed mutagenesis (Thermo Scientific).

**Caspase-7 Expression and Purification**

Plasmids encoding human caspase-7 and all variants were transformed into BL21(DE3) E. coli cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD600 of 0.6. The temperature was reduced to 18°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and 50 mM imidazole. Caspase-7 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT to reduce the salt concentration. This protein was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Protein eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

For several sets of experiments it was imperative that the caspase-7 retained its full-length uncleaved zymogen form, however, during expression in E. coli the prodomain is
cleaved off over time. Therefore, full-length caspase-7 was induced for only 10 min at 37°C before harvesting the cells.

**Caspase-8 Expression and Purification**

The expression construct encoding human caspase-8 was transformed into BL21(DE3) *E. coli* cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD<sub>600</sub> of 0.6. The temperature was reduced to 25°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 8 mM imidazole. Caspase-8 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT to reduce the salt concentration. This sample was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Caspase-8 eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

**Caspase-9 Expression and Purification**

An expression construct for full-length human caspase-9 was transformed into BL21 (DE3) *E. coli* cells. The cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD<sub>600</sub> of 0.9. The temperature was reduced to 15°C and cells were induced with 1 mM IPTG to express soluble His-tagged caspase-
9. Cells were harvested after 3 hrs to obtain single site processing at D315. Cell pellets stored at -80°C were freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The filtered supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM imidazole until the absorbance returned to baseline. The protein was eluted using a 2-100 mM imidazole gradient over the course of 270 mL. The eluted fractions containing protein of the expected molecular weight and composition were diluted 10-fold into a buffer containing 20 mM Tris pH 8.5 and 5 mM DTT to reduce the salt concentration. This caspase-9 sample was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient and eluted in a buffer containing 20 mM Tris pH 8.5, 180 mM NaCl, and 5 mM DTT. The eluted protein was stored at -80°C in the elution buffer and was analyzed by SDS-PAGE for purity.

PAK2 T402E Expression and Purification

An expression construct for human p21 activated kinase 2 (PAK2) T402E was transformed into BL21(DE3) E. coli cells. Cultures were grown in 2xYT media with kanamycin (40 µg/mL, ThermoFisher) at 37°C until they reached an OD₆₀₀ of 0.6. The temperature was reduced to 25°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.05% Tween-20, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was
loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.05% Tween-20, and 10 mM imidazole. PAK2 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT, to reduce the salt concentration. This protein sample was then loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Protein eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

**Activity Assays**

For kinetic measurements of caspase activity, 100 nM freshly purified protein was assayed over the course of 7 minutes in a caspase-7 activity-assay buffer containing 100 mM HEPES pH 7.5, 5 mM CaCl₂, 10% PEG 400, 0.1% CHAPS, and 5 mM DTT. For substrate titrations, a range of 0-200 µM of the fluorogenic substrate DEVD-AMC, (N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin), Enzo Lifesciences; Ex. 365 nm / Em. 495 nm), was added to initiate the reaction. Assays were performed in triplicate at 37°C in 100 µL volumes in 96-well microplate format using a Molecular Devices Spectramax spectrophotometer. Initial velocities versus substrate concentration were fit to a rectangular hyperbola using GraphPad Prism (Graphpad Software) to determine the kinetic parameters $K_M$ and $k_{cat}$. Enzyme concentrations were determined by active-site titrations with the quantitative inhibitor DEVD-CHO (N-Acetyl-Asp-Glu-Val-Asp-Aldehyde; Enzo Lifesciences) or z-VAD-FMK (Z-Val-Ala-Asp-fluoromethylketone; Enzo Lifesciences). Active-site titrations were incubated over a period of 2 hours in activity assay buffer. Optimal labeling was observed when protein was added to DEVD-
CHO or z-VAD-FMK solvated in DMSO in 96-well V-bottom plates, sealed with tape, and incubated at room temperature in a final volume of 200 µL. 90 µL aliquots were transferred to black-well plates in duplicate and assayed with 50-fold molar excess of substrate. The protein concentration was determined to be the lowest concentration of DEVD-CHO or z-VAD-FMK inhibitor at which full inhibition was observed.

Caspase-8 and caspase-9 kinetics were assessed as described above, using the peptide substrate LEHD-AFC (N-acetyl-Leu-Glu-His-Asp-AFC (7-amino-4-trifluoromethylcoumarin), Enzo Life Sciences; Ex. 380 nm/ Em. 505 nm). In addition each assay was performed in the enzyme’s preferred activity assay buffer (see Materials and Methods).

**In vitro Phosphorylation Assays**

Purified recombinant PAK2 T402E was first allowed to auto-activate by incubation with 1 mM ATP in PAK2 activity buffer (50 mM Tris pH 7.5, 20 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT) for 1 hour at 30°C. This activated kinase was then incubated with caspase-7 variants at a 20:1 caspase:kinase ratio. Additionally, [γ-³²P] ATP (10 µCi/µL) was added to radioactively label phosphorylation on the caspase subunits. Reactions were resolved by SDS-PAGE and visualized by autoradiography using a Typhoon FLA 7000 (GE Healthcare). Band quantification was carried out by ImageQuant software (GE Healthcare) using a set of [γ-³²P] ATP standards.

PAK2 is also a substrate of caspase-7, and under certain conditions caspase-7 will cleave PAK2. In order to silence this enzymatic activity, but still run the in vitro phosphorylation assay, active forms of caspase-7 could be silenced by incubation with a protecting group (PG, L-cysteiny1-2-pyridyl disulfide)⁴¹ that targets free activated...
cysteines on the caspase. Prior to the in vitro phosphorylation assay, 20 µM caspase-7 was incubated with 2 mM PG for 1 hour in 20 mM Tris pH 7.5, 100 mM NaCl, and was buffer exchanged to reduce the concentration of reductant (DTT) remaining after purification. This inactive caspase-7 was then used in an identical fashion to the in vitro phosphorylation assay described above.

**Caspase-7 S239E Crystallization and Data Collection**

Purified caspase-7 S239E (CTC construct) in a buffer containing 120 mM NaCl and 20 mM Tris buffer, pH 8.0, was concentrated using Millipore Ultrafree 3K NMWL membrane concentrators (Millipore) to 18 mg/mL as assessed by absorbance at 280 nm. Crystal trays were setup at room temperature and grown in 3 µL hanging drop trays with mother liquor consisting of 2.1 M sodium formate and 300 mM sodium citrate, pH 5.5, in a 2:1 ratio of protein to mother liquor. Crystals grew to a maximum of 240 µm × 340 µm in 3 days at 20°C. Crystals were cryoprotected in 20% ethylene glycol in mother liquor with a 60 second incubation, and then frozen by rapid immersion in liquid N2. Diffraction data was collected at Brookhaven National Laboratories National Synchrotron X6A beamline (Upton, NY).

**Structure Determination**

X-ray data was indexed, integrated, and scaled using HKL2000 software and well defined diffraction spots were observed to 2.2 Å. Crystallographic phases were generated by molecular replacement using 3IBF as a search model for PhaserMR, part of the CCP4 software suite. To avoid bias, substrate binding loops were omitted from the search model and rebuilt into unambiguous electron density. Structures were refined using iterative rounds of rebuilding in Coot and refinement in Refmac5 using
individual B-factors. Water and formate molecules were modeled into the structures, verified with refinement, and checked by stereo-chemical viability. A final round of refinement with NCS restraints imposed was performed in Phenix.\textsuperscript{73}

**Caspase Cleavage Assays**

To monitor the impact of caspase-7 phosphorylation on cleavage of full-length caspase-7 by the initiators caspase-8 or -9, caspase-7 zymogen was first incubated with PAK2. In this experiment all of the caspase-7 variants had the catalytic cysteine mutated to alanine (C186A) in order to ensure that all observed cleavage was from the initiator caspases. Caspase-7 full-length C186A and phosphoknock-outs (S30A, T173A) were first incubated with pre-activated PAK2 (as described above) or with PAK2 in the absence of ATP, in PAK2 activity buffer for 3 hours at 30°C. Additionally, $[^\gamma-32P]$ATP (10 µCi/µL) was added to radioactively label phosphorylation on the caspase subunits. The equivalent volume of water was added to each reaction in the “no ATP” controls. Treated caspase-7 was then diluted to 3 µM in caspase-9 activity assay buffer (100 mM MES pH 6.5, 20% PEG 400, and 5 mM DTT) or in caspase-8 activity assay buffer (10 mM PIPES pH 7.2, 0.1 M NaCl, 1mM EDTA, 10% sucrose, 0.05% CHAPS, and 5 mM DTT). The corresponding initiator caspase was added at a final concentration of 500 nM. The reaction was quenched at each time point by the addition of SDS sample buffer and boiled for 10 minutes. Cleavage was assessed by SDS PAGE analysis and band quantification was determined using Image Lab software (Bio-Rad).

**Fluorescence Anisotropy**

Fluorescence anisotropy was monitored using a SpectraMax M5 plate reader (Molecular Devices) with a fixed excitation wavelength set to 485 nm and an emission wavelength
set to 525 nm. Full-length caspase-7 C186A or full-length caspase-7 S30A/C186A was labeled with fluorescein isothiocyanate isomer 1 (FITC; Sigma) in 0.1 M sodium bicarbonate buffer at pH 9.0. After labeling, unreacted FITC was removed from labeled caspase-7 by buffer exchanging with 3K MWCO filters into 100 mM Tris pH 7.5 and 100 mM NaCl. A fixed concentration of 20 nM labeled protein was subject to a serial dilution of caspase-9 constitutive dimer C287A from 40 µM to 10 nM. All measurements were taken at 25°C after a one hour incubation.

**Cell Culture and Transfection**

MCF7 breast cancer cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L D-glucose, 1 mM sodium pyruvate, 100 mg/mL streptomycin, 100 units/mL penicillin, and 1% Glutamax. Cells were incubated at 37°C in a humidified atmosphere maintained at 5% CO₂. Cells were transiently transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer instructions. Transfection was assessed by comparative immunoblotting of transfected and non-transfected cells.

**Immunoblotting**

Cellular proteins from transfected MCF lysates were first separated by SDS-PAGE. Each well of a 12% acrylamide gel was loaded with 20 µg of total protein from lysates. Proteins were then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were washed overnight in a 3% BSA blocking solution containing Tris-buffered saline with 0.1% Tween-20. The following day the membrane was probed with primary antibodies. Both the caspase-7 antibody (recognizing the large subunit) and the HDAC-1 antibody (loading control) were obtained from Cell Signaling
Technologies. Antibody-antigen complexes were then washed and blotted with goat anti-rabbit IgG-peroxidase conjugates (Jackson ImmunoResearch Labs). After a final washing in TBST, the membrane was introduced to the enhanced chemiluminescent substrate for horseradish peroxidase (HRP) and detected according to the manufacturer’s instructions (ThermoFisher Kit) using a ChemiDocMP (Biorad Laboratories Inc.).

**MCF7 Lysate Cleavage Assay**

MCF cells were first washed twice with ice-cold 1x PBS and were then lysed in 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, and protease inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 µM aprotinin, 29 µM bestatin, 10 µM pepstatin, 1.3 mM EDTA) and phosphatase inhibitors (5 mM β-glycerophosphate, 20 mM sodium fluoride and 200 µM sodium orthovanadate) on ice for 30 minutes, then were centrifuged at 20,000 rcf for 20 minutes at 4°C. Quantification of total protein levels in the supernatant were determined using the bicinchoninic acid assay (Pierce). An equal amount of total protein was then subject to caspase-9 cleavage by the addition of 0.93 µg/µL of purified recombinant human caspase-9.

**MCF7 Whole Cell Caspase-7 Cleavage Assay**

MCF7 cells were grown to ~90% confluence and then plated in six well plates with one million cells per well. Cells were transiently transfected with caspase-7 C186S DNA or caspase-7 S30A/C186S DNA using Lipofectamine 3000 (Invitrogen) according to the manufacturer instructions. Cells were then treated with DMSO, staurosporine (STS; 1 µM), or apoptosis inducing compounds (ABT-263 and A1210477; 10 µM) for three hours. After incubation with compounds, cells were trypsinized and washed twice with ice-cold 1x PBS buffer. Cells were then lysed as described previously and lysates were
quantified using the bicinchoninic acid assay (Pierce). An equal amount of total protein was loaded into each well of a 12% acrylamide gel and cleaved caspase-7 was detected by western blot analysis. Protocol for Immunoblotting is in the supplementary information.

Author Contributions
S.J.E. initiated and performed all experimental aspects of the study, prepared all figures and was the principal author for the manuscript. K.R. synthesized the cysteinyl-2-pyridyl disulfide protecting group. J.A.H conceptualized the project, secured funding, directed the research project, wrote parts of the manuscript and edited the manuscript.
References


CHAPTER III

ZINC INHIBITION OF THE APOPTOTIC CASPASES -3, -6, -7, AND -8

The majority of this chapter will be submitted: Eron, S.J., MacPherson, D.J., Dagbay, K., Hardy, J.A. Zinc Inhibition of the Apoptotic Caspases -3, -6, -7, and -8. (2016).

Introduction

Until recently the magnitude of the impact of zinc on a wide array of biological processes was vastly underappreciated. It has now become clear that zinc plays fundamental roles in growth, development, metabolism, gene transcription, and signaling. A large body of work has established both structural and catalytic roles for zinc in biology, and more recent investigations have observed zinc acting as a signaling factor.\textsuperscript{1,2} In addition, sequence analysis suggests that up to 10\% of the human proteome has potential zinc binding sites.\textsuperscript{3} The functional roles of these sites remain a large unsolved mystery. In this chapter we undertake uncovering the roles of a few of these zinc-binding sites in caspases.

The regulation of intracellular zinc is imperative for maintaining proper homeostasis, and the extent of this regulation is remarkable. Whereas levels of other metals are controlled by a small number of transporters (e.g. one for iron), levels of zinc are tightly controlled by no fewer than 14 zinc importers and 9 zinc exporters in humans, as well as metallothioneins as intracellular zinc buffering proteins (for review see Kimura and Kambe\textsuperscript{4}). The complexity of this underscores the importance of tightly regulating and controlling zinc levels. It is estimated that total cellular zinc concentrations reach hundreds of micromolar, but the “free” or “available” zinc pool is estimated to be in the high picomolar to low nanomolar range.\textsuperscript{5–8} This stark contrast between high total zinc
levels and low available zinc further emphasizes the tight regulation involved in maintaining zinc homeostasis. Small fluxes in the available pool of zinc can have dramatic consequences. This has been observed during fertilization and early human development with the detection of zinc sparks, as well as the zinc wave acting as a signaling event. This zinc wave is mediated by zinc importers that pump excess zinc into the cytosol, directly inactivating phosphatases, which as a result also leads to activation of kinases. In addition, ZIP7, a zinc importer, has been identified as being overexpressed in breast cancer, suggesting a link between cancer progression and excess zinc. This is an example of how small changes in available zinc have substantial cellular implications and motivates our pursuit of detailed biochemical characterizations of zinc binding to important cellular targets.

Apoptosis is a crucial biological pathway that is highly susceptible to regulation by zinc (for review see Truong-Tran et al). Caspases are at the heart of this cell death process and their activity is essential in order for apoptotic programmed cell death to occur. There has been mounting evidence that zinc plays an inhibitory role by blocking caspase activity and thus serving an anti-apoptotic function. Caspases are proteases that use a cysteine-histidine catalytic dyad, both of which are necessary components for hydrolysis of protein substrates. Apoptotic caspases are separated into two types, the upstream initiator caspases and their substrates, the downstream executioner caspases. The executioner caspases -3, -6, and -7 exist as homodimers and require cleavage at their intersubunit linker in order to achieve full activity. In contrast, the activity of the initiator caspases -8, -9, and -10 is dependent on a trigger, such as dimerization or binding to an activating platform. Caspase-9 is activated on the heptameric apoptosome. In the case
of caspase-8, a death inducing signaling complex (DISC) on the cytosolic face of the cell membrane recruits monomeric procaspase-8 via its death effector domain (DED). Caspase-8 is subsequently activated in an oligomeric dependent manner. Caspase-8 requires both dimerization and cleavage in order to be activated.

Because zinc functions as a regulator and cellular signal, dysregulation of zinc homeostasis can have dramatic effects, and links to Alzheimer’s, cancer, and diabetes have been reported. Thus, it is imperative to uncover the molecular details of zinc-mediated inhibition as potential therapeutic avenues. There is a notable benefit to interrogating zinc regulation of caspases due to their direct role in maintaining cell life or death. Previous investigations have defined inhibitory sites for zinc on both caspase-9 and caspase-6, and caspase-3 has been accumulating information for several years regarding potential zinc-binding sites. However, details about zinc binding in caspase-7 and -8 are entirely lacking. The inhibition of caspases by zinc has potential to occur at the catalytic cysteine-histidine dyad since both residues are robust zinc ligands. Indeed, caspase-9 is inhibited by zinc through binding at the active site cysteine. However, further analysis revealed a second zinc binding to caspase-9 at an exosite below the 210s helix (Figure 19). In addition, caspase-6 crystallographic studies established that zinc binds to an allosteric site and not the catalytic dyad in the active site (Figure 19). Meanwhile, caspase-3 has been proposed to bind zinc via its catalytic histidine and a proximal methionine, rather than the reactive cysteine. However, it has been shown that caspase-3 is inhibited at extremely low concentrations down to 1.7 nM. These previous studies provide evidence that zinc inhibition may be nuanced for each caspase and may occur at biologically relevant, low levels of available zinc.
In this investigation we have pursued detailed biochemical characterization of zinc inhibition for caspase-3, -6, -7, and -8. We have evidence that zinc selectively inhibits these caspases amongst a panel of relevant metals. Moreover, the zinc inhibition of these caspases occurs at biologically relevant levels of available zinc, in the low nanomolar range. We also interrogated the mechanisms of inhibition and found zinc does indeed have an affinity for the active site of caspase-7 and the catalytic cysteine for caspase-8. In addition, zinc has a destabilizing effect on caspase-8 and its oligomeric state by disrupting its ability to form the dimer necessary for activity. This provides key molecular details that establish a deeper understanding of zinc regulating caspases and apoptosis.

Figure 19: Known Zinc Binding Sites in Caspases
Previous investigations have found that caspase-9 (represented by PDB 1JXQ) binds zinc at the active site as well as at an exosite at the bottom of the 210s helix. Conversely, caspase-6 binds a single zinc at a defined allosteric site, but does not bind zinc at the active site (PDB 4FXO).
Results

Zinc Inhibits Caspases from Cleaving Peptide and Protein Substrates

Zinc has been shown to have an inhibitory effect on apoptosis, with a specific negative influence on the caspase activity.\textsuperscript{16} Previous investigations have taken a mechanistic approach to zinc inhibition of caspase-6\textsuperscript{19} and -9,\textsuperscript{18} while others have interrogated certain aspects of caspase-3 binding zinc.\textsuperscript{28,29,31} However, each individual caspases has numerous interactive surface elements as well as a reactive cysteine nucleophile in the active site, both of which create potential for binding zinc or a variety of biologically relevant metals. Therefore, the effect of a panel of biologically relevant metals was tested on the two primary executioner caspases (caspase-3 and -7) as well as the initiator caspase-8, which have not been investigated previously. Each of these apoptotic caspases were treated with a panel metals at 250 nM for one hour, and their activity was subsequently assessed by the ability to cleave a preferred fluorogenic tetrapeptide substrate (Figure 20 A-C). Both the executioner caspases-3 and -7 were tested with their preferred substrate DEVD-AMC (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) and caspase-8 was tested with LEHD-AMC (acetyl-Leu-Glu-His-Asp-aminomethylcoumarin). Among the biologically relevant metals, zinc and only zinc was able to inhibit all three caspases under investigation. Due to the variation of the counter ions in the panel, both ZnCl\textsubscript{2} and ZnSO\textsubscript{4} were tested in order to eliminate the counter ion as a possible reason for inhibition. In addition, cadmium was tested for all three caspases because it shares similar properties to zinc: a group 12 metal with a d\textsuperscript{10} valence electron configuration. Cadmium had a marginal effect on the ability of caspase-7 to cleave its peptide substrate (84% activity remained), a more significant effect on
caspase-3 (39% activity remained), and an almost complete inhibitory effect on caspase-8 (12% activity remained) (Figure 20 A-C).

Peptide substrates such as DEVD-AMC and LEHD-AMC occupy only the substrate-binding groove of the respective caspase. In addition to peptide substrates, it is important to understand the effect of each metal on the ability of the caspase to cleave a biologically relevant protein substrate. While hydrolyzing a protein substrate, the caspase is predicted to make contacts beyond the amino acids proximal to the active site, at exosites such as those that have been identified in the caspase-7 N-terminal region.\textsuperscript{32,33} It would be informative to ascertain whether or not these additional contacts have an effect on the metal-mediated caspase inhibition, as this may be a means to identify other
caspase exosites. First, caspase-3 was incubated with each metal for one hour, and the cleavage reaction was then initiated by addition of the protein substrate, PAK2 (Figure 20D). Both ZnCl₂ and ZnSO₄ completely inhibited substrate cleavage and cadmium had a similar effect. Previous reports have suggested that other metals have an inhibitory effect on caspase-3, including copper and cobalt.²⁸,³¹ We did not observe a copper or cobalt-mediated inhibition. To confirm this observation, the assay was repeated with another caspase-3 substrate, PARP (Figure 20E), as done in previous work.²⁸ Similar to the PAK2 substrate, only zinc and cadmium had an inhibitory effect under these conditions.

Caspase-7 and caspase-8 were subjected to the metal panel in an identical fashion to caspase-3. Caspase-7 cleavage of the protein substrate PAK2 was inhibited by both ZnCl₂ and ZnSO₄, but no other biologically relevant metal (Figure 20F). However, cadmium had an inhibitory effect. This was a surprising result based on the previous observation that cadmium had only a slight effect on caspase-7 cleaving a peptide substrate. Lastly, caspase-8 was tested by incubation with the downstream protein substrate caspase-7 (Figure 20G). The caspase-7 substrate was the full-length zymogen protein with the active site cysteine mutated to an alanine (C186A) in order to prevent any self-processing. For caspase-8, zinc and only zinc had a significant impact on cleavage of a protein substrate. However, copper and cadmium seemed to have a minor impact on cleavage. This result for cadmium is counter to what was observed with the peptide substrate (Figure 20C), suggesting that multiple metal sites may be relevant in caspase-8 function and inhibition.
Biologically Relevant Zinc Concentrations Inhibit Caspase-3, -6, -7, and -8

Although it is clear that zinc selectively inhibits caspase-3, -7, and -8 (Figure 20) as well as caspase-6, this inhibition will only be biologically relevant at certain affinities. While total intracellular zinc concentrations are estimated to be in the micromolar range, available zinc concentrations are tightly controlled at much lower levels, estimated to be high picomolar or low nanomolar. Therefore, it is imperative that while making in vitro measurements at low zinc concentrations that the system be carefully managed so that the available zinc concentration can be tested. In order to achieve such control, we used zinc-buffering systems and calculated the available zinc concentration utilizing the online calculator, MaxChelator. By deliberately choosing each buffer component and taking advantage of known zinc binding constants we were able to set specific free zinc ion levels and prevent large fluctuations in free zinc concentration. HEPES buffer at pH 7.5 was used due its low binding capacity for zinc and TCEP as the reducing agent in order to avoid excess thiols that can coordinate heavy metals. In addition, the zinc buffering reagent was carefully chosen based on the buffering capacity and the concentration range of zinc under investigation. The experiment was also performed in the absence of a buffering agent in order to assess the effect of zinc buffering on the catalytic activity of these caspases.

After careful consideration of the buffer, caspase-3, -6, -7, and -8 were subjected to a titration of increasing zinc concentrations and assayed for remaining activity. This was done in the absence of zinc buffering (Figure 21, gray lines) and the presence of a zinc buffer (Figure 21, black lines), which allowed the available zinc concentration to be reported. Under zinc-buffering conditions there was a significant shift in IC\textsubscript{50} values.
Figure 21: Buffered Zinc Conditions Reveal Biologically Relevant Inhibition

Dose response curves of caspase activity (10 nM) with increasing concentrations of zinc. A preferred tetrapeptide substrate was used for each caspase. Titrations were carried out under non-buffering conditions for zinc (gray lines) and repeated under zinc buffering conditions (black lines). Each titration was done in duplicate on three separate days with values represented as means ± SEM and fit for the IC₅₀ value.

<table>
<thead>
<tr>
<th>IC₅₀ Values (nM)</th>
<th>Unbuffered (nM)</th>
<th>Buffered (nM)</th>
<th>Kᵢ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>104 ± 26</td>
<td>12.5 ± 0.5</td>
<td>6.9</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>4531 ± 1100</td>
<td>2.61 ± 0.4</td>
<td>2.6*</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>1580 ± 750</td>
<td>141 ± 2.1</td>
<td>76.1</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>367 ± 38</td>
<td>9.25 ± 1.2</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Table 2: Parameters for Zinc Inhibition of Caspases under Metal Buffering Conditions and Unbuffered Conditions

The zinc buffer utilized for caspase-3, -6, and -8 was NTA and citrate was used for caspase-7. All zinc calculations were done using MaxChelator. Inhibition constants were calculated assuming a mixed model of inhibition, with the exception of caspase-6 (*), which was assumed to be noncompetitive based on previous determination of allosteric binding of zinc.
relative to the unbuffered conditions. With zinc buffering the inhibition constants for all caspases were in the biologically relevant range of zinc concentrations (Table 2). Caspase-3, -7, and -8 were then assayed, under buffered conditions, at various concentrations of zinc to determine the zinc inhibition constants (Figure 22). The curves for all three caspases best fit to a mixed model of inhibition, and $K_i$ values were calculated based on a global fit.

![Figure 22: Kinetic Fits to Determine Inhibition Constants](image)

Zinc inhibition of caspase-3 (top), caspase-7 (middle), and caspase-8 (bottom). A global fit of Michaelis-Menten hyperbolas was used to determine the mode of inhibition and extract the inhibition constant $K_i$. The mixed model of inhibition represented the best fit for each caspase.
Determining the Stoichiometry of Zinc Binding in Caspases

Zincon, a colorometric zinc indicator, was used to determine Zn:Caspase stoichiometries. Zincon is a low affinity zinc chelator ($K_D = 12 \mu M$)\textsuperscript{36,37} that undergoes a change in absorbance spectra as it binds zinc. Zinc binding can be detected by monitoring the absorbance increase at 620 nm. Thus, zinc was titrated into a sample containing zincon and caspase at increasing molar equivalents to determine the zinc-binding stoichiometry for each caspase (Figure 23). Only after a colorometric response is detected have all the caspase zinc-binding sites been occupied, indicating the number of zins binding per caspase monomer. Caspase-3, -7, and -8 were found to bind three, one, and two zins respectively. Stoichiometries for caspase-3 and -7 agree with previously reported results.\textsuperscript{19} Caspase-8 was observed to bind two zins, although the shape of the zincon response curve suggested that the two zins bind with different affinities. A slight rise in the

Figure 23: Stoichiometry of Zinc Binding
Zincon was used as a colormetric zinc indicator in order to determine the stoichiometries of zinc binding for caspase-3, -7, and -8. Molar equivalents of zinc were added to a mixture of caspase and the zincon indicator. Absorption spectra recorded at 620 nm indicate a zincon response after three molar equivalents for caspase-3 (top), one equivalent for caspase-7 (middle), and two equivalents for caspase-8 (bottom). Each graph represents the mean ± SEM for three separate experiments on three separate days.
absorbance at two molar equivalents of zinc could indicate a competition with the zincon reporter. This suggests that caspase-8 and the zincon bind the zinc with similar affinities around 12 µM.

**Zinc Binds to the Caspase-8 Catalytic Dyad and Impacts Oligomeric Structure**

The cysteine-histidine catalytic dyad is well conserved across the caspase family. These two residues are also very frequently found together in zinc-binding sites, and so it has been postulated that the caspase catalytic dyad is a likely zinc binding site.\(^{18,19,27}\) To test this hypothesis in caspase-8, the catalytic cysteine (C360) was replaced by alanine in order to ablate zinc-binding properties at this site. The stoichiometry of zinc binding for the caspase-8 wild-type (Figure 24A) and the C360A variant (Figure 24B) were assessed by zincon assay. Replacement of the catalytic cysteine of caspase-8 with the non-zinc binding residue alanine significantly altered its ability to bind zinc. The stoichiometry shifted to less than two zins per monomer of caspase-8, suggesting that the catalytic dyad is responsible for binding one of the zins.

Size exclusion chromatography and analytical ultracentrifugation sedimentation velocity experiments have demonstrated that aspase-8 exists in an equilibrium between a monomeric and dimeric state.\(^{38,39}\) At a concentration of 30 µM caspase-8 is approximately 69% monomer. In addition, dimerization is required in order for the protein to be active.\(^{22}\) Active site-binding inhibitors, like substrate mimics, can strongly influence the equilibrium to favor the dimeric state.\(^{38}\) We sought to determine if zinc has any influence on the oligomeric state of the caspase-8 through size exclusion chromatography. When caspase-8 was incubated alone and loaded onto a sizing column, it eluted with 67% monomer and 33% dimer (Figure 24C). This observation agrees well
with previous investigations of caspase-8 oligomeric state distributions. After incubation with an active site-binding inhibitor, zVAD-FMK (carbobenzoxy-Val-Ala-Asp-fluoromethylketone), the ratio shifted as expected to 37% monomer and 63% dimer (Figure 24D). However, after incubation with zinc, the distribution favored the monomer (Figure 24E). The caspase-8 monomer peak represented 90% of the total protein load, with only 10% eluting in the dimeric state (Figure 24E,F). This observation suggests that the mechanistic action for zinc inhibition of caspase-8 involves preventing the protein from reaching the dimeric state necessary to enable substrate cleavage.

Figure 24: Zinc Binds to the Active Site of Caspase-8 and Influences the Oligomeric State
Zinc binding to caspase-8 (A) and caspase-8 C360A (B) was measured by monitoring the absorbance at 620 nm in the presence of the colorimetric zinc indicator, zincon, and increasing concentrations of zinc. (C) The oligomeric state of caspase-8 was monitored by elution from a size exclusion column, with two dominant peaks correlating to the caspase-8 dimeric state and monomeric state. (D) Caspase-8 was incubated with an inhibitor, zVAD-FMK, which is known to increase the concentration of the dimeric state. The distribution of monomer and dimer was monitored by SEC. (E) The distribution of caspase-8 monomeric and dimeric states in the presence of ZnCl₂ assessed by SEC. (F) A graphical representation of quantified volumes under each curve representing the monomeric or dimeric states of caspase-8 alone, in the presence of zVAD, or in the presence of ZnCl₂. These quantifications represent the mean ± SEM for three separate experiments on three separate days.
Zinc Binding Destabilizes Caspase-8

Due to the fact that zinc binding shifts the caspase-8 conformation to favor the monomeric state, we hypothesized that zinc destabilizes caspase-8. Thermal stability assays were conducted using differential scanning fluorimetry in order to assess caspase-8 stability in the presence and absence of zinc. Fluorescence of a protein-binding dye (SYPRO Orange), which increases in fluorescence when bound to hydrophobic residues that become exposed during denaturation, was monitored as a function of temperature. Wild-type caspase-8 was subjected to a melting curve in the absence of zinc or after incubation with ZnCl$_2$ and ZnSO$_4$ at 50 µM (Figure 25A). Both zinc salts destabilized caspase-8 by approximately 3°C (Table 3). Likewise, both zinc salts destabilized caspase-8 C360A by a similar amount (5°C, Figure 25B). Given that removing the active site cysteine (C360A) had a significant effect on the stoichiometry of zinc binding (Figure 24A,B), this suggests that the zinc binding site that leads to destabilization, and loss of dimer, is not at the catalytic dyad.

In contrast to the impact of zinc, caspase-8 binding to the tetrapeptide substrate-like inhibitor IETD (acetyl-Ile-Glu-Thr-Asp-aldehyde), has an extremely stabilizing effect, with an observed $T_m$ increase of 27°C (Figure 25C). This increase in stability in the presence of substrate-like inhibitors has been observed previously as caspase-7 is stabilized by 17°C upon binding of DEVD-aldehyde.$^{40}$ This is likely a result of stabilizing the dynamic active site loops upon formation of a caspase-8 dimer. After observing this extreme stabilization event, we probed the effect of zinc on dimerization of caspase-8. After incubation with the inhibitor, the caspase-8 was exposed to increasing concentrations of zinc. Substrate-bound caspase-8 was destabilized by zinc exposure, and
Figure 25: Zinc Destabilizes Caspase-8

The thermal stability of caspase-8 was interrogated by differential scanning fluorimetry using the SYPRO Orange fluorescent dye. A melting curve was generated for (A) caspase-8 wild-type alone, after incubation with ZnCl₂, and after incubation with ZnSO₄ and repeated for (B) caspase-8 C360A, a catalytically inactive variant. (C) Caspase-8 thermal stability was then investigated after incubation with a known peptide substrate, IETD, which contained an aldehyde moiety that reversibly reacts with the active site cysteine of caspase-8. This caspase-8-IETD was then incubated with zinc. (D) Caspase-8 C360A thermal melts after incubation with the aldehyde IETD inhibitor.

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>C360A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casp8 only</td>
<td>48.1 ± 0.5</td>
<td>46.7 ± 0.2</td>
</tr>
<tr>
<td>+ZnCl₂</td>
<td>44.5 ± 0.4</td>
<td>41.5 ± 0.9</td>
</tr>
<tr>
<td>+ZnSO₄</td>
<td>44.8 ± 0.3</td>
<td>41.6 ± 0.6</td>
</tr>
<tr>
<td>+IETD</td>
<td>75.5 ± 0.3</td>
<td>46.4 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3: Thermal Stability Tₘ Measurements
a slight increase in the monomer population was observed. Meanwhile, caspase-8 with the C360A substitution at the catalytic cysteine was unable to attain the stabilization shift from incubation with the substrate-like inhibitor IETD (Figure 25D) because it is unable to covalently bind to the IETD inhibitor. Nevertheless, we consistently observe that both wild-type and C360A caspase-8 are destabilized by zinc. This correlates with the ability of zinc to promote monomerization of caspase-8 and suggests that the zinc binding site that impacts dimerization does not include C360.

Due to the modest destabilizing effect in $T_m$ upon zinc binding to the caspase-8 C360A variant, we aimed to further interrogate this shift by incubation with other metals (Figure 26). Thermal stability measurements after incubation with zinc continued to display a 5°C destabilizing effect, however, incubation with iron, calcium, and copper failed to significantly alter the $T_m$ for caspase-8 C360A. This result confirmed our previous observation that the destabilizing effect shown by a shift in $T_m$ is due to zinc binding and is not simply due to the presence of cations in solution.

**Figure 26: Caspase-8 C360A is Only Destabilized by Zinc**
Thermal stability assay for the caspase-8 catalytically inactive variant (C360A) after incubation with zinc, iron, calcium, or copper. Only incubation with zinc destabilized the caspase-8 C360A.
Caspase-7 Binds Zinc at the Catalytic Dyad

Caspase-9 was observed to bind two zinccs, one at the active site and a second at an exosite at the base of the 210s helix. A sequence alignment of this caspase-9 exosite (H224 and C272 in caspase-9 numbering) with caspase-7 amino acids (H131 and C171) suggests caspase-7 lacks a critical cysteine necessary to bind zinc in the same manner as caspase-9 at an exosite. However, an investigation of the caspase-7 structure reveals that there is indeed a cys-his cluster with potential for zinc binding. To determine if this site is important in caspase-7, both the histidine (H131 in caspase-7 numbering) and the cysteine (C171) were replaced with alanine in caspase-7. This variant was then incubated in the presence and absence of ZnCl$_2$ (Figure 27A). This caspase variant was inhibited by zinc in a similar fashion to wild-type, suggesting this exosite has no inhibitory potential for caspase-7 and may be unique to caspase-9. As a result, we next interrogated the active site of caspase-7 as the binding site for the singular zinc.

The cysteine-histidine dyad comprising every caspase active site is also a potential zinc binding site. However, when a caspase is bound to a peptide-based active-site inhibitor, the substrate binding groove is sterically blocked and the cysteine-histidine dyad is covalently blocked, and therefore unavailable to bind zinc. To determine if caspase-7 and caspase-3 bind zinc using active-site residues, we first incubated each caspase with a known covalent inhibitor, zVAD-FMK, which occupies the caspase active site. After a two hour incubation zVAD-FMK completely inhibited both caspase-7 and caspase-3 (Figure 27B). After incubation with inhibitor, each caspase was subjected to increasing molar equivalents of zinc in the presence of zincon to assess zinc stoichiometry in the presence of a sterically filled substrate-binding groove active site.
Caspase-7 was unable to bind the single zinc that we observed in the absence of zVAD-FMK (Figure 27C). However, caspase-3 showed only a modest alteration in the zincon response in the presence of zVAD-FMK (Figure 27D). This suggests that caspase-3 can bind three equivalents of zinc and at the same time interact with a covalent active site inhibitor. This result is consistent with a previous report that zinc and a fluorescently labeled caspase-3 active site inhibitor are able to simultaneously react with caspase-3. This prior analysis went on to suggest that the active site histidine was a zinc ligand but the active site cysteine is not.

Figure 27: Substrate-Mimic Occupying the Active Site Disrupts Zinc Binding to Caspase-7 but Not to Caspase-3
(A) The activity of the caspase-7 variant H131A/C171A was tested in the absence and presence of ZnCl₂. (B) Activity of caspase-7 and caspase-3 after a two hour incubation alone, or in the presence of an active site inhibitor zVAD-FMK. (C) Caspase-7 alone (top) and caspase-7 with its active site occupied by zVAD (bottom) were analyzed by increasing equivalents of zinc and stoichiometry of zinc binding was determined by measuring zincon absorbance at 620 nm. (D) Zincon measurements were repeated for caspase-3 (top) and caspase-3 incubated with zVAD (bottom). Data represent the mean ± SEM for three separate experiments on three separate days.
Buffer Components Cloud Interpretation of Caspase-7 Thermal Melts

Determining that the caspase-7 active site is responsible for binding zinc is an important discovery. We recognize that it is also important to understand the residues responsible for this interaction in order to fully comprehend the inhibitory mechanism. We aimed to use differential scanning fluorimetry to test a panel of variants in which potential zinc-liganding residues had been replaced by alanine to assess the effect of zinc on their thermal stability. Initial experiments exhibited difficulties with data interpretation for caspase-7 based on a high background signal from the SYPRO orange fluorescent dye. Therefore, we tested three buffers in order to optimize detection of the melting properties (Figure 28A). Unfortunately, minimal buffers such as the standard zinc assay buffer (100 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM TCEP) or zinc assay buffer including 10% sucrose, had detrimental effects on data interpretation due to an usual baseline and multiple transitions, which has been reported for some buffers. However, caspase-7 activity assay buffer (100 mM HEPES pH 7.4, 5 mM CaCl$_2$, 10% PEG 400, 0.1% CHAPS, and 1mM TCEP) seemed to give reasonable raw data, despite the presence of two transitions. Differential scanning fluorimetry was performed using the SYPRO Orange dye on full-length caspase-7 and catalytic dyad variants C186A, H144A or H144A/C186A (Figure 28 B-E). The raw data for all four experiments showed an elevated baseline reading even at 20°C with the addition of zinc, an outcome possibly due to an interaction of the buffer with these particular variants. In addition, thermal shift assays were repeated with cleaved wild-type caspase-7 in the presence and absence of zinc (Figure 28F) and there appears to be two transitions. The caspase-7 data (Figure 28 A-F) was then compared to previous differential scanning fluorimetry data for caspase-8.
Caspase-8 exemplifies a very typical and easily interpretable thermal melt. Both the caspase-8 and the caspase-8 incubated with zinc have a comparable baseline with a single transition, as observed for typical differential scanning fluorimetry melting curves. It is also important to note the loss of fluorescence after the melting transition, which is observed in every sample (Figure 28 A-G), is a typical observation for these types of experiments.\textsuperscript{42,43} This decreasing signal at high temperatures is due to dye dissociation and protein aggregation and is commonly observed after the protein melts in differential scanning fluorimetry.\textsuperscript{42,43}

Figure 28: Comparison of Raw Data for Caspase-8 and Caspase-7 Differential Scanning Fluorimetry (A) Caspase-7 thermal stability assay using three different buffers. Raw data for relative fluorescent units was plotted for thermal stability assays of full-length caspase-7 in the absence (black lines) and presence of zinc (blue lines). This included the (B) wild-type and mutants of the catalytic dyad (C) C186A, (D) H144A, and (E) H144A/C186A. (F) Cleaved caspase-7 in the absence (black line) and presence of zinc (blue line). (G) Caspase-8 wild-type thermal stability assay raw data in the absence (black line) and presence of zinc (blue line).
Despite the unusual raw data from the caspase-7 melts, the transitions can be plotted and $T_m$ measurements can be calculated at the midpoint of each curve. The data for caspase-7 full-length wild-type as well as the active site variants in the presence and absence of zinc was plotted (Figure 29 A-D) and the thermal stabilities tabulated (Table 4). The result was unexpected, with zinc having a stabilizing effect on each sample, despite the removal of two potential zinc binding ligands (H144A/C186A). It would be beneficial to interrogate the effect of zinc on these caspase-7 active site variants by a complimentary approach, such as circular dichroism or perhaps tryptophan fluorescence. These experiments will be carried out by another lab member in the future.

Figure 29: Thermal Shift Assays for Full-Length Caspase-7 and Active Site Variants
Thermal shift assays for the full-length caspase-7 variants were processed at the transition, normalized, and fit to a boltzmann distribution in order to determine the $T_m$. This was done in the absence (black lines) or presence of ZnCl$_2$ (dark blue) or ZnSO$_4$ (light blue) on (A) caspase-7 full-length wild-type (B) C186A (C) H144A and (D) H144A/C186A.
The thermal stability of caspase-3 was also investigated by differential scanning fluorimetry. ZnCl$_2$ and ZnSO$_4$ both had a stabilizing effect of approximately 13°C on the cleaved caspase-3 wild-type. However, this stabilization effect was diminished to only 2.4°C when the full-length caspase-3 catalytically inactive (C163A) variant was incubated with zinc. It is interesting to note the fact that the C163A variant showed a 6°C stabilization over the wild-type cleaved caspase-3 when melting the caspase alone. Nonetheless, the addition of zinc continued to stabilize the C163A version of caspase-3. Due to the fact that three zincs bind caspase-3, it is difficult to attribute the increase in stability to a zinc binding the catalytic cysteine or if there is simply a smaller effect using the full-length caspase-3. In order to interrogate this query, current experiments are being performed on the full-length catalytic histidine to alanine variant. In addition, the processing sites on caspase-3 are being mutated to alanine in order to preserve the full-length protein (D169A/D175A). Thermal stability measurements carried out on variants in this construct would determine differences in zinc binding between the full-length and cleaved forms of caspase-3.

<table>
<thead>
<tr>
<th></th>
<th>Full-Length</th>
<th>Cleaved</th>
<th>+ZnCl$_2$</th>
<th>+ZnCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>59.0 ± 0.4</td>
<td>62.4 ± 0.3</td>
<td>69.4 ± 0.3</td>
<td>65.2 ± 0.7</td>
</tr>
<tr>
<td>C186A</td>
<td>55.8 ± 1.1</td>
<td>55.4</td>
<td>71.2 ± 0.2</td>
<td>71.9</td>
</tr>
<tr>
<td>H144A</td>
<td>55.7 ± 1.3</td>
<td>64.6 ± 1.1</td>
<td>70.9 ± 0.4</td>
<td>69.4 ± 0.1</td>
</tr>
<tr>
<td>H144A/C186A</td>
<td>55.4</td>
<td></td>
<td>71.9</td>
<td>66.5</td>
</tr>
</tbody>
</table>

Table 4: Thermal Stability Measurements for Caspase-7
As our understanding of zinc in biology continues, it has become increasingly important to dissect the interactions of this essential metal with the diverse array of proteins it appears to regulate. The discovery of zinc transporters and intracellular buffering capabilities has shifted the spotlight of zinc biology toward the role of zinc as a signaling moiety. The careful regulation of the intracellular pool of available zinc by ZIPs and ZnTs can affect a variety of pathways including gene expression of MTF1 and the balance of phosphorylation (Figure 31). Maintaining proper regulation of this intracellular zinc pool is imperative. This is evident in the fact that overexpression of particular zinc importers have been linked to cancer, including ZIP6, ZIP7, ZIP8, and ZIP10. The recent appreciation for zinc as a signaling moiety creates an important need to identify zinc targets, the resulting effect upon zinc binding, the relative affinity, and the mechanism by which zinc alters protein function.

The family of apoptotic caspases are known to be responsive to zinc, linking zinc signaling to a powerful cell death pathway. Zinc inhibition extends to both the extrinsic
and intrinsic apoptotic pathways, with evidence that zinc inhibits apical caspases -8\textsuperscript{16} and -9\textsuperscript{18}. In addition, zinc also can act downstream and silence the executioner caspases -3, -6, and -7\textsuperscript{16} (Figure 31). Previous investigations have uncovered the molecular details of zinc binding and inhibiting both caspase-6\textsuperscript{19} and caspase-9\textsuperscript{18}. In addition, zinc binding to caspase-3 has been shown to be extremely potent\textsuperscript{27} and another study has proposed a

---

**Figure 31: The Influence of Zinc on Caspases and Apoptosis**

The pool of available intracellular zinc is dynamically regulated by both zinc importers (ZIPs) and zinc exporters (ZnTs). These membrane associated ZIPs and ZnTs move zinc to and from the cytosol from the extracellular space or intracellular compartments. This fluctuating pool of available zinc can affect a variety of proteins with numerous downstream effects including the inhibition of phosphatases and thus the increase in kinase activity, as well as regulating transcription factors such as MTF1. The available zinc is also able to regulate caspase activity in an anti-apoptotic fashion. Zinc inhibition of caspase-8 blocks its ability to contribute to the extrinsic apoptotic pathway while zinc interactions with caspase-9 interfere with the intrinsic apoptotic pathway. These apoptotic pathways converge on the executioner caspases -3, -6, and -7, which are also directly inhibited by zinc. Zinc targets multiple junctions of the apoptotic cascade and as a consequence the caspases cannot cleave their substrates and apoptotic cell death is obstructed.
unique binding site on caspase-3. However, there was a paucity of information regarding the inhibition of caspase-7 and caspase-8 by zinc. We have shown here that zinc is the only biologically relevant metal to inhibit caspase-3, -7, and -8. In addition, this inhibition is evident for cleavage of tetrapeptide substrates as well as known apoptotic protein substrates.

Zinc binding to the apoptotic caspases -3, -6, -7, and -8 is an extremely tight interaction. The IC$_{50}$ and inhibitory constants determined here reveal nearly a 1:1 stoichiometry of added zinc correlating to inhibited caspase. This was true for caspase-3, -6, and -8, while caspase-7 had a weaker response by approximately 1.5 orders of magnitude. In addition, the measurement for caspase-3 agrees well with previous studies. It is extremely important to carefully consider the conditions under which these assays are performed (for review see Patton et al) in order to fully understand the zinc binding affinity. It is crucial to use a pH buffering component with low metal binding affinity, a reducing agent lacking a thiol to minimize metal chelation, and a zinc buffering agent with the appropriate zinc buffering capacity for the interaction intended to be measurable. Here we deliberately used HEPES as the low affinity pH buffer, TCEP as the reducing agent, and NTA for the tight binding caspases -3, -6, and -8 and citrate for caspase-7. This approach has been utilized previously, with one specific example being the protein-tyrosine phosphatase β. In this study they observed that zinc indeed inhibits this phosphatase at orders of magnitude lower than previously reported. This discovery was owed to attentive and deliberate formulation of the zinc buffering during kinetic analysis. Similarly, we observe a dramatic increase in affinity for zinc with the apoptotic caspases, observing inhibition undoubtedly in the biologically relevant range of
intracellular available zinc. In addition, the inhibition measured here is at the upper limit of detection.

Small fluctuations in the available zinc pool can have dramatic downstream consequences. Therefore, uncovering the means by which zinc inhibits each caspase is essential to further our understanding of zinc and its role in apoptosis. Caspase-6 and caspase-9 are inhibited by zinc through two very different mechanisms, suggesting that zinc inhibition may be nuanced to each caspase. Deciphering these differences would provide salient information in the pursuit of therapeutic intervention. Our examination regarding caspase-8 inhibition by zinc has unique details compared to previously determined zinc-caspase inhibition mechanisms. Caspase-8 binds two zinbes with an observed threefold effect: complete inhibition of the enzyme, alterations to the oligomeric state, and an overall destabilization of the enzyme. Changing the active site cysteine residue to alanine altered the stoichiometry of zinc binding, reducing it to approximately one zinc per caspase-8 monomer. This suggests that zinc binds the catalytic dyad, similar to caspase-9. Caspase-8 exists in a concentration dependent equilibrium between the monomeric and dimeric state. Dimerization is necessary in order for caspase-8 to be active. Zinc binding prevents caspase-8 dimerization and heavily shifts the equilibrium towards the monomeric, inactive state. This observation is in contrast to zinc inhibition of caspase-9, which had no influence on oligomeric state. In addition, zinc had a destabilizing effect on the thermal stability of caspase-8 and the caspase-8 catalytically inactive C360A. If destabilization by zinc is concomitant with the alteration of the oligomeric state, then this data suggests a second zinc binding site, beyond the C360 active site, is responsible for blocking caspase-8 dimerization. This would offer two
different mechanisms for zinc inhibition for the same enzyme, offering the hypothesis that one site targets procaspase-8 by blocking dimer formation and the other targets any form in which the active cysteine is exposed.

The catalytic dyad for the dimeric caspase-3 and caspase-7 is also a potential zinc binding site, and a previous work has suggested that zinc binding to these two executioners could involve the catalytic histidine but not the cysteine.\textsuperscript{29} We targeted the active site for both of these molecules as a possible inhibitory site for zinc. Blocking the active site with a known peptide inhibitor completely abolished zinc binding to caspase-7. This suggests that perhaps zinc simply binds the catalytic dyad in the caspase-7 active site. However, blocking the active site of caspase-3 did not change the stoichiometry of zinc binding as determined by a colorometric zincon assay. This results suggests (1) substrate and zinc can bind concurrently and (2) perhaps zinc binding does not involve the catalytic cysteine, as suggested previously.\textsuperscript{29}

It is also important to note that caspase-3 binds three zincs per monomer, an observation in agreement with previous ICP-OES analysis.\textsuperscript{19} It is intriguing to consider that, despite the previous proposal for zinc blocking the catalytic histidine, there are still two more zinc binding sites. If zinc simply binds the histidine and prevents substrate turnover, what is the need for two additional zinc binding sites or do these other sites play roles in caspase-3 biology? This is also observed for caspase-9, wherein the activity is ablated by zinc binding to the active site, yet there remains a second site established to bind zinc. These extra binding sites could be exosites that alter the ability of the caspase to recognize or interact with other proteins, or may have no structural or functional impact. For example, if we assume that cadmium and zinc share binding sites on caspase-
3, then binding an exosite would inhibit cleavage of a protein substrate more compared to a small peptide substrate. In fact, we observed this with caspase-3 and the protein substrate PAK2 (Figure 20). Cadmium inhibits caspase-3 from cleaving peptide substrates approximately 61%, but fully inhibits the cleavage of the protein substrate PAK2. Further investigations, such as crystallography, could provide meaningful information about the nature of these additional zinc binding sites.

We have shown in this work that zinc inhibits caspase-3, -6, -7, and -8 in the low nanomolar range in correlation with biologically relevant levels of available zinc. This inhibition by zinc is specific and does not apply to other metals found in biology. We specifically interrogated the zinc mechanism of action against caspase-8, which had not been investigated previously. Caspase-8 binds two zincs, including one at the active site cysteine C360. The zinc binding also destabilizes caspase-8 and alters the oligomeric state to favor the inactive monomer. Such a mechanism of inhibition by zinc is thus far unique to caspase-8. In addition, caspase-7 binding zinc is in direct competition with substrate binding at the active site. This data, taken together unveils an important target for zinc and its regulation of the apoptotic cascade. The biochemical details discerned from this work provide essential information regarding caspase inhibition by zinc that adds fuel to the ever-expanding discoveries centered around zinc biology.

**Materials and Methods**

**DNA Expression Constructs**

Expression constructs for human caspase-3 and caspase-9 both in pet23b, were obtained from Addgene. The expression construct for human caspase-7 in pET 23b was a gift from Guy Salvesen and human caspase-8 in pET 15b was a gift from JB Denault. The wild-type caspase-6 human gene was a constitutive two-chain (CT) construct in
pET11a. The expression construct for human p21 activated kinase 2 (PAK2), which was used as a caspase substrate, was in pET28b and was a gift from John Kuriyan’s lab. The expression construct for Human Rhinovirus 3c protease (HR3cPro) in the pGEX vector was a gift from M. Romanowski.

**Caspase Cleavage of Peptide Substrates with Various Metals**

Caspase-3, -7, and -8 were incubated with a panel of metal salts to test the effect of each metal on catalytic activity. Caspase-3 (10 nM enzyme) was incubated in minimal cleavage buffer (100 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM TCEP) along with 250 nM of each metal for 30 minutes at room temperature. The activity was assessed by the addition of 100 µM of the fluorogenic peptide substrate DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin), Enzo Lifesciences; Ex. 365 nm / Em. 495 nm). Assays were performed in duplicate at 37°C in 100 µL volumes in a 96 well black bottom microplate, using a Molecular Devices Spectramax spectrophotometer. Initial velocities were measured as a function of time in order to determine the relative activity. Caspase-7 and caspase-8 (100 nM enzyme) were incubated in minimal cleavage buffer with 2.5 µM metal in an identical fashion to the that described above for caspase-3. Caspase-7 activity in the presence of each metal was assessed by the addition of DEVD-AMC, while caspase-8 activity was assessed using the fluorogenic substrate LEHD-AMC (N-acetyl-Leu-Glu-His-Asp-AMC (7-amino-4-methylcoumarin), Enzo Lifesciences; Ex. 365 nm / Em. 495 nm).

**Caspase Cleavage of Protein Substrates with Various Metals**

Caspase-3, -7, and -8 were each incubated with a preferred protein substrate under the influence of a panel of metals in order to determine an effect on the ability to cleave a
folded protein. Each caspase (all 1 µM) was incubated in minimal cleavage buffer (100 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM TCEP) with 25 µM of each metal for 30 minutes at room temperature. The protein cleavage reactions were initiated with the addition of 2 µM of a protein substrate. Caspase-3 was incubated with the protein substrate PAK2 for 60 minutes at 37°C; caspase-7 was incubated with the protein substrate PAK2 for 120 minutes at 37°C; and caspase-8 was incubated with the full-length caspase-7 C186A catalytically inactive mutant as a substrate for 120 minutes at 37°C. Reactions were stopped by the addition of Laemmli buffer and heating at 95°C for 10 minutes. Cleavage was assessed by SDS-PAGE.

**Zn Inhibition Assays using Zinc Buffering**

The conditions for achieving each free Zn concentration were calculated using Maxchelator.\(^5^\) For caspase-3, -6, and -8, 1 mM NTA was incubated with the calculated amount of zinc in minimal assay buffer (100 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM TCEP) for 15 minutes at room temperature. Due to the difference in inhibition for caspase-7, 1 mM citrate was used to buffer the free Zn to achieve the desired free Zn concentrations. Each caspase enzyme was added to the reaction mixture at a final concentration of 10 nM. The enzyme was allowed to incubate with the free Zn for 1 hour at 25°C. The fluorogenic peptide substrate was then added to the reaction to a final concentration of 100 µM, and the activity was measured by monitoring fluorescence as a function of time. Caspase-3 and -7 were assayed with the fluorogenic peptide substrate DEVD-AMC (see above). Caspase-6 was monitored with VEID-AMC (N-acetyl-Val-Glu-Ile-Asp-AMC (7-amino-4-methylcoumarin), Enzo Lifesciences; Ex. 365 nm / Em. 495 nm). Caspase-8 was monitored using LEHD-AMC (see above).
**Determination of Zinc Stoichiometry using Zincon**

Zincon was used as a spectrophotometric reagent to determine the stoichiometry of zinc binding to caspases. A 1-mL reaction containing 10 µM caspase, 25 µM Zincon, and 1 mM TCEP was equilibrated at room temperature in a buffer containing 100 mM HEPES pH 7.5 and 100 mM NaCl. Each reaction was placed in a cuvette and titrated with ZnCl₂ where the optical absorption spectrum was taken after each addition of Zn(II). The change in absorbance at 620 nm was recorded and plotted against the molar equivalents of Zn(II) added per caspase monomer. Assays were performed at 25°C on three separate days and monitored in a Molecular Devices Spectramax spectrophotometer. Zincon has been reported to bind Zn(II) with a $K_d$ value 12.6 µM.\(^{37,60}\)

**Caspase-8 Size Exclusion Chromatography**

Caspase-8 was concentrated using Amicon Ultra – 0.5 mL Centrifugal Filters 3K MWCO (EMD Millipore) and the buffer was exchanged in the same device three times to remove residual DTT present from the purification using a sizing column buffer (100 mM HEPES pH 7.5, 100 mM NaCl, 1 mM TCEP). A Superdex 200 increase 10/300 GL (GE Lifesciences) was equilibrated with 7 column volumes of buffer at room temperature with a flow rate of 0.5 mL/min. 100 µL of Blue Dextran (2,000 kDa) was injected over the column with 0.5 mL of sizing column buffer at 0.5 mL/min. The void volume of the column was determined to be 8.56 mL. 100 µL of a standard mixture of ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa) and ribonuclease A (13.7 kDa) was injected with 0.5 mL of sizing column buffer at 0.5 mL/min. Caspase-8 wild-type (10 µM) was incubated alone, or with 50 µM covalent inhibitor zVAD-FMK (carbobenzoxy-Val-Ala-Asp-fluoromethylketone; known to induce dimerization), or with
50 µM zinc chloride for 1 hour at room temperature. 100 µL of each sample was injected with 0.5 mL of sizing column buffer at 0.5 mL/min and eluted with 1.5 column volumes of sizing column buffer. Chromatograms of both standards and unknowns were integrated using Unicorn 5.10 software (GE Healthcare) to determine retention times and peak elution volumes. The molecular weight of each peak (monomer and dimer of caspase-8) was determined using the standard curve generated by the mixture of standards. The total volume of caspase-8 was calculated by adding the volumes from the area under the curves of both monomeric and dimeric caspase-8. The ratio of monomer to dimer was determined by dividing the volume of either monomer/dimer by the total volume of caspase-8. Three trials were performed on three separate days, and data were plotted using GraphPad Prism. The column was cleaned between replicates with 5 column volumes of 1 mM EDTA to remove any residual zinc, and then re-equilibrated with 7 column volumes of sizing column buffer.

**Thermal Stability Analysis by Differential Scanning Fluorimetry**

The thermal stability of each caspase and respective variants was measured in the presence and absence of zinc or other metals. Caspases were incubated at 5 µM in minimal assay buffer (100 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM TCEP) in the presence or absence of 10x molar excess zinc or other metal. Stability was measured in the presence of 2x SYPRO® Orange dye (ThermoFisher) using a CFX Connect Real-Time PCR detection system (BioRad). Reactions were 50 µL in a 96 well plate. Fluorescence intensity was recorded at increasing temperatures by 0.5°C intervals from 25 to 95°C. Thermal melting points (Tₘ) values were calculated by curve fitting analysis using Prism (GraphPad) software.
Caspase-3 Expression and Purification

Plasmids encoding human caspase-3 and all variants were transformed into BL21(DE3) *E. coli* cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.6. The temperature was reduced to 30°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and 50 mM imidazole. Caspase-3 was eluted with a step gradient to 300 mM imidazole. The eluted caspase-3 was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT to reduce the salt concentration. This protein was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Protein eluted in 110 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C.

Caspase-7 Expression and Purification

Plasmids encoding human caspase-7 and all variants were transformed into BL21(DE3) *E. coli* cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.6. The temperature was reduced to 18°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH
8.0, 300 mM NaCl, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and 50 mM imidazole. Caspase-7 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT to reduce the salt concentration. This protein was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Protein eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

For several sets of experiments it was imperative that the caspase-7 retained its full-length uncleaved zymogen form, however, during expression in *E. coli* the prodomain is self-proteolyzed as a function of time. Therefore, full-length caspase-7 was produced by a 10 min induction at 37°C. The 6x His tag was removed off the C-terminus of caspase-7 by HR3cPro Prior to the His tag, a linker for the preferred precision protease cleavage site was inserted as amino acids –GGGSLEVLFQGP. After the Q column purification step the pooled fractions were subjected to a HR3cPro digestion in a suitable buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 1 mM EDTA). A total of 1 mg of HR3cPro was added to 55 mg of purified caspase-7 protein and allowed to incubate overnight. Complete digestion was confirmed via differential migration of the small subunit assessed by SDS PAGE. To further purify the protein and remove the HR3cPro, the digested caspase-7/HR3cPro mixture was concentrated to 5 mL and loaded on to a HiLoad Superdex 75 26/60 size exclusion column equilibrated a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, and 2 mM DTT. Samples were eluted with lysis buffer
and fractions corresponding to the elution peak on the chromatogram were pooled and concentrated. Purity was assessed via SDS-PAGE and aliquots were stored at -80°C.

**Human Rhinovirus 3C protease (HR3cPro) Expression and Purification**

The expression construct encoding HR3cPro was transformed into BL21(DE3) *E. coli* cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.6. The temperature was reduced to 30°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in 1x PBS. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a packed GST column (GE Healthcare) and washed with 1x PBS until the absorbance reached baseline. The column was subsequently washed with wash buffer (50 mM Tris pH 8.5, 150 mM NaCl) and protein was eluted in 50 mM Tris pH 8.5, 150 mM NaCl, 10 mM reduced glutathione, and 1 mM DTT. The eluted HR3cPro was analyzed by SDS-PAGE for purity and stored at -80°C.

**Caspase-8 Expression and Purification**

The expression construct encoding human caspase-8 was transformed into BL21(DE3) *E. coli* cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.6. The temperature was reduced to 25°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity
column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 8 mM imidazole. Caspase-8 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT to reduce the salt concentration. This sample was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Caspase-8 eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

Caspase-9 Expression and Purification

An expression construct for full-length human caspase-9 was transformed into BL21 (DE3) *E. coli* cells. The cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.9. The temperature was reduced to 15°C and cells were induced with 1 mM IPTG to express soluble His-tagged caspase-9. Cells were harvested after 3 hrs to obtain single site processing at D315. Cell pellets stored at -80°C were freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The filtered supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM imidazole until the absorbance returned to baseline. The protein was eluted using a 2-100 mM imidazole gradient over the course of 270 mL. The eluted fractions containing protein of the expected molecular weight and composition were diluted 10-fold into a buffer containing 20 mM Tris pH 8.5 and 5 mM DTT to reduce the salt concentration. This caspase-9 sample was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient.
gradient and eluted in a buffer containing 20 mM Tris pH 8.5, 180 mM NaCl, and 5 mM DTT. The eluted protein was stored at -80°C in the elution buffer and was analyzed by SDS-PAGE for purity.

Caspase-6 Expression and Purification

Plasmids encoding human caspase-6 and all variants were transformed into BL21(DE3) E. coli cells. Cultures were grown in 2xYT media with ampicillin (100 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.6. The temperature was reduced to 20°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 hours to express soluble proteins. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Tris pH 8.5, 300 mM NaCl, 5% glycerol, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM Tris pH 8.5, 300 mM NaCl, 5% glycerol, and 50 mM imidazole. Caspase-6 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT to reduce the salt concentration. This protein was loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Protein eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

PAK2 T402E Expression and Purification

An expression construct for human p21 activated kinase 2 (PAK2) T402E was transformed into BL21(DE3) E. coli cells. Cultures were grown in 2xYT media with kanamycin (40 µg/mL, ThermoFisher) at 37°C until they reached an OD$_{600}$ of 0.6. The
temperature was reduced to 25°C and cells were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were stored at -80°C, freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.05% Tween-20, and 2 mM imidazole. Lysed cells were centrifuged at 27,000 rcf to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare). The column was washed with a buffer of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.05% Tween-20, and 10 mM imidazole. PAK2 was eluted with a step gradient to 300 mM imidazole. The eluted fraction was diluted 6-fold into a buffer containing 20 mM Tris pH 8.5 and 2 mM DTT, to reduce the salt concentration. This protein sample was then loaded onto a 5 mL HiTrap Q column (GE Healthcare). The column was developed with a linear NaCl gradient. Protein eluted in 120 mM NaCl and was assessed for purity by SDS-PAGE and stored at -80°C in elution buffer.

References


CHAPTER IV

REACTIVE SELF-ASSEMBLY OF POLYMERS AND PROTEINS TO REVERSIBLY SILENCE A KILLER PROTEIN


Abstract

Conjugation of biologically active proteins to polymeric materials is of great interest in the treatment of cancer and other diseases of protein deficiency. The conjugation of such biomacromolecules is challenging both due to their hydrophilicity and propensity to denature under non-native conditions. We describe a novel reactive self-assembly approach to “wrap” a protein with polymers, simultaneously protecting its delicate folded state and silencing its enzymatic activity. This approach has been demonstrated using caspase-3, an apoptosis-inducing protein, as the first case study. The protein-polymer conjugation is designed to be reversed under the native conditions for caspase-3, that is, the reducing environment found in the cytosol. The current strategy allowed release and recovery of up to 86% of caspase activity and nanogel-caspase-3 conjugates induced 70-80% apoptotic cell death shortly thereafter. This approach is widely generalizable and should be applicable to the intracellular delivery of a wide range of therapeutic proteins for treatment of complex and genetic diseases.
**Introduction**

Proteins perform vital biological functions, ranging from gene regulation to catalysis of metabolic reactions and cell signaling to programmed cell death. Proteins are widely used as therapeutics (‘biologics’), because they exhibit higher specificity and offer more nuanced functions than can be achieved by small synthetic molecules.\(^1,2\) For example, small-molecule-based drugs often suffer from off-target activities, especially because of their inability to differentiate within protein sub-classes. These off-target effects can be avoided by using a deficient, repressed or down-regulated protein as a drug directly. However, *in vivo* stability of proteins has been a significant issue with this approach. PEGylation of proteins has been effective in the *in vivo* stabilization of protein-therapeutics,\(^3,4\) but complementary approaches are necessary as this approach can result in irreversible modification of the surfaces of the protein cargo. Many of the biologically important proteins also have inherent liabilities for manipulation and direct administration, including conformational flexibility, a metastable “folded” state, large size, propensity to aggregate and susceptibility to oxidation or degradation. As a result, the development of new protein-polymer nanoconjugates to stabilize and deliver proteins is gaining tremendous interest.\(^5–8\) Traditionally, conjugation of proteins to polymeric nanocarriers has been quite challenging due to the propensity of proteins to denature during polymer conjugation, which often requires using organic solvents and harsh conditions for synthesis.\(^9,10\)

The past decade has seen some brilliant contributions to address this need. Conjugation of proteins to telechelic, branched, star polymers and polymeric supramolecular molecules has been achieved without compromising the activity or
integrity of certain proteins.\textsuperscript{11–15} Since most of the current approaches use irreversible covalent conjugation to reactive, surface exposed residues of proteins (such as lysines and cysteines), and since multiple copies of these amino acids might be present on the surface, there have been efforts to genetically modify proteins whereby reactive functional groups can be placed in specific locations,\textsuperscript{16–21} but not without risks to the inherent immunogenicity. We are interested in developing a strategy that provides for complete reversibility in the protein-polymer conjugation and utilizes native proteins. More specifically, we are also interested in an approach that allows for turning-off the protein activity, and then turning the activity ‘on’ when it reaches its target environment using a specific biological trigger.

A commonly used strategy for reversibility involves the utilization of electrostatic complementarity, which has advantages because of the simplicity in obtaining the formulation.\textsuperscript{22–25} Since there is a significant literature that suggests that charge-neutral nanoscopic systems are desired for long circulation times, we particularly focus on methods that can conveniently provide a charge-neutral surface. Inverse mini-emulsion methods use large volumes of organic solvents, where the aqueous phase is the dispersed phase. This dispersed phase can be utilized to trap water-soluble monomers, crosslinkers, and proteins and then encapsulate the protein using a polymerization reaction.\textsuperscript{9,10,26–29} We are interested in developing a reactive self-assembly of a precursor polymer to conjugate proteins, where: (i) the conjugation is covalent, but reversible; (ii) the process does not use organic solvents; (iii) the protein is in its native form; (iv) the protein is encapsulated to be protected from proteases in order to avoid premature degradation; (v) the protein activity is turned-off in the encapsulated form; and (vi) the protein activity is recovered in
its native environment. In this manuscript, we disclose a reactive self-assembly strategy that provide a polymer-protein nanoassembly with all these characteristics.

We use a caspase protein to demonstrate the utility of the approach outlined here. Caspases are cysteine proteases that are known for their exquisite specificity for cleaving after particular aspartic acid residues and rapidly inducing apoptotic cell death.\textsuperscript{30,31} Their ability to trigger apoptosis makes them attractive cargo for selective cell killing, especially in cancer therapeutics. However, caspases are inherently fragile because they contain dynamic loops, reactive cysteines poised for chemistry at their active site, and are prone to aggregation due to their tetrameric composition. These qualities therefore necessitate a delivery system that will not damage the protein and protect it simultaneously. Among the family of apoptotic caspases, caspase-3 is of special interest due to its major role in cleaving substrates during apoptosis\textsuperscript{32} as well as its high catalytic rate.\textsuperscript{33} Reversible conjugation with silenced activity, targeted in this study, is also ideal to study with this protein, because accidental release of active caspases can lead to irreversible proteolytic damage or even unwanted apoptotic death. Thus, the therapeutic potential of caspases for inducing cell death using intrinsic biological pathways could be ultimately harnessed, if it were possible to silence caspase activity through polymer conjugation and then recover its activity in response to a specific trigger.

**Results and Discussion**

Since caspases are active in the cytosol, it is clear that these proteins are active under the reducing conditions of high glutathione (GSH) concentrations. Therefore, we were interested in developing a nanogel where the crosslinkers are based on disulfides. In this case, the protein will be stably encapsulated in the low GSH concentration (µM)
of the serum, but will be released at high GSH concentration of the cytosol (mM) due to the disulfide bond cleavage in the polymeric nanogel. Accordingly, we attempted the preparation of caspase-encapsulated nanogels using inverse emulsion polymerization. In this case, free radical polymerization of a variety of PEG-functionalized acrylates was carried out in the presence of caspase-3 as the cargo and a diacrylamide derived from cystine as the crosslinker. Although we were able to achieve protein encapsulation, we also found that the process had an irreversibly detrimental effect on caspase-3 activity (data not shown). A number of experiments were then performed to assess the effect of the photoinitiator (Igracure) with light, the acrylate monomer alone, and another common initiator/catalyst combination (APS/TEMED) on caspase-3 activity. The results (Figure 32a) led us to conclude that an irreversible reaction between the surface functional groups of caspase-3 and the reactants utilized for this inverse emulsion polymerization, specifically the acrylic monomers, destroyed the protein’s activity. In fact, incubation of caspase-3 with the PEG-acrylate monomer alone indeed irreversibly damaged the activity of the protein.

This observation of irreparable damage to the protein function led us to reassess our polymer linkage to this fragile enzyme. We hypothesized that the reactive cysteines at the active site of the protein were interacting with the polymerization agents and asked if we could use reversible thiol chemistry to our advantage. Since pyridyldisulfide undergoes a selective thiol-disulfide exchange with reduced thiols of cysteines, we treated caspase-3 with cysteinyl-2-pyridyl disulfide. The product disulfide acts as a protecting group to completely silence the caspase-3 activity in an oxidized state, but
upon exposure to a reducing environment the protein is able to regain full activity (Figure 32b).

![Figure 32: Recovery of caspase-3 activity after treatment with various polymerization agents.](image)

We were inspired by these results at the possibility of a reactive self-assembly approach to encapsulate proteins in a way that would turn-off its activity and then reversibly recover the activity under the native conditions of the enzyme. There are five reduced cysteines that are surface exposed in a caspase monomer (Figure 33). If we were to mix caspase-3 with a copolymer that contains pyridyldisulfide moieties as side chains, then we hypothesized that the cysteines in the protein would react with the polymer to afford a self-assembled protein-polymer conjugate due to the thiol-disulfide exchange reaction. Pyridyldisulfide (PDS) is a hydrophobic functional group and when
copolymerized with a PEG-containing monomer, the copolymer forms amphiphilic assembly. We have previously used a 7:3 ratio of PDS and oligoethyleneglycol (OEG) based monomers to obtain robust amphiphilic assemblies to sequester hydrophobic guest molecules.\textsuperscript{34} However, for the reactive self-assembly with a protein that we envision here, it is essential that the polymer is more dynamic. Therefore, we targeted a polymer that contains a lower percentage of the hydrophobic PDS-based monomer and used a 1:1 ratio of these two monomers for the self-assembly.

The random copolymer nanogel precursor was obtained by the reversible addition-fragmentation chain transfer (RAFT) polymerization of OEG-methacrylate and PDS-methacrylate. The feed ratio of the monomers was 50:50 and experimentally the resulting copolymer was found to contain 48\% OEG units and 52\% PDS groups, as discerned by NMR (Figure 34). Polydispersity and M\textsubscript{n} was found to be 1.5 and 20K, respectively. To encapsulate the protein using the reactive self-assembly strategy, caspase-3 and the amphiphilic polymer were added simultaneously to aqueous media. The protein was presumably wrapped in the assembly by initiating thiol-disulfide exchange reactions between PDS groups; the assembly was further locked-in with the addition of a precise amount of DTT (Scheme 1, NG-Casp-In). It is likely that the
exposed cysteines on the caspase play a critical role in this reactive self-assembly process.

Concurrently, we also targeted a control nanogel, where the nanoassembly did not contain any encapsulated protein (NG-empty). In an aqueous solution, the polymer forms an aggregate, which was locked-in using a self-crosslinking process enabled by intra- and inter-chain disulfide cross-linking of the PDS groups in the presence of the reducing agent dithiothreitol (DTT). The nanogel formation process was monitored by tracing the absorption spectra of 2-pyridinethione (byproduct of the disulfide crosslinking) at 343 nm (Figure 35). Based on the 2-pyridinethione released, the crosslink density of these nanogels was 18% (Figure 35A). As an additional control, we also prepared a nanogel, where the caspase-3 is covalently conjugated to the surface of the nanogel. Here, we first prepared the cross-linked nanogels (similar to NG-empty); caspase-3 was then covalently attached on its surface using the cysteine residues of the protein and unreacted PDS
groups (Scheme 1, NG-Casp-Out). This effectively decorated the nanogel with caspase-3 attached to the outside. Due to their hydrophobic nature, we suspected that the unreacted PDS groups might collapse into the core of the nanogels and therefore might not be available for surface functionalization. This seems to be not the case and surface functionalization can indeed be achieved with reasonable efficiency.

To ultimately evaluate these nanogels in both passive and activated cellular uptake pathways, we also prepared nanogels with cell penetrating capabilities by incorporating a cysteine-containing tri-arginine peptide on the surface of these nanogels to generate NG-Empty RRR, NG-Casp-In RRR, NG-Casp-Out RRR (Scheme 1). Incorporation of the peptide on the surface was confirmed by further increase in the 2-pyridinethione absorption spectrum (Figure 35 D, E, and F). Based on the 2-
pyridinethione released, we found that the presence or absence of caspase had no overall impact on the ability to conjugate a second molecular entity. The crosslinking densities of these nanogels NG-Empty\textsuperscript{RRR}, NG-Casp-In\textsuperscript{RRR}, NG-Casp-Out\textsuperscript{RRR} were each found to be $\sim 18\%$, suggesting that peptide had been incorporated.

**Figure 35:** Absorption spectra of nanogel-caspase conjugates (A) NG Empty (0.1 mg/mL) (B) NG-FITC-Casp-In (0.1 mg/mL) (C) NG-FITC-Casp-Out (0.1 mg/mL) (D) NG EmptyRRR (0.05 mg/mL) (E) NG-FITC-Casp-InRRR (0.05 mg/mL) (F) NG-FITC-Casp-OutRRR (0.05 mg/mL).
To further characterize these nanogel-protein conjugates, we evaluated their size by dynamic light scattering (DLS) and found that the hydrodynamic diameter of free caspase-3, NG-Empty and NG-Empty$^{RRR}$ were 6, 12, and 10 nm respectively (Figure 36). After the conjugation of caspase to the nanogels, there was an increase in the size of the conjugates, indicative of protein conjugation (12 nm – 18 nm, Figure 36a and b). Caspase-3 is negatively charged (pI 6.1), whereas CRRR is positively charged due to the arginine residues. These differences led us to evaluate the change in the surface charge of these conjugates by zeta potential measurements. After conjugation of caspase-3, the surface charge of NG-Casp-In (-19 mV) did not change significantly from the value observed in the original NG-Empty (-17 mV, Figure 36c). In the case of NG-Casp-Out, the zeta potential value obtained shifted towards -7 mV, similar to the value observed for free caspase-3 (-8 mV). Although these are PEG-functionalized surfaces, these studies suggest that the nanogels carry a slight, apparent negative charge, which has been previously seen with PEG-functionalized surfaces. After surface decoration of nanogels with CRRR, we expected that NG-Empty$^{RRR}$ would show a positive zeta potential value since these nanogels do not have caspase-3 conjugated. As expected, the surface charge for NG-Empty$^{RRR}$ was found to be +18 mV. With caspase-3 conjugation, the surface charge became less positive: +4 mV for NG-Casp-In$^{RRR}$ and +9 mV for NG-Casp-Out$^{RRR}$ (Figure 36d). These results, along with the UV-vis absorption spectra recorded during the nanogel-caspase conjugates synthesis (Figure 35), suggest that unreacted PDS groups from the nanogels were sterically available to covalently react with the surface exposed cysteine residues from caspase-3. Furthermore, despite the presence of a large and bulky biomacromolecule on these polymeric nanogels,
subsequent surface functionalization by CRRR addition can also be achieved, demonstrating the prevalent accessibility and reactivity of the PDS groups. The properties of these nanogel-caspase conjugates are summarized in Figure 36e.

To further assess the covalent protein conjugation to the nanogels, we utilized SDS-PAGE to assess caspase dissociation from the nanogels in a reducing environment (Figure 37). When treated with sodium dodecyl sulfate (SDS) and boiled, caspase-3
dissociates into the constituent large and small subunits (17 and 12 kDa, respectively), which migrate as two characteristic bands on SDS-PAGE. We expected that caspase-3 conjugated to the inside or outside of nanogels should not be observable by SDS-PAGE, because it will remain bound to and migrate (or fail to migrate) with the nanogel. We expected to observe the two caspase-3 bands only after treatment of the nanogels with a reducing agent. As anticipated, we did not observe the bands corresponding to caspase-3 in the NG-Casp-In or in the NG-Casp-Out samples in the absence of reductant (Figure 37a). This suggests that caspase-3 is covalently conjugated through disulfide bonds and not simply associated through physical adsorption. Since disulfide bonds can be cleaved at high concentrations of the reducing agent, we hypothesized that we should be able to “unlock” the protein from the assembly by exposing the conjugates to DTT. The appearance of the characteristic caspase-3 bands for the large and small subunits in both samples, NG-Casp-In and NG-Casp-Out, indicated that the protein was released after the reduction of disulfide bonds. This confirmed the conjugation of caspase-3 in both the “in” and “out” configurations (Figure 37b) and demonstrated that these nanogels respond to a

**Figure 37:** SDS-PAGE gel validating the nanogel-caspase conjugation through reducible disulfide linkages. (a) Nanogel-caspase conjugates under non-reducing conditions (b) Nanogel-caspase conjugates under reducing conditions (c) Nanogel-caspase\textsuperscript{RRR} conjugates under non-reducing conditions (d) Nanogel-caspase\textsuperscript{RRR} under reducing conditions.
specific redox stimulus, namely DTT. We estimated the concentration of caspase-3 released from each conjugate by comparing the intensity of the bands from nanogel-released caspase to known concentrations loaded into neighboring wells. Both nanogel-caspase conjugates (50 µg) released about 2 µg of caspase-3. Similar results were observed for the nanogels functionalized with the cell-penetrating peptide, RRR. Under non-reducing conditions, no caspase bands were observed for the NG-Casp-In$_{RRR}$ and NG-Casp-Out$_{RRR}$ samples (Figure 37c). When the samples were exposed to reducing conditions, we observed the appearance of the bands indicative of free caspase-3 released from NG-Casp-In$_{RRR}$ and NG-Casp-Out$_{RRR}$ (Figure 37d). The concentration of released caspase-3 from 50 µg NG-Casp-In$_{RRR}$ and NG-Casp-Out$_{RRR}$ was found to be 1 µg and 0.5 µg, respectively.

We had hypothesized that the encapsulation of proteins would protect these cargo from protease degradation. We had also hypothesized that the proteins could be encapsulated within the interior of these nanogels or conjugated to the surface of the nanogels by simply altering the order of protein conjugation and crosslinking steps. To test both of these hypotheses, we performed an enzymatic degradation study. The conjugates were exposed to acetonitrile (20% of the total volume) to denature the protein, then added trypsin, a serine protease that hydrolyzes peptide bonds strictly after the basic residues arginine and lysine. These fragments were then analyzed by mass spectrometry (MS). First, caspase-3 itself was subjected to trypsin digest and the MS analysis displayed seven major peaks (Figure 38a). The caspase-3 fragments that each of these peaks represent are shown in Figure 38e.
Figure 38: Mass Spectrometry of nanogel-caspase conjugates. Mass spectra of (a) caspase-3, (b) NG-Empty, (c) NG-Casp-In, and (d) NG-Casp-out. A summary of the peptides and their m/z values are tabulated in (e).

<table>
<thead>
<tr>
<th>m/z (av)</th>
<th>Start</th>
<th>End</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1018.5238</td>
<td>66</td>
<td>73</td>
<td>(R)EEIVELMR(D)</td>
</tr>
<tr>
<td>1118.5436</td>
<td>37</td>
<td>47</td>
<td>(R)SGTDVAANLR(E)</td>
</tr>
<tr>
<td>1617.8265</td>
<td>61</td>
<td>73</td>
<td>(K)NDLTREEIVELMR(D)</td>
</tr>
<tr>
<td>1764.9102</td>
<td>197</td>
<td>210</td>
<td>(K)QYADKLEFMHILTR(V)</td>
</tr>
<tr>
<td>1854.861</td>
<td>30</td>
<td>47</td>
<td>(K)STGMTSRSGTDVAANLR(E)</td>
</tr>
<tr>
<td>1933.8967</td>
<td>215</td>
<td>231</td>
<td>(K)VATFESFSFDATFHAK(K)</td>
</tr>
<tr>
<td>1963.9908</td>
<td>233</td>
<td>248</td>
<td>(K)QIPCIVSMLTXELFY(-)</td>
</tr>
</tbody>
</table>
We also analyzed empty nanogels by the same method; as expected, no peaks were observed in the MS (Figure 38b). In the case of NG-Casp-In, we expected that if the caspase-3 was in fact encapsulated within the crosslinked core of the assembly, trypsin would not be able to reach the caspase-3 and therefore no signal would be observed. Indeed, none of the peaks observed previously for caspase-3 were present, suggesting that the protein is “wrapped” and protected from proteolytic digestion within the nanogels (Figure 38c). On the other hand, in the case of NG-Casp-Out, peaks with \( m/z \) matching those observed for caspase-3 were detected, demonstrating that these caspase molecules are indeed on the outside of the nanogel assemblies and are not protected from proteolysis like those in the NG-Casp-In state (Figure 38d). These results demonstrate the versatility of the reactive self-assembly to covalently bind proteins and “cage” the protein cargo within the nanogel, protecting it from enzymatic degradation.

A unique aspect of this system is the versatility of these polymeric nanogels to silence enzymatic activity, when conjugated. This capability is only useful if protein cargos retain enzymatic activity upon release from the nanogels. The enzymatic activities of caspase-3 conjugated to the nanogels or released after redox stimulus were assessed using a fluorogenic substrate cleavage assay. Based on quantification of caspase release (Figure 37), the activity from the precise quantity of nanogels encapsulating 50 nM caspase-3 was measured under non-reducing conditions (Figure 39). The catalytic cysteine of caspase-3 requires a reducing environment to be active, so a small amount of DTT (0.5 mM) was added for the “non-reducing conditions.” This was enough reductant to assess activity of any unbound caspase, but not enough to promote disassembly of the nanogels. Under these conditions, none of the conjugates exhibited caspase-3 activity,
suggesting that there is no unbound active caspase and the enzyme conjugated to the nanogel is effectively catalytically silenced. This silencing could be due to the structural constraints imposed by the nanogels and/or the lack of accessibility to the peptide substrate. The fact that no caspase-substrate cleavage was observed, even in the NG-Casp-Out nanogels, suggests that even non-encapsulated caspase have been catalytically silenced. Caspase-3 contains a free and highly reactive surface cysteine as part of its catalytic diad (Cys-285). Due to its reactivity, it is likely that this cysteine is involved in the conjugation to the nanogel. Thus, the surface incorporated caspase could be catalytically silent due to covalent conjugation of the active site cysteine-285.

The nanogel-caspase conjugates were then incubated in the presence of 100 mM DTT to release the caspase-3 cargo. Recovery of enzymatic activity under these conditions confirmed that the protein is active upon release from the nanogel (Figure 39a and c). Released caspase-3 activity reached 17% (Figure 39b) upon release from NG-Casp-In and 15% for NG-Casp-Out and 74% for NG-Casp-In\textsuperscript{RRR} and 60% for NG-Casp-Out\textsuperscript{RRR} (Figure 39d). An earlier protocol for the construction of caspase-containing nanogels utilized lyophilization. Caspase-3 is an obligate heterotetramer, which does not refold spontaneously with high yields, so it is not surprising that dramatically lower yields of functional protein (0.24-3%) were released from lyophilized nanogels using the older protocol (Figure 40). Given the 15-74% recovery rates observed currently, it is safe to conclude that the conjugation process itself is mild and that the nanogels protect the caspase, helping it to avoid denaturation and thus retain enzymatic activity. We were surprised to note the higher fraction of recovered caspase-3 activity upon release from the triarginine-containing nanogels, NG-Casp-In\textsuperscript{RRR} and NG-Casp-Out\textsuperscript{RRR} (Figure 39d),
compared to those from NG-Casp-In and NG-Casp-Out (Figure 39b). This result was puzzling, since both these types of nanogels had very similar abilities to induce cellular apoptosis (*vide infra*). A key difference between these nanogels is that the non-functionalized nanogels (NG-Casp-In, NG-Casp-Out) contain unreacted PDS moieties, while these functional groups have been consumed during conjugation of the RRR peptide in the functionalized nanogels (NG-Casp-In<sup>RRR</sup> and NG-Casp-Out<sup>RRR</sup>). We hypothesized that, during the release from the non-functionalized nanogels, the added DTT is initially increasing the percent crosslinking (toward 100%) leaving only a fraction
of DTT to liberate the caspase from what is then a much more extensively crosslinked nanogel. To test this hypothesis, we prepared the non-functionalized nanogel-conjugates as before, with an 18% crosslinking density and then reacted away the remaining PDS groups using thiol-terminated PEG (MW 1k) to generate NG-Casp-In$^\text{PEG}$ and NG-Casp-Out$^\text{PEG}$. Next, the enzymatic activity of capase-3 was assessed after exposing the conjugates to 100 mM DTT. SDS-PAGE of the nanogel-caspase PEG-thiol conjugates validated the caspase conjugation (Figure 41a and b). Once the non-functionalized nanogel-conjugates were protected from additional crosslinking by the addition of PEG groups, it was possible to release and recover a high percentage of caspase activity (79% and 86%, Figure 41c) comparable to those observed for the nanogel-caspase$^{RRR}$ conjugates. The extent of recovered caspase-3 activity is quite remarkable considering the lability of the caspase heterotetramer and its dependence on proper formation of the active-site loop bundle for activity. This high yield indicates that these nanogels incorporate their cargo without damaging it, while at the same time remaining robust and responding specifically to a redox stimulus.

The ultimate goal for the use of these nanogel-protein conjugates is to deliver enzymes in their inactive form and activate them using the innate intracellular environment in mammalian cells. In this case, such caspase delivery is expected to result

Figure 40: Caspase-3 activity recovered after an early protocol for the construction of caspase-containing nanogels. Both the control and the released caspase are estimated to be 50 nM.
in cell killing. To determine whether caspase-conjugated nanogels are capable of internalization in living cells, we monitored the cellular uptake of nanogels upon incubation of the conjugates with HeLa cells. Caspase-3 was labeled with fluorescein isothiocyanate (FITC) to enable intracellular visualization; the cell nucleus was stained with DRAQ5. The fluorescence distribution of FITC and DRAQ5 was observed by confocal fluorescence microscopy (Figure 42). FITC-caspase-3 was observed for nanogels caspase on the surface or inside of nanogels (Figure 42a, b), whereas no fluorescence was visible in cells treated with FITC-labeled caspase-3 (Figure 43), suggesting that the protein is not capable of penetrating the cells by itself and requires nanogel conjugation for efficient internalization. Cellular uptake of nanogel conjugates is slow unless peptides for uptake are attached. This trend mirrors previous reports showing no significant internalization of polymeric nanogels at doses of 0.1 mg/mL after 6 hours in HeLa cells. As expected, incorporation of triarginine containing peptides onto caspase-containing nanogels (NG-Casp-Out<sup>RRR</sup> and NG-Casp-In<sup>RRR</sup>; Scheme 1)
**Figure 42:** Cellular internalization of nanogel-caspase conjugates with FITC labeled caspase-3. (a) NG-FITC-Casp-In (b) NG-FITC-Casp-Out (c) NG-FITC-Casp-In<sup>RRR</sup> (d) NG-FITC-Casp-Out<sup>RRR</sup> at 0.5 mg/mL on HeLa cells. Within each image set, the top left panel is the FITC channel (green; caspase-3), top right is the DRAQ5 channel (red; nucleus), bottom left is the DIC image and bottom right is the overlap of all three. This experiment was performed with triplicate visualization on one day. One representative field is shown for each condition.
displayed higher accumulation of caspase-3 both on the membrane and in the cell within this short time frame (Figure 42c, d). This is likely due to the increased local concentration of the positively charged RRR and the negatively charged cellular membrane.

Caspase-3 plays a critical role during the apoptotic process, so we anticipated strong cell death-inducing potential of these nanogel-caspase conjugates, which release up to 75% of the caspase-3 cargo in an active form. The extent of cell death was measured in HeLa cells treated with increasing doses of the nanogel conjugates (Figure 44). Staurosporine, a protein kinase inhibitor known to induce apoptosis was used as a positive control. After 24 hours, cell viability was measured using an Alamar Blue assay. Apoptosis is characterized by the marked changes in cell morphology such as cell shrinkage and membrane blebbing. HeLa cells treated with nanogel caspase conjugates appeared to be rounded and shrinking similar to those undergoing apoptosis induced by staurosporine, suggesting that killing was via an apoptotic route.

We anticipated that bare nanogels would be relatively non-toxic and that those conjugated to caspase-3 should exhibit significantly higher rates of cell death induction. NG-Empty exhibited low cellular toxicity at concentrations up to 1 mg/mL (Figure 44a),

Figure 43: No observed cellular internalization of FITC-caspase-3 alone after 4 hours. Top left is the FITC channel, top right is the DRAQ5 channel showing red for the nucleus. Bottom left is the DIC image and the bottom right is all three images merged.
whereas nanogel-caspase conjugates displayed a strong dose response for cell death. At a concentration of 1 mg/mL, the cell viability for both NG-Casp-In and NG-Casp-Out was reduced to nearly 20%. To confirm that the cell death observed in the nanogel-caspase conjugates was induced by the intracellular release of active caspase-3 aided by the nanogels and not by the action of caspase-3 in solution, cells were exposed to free caspase-3 utilizing the amount of protein fed during the synthesis of 0.1, 0.5 and 1.0 mg/mL nanogels (50:1 weight ratio, nanogel:caspase-3). Since caspase-3 alone is not expected to effectively penetrate the cell membrane, we anticipated that the protein itself should not induce cell death. Indeed, the cell viability observed for caspase-3 was approximately 80% for a concentration up to 1 mg/mL, indicating that the vast majority of the cell death observed corresponded to apoptosis induced by the intracellular release of active caspase-3 from the polymeric nanogels. Similar results were observed for the case of nanogels decorated with RRR peptide (Figure 44b). At a concentration of 1 mg/mL, the cell viability for NG-Empty$^{RRR}$ was about 80% (Figure 44b); this may be because positively charged RRR peptides directly penetrate the cell membrane, causing rupture or damage, thus introducing slightly higher toxicity. Similar to the nonfunctionalized nanogel caspase conjugates, the RRR caspase nanogels induced cell death in a dose responsive manner. Cell viability for NG-Casp-In$^{RRR}$ and NG-Casp-Out$^{RRR}$ was reduced to about 30-35% at a concentration up to 1 mg/mL. Although nanogels lacking any targeting peptides (Figure 42a, b) show much less cell internalization than the RRR nanogels, they are more capable of inducing cell death. At first this result was perplexing, with cell internalization studies seemingly uncorrelated with increases in cell death. However, upon further investigation we observed that the
RRR-decorated nanogels appear to be accumulating on cell membranes, rather than being fully incorporated in the cytoplasm where caspases can be released and activated. Note that caspases work optimally under the reducing and the neutral pH conditions of the cytosol. The activity of these enzymes are substantially reduced under non-native conditions. For examples, although caspase-3 is optimally active at pH 7.4, its activity significant decreases at lower pH values, dropping to just 10% remaining activity at pH 6.0. Thus, although far fewer NG-Casp exist in cells and NG-CaspRRR are more abundant overall, the NG-Casp have greater cell-killing potential, presumably due to their more favorable intracellular localization. This points to an important finding. Optimal delivery of caspases ultimately require localization in the cytosol. The fact that the nanogels (without the cell penetrating peptides) exhibit excellent apoptotic efficiency suggests that these nanocarriers are already doing well in this capacity. Future generations of caspase-containing nanogels that utilize alternate targeting ligands that are capable of cytosolic delivery, rather than the triarginine peptides, should yield even more

**Figure 44:** Cell viability after 24 hr exposure of HeLa cells with the conjugates. (a) nanogel-caspase conjugates; (b) nanogel-caspaseRRR. The concentration in the caspase-3 samples is the feed amount of caspase-3 used when preparing 0.1, 0.5, and 1 mg/mL solutions. Nanogel/caspase-3 (50:1). Data in (a) were collected from a single experiment performed in triplicate on one day. Data in (b) were collected from two experiments in triplicate on two separate days. Data from day 1 is shown.
productive delivery systems, capable of selective cell killing via apoptosis at even lower doses.

**Conclusion**

We report a novel reactive self-assembly strategy to conjugate active enzymes to polymeric nanogels with responsive characteristics, where caspase-3 has been used as the active protein cargo. In this study, we show that: (i) the proteins can be effectively encapsulated inside a polymeric nanogel by simply mixing the polymer and the protein in this reactive self-assembly strategy. (ii) Conjugation of the protein after the nanogel formation affords a control conjugate, where the protein is attached to the surface of the nanogel. (iii) The activity of the protein is completely turned off in both these approaches, a feature that is critical when delivering cargos that could have deleterious consequences in off-target locations. (iv) The redox sensitive unlocking event causes the protein to be reactivated, allowing recovery of ~80% of the activity. In addition to showing the versatility of these approaches, the fact that these have been demonstrated with a protein that is very prone to irreversible unfolding suggests that this approach is broadly applicable to other proteins. (v) The recovery of activity under reducing conditions can be utilized to recover the enzymatic activity of caspases inside cells, where the reducing environment of the cytosol due to high glutathione concentrations is targeted as the triggering mechanism. This was discerned by the fact that both conjugation approaches provide robust cellular entry, protein release, and apoptotic activity. (vi) The nanogel-protein conjugate was able to gain cellular entry, while the protein by itself did not enter the cells. These results, combined with the fact that the nanogel by itself is not cytotoxic, suggest that these conjugates are promising protein
Carriers. (vii) The protein conjugated to the surface of the nanogel is prone to proteolysis, while the encapsulated protein is not accessible to proteolytic enzymes. These results suggest that while both approaches are versatile for in vitro protein delivery, the encapsulation approach is more promising for future in vivo applications. (viii) The nanogel-protein conjugates, decorated with cell-penetrating peptides, gain cellular entry much more rapidly compared to the unfunctionalized nanogels. However, the overall apoptotic efficiency of the unfunctionalized nanogels is comparable to (or even better than) those functionalized with RRR. These results show that the unfunctionalized nanogels end up in the cytosol more effectively, as these are not hampered by electrostatic association with cellular membranes. The fact that the unfunctionalized nanogels are slow in cellular uptake, but are more effective in releasing the cargo, bodes well for utilizing these vehicles for targeted delivery of a broad range of protein cargos. These efforts are currently underway in our laboratories.

Acknowledgment
Support from the NCI of the National Insitutes of Health (CA169140) is acknowledged. Support from National Science Foundation (CHE-1307118 to ST) and a fellowship for JV and DGT (DGR-0654128) are gratefully acknowledged.

Materials and Methods
Materials
Polyethylene glycol monomethyl ether methacrylate (PEGMA; MW 475), 2,2’-dithiodipyridine, 2,2’-azobis-(2-methylpropionitrile) (AIBN), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (chain transfer agent), D,L-dithiothreitol (DTT),
and other conventional reagents were obtained from commercial sources and were used without further purification, except for AIBN, which was purified by recrystallization. Pyridyl disulfide ethyl methacrylate (PDSMA) was prepared using the previously reported procedure (Macromolecules 2006, 39, 5595-5597). $^1$H NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Chemical shifts are reported in parts per million (ppm). Molecular weight of the polymer was estimated by gel permeation chromatography (GPC) in THF using poly(methyl methacrylate) (PMMA) standards with a refractive index detector. Dynamic light scattering (DLS) measurements were performed using a Malvern NanoZetasizer. UV-visible absorption spectra were recorded on a Varian (Model EL 01125047) spectrophotometer. Size exclusion chromatography was performed on Amersham Biosciences chromatography system equipped with a GE health care life sciences superdex 75 10/300 GL column. Activity assay was performed utilizing Spectramax M5 spectrophotometer. MALDI-MS analyses were performed on a Bruker Autoflex III time-of-flight mass spectrometer. All mass spectra were acquired in the reflectron mode, and an average of 200 laser shots at an optimized power (60%) was used. Cell imaging was performed using Zeiss 510 META confocal microscope.

**Synthesis of p(PEGMA-co-PDSMA) (P1)**

A mixture of PDSMA (536.8 mg, 2.1 mmol), PEGMA (1 g, 2.1 mmol), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (21 mg, 0.0756 mmol), and AIBN (1.2 mg, 0.00756 mmol) were dissolved in 3 mL of THF in a 10 mL Schlenk flask and degassed by performing three freeze-pump-thaw cycles with an argon inflow into the reaction. The reaction vessel was sealed and placed in a preheated oil bath at 70 °C for 12 h. The product polymer P1 was then purified by precipitation in cold ether (20 mL) to yield the
random copolymer. Yield: 90%. GPC (THF) M₆: 20 K. Đ: 1.5. ¹H NMR (400 MHz, CDCl₃): δ 8.45, 7.66, 7.09, 4.20-4.06, 3.80-3.42, 3.01, 2.10-1.65, 1.10-0.80. The molar ratio between the two monomers was determined by the integration of the methoxy proton in the polyethylene glycol unit and the aromatic proton in the pyridine and found to be 48:52% (PEG/PDS).

**Synthesis of Nanogel-Caspase Conjugates**

*NG Empty:* The polymer (3 mg) was dissolved in 1 mL of 1× PBS buffer pH 7.4 and the solution was left stirring at 20 °C for 15 min. A calculated amount of DTT was added to the micellar aggregates and the solution was allowed to cross-link for 1 h at 20 °C. The resulting nanogels were dialyzed at 20°C using a 7000 Da MWCO membrane.

*NG-Casp-In:* The polymer (3 mg) was dissolved in 1 mL of 1× PBS buffer pH 7.4 and the solution was left stirring at 20 °C for 15 min. To this micellar aggregate solution, 0.06 mg of caspase-3 was added, and the mixture was left reacting for 1 h at 20°C. Then a calculated amount of DTT was added to the solution and was stirred for another hour at 20°C to allow for cross-linking. The resulting nanogels were dialyzed at 20°C using a 7000 Da MWCO membrane and unbound caspase-3 was removed by Amicon Ultra Centrifugal Filters MWCO 100000.

*NG-Casp-Out:* The polymer (3 mg) was dissolved in 1 mL of PBS buffer pH 7.4, and the solution was left stirring at 20°C for 15 min. A calculated amount of DTT was added to the micellar aggregates and the solution was allowed to cross-link for 1 h at 20°C. Then, 0.06 mg of caspase-3 was added, and the mixture was left reacting for another hour at 20°C. The resulting nanogels were dialyzed at 20°C using a 7000 Da MWCO membrane and unbound caspase-3 was removed by Amicon Ultra Centrifugal Filter MWCO 100k.
NG Empty\textsuperscript{RRR}: To functionalize the surface of the nanogels, the same procedure described above for nanogels was followed. In addition, CRRR peptide (50% by weight compared to the polymer) was added and then stirred for another hour at 20°C. The resulting nanogels were dialyzed at 20°C using a 7000 Da MWCO membrane. To functionalize the surface of the nanogels, the same procedure described above for NG-Casp-In was followed. In addition, an excess of the ligand (5 mg), CRRR, was added and then stirred for another hour at 20°C. The resulting nanogels were dialyzed at 20°C using a 7000 Da MWCO membrane and unbound caspase-3 was removed by Amicon Ultra Centrifugal Filters MWCO 100000 Da.

NG-Casp-Out\textsuperscript{RRR}: To functionalize the surface of the nanogels, the same procedure described above for NG-Casp-Out was followed. In addition, CRRR peptide (50% by weight compared to the polymer) was added and then stirred for another hour at 20°C. The resulting nanogels were dialyzed at 20°C using a 7000 MWCO membrane and unbound caspase-3 was removed by Amicon Ultra Centrifugal Filters MWCO 100000.

Activity Assay

For measurement of caspase-3 activity, various amounts of nanogel, ranging from 13 to 230 µg, were used in order to release 50 nM caspase-3 in each experiment. These nanogel-caspase conjugates were incubated in 100 mM DTT for 1 h in order to fully release the cargo caspase-3. Identical samples were subject to 0.5 mM DTT treatment in an identical fashion in order to assay for any free, or unbound, caspase-3. The caspase-3 activity was then assayed over a 7 min time course in caspase-3 activity assay buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM CaCl\textsubscript{2}, and 10% PEG 400. In this experiment, caspase-3 hydrolyzes the peptide substrate, N-acetyl-
Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, resulting in the release of the 7-amino-4-methylcoumarin (AMC) moiety as a fluorophore that can be quantified over time. The fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-AMC, Enzo Lifesciences (Ac-DEVD-AMC), Ex 365/ Em495, was added to a final concentration of 100 µM to initiate the reaction. These 100 µL assays were performed in duplicate in a 96-well microplate at 37°C using a spectramax M5 spectrophotometer.

References


CHAPTER V

HARNESSING THE APOPTOTIC POTENTIAL: INTERROGATING CASPASE STRUCTURE TO CONTROL FUNCTION

Investigating Regulation of the Apoptotic Caspases

The meticulous nature by which our human cells are able to manage a multitude of intersecting and continuous biological processes is both fascinating and near impossible to fully imagine. The scores of machinery and tiers of regulation seem to extend beyond comprehension; but within this labyrinth scientists have managed to decipher information bit by bit, slowly building a wealth of knowledge. It is the exploitation of this information that has allowed development of drugs and therapeutics that attempt to counteract any malfunctions within such a complex system.

One of the most pertinent and powerful pathways for therapeutic intervention is that of controlled cell death. This process, governed by a family of cysteine proteases, must be regulated flawlessly to prevent disease or even organismal death. Dysregulation has been implicated as a cause or a contributing factor to a myriad of diseases. This dissertation has focused on understanding the regulation of the apoptotic caspases that are at the heart of this gene-directed suicide pathway. It is imperative to understand caspases from a mechanistic perspective in order to fully comprehend their regulation, activation, and subsequent activity. Only then can we effectively exert any therapeutic control.

The idea of targeting caspase activity for drug development has been pursued by many pharmaceutical companies and academics, but certain challenges have not yet been overcome. This fact only underscores the continued need to uncover further details concerning the function and regulation of these proteases. These efforts have been
twofold: in chapters II and III I have recorded the attempt to enable regulated inhibition of caspases in order to prevent unwanted cell death and in chapter IV I have recorded delivery of active levels of caspases to terminate aberrant or problematic cells. The former has applications in diseases such as neurodegenerative disorders, stroke, liver and heart disease. Meanwhile, the latter directly applies to the lack of apoptosis in both cancer and autoimmune disease.

The work presented here chronicles detailed structural and mechanistic investigations of caspase regulation in order to provide information for future therapeutic applications. Uncovering the dual regulatory mechanism of caspase-7 phosphorylation (Chapter II) provides much needed details surrounding regulation of a powerful executioner caspase. Meanwhile, studies regarding zinc specific inhibition (Chapter III) took a more global approach to caspase metal-mediated regulation. Kinetic and mechanistic strategies revealed biologically relevant levels of zinc have an effect on caspase activity. Furthermore, seeking to exploit our detailed knowledge of caspase activity, we aimed to utilize the cell killing potential of caspases by packaging and delivering these enzymes to cancer cells (Chapter IV). A delivery system was developed wherein a caspase cargo could be encapsulated by a polymeric nanogel and kept enzymatically silent until subjected to a redox trigger. Caspase-3 was successfully incorporated into a nanogel vehicle, delivered to cancer cells, released intracellularly, and activated to produce an apoptotic response.

**Caspase-7 and PAK2 Co-Regulation**

The duality of the caspase-kinase relationship has momentous consequences when considering the effect of each posttranslational modification. The dynamic relationship
between these two enzymes is remarkable, where phosphorylation by the corresponding kinase results in cell life while conversely, cleavage by the caspase culminates in apoptotic programmed cell death.

Deciphering the mechanism of inhibition of caspase-7 by PAK2 phosphorylation provides crucial details about both enzymes. First and foremost, phosphorylation of caspase-7 identifies two sensitive sites within the protein that could be exploited for control over caspase activity. This interrogation is twofold: the first relates to caspase-7 specifically, and the second reveals information that can be applied to the family of apoptotic caspases. The effects of phosphorylation at S30 of caspase-7 is a critical discovery. Chapter II dives into detail about how this modification slows processing by the upstream initiator caspases -8 and -9. It further outlines how phosphorylation at this site blocks interactions with caspase-9, thus inhibiting activation of the executioner caspase-7. The N-terminal region of caspase-7 has been shown to be a significant factor in aiding recognition of specific apoptotic substrates. A basic lysine patch, just eight residues from S30, directly contributes to substrate recognition. Additionally, phosphorylation at S30 is far in sequence from the linker region that is recognized and cleaved by the initiator caspase-9. This provides a compelling argument that S30 acts as an exosite for recognition that promotes cleavage and thus, activation. This information reveals a sensitive site on the executioner caspase-7 that could be targeted as a means to prevent caspase-7 activation, and perhaps prevent or slow apoptosis. For example, if a small molecule targeted the S30 region, it could mask the necessary interactions that promote caspase-9 mediated cleavage, thus keeping caspase-7 uncleaved and catalytically inactive. This could also be applied to the S239 phosphorylation site. However, S239 is
housed on loop 3, a key active site loop that must attain a particular conformation in order to properly bind and hydrolyze the substrate. PAK2 phosphorylation at this residue is a more obvious disruption to caspase activity.

A second detail that stands out from the structural aspect of the phosphorylation study (Chapter II) of caspase-7 is the importance of the caspase-7 conformation. In the full-length form, activated PAK2 recognizes a set of substrates and plays a role in cell motility, mitosis, and survival. However, upon cleavage by caspases, the PAK2 kinase domain is translocated to the nucleus where it phosphorylates substrates that propagate the apoptotic response.  

We have found that the conformational state of caspase-7, particularly the orientation of the active site loops, can dictate phosphorylation of S239 by PAK2. This is an extremely important observation when considering caspase-7 regulation, and can be applied to other apoptotic caspases as well. One could speculate that cancerous cells with hyperactive PAK2 might also overproduce a naturally-occurring regulatory small-molecule that might bind caspase-7 and hold the loops in a conformation favorable for PAK2 recognizing and phosphorylating S239. This potential allosteric interaction could operate in tandem with PAK2 working synergistically to inactivate caspase-7 and prevent an apoptotic response.

PAK2 is not the only kinase to have significant cross-talk with its caspase substrates.  

Interrogating the caspase-kinase battle for determining the fate of the cell has revealed that when caspases triumph, they often leave remnants of a functioning kinase. For example, caspase cleavage of receptor tyrosine kinases abrogates their ability to bind signaling proteins that help produce a prosurvival response. However, much like PAK2 after caspase-mediated cleavage, the cleaved kinase domain of the receptor
tyrosine kinases often exhibit proapoptotic behavior in the cytosol. It would be very interesting to interrogate whether the proapoptotic kinase domain liberated after caspase processing has the same affinity for phosphorylating caspase substrates following cleavage. It has been shown that cleavage of kinases can result in transporting the kinases to a new cellular compartment, but perhaps cleavage also exposes some exosites that completely redefine the kinase substrate profile. Both possibilities open the door for the potential to control kinase location and substrate recognition.

In addition to S30 and S239 phosphorylation of caspase-7, it had been suggested that T173 is also phosphorylated by PAK2. Our in vitro assays never detected phosphorylation at this threonine, nor did the T173E phosphomimic show any functional effect. While a number of possibilities can explain this observation (see Chapter II, Discussion), it raises the question whether or not there is some caspase conformation or condition not yet explored, which might lead to a functional phosphorylation on caspase-7 at T173. For example, the base of the helix where T173 resides contains potential zinc binding residues. In addition, caspase-9 binds a zinc at a very similar location at the base of an analogous helix. Perhaps under certain conditions PAK2 phosphorylates caspase-7 at T173 and creates a zinc binding site. Posttranslational modifications can contribute to metal binding, as has been seen with (1) vitamin-K dependent carboxylase creating γ-carboxyglutamic acid residues for the chelation of calcium, (2) hydroxylation by α-ketoglutarate-dependent dioxygenase acting on substrates to convert aspartates and asparagines to their β-hydroxy counterparts for metal binding, and (3) kinases that convert serine to phosphoserine. There are several cases specific to a phosphoserine/threonine/tyrosine modification generating a metal binding site, including
phosphoproteins storing calcium during tooth and bone development,\textsuperscript{11} two adjacent phosphoserines in the egg yolk protein phosvitin that act as ligands binding iron at close to attomolar affinity,\textsuperscript{12} and a phosphotyrosine in an α-synuclein peptide which enhances its affinity for calcium binding.\textsuperscript{13} In addition, a free phosphate has been observed crystallographically as a zinc ligand at the base of a helix in ZntR.\textsuperscript{14} It would be interesting to explore the possibility of regulation by phosphorylation and zinc crossing paths at the base of this caspase-7 helix.

Overall, the structural and mechanistic analysis of caspase-7 phosphorylation has provided a wealth of information regarding caspase-7 structure-function relationships. It has identified the molecular basis for inhibition at two sensitive sites on the caspase-7 protease that can be targeted for therapeutic control of such an enzyme. The information obtained here can be applied to caspase-7 and its role in apoptosis, as well as its other emerging non-apoptotic roles.\textsuperscript{15} More broadly, it is clear that the conformational state of caspase-7 plays a significant role in its ability to be posttranslationally modified. Nature found a way to maximize the silencing of caspase-7 activity by phosphorylating at two different locations with two very different inhibitory mechanisms, yet generating the same overall biological result at two different points within apoptosis.

**Apoptotic Caspases: Inhibition by Zinc**

The cell biology of zinc and its substantial role in numerous cellular pathways is an evolving field that has gained considerable interest in recent years.\textsuperscript{16} The influence of this metal has proven to be essential for growth, development, metabolism, gene transcription and many more biological processes. As a means of maintaining tight control over zinc concentrations, there exists a pool of labile intracellular zinc as well as
stores of zinc in various proteins and membrane structures. The pool of available zinc is extremely small, but minor fluctuations have incredible significance. This is highlighted when considering zinc as a signaling molecule, with a prominent example being the regulation of transcription factors such as metal-regulatory transcription factor 1 (MTF1). Small fluxes in zinc can activate MTF1 and alter the transcription of a number of genes. In addition, zinc ‘waves’ have been shown to release an excess pool of intracellular zinc via particular zinc transporters, and can have lasting results. One such result is the inactivation of certain tyrosine phosphatases and the resulting increase in kinase activity. When considering the effects of zinc as a regulatory or signaling substance, dysregulation of zinc homeostasis can have dramatic effects, with links to Alzheimer’s, cancer, and diabetes.

Studies centering on zinc inhibition of tyrosine phosphatases have uncovered a number of details with far-reaching implications. Originally, it was thought that these tyrosine phosphatases were inhibited by zinc at high zinc concentrations that were not biologically relevant. However, careful kinetic studies revealed that PTP1B, a centrally situated phosphatase, has an affinity for zinc around 5 nM. This discovery forced further investigations that now implicate PTP1B in cancer. The overexpression of certain zinc transporters in cancer could be releasing a small excess of zinc that is turning off tyrosine phosphatases and amplifying kinase activity, resulting in a twofold effect favoring the cancerous phenotype. This work stresses the need to mechanistically and kinetically interrogate zinc regulation. The PTP1B example underscores the fact that careful biochemical studies must be carried out in order to understand the true effect of zinc. This dissertation has followed a similar approach with caspases. The work in
Chapter III highlights the zinc inhibition of caspases and reveals biologically relevant levels of zinc affect the apoptotic caspases.

Investigating zinc inhibition of caspase-3, -6, -7, and -8 will have implications in drug discovery. The effect of zinc inhibiting apoptosis is well established, and the fact that caspases are affected by zinc at biologically-relevant levels further emphasizes them as targets for apoptotic control. This knowledge can be integrated with drug discovery efforts aiming to activate the apoptotic cascade. Many attempts of turning on apoptotic programmed cell death, in cancer applications for example, have focused upstream of caspases but have had little success. However, both the intrinsic and extrinsic apoptotic pathways converge on the executioner caspases, so it would be advantageous to target their activation in order to evade the complicated upstream regulatory elements. Relief of zinc-mediated inhibition is one mechanism by which others have attempted to activate executioner caspases, in particular procaspase-3. The small molecule PAC-1 chelates zinc and as a result allows the activation of procaspase-3.26 This has proven to be an effective strategy in a number of cancers and has excellent potential with certain combination therapies. Perhaps the most interesting aspect is lack of toxicity thus far due to the fact that the affinity of PAC-1 for zinc does not exceed that of essential zinc-binding proteins.27

In addition to uncovering the biologically relevant levels of zinc inhibition of apoptotic caspases, Chapter III also focused on other important biochemical aspects of zinc binding to apoptotic caspases. Much of the information regarding the mechanism of zinc inhibition of caspase-8 has potential for directing future studies. For example, the discovery that two zinscs bind to caspase-8 with seemingly different affinities suggests
there are two sensitive sites that quite possibly have two different effects when bound to zinc. The fact that zinc destabilizes the dimer population of caspase-8 reveals that zinc binding has a distinct effect on the oligomeric state of caspase-8. In addition to its apoptotic roles, caspase-8 has non-apoptotic roles in embryonic development and differentiation, as well as cell motility. It is perhaps possible that zinc functions as a modulator of caspase-8 activity under these non-apoptotic conditions.

Another very interesting observation from the work presented in this dissertation pertains to the effect of cadmium on caspase activity. Cadmium sits just below zinc as a group 12 metal with a d^{10} valence electronic configuration. Given such similar chemical properties it was assumed that cadmium and zinc would bind and inactivate caspases in an identical fashion, however, that is not what was observed (Figure 20). Cadmium seems to have a nuanced inhibition for caspase-3, -7, and -8 for both peptide and protein substrates that differs from the global inhibition seen with zinc. For caspase-3, cadmium blocks both peptide and substrate cleavage, while cadmium has no inhibitory effect on peptide substrates for caspase-7. Interestingly, cadmium completely inhibits caspase-8 from cleaving peptide substrates but does not have nearly the same effect on protein substrates. Information derived from this data could shed light on where zinc could be binding, assuming zinc and cadmium share a binding site. For example, one might hypothesize that because cadmium blocks peptide substrate cleavage by caspase-8, it could be active site based inhibition. Meanwhile, caspase-8 binding a protein substrate could shift the conformation of the enzyme to favor dimerization, which could compete with the metal and result in marginal activity against a protein substrate.
In addition, caspase-3 binds three zins per monomer (Figure 22 and Velázquez-Delgado et al.\textsuperscript{30}). This begs the question why nature would select for what appears to be such a redundant function. Why would an enzyme evolve three metal binding sites when just binding one at the active site would be sufficient for silencing activity? One could speculate that (1) each zinc binding site plays a non-redundant functional role in different aspects of the caspase activity, whether it be in apoptosis or other non-apoptotic pathways, or even blocking a specific exosite (2) each zinc binding site has varying affinity for zinc and act as a slow activation mechanism where loss of all three is required to achieve full caspase-3 activity (3) three zins are needed to act together in order to hold the procaspase-3 enzyme completely in the off-state and shift the equilibrium so that it heavily favors a conformation that is unable to recognize substrate. In any of these hypotheses or any other imagined scenario, it would be extremely beneficial to obtain a crystal structure of caspase-3 bound to zinc(s). Crystallographic avenues for both caspase-3 and caspase-7 are currently under pursuit by another talented graduate student, Derek MacPherson, in the Hardy lab.

**Exploiting Caspase Cell Death Potential via Nanogel Delivery**

The successful delivery of an active caspase-3 that was then able to terminate cancer cells represents an important advance for protein delivery utilizing this nanogel strategy. This delivery data (Chapter IV) solidified an approach wherein the cell-killing potential of the executioner caspase-3 was exploited in an application to execute cancer cells. From the encapsulation perspective, this study has established a platform with extensive potential for use in a variety of therapeutic applications. The apoptotic caspases are a class of enzymes that are difficult to manipulate and control. They have very
flexible active site loops, rigid requirements for maintaining catalytic activity such as pH sensitivity, and susceptibility to misfold under the wrong conditions. Despite these challenges, the nanogel delivery vehicle was able to easily encapsulate the caspase cargo, silence its enzymatic activity while conjugated, and recover up to 86% caspase activity after a specific redox trigger. In addition, these nanogel assemblies were able to gain cellular entry and dose-dependently kill a population of cancer cells.

The successes of this nanogel-caspase conjugate in Chapter IV pave a path forward to utilize this platform in what seems like endless possibilities. First and foremost, it provides a means to deliver a cell-killing enzyme, something that could be incredibly useful in the treatment of diseases like cancer that could benefit from a controlled programmed cell death. In addition, these nanogels can be easily functionalized in order to target specific populations of cells. One example (currently under investigation by Kishore Raghupathi in the Thayumanavan group) is targeting a cancer overexpressing a certain receptor, such as the folate receptor, for a more focused delivery. In addition, exploring other apoptotic inducing proteins and small molecules in combination could be a powerful avenue to optimize the cell-killing potential of these nanogel conjugates (currently being explored by the skillful Francesca Anson). The beauty of this encapsulation method is that it is not limited to caspase delivery and inducing cell death. One could imagine this approach being utilized for the intracellular delivery of a variety of therapeutic proteins to treat a number of diseases. This could further be expanded to other biomacromolecules, such as nucleic acids, which creates a tempting platform for other applications such as delivering CRISPR components.
The success found with caspases in this system was in part due to the clever thiol chemistry engineered by the Thayumanavan lab. The five surface exposed cysteines in each monomer of caspase-3 were targeted by the polymer during the reactive self-assembly, facilitating its encapsulation. Using this system with non-cysteine containing proteins may require a variation on the encapsulation method. A current collaboration with Kishore Raghupathi has adopted an encapsulation method that utilizes an inversion emulsion polymerization approach to incorporate hydrophilic biomolecules within a nanogel. This has a unique advantage because it forces the cargo inside the hydrophilic center of the polymer aggregate during assembly, thus entrapping cargo with a high efficiency. Ongoing work has established this inverse emulsion method and successfully entrapped a variety of proteins including caspase-3, PAK2, lysozyme, and GFP. These protein cargoes maintain their activity and interestingly, the GFP remains fluorescent even after encapsulation within the nanogel.

Future applications of this delivery system will surely require nuances to the nanogel polymerization method in order to take full advantage of the diverse set of proteins under investigation. After these adjustments are made to the system as a whole, it is important to confirm that intracellular release of the protein cargo is maintained under the stringent control of the intracellular redox trigger. It would be of great benefit to develop a tool in order to predetermine (1) if the cargo stays inside the nanogel, (2) if the nanogel can release its contents unperturbed, and (3) if the cargo can reach the cytosol. A tool that will satisfy these requirements is an adapted version of the split GFP. The cells in question could be transfected with the short 11\textsuperscript{th} strand of GFP, and the newly formed nanogels in question could entrap GFP 1-10. When the transfected cells are
exposed to the nanogel conjugate, there will be GFP fluorescence if and only if the nanogel was taken up by the cells, the GFP 1-10 was properly released, and the GFP 1-10 reaches the cytosol. A tool of this nature would be extremely beneficial when interrogating different nanogels with variable synthetic routes.

**The Killing Blow**

Ultimately it is the apoptotic caspases that deliver the killing blow in the controlled termination of cells amongst all eukaryotes. The need to fully understand caspase activation, regulation, structure, and function has become essential in order to therapeutically harness the true potency of programmed cell death. By interrogating phosphorylation, zinc binding, and caspases themselves as biologics, this dissertation hopes to add to the biochemical knowledge regarding caspase activity and positively influence further studies targeting this powerful class of enzymes.

**References**


BIBLIOGRAPHY


Sprick, M.R. (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. EMBO J. 21, 4520–4530.


