Investigating Structures and Functions of Apoptotic Caspases

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INVESTIGATING STRUCTURES AND FUNCTIONS
OF APOPTOTIC CASPASES

A Dissertation Presented
by
ISHANKUMAR VIJAYKUMAR SONI

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

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Chemistry Department
INVESTIGATING STRUCTURES AND FUNCTIONS
OF APOPTOTIC CASPASES

A Dissertation Presented

by

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ABSTRACT

INVESTIGATING STRUCTURES AND FUNCTIONS
OF APOPTOTIC CASPASES

MAY 2022

ISHANKUMAR VIJAYKUMAR SONI,
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Caspases are cysteine aspartate proteases involved in various cellular pathways including apoptosis, inflammation, and neurodegeneration. Caspase-9 is classified as an initiator apoptotic caspase that is activated upon intrinsic stress. Caspase-9 is composed of two domains: an N-terminal CARD domain and a catalytic core domain. We have employed hydrogen deuterium exchange mass spectrometry (H/DX-MS) to determine the 1) dynamics of the full-length caspase-9, 2) dynamic impacts on caspase-9 upon substrate-induced dimerization, and 3) regions involved in the CARD: catalytic core domains interactions.

Upon intrinsic stress, caspase-9 activates executioners, procaspase-3 and -7 but not procaspase-6. We have employed site-directed mutagenesis technique and substrate-digestion assays to identify the factors (e.g., sequence and the local context of the cleavage sites) that facilitate caspase-9 to cleave the intersubunit linker (ISL) of procaspase-3 but not the ISL of procaspase-6.

One prime question related to caspase-9 is – Is caspase-9 only an initiator of apoptosis, or it has any others proteolytic roles? We employed reverse N-terminomics on caspase-3 and -9, and identified 124 and 906 putative substrates, respectively. We also determined which substrates are proteolyzed upon staurosporine induced apoptosis using immunoblotting.

Caspase-6, classified as an executioner of apoptosis, is involved in neurodegenerative pathways. Using a chemoproteomics study, an acyl phosphate ATP probe was shown to bind to
procaspase-6 but not active caspase-6. By using site-directed mutagenesis and IC$_{50}$ determination by SDS-PAGE assays, we found that 1) ATP attenuates auto-activation of procaspase-6 with an IC$_{50}$ = 19 mM, and 2) dimer interface of procaspase-6 is a putative ATP-binding site.
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CHAPTER I:
INTRODUCTION

Apoptosis: Death for Survival

Apoptosis – programmed cell death – is a vital cellular process that occurs in multicellular organisms, and it is indispensable in development, morphogenesis, regeneration, and homeostasis (for review see1–3). During early development of multicellular organisms, large quantity of cells die via apoptosis4,5 to delete unwanted tissues (e.g., removal of a tadpole tail during metamorphosis time into a frog) and to sculp organs (e.g., individualization of digits by removing interdigital webs) (Figure 1.1.A).

![Figure 1.1. Apoptosis: death for survival.](image)

(A) Apoptosis is vital in early development. Figure adapted from.1 (B) Morphology of viable and apoptotic cells. Figure adapted from.2

The signature mark of cells undergoing apoptosis is their morphology. Apoptotic cells display cell blebbing and cell shrinkage (Figure 1.1.B). Moreover, nuclear condensation6 and DNA fragmentation7 are also observed in apoptotic cells. In general, apoptotic cells are recognized by macrophages before their intracellular contents are leaked. Unlike necrosis, cells dying via apoptotic mechanism do not release immunostimulatory molecules (denoted as danger associated molecular patterns) that trigger immune response. Thus, one advantage of apoptosis is that it is a “clean death” without inflammation.

Billions of cells in an average healthy human body die via apoptosis every day. The role of apoptosis is not limited to development and regeneration, but it is also crucial in maintaining homeostasis – a state in which the ratio between the newly generated cells and dying cells is balanced (Figure 1.2). Due to apoptosis, cells that are damaged or potentially harmful to other
cells, die in a controlled manner. Thus, proper execution of apoptosis is essential in culling unwanted cells. Imbalance in apoptosis can disrupt homeostasis which is a hallmark of several diseases. For example, too much cell death will cause disorders such as neurodegeneration, immunodeficiency, and infertility. On contrary, less cell death will cause cancers and autoimmunity.

![Figure 1.2. Imbalance in apoptosis is a hallmark of several diseases.](image)

A fine balance in the ratio of newly generated cells and dead cells is required to maintain homeostasis. Imbalance in apoptosis causes development of several diseases as listed here. Figure adapted from.\(^8\)

**Caspases Regulate Multiple Cellular Pathways Including Apoptosis**

Caspases, cysteine-aspartate proteases, tightly regulate multiple cellular pathways including apoptosis and pyroptosis. Dysfunctional caspases are associated with multiple diseases such as cancers,\(^9,10\) autoimmune disorders,\(^11,12\) and neurodegeneration.\(^13,14\) Thus, in the development of therapeutics to treat these diseases, understanding the structures and functions of caspases at molecular level is crucial. There are 12 known human caspases. Based on their cellular roles, most members of caspase family can be categorized into two major groups: apoptotic caspases, caspase-3, -6, -7, -8, -9, and -10, and inflammatory caspases, caspase-1, -4, -5, and -12. Caspase-2 is activated via PIDDosome, and it has a crucial role in cellular differentiation during organogenesis and regeneration.\(^15\) Caspase-14 is involved in the terminal differentiation of epidermal keratinocytes.\(^16\) Apoptotic caspases are classified as initiators (caspase-8, -9, and -10) and
executioners (caspase-3, -6, and -7). Caspases are expressed as full-length, inactive zymogens termed as ‘procaspases’, and are activated through series of cleavage event due to “intrinsic” or “extrinsic” cellular stress such as hypoxia, radiation, growth factor withdrawal, and DNA damage (Figure 1.3).

**Extrinsic and Intrinsic Apoptotic Pathways Are Governed by Caspases**

Cells can undergo apoptosis via two different pathways: 1) the extrinsic apoptotic pathway initiated by death receptors, and 2) intrinsic apoptotic pathway initiated by mitochondrial stress (Figure 1.3).

![Figure 1.3. Extrinsic and intrinsic apoptotic pathways are governed by caspases.](image)

Initiator caspases, caspase-8 (due to extrinsic stress) and -9 (due to intrinsic stress) are recruited to their activation platforms death-inducing signaling complex (DISC) and apoptosome, respectively. The executioners, caspase-3 and -7 are then activated which subsequently activates caspase-6. Eventually, caspase protein substrates are cleaved, evoking apoptosis.

In an extrinsic apoptotic pathway, proapoptotic signals cause extracellular receptors (e.g., Fas and tumor necrosis factor-a (TNF-a)) to bind to their specific ligands (e.g., FasL and TNF-
related apoptosis-inducing ligand (TRAIL))\(^\text{17}\) (Figure 1.3). This ligand binding forces accumulation of the death receptors recruiting Fas associated death domain (FADD) and multiple monomeric procaspase-8 intracellularly to form a complex named death-inducing signaling complex (DISC).\(^\text{18}\) The DISC complex induces dimerization of monomeric procaspase-8 ultimately causing the activation of caspase-8. Caspase-8 then activates the executioners, caspase-3 and -7. Activated caspase-3 and -7 then activate executioner caspase-6. These activated executioners then cleave substrates (e.g., lamin A/C and Poly (ADP-ribose) polymerase – PARP) evoking apoptosis.

In the intrinsic apoptotic pathway, mitochondrial stress results in release of cytochrome c from the mitochondria (Figure 1.3). Cytochrome c interacts with apaf-1 (apoptotic protease activating factor-1), and in the presence of dATP apoptosome complex is formed, in which the CARD domain (caspase activation and recruitment domain) of apaf-1 then recruits procaspase-9 monomers\(^\text{19,20}\) via CARD:CARD interactions. Procaspase-9 recruitment of the apoptosome enhances its activity. Activated caspase-9 then proteolytically activates its substrates, procaspase-3 and -7 to caspase-3 and -7. Subsequently, caspase-3 and -7 activates caspase-6.\(^\text{21-23}\) Eventually, these executioners cleave their protein substrates to induce apoptosis. Thus, caspases are central in regulating apoptosis in either extrinsic or intrinsic pathway, and their investigation at molecular level is essential.

**Chemistry of Caspases**

Caspases are cysteine proteases which cleave their substrates at sites containing aspartate at P1 position (please see Caspases Substrate Cleavage Preference below for more details). Although all caspase family members have distinct substrate-binding grooves which play a major role in substrate selectivity, each of them possesses a Cys-His dyad in their active site pocket which promotes substrate hydrolysis (Figure 1.4). The presence of His polarizes the adjacent Cys, making it a good nucleophile. Nucleophilic attack leads
to hydrolysis of the C-terminal backbone of adjacent to an Asp in a target protein substrate. Depending on the location of the cleavage, the caspase substrate is either activated or inactivated.

**Molecular Architecture of Caspases**

Since dysfunction or dysregulation of caspases are involved in many diseases including cancers and neurodegenerative diseases, determining their structures as well as studying them at a molecular level is vital in developing therapeutical treatments. In caspase family, the initiators (e.g., caspase-8 and -9) are expressed as monomeric zymogens and require an activation platform to

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**Figure 1.5. Conformational changes are observed upon activation of procaspase-6 to mature caspase-6.**

Structures of (A) procaspase-6 (PDB: 3NR2) and (B) active caspase-6 (PDB: 3OD5) are shown where large subunit (Lg) is colored magenta; small subunit (Sm) is colored cyan; inter-subunit linker (L) is colored wheat; and active site Cys-163 is colored grey. The prodomain in procaspase-6 is outlined in black. The proteolytic cleavage event in procaspase-6 occurs after the Asp-23, Asp-179, and Asp-193 residues. Loop-1, -2, -3, -4, -1’, -2’, -3’, and -4’ are abbreviated as L1, L2, L3, L4, L1’, L2’, L3’, and L4’, respectively. Due to crystallization limitations, the pro-domain, inter-subunit linker, and part of loop-2/2’ as well as loop-4/4’ are missing in the structure of procaspase-6. Dotted curves are used to represent missing sections of the loops.
dimerize and ultimately achieve maturation. In contrast, the executionary caspases, caspase-3, -6, and -7 are translated as dimeric zymogen and undergo series of cleavage events to achieve full maturation.

Each member of caspase family is composed of an N-terminal prodomain and a catalytic core domain that contains the active site Cys-His dyad. The prodomain of initiators (e.g., CARD domain of caspase-9 and two DED domains of caspase-8) is longer and well-structured. The prodomain of executioners are short in length and at least for caspase-6\textsuperscript{24} and -7\textsuperscript{25} are intrinsically disordered. Excluding mobile loops, the catalytic core domains of all caspases have highly similar structural folds. Therefore, structures of procaspase-6 and caspase-6 are shown here as an example of all caspases (Figure 1.5).

Each monomer of dimeric procaspase-6 possesses a prodomain (residues 1–23), a large subunit (residues 24–179), an intersubunit linker (ISL) (residues 180–193), and a small subunit (residues 194–296) (Figure 1.5.A).\textsuperscript{24,26} The substrate binding groove is surrounded by loops which are denoted as, L1, L2 (consisting ISL), L3, and L4 for one monomer, and L1’, L2’, L3’, and L4’ for another. Due to the presence of prodomain and ISL, the active sites containing catalytic cysteines, Cys-163 in procaspase-6, are buried and therefore unable to cleave the substrates in trans.\textsuperscript{24,26,27} Removal of both the prodomain and ISL (Figure 1.5.B) is a pre-requisite for procaspase-6’s full maturation. However, an ISL cleavage at D193 is a prerequisite for procaspase-6 activation, and this cleavage is sufficient to activate procaspase-6.\textsuperscript{26,28} Upon ISL cleavage, L2’ which is parallel to the L2 at the dimer interface of procaspase-6 (Figure 1.5.A) undergoes dramatic conformational rearrangement (by folding against L2 and L4) to bind and cleave substrates (Figure 1.5.B).\textsuperscript{24,26,27}
Structure of Caspase-9

Caspase-9 has two domains, an N-terminal CARD domain (residues: 1-138) possessing a CARD domain linker (CDL) and a catalytic core domain possessing a large subunit (residues: 139-306), small subunit (residues: 331-416), and intersubunit linker (ISL) (residues: 307-330) (Figure 1.6.A). As for caspases generally, the active site cysteine, C287, resides in the large subunit of catalytic core. There are three ISL cleavage sites present in caspase-9: E306, D315, and D330. Self-cleavage of caspase-9 occurs at D315 (the primary site where most cleavage is observed) and E306

Figure 1.6. Structure of caspase-9.
(A) Linear schematic diagram of procaspase-9, which is composed of a CARD domain and a catalytic core domain. CARD domain has structural region followed by a linker termed as CARD domain linker, CDL. The catalytic core domain comprises of a large subunit (Lg), a small subunit (Sm), and an intersubunit linker, ISL. Active cysteine, C287 resides in the large subunit of the catalytic core domain. Caspase-9 has three ISL cleavage sites: E306, D315, and D330. (B) Structure of procaspase-9 CARD obtained from the complex of apaf-1 CARD: procaspase-9 CARD (PDB: 3YGS). CARD domain has of six helices H1-H6. The CDL is missing in all caspase-9 structures. (C) A dimeric catalytic core domain of caspase-9 with an active site inhibitor, z-EVD-dcbmk, present in the substrate-binding groove (PDB: 1JXQ). Loops surrounded to z-EVD-fmk are denoted as L1, L2, L3, and L4 from one monomer, and L2' from another monomer. (D) of apoptosome consisting of heptameric hub of apaf-1 core (in light orange), cytochrome c (cyt c; in blue), apaf-1 CARD (in dark orange), and procaspase-9 CARD domain (in green); however, the catalytic core domain of caspase-9 is missing from the structure (PDB: 5WVE).
(secondary site where less cleavage is observed). Through a feedback mechanism, caspase-3 cleaves caspase-9 at D330.

Caspase-9 plays crucial role in an intrinsic apoptosis. Defects in caspase-9 functionality is involved in multiple diseases including neurodegeneration (see Caspase-9: More Than an Apoptotic Initiator section below for more details). Thus, determining the structural as well as dynamics information of different states of caspase-9 is vital in 1) understanding intrinsic apoptotic pathway at molecular level and 2) designing therapeutics for the treatment of the diseases in which caspase-9 dysfunctionality is involved. It is worth mentioning that the field lacks structural dynamic information on the full-length uncleaved monomeric procaspase-9. A structure procaspase-9 CARD domain in a complex with apaf-1 CARD has been reported (PDB: 3YGS). From this structure, procaspase-9 CARD is composed of six structured helices, H1, H2, H3, H4, H5, and H6 (Figure 1.6.B). There is no structural information of the CDL. The structure of dimeric cleaved (activated) caspase-9 was obtained, in which dimerization was promoted by an active site inhibitor, z-EVD-dcbmk, benzoxy carbonyl-Glu-Val-Asp-dichlorobenzylmethylketone (Figure 1.6.C).29 The catalytic core domain is composed of a large subunit (Lg), a small subunit (Sm), and an intersubunit linker (ISL) which becomes L2/L2’ upon cleavage. The substrate-binding groove of caspase-9 is surrounded by the loops, L1, L2, L3, L4, and L2’. Notably, the field lacks structural/dynamic information on the ISL of caspase-9. Moreover, there is no information on how substrate-binding and dimerization impact the dynamics of caspase-9. Earlier lower resolution cryoelectron microscopy (cryo-EM) data of the apoptosome:procaspase-9 complex provided the structure of the heptameric apoptosome in which lobes of density at the center and at the edge of the complex were thought to represent apaf-1 CARD: procaspase-9 CARD domains and a catalytic core domain, respectively.30,31 However, a subsequent higher resolution cryo-EM structure revealed that the lobes of density present at the edge are two apaf-1 and one procaspase-9 CARD domains (Figure 1.6.D).32 Thus, to date, there is no structural information on holoenzyme consisting of the catalytic core domain of caspase-9.
Previous studies have demonstrated that caspase-9 can be activated without associating with apoptosisome through different pathways.\textsuperscript{33-35} Therefore, studying caspase-9 functions in the presence of its CARD domain is necessary. Removal of the CARD domain has been shown to impact on the caspase-9 activity.\textsuperscript{36} Moreover, the CARD domain appears to interact with the catalytic core domain upon z-VAD-fmk (carbobenzoxy-Val-Ala-Asp-fluoromethylketone) – a substrate mimic – binding at the active site.\textsuperscript{36} Although the study\textsuperscript{36} provides evidences that the CARD domain interacts with the core domain upon substrate binding, the regions of the CARD and the core domain involved in this interaction remain unknown.

Hydrogen Deuterium Exchange Mass Spectrometry (H/DX-MS) is a powerful tool which can provide the missing structural as well as dynamics information of a protein of interest. Such information is important in 1) suggesting the behavior of different regions of a protein (e.g., which regions of the protein are more or less flexible), 2) identifying the regions which can be targeted (e.g., allosteric sites), and 3) designing therapeutics to target particular site of the protein. Previously, our laboratory has used this technique to successfully assess some dynamic information about the procaspase-6 prodomain and the ISL.\textsuperscript{24} Moreover, H/DX-MS also identified the regions of caspase-6 and -7 that undergo confirmational changes upon substrate binding.\textsuperscript{37} Thus, in the case of caspase-9, employing H/DX-MS can provide the information on 1) the dynamics of the full-length monomeric caspase-9 possessing the CARD domain and the ISL, 2) the regions that undergo huge confirmational changes upon substrate-induced dimerization, and 3) the regions involved in CARD: core domain interaction.

**Caspases Substrate Cleavage Preference**

To determine the cellular roles of any protease, identifying its substrate cleavage preference is required. One very popular way of showcasing the interactions between the protease active site and the cleavage site motif is by using Schechter-Berger nomenclature (Figure 1.7).\textsuperscript{38} In this schematic representation, the peptide residues of substrate are denoted as P (e.g., P1), the sub-sites
of protease active site are denoted as S (e.g., S1), and he proteolytic cleavage (↓) occurs between P1 and P1’.

![Peptide residues of substrate](image)

**Figure 1.7. Schechter-Berger schematic diagram representing protease cleavage specificity.** P4, P3, P2, P1, P1’, P2’, P3’, and P4’ are the peptide residues of substrates. S4, S3, S2, S1, S1’, S2’, S3’, and S4’ are the sub-sites of protease active site.

Caspases cleave their substrates at sites containing aspartate at the P1 position, although glutamate and phosphoserine can also be recognized, albeit at low frequencies. From a screening using peptide-based substrate library, the cleavage preference (P4-P1) was determined (Table 1.1). These data demonstrate that inflammatory caspases, caspase-1, -4, and -5 prefer WEHD, (W/L)EHD, and (W/L)EHD as P4-P1 position, respectively. Initiator caspases, caspase-8 and -9, prefer LETD and LEHD as P4-P1 position, respectively. It is important to note that these most frequent recognition sites, while useful in communicating differences in substrate specificity can also be misleading if taken out of context as these "consensus" sequences (sequence logos) typically represent just 3-5% of the observed cleavage sites (discussed in detail in the next paragraph). The S4 sub-site of inflammatory caspases and apoptotic prefers hydrophobic residue as P4 position. Executioners, caspase-3 and -7 prefer DEVD as P4-P1 positions, while caspase-6 has a preference towards VEH(V)D. Thus, the S4 sub-site of apoptotic executioners, caspase-3/-7 prefers Asp residue as P4 position, while caspase-6 active site prefers a small hydrophobic residue, Val. All caspases have high preference to Glu residue as P3 position.

**Table 1.1: The P4-P1 selectivity of caspases based on peptide substrate screening.**

<table>
<thead>
<tr>
<th>Caspase</th>
<th>P4-P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>WEHD</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>DEHD</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>DEVD</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>(W/L)EHD</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>(W/L)EHD</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>VEH(V)D</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>DEVD</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>LETD</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>LEHD</td>
</tr>
</tbody>
</table>

Data were gathered from 41.
Thus, major differences among the substrate-binding grooves of caspases are observed in S4 and S2 sub-sites. These data are beneficial in determining the active site specificity of all caspases towards their peptide substrates and synthesize peptide-based active site-specific inhibitors. However, these data are not sufficient in identifying which protein will be a substrate of caspase(s).

**Figure 1.8. Cleavage preference of caspase-7 and -6 derived from N-terminomics.** Sequence alignment of substrates cleavage site represented by IceLogo for (A) caspase-7\(^{43}\) and (B) caspase-6.\(^{42}\) The size of residue is related to how frequent it appeared in proteome.

Using proteomics studies (e.g., N-terminomics), the list of caspase putative substrates can be determined. Sequence alignment of the substrates’ cleavage site motifs can give an estimate of caspase cleavage preference. For example, N-terminomics of caspase-7 and -6 revealed their P4-P1’ preference as DEVD↓G (Figure 1.8.A) and VEVD↓G (Figure 1.8.B), respectively.\(^{42,43}\) This data agreed with the peptide-based screening data (Table 1.1). From the same proteomics study’s data, of 128 peptides cleaved by caspase-7, the cleavage sites of DEVD↓G, DEVD↓X, DEXD↓X and DXXD↓X were only 2, 4, 9, and 36, respectively.\(^{43}\) Of 1402 peptides cleaved by caspase-6, the cleavage sites of VEVD↓G, VEVD↓X, VEXX↓X and VXXD↓X were only 6, 16, 56, and 244, respectively.\(^{42}\) These data strongly suggest that the sequence of substrate cleavage site (e.g., P4-P1’) is not the only governing factor of proteolysis, and other factors such as the local context surrounding may have a role in a successful proteolytic cleavage. Caspase-9 activates procaspase-3 and -7 but not procaspase-6 (Figure 1.3). Thus, a molecular level investigation is needed to determine whether the sequence or the local contexts (or both) of procapse-6 cleavage site plays role in the ISL cleavage protection from caspase-9.
**Caspase-9: More Than an Apoptotic Initiator**

Caspase-9 is classified as an initiator of apoptosis triggered via intrinsic stress. Caspase-9 has been implicated in nonapoptotic pathway called paraptosis – an apaf-1 independent and caspase-9 dependent cell-death mechanism. Interestingly, in paraptosis caspase-3 and -7 are not activated, caspase-9 also acts as an executioner. Moreover, in immortalized bone-marrow-derived macrophages (iBMDMs) caspase-1 activated by *Salmonella* infection, directly activates caspase-9 which acts as an effector caspase and induces cell-death without any need of caspase-3 and -7. Thus, caspase-9 is not only an initiator of apoptosis and it has diverse cellular roles. An earlier study of embryonic stem cells and mouse embryonic fibroblast demonstrated that a lack of caspases-9 functionality leads to defective brain development and embryonic lethality. Recent mouse studies have shown that endothelial caspase-9 activity regulates severity of retinal detachment and subsequent photoreceptor injury. Moreover, pharmacological inhibition of caspase-9 by Pen1-XBir3 reduces severity of retinal detachment in mouse retinal vein occlusion.

Since caspase-9 dysfunction is implicated in a number of diseases, understanding its proteolytic role – identifying substrates of caspase-9 – is crucial.

**N-terminomics of Caspases**

Since caspses play major role in apoptosis and pyroptosis, many members of this protease family have common substrates (e.g., PARP is cleaved by caspase-3 and -7). Although belonging to the same family, each caspase may have unique substrates (e.g., lamin A/C for caspase-6) and unique cleavage site indicating its unique role. Thus, in any case, determining the proteolytic roles of any individual caspase heavily depends on the identification of its substrates. Due to structural similarities amongst the caspase family members, targeting a particular caspase with high specificity is challenging. Knowing the substrate/s of caspases can open a new door of target selection. For example, gasdermin-D that was found to be a substrate of caspase-1, is a potential therapeutic target for many autoimmune diseases.
N-terminomics, a protease-directed type of proteomics study that identifies new N-termini, can be employed to detect 1) the substrates of an individual protease and 2) substrates’ cleavage sites which have been subject to protease hydrolysis. One advantage of this technique is that substrates in the cell lysate treated with recombinant caspase are in their native state (meaning the substrates are folded and the interactions are maintained). N-terminomics is also a rapid approach which can provide a list of hundreds of putative caspase substrates in a single run. From this list of the substrates, unique and common substrates of a caspase can be derived. Moreover, based on the literature as well as experimental data generated by cell culture and immunoblotting, a substrate’s role in particular pathway can be determined (e.g., is the substrate apoptotic or nonapoptotic?). Since N-terminomics also provides sequence information for cleavage sites, the significance of a particular substrate cleavage can be further studied. Therefore, performing N-terminomics on caspase-9 is crucial since only few caspase-9 substrates (less than 10) were known prior to the onset of the work described in this dissertation.50–55

**Caspase-6 Is Involved in Various Apoptotic and Non-apoptotic Pathways**

Caspase-6’s involvement in multiple biological pathways such as apoptosis, inflammation and neurodegeneration designate it as unique amongst the caspase family. Functioning as an executioner caspase, caspase-6 is the only known caspase to cleave the nuclear lamellar protein – lamin A/C.56 Caspase-6 can cleave executioner caspase (e.g., procaspase-3)57 and initiator caspase (e.g., procaspase-8)58 illustrating that its role is not limited to an executioner of apoptosis. In a canonical apoptotic route, procaspase-6 is often activated by caspase-3 rather than by the initiator caspases.21–23 Moreover, procaspase-6 can undergo auto-activation by intramolecular cleavage into the active state composed of large and small subunits.13 This self-proteolytic feature of caspase-6 distinguishes it from other executioner caspases. Activation by caspase-1 (an inflammatory caspase)59 and subsequent cleavage of its neuronal substrates (e.g., tau, huntingtin, and amyloid precursor protein)14 causing axonal degeneration suggest that caspase-6 is involved in inflammatory as well as neurodegenerative pathways. Thus, caspase-6 is an important drug target
protein in the treatment of neurodegenerative disorders including Alzheimer’s Disease, Huntington’s Dieses, and Parkinson’s Disease.

The Dimer Interface of Procaspase-6 and Caspase-6 Is a Known Allosteric Site

The executioner caspases (caspase-3, -6, and -7) have a cavity at their dimer interface to allosterically bind to their substrates. FICA and DICA (Figure 1.9.A) are the first synthesized small-molecule inhibitors that inhibit mature executioner caspases with a disulfide bond formed with a conserved cysteine residue available in the dimer interface (Cys-264 for mature caspase-3, which is homologous to Cys-277 for mature caspase-6 and Cys-290 for mature caspase-7, shown in Figure 1.9.B). Moreover, a recent study has demonstrated that caspase-6 is inhibited upon palmitoylation at Cys-277 (present in the dimer interface) and Cys-264 (present in the L4 loop).
residues by the palmitoyl acyltransferase – HIP14. Thus, the cysteine residues at dimer interface are highly sensitive target sites for chemically synthesized as well as biological molecules.

Murray et. al. likewise observed that the dimer interface of procaspase-6 is a binding pocket for various chemically synthesized small-molecules. Figures 1.9.C and 1.9.D include the compounds that were crystallized with procaspase-6 and surface structure of procaspase-6 with highlighted binding pocket at the dimer interface, respectively. The crystal structures of procaspase-6 binding to small cyclic molecules shown in Figure 1.9.C revealed Tyr-198 (present in the L2/2’ at the dimer interface) as a major binding site. However, the biological relevance of the Tyr-198 and other residues in the dimer interface of procaspase-6 is obscure and needs to be investigated to understand their allosteric functions.

**Investigating Structures and Functions of Apoptotic Caspases**

In this dissertation, our focus is to address some of the key questions in the field of apoptotic caspases. As described, apoptotic caspases play important role in many cellular processes, in and out of apoptosis. Moreover, dysfunctionality in apoptotic caspases can cause several diseases such as cancers and neurodegenerative diseases. To design the therapeutics which can treat these diseases, a deep knowledge on the structures as well as the functions of apoptotic caspases is required. Studies conducted in this dissertation focus on eliminating the gaps in our understanding of apoptotic caspases particularly caspase-3, -6 and -9. By employing H/DX-MS, we are attempting to understand the dynamics of different states of caspase-9 (see Chapter II). We found that distinct regions of caspase-9 including the ISL and the CDL undergo huge confirmational changes in different cleavage states including zymogen, active cleaved at the ISL, and ISL-cleaved bound to a substrate. Our findings suggested CARD may bind to the core domain to different extents for these different states of caspase-9. In Chapter III, we engineered various constructs of procaspase-3 and -6 to study the cleavage preference of caspase-9. We found that both the sequence as well as the local context of the substrate cleavage site motifs play important role in protecting direct activation of procaspase-6 by caspase-9. Using N-terminomics and immunoblotting, we are trying
to deorphanize several putative protein substrates of caspase-3 and -9 (see Chapter IV). We deorphanized 124 and 906 substrates of caspase-9 and -3, respectively. Our immunoblotting results suggested both apoptotic as well as nonapoptotic roles of caspase-9. Employing ATP acyl probe and procaspase-6 self-proteolysis assays, we are investigating the location and the function of procaspase-6 ATP-binding (see Chapter V). The determined IC$_{50}$ of procaspase-6 auto-activation by ATP was 19 mM. We also found that the Y198 present in the dimer interface of procaspase-6 plays an important role in ATP probe binding. The overall theme that ties this dissertation together is providing a molecular understanding of the similarities and differences in several key apoptotic caspases.
References


CHAPTER II:
HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY REVEALS
INTERACTIONS BETWEEN CASPASE-9 CARD AND CORE

This chapter is a manuscript being prepared as: Soni, I. V., Eyles, S. J., and Hardy, J. A. Hydrogen/Deuterium Exchange Mass Spectrometry Reveals Interactions Between Caspase-9 CARD and Core. manuscript in preparation

Authors’ Contributions: JAH obtained funding and directed the project. IVS and JAH strategized and designed all experiments. IVS performed protein expression, purification, LEHDase activity assays, and substrate-digestion assays. IVS also prepared samples for H/DX-MS. SJE helped IVS in all aspects of running and troubleshooting H/DX-MS. IVS analyzed H/DX data. IVS and JAH prepared the figures and wrote the manuscript.

Note: Our collaborators at Roivant Sciences are currently in the process of performing molecular dynamics with weighted ensemble simulations using the H/DX-MS data presented here as constraints to calculate a structure of the holo caspase-9 enzyme. These structures will be added to the manuscript prior to publication.

Abstract

Caspase-9, an initiator caspase, plays major role in the intrinsic apoptotic pathway. Defects in caspase-9 are involved several diseases such as acute and chronic neurodegeneration, retinal neuropathy, and dilated cardiomyopathies. Thus, determining the structure and the dynamics of caspase-9 is important. In the past, various studies have been performed to determine the structure of caspase-9. Although these studies have provided some meaningful structural information on the constituent domains of caspase-9 in isolation, the field lacks the structure and dynamics of full-length (FL) caspase-9 consisting of both the CARD (caspase activation and recruitment domain) and catalytic core domain. The structure of caspase-9 catalytic core domain illustrated that caspase-9 dimerizes in the presence of an active site inhibitor, z-EVD-dcbmk (a substrate mimic); however,
dynamics information is missing. Our previous work suggests that the CARD of caspase-9 interacts with the catalytic core domain upon z-VAD-fmk (a substrate mimic) binding. Nevertheless, the regions involved in this interaction remained undefined. In this work, we employed hydrogen/deuterium exchange – mass spectrometry (H/DX-MS) on seven different activation states of caspase-9, representing the various cleavage forms of caspase-9. H/DX-MS provided insight into the dynamics of the FL caspase-9 including the CARD linker (CDL) and ISL. H/DX also revealed caspase-9 regions (e.g., 130’s helix: strand region) which undergo huge conformation changes upon z-VAD-fmk binding. These data suggest that the CDL and the loops surrounded to the substrate binding groove (including ISL) undergo conformational changes upon ISL cleavage, which are enhanced upon z-VAD-fmk binding, suggesting these linkers play key role in the CARD: core domain interactions.

**Introduction**

Apoptosis – programmed cell death – is a vital cellular process that occurs in multicellular organisms, and it is indispensable in early development, morphogenesis, regeneration, and homeostasis (for review, see1–3). In healthy organisms, cells that are damaged or potentially harmful to other cells, die by apoptosis in a controlled manner without triggering the immune response. Defects in apoptosis disrupt homeostasis, which is a hallmark of several diseases such as cancers4,5 and neurodegeneration.6,7 The faithful implementation of apoptosis is ultimately dependent on the activation of cysteine-aspartate proteases termed caspases. These proteases hydrolyze particular protein substrates with a high preference for cleavage after an aspartate residue at the P1 position; however, glutamate and phosphoserine are also recognized by caspases, albeit, at lower frequencies.8,9 Apoptotic caspases are classified as the initiators (caspase-8, -9, and -10) or executioners (caspase-3, -6, and -7). Since highly active caspases are lethal to cells, these proteases are translated as inactive zymogens denoted as procaspases. Cells can undergo apoptosis via two different pathways: 1) the extrinsic apoptotic pathway initiated by death receptors and 2) intrinsic apoptotic pathway initiated by mitochondrial stress. Caspase-9, categorized as an initiator, plays
critical role in intrinsic apoptosis. In a canonical intrinsic apoptotic pathway, mitochondrial stress results in release of cytochrome c from the mitochondria. Cytochrome c interacts with apaf-1 (apoptotic protease activating factor-1), and in the presence of dATP, the apoptosome complex is formed, in which the CARD (caspase activation and recruitment domain) of apaf-1 recruits procaspase-9 monomers via apaf-1 CARD: caspase-9 CARD interactions. Procaspase-9 recruitment of the apoptosome dimerizes and enhances its activity. Caspase-9 then activates the executioners, procaspase-3 and -7 but not procaspase-6.\textsuperscript{10–12} Subsequently, caspase-3 activates procaspase-6.\textsuperscript{10} Eventually, the activated executioners cleave their particular protein substrates, evoking apoptosis. Thus, for proper execution of intrinsic apoptosis, caspase-9 must function correctly. Our recent work deorphanized more than 100 substrates of caspase-9 demonstrating caspase-9 functions not only as an apoptotic initiator but also as an “executioner” directly cleaving a number of key protein substrates.\textsuperscript{13} Lack of caspase-9 functionality contributes to multiple diseases including acute and chronic neurodegeneration,\textsuperscript{14–16} retinal neuropathy,\textsuperscript{17} and dilated cardiomyopathies\textsuperscript{18} (for a review, see\textsuperscript{19}). To design the therapeutics for the treatment these diseases as well as to understand the intrinsic apoptotic pathway at molecular level, an in-depth study of the structure of caspase-9, which will enhance understanding of how caspase-9 recognizes, binds, and hydrolyzes substrates and how caspase-9 is regulated molecularly, is crucial.

Caspase-9 is composed of two domains, an N-terminal CARD (residues: 1-138) including a CARD linker (CDL; residues: 96-138) and a catalytic core domain possessing a large subunit (residues: 139-306), small subunit (residues: 331-416), and an intersubunit linker (ISL) (residues: 307-330) (\textbf{Figure 2.1.A}). As for caspases generally, the active site cysteine, C287, resides in the large subunit of the catalytic core. There are three ISL cleavage sites present in caspase-9: E306, D315, and D330. Self-cleavage of caspase-9 occurs at D315 (the primary site where most cleavage is observed) and E306 (secondary site where less cleavage is observed).\textsuperscript{20,21} Through a feedback mechanism, caspase-3 cleaves caspase-9 at D330.\textsuperscript{10}
It is worth mentioning that the field lacks structural dynamic information on the full-length (FL) monomeric procaspase-9. A structure of the caspase-9 CARD in a complex with the apaf-1 CARD has been reported (PDB: 3YGS).\textsuperscript{22} From this structure, it is clear that the caspase-9 CARD is composed of six structured helices, H1, H2, H3, H4, H5, and H6 (Figure 2.1.B). There is no structural information from any source describing the CDL. The structure of dimeric cleaved (activated) caspase-9 was obtained (PDB: 1JXQ), in which dimerization was promoted by an active site inhibitor, z-EVD-dcbmk, benzoxy carbonyl-Glu-Val-Asp-dichlorobenzylmethylketone (Figure 2.1.C).\textsuperscript{23} The catalytic core domain is composed of a large subunit, a small subunit, and an ISL which becomes L2/L2' (loops involved in formation of the substrate binding groove) upon cleavage. The residues composing the catalytic dyad, C287 and H237, reside in the large subunit. The substrate-binding groove of caspase-9 is composed entirely of the mobile loops, L1, L2, L3, L4, and L2'. The field lacks structural/dynamic information on the ISL (which becomes L2 and L2' upon caspase activation a the ISL) of caspase-9. Moreover, there is no information on how substrate-binding and dimerization impact the overall conformational dynamics of caspase-9 at either the atomic/residue/molecular level or the level of domain-domain interactions. In the only existing crystal structure of caspase-9, which is a structure of dimeric caspase-9 (PDB: 1JXQ), the covalent active-site inhibitor, z-EVD-dcbmk, binds to only one active site, while another active site remains unbound and appears to exhibit an “inactive site”\textsuperscript{23}. Previous cryoelectron microscopy studies of the apoptosome:procaspase-9 complex provided the structure of the heptameric apoptosome in which lobes of density at the center and at the edge of the complex were modelled as apaf-1 CARD: procaspase-9 CARD domains and a catalytic core domain, respectively.\textsuperscript{24,25} However, a subsequent higher resolution cryoelectron microscopy structure revealed that the lobes of density present at the edge are two apaf-1 and one procaspase-9 CARD domains (PDB: 5WVE).\textsuperscript{26} Thus, to date, there is no structural information on the holoenzyme consisting of the catalytic core domain of caspase-9.
Figure 2.1. Caspase-9 constructs employed for H/DX profiling.
(A) Linear schematic diagram of procaspase-9, which is composed of a CARD and a catalytic core domain. CARD comprises a structured helical region followed by a linker termed the CARD linker, CDL. The catalytic core domain comprises of a large subunit (Lg), a small subunit (Sm), and an intersubunit linker (ISL). The active-site catalytic cysteine, C287 resides in the large subunit of the catalytic core domain. Caspase-9 has three ISL cleavage sites: E306, D315, and D330. (B) Structure of procaspase-9 CARD obtained from the complex of apaf-1 CARD: procaspase-9 CARD (PDB: 3YGS). The CARD is composed of six helices (H1-H6). Density for the CDL is missing in all extant caspase-9 structures. (C) The dimeric catalytic core domain of caspase-9 with an active site inhibitor, z-EVD-dcbmk, present in the substrate-binding groove (PDB: 1JXQ). Loops surrounding z-EVD-dcbmk are denoted as L1, L2, L3, and L4 from one monomer, and L2’ from the other monomer. (D) Caspase-9 constructs on which H/DX-MS analysis was performed. Active-site cysteine C287 was substituted by alanine to obtain the uncleaved constructs of caspase-9. FL indicates full-length. ΔCARD indicates the constructs lacking the CARD domain. WT (wild-type) indicates caspase-9 cleaved at D315. Similarly, ΔCARD caspase-9 is cleaved at D315. An active site inhibitor, z-VAD-fmk was employed as a substrate mimic. Caspase-9 dimerizes in the presence of z-VAD-fmk. (E) Caspase-9 constructs analyzed by SDS-PAGE illustrating size. (F) Catalytic properties of WT caspase-9 and ΔCARD caspase-9 to hydrolyze a peptide substrate, Ac-LEHD-afc. (G) Comparing procaspase-7 activation (cleavage) by WT caspase-9 and ΔCARD caspase-9.
Some studies on the apoptosome complex,\textsuperscript{26,27} have reported that the removal of the CARD increases caspase-9 activity. In contrast, other studies demonstrate that the CARD enhances caspase-9 activity.\textsuperscript{28,29} Irrespective of the different outcomes from the different studies, the CARD impacts the proteolytic activity of caspase-9. Various studies from different groups have demonstrated that caspase-9 can be activated without associating with apoptosome through different pathways.\textsuperscript{30–34} Therefore, studying caspase-9 functionality alone without the other components of apoptosome is equally important. From the previous work by our group,\textsuperscript{35} the CARD appears to interact with the catalytic core domain upon z-VAD-fmk (carbobenzoxy-Val-Ala-Asp-fluoromethylketone) – a substrate mimic – binding to the substrate-binding groove; however, the regions involved in this interaction remain unknown.

Hydrogen/Deuterium Exchange-Mass Spectrometry (H/DX-MS) is a powerful tool which can provide the structural as well as dynamics information of proteins. In the past, our group has used this technique to successfully assess some dynamic information about the different maturation states of caspase-6.\textsuperscript{36} We also provided some structural insights on the different regions of caspase-6 that are missing from the crystal structures such as the prodomain and the ISL.\textsuperscript{36} Moreover, H/DX-MS also identified the regions of caspase-6 that undergo conformational changes upon substrate-binding.\textsuperscript{36,37} In this work, we performed H/DX-MS on seven different constructs and oligomeric states of caspase-9 (\textbf{Figure 2.1.D}) to obtain the information on 1) the dynamics of the full-length monomeric caspase-9 possessing the CARD and the ISL, 2) the regions that undergo conformational changes upon substrate-induced dimerization, and 3) the regions involved in the CARD: catalytic core domain interactions. We found that the CDL and the ISL of caspase-9 were among the most dynamic regions for all caspase-9 constructs/states. From our results, we observed that the dimer interface and the 130’s region are more protected upon substrate binding suggesting conformational changes and/or dynamics changes in these regions. Finally, we also found that the CDL and the ISL regions are highly protected upon substrate binding, suggesting their putative role in the CARD: core interactions.
Results

H/DX profiles of caspase-9 constructs/states.

Figure 2.2. Peptide coverage of caspase-9 constructs, FL caspase-9 C287A, caspase-9 CARD, and ΔCARD caspase-9 C287A, identified from H/DX-MS.

The coverage of peptic peptides from (A) FL caspase-9 C287A (B) caspase-9 CARD, and (C) ΔCARD caspase-9 C287A. All caspase-9 constructs including (B) caspase-9 CARD have a 6xHis-tag at C-terminus. Therefore, the peptide containing residues 131-144 of caspase-9 CARD was discarded when comparing the deuterium uptake to the other constructs. For convenience, while comparing to other caspase-9 constructs, the numberings of ΔCARD caspase-9 C287A in (C) are adjusted to (A). Each experiment was performed twice, on each of two separate days.
In this study, we performed H/DX on seven different constructs and oligomeric states of caspase-9 (Figure 2.1.D). The first construct, FL caspase-9 C287A is a constitutive uncleaved zymogen. The exact mass of this construct is 47.1 kDa (Figure 2.1.E). The second construct, caspase-9 CARD is only the CARD possessing structured region (six helices; residues 1-95) followed by the CDL. The exact mass of this construct is 16.6 kDa (Figure 2.1.E). The third construct, ΔCARD caspase-9 C287A is an uncleaved monomeric caspase-9 expressed without the CARD. The exact mass of this construct is 31.5 kDa (Figure 2.1.E). The fourth construct, wild-type (WT) caspase-9, is a FL caspase-9 with an ISL cleavage at D315. We prevented the self-proteolytic cleavage of caspase-9 at E306 by inducing this construct for only 3 hours. The exact mass of this construct are 34.8 kDa (for CARD with large subunit; CARD+Lg) and 12.4 kDa (for small subunit, Sm) (Figure 2.1.E). The fifth sample, WT caspase-9 with z-VAD-fmk (substrate mimic), has an ISL cleavage at D315 and is dimerized due to z-VAD-fmk binding. The sixth construct, ΔCARD caspase-9, has an ISL cleavage at D315 and is expressed without the CARD. The exact mass of this construct is 19.1 kDa (for Lg) and ~ 12.4 kDa (for Sm) (Figure 2.1.E). The seventh sample, ΔCARD caspase-9 with z-VAD-fmk, has an ISL cleavage at D315, is dimerized due to z-VAD-fmk binding, and is missing the CARD. Catalytic parameters of caspase-9 constructs are shown (Figure 2.1.F). From the LEHD-ase activity of WT caspase-9 and ΔCARD caspase-9, we found that the WT is greater than 10-fold more active (Figure 2.1.F). These data are consistent with previous results from our group.35 Using a time-point digestion assay, we also compared the activities of WT caspase-9 and ΔCARD caspase-9 to cleave the protein substrate, FL caspase-7 C186A (a mimic of procaspase-7) (Figure 2.1.G). We observed less cleaved caspase-7 as a product while incubating with the ΔCARD caspase-9. Thus, WT caspase-9 cleaves procaspase-7 with a higher efficiency than ΔCARD caspase-9 does. These data complemented our previous results illustrating that WT caspase-9 cleaves procaspase-3 with a higher efficiency that ΔCARD caspase-9 does,35 suggesting a key role for the CARD on both peptide and protein substrates.
H/D exchange analysis of these seven states of caspase-9 were performed in deuterium with incubation times of 0 min, 0.17 min, 1 min, 10 min, and 60 min. Here, 0 min time point has no incubation in deuterium. Each exchange reaction was stopped by quenching at pH = 2.5. We ran the samples through a pepsin column, generating peptic peptides that were detected via LC/MS.

Figure 2.3. Peptide coverage of WT caspase-9 and WT caspase-9 with z-VAD-fmk, identified from H/DX-MS.

The coverage of peptic peptides from (A) WT caspase-9 and (B) WT caspase-9 with z-VAD-fmk (a peptide-based inhibitor which induces dimerization). Each experiment was performed twice, once on each of two separate days.
From the peptide coverage maps (Figure 2.2-2.4), we obtained the coverage of > 92% of the protein sequences with > 2 observed peptide redundancy across all caspase-9 constructs and states.

Figure 2.4. Peptide coverage of ΔCARD caspase-9 and ΔCARD caspase-9 with z-VAD-fmk, identified from H/DX-MS. The coverage of peptic peptides from (A) caspase-9 ΔCARD and (B) caspase-9 ΔCARD with z-VAD-fmk. For convenience, while comparing to other caspase-9 constructs, the numberings of (A) and (B) are adjusted to Figure 2.3.A. Each experiment was performed twice, once on each of two separate days.
We generated heatmaps of each caspase-9 construct/state illustrating their dynamics (Figure 2.5). To make these heatmaps, we used the fractional deuterium uptake (%) of the most

**Figure 2.5. H/D exchange heatmap illustrating the dynamics of caspase-9 constructs/states.**

The fractional deuterium uptake (%) of (A) FL caspase-9 C287A, (B) caspase-9 CARD, (C) ΔCARD caspase-9 C287A, (D) WT caspase-9, (E) WT caspase-9 with z-VAD-fmk, (F) ΔCARD caspase-9, and (G) ΔCARD caspase-9 with z-VAD-fmk for the H/D exchange time points: 0.17, 1, 10, and 60 min. The fractional deuterium uptake for each peptide (%) was calculated by normalizing relative deuterium uptake with the maximum (theoretical) deuterium uptake of the peptide. Here, maximum (theoretical) deuterium uptake = (total residue number of a peptide) minus 1, (to reflect the lack of a proton on the N-terminus) minus number of proline residues present in the peptide. The secondary structure of caspase-9 (highlighting H1-H6, CDL, ISL, L1, L3, L4, 130’s region (based on caspase-6 numbering), active site C287 and H237, and the dimer interface) is shown to the left of the peptides for a reference. Deuterium uptake is color coded (in accordance with the legend on the right side) representing the dynamics of the caspase-9 constructs/states.
representative observed peptides (Figure 2.6-2.10).

Figure 2.6. Fractional deuterium uptake plots for caspase-9 constructs/states over the course of the H/D exchange experiment. Fractional deuterium uptake for the peptides derived from residues from 1 to 163 of caspase-9 constructs/states are represented as different colors. The fractional deuterium uptake (%) was calculated by normalizing the relative deuterium uptake of peptide by the theoretical maximum deuterium uptake as described in the Materials and Methods section. The error bars are the SD from the duplicate H/DX measurements performed on two separate days.
Figure 2.7. Fractional deuterium uptake plots of caspase-9 constructs/states over the course of the H/D exchange experiment (continued).

Fractional deuterium uptake for the peptides derived from residues from 153 to 240 of the caspase-9 constructs/states are represented as different colors. The fractional deuterium uptake (%) was calculated by normalizing the relative deuterium uptake for peptide by the theoretical maximum deuterium uptake as described in the Materials and Methods section. The error bars are the SD from the duplicate H/DX measurements performed on two separate days.
Figure 2.8. Fractional deuterium uptake plots of caspase-9 constructs/states over the course of the H/D exchange experiment (continued).

Fractional deuterium uptake for the peptides derived from residues from 234 to 322 of the caspase-9 constructs/states are represented as different colors. The fractional deuterium uptake (\%) was calculated by normalizing the relative deuterium uptake for peptide by the theoretical maximum deuterium uptake as described in the Materials and Methods section. The error bars are the SD from the duplicate H/DX measurements performed on two separate days.
Figure 2.9. Fractional deuterium uptake plots of caspase-9 constructs/sates over the course of the H/D exchange experiment (continued).

Fractional deuterium uptake of the peptides belonging to the residues from 300 to 404 of caspase-9 constructs are represented as different colors. The fractional deuterium uptake (%) was calculated by normalizing the relative deuterium uptake of peptide with the theoretical maximum deuterium uptake as described in the Materials and Methods section. The error bars are the SD from the duplicate H/DX measurements performed on two separate days.
The selection was based on using the peptic peptides that were common amongst all caspase-9 constructs, so that the dynamics of one caspase-9 construct/state can be compared with the others. The fractional deuterium uptake (%) was calculated by normalizing the relative deuterium uptake of a peptide with the maximum theoretical deuterium uptake for a peptide of the observed length (see Materials and Methods section for details). Looking at the exchange dynamics of procaspase-9 (Figure 2.5.A), we observed that the loops connecting the helices in the CARD, the CDL, the ISL, residues after the ISL (cleavage site at D330), and the dimer interface were the most exchangeable. These observations were expected because the CDL and the ISL might be predicted to be highly dynamic given that the CDL has not been observed in any crystal structure and given that the ISL appears in many different conformations in structures of various caspases, and procaspase-9 is predominantly in a monomeric state. With the ISL intact and no substrate bound, we observed similar dynamics in both the CARD and the core domain regardless of whether the CARD was attached (Figure 2.5.A) or detached from the core domain (Figure 2.5.B and C), suggesting that there are few or no interactions mediated by CARD attachment to the core domain, as occurs in zymogen. Irrespective of the cleavage in the ISL at D315, the presence or absence of

Figure 2.10. Fractional deuterium uptake plots of caspase-9 constructs/states over the course of the H/D exchange experiment (continued).

Fractional deuterium uptake of the peptides belonging to the residues from 398 to 422 of caspase-9 constructs/states are represented as different colors. The fractional deuterium uptake (%) was calculated by normalizing the relative deuterium uptake of peptide with the theoretical maximum deuterium uptake as described in the Materials and Methods section. The error bars are the SD from the duplicate H/DX measurements performed on two separate days.

The selection was based on using the peptic peptides that were common amongst all caspase-9 constructs, so that the dynamics of one caspase-9 construct/state can be compared with the others. The fractional deuterium uptake (%) was calculated by normalizing the relative deuterium uptake of a peptide with the maximum theoretical deuterium uptake for a peptide of the observed length (see Materials and Methods section for details). Looking at the exchange dynamics of procaspase-9 (Figure 2.5.A), we observed that the loops connecting the helices in the CARD, the CDL, the ISL, residues after the ISL (cleavage site at D330), and the dimer interface were the most exchangeable. These observations were expected because the CDL and the ISL might be predicted to be highly dynamic given that the CDL has not been observed in any crystal structure and given that the ISL appears in many different conformations in structures of various caspases, and procaspase-9 is predominantly in a monomeric state. With the ISL intact and no substrate bound, we observed similar dynamics in both the CARD and the core domain regardless of whether the CARD was attached (Figure 2.5.A) or detached from the core domain (Figure 2.5.B and C), suggesting that there are few or no interactions mediated by CARD attachment to the core domain, as occurs in zymogen. Irrespective of the cleavage in the ISL at D315, the presence or absence of
the CARD domain, or the substrate (z-VAD-fmk) binding, the exchange observed in the CDL and the ISL suggests that these regions are highly dynamic compared to the other regions of all caspase-9 constructs (Figure 2.5.A-G). The dimer interface of WT caspase-9 and ΔCARD caspase-9 appear to be highly exposed (Figure 2.5.D and 2.5.F). Upon z-VAD-fmk binding, the dimer interface of both WT caspase-9 and ΔCARD caspase-9 show exchange consistent with a much greater degree of protection (Figure 2.5.D-F), which is consistent with the expected substrate-induced dimerization, which has been observed previously.23,35 To elucidate these observations and determine the regions that are impacted upon the z-VAD-fmk binding, further analysis comparing the difference in the fractional deuterium uptake (%) was required.

**Substrate binding enhances overall protection of WT and ΔCARD caspase-9.**

To visualize difference that occur upon substrate binding and the associated dimerization, we calculated heatmaps (Figure 2.11) by subtracting the fractional deuterium uptake (%) of WT caspase-9 or ΔCARD caspase-9 from the z-VAD-fmk bound versions of the same constructs (Figure 2.6-2.10). To simplify our analysis, we first focused on the impact in the CARD of WT caspase-9 as a consequence of z-VAD-fmk binding (Figure 2.11.A). Using the difference in fractional (%) values after 10 min of labeling time, we illustrated the changes in exchange observed in the CARD upon z-VAD-fmk binding, and mapped them onto both cartoon and surface structures (Figure 2.11.B). We employed the structure of the caspase-9 CARD (PDB: 3YGS) and appended and calculated a potential structure for the CDL (residues 96-142; although residues of the CARD are 1-138, we included residues 139-142 to correspond to regions covered by the H/D exchange data for one of the observed peptides: 131-142) (see Materials and Methods section). The H/D exchange data were mapped onto the CARD and modeled CDL. Overall, we observed some protection (between 6% and 12.5 %) in some regions (the loop between the H2 and H3 helices, the
loop between the H4 and H5 helices, and the CDL) of the CARD upon z-VAD-fmk binding,
suggesting that substrate binding may enhance stability of the CARD.

To examine the dynamics change upon z-VAD-fmk binding to the catalytic core domain of WT caspase-9 and ΔCARD caspase-9, we generated heatmaps exemplifying the difference in the fractional deuterium uptake (%) (Figure 2.11.C and D). Using the difference in the fractional uptake (%) values at 10 min of labeling time, we illustrated the dynamic changes upon z-VAD-fmk binding as cartoon and surface structures for WT caspase-9 (Figure 2.11.E) and ΔCARD caspase-9 (Figure 2.11.F). The dimer interface of both WT and ΔCARD caspase-9 was highly protected (>15%) upon z-VAD-fmk binding. This was expected, as earlier studies demonstrated that both WT and ΔCARD caspase-9 dimerizes upon z-VAD-fmk binding.23,35 Intriguingly, we observed the 130’s region (numbering based on caspase-6) underwent significant changes in protection (>15%), suggesting conformational changes upon z-VAD-fmk binding to both WT and ΔCARD caspase-9.

It is noteworthy that in an earlier study by our group, we observed protection in the 130’s region for caspase-6 but not for caspase-7 upon substrate binding.37 Overall, our data show that substrate binding increases the protection of the catalytic core domain of both WT and ΔCARD caspase-9, suggesting significant conformational changes and/or dynamic changes upon substrate binding.

Our previous findings suggest that the CARD only significantly interacts with the catalytic core domain upon z-VAD-fmk binding to active cleaved caspase-9.35 Therefore, we sought to identify regions involved in the CARD: catalytic core domain interactions. By looking at the heatmaps and structures of the CARD (Figure 2.11.A and B) and catalytic core domain (Figure 2.11.C and E) of WT caspase-9, comparatively more protection was observed for the catalytic core domain than for the CARD. This may the result of to two factors: 1) z-VAD-fmk binding to the substrate binding groove and 2) dimerization, as we also observed high protection in many regions of ΔCARD caspase-9 upon z-VAD-fmk binding (Figure 2.11.D and F). Since both the dimer interface as well as the 130’s regions are protected with a similar difference in the fractional deuterium uptake (>15%) (Figure 2.11.C-F), these two regions may not be directly involved in
the CARD: catalytic core domain binding. By comparing the catalytic core domains of WT and ΔCARD caspase-9 upon z-VAD-fmk binding (comparing Figure 2.11.C with D and comparing Figure 2.11.E with F), the loops surrounding the substrate-binding groove, (L1, L3, L4 and the ISL which converts into L2 and L2’ upon cleavage at D315) were more protected for WT caspase-9 than for ΔCARD caspase-9. This suggests that these loops might be participating in CARD:

![Image](image-url)

**Figure 2.12.** Distinct regions of the CARD undergo substantial conformational changes from zymogen → active cleaved → substrate bound forms of caspase-9.
Heatmap of the comparative difference in the fractional deuterium uptake (%) between the caspase-9 CARD only and the CARD of (A) FL caspase-9 C287A (B) WT caspase-9 and (C) WT caspase-9 with z-VAD-fmk. The secondary structure of the CARD is placed left to the peptide numbers for a reference. (D), (E), and (F) are the cartoon and surface representation of the difference in the fractional deuterium uptake (%) at 10 min of deuterium labeling of (A), (B), and (C), respectively. The CDL of these structures was modeled (see the Materials and Methods section) by inserting residues 96-142 to the structural region of the CARD (residues 1-95) (PDB: 3YGS). Heatmaps and the structures are color coded with an accordance with the legend shown illustrating exposure and protection.

catalytic core domain interactions upon z-VAD-fmk binding. By comparing the differences in the
fractional deuterium uptake (%) of different states of caspase-9 (e.g., zymogen, active cleaved, and substrate bound), we further investigated to examine previous finding by our group that CARD only binds to the catalytic core domain upon z-VAD-fmk binding as well as to determine the regions involved in CARD: catalytic core domain interactions.

**Distinct regions of the CARD are protected during the transition from zymogen → active cleaved → substrate bound.**

We produced heatmaps (Figure 2.12.A-C) by subtracting the fractional deuterium uptake (%) of caspase-9 CARD from the CARD in FL caspase-9 C287A, WT caspase-9, and WT caspase-9 bound to z-VAD-fmk (Figure 2.6). Using the difference in fractional uptake (%) values from the 10 min of labeling time (Figure 2.12.A-C), we highlighted the changes in exposure on both the cartoon and surface structures (Figure 2.12.D-F). We observed no significant protection when comparing the caspase-9 CARD to the CARD of zymogen (Figure 2.12.A and D). In fact, some exposure (between -6% to -15%) were observed in some regions (H1 helix, the loop between H2 and H3 helices, the loop between H3 and H4 helices, and the loop between H4 and H5 helices) for the labeling time point of 1 min (and 0.17 min for H1 helix) (Figure 2.12.A). Thus, our data suggested that the CARD of the zymogen is subtly more dynamic changes and/or unfolded compared to the caspase-9 CARD in isolation. Since no significant protection was observed, our data also suggest that there is little to no interaction between the CARD and the catalytic core domain in procaspase-9. We performed similar analysis on the H/D exchange data to comparing the caspase-9 CARD with the CARD of WT caspase-9 (Figure 2.12.B and 2.12.E). As observed for the CARD of procaspase-9 (Figure 2.12.A), some regions (the loop between H2 and H3 helices and the loop between H4 and H5 helices) showed some modest exposure (between -6% to -15%) for the CARD of WT caspase-9 for the labeling time of 0.17 min and 1 min (Figure 2.12.B). We also observed some protection (between 6% to 15%) for many regions when the CARD is attached to the caspase-9 core: H1 helix for the labeling time of 10 and 60 min, the loop between H2 and
H3 helices for the labeling time of 60 min, the loop between H3 and H4 helices for the labeling time of 10 and 60 min, H4 helix for the labeling time of 1, 10 and 60 min, the loop between H5 and H6 helices for the labeling time of 60 min. Since we did not detect the peptide 19-25 for the WT caspase-9 (*Figure 2.6*), we could not derive the difference in the dynamics for that region. Interestingly, for all labeling time points, we observed protection (between 6% to 10%) for the peptides, 88-103 and 104-121, within the CDL. Even more protection (between 10% to 12.5%) in the CDL region for the peptide 112-130, was observed. These data prompted us to reason that the CARD of the active cleaved caspase-9 (WT caspase-9) may interact with the catalytic core domain. Similar analysis comparing caspase-9 CARD alone with the CARD of the WT caspase-9 bound to z-VAD-fmk was likewise performed (*Figure 2.12.C and F*). We did not observe any significant amount of exposure at any timepoint, in contrast to what we had observed for WT caspase-9 and procaspase-9. Intriguingly, we observed more protection throughout the entire CARD for WT caspase-9 bound to z-VAD-fmk than unbound WT caspase-9 (comparing *Figure 2.12.C* with *2.12.B* and comparing *2.12.F* with *2.12.E*). Most regions of the structured region of the CARD (residues 1-95) were protected between 6% to 12.5% for many labeling time points. Enhanced protection (more than 12.5%) for the CDL region (residues 96-130) for all labeling time points was observed. Thus, these data suggested us that the CARD of active cleaved caspase-9 may engage in more interactions with the catalytic core domain when the substrate (in this case, z-VAD-fmk) is bound. Overall, our H/D exchange data provided evidence that distinct regions of the caspase-9 CARD, especially the CDL, undergo major conformational changes when going from the zymogen (here, FL caspase-9 C287A) to the active cleaved (here, WT caspase-9) and going from the active cleaved to the substrate bound (here, WT caspase-9 bound to z-VAD-fmk) version of caspase-9, suggesting CARD:catalytic core interactions.
The CARD binds the catalytic core domain to different extents dependent on the ISL cleavage and substrate-bound state of caspase-9.

Figure 2.1. The CARD binds the catalytic core domain to different extents dependent on the ISL cleavage and substrate-bound state of caspase-9. Heatmap of the comparative difference in the fractional deuterium uptake (%) between (A) ΔCARD caspase-9 C287A and FL caspase-9 C287A (B) ΔCARD caspase-9 and WT caspase-9, and (C) ΔCARD caspase-9 bound to z-VAD-fmk and WT caspase-9 with z-VAD-fmk. The secondary structure of the catalytic core domain is denoted left to the peptide numbering for reference. (D), (E), and (F) are the cartoon and surface representation of the difference in the fractional deuterium uptake (%) at 10 min of deuterium labeling of (A), (B), and (C), respectively. (D) and (E) are drawn as monomers derived from the dimeric original structure (PDB: 1JXQ) possessing an “inactive” active site. Structures in (F) have z-VAD as P4-P1 positions which were substituted from z-EVD of the original structure (PDB: 1JXQ) via mutagenesis tool of PyMOL. All structures lack the ISL. Changes in the fractional deuterium uptake (%) for the heatmaps and structures are color coded in accordance with the legend.
The next analysis aimed to uncover which regions of the catalytic core domain were protected by binding to the CARD in different caspase-9 maturation states. We created heatmaps (Figure 2.13.A-C) by subtracting the fractional deuterium uptake (%) of ΔCARD caspase-9 C287A, ΔCARD caspase-9, and ΔCARD caspase-9 bound to z-VAD-fmk from the fractional deuterium uptake (%) of FL caspase-9 C287A, WT caspase-9, and WT caspase-9 bound to z-VAD-fmk (Figure 2.6-2.10), respectively. As in our earlier analyses, cartoon and surface structures illustrating the difference in the fractional deuterium uptake (%) for 10 min of labeling time were prepared (Figure 2.13.D-F). Overall, we did not observe any significant exposure in the catalytic core domain of caspase-9 constructs – zymogen, active cleaved, and active cleaved bound to z-VAD-fmk – when the CARD is intact (Figure 2.13.A-C). For these constructs/state, we saw high protection in multiple different regions suggesting the site of CARD interaction. Thus, irrespective of the state of caspase-9, removing the CARD decreased overall protection. Surprisingly, we observed protection (~ < 15%) in some regions (130’s region and the regions nearby 130’s such as L1) of the catalytic core domain when comparing ΔCARD caspase-9 C287A with procaspase-9 (Figure 2.13.A and D). Since we did not observe significant protection in the CARD when comparing the caspase-9 CARD alone with the CARD of procaspase-9 (Figure 2.12.A and D), we anticipate that the protection we observed in the 130’s region (as well as its surrounded regions) (Figure 2.13.A and D), are not due to the CARD: catalytic core domain interaction. We predict that 130’s region (as well as the surrounding regions) become more dynamic upon the removal of the CARD because we observed protection in these regions for active cleaved caspase-9 (Figure 2.13.B and E) and active cleaved caspase-9 bound to z-VAD-fmk (Figure 2.13.C and F). When comparing the catalytic core domain of the ΔCARD caspase-9 with WT caspase-9, we observed protection (~ < 15%) in some additional regions (N-terminal region of the catalytic core domain represented by a peptide spanning residues: 143-152, the C-terminus of L1 represented by a peptide spanning residues: 178-187, the C-terminus of 130’s region represented by a peptide spanning
residues: 266-272, the residues of the ISL that belong to L2 but not L2’, and subtle protection in the dimer interface) (Figure 2.13.B and E). We anticipate that these regions are involved in the CARD: catalytic core domain interactions (or allosterically impacted due to CARD: catalytic core domain interactions) because we observed a similar amount of protection (~ < 15%) in the CARD when comparing caspase-9 CARD alone with the CARD of WT caspase-9 (Figure 2.12.B and E).

When comparing the catalytic core domain of ΔCARD caspase-9 bound to z-VAD-fmk with WT caspase-9 bound to z-VAD-fmk (Figure 2.12.C and F), we observed significant enhancement in the protection in the same regions that we observed for active cleaved caspase-9 (Figure 2.12.B and E). Moreover, we observed additional protected regions (loops surrounding the substrate binding groove: L3, L4, and L2’) with a value of protection between 6% to 15%. Thus, z-VAD-fmk binding to the active cleaved caspase-9 enhances protection of the entire ISL, both L2 and L2’.

Overall, our H/D exchange data provided evidence that distinct regions of the caspase-9 catalytic core domain, especially the ISL, undergo major conformational changes when going from the zymogen to the active cleaved and going from the active cleaved to the substrate bound version of caspase-9. In addition, we observed clear evidence for interaction between CARD and core domain which are prevalent when caspase-9 is dimeric and bound to substrate.

Discussion

Since caspase-9 plays critical role in the intrinsic apoptosis, and its dysfunctionality can cause various diseases, we sought to determine missing structural details of this protease. Our H/D exchange data revealed some dynamics information of full-length (uncleaved, cleaved, and substrate bound) caspase-9 possessing the CARD and the catalytic core domain. This information is important as, to date, there is no single structure of caspase-9 possessing both domains. Moreover, our H/D exchange data also provided information on the CDL and the ISL. This information is crucial because both linkers play major roles in caspase-9 function, and the field lacks structural details of them. By analyzing the dynamics of all caspase-9 constructs/ states (Figure 2.5.A-G), we found that the CDL and the region C-terminal to the ISL cleavage site D330
(residues 330 to 342) are amongst the most exposed regions with the fraction deuterium uptake value of > 48% for all labeling times. The dimer interface of the caspase-9 constructs (FL caspase-9 C287A, ΔCARD caspase-9 C287A, WT caspase-9, and ΔCARD caspase-9) was also found to be highly exposed to the solvent with the fraction deuterium uptake value between 72% to 48% for all labeling times in the absence of active-site binding, which causes dimerization (Figure 2.5.A, C, D, and F). The dimer interface of caspase-9 (ΔCARD caspase-9 bound to z-VAD-fmk and WT caspase-9 bound to z-VAD-fmk) underwent protection with the fraction deuterium uptake value reduced to < 48% for all labeling time (Figure 2.5.E and G) when it was induced to dimerization by binding of an active site peptide inhibitor. Thus, our data supported previous findings that z-VAD-fmk binding induces dimerization of active cleaved caspase-9 constructs. More than 75% of the amide backbones of all caspase-9 constructs (except WT caspase-9 bound to z-VAD-fmk) employed in this study, had a fractional uptake values between 24% to 36% for 0.17 min time point. For WT caspase-9 bound to z-VAD-fmk, more than 75% of amide backbones had fractional uptake values below 24% for 0.17 min time point. Thus, z-VAD-fmk binding to the full-length active cleaved caspase-9 drastically increased the protection. Overall, our H/D exchange data (Figure 2.5.A-G) enhanced our knowledge in understanding the dynamics of different states of caspase-9.

To determine the impact of substrate binding impact on the structure of caspase-9, we compared the fractional deuterium uptake (%) of WT caspase-9 and ΔCARD caspase-9 to the z-VAD-fmk bound versions (Figure 2.11). From our analysis, we found some protection in some regions (e.g., the CDL) of the CARD of WT caspase-9 upon z-VAD-fmk binding. Our data also showed that the dimer interface of both WT caspase-9 and ΔCARD caspase-9 underwent major protection upon z-VAD-fmk binding. Surprisingly, we found that the 130’s region of both WT caspase-9 and ΔCARD caspase-9 is highly protected upon substrate (z-VAD-fmk) binding. From a previous study by our group, we demonstrated that 130’s region of caspase-6 but not caspase-7 is more protected upon substrate binding. Based on the H/DX data and the structure solved by
our group,\textsuperscript{38} we proposed that the 130’s region of caspase-6 unbound to a substrate constantly interchanges to helix: strand confirmation, and upon substrate binding, caspase-6 prefers the strand confirmation.\textsuperscript{37} Due to this confirmation change, protection in the 130’s region of caspase-6 is observed upon substrate binding. In the case of caspase-9, the 130’s region is larger, consisting of more residues than any executioner caspase, caspase-3, -6, and -7. By looking at the substrate bound structure of caspase-9 (PDB: 1JXQ), the 130’s region of one monomer reaches nearby some parts of the 130’s region of another monomer placing them right above the dimer interface (cartoon version in Figure 2.11.E and F). We anticipate this type of interaction (130’s region interacting to the 130’s region of another monomer) which is missing in monomeric caspase-9 forms but present in the dimeric caspase-9 forms enhances stability in the 130’s region upon substrate binding. Overall, in addition to identifying predicted regions (e.g., dimer interface), our H/D exchange data analyses (Figure 2.11) also identified some new regions (e.g., the CDL and the ISL) of caspase-9 that undergo conformational changes upon substrate-induced dimerization.

Upon comparing caspase-9 CARD alone with the CARD of procaspase-9, we found no major protection (Figure 2.12.A and D). Because of this result, we anticipate that the protection observed at the 130’s region by comparing ΔCARD caspase-9 C287A with the catalytic core domain of procaspase-9 (Figure 2.13.A and D), is not due to CARD: catalytic core interaction. This result supported our previous finding in which CARD of procaspase-9 was unable to bind to the catalytic core domain.\textsuperscript{35} When comparing caspase-9 CARD alone with WT caspase-9, we saw some protection in different regions (Figure 2.12.B and E). Similarly, when comparing ΔCARD caspase-9 with WT caspase-9, we observed some protection in several regions (Figure 2.13.B and E). In these analyses, we found that many regions (e.g., the CDL and the L2 of ISL) of the CARD and catalytic core domains might be involved in this interaction. Our H/D exchange results suggest that the CARD of WT caspase-9 may interact with the catalytic core domain. These results suggest refinements to our previous model, that CARD binding to the catalytic core domain of WT caspase-
9, to such an extent that widespread protection is observed. Our prior model was based on the thermal denaturation curve data of WT caspase-9 monitored by circular dichroism (CD). This CD spectra provided two melting temperatures of WT caspase-9 illustrating two domains: CARD and catalytic core domain. This type of dual melting curve with two melting temperature was not observed in the CD spectra of WT caspase-9 bound to z-VAD-fmk. Therefore, we generated a model illustrating the CARD interacts with the catalytic core domain of active cleaved caspase-9 only upon substrate (z-VAD-fmk) binding. Since our H/DX-MS results revealed protected regions from the CARD as well as the catalytic core domain, we anticipate that CARD of WT caspase-9 can interact with the catalytic core domain to some extent even in the absence of z-VAD-fmk, although the H/DX model would not predict as much interaction as the prior analysis. When we compared caspase-9 CARD alone with the CARD of WT caspase-9 bound to z-VAD-fmk, we observed an increase in the protection in many regions including the CDL (Figure 2.12.C and F). Similarly, upon comparing ΔCARD caspase-9 bound to z-VAD-fmk with the catalytic core domain of WT caspase-9 bound to z-VAD-fmk, we found overall enhancement in protection (Figure 2.13.C and F). We found that regions such as the L2 of ISL and C-terminal of 130’s region are more protected upon z-VAD-fmk binding (comparing Figure 2.13.B with 2.13.C and comparing Figure 2.13.E with 2.13.F). Moreover, we found some additional regions (e.g., loops surrounded to the substrate binding groove: L1, L3, L4, and L2’) involved in CARD: catalytic core interactions. Thus, in addition to the L2 loop, other loops surrounded to the substrate binding groove (L1, L3, L4, and L2’) also participate in CARD:catalytic core domain interactions upon z-VAD-fmk binding. Previously, in the case of caspase-7, we demonstrated the importance L2’ in formation of a properly aligned substrate binding groove, in which not allowing L2’ to interact with L2 causes allosteric inhibition of caspase-7.39 Similarly, our H/DX-MS data on caspase-9 suggested the importance of L2/L2’ interaction to facilitate CARD binding to the catalytic core domain. Monomeric WT caspase-9 lacks L2/L2’ interaction. In contrast, its substrate bound dimeric version is expected to possess the L2/L2’ bundle. Our H/DX-MS data also backed our previous finding that
CARD binds to the catalytic core domain upon substrate (z-VAD-fmk) binding. We believe that this distinct feature of caspase-9 substrate bound state is one of the factors in facilitating CARD:catalytic core domain interactions.

Our H/DX-MS demonstrated that the highest amount of protection in the CARD (Figure 2.12.C) and core domain (Figure 2.13.C) is observed when z-VAD-fmk is bound, suggesting

![Diagram](image)

**Figure 2.14. Regions involved in the interactions between the CARD and the substrate-bound core domain.**

(A-B) Modeled L2 and L2’ of dimeric caspase-9 (See Materials and Methods) are illustrated as (A) cartoon and (B) surface. The values of difference in fractional uptake are taken from the analysis of ΔCARD caspase-9 bound to z-VAD-fmk minus WT caspase-9 bound to z-VAD-fmk (Figure 2.13.C). (C) List of CARD and core domain residues undergoing > 12.5% of protection at 10 min of labeling time. Residues with >15% of protection are bolded. The residues of CARD are taken from the analysis of ΔCARD caspase-9 bound to z-VAD-fmk minus WT caspase-9 bound to z-VAD-fmk (Figure 2.13.C).

CARD: core domain interactions. We visualized the regions of the CARD undergoing protection (suggesting binding with the core domain) as cartoon and surface (Figure 2.12.F). To visualize the ISL residues of catalytic core domain undergoing protection due to the presence of the CARD, we
employed caspase-9 structure (PDB: 1JXQ) and modeled missing residues of the ISL, L2 from active monomer and L2’ from inactive monomer (see Materials and Methods). The cartoon and surface representation of this model are shown in Figure 2.14.A and 2.14.B, respectively. Both the L2 and L2’ undergo a huge increase protection (Figure 2.14.A and 2.14.B), suggesting that they play a significant role in the CARD:core interaction. To summarize the interactions, we created a list (Figure 2.14.C) of caspase-9 residues that undergo > 12.5% of protection, and which therefore appear to play key roles in CARD:core binding directly or may undergo allosteric changes upon CARD: core interaction. From this list, it is clear that both the ISL and the CDL of caspase-9 play a significant role in CARD:core interactions. Previously, we had performed docking studies to identify the regions involved the CARD:core interactions using RosettaDock server.35 We proposed two models: 1) H1 of the CARD (residues: R7 and R11) binds to the α4 (residues: E365, D368, and E372) of the core domain and 2) the loop between H1 and H2 (residue: D23) and the loop between H4 and H5 (residue: R51) bind to the α1 (residue: R193) and the α4 (residue: E365) of the core domain, respectively. Except H1 helix of the CARD, we do not observe high protection in the regions that were previously predicted to be involved in the CARD:core interactions (Figure 2.14.C). Moreover, our model in the prior analysis lacked two regions, the ISL and the CDL, which show significant protection (and therefore, higher chance of involvement in the CARD: core interaction) in H/D exchange studies in this work. Our model generated from the H/DX data (Figure 2.14) has enhanced our understanding of the regions involved in the CARD: core interaction, and our collaborators at Roivant Sciences will perform future calculations on our H/DX data using molecular dynamics with weighted ensemble simulations.

In summary, our H/DX-MS data revealed many new details about the dynamics of different states of caspase-9. Importantly, we gained dynamic information of full-length versions caspase-9: procaspase-9, active cleaved caspase-9, and substrate bound active cleaved caspase-9. These data also provided the dynamic information on the CDL and the ISL which are missing from all caspase-
9 structures. The H/D exchange data also identified the caspase-9 regions (e.g., the dimer interface and the 130’s region) undergoing huge conformational changes upon substrate binding. Lastly, our data also improved our knowledge in determining the caspase-9 regions that are impacted because of CARD: catalytic core domain interactions.

**Materials and Methods**

**DNA plasmids and generation of caspase constructs**

Plasmids of wild-type (WT) caspase-9 (pET23b-Casp9-His) and WT caspase-7 (pET23b-Casp7-His) were gifts from Guy Salvesen, and obtained from Addgene as plasmids, 11829 and 11825, respectively.\(^{21,40}\) As described previously,\(^{35}\) using WT caspase-9 as a template, other caspase-9 constructs were generated via QuikChange mutagenesis (Stratagene). As described previously,\(^{41}\) using WT caspase-7 as a template, full-length (FL) caspase-7 C186A was generated via QuikChange mutagenesis (Stratagene). All templates expressed constructs that have a 6xHis-tag at the C-terminus.

**Expression and purification of recombinant caspase-9 constructs**

We transformed DNA plasmids of individual caspase-9 construct into the BL21 (DE3) T7 express strain of *E. coli* (New England Biolabs). Seed cultures expressing each construct were grown by transferring a single colony into 50 mL LB media (Research Products International) containing 0.1 mg/mL ampicillin (Fisher BioReagents) and incubating at 37 °C for overnight. The next day, we transferred 3 mL of seed culture into 1 L of LB media supplemented with 0.1 mg/mL ampicillin. We incubated these cultures at 37 °C until the desired Abs\(_{600}\) (0.6 for caspase-9 CARD and 1.0 for the other caspase-9 constructs). We then induced protein expression by supplementing 1 mM IPTG (GoldBio) and lowered the temperature at 15 °C for 3 hours. Then, cells were harvested by centrifuging at 5000 rcf for 7 minutes at 4 °C and collecting cell pellets. We stored these pellets at -80 °C until purification.

We thawed frozen pellets and diluted them by adding lysis buffer (50 mM sodium phosphate pH 7.0, 300 mM NaCl, 5% glycerol, and 2 mM imidazole). We then lysed the cells using
a microfluidizer (Microfluidics, Inc.). Lysed cells were centrifuged at 50,000 rcf for 1 hour at 4°C, and cell lysates were separated from the cell debris. Cell lysates were purified using a Hi-Trap™ chelating HP column charged with Ni²⁺ (Cytiva). Using lysis buffer and elution buffer (50 mM sodium phosphate, pH = 7.0, 300 mM NaCl, 5% glycerol, and 300 mM imidazole), we developed the column a linear gradient (0 to 33% of elution buffer) to elute caspase-9 constructs. Eluted proteins were diluted 5 times by buffer A (20 mM Tris pH 8.5, 5% glycerol and 2 mM DTT). For further purification, we employed Hi-Trap™ Q HP column (Cytiva) for anion exchange chromatography. Using buffer A and buffer B (20 mM Tris pH 8.5, 1 M NaCl, 5% glycerol, and 2 mM DTT), we developed the column with a linear gradient (0 to 30%) to elute caspase-9 variants. We assessed the purity and concentration of purified caspase-9 constructs by using SDS-PAGE. The most pure and concentrated fractions were aliquoted and stored at -80°C for further usage.

**Expression and purification of recombinant FL caspase-7 C186A**

The FL caspase-7 C186A was expressed, purified, and stored at -80°C by following the exact protocol reported earlier by our group.¹¹

**LEHD-ase activity of ΔCARD caspase-9**

We followed previously described protocol (in which LEHD-ase activity of WT caspase-9 was determined) to derive the LEHD-ase activity of ΔCARD caspase-9, and derive the kinetic parameters.¹²

**Procaspsae-7 digestion by WT caspase-9 and ΔCARD caspase-9**

3 µM of procaspase-7 (FL caspase-7 C186A) was separately incubated with 0.3 µM of WT caspase-9 or 0.3 µM ΔCARD caspase-9 in an activity assay buffer (100 mM MES, pH = 6.5, 20% PEG 400, 5 mM DTT) at 37 °C over the course of different time points: 0 min, 5 min, 15 min, 30 min, and 60 min. Each reaction was stopped by adding 1x SDS loading dye (New England Biolabs). The samples were denatured at 90 °C for 5 minutes and analyzed by 16% SDS-PAGE. These gels were imaged using a ChemiDoc™ MP imaging system (Bio-Rad Laboratories).
**Hydrogen/Deuterium Exchange Mass Spectrometry**

We followed previously described protocols,\textsuperscript{36,37} with some modifications, to perform H/DX-MS. To prepare the samples containing z-VAD-fmk, an initial stock of 30 µM of caspase-9 (in an H\textsubscript{2}O buffer: 10 mM sodium phosphate, pH = 7.5) was incubated with 120 µM of z-VAD-fmk (dissolved in DMSO) at 25 °C for 1 hour. We did not observe significant LEHD-ase activity for these samples, suggesting caspase-9 was saturated by excess z-VAD-fmk. To prepare the samples not containing z-VAD-fmk, an initial stock of 30 µM of caspase-9 construct (in a H\textsubscript{2}O buffer: 10 mM sodium phosphate, pH = 7.5) was supplemented with just DMSO at 25°C for 1 hour. The samples were then introduced to the nanoACQUITY system equipped with H/D exchange technology for ultra-performance liquid chromatography (UPLC) separation\textsuperscript{42} (Waters Corp.), which performed all subsequent manipulations for the H/D exchange. Using the nanoACQUITY system, samples were incubated in D\textsubscript{2}O buffer: 10 mM sodium phosphate (dissolved in 99.9% D\textsubscript{2}O (Cambridge Isotope Laboratories, Inc.)), pD = 7.5 at 25 °C for different time points: 0, 0.17, 1, 10, and 60 min. Here, for 0 min time point, we used H\textsubscript{2}O buffer. The deuterium labeling for each sample was stopped by supplementing the samples with a 1:1 ratio of quenching buffer (100 mM sodium phosphate, pH = 1.9, and 1M guanidine-HCl) at 4 °C. As described in the previous protocols,\textsuperscript{36,37} we used Protein Lynx Global Server (PLGS) 3.0 software (Waters Corp.) to determine the coverage of peptic peptides, and then these data, along with data from the deuterated samples were imported and manually inspected in DynamX 3.0 (Waters Corp.) to determine the relative deuterium uptake. The relative deuterium uptake values for each peptic peptide for different time points were exported from DynamX 3.0 and were divided by the maximum theoretical uptake to derive the fractional deuterium uptake (%). Maximum theoretical deuterium uptake = (total residue number of a peptide) minus 1, (to reflect the lack of a proton on the N-terminus) minus number of proline residues present in the peptide. The heatmaps of each construct and the comparison of two constructs were generated in Prism (GraphPad) software by using the fractional deuterium uptake or the difference in the fractional deuterium uptake, respectively. The cartoon

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and surface structures of caspase-9 constructs representing changes in two states were generated with preference given to data from shorter peptides, as those provide more finely spaced data, using PyMOL (Schrödinger, LLC).

**Generation of CARD by adding the missing residues of CARD linker (CDL)**

We generated a model of the full CARD including the missing residues of the CDL (residues 96-142) by employing the structure of procaspase-9 CARD from PDB: 3YGS (possesses structural helical region from residues 1-95). The missing residues of the CDL were modeled using UCSF Chimera/MODELLER integrated system.\(^{43,44}\)

**Generation of the ISL by adding the missing residues of L2 and L2’**

We employed PDB: 1JXQ and inserted missing residues of the ISL, L2 residues: 299-315 for active monomer and L2’ residues: 316-332 for inactive monomer by using Builder feature of PyMOL (Schrödinger, LLC).

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References


CHAPTER III:
CASPASE-9 ACTIVATION OF PROCASPASE-3 BUT NOT PROCASPASE-6 IS BASED ON THE LOCAL CONTEXT OF CLEAVAGE SITE MOTIFS AND ON SEQUENCE

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Authors’ Contributions: JAH obtained funding, directed the project, and wrote parts of the manuscript. IVS performed all experiments, prepared figures, and wrote the manuscript.

Abstract

Studying the interactions between a protease and its protein substrates at a molecular level is crucial for identifying the factors facilitating selection of particular proteolytic substrates and not others. These selection criteria include both the sequence and the local context of the substrate cleavage site where the active site of the protease initially binds and then performs proteolytic cleavage. Caspase-9, an initiator of the intrinsic apoptotic pathway, mediates activation of executioner procaspase-3 by cleavage of the intersubunit linker (ISL) at site 172IETDS. Although procaspase-6, another executioner, possesses two ISL cleavage sites (site 1, 176DVVDN; site 2, 190TEVD4A), neither is directly cut by caspase-9. Thus, caspase-9 directly activates procaspase-3 but not procaspase-6. To elucidate this selectivity of caspase-9, we engineered constructs of procaspase-3 (e.g., swapping the ISL site, 172IETDS, with DVVDN and TEVDA) and procaspase-6 (e.g., swapping site 1, 176DVVDN, and site 2, 190TEVD4A, with IETDS). Using the substrate digestion data of these constructs, we show here that the P4–P1’ sequence of procaspase-6 ISL site 1 (DVVDN) can be accessed but not cleaved by caspase-9. We also found that caspase-9 can recognize the P4–P1’ sequence of procaspase-6 ISL site 2 (TEVDA); however, the local context of this cleavage site is the critical factor that prevents proteolytic cleavage. Overall, our data have
demonstrated that both the sequence and the local context of the ISL cleavage sites play a vital role in preventing the activation of procaspase-6 directly by caspase-9.

**Introduction**

Proteolysis, a process of enzymes catalyzing the hydrolytic cleavage of their substrates, drives various biological pathways (e.g., cell cycle, cell differentiation, and cell death) to maintain homeostasis in all living organisms. Examining the interactions between a particular protease and its protein substrate at a molecular level is beneficial in understanding their involvement in particular biological pathways. The Schechter-Berger convention has been widely adopted by the protease community to showcase the interactions between the peptide residues in the substrate cleavage-site (denoted as P) and sub-sites of protease active-site (denoted as S), where the cleavage (↓) occurs between P1 and P1’ (Figure 3.1.A). The nature of the sub-sites on the enzyme together with the cleavage site motif within the substrate is considered to define protease preference. This notion is reinforced when the broad range of sequences detected by protein or peptide-based protease substrate profiling are reduced to a single “preferential” cleavage motif. In contrast to this narrow view of recognition specificity, detailed analyses of aggregate data on proteolysis suggest that reducing the concept of recognition motif to a single sequence is overly simplistic. For example, the preferred cleavage motif of caspase-7 protease using the peptide-based screening and the proteomics study was identified as DEVD↓ (P4-P1) and DEVD↓G (P4-P1’), respectively. However, from the same proteomics study’s data, we found that, out of 128 cleaved peptides by caspase-7, the cleavage sites of DEVD↓G, DEVD↓X, and DXXD↓X were only 2, 4, and 36, respectively. Another example is the family of ClpP proteases (from *Escherichia coli*, *Staphylococcus aureus*, and human mitochondria) which favor peptide substrates composed of specific residues at the P3-P1 positions (e.g., natural amino acids preferred by human mitochondria ClpP at P3-P1 positions are F/W-M/T/L-M/L); nonetheless, proteomics studies on these proteases revealed very low cleavage preference. In fact, it was later discovered that proteolysis by ClpP protease (from *Caulobacter crescentus*) complexed with ClpX (an unfoldase which feeds ClpP) is
mainly governed by local context (substrate cleavage occurred after every 10-13 residues) and not sequence dependent. Looking at the cleavage preferences for any protease and its suite of cleaved

Figure 3.1. Procaspase-3 and -6 share the same core structural fold, and intersubunit linker (ISL) cleavage is a pre-requisite for their activation.

(A) Schechter-Berger schematic diagram representing protease-specificity towards the cleavage sequence of the substrates. Peptide residues of the substrates and sub-sites of protease active-site are denoted as P and S, respectively. The cleavage of substrate occurs at the amide bond between the residues at P1 and P1'. Caspases prefer aspartate at the P1 position. (B) Procaspase-3 (light pink) and procaspase-6 (light cyan) structures with the missing loops/regions shown as dashes, which were modeled as described in the materials and methods. The PDB IDs used to model procaspase-3 and -6 were 4JQY and 3NR2, respectively. The active-site cysteines of both procaspases were substituted by alanine (C163A) to determine the structures of full-length uncleaved zymogen. Active-site cysteines (substituted from alanine present in the original structures by using mutagenesis feature of PyMOL) of procaspase-3 and -6 are shown as spheres. Both procaspases possess a similar overall structural fold in the core. The greatest differences are present in the mobile loops. Loops containing the ISL are highlighted (dark pink for procaspase-3 and dark cyan for procaspase-6). Procaspase-3 has a known ISL cleavage after D175 (P1 position), and procaspase-6 has two known ISL cleavages after D179 and D193. (C) Linear cartoons of procaspase-3 and -6 illustrating their prodomain (N), large subunit (Lg), ISL and small subunit (Sm). Simply removing the prodomain for both procaspases is not sufficient for their activation. ISL cleavage is required for both procaspases for activation.
substrates, it becomes clear that i) no single peptide sequence can fully account for the cleavage properties observed for a natural protease, and ii) the sequence specificity alone cannot completely account for the selection of substrates that are cleaved. Thus, we hypothesized at the outset of this work that both the sequence and the local context of the substrate cleavage site may be important factors governing recognition by a protease.

One prime example of the gulf between canonical recognition sequence and the actual cleavage propensity is exemplified in the caspases. Caspases are cysteine-aspartate proteases that play key roles in regulating multiple cellular pathways including apoptosis and inflammation. Dysfunction in caspase regulation is a hallmark of several diseases such as cancers, autoimmune disorders, and neurodegeneration. Therefore, it is important to understand their cellular pathways at a molecular level. The defining feature of this family of proteases is their ability to cleave substrates at sites containing aspartate at the P1 position (Figure 3.1.A), although glutamate and phosphoserine can also be recognized albeit at low frequency. Apoptotic caspases are categorized into two groups: initiators (caspase-8, -9, and -10) and executioners (caspase-3, -6 and -7). Caspases are translated as full-length, inactive zymogens termed as procaspases. Initiator zymogens are recruited to activation platforms upon either intrinsic (e.g., procaspase-9 to form apoptosome) or extrinsic (e.g., procaspase-8 to form death-inducing signaling complex) cell-signaling to dimerize and ultimately achieve maturation. Excluding the mobile loops, executioner zymogens possess similar structural folds (Figure 3.1.B) and are activated by a cleavage event at their intersubunit linker (ISL) generating large and small subunits (Lg and Sm) from each chain of the procaspase dimer (Figure 3.1.C). Procaspase-3 has one ISL cleavage site at D175, while procaspase-6 has two ISL cleavage sites, D179 and D193 (Figure 3.1.B and 3.1.C). In an intrinsic apoptotic pathway, upon its activation, caspase-9 cleaves the ISL of procaspase-3 and -7, thereby activating them to the mature form. Activated caspase-3 mediates procaspase-6 ISL cleavage to activate caspase-6. Activated executioners proteolyze their respective and common substrates evoking apoptosis. In this manuscript, we sought to address a
key question in the field: Why can caspase-9 cleave the ISLs of procaspase-3 and -7 but not the procaspase-6 ISL cleavage sites? Addressing this question, while providing insights for caspase-9, is also applicable to proteases and their substrate selection generally.

**Results**

**Caspase-9 cleaves the ISL of procaspase-3 but not procaspase-6.**

To examine the behavior of procaspase-3 and -6 as substrates of wild-type caspase-9 in vitro, we performed an SDS-PAGE-based substrate-digestion assay. Caspase-9 cleaved caspase-3 C163A (Figure 3.2A); however, it was unable to proteolyze caspase-3 C163A D175A (Figure 3.2B). Thus, caspase-9 cleaved procaspase-3 ISL at D175 resulting in two cleavage products: prodomain + large subunit (N+Lg) and small subunit (Sm) (Figure 3.2A). Caspase-9 cleaved the prodomain of caspase-6 C163S resulting in caspase-6 ΔN C163S; nevertheless, no significant ISL cleavage was observed (Figure 3.2C). A previous study showed that removal of the prodomain while the ISL remained intact was not sufficient to achieve procaspase-6 activation. Thus, caspase-9 cannot activate procaspase-6 directly (Figure 3.2C). From a peptide-based profiling study, caspase-9 canonical recognition sequence is LEHD (P4-P1). Therefore, we carried out an LEHD-ase activity assay to determine the kinetic parameters ($k_{cat}$ and $K_m$) of caspase-9 using a fluorogenic peptide substrate, Ac-LEHD-afc (Figure 3.2D). The catalytic efficiency, $k$ ($k_{cat}/K_m$), of caspase-9 to hydrolyze Ac-LEHD-afc is $(12.8 \pm 1.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Figure 3.2E). For the experiments (Figure 3.2A, 3.2B, and 3.2C), the substrate (caspase-3 or -6) concentration (3 µM) was in excess over the enzyme (caspase-9) concentration (0 – 1 µM). Limitations of the slow kinetics of caspase-9 coupled with the sensitivity of detecting cleaved products makes it impossible to monitor the reaction under pseudo-first order conditions as has been described previously. Nevertheless, we used a similar approach to estimate a relative rate ($\tilde{k}$) for caspase-9 cleavage of the ISLs of procaspase-3, caspase-3 D175A C163A, and procaspase-6 (Figure 3.2E). These apparent relative cleavage rates ($\tilde{k}$) for proteolysis of the ISL of procaspase-3, caspase-3 D175A C163A, and procaspase-6 by caspase-9 were $390 \pm 30 \text{ M}^{-1}\text{s}^{-1}$, $< 25 \text{ M}^{-1}\text{s}^{-1}$, and $< 25 \text{ M}^{-1}\text{s}^{-1}$, respectively (Figure
We used same approach throughout this manuscript to approximate rate ($k$) values. Our results complemented previous findings by various research groups demonstrating caspase-9 does not directly activate procaspase-6. Excluding its prodomain and the ISL, procaspase-6 is about 43% identical in protein sequence to procaspase-3 (Figure 3.3). Due to the fact that they are
the most divergent segments of caspase-6, we hypothesized that the prodomain and/or the ISL of procaspase-6 might protect procaspase-6 direct activation by caspase-9.

<table>
<thead>
<tr>
<th>Prodomain</th>
<th>Large Subunit</th>
<th>Loop-2 possessing the ISL</th>
<th>Small Subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% identity = 30%</td>
<td>% identity = 38%</td>
<td>% identity = 7%</td>
</tr>
<tr>
<td></td>
<td>% identity of the full-length = 38%</td>
<td>% identity = 54%</td>
<td></td>
</tr>
</tbody>
</table>

| % identity without the prodomain and ISL = 43% |

**Figure 3.3. Procaspase-6 and -3 are sequentially more identical when the prodomain and ISL are ignored.**

We aligned the sequence of procaspase-3 and -6 where the identical residues are marked with *. From this sequence alignment, we found that the procaspase, large subunit, loop-2 possessing the ISL, and small subunit of procaspase-6 are 30%, 38%, 7%, and 54% identical to procaspase-3, respectively. Excluding the prodomain and the ISL regions of procaspase-6 results in more sequential identity to procaspase-3 (increasing to 43% from 38% identical).

**Prodomain of procaspase-6 does not play a significant role in protecting direct activation by caspase-9.**

To test the hypothesis that the procaspase-6 prodomain might protect the procaspase-6 ISL from cleavage, we used caspase-6 ΔN C163S, an uncleaved form of caspase-6 lacking the prodomain. If the prodomain prevents procaspase-6 ISL cleavage by caspase-9, then we should observe more ISL cleavage for caspase-6 ΔN C163S than what we observed for caspase-6 C163S (Figure 3.2.C). The ISL cleavage of caspase-6 ΔN C163S by caspase-9 was almost undetectable (Figure 3.4.A). From the comparison, we observed no significant difference in the ISL cleavage of caspase-6 C163S as compared to caspase-6 ΔN C163S cleavage by caspase-9 (Figure 3.4.B). The estimated rate \( k \) values of caspase-9 to cleave the ISL of both constructs were \( < 25 \text{ M}^{-1}\text{s}^{-1} \) (Figure 3.4.C). These data imply that the prodomain does not play a significant role in preventing cleavage of the procaspase-6 ISL by caspase-9. This suggests that the procaspase-6 ISL cleavage
sites, site 1: $^{176}\text{DVVD}\downarrow\text{N}$ and site 2: $^{190}\text{TEVD}\downarrow\text{A}$ may contribute to substrate selection and should be tested individually.

**Caspase-9 does not recognize the procaspase-6 ISL site 1, $^{176}\text{DVVD}\downarrow\text{N}$.**

Using sequence alignment, we compared the procaspase-6 ISL site 1 ($^{176}\text{DVVD}\downarrow\text{N}$) with the procaspase-3 ISL site ($^{172}\text{IETD}\downarrow\text{S}$) (Figure 3.5.A).

To understand the inability of caspase-9 to hydrolyze the procaspase-6 ISL site 1, we engineered a construct in which we introduced the site 1 sequence from procaspase-6 into procaspase-3 to generate caspase-3 C163A $^{172}\text{IETD}\downarrow\text{S}$ to DVVDN. Using this construct, we asked whether the DVVDN sequence could be cleaved by caspase-9 if it was presented in the background of procaspase-3. We also engineered another construct by introducing the procaspase-3 ISL site into procaspase-6, caspase-6 C163S $^{176}\text{DVVD}\downarrow\text{N}$ to IETDS. Using this construct, we can determine whether caspase-9 can recognize an appropriate procaspase-3 cleavage site sequence in the context of procaspase-6. Caspase-9 did not proteolyze caspase-3 C163A $^{172}\text{IETD}\downarrow\text{S}$ to DVVDN (Figure 3.5.B) suggesting that the sequence DVVDN is not optimized for cleavage by caspase-9. In contrast, caspase-9 can cleave the ISL of caspase-6 C163S $^{176}\text{DVVD}\downarrow\text{N}$ to IETDS resulting in generation of cleavage products: Lg and small subunit + intersubunit linker (Sm+ISL) (Figure 3.5.C). This indicated that the context of the 176 to 180 region is available to caspase-9 for recognition. A previous peptide-based profiling study$^5$ demonstrated that the most preferred...
peptide-substrate sequence of caspase-9 is LEHD↓ (P4-P1). Therefore, we constructed a third construct, caspase-6 C163S 176DVVD↓N to LEHDS, to analyze how efficiently caspase-9 can proteolyze the canonical consensus sequence. This construct was cleaved at the ISL by caspase-9 resulting in the formation of Lg and Sm+ISL (Figure 3.5.D). No significant difference in the ISL cleavage was detected between caspase-6 C163S and caspase-3 C163A 172IETD↓S to DVVDN (Figure 3.5.E). The cleavage rate (k) of caspase-9 to proteolyze the ISL of caspase-3 C163A 172IETD↓S to DVVDN was < 25 M⁻¹s⁻¹ (Figure 3.5.F). Thus, it appears that the caspase-9 active-
site is unable to recognize the sequence of procaspase-6 ISL site 1, \textsuperscript{176}DVVD\textsubscript{↓}N. There was a significant difference in the ISL cleavage when procaspase-6 site 1 was replaced from \textsuperscript{176}DVVD\textsubscript{↓}N to IETD/LEHD (Figure 3.5.E). We did not observe significant changes in the ISL cleavage among the constructs, caspase-3 C163A, caspase-6 C163S \textsuperscript{176}DVVD\textsubscript{↓}N to IETDS, and caspase-6 C163S \textsuperscript{176}DVVD\textsubscript{↓}N to LEHDS (Figure 3.5.E). Moreover, the ISLs of these three constructs were cleaved by caspase-9 with a similar estimated cleavage rate, \( k \) (Figure 3.2.E and 3.5.F). These results demonstrated that caspase-9 active-site can access the procaspase-6 ISL site 1; however, the sequence, DVVDN (P4-P1') is unfavorable for proteolytic cleavage.

**Caspase-9 does not cleave the procaspase-6 ISL site 2, \textsuperscript{190}TEVD\textsubscript{↓}A, mainly due to the local context.**

We compared the procaspase-3 ISL site (\textsuperscript{172}IETD\textsubscript{↓}S) and procaspase-6 ISL site 2 (\textsuperscript{190}TEVD\textsubscript{↓}A) by aligning the sequences (Figure 3.6.A). To investigate why caspase-9 does not cleave the procaspase-6 ISL site 2, which is the sequence first recognized by caspase-6,\textsuperscript{9} we engineered constructs, caspase-3 C163A \textsuperscript{172}IETD\textsubscript{↓}S to TEVDA, caspase-6 C163S \textsuperscript{190}TEVD\textsubscript{↓}A to IETDS, and caspase-6 C163S \textsuperscript{190}TEVD\textsubscript{↓}A to LEHDS (the canonical caspase-9 recognition motif\textsuperscript{5}). Caspase-9 cleaved the ISL of caspase-3 C163A \textsuperscript{172}IETD\textsubscript{↓}S to TEVDA resulting in the formation of cleavage products: N+Lg and Sm (Figure 3.6.B). Though we see a band indicating removal of the prodomain (ΔN), the ISL cleavages for caspase-6 C163S \textsuperscript{190}TEVD\textsubscript{↓}A to IETDS and caspase-6 C163S \textsuperscript{190}TEVD\textsubscript{↓}A to LEHDS were not observed (Figure 3.6.C and 3.6.D). We observed a significant difference in the ISL cleavage between the constructs, procaspase-6 and caspase-3 C163A \textsuperscript{172}IETD\textsubscript{↓}S to TEVDA (Figure 3.6.E). Thus, caspase-9 can recognize the procaspase-6 ISL site 2 in the context of the procaspase-3 background. We observed a significant difference in the ISL cleavage between the constructs, caspase-3 C163A and caspase-3 C163A \textsuperscript{172}IETD\textsubscript{↓}S to TEVDA (Figure 3.6.E). Caspase-9 cleaved the ISL of procaspase-3 with much
higher efficiency than caspase-3 C163A 172IETD↓S to TEVDA (Figure 3.2.E and 3.6.E). Thus, caspase-9 cannot efficiently recognize the sequence of procaspase-6 ISL site 2, 190TEVD↓A, even when it was presented in an optimal context in procaspase-3. No significant change in the ISL cleavage was observed among the constructs: caspase-6 C163S, caspase-6 C163S 190TEVD↓A to IETDS, and caspase-6 C163S 190TEVD↓A to LEHDS (Figure 3.6.E). Caspase-9 cleavage rates, \( k \), to cleave the ISL of these three constructs were \(< 25 \text{ M}^{-1}\text{s}^{-1} \) (Figure 3.2.E and 3.6.F). Thus, even...
replacing procaspase-6 ISL site 2 (190TEVD↓A) to IETDS or LEHDS (the most preferred sequence of caspase-9 active-site for P4-P1 positions), resulted in almost no ISL cleavage. These results strongly suggest that in addition to the sequence, the local context of procaspase-6 ISL site 2 (190TEVD↓A) plays a vital role in blocking the access of caspase-9.

**Caspase-9 active-site preference for P4-P1’ positions is IETDS > TEVDA > DVVDN.**

We used a previously solved crystal structure (PDB: 1JXQ) to model the caspase-9 active-site bound to different peptides. This reported crystal structure has a peptide-based inhibitor, z-EVD-Dcbmk (benzoxycarbonyl-Glu-Val-Asp-dichlorobenzylmethylketone) bound at the active-site pocket occupying the P4-P1 positions (Figure 3.7.A). Using the mutagenesis feature of PyMOL, we generated structural models of caspase-9 active-site binding to the peptides, IETD, TEVD, and DVVD (Figure 3.7.B, 3.7.C, and 3.7.D). The S4 sub-site of the caspase-9 active-site contains residues, I396, Y397, W354, and W362, rendering it hydrophobic. Therefore, isoleucine, a hydrophobic residue, can be accommodated at the P4 position (Figure 3.7.B). Aspartic acid present in DVVDN is a very hydrophilic residue that may resist residing inside the S4 sub-site (Figure 3.7.C). Moreover, the presence of D356 nearby S4 sub-site may further decrease affinity for a negatively charged aspartic acid at the P4 position (Figure 3.7.C). Threonine is uncharged and less hydrophilic than aspartic acid. Thus, the S4 sub-site may more readily accept threonine (Figure 3.7.D). Peptides IETD and TEVD have an advantage of possessing glutamic acid as P3 position, which can interact with R355 via hydrogen bonding (Figure 3.7.B and 3.7.D). In contrast, valine, a hydrophobic residue, is not suitable for S3 sub-site binding (Figure 3.7.C). The threonine of IETD peptide can interact with K292 by making a hydrogen bond (Figure 3.7.B). Looking at the cavity of the S1’ sub-site, small sized residues such as glycine, serine and alanine are more preferred than asparagine as P1’ position (not shown in the figure because P1’ was not present in the original structure, Figure 3.7.A). Therefore, the caspase-9 active-site preference for P4-P1’ positions is IETDS > TEVDA > DVVDN. Calculation
of the solvent accessible surface area (SASA) also provides insights into recognition preferences (Table 3.1). While the cleavage site in procaspase-3 and site 1 in procaspase-6 are both highly accessible, site 2 in procaspase-6 is much less accessible. The structural models (Figure 3.7) and SASA analysis support our cleavage assays data that caspase-9 i) cleaves the procaspase-3 ISL site (\(^{172}\text{IETD} \downarrow \text{S}\)) with a higher cleavage rate (Figure 3.7), ii) does not recognize the procaspase-6 ISL.
Table 3.1: Solvent Accessible Surface Area (SASA) of procaspase-3 and -6 ISL cleavage sites.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Description</th>
<th>SASA (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>172IETDS</td>
<td>Procaspaose-3 ISL site</td>
<td>474.2</td>
</tr>
<tr>
<td>176DVVDN</td>
<td>Procaspaose-6 ISL site 1</td>
<td>503.7</td>
</tr>
<tr>
<td>190TEVDA</td>
<td>Procaspaose-6 ISL site 2</td>
<td>164.0</td>
</tr>
<tr>
<td>115VCVFL</td>
<td>Beta strand in the core region of procaspaose-6</td>
<td>8.9</td>
</tr>
<tr>
<td>1MSSAS</td>
<td>Part of the prodomain of procaspaose-6</td>
<td>621.3</td>
</tr>
</tbody>
</table>

We employed full-length models of procaspaose-3 and -6 (described in Figure 3.1) to perform SASA analysis on the ISL cleavage sites using the “get area” feature of PyMOL (Schrodinger, LLC). The SASA of 115VCVFL (buried in core beta strand residues) and 1MSSAS (highly solvent exposed prodomain residues) were calculated as controls. The ISL site of procaspaose-3 (172IETDS) and site 1 of procaspaose-6 (176DVVDN) are highly exposed to the solvent illustrating they are accessible to proteases (e.g., caspase-9). In contrast, the site 2 of procaspaose-6 (190TEVDA) is not very exposed to the solvent, illustrating that it is not as accessible to proteases (e.g., caspase-9).

Discussion

The order of caspases activation during the intrinsic apoptotic pathway has been studied in-depth previously.26,34 Based on these studies, we created a schematic diagram of intrinsic apoptosis focusing on caspase-9 activation via apoptosome complex and the order of activation of the executioners (caspase-3, -6, and -7) (Figure 3.8A). Once triggered via formation of the apoptosome, caspase-9 mediates the activation of procaspaose-3 and -7. In the case of procaspaose-3, caspase-9 cuts the ISL site (172IETDS) (Figure 3.2A and 3.2.B). This proteolytic cleavage event at the ISL is required to activate procaspaose-3 35,36 (Figure 3.8.B). After this ISL cleavage, to achieve complete maturation, the prodomain of caspase-3 is removed (first cleavage at D9 and then at D28) likely via self-proteolysis or caspase-3-like activity.14,15 Activated caspase-3 further processes caspase-9 via a feedback mechanism – a cleavage event at D33026 (Figure 3.8.A).
Procaspase-7 contains three cleavage sites: D23, D198, and D206. In order to activate the intrinsic apoptotic pathway, caspase-9 cannot directly activate caspase-6 due to the sequence of ISL cleavage site 1 and the local context of ISL cleavage site 2.

(A) Mitochondrial stress releases cytochrome c, which interacts with Apaf-1 (apoptotic protease activating factor 1, colored orange) to form the apoptosome complex which recruits procaspase-9 to mediate activation of caspase-9. Caspase-9, then activates caspase-3 and -7; however, it is unable to activate caspase-6 directly. Activated caspase-3 processes procaspase-9 via feedback mechanism. Caspase-3 also cleaves and activates procaspase-6. In the absence of active caspase-3, activated caspase-7 activates procaspase-6. The apoptotic executioners (caspase-3, -6, and -7) cleave their respective downstream substrates to evoke apoptosis. (B) Schematic diagram showing caspase-9 activating procaspase-3 by the ISL cleavage at D175. (C) Schematic diagram showing caspase-9 can access D179 of procaspase-6 but the sequence, DVVDN, is uncleavable (P4-P1' cleavage sites for each monomer are shown as transparent blue spheres). Caspase-9 is unable to hydrolyze D193 due to the local context (inaccessibility is shown as clashes). Though caspase-9 can remove the prodomain of procaspase-6, the resultant product (ΔN caspase-6) is inactive which cannot hydrolyze downstream substrates.
procaspase-7, prodomain (residues: 1-23)\textsuperscript{38} and then N-terminal peptide (residues: 23-28)\textsuperscript{39} are removed perhaps by active caspase-3. These cleavage events facilitate procaspase-7 activation via ISL cleavage by initiator caspases (e.g., caspase-9 and -8). Caspase-9 activates procaspase-7 (\textbf{Figure 3.8.A}) via cleavage at ISL site 1 (\textsuperscript{195}IQAD↓S) and ISL site 2 (\textsuperscript{203}NDTD↓A).\textsuperscript{39} The cleavage rates (\(k\)) for caspase-9 to cut procaspase-7 ISL site 1 (\textsuperscript{195}IQAD↓S) and ISL site 2 (\textsuperscript{203}NDTD↓A) were derived to be \(\sim 0.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\) and \(< 200 \text{ M}^{-1}\text{s}^{-1}\), respectively.\textsuperscript{30} Thus, caspase-9 proteolyzes procaspase-7 ISL site 1 with a much higher cleavage rate than ISL site 2. Swapping the procaspase-7 ISL site 2 from \textsuperscript{203}NDTD↓A to LEHDA robustly enhanced the cleavage rate of caspase-9 (from \(< 200 \text{ M}^{-1}\text{s}^{-1}\) to \(~0.1\times 10^4 \text{ M}^{-1}\text{s}^{-1}\)\textsuperscript{30} exemplifying the importance of the cleavage site motif sequence in proteolysis. Nevertheless, this improved cleavage rate of caspase-9 for this swapped construct of procaspase-7 was about four times less than the cleavage rate to hydrolyze at ISL site 2, \textsuperscript{195}IQAD↓S.\textsuperscript{30} Thus, the ISL site 1 of procaspase-7 appears to be more accessible to caspase-9 active-site than the ISL site 2, illustrating the importance of local context of a cleavage site in the process of proteolysis.

The inability of caspase-9 to directly activate procaspase-6 (\textbf{Figure 3.8.A}) has been reported in previous studies.\textsuperscript{26-28.32} Proteolytic cleavage at any of the ISL cleavage sites (either at D179 or D193) of procaspase-6 is a prerequisite to activate procaspase-6.\textsuperscript{13.29} Moreover, ISL cleavage of procaspase-6 results in structural-dynamics changes rendering the active-site more accessible to solvent.\textsuperscript{40} Based on our results from this manuscript (\textbf{Figure 3.2, 3.5, and 3.6}), we prepared a schematic diagram showcasing the interactions between caspase-9 and procaspase-6 (\textbf{Figure 3.8.C}). We demonstrate that the procaspase-6 ISL site 1 (\textsuperscript{176}DVVD↓N) is accessible (in other words, the local context of this cleavage site is not creating hindrance) to the active-site of caspase-9. However, the sequence of this cleavage site (DVVDN as P4-P1'), illustrated as transparent blue spheres for each monomer (\textbf{Figure 3.8.C}), is unrecognizable (\textbf{Figure 3.5}). We also found that the active-site of caspase-9 can recognize the sequence of the procaspase-6 site 2 (\textsuperscript{190}TEVD↓A). Nonetheless, the local context of this ISL cleavage site, shown as clashes (\textbf{Figure 3.8.C}).
3.8.C), is blocking the access of caspase-9 active-site (Figure 3.6). Thus, both the sequence as well as the local context of procaspase-6 ISL cleavage sites play vital roles in preventing direct activation by caspase-9. We observed that caspase-9 only partially cleaved the prodomain of procaspase-6, but not any other sites, although caspase-9 was present at concentrations above those expected physiologically (Figure 3.2.C). The removal of only the prodomain is not sufficient for procaspase-6 activation. Thus, procaspase-6 proteolysis by caspase-9 simply results in an inactive ΔN version of caspase-6 (Figure 3.8.C).

Upon its activation by caspase-9, caspase-3 hydrolyzes procaspase-6 into mature caspase-6\(^{26,27}\) (Figure 3.8.A). Active caspase-3 proteolyses procaspase-6 at all three cleavage sites: D23, D179, and D193.\(^9,27\) Among these three cleavage sites of procaspase-6, caspase-3 directly cuts at D23 and D179, and only then proteolysis at D193 can occur.\(^9,27\) These findings strongly suggested that the procaspase-6 ISL site 1 (\(^{176}\)DVVD↓N) is readily accessible and recognizable to the active-site of caspase-3. Moreover, the local context of procaspase-6 ISL site 2 (\(^{190}\)TEVD↓A) initially blocks the caspase-3 active-site, and only allows the access after prior cleavage event at D179,\(^27\) although is it difficult to disentangle the contribution of a more favorable recognition sequence. Activated caspase-6 can also proteolyze procaspase-6 intermolecularly at all three cleavages sites: D23, D179, and D193.\(^9\) Among these three cleavage sites, active caspase-6 robustly cleaves at D23 but inefficiently proteolyzes at D179 and D193.\(^9,27\) Active caspase-6 was able to hydrolyze the procaspase-6 ISL site 1 with much higher efficiency when the sequence \(^{176}\)DVVD was substituted with TETD (P4-P1 sequence of the procaspase-6 prodomain).\(^9\) Thus, as we determined for caspase-9 (Figure 3.5), the sequence of procaspase-6 site 1 is not well recognized by caspase-6 via intermolecular recognition. Nevertheless, when it engages in an intermolecular interaction, in contrast to caspase-3, active caspase-6 can cut procaspase-6 at D193 without any need for prior cleavage at D179.\(^9,27\) Thus, unlike active caspase-9 (Figure 3.6) and caspase-3,\(^9,27\) active caspase-6 can access and directly cleave the procaspase-6 ISL site 2 (\(^{190}\)TEVD↓A). Despite the fact that caspase-3 hydrolyzes its preferred fluorogenic peptide substrate (DEVD-ase activity)\(^41\) with ~5-
fold higher $k_{\text{cat}}/K_m$ value than caspase-6 (VEID-ase activity),\textsuperscript{42,43} the inability of caspase-3 and the ability of caspase-6 to directly proteolyze the procaspase-6 ISL site 2 via intermolecular interactions signifies the importance of the local context of cleavage sites in the process of proteolysis. Early work in the field showed that caspase-3 but not caspase-7 activated caspase-6.\textsuperscript{44} More recently, in at least one study of the intrinsic apoptotic pathway,\textsuperscript{34} in the absence of caspase-3, activated caspase-7 was able to activate procaspase-6 to caspase-6 (Figure 3.8.A); however, the molecular factors facilitating this activation remain to be identified.

Peptide-based screening and proteomics-wide studies of an individual protease are insightful in designing active-site inhibitors/peptide substrates\textsuperscript{5,10,45} and obtaining the list of protein substrates,\textsuperscript{46–48} respectively. However, this information is not enough to completely understand the substrate preference of an individual protease. Such knowledge at a molecular level can be determined by performing biochemical and/or biophysical studies on a protease and its substrates. Interactions between many proteases and their protein substrates (e.g., caspase-9 and the executioners, procaspase-3, -6, and -7) are transient; therefore, deriving the structural information of their complexes using biophysical techniques such as X-ray crystallography, NMR, and cryo-EM is challenging (e.g., currently, there is no structure deposit of the complex of caspase-9 and any of its protein substrates in the Protein Data Bank, https://www.rcsb.org/). In such cases, using site-directed mutagenesis coupled with protein substrate digestion assays, as we have undertaken herein, can help in elucidating the mechanisms of biological pathways at a molecular level. This study demonstrates two key points: i) That the molecular architecture of procaspase-6 renders it insensitive to caspase-9 activation, although its ISL cleavage sites are similarly positioned (within the ISL) to those in procaspase-3/-7. ii) That sequence as well as the local context of the cleavage sites enables productive proteolytic activation.
Materials and Methods

Generation of full-length procaspase-3 and -6 models by adding missing residues

We generated full-length models of procaspase-3 and -6 by employing structures from the Protein Data Bank, PDB: 4JQY and PDB: 3NR2, respectively. The missing residues of procaspase-3 (1-31, 54-64, 165-185, 201-210, and 251-260) as well as procaspase-6 (1-30, 167-186, and 262-270) were modeled using UCSF Chimera/MODELLER integrated system. The illustrations of procaspase-3 and -6 models were created using PyMOL (Schrödinger, LLC).

Sequence alignment of procaspase-3 and -6

We used Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to align the sequences of procaspase-3 (Uniprot ID: P42574) and procaspase-6 (Uniprot ID: P55212).

DNA plasmids and generation of caspase constructs

Plasmids for expression of wild-type caspase-3 (pET23b-Casp3-His) and wild-type caspase-9 (pET23b-Casp9-His) were gifts from Guy Salvesen, and obtained from Addgene as plasmids, 11821 and 11829, respectively. An E. coli codon-optimized wild-type caspase-6 gene (with a 6xHis at C-terminus) was synthetically produced (Celtek Bioscience) and ligated into the NdeI/BamHI sites of the pET11a vector. Wild-type caspase-3 and wild-type caspase-6 plasmids were used as templates to generate their respective constructs (substitutions and deletions) employing QuikChange mutagenesis (Agilent Technologies).

Expression and purification of recombinant caspase constructs

DNA plasmids for expression of caspase constructs were transformed into BL21 (DE3) T7 express strain of E. coli (New England Biolabs). A single colony was picked for each construct and overnight seed cultures were grown in 50 mL LB media (Research Products International) supplemented with 0.1 mg/mL ampicillin (Fisher BioReagents) by incubating at 37 °C. For large-scale growth, 3 mL of seed culture of each caspase construct was transferred into 1 L of LB medium containing 0.1 mg/mL ampicillin. Incubation at 37°C was carried until desired Abs600 (0.6 for caspase-3 constructs, 0.8 for caspase-6 constructs, and 1.0 for caspase-9) was achieved. In each
case, protein expression was induced by adding 1 mM of IPTG (GoldBio), and the temperatures were lowered (30 °C for caspase-3 constructs, 25 °C for caspase-6 constructs, and 25 °C for caspase-9). After 4 hours, cells were centrifuged at 5000 rcf for 7 minutes at 4 °C, and cell pellets were collected and stored at -80 °C until they were thawed and used for purification.

To purify all caspase constructs, we employed Ni²⁺-ion affinity chromatography followed by anion exchange. Caspase-3 constructs were purified as previously described wild-type caspase-3 purification protocol. Caspase-6 constructs were purified as previously described procaspase-6 (caspase-6 C163S) purification protocol. Caspase-9 was purified as previously described caspase-9 full-length purification protocol. Purity and concentrations of purified caspases were assessed by SDS-PAGE gel electrophoresis, and aliquots were stored at -80 °C until further usage for different assays.

**Protein substrate digestion and determination of cleavage rate (k)**

Different concentrations (0, 0.125, 0.25, 0.5, and 1 µM) of caspase-9 were individually incubated with 3 µM of an individual caspase-3 or -6 construct in an activity assay buffer (100 mM MES, pH = 6.5, 20% PEG 400, 5 mM DTT) for 1 hour at 37 °C. Each reaction was stopped by adding 1x SDS loading dye (New England Biolabs). These samples were denatured at 90 °C for 10 minutes. Each sample was analyzed using a 16% SDS-PAGE. SDS-PAGE gels were imaged using a ChemiDoc™ MP imaging system (Bio-Rad Laboratories). Band intensities were quantified using Image Lab software (Bio-Rad Laboratories). The cleavage rates of caspase-9 to proteolyze each of the caspase-3 and -6 constructs were determined by following the previously described method.

**Caspase-9 LEHD-ase activity and determination of kinetic parameters**

We followed previously described protocol to derive the LEHD-ase activity of caspase-9. For a substrate titration, 10 µL of fluorogenic substrate, Ac-LEHD-afc (N-acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin; Enzo Life Sciences, Inc.) with concentrations ranging from 0 to 3000 µM were placed into a 96-well black plate. Recombinant caspase-9 (90 µL of 800
nM) in an activity assay buffer (100 mM MES, pH = 6.5, 20% PEG 400, 5 mM DTT) was added into each well to make final volume of 100 µL. Immediately, fluorescence kinetics were measured (λ_ex of 400 nm and λ_em of 505 nm) at 37 ºC for 7 minutes using a microplate reader (SpectraMax M5; Molecular Devices). Initial velocities versus substrate concentrations were plotted to a Michaelis-Menten curve using GraphPad Prism software, and K_m was determined. To derive the exact concentration of caspase-9, an active-site titration was performed using a covalent inhibitor, z-VAD-fmk (carbobenzoxy-Val-Ala-Asp-fluoromethylketone; Enzo Life Sciences, Inc.). For that, 2 µL of z-VAD-fmk (diluted in DMSO) with the concentrations ranging from 0 to 2 mM was added into 96 black-well plate containing 90 µL of 800 nM caspase-9 in an activity assay buffer. The plate was sealed using aluminum foil and incubated at 25 ºC for 1.5 hours. Each aliquot (92 µL) was transferred in a duplicate 96 black-well plate containing 1 mM Ac-LEHD-afc (to make 100 µL as a total volume), and fluorescence kinetics were measured using a microplate reader as done for substrate titration. The exact concentration of caspase-9 (total enzyme concentration, E_T) was determined as the lowest concentration at which full inhibition was observed, and using this value, k_cat was calculated. Finally, caspase-9 LEHD-ase activity (k_cat/K_m) was calculated. Both the substrate titration and active-site titration assays were performed twice using two different aliquots on two separate days.

**Generation of structural models of caspase-9 active-site accommodating peptide substrates**

To generate models of caspase-9 active-site bound to peptides, IETD, DVVD and TEVD as P4-P1 positions, we employed a crystal structure of caspase-9 bound to a peptide substrate, z-EVD-Debmk (benzoxycarbonyl-Glu-Val-Asp-dichlorobenzylmethylketone) (PDB: 1JXQ). We used the mutagenesis feature of PyMOL (Schrödinger, LLC) to substitute the relevant residues. Rotamers of each substituted residue with the minimal clashes were selected to generate models.

**Accession Codes**

UniprotKB: Caspase-3 (CASP3_HUMAN, P42574); Caspase-6 (CASP6_HUMAN, P55212); Caspase-7 (CASP7_HUMAN, P55210); Caspase-8 (CASP8_HUMAN, Q14790); Caspase-9
(CASP9_HUMAN, P55211); Caspase-10 (CASPA_HUMAN, Q92851); ClpP E. Coli. (CLPP_ECOLI, P0A6G7); ClpP S. aureus (CLPP_STAAR, Q6GIM3); ClpP human mitochondria (CLPP_HUMAN, Q16740); ClpP Caulobacter crescentus (CLPP_CAUVN, B8GX16); ClpX Caulobacter crescentus (CLPX_CAUVN, B8GX14).

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References


CHAPTER IV:
DEORPHANIZING CASPASE-3 AND CASPASE-9 SUBSTRATES
IN AND OUT OF APOPTOSIS WITH DEEP SUBSTRATE PROFILING


‡Authors contributed equally.

Authors’ Contributions: JAH obtained funding to support IVS and directed the caspase-9 aspects project. IVS expressed and purified caspase-9 and performed LEHDase assays. IVS prepared all samples for caspase-9 N-terminomics. IVS performed and analyzed all immunoblotting assays. IVS and JAH prepared immunoblotting figures and wrote the manuscript. OJ obtained funding to support LEA. LEA expressed and purified caspase-3 and performed DEVDase assays. LEA prepared samples of caspase-3 N-terminomics. LEA and OJ performed caspase-3 and -9 N-terminomics and analyzed data. LEA and OJ prepared N-terminomics figures, created tables of N-terminomics data, and contributed sections of the manuscript.

Abstract
Caspases are a family of enzymes that regulate biological processes such as inflammation and programmed cell death, through proteolysis. For example, in the intrinsic pathway of apoptosis, cell death signaling involves cytochrome c release from the mitochondria, which leads to the activation of caspase-9 and eventually the executioners caspase-3 and -7. One key step in our understanding of these proteases is to identify their respective protein substrates. Although hundreds of substrates have been linked to caspase-3, only a small handful of substrates have been reported for caspase-9. Employing deep profiling by subtiligase N-terminomics, we present here an unbiased analysis of caspase-3 and caspase-9 substrates in native cell lysates. We identified 906 putative protein substrates associated with caspase-3 and 124 protein substrates for caspase-9. This
is the most comprehensive list of caspase substrates reported for each of these proteases, revealing a pool of new substrates that could not have been discovered using other approaches. Over half of the caspase-9 substrates were also cleaved by caspase-3, but often at unique sites, suggesting an evolved functional redundancy for these two proteases. Correspondingly, nearly half of the caspase-9 cleavage sites were not recognized by caspase-3. Our results suggest that in addition to its important role in activating the executioners, the role of caspase-9 is likely broader and more complex than previously appreciated, which includes proteolysis of key apoptotic substrates other than just caspase-3 and -7 and involvement in non-apoptotic pathways. Our results are well poised to aid the discovery of new biological functions for these two caspases.

**Introduction**

Caspases are a family of cysteine aspartyl proteases involved in cell fate, most notably as being key drivers of apoptosis and pyroptosis.\(^1\,^2\) They also participate in non-apoptotic biological processes, such as tissue differentiation,\(^3\) cell proliferation,\(^4\) and neurodegeneration.\(^5\) Caspases cleave almost exclusively C-terminal to aspartic acid residues, though each caspase has its own highly tuned substrate specificity.\(^6\,^7\) There are 12 human caspases, most of which can be classified as initiator (caspase-2, -8, -9, and -10), executioner (caspase-3, -6, and -7), or inflammatory (caspase-1, -4, -5, and -12), which indicate their role in cell death,\(^8\) while caspase-14 is known to play a role in terminal differentiation of epidermal keratinocytes.\(^9\) In this study, we focus our attention on one executioner, caspase-3, and one initiator, caspase-9. The outcomes from our investigation reveal new roles for both caspases and deorphanize many apoptotic substrates.

Caspase-9 is an initiator caspase that functions upstream of caspases-3/-7. In the intrinsic apoptotic pathway, intracellular injury (such as radiation, chemical insults or growth factor withdrawal) leads to cleavage of BID, triggering a signaling cascade which releases cytochrome c from the mitochondria into the cytoplasm prompting association with apoptotic protease activating factor-1 (apaf-1) to form the apoptosome (**Figure 4.1**).\(^10\) Procaspase-9 binds to the apoptosome and is activated. Activated caspase-9 then cleaves procaspases-3 and -7 at the intersubunit linker
between the large and small subunits, activating both caspases.\textsuperscript{11,12} Procaspase-3 can also be activated via the extrinsic apoptotic pathway. In the extrinsic pathway, pro-apoptotic signals bind to extracellular receptors, leading to formation of the Death Induced Signaling Complex (DISC). Oligomerization and autocatalytic activation of the initiator caspase-8 occurs on the DISC. Once

\textbf{Figure 4.1. Procaspase-3 and -6 share the same core structural fold, and intersubunit linker (ISL) cleavage is a pre-requisite for their activation.}

In healthy cells, cytochrome c is mostly restrained to the mitochondria and XIAP (X-linked inhibitor of apoptosis protein) is blocking caspase function. During apoptosis, the intrinsic pathway is activated by stresses (e.g., radiation, chemical insults, and growth factor withdrawal). This leads to the transcriptional repression of anti-apoptotic proteins, such as BCL-2, BCL-X and MCL1, and to the activation of BH3-only proteins, which will in turn activate BAX and BAK. This causes cytochrome c (pink) and SMAC (blue) (second mitochondria-derived activator of caspase) release from the mitochondria. SMAC interacts with XIAP to prevent caspases inhibition. The cytochrome c associates with apaf1, leading to the formation of the apoptosome, ultimately activating caspase-9. The main established role of the initiator caspase-9 (PDB: 1JXQ) is to activate the executioner caspase-3 (PDB: 1QX3). In this study, we address the specificity of caspase-3 and caspase-9 and report their unique set of respective protein substrates.
activated, caspase-8 can directly activate procaspase-3 by cleavage of the intersubunit linker or indirectly stimulate caspase-3 activation by cleaving BID and inducing caspase-9.\textsuperscript{13}

Knowledge of substrates that caspases can cleave is extensive and yet is still incomplete. Some caspases, including caspase-3 and -7, have hundreds of known substrates.\textsuperscript{14,15} In contrast, little is known about the substrates of caspase-9, other than downstream procaspase-3 and -7 proteolysis. In fact, procaspase-6, which is highly homologous to procaspase-3 and -7, and often likewise classified as an executioner, is not directly activated by caspase-9.\textsuperscript{16–19} The first non-procaspase substrate of caspase-9 identified was vimentin,\textsuperscript{20} which was determined to be cleaved in apoptotic cells. Since then, there have been just six other substrates identified: Semaphorin 7A,\textsuperscript{21} SNX1 and SNX2,\textsuperscript{22} Major Vault Protein,\textsuperscript{23} HDAC7,\textsuperscript{24} and RING2 (also known as RING1B).\textsuperscript{25}

Several methods have been developed over the years to study proteolysis in complex mixtures: subtiligase N-terminomics,\textsuperscript{26} COFRADIC,\textsuperscript{27} TAILS,\textsuperscript{28} and CHOPS.\textsuperscript{29} COFRADIC and subtiligase N-terminomics,\textsuperscript{30} in particular, have been extensively used to study caspases. Subtiligase N-terminomics takes advantage of the enzyme subtiligase, an engineered subtilisin enzyme capable of ligating a peptide ester tag to the N-termini of proteins. This technology is useful in determining proteolytic substrates as \(>80\)% of the N-termini of mammalian proteins are naturally acetylated,\textsuperscript{31} so subtiligase predominantly ligates peptide ester tags to the neo N-termini created by proteolysis. Thus, subtiligase N-terminomics is a powerful technique to detect cleaved proteins in complex mixture. This method can be employed in either a \textit{forward} or a \textit{reverse} mode. Each mode has its own benefits. \textit{Forward} N-terminomics involves inducing a biological process, such as apoptosis, in cells or tissue to generate cleavage products. However, the identification of the proteases responsible for the proteolytic activity is usually unknown. In \textit{reverse} N-terminomics, a native lysate is incubated with an enzyme to generate cleavage products. This experiment generates cleavages which can be attributed to the added enzyme, but their biological roles are not explicitly revealed.
Subtiligase N-terminomics has been utilized to discover substrates for many human caspases, using both forward and reverse methods. The forward mode has been used to study cell death pathways, which led to the creation of the DegraBase, a repository of cleaved substrates in both healthy and apoptotic cells. The reverse mode has been used to profile substrates of caspases-1, 4 and -5, caspase-2 and -6, caspase-7, as well as caspase-3, -7 and -8, but analyzed substrates were limited to those observed under apoptotic conditions in prior forward N-terminomics analysis. These types of analyses have identified critical substrates and opened important new fields of research, such as the discovery of gasdermin D, interdomain cleavage of which was found to be sufficient to trigger pyroptosis. Reverse N-terminomics of caspase-9 was performed more than a decade ago, however, no apoptotic substrates were identified, perhaps due to the lower sensitivity of mass spectrometers at that time, although a few caspase-9 substrates are known, suggesting that other key substrates remain to be identified.

It is abundantly clear that caspases play major controlling roles in many cell death pathways. The designations as apoptotic initiator (upstream) and executioner (downstream) caspases were provided over two decades ago when the molecular underpinnings of apoptosis began to be reported, and little was known about other mechanisms of cell death. During the ensuing decades a great deal about cell death pathways has been uncovered. In addition, tremendous advances in mass spectrometry and biotechnology methods available to assess protease substrates have been developed. This convergence makes possible to address the roles of individual caspases more systematically and more comprehensively, and to understand the complex network of interactions in various pathways of cell death. Thus, the goal of this study has been to assess, using the most sensitive cutting-edge approaches, the interplay, redundancy, and substrate pool of one initiator caspase, caspase-9 and one executioner, caspase-3, to distinguish the roles of each caspase in the myriad of cellular pathways. We present here the most comprehensive datasets of caspase-3 and caspase-9 substrates to date. Importantly, we found more than fifty caspase-9
cleavage sites that are not cleaved by caspase-3, suggesting a unique role of caspase-9 in addition to its known canonical role for activating the executioner caspases, caspase-3 and -7.

Results

We report 906 and 124 protein substrates targeted by caspase-3 and caspase-9, respectively. These substrates were observed by using a subtiligase-based reverse N-terminomics enrichment method (Figure 4.2). For the analyses of caspase-3 and -9 substrates to play the intended role in providing insights into their respective functions, it is critical that we assess the proteolysis of these caspases in a native environment (substrates folded and interactions maintained), while inhibiting activation of endogenous proteases.33,34

Figure 4.2. Deep substrates profiling workflow used to deorphanize caspase-3 and -9 substrates. Subtiligase N-termini enrichment was used to label and capture caspase substrates in Jurkat cell lysates. This reverse N-terminomics workflow involves lysing cells in a buffer containing endogenous protease inhibitors, subsequent incubation with either active caspase-3 or -9, enzymatic substrate labeling with a biotin tag at the N-termini, enrichment on agarose beads, trypsinization, and TEV cleavage to release peptides from the beads. The eluted peptides can then be analyzed via LC-MS/MS. Key to this method is the use of a biotin tag featuring a non-standard residue engineered in the TEV cleavage site (ENLYFQ) with a unique mass, aminobutyric acid (abu), which will remain on the N-termini of proteolytic cleavage sites generated in the lysate, allowing unambiguous identification of caspase cleavage sites.
Using reverse N-terminomics, the activities of caspase-3 and -9 were assessed and optimized in Jurkat lysates (Figure 4.3). The general schematic diagram of reverse N-terminomics is shown (Figure 4.2). During cell lysis, background proteolysis was minimized by the addition of protease inhibitors including iodoacetamide which attenuates the activity of endogenous cysteine proteases such as caspases and cathepsins. Dithiothreitol was subsequently supplemented to neutralize excess iodoacetamide, prior to adding purified caspase. In addition, extra precautions were taken for the initiator caspase-9 assay. Jurkat JMR, a caspase-9 deficient cell line, was used.

![Graph A](image1.png)

**Figure 4.3. Recombinant caspase-9 and -3 are active in cell lysates.**

(A) Optimization of caspase-9 concentration for N-terminomics was performed using a LEHDase activity assays *in vitro* in caspase-9 activity assay buffer. Caspase-9 concentration from 0 to 15 µM was assayed to determine that the optimal LEHDase activity (in RFU/s) for 3 mM of the fluorogenic peptide substrate (Ac-LEHD-afc) is 8 µM of caspase-9. (B) LEHDase activity of caspase-9 is higher in Jurkat JMR cells lysate than in the standard caspase-9 activity assay buffer. The increase of LEHDase activity in Jurkat JMR cells lysate over time is likely due to the activation of endogenous caspases in the lysate including caspase-3/-7/-8. (C) To quench the background caspase activity seen in B, 25 µM of Ac-DEVD-fmk was added in Jurkat JMR cells lysate. This is sufficient to inhibit caspase-3/-7. (D) Relative fluorescence of Ac-DEVD-amc 5 minutes after adding caspase-3 to Jurkat lysate. Caspase-3 activity was measured in Jurkat lysate and compared to the activity in buffer (*in vitro*) and background proteolysis in lysate alone. The results show that caspase-3 activity in lysate is almost as active as in optimal buffer. (E) Endpoint fluorescence assay outputs for all monitored timepoints. DEVDase activity is significantly higher when exogenous caspase-3 is added to the lysate (3x10^3 RFU for lysate alone vs 1x10^5 RFU for induced lysate).
to ensure the measurement of exogenously added caspase-9-cleaved substrates only. To ensure that
the executioners caspase-3/-7 were fully inhibited and not contributing to the observed cleavage, Ac-DEVD-fmk was added to the lysate (Figure 4.3C). Subtiligase and biotin ester peptide tag were then added to label the newly generated N-termini of the cleaved products. Biotinylated protein fragments were then captured on neutravidin beads, trypsinized, and released by TEV cleavage. The eluted peptides were then identified using tandem mass spectrometry (LC-MS/MS) (Figure 4.2). Importantly, the peptides that have been labeled with the biotin ester peptide tag and released by TEV retain a nonstandard amino acid, aminobutyric acid (abu), that allows for unambiguous identification of proteolytic products and precise location of the cleavage sites.

**Caspase-3 and -9 cleave new and expected apoptotic substrates, enabling deorphanization.**

Prior subtiligase N-terminomics analysis for caspase-3 observed 180 substrates linked to apoptosis, whereas no substrates were found for caspase-9. This is possibly due to the low intrinsic activity of caspase-9 and the lower sensitivity of the mass spectrometers used in prior N-terminomics assessments. Thus, all caspase-9 substrates known have been reported via individual biological investigations.

In our N-terminomics analyses across two biological replicates, we found 1126 cleavage sites featuring an aspartate at P1 position (P1 = D) in 906 proteins for caspase-3 (1.2 cleavage sites per protein), and 137 cleavage sites in 124 proteins (1.1 cleavage sites per protein) for caspase-9 (Figure 4.4A). The caspase-3 reverse N-terminomics experiments exhibited a P1 = D cleavage in 46% of its labeled N-termini (1126 out of 2437), while the caspase-9 experiment exhibited 32% (137 out of 428) (Figure 4.4A). This is significantly higher than found in non-treated cell lysate, where we typically observe 6.5% of N-termini featuring a P1 = D, confirming strong caspase substrate proteolysis induced by the addition of exogenous caspase.

We then aligned each P1 = D peptide from the caspase-3 and caspase-9 datasets to determine the specificity of each protease in human cell lysates, where we anticipate that potential
substrates are intact and properly folded (Figure 4.4.B). The caspase-3 cleavage sites revealed a clear DEVD↓(G/S/A) cleavage motif for amino acids P4-P1↓(P1'), (Figure 4.4.B) as expected.

Figure 4.4. Caspase-3 and -9 substrate discovery. (A) We identified 906 caspase-3 protein substrates (1126 peptides featuring an aspartate at P1 position) and 124 caspase-9 protein substrates (137 peptides featuring an aspartate at P1 position). Some of these substrate proteins are cleaved at multiple sites. (B) The iceLogo revealed a clear DEVD↓(G/S/A) motif for caspase-3 and an LESD↓(G/S) motif for caspase-9. (C) Venn diagram showing the overlap between the peptide cleavage sites found in caspase-3 (red) and caspase-9 (grey), showing a set of cleavage sites unique to caspase-9.

The full list of cleavage sites recognized shows considerably greater breadth of recognized sequences than may be reflected in the sequence logo, suggesting that context of the cleavage site, in addition to the sequence, is critical to substrate recognition (Supplemental File S1). The caspase-9 cleavage sites on the other hand revealed a LESD↓(G/S) cleavage motif for P4-P1↓(P1') (Figure 4.4.B), similar to its reported cleavage specificity for P4-P1↓ as LEHD. More importantly, there is no evidence of a DEVD cleavage site motif (Supplemental File S1), indicating any DEVDase activity has been fully blocked by the Ac-DEVD-fmk inhibitor (Figure 4.4.C). Thus, these results (Figure 4.4.B) strongly suggest that we succeeded in inducing selective caspase proteolysis in each of our caspase-3 and caspase-9 experiments, with no or limited contamination from other activated caspases.

To determine which substrates have already been previously observed in apoptosis, we compared our results with the DegraBase, a repository containing >6000 unique N-termini (>1700 caspase cleavage sites) identified in subtiligase-based N-terminomics of cells undergoing apoptosis (Supplemental File S1).14 Derived from previous studies, the DegraBase includes the list of proteolytic substrates from many different inducers of apoptosis, including etoposide, staurosporine, TRAIL, bortezomib and doxorubicin. This important resource does not, however, identify which protease is responsible for each cleavage event. By cross-referencing our substrates with the DegraBase, we found that many of the caspase-3 and -9 substrates identified are cleaved during apoptosis (Supplemental File S1). We can now deorphanize these proteolytic events, linking them to their respective caspases. We found that 577 cleavage sites from caspase-3 (51% of observed cleavages) and 52 cleavage sites from caspase-9 (38% of observed cleavages) had not been previously identified in the DegraBase. We also looked for new substrates that were not previously reported in the DegraBase. We found 257 new caspase-3 and 20 new caspase-9 substrates (Supplemental File S1). This suggests that these new substrates of caspase-3 and -9 may be present at low abundance or play roles in pathways other than apoptosis.
Comparing the results obtained in these experiments, 43% of the cleavage sites (and 40% of the substrates) observed in the caspase-9 reverse N-terminomics experiment were not observed in the caspase-3 experiment (Figure 4.4C). This suggests that many of the substrates and the cleavage sites observed in the caspase-9 reverse experiments are unique to caspase-9, especially considering that caspase-3 is much more active ($k_{cat}/K_m = 7.6 \times 10^5 \text{M}^{-1}\text{s}^{-1}$) than caspase-9 ($k_{cat}/K_m = 3.3 \times 10^3 \text{M}^{-1}\text{s}^{-1}$). Furthermore, we observed none of these caspase-9 cleavages in either of the

**Figure 4.5.** Substrate subcellular localization and pathway enrichment in caspase-3 and caspase-9 reverse experiments. (A and B). Reactome (https://reactome.org) analysis of cellular pathways enriched in either caspase-3 or caspase-9 experiments, with their p-values in parentheses. The number of proteins for each pathway is reported in brackets. Selected examples of pathway members are shown. Bolded outlined proteins indicate substrates found in both caspase-3 and caspase-9 reverse N-terminomics datasets. The full data are provided in Supplementary File S1.
two replicate experiments of caspase-3 N-terminomics. These results are further evidenced by our subsequent characterization (see below).

The distribution of subcellular localization of caspase-3 and -9 substrates is similar, with the majority of substrates being localized to either the cytoplasm or the nucleus. Our N-terminomics method is less suited to detect secreted and membrane proteins, which likely contributes to their lower appearance in our datasets. Several substrate proteins have been reported in more than one subcellular location. Within the caspase-3 dataset, 49% of the substrate proteins have been reported to be present in the cytoplasm, 48% in the nucleus, 6% in the mitochondria, 7% in the endoplasmic reticulum, 7% in the cell membrane, 4% in other organelles and 2% were reported to be secreted. Within the caspase-9 dataset, 47% of substrate proteins were found in the cytoplasm, 55% in the nucleus, 1% in the mitochondria, 6% in the endoplasmic reticulum, 7% in the cell membrane, 4% in other organelles and 2% were found to be secreted. Compared to the proteome, there is a higher proportion of proteins localized in the nucleus or cytoplasm (50% in caspase datasets and 25% in proteome), with lower representation of other subcellular locations (1-10% in datasets and 5-15% in proteome).

We also carried out a Reactome\textsuperscript{50} pathway analysis to identify cellular pathways enriched in our datasets. Across both datasets, pathways with p-values below 5.0x10^{-3} were related to mRNA splicing, RNA metabolism and SUMOylation of proteins, including the expected enrichment of
proteins associated with apoptosis (Figure 4.5A and 4.5B). We observed more enrichment for Notch-HLH transcription and HIV infection pathways in the caspase-3 but not in the caspase-9 dataset (Figure 4.5.A and 4.5.B). For caspase-9, we saw enrichment for mitotic prometaphase, Rho GTPase signaling and membrane trafficking (Figure 4.5.B). Broadly, we observed enrichment of pathways often found in prior caspase N-terminomics analyses.26,33

We also extracted the location of the caspase cleavage sites from the secondary structure of each substrate, if available (Figure 4.6). As expected, the majority of the caspase cleavage sites occur in loop or disordered regions (58% for caspase-3 and 65% for caspase-9), but proteolysis also is observed in regions of α-helices (31% for caspase-3 and 23% for caspase-9) and β-sheets (11% for caspase-3 and 12% for caspase-9) suggesting that local unfolding may also be involved in substrate recognition. We further compared our results to previously published machine learning algorithms used to predict caspase cleavage sites in the human proteome based on protein surface accessibility and secondary structure.51 Overall, almost all observed cleavage sites reported here scored above the average aspartate site found in the proteome (Figure 4.7), supporting the predictions.51
From the N-terminomics results, we individually examined each substrate, and selected ten targets to further investigate the cleavage sites we observed (Table 4.1). The intention in selecting these ten targets was to pick a mixture of cleavage sites within and outside the DegraBase. Some of these cleavage sites were observed only in the caspase-3 experiments, some only in the caspase-9 experiments, and some in both experiments. The aim of these studies is to further understand the unique and overlapping roles of these caspases. We also sought to probe in detail substrates featuring multiple caspase cleavage sites. Moreover, for diversification, we selected substrates that belong to different functional protein families: a DNA helicase, three E3 ubiquitin ligases, a nucleoporin, a pore-forming membrane protein, a GTPase, a kinase, a ribonuclease, and a protein involved in RNA processing. It is important to mention that we reported here all our validation attempts and did not withhold any data.

**Table 4.1: Caspase-3 and -9 N-terminomics substrates selected for deep interrogation**

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>Protein Name</th>
<th>Cleavage site (P1 = D)</th>
<th>Caspase-3</th>
<th>Caspase-9</th>
<th>DegraBase</th>
<th>Localization</th>
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<tr>
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<td>ATP-dependent DNA helicase Q5</td>
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<td>E3 ubiquitin–protein ligase RNF126</td>
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<td>+</td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>GSDMD</td>
<td>Gadd45α-D</td>
<td>D&lt;sup&gt;37&lt;/sup&gt;MQQIQ</td>
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<td></td>
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<td>N</td>
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<td>MFN2</td>
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<td>D&lt;sup&gt;396&lt;/sup&gt;IQLKP</td>
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<td></td>
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<td>+</td>
<td>+</td>
<td>N, C</td>
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<tr>
<td>PAK2</td>
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<td>VGF&lt;sup&gt;38&lt;/sup&gt;DAVTG</td>
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<td></td>
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<td>PARN</td>
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<td>EQTD&lt;sup&gt;98&lt;/sup&gt;ICSAE</td>
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<td>+</td>
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<td>D&lt;sup&gt;38&lt;/sup&gt;ITMVF</td>
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<td>+</td>
<td>+</td>
<td>N</td>
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</tr>
</tbody>
</table>

**Note**: Complete data sets available online in Supplemental File S1. N = nucleus, C = cytoplasm, CM = cell membrane, M = mitochondrion.

**Caspase-9 cleaves distinct substrates from caspase-3.**

As mentioned above, of the 124 caspase-9 substrates observed, 50 of them (43%) were not observed in the caspase-3 experiment (Figure 4.8), although these experiments were run using the same protocol. For further investigation into novel roles of caspase-9, we selected three caspase-9
substrates that are not cleaved by caspase-3: ATP-dependent DNA helicase Q5 (RECQL5), nucleoporin 43 (NUP43), and E3 ubiquitin-protein ligase - Ring Finger Protein 126 (RNF126).

Figure 4.8. RECQL5, RNF126, and NUP43 are the substrates of caspase-9 but not caspase-3. Staurosporine (STS) induced apoptosis results in the proteolysis of RECQL5 but not RNF126 and NUP43.

(A) Domain organization of RECQL5 comprises a helicase domain, zinc-binding domain, wedge domain, IRI domain, internal RNAPII-interacting domain, and SRI domain, Set2-Rpb1-interacting domain. Caspase-9 cleaves at D809 (N-terminomics). (B) RECQL5 is cleaved by recombinant caspase-9 but not caspase-3. (C) Treating Jurkat cells with 0.5 µM of STS for 3 hours revealed that RECQL5 is proteolyzed during apoptosis. (D) RNF126 comprises a NZF domain, N-terminal zinc finger domain and RING (Really Interesting New Gene) domain. Caspase-9 cleaves at D253 (N-terminomics). (E) RNF126 is cleaved by recombinant caspase-9 but not caspase-3. (F) RNF126 is not cleaved during STS induced apoptosis suggesting caspase-9 has a putative non-apoptotic role. (G) NUP43 has seven WD40 repeat (also known as Trp-Asp 40 repeat) domains. Caspase-9 cleaves at D58 (N-terminomics). (H) NUP43 is cleaved by recombinant caspase-9 but not caspase-3. (I) NUP43 is not cleaved during STS-induced apoptosis suggesting caspase-9 has a putative non-apoptotic role. As a loading control, each immunoblot was stripped using a stripping buffer, and then immunoblotted using an anti-GAPDH antibody. Each experiment was performed twice using two different samples on two different days.

RECQL5 is a DNA helicase involved in chromosomal and genome stability, DNA replication and double strand break repair.\textsuperscript{52-54} The full-length RECQL5 can be divided into two
parts: the N-terminal region (primarily responsible for helicase activity) composed of a helicase domain, a zinc-binding domain, and a wedge domain, and the C-terminal region (mainly involved in DNA repair during transcription) containing an internal Pol II–interacting (IRI) domain and a Set2-Rpb1–interacting (SRI) domain (Figure 4.8.A). As was observed in our N-terminomics experiments, RECQL5 was robustly cleaved in Jurkat cell lysates treated with caspase-9, but not caspase-3 (Figure 4.8.B). Importantly, RECQL5 was also cleaved in Jurkat cells treated with the general kinase inhibitor and apoptosis inducer, staurosporine (STS), which led to expected phenotypic changes associated with apoptosis (Figure 4.8.C). From our N-terminomics data, caspase-9 cleaves RECQL5 at D809 removing the SRI domain (Table 4.1 and Figure 4.8.A). To maintain genome stability, the SRI domain of RECQL5 directly interacts with multiple binding partners, such as RNA polymerase I, RNA polymerase II, and proliferating cell nuclear antigen. Thus, caspase-9 cleavage would prevent these interactions. From our N-terminomics data, both RECQL5 and DNA topoisomerase II alpha, which is likewise involved in DNA decatenation and cell cycle progression, were observed to be cleaved by caspase-9 but not caspase-3 (Supplemental File S1). Given the fact that the cell cycle, DNA replication and DNA repair should be halted in the initial stage of apoptosis, our results suggest that caspase-9 also cleaves critical early apoptotic substrates. These data strongly suggested that caspase-9 can act as an executioner, and its role is not limited to only serve as an initiator of apoptosis through cleavage of caspase-3 and -7.

RNF126 is an E3 ubiquitin ligase known to target the p21 tumor suppressor, and as such is considered a potentially useful cancer biomarker or therapeutic target. RNF126 possesses two domains, an N-terminal zinc finger domain and a C-terminal RING (Really Interesting New Gene) domain (Figure 4.8.D). RNF126 was readily identified in the caspase-9 N-terminomics analysis; however, it was not found as a caspase-3 substrate in our analysis nor in the DegraBase (Table 4.1). Consistent with the N-terminomics findings, RNF126 was cleaved by caspase-9, but not by caspase-3 (Figure 4.8.E). In addition, when apoptosis was induced by STS, no RNF126
cleavage was observed (Figure 4.8.F). Thus, RNF126 could be a new non-apoptotic substrate of caspase-9. One of the known functions of RNF126 is that its RING domain directly interacts and ubiquitinates AICDA (activation-induced cytidine deaminase), an enzyme that deaminates deoxycytidines in single-stranded DNA. The exact outcome of this ubiquitination (whether AICDA is degraded or not) remains to be determined in vivo. AIDCA is predominantly expressed in germinal centers, and as an immune response, it produces and distributes high affinity antibodies against foreign antigens. Because caspase-9 cleaves RNF126 in the RING domain at D253 (Table 4.1 and Figure 4.8.D), this cleavage is likely to disrupt the ability of RNF126 to ubiquitinate AICDA.

While a number of nucleoporins have previously been reported as caspase substrates, NUP43, a component of the nucleoporin complex (NPC), was also identified as a new substrate of caspase-9 that had not been observed in previous studies. Cleavage of nucleoporins is critical as it allows entry of caspases lacking a nuclear localization signal into the nucleus. NUP43 is composed of seven WD40 repeat domains, WD1 to WD7 (Figure 4.8.G). Although caspase-9 was robustly able to cleave NUP43 in Jurkat cell lysates, no cleavage by caspase-3 was observed (Figure 4.8.H). These findings are consistent with our N-terminomics analyses (Table 4.1). In contrast, when apoptosis was induced by STS, no NUP43 cleavage was observed (Figure 4.8.I) suggesting that NUP43 is not an apoptotic substrate cleaved in STS-treated cells. Induction of apoptosis by etoposide has been previously shown to result in cleavage of other nucleoporins including NUP93 and NUP96, but also did not result in cleavage of NUP43. This provides increased evidence that NUP43 could be a substrate of caspase-9 under non-apoptotic conditions. The caspase-9 cleavage of NUP43 occurs in the WD1 domain at D58 (Table 4.1 and Figure 4.8.G). In the NPC, NUP43 has been shown to interact with other nucleoporins, NUP85 and Seh1. Therefore, it remains to be discovered how the overall structure and function of NPC are changed upon caspase-9 cleavage of NUP43.
The majority of caspase-3 substrates are not recognized by caspase-9.

Figure 4.9. GSDMD, MFN2, RNF4, and PAK2 are the substrates of caspase-3 but not caspase-9, and are proteolyzed during STS induced apoptosis.

(A) GSDMD is composed of two domains: GSDMD-N (N-terminal domain also known as pore forming domain) and GSDMD-C (C-terminal domain also known as auto-inhibitory domain). N-terminomics reveals caspase-3 cleavage at D87 and D275. (B) GSDMD is cleaved by recombinant caspase-3 but not caspase-9. (C) Jurkat cells treated with 0.5 µM of STS for 3 hours revealed that GSDMD is proteolyzed during apoptosis. (D) MFN2 has a GTPase domain, HR1 (first coiled-coil heptad repeat region), PR (proline-rich) domain, TM (transmembrane) domain, and HR2 (second coiled-coil heptad-repeat region) domain. N-terminomics revealed caspase-3 cleavage at D499. (E) MFN2 is cleaved by recombinant caspase-3 but not caspase-9. (F) MFN2 is cleaved during STS-induced apoptosis. (G) RNF4 has four tandem SIMs [SUMO (small ubiquitin-like modifier)-interaction motifs] in the N-terminal domain between residues 32-82, and a RING domain at the C-terminal. N-terminomics revealed caspase-3 cleavage at D89 and D137. (H) RNF4 is cleaved by recombinant caspase-3 but not caspase-9. (I) RNF4 is cleaved during STS-induced apoptosis. (J) PAK2 is composed of two domains: Auto-inhibitory (regulatory) and kinase domains. N-terminomics revealed caspase-3 cleavage at D89 and D148. (K) Immunoblot analysis resembled N-terminomics data that PAK2 is cleaved by recombinant caspase-9 but not caspase-3. (L) PAK2 is proteolyzed during STS-induced apoptosis. As a loading control, each immunoblot was stripped using a stripping buffer, and then immunoblotted using an anti-GAPDH antibody. Each experiment was performed twice using two different samples on two different days.
Of the 906 substrate proteins cleaved by caspase-3 in our analysis, 832 of the proteins were not cleaved by caspase-9. We selected four unique caspase-3 substrates for further analysis: gasdermin D (GSDMD), mitofusin 2 (MFN2), E3 ubiquitin-protein ligase - RING finger protein 4 (RNF4), and serine/threonine protein kinase PAK 2 (PAK2). All of these substrates were also cleaved at the expected sites when apoptosis was initiated by STS in Jurkat cells (Figure 4.9), strongly suggesting that these proteins are bonafide apoptotic caspase-3 substrates.

GSDMD, a pore-forming membrane protein, controls membrane permeabilization during pyroptosis. GSDMD is composed of two domains, GSDMD-N (N-terminal pore-forming domain) and GSDMD-C (C-terminal auto-inhibitory domain) (Figure 4.9A). The full-length GSDMD remains inactive by an autoinhibitory mechanism due to the presence of GSDMD-C. To induce pyroptosis, GSDMD is recognized by the inflammatory caspases (caspase-1, -4, -5 and -11) which cleave a linker between GSDMD-N and GSDMD-C at D275. This cleavage facilitates GSDMD-N domains to oligomerize and form pores in the cell membrane. In contrast, prior work has shown that GSDMD is readily cleaved by caspase-3 in GSDMD-N at D87, instead of D275. Cleavage of GSDMD-N at D87 by caspase-3 is critical for faithful execution of apoptosis, as it is sufficient to prevent pyroptosis. Our N-terminomics datasets showed that GSDMD can be cleaved by caspase-3 at both D87 and D275, consistent with both sites reported in the DegraBase (Table 4.1). Mirroring the N-terminomics results, we also observed cleavage of GSDMD in Jurkat lysates incubated with caspase-3 but not caspase-9 (Figure 4.9B). GSDMD was also proteolyzed during STS-induced apoptosis (Figure 4.9C).

MFN2 is present in the outer mitochondrial membrane and is essential for fusion of mitochondria. MFN2 has also been implicated in the regulation of mitochondrial metabolism, apoptosis, shape of other organelles (e.g. endoplasmic reticulum), and cell cycle progression. MFN2 possesses a GTPase domain, a first coiled-coil heptad-repeat region domain, a proline-rich domain, transmembrane domains, and a second coiled-coil heptad-repeat region domain (Figure 4.9D). MFN2 is cleaved by caspase-3, but not caspase-9 (Table 4.1 and Figure 4.9E). Cleavage
of MFN2 in Jurkat cells after induction of apoptosis (Figure 4.9.F) further suggests that MFN2 is a bonafide apoptotic substrate solely of caspase-3, although this cleavage has not been previously reported in earlier studies (Table 4.1). Caspase-3 cleaves MFN2 at D499 (Table 4.1 and Figure 4.9.D). This cleavage severs the GTPase domain from the second coiled-coil heptad-repeat region domain, important components of MFN2 required to initiate and induce the fusion of mitochondria (Figure 4.10).83–85 Thus, caspase-3 cleavage of MFN2 can prevent mitochondrial fusion. Another role of MFN2 is that it interacts with BAX (Bcl-2-associated X protein) under non-apoptotic conditions, preventing apoptosis.86 Furthermore, reduction of MFN2 levels has been shown to render cells more sensitive to mitochondrial Ca2+-dependent cell death.87 Thus, we anticipate that caspase-3-mediated cleavage of MFN2 likewise increases the release of cytochrome c, evoking apoptosis.

RNF4 is an E3 ubiquitin ligase that recognizes SUMO-modified proteins and degrades them via ubiquitination.88 RNF4 accumulates at the foci of DNA double strand break repair, so its deficiency leads to increased DNA damage.89 RNF4 can be divided into two parts: the N-terminal region possessing four tandem SUMO-interacting motifs (SIMs) and the C-terminal RING domain (Figure 4.9.G).90–92 We identified RNF4 as a substrate of caspase-3, but not of caspase-9 (Table 4.1 and Figure 4.9.H). Moreover, RNF4 was found to be cleaved during STS-induced apoptosis in Jurkat cells (Figure 4.9.I). RNF4 was cleaved by caspase-3 at D89 and D137 (Table 4.1 and Figure 4.9.G) causing the removal of the N-terminal region that recognizes SUMO-modified proteins. The majority of SUMO-modified proteins regulated by RNF4 are involved in nucleic acid metabolism.

Figure 4.10. MFN2 cleavage by caspase-3 can disrupt mitochondrial fusion.
(A) Linear schematic of MFN2 illustrating domains, GTPase, HR1 (first coiled-coil heptad-repeat region), HR2 (second coiled-coil heptad-repeat region), and caspase-3 cleavage site D499. Caspase-3 cleavage removes the HR2 domain. (B) Schematic diagram of mitochondrial fusion mediated by MFN2. The fusion activity is regulated by MFN2, where tethering occurs due to the dimerization of HR2-HR2 domains and GTPase-GTPase domains of two individual mitochondria-MFN2 complexes. Thus, this is a clear mechanism by which caspase-3 cleavage can impact the tethering and therefore the fusion activity of MFN2 can be envisioned.
with a particular emphasis on SUMOylation, transcription, DNA repair, and chromosome segregation. Since these types of cellular procedures must be halted during apoptosis, it is understandable that RNF4 emerged as an apoptotic substrate of caspase-3.

PAK2 is known to play a role in regulating apoptosis through reciprocal interactions with caspase-7. PAK2 is composed of two domains, an auto-inhibitory domain and a kinase domain (Figure 4.9.J). PAK2 was identified as a substrate of caspase-3, but not of caspase-9 (Table 4.1 and Figure 4.9.K). Moreover, it was proteolyzed after STS-induced apoptosis in Jurkat cells (Figure 4.9.L). In its full-length form, PAK2 stimulates cell survival by the phosphorylation and inactivation of caspase-7. PAK2 is also a known substrate of caspase-3 and -7. Cleavage of PAK2 by caspase-3 or -7 at D212 removes the autoinhibitory domain. As a result, the kinase domain translocates from the cytoplasm to the nucleus, and phosphorylates a new set of substrates contributing to apoptosis. Intriguingly, in the caspase-3 N-terminomics analysis, we did not observe cleavage at D212, but rather observed two cleavages at D89 and D148 (Table 4.1 and Figure 4.9.J). These results appear to be in line with our observation of PAK2 cleavage in vitro which demonstrates that PAK2 is such an excellent substrate of caspase-3 that it completely disappears on the immunoblot after incubation with caspase-3 (Figure 4.9.K) as well as during STS-induced apoptosis (Figure 4.9.L).

Caspase-3 and -9 share some common substrates.

Of the 906 caspase-3 substrate proteins and 124 caspase-9 substrate proteins, 74 were cleaved by both caspases. These 74 represent 57% of all caspase-9 substrate proteins identified, suggesting that there is significant redundancy between caspase-9 and -3 substrates. We selected three common substrates of caspase-3 and -9, poly (A)-specific ribonuclease (PARN), ataxin-2-like protein (ATXN2L), and E3 ubiquitin-protein ligase RING1, for further investigation.

PARN is a deadenylating nuclease that regulates mRNA turnover and non-coding RNA maturation. PARN possesses three well-structured RNA binding domains (catalytic nuclease domain, an R3H domain, and an RRM domain) and an intrinsically disordered C-terminal
domain (CTD) (Figure 4.11.A).\textsuperscript{101,102} From our N-terminomics analyses, PARN was observed to be a substrate of both caspase-3 and -9, in which both caspases cleaved PARN in the CTD at the same site, D595 (Table 4.1 and Figure 4.11.A). These results mirrored our immunoblotting analysis (Figure 4.11.B). The CTD interacts with the other regions of PARN and enhances the

Figure 4.11. PARN, ATXN2L, RING1 are the substrates of both caspase-9 and -3, and they are proteolyzed during STS induced apoptosis.

(A) PARN is composed of a catalytic nuclease domain (gray regions), R3H domain, RRM (RNA recognition motif) domain, and CTD, C-terminal domain. Caspase-9 cleaves at D595 (N-terminomics). (B) PARN is cleaved by recombinant caspase-9 and -3. (C) Treating Jurkat cells with 0.5 µM of STS for 3 hours revealed that PARN is proteolyzed during apoptosis. (D) ATXN2L comprises a LSm (like-Sm protein) domain, LSmAD (LSm associated domain) and PAM2 (PABP-interacting motif 2) domain. N-terminomics revealed that caspase-9 cleavage occurs at D246, and caspase-3 cleavage occurs at D181 and D584. (E) ATXN2L is cleaved by recombinant caspase-9 and -3. (F) ATXN2L is cleaved during STS induced apoptosis. (G) RING1 comprises a RING domain, and a Ring finger and WD40 Ubiquitin-Like Domain. N-terminomics revealed that caspase-9 cleavage occurs at D246, and caspase-3 cleavage occurs at D193 and D584. (H) RING1 is cleaved by recombinant caspase-9 and -3. (I) RING1 is cleaved during STS-induced apoptosis. As a loading control, each immunoblot was stripped using a stripping buffer, and then immunoblotted using an anti-GAPDH antibody. Each experiment was performed twice using two different samples on two different days.
overall thermal stability of this protein. Moreover, the CTD of PARN contains a nucleolar localization signal (residues: 598-624), and interacts with the nuclear non-coding RNAs in response to DNA damage. Thus, PARN cleavage by caspase-3 and -9 at D595 can not only prevent PARN access to the nucleolus but also destabilize the protein. Cleavage in the CTD appears to be crucial, as both caspase-3 and -9 execute this apoptotic role (Figure 4.11.B), and it is cleaved during STS-induced apoptosis (Figure 4.11.C). Deficiency in PARN leads to shortening of telomeres. Thus, PARN inactivation would likewise be associated with the DNA fragmentation that is observed during apoptosis.

ATXN2L, a component of stress granules, plays a role in RNA processing, and possesses three domains: LSm domain, LSmAD domain, and PAM2 domain (Figure 4.11.D). ATXN2L is cleaved by both caspase-3 and -9, which recognize different cleavage sites: D181 and D584 for caspase-3 and D246 for caspase-9 (Table 4.1 and Figure 4.11.D). These outcomes from N-terminomics analyses mirrored our immunoblotting results (Figure 4.11.E). Moreover, ATXN2L was readily proteolyzed during STS-induced apoptosis demonstrating that it is an apoptotic substrate (Figure 4.11.F). ATXN2L appears to play a similar role to its paralog, Ataxin-2, as it interacts with Ataxin-2 itself and with Ataxin-2 interacting proteins, an RNA helicase, DDX6 (perhaps through the LSm and LSmAD domains) and Poly(A)-binding protein, PABP (perhaps through the PAM2 domain). Caspase-9 cleavage at D246 removes the LSm domain of ATXN2L (Table 4.1 and Figure 4.11.D) which may prevent ATXN2L interactions with DDX6. Since caspase-3 cuts ATXN2L at two distinct sites, D181 and D584 (Table 4.1 and Figure 4.11.D), these cleavages may disrupt the ability of ATXN2L to interact with DDX6 as well as PABP. Since RNA helicases (e.g., DDX6) are involved in the production of virtually all RNA types, targeting ATXN2L provides a means to block RNA production and function globally in roles including translation. These analyses underscore the observation that redundancy for key apoptotic substrates (such as global regulators of RNA metabolism) may be built into multiple caspases.
RING1 (also known as RING1A) is an E3 ubiquitin ligase that we observed to be a substrate of both caspase-3 and -9, which cleave RING1 at independent sites (Table 4.1). RING1 is cleaved by caspase-3 at D189 and by caspase-9 at D193 (Table 4.1 and Figure 4.11.G) which are both between the RING domain and a ubiquitin-like domain. We observed RING1 cleavage in our in vitro cleavage assay for both caspase-3 and -9, correlating with our N-terminomics data (Figure 4.11.H). RING1 is known to degrade p53 protein causing proliferation of cancerous cells. For this reason, RING1 is perhaps an unsurprising apoptotic substrate (Figure 4.11.I). Caspase cleavage of RING1 should protect p53 from degradation resulting in the needed ability to induce apoptosis. RING2 (also known as RING1B), which is highly homologous to RING1, was reported as a direct substrate of caspase-3 (cleaves at D175) and caspase-9 (cleaves at D208). Interestingly, these cleavages are also occurring between the RING domain and the ubiquitin-like domain of RING2. These cleavages by caspase-3 and -9 lead to the redistribution of RING2 from nuclear localization to even distribution throughout the entire cell. The N-terminomics identification of RING1 as a substrate of caspase-3 and -9 (cleaving at different sites), may suggest that RING1 cleavage may lead to similar impacts on cellular localization. As was the case for ATXN2L, it is tempting to speculate that a key role for RING1 in apoptosis led to evolution of cleavage sites for redundant cleavage by both caspase-3 and -9. In addition, the observation that these two caspases cleave at different sites within the same local region (D189/193) further underscores the importance of this cleavage event, perhaps even under different mechanisms of cell death that engage caspases uniquely.

**Discussion**

In this study, we sought to discover and compare all possible caspase substrates cleaved by the executioner caspase-3 and the initiator caspase-9. We report 906 and 124 protein substrates targeted by caspase-3 and caspase-9, respectively. Of the 124 caspase-9 substrates, 50 of them were not observed in the caspase-3 experiment. Our results clearly show that caspase-3 and caspase-9 possess both common and distinct pools of protein substrates. We found that some of these
substrates are cleaved during apoptosis, while others are not, suggesting new non-apoptotic roles in the biology of these two caspases.

A strength of the reverse N-terminomics approach is that it allows identification of new substrates that could not be identified by other means. GSDMD, MFN2, RNF4, and PAK2 are all substrates of caspase-3, but not caspase-9 (Table 4.1 and Figure 4.9). Neither RNF4 nor MFN2 had been observed as apoptotic substrates previously. GSDMD was cleaved by caspase-3 (Table 4.1 and Figure 4.9) and showed detectable cleavage product in apoptotic cells (Figure 4.9), consistent with the DegraBase. In our immunoblot analyses, PAK2 and RNF4 seemed to be both fully degraded when incubated with caspase-3 and during STS-induced apoptosis, as no cleavage products were observed (Figure 4.9). However, reverse N-terminomics provided an exact location of the cleavage sites. We likewise interrogated three unique caspase-9 substrates, RECQL5, NUP43, and RNF126. We observed RECQL5 proteolysis during STS-induced apoptosis, but no cleavage of NUP43 and RNF126 (Figure 4.8), indicating that caspase-9 plays putative non-apoptotic roles involving these substrates. Detection of PARN, ATXN2L and RING1 as the common substrates of caspase-3 and -9, again illustrates advantages of employing reverse N-terminomics. Both ATXN2L and RING1 were reported as apoptotic substrates in the DegraBase; nonetheless, it is our reverse N-terminomics analyses which revealed they are proteolyzed by caspase-3 and -9, at distinct cleavage sites. Our analyses also discovered new caspase cleavage sites for these three substrates, PARN (D595 by caspase-3 and -9), two additional cleavage sites of ATXN2L (D181 by caspase-3 and D246 by caspase-9) and one additional cleavage site of RING1 (D189 by caspase-9) (Table 4.1). Together these observations underscore the complementarity of both forward and reverse N-terminomics experiments to capture the nuanced suite of substrates of these proteases.

The comprehensive list of caspase-3 and caspase-9 substrates we provide here allowed us to finally deorphanize more than a thousand caspase substrates (Supplemental File S1). Not surprisingly, hundreds of them have been identified before as apoptotic substrates (649 out of 906
for caspase-3 and 103 out of 124 for caspase-9). However, until now, it was unknown which caspase was most responsible for these proteolytic events. Not surprisingly, the executioner caspase-3 cleaves hundreds of apoptotic substrates. The fact that prior studies did not identify any caspase-9 substrates, and our literature search only found seven non-caspase substrates, our work in deorphanizing more than hundred caspase-9 substrates represents a significant milestone in the field of caspase-9 biology. Our findings clearly demonstrate that the role of caspase-9 is not only to activate caspase-3 and -7, but also to target its own set of protein substrates.

We curated the caspase-9 substrate list, searching the literature for any references to caspase or apoptosis. Of the 124 substrate proteins (137 cleavage sites) from N-terminomics, we found literature references for only 28 proteins which are reported as caspase substrates and/or are involved in apoptosis (Table S1; This table is available in the supporting PDF of Araya, L. E., Soni, I. V., Hardy, J. A, and Julien, O. Deorphanizing Caspase-3 and Caspase-9 Substrates In and Out of Apoptosis with Deep Substrate Profiling. ACS Chemical Biology 16, 11, 2280-2296 (2021.). In contrast, of those 124 caspase-9 substrates, only 20 were not in the DegraBase (Supplemental File S1), which catalogues apoptotic substrates derived from 33 different experiments ranging over 7 different apoptosis inducers in 5 independent cell lines, reporting a total of 1706 cleavage sites in 1268 proteins. Thus, most of the caspase-9 substrates identified in this study (84%) are, in fact, apoptotic substrates that were orphaned prior to this work. It is possible or even likely that proteolysis of the 20 proteins that were not found in the DegraBase (Supplemental File S1) is mediated by caspase-9 in a non-apoptotic context. Of those 20 proteins, that were not found in the DegraBase (Supplemental File S1), references in the literature were present for only 3 substrates (ATP-dependent DNA helicase Q5, chromosome transmission fidelity protein 18 homolog, and poly(A)-specific ribonuclease PARN) as a caspase substrate and/or involved in apoptosis (Table S1), underscoring the fact this analysis had contributed to identify 17 entirely new caspase substrates which are proteolyzed by caspase-9 (Table S2; This table is available in the supporting PDF of Araya, L. E., Soni, I. V., Hardy, J. A, and Julien, O. Deorphanizing Caspase-3

The discovery and deorphianization of more than a hundred new caspase-9 substrates provides a critical repository of information of other functions that caspase-9 activation plays, in addition to its role as a canonical apoptotic initiator. Interestingly, caspase-9 has been implicated in a non-apoptotic form of cell death, called paraptosis. Paraptosis, an Apaf-1-independent but caspase-9-dependent form of programmed cell death, was first termed over two decades ago, which can occur during development and neurodegeneration. It was shown that human insulin-like growth factor I receptor (IGFIR) stimulates paraptosis in HEK293T cells as well as in mouse embryonic fibroblasts. Caspase-9, but not caspase-3/-7, was also shown to play a non-apoptotic role in primitive erythropoiesis. Thus, it is possible that some of the caspase-9 substrates we identified play crucial roles in non-apoptotic pathways such as paraptosis and/or primitive erythropoiesis.

We also investigated 74 substrates that were cleaved by both caspase-3 and caspase-9. These proteins were cleaved at the same site by both caspases in 45% of the cases, whereas 55% were cleaved at different sites. Amongst those 74 substrates, 35 were cleaved at just one site by caspase-3 whereas 29 were cleaved at two sites and 10 were cleaved at three or more sites (such as enhancer of mRNA-decapping protein 4, which is cleaved at six sites). In contrast, 64 of the overlapping 74 substrates were cleaved at just one site by caspase-9, whereas 9 were cleaved at two sites and only 1 substrate (U2 snRNP-associated SURP motif-containing protein) was cleaved at four sites. The canonical view is that caspase cleavage at a single site leads to changes in function or localization that contribute to apoptosis. The observation of large numbers of cleavage sites in a single substrate begs the question of whether caspases play degradative roles for some key substrates.

We also observed that multiple members of the same functional complex are often targeted by caspases. For example, it’s been shown that the proteasome, the condensin I complex and the
spliceosome are heavily targeted by caspases during apoptosis. Consistently, we found an enrichment for RNA splicing in both sets of caspase-3 and caspase-9 substrates. We also found that caspase-3 and caspase-9 can cleave the same substrate in the same region, but at different aspartate residues (e.g., RING1 is cleaved at D189 by caspase-9 and at D193 by caspase-3). Similarly, synapse associated protein 1 (SYAP1) was shown to be cleaved by caspase-1 at D278 and caspase-3 and -7 at D281, with a cleavage site motif, FVSD↓AFD↓A. Interestingly, in this study, we found that SYAP1 is also cleaved by caspase-9 at the same site as caspase-1 (D278). We hypothesize that this redundancy, cleavage by different caspases that function in different biological pathways at adjacent and therefore likely functionally similar sites, may provide a means of identifying proteolytic events that are critical in multiple contexts.

To conclude, our study has deorphanized hundreds of caspase-3 and -9 protein substrates. Most of these proteolytic events obviously play key roles in apoptosis, induced by caspase-3 and/or caspase-9 proteolysis. However, we anticipate that these datasets will provide a powerful resource for the future investigation of the roles of these caspases in apoptotic as well as non-apoptotic pathways.

**Materials and Methods**

**DNA plasmids.**

The expression construct for WT caspase-3 (pET23b-Casp3-His) was a gift from Guy Salvesen, and obtained from Addgene (plasmid 11821). The expression construct for WT Caspase-9 (pET23b-Casp9-His) was a gift from Guy Salvesen, and obtained from Addgene (plasmid 11829).

**Protein expression and purification.**

Recombinant His-tagged caspase-3 was expressed in BL21 (DE3) pLysS E. coli. One colony was added to 50 mL 2xYT media supplemented with ampicillin and chloramphenicol. The following morning, 25 mL of the starter culture was used to inoculate 3 L of 2xYT (Fisher Scientific) supplemented with 100 μg/mL ampicillin and 12.5 μg/mL chloramphenicol at 37°C until
the O.D. of 0.6 was reached. We then induced expression for 5 h at 30°C using 0.3 mM IPTG (Fisher Scientific). Cells pellets were collected by centrifugation of the culture at 4,000 g for 20 minutes at 4°C. The supernatant was discarded, and the pellet was frozen at -80°C. The following day, the pellet was thawed on ice and resuspended in 45 mL of Lysis buffer (100 mM NaCl and 100 mM Tris pH 8.0). Cells were lysed through high-pressure homogenization (Avestin Emusiflex C3), then centrifuged at 40,000 g for 45 minutes at 4°C. The clarified protein supernatant was passed through a 1-mL Ni\(^{2+}\) affinity column (Cytiva Inc.), following which protein was eluted from the column using a linear gradient (Elution buffer: 500 mM NaCl, 20 mM imidazole and 100 mM Tris pH 8.0). The eluted protein purity was confirmed via SDS-PAGE. Eluted fractions were pooled and buffer exchanged through size exclusion chromatography (HiLoad 16/600 Superdex 200 pg, Cytiva Inc.), eluting in storage buffer (100 mM NaCl, 25 mM Tris pH 7.5, and 2 mM DTT). Fractions were assayed for DEVDase activity. The fractions with the highest activity were pooled and concentrated using a 10K MWCO filter spin concentrator (Cytiva Inc.)

The DNA plasmid encoding caspase-9 was transformed into BL21 (DE3) T7 express strain of E. coli (New England Biolabs), and plated on a LB+agar petri dish to grow overnight at 37°C. We picked a single colony and transferred it into a flask containing 50 mL LB media (Research Products International). We incubated this media at 37°C overnight to grow a seed culture. Next day, we diluted 1 mL of the seed culture one thousand times using 1 L of LB media containing 0.1 mg/mL ampicillin (Fisher BioReagents). We incubated this culture at 37°C until the desired O.D. of 1.0. We induced the culture by adding added IPTG (GoldBio) to a final concentration of 1 mM and lowered the temperature to 25°C for 4 hours. We centrifuged this culture at 5000 g for 7 minutes at 4°C, and cell pellets were collected and stored at -80°C until their use for purification. The frozen pellet was thawed and diluted in 200 mL of lysis buffer (50 mM Sodium Phosphate pH 7.0, 300 mM NaCl, 5% glycerol, and 2 mM Imidazole). Cells were lysed using a microfluidizer (Microfluidics, Inc.), and cell lysate was separated from the cell debris by centrifugation at 50,000 g for 1 hour at 4°C. Cell lysis was purified using a Hi-Trap™ chelating HP column charged with
Ni\textsuperscript{2+} (Cytiva inc.). We performed a linear gradient (0 to 33% of elution buffer) and eluted protein by using an elution buffer (50 mM Sodium Phosphate, pH = 7.0, 300 mM NaCl, 5% glycerol, and 300 mM Imidazole). Eluted protein was diluted 5 times using buffer A (20 mM Tris pH 8.5, 5% glycerol, and 2 mM DTT). Then, for further purification by anion exchange chromatography, we applied protein to Hi-Trap\textsuperscript{TM} Q HP column (Cytiva inc.). The column was washed and then developed with a linear gradient (0 to 30% of buffer B: 20 mM Tris pH 8.5, 1 M NaCl, 5% glycerol, and 2 mM DTT). Purity and concentration of purified caspase-9 were assessed using SDS-PAGE. The most concentrated fractions were aliquoted to store at -80°C for further usage.

**Mammalian cell culture.**

Jurkat cells were used for caspase-3 experiments, while Jurkat JMR (caspase-9 knockout) were used for caspase-9 experiments.\textsuperscript{43} Caspase activity was assayed in lysate compared to buffer (Figure 4.3) (see caspase activity assays *in vitro* and in cell lysate for detailed procedures). Jurkat and Jurkat JMR cell pellets were thawed from frozen stocks and grown in RPMI cell culture media (Gibco), supplemented with 10% fetal bovine serum (Sigma inc.), 100 µg/mL penicillin/streptomycin (Gibco) and 2 mM L-glutamine (Gibco). Cells were grown at 37°C, passing stepwise from growing in culture dishes to 4 L spinner flasks. Cells were harvested by centrifugation at 800 g for 5 minutes, washed with cold PBS and centrifuged again at 800 g for 5 minutes. Pellets were kept frozen at -80°C until required for reverse N-terminomics experiments.

**Caspase activity assays *in vitro* and in cell lysate.**

The caspase-3 activity in cell lysates was monitored over the course of the N-terminomics experiment by measuring DEVDase activity using an Ac-DEVD-aflc fluorogenic probe (excitation/emission at 400 nm/505 nm). This assay was initiated upon addition of 0.5 µM of recombinant caspase-3 to the Jurkat lysate. Aliquots were removed and assayed at 5 minutes, 1 hour and 2 hours (see caspase-3 reverse N-terminomics for lysate preparation conditions).

We analyzed the LEHDase activity of caspase-9 *in vitro* (activity assay buffer: 100 mM MES pH 6.5 and 10 mM DTT) to optimize the concentration needed for cell lysate experiments. Caspase-9
concentrations ranging from 0 to 15 µM were prepared in a 96-well black plate using activity assay buffer. We immediately transferred 3 mM of caspase-9 fluorogenic peptide substrate, Ac-LEHD-afc (Enzo Life Sciences, Inc.). The lowest concentration at which we observed an acceptable signal to noise ratio was 8 µM of caspase-9; therefore, we used that concentration for all subsequent assays performed in cell lysates. We lysed 2.4 x 10^6 Jurkat JMR cells using a lysis buffer (5 mM EDTA, 1 mM PMSF, 4 mM Iodoacetamide (IAM), 1 mM AEBSF and 0.1% Triton X-100 in 100 mM HEPES pH 7.4) on ice for 30 minutes. Cell lysate was separated from cell debris by centrifugation at 4,000 g for 15 minutes. Then, cell lysate was incubated with 8 µM of caspase-9 in activity assay buffer for 4 hours. Meanwhile, we examined LEHDase activity of caspase-9 in cell lysate at 0, 0.5, 1, 2, and 4 hours. To quench DEVDase activity (that is expected to be generated by an activation of endogenous caspase-3/-7 by added recombinant caspase-9), we supplemented the lysis buffer with 25 µM Ac-DEVD-fmk (Enzo Life Sciences, Inc.), and then examined both DEVDase and LEHDase activity.

**Caspase-3 reverse N-terminomics lysate preparation.**

7.5 x 10^8 harvested Jurkat cells were thawed on ice and resuspended in lysis buffer (5 mM EDTA, 1 mM PMSF, 4 mM IAM, 1 mM AEBSF and 0.1% Triton X-100 in 100 mM HEPES pH 7.4). Sample was incubated for 45 minutes in the dark at room temperature, then sonicated using a probe tip sonicator at 20% amplitude, 2 seconds on 5 seconds off for 5 minutes. 20 mM DTT was added to quench the IAM, to retain the activity of the caspase-3 that is added subsequently. Total protein concentration in the cell lysate was determined to be 7 mg/mL using a Bradford assay kit (Bio-Rad Laboratories). The lysate was centrifuged for 10 minutes at 4,000 g to remove cell debris. A concentrated caspase activity buffer (10x) was added to the lysate (for final concentrations of 10 mM HEPES pH 7.4, 50 mM KCl, 1.5% sucrose, 0.1% CHAPS, and 10 mM DTT). Lysates were incubated with 0.5 µM caspase-3 in the dark at room temperature for 2 hours and assayed for DEVDase activity. After 2 hours had elapsed, enzyme activity was irreversibly inhibited by treating
with 100 µM z-VAD-fmk which quantitatively blocks the function of all caspases. We then proceeded with N-terminomics labeling (see below).

Caspase-9 reverse N-terminomics lysate preparation.

We grew and harvested 5 x 10^8 of Jurkat JMR cells. To lyse the cells, we directly suspended the cell pellet (without freezing) in a lysis buffer (100 mM HEPES pH 7.4, 5 mM EDTA, 1 mM PMSF, 4 mM IAM, 1 mM AEBSF, 25 µM Ac-DEVD-fmk, and 0.1% Triton X-100), and then incubated on ice in the dark for 30 minutes. Total protein concentration in the cell lysate was determined to be 10 mg/mL using a BCA assay kit (ThermoFisher Scientific). We added 20 mM of DTT to the cell lysate to quench the IAM. The lysate was centrifuged for 10 minutes at 4,000 g to remove cell debris. A concentrated caspase-9 activity assay buffer (for final concentrations of 100 mM MES, pH = 6.5, and 10 mM DTT) was added to the lysate, following which 8 µM purified caspase-9 was added. Lysates were incubated with caspase-9 in the dark at room temperature for 4 hour and assayed for LEHDase activity. Then, the enzyme activity in treated lysates was irreversibly inhibited by treating with 100 µM z-VAD-fmk. We stored this sample at -80°C for the further usage in reverse N-terminomics.

Caspase-3 and -9 reverse N-terminomics.

Lysates were incubated with 1 mM TEVest6 biotin peptide ester tag and subtiligase (1 µM wild-type and 1 µM M222A mutant) for 2 hours. Labeling was monitored by immunoblot using a streptavidin IRDye 800CW (LI-COR inc.). Protein was precipitated in acetonitrile at -20°C overnight. The precipitate was recovered by centrifugation at 12,000 g, resuspended in 8 M guanidine hydrochloride and boiled with 100 mM tris(2-carboxyethyl)phosphine (TCEP) for 15 minutes. Once cooled, resuspension was treated with 4 mM IAM and incubated in the dark for 1 hour. The sample was then reduced with 20 mM DTT and precipitated in ethanol (100 proof) at -80°C overnight. The following morning, the precipitate was recovered by centrifugation and resuspended in 8 M guanidine hydrochloride, which was diluted to 4 M with water once the precipitate was dissolved. Neutravidin agarose beads were added to the resuspension and incubated.
overnight at room temperature on a rotator. Capture efficiency was measured by dot blot using the same streptavidin IRDye 800CW. When a 90% capture efficiency was observed, the beads were washed with a biotin wash solution (1 mM biotin, 10 mM bicine pH 8.0), then washed with 4 M guanidine hydrochloride, then washed and resuspended in a trypsin buffer (100 mM bicine pH 8.0, 200 mM NaCl, 20 mM CaCl₂, and 1 M guanidine hydrochloride). 20 µg trypsin (Promega) was added and incubated overnight at room temperature on a rotator. The next day, the trypsin in buffer was washed from the beads using 4 M guanidine hydrochloride. The neutravidin beads were then resuspended in Tobacco Etch Virus (TEV) protease buffer (100 mM ammonium bicarbonate pH 8.0, 2 mM DTT, and 1mM EDTA). 65 µM TEV protease was added to the resuspension before they were incubated overnight at room temperature on a rotator. The next day, the supernatant was recovered and dried on a Genevac® solvent evaporator (SP Scientific). When dry, the samples were resolubilized in 5% trifluoroacetic acid (TFA) and incubated for 10 minutes at room temperature to precipitate the TEV protease. Samples were centrifuged and the supernatant was desalted using C18 desalting resin tips (PureSpeed Rainin). The eluted solution from the desalting was dried on a Genevac®.

**Mass spectrometry.**

Dried samples were resuspended in 10 µL 0.1% formic acid. Peptides were analyzed using a nanoflow-HPLC (Thermo Scientific EASY-nLC 1000 system) coupled to a Lumos (Thermo Fisher Scientific) mass spectrometer. Peptides were eluted using a 120 minute 0 – 42% linear acetonitrile gradient, followed by elution with 80% acetonitrile. Data were analyzed using ProteinProspector (v5.22.1) software against the human proteome (2017-11-01 human proteome sequence downloaded from [https://www.uniprot.org](https://www.uniprot.org)). Search parameters included allowing non-tryptic cleavage at N-termini, a maximum of three missed trypsin cleavages, a precursor mass tolerance of 10 ppm as well as a fragment mass tolerance of 0.8 daltons. Carbamidomethylation of Cys was searched as a static modification and oxidation of Met, deamidation of Asn and Gln and addition of aminobutyric acid (abu) were searched as variable modifications. The TEVest6 biotin
ester peptide tag contains an unnatural residue, abu that is retained on labeled peptides following TEV protease cleavage of peptides from the neutravidin beads. Search parameters included a strict false discovery rate (FDR) of 5% for proteins, 1% for peptides. The maximum number of variable modifications was set to 2.

**Sample preparation for immunoblotting.**

For the cleavage assays using recombinant caspase-3 and -9, we followed the same procedure to prepare the samples for immunoblotting as we did for reverse N-terminomics. After the incubation with the respective caspase (or only buffer A without caspase as a control) and addition of z-VAD-fmk, we added 1xSDS blue loading dye (New England Biolabs) and denatured the samples by heating at 90°C for 10 minutes. Samples were aliquoted and stored at -80°C until the further usage.

For staurosporine (STS) induced apoptosis assays, we treated Jurkat cells ($5 \times 10^7$) with either 0.5 µM of STS or DMSO (control). We incubated these cultures at 37°C for 3 hours to induce apoptosis. Then, we lysed cells following the same protocol as for N-terminomics. At last, we added 1xSDS blue loading dye (New England Biolabs) and denatured the samples by heating at 90°C for 10 minutes. These samples were also aliquoted and stored at -80°C until further usage.

**Immunoblotting analysis.**

The samples were thawed, and the proteins separated by molecular weight using gel electrophoresis. We used a 12% SDS-PAGE gel for RECQL5, GSDMD, MFN2, PAK2, PARN, and ATXN2L and a 16% SDS-PAGE gel for NUP43, RNF126, RNF4, and RING1. The proteins were transferred from the SDS-PAGE gel to an Immobilon-P PVDF membrane (MilliporeSigma). These membranes were washed five times with TBST over a period of 30 minutes. The membranes were then blocked using OneBlock™ Western-CL Blocking Buffer (Genesee Scientific Corporation) at 4°C for 1 hour. Primary antibody solutions were prepared as follows: RECQL5 [ThermoFisher Scientific (PA5-56315); final concentration: 0.1 µg/mL], NUP43 [ThermoFisher Scientific (A303-976A); 1:5000 dilution], RNF126 [abcam (ab183102); 1:700 dilution], GSDMD
[MilliporeSigma (G7422); 1:1000], MFN2 [abcam (ab205236); 1:5000 dilution], RNF4 [R&D Systems (AF7964); final concentration: 1 µg/mL], PAK2 [Cell Signaling Technology (2608); 1:5000 dilution], PARN [abcam (ab188333); 1:5000 dilution], ATXN2L [Proteintech Group, Inc. (24822-1-AP); 1:5000 dilution], and RING1 [Cell Signaling Technology (13069); 1:5000 dilution] into 10 mL of blocking buffer. After removing the blocking buffer from the membranes, they were incubated with primary antibodies solutions at 4°C overnight. The next day, the membranes were washed five times with TBST over a period of 30 minutes and subsequently incubated with secondary antibodies: RNF4, rabbit anti-goat IgG HRP antibody [R&D Systems (HAF017); 1:10,000 dilution], and for all the other antibodies, goat anti-rabbit IgG H L HRP [Genesee Scientific Corporation (20-303); 1:10,000 dilution]. The incubation for secondary antibodies was performed for 1 hour, and then the membranes were washed five times using a TBST buffer over the period of 30 minutes. At last, the signal was detected using a SuperSignal™ West Dura Extended Duration Substrate kit (ThermoFisher Scientific). The images of immunoblots were taken using ChemiDoc™ MP imaging system (Bio-Rad Laboratories) and analyzed using Image Lab software (Bio-Rad Laboratories). GAPDH protein levels were used as loading control. Each membrane was stripped by incubating with a mild stripping buffer (1.5% glycine pH 2.2, 0.1% SDS, and 1% Tween 20) for 10 minutes. The membranes were washed twice with PBS, then twice with TBST and incubated with GAPDH primary antibody [ThermoFisher Scientific (MA5-15738); 1:5000 dilution], and secondary antibody, goat anti-mouse IgG H L HRP [Genesee Scientific Corporation (20-304); 1:10,000 dilution], as described above.

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GM008515. This work was supported by NIH GM 008532. **Figure 4.1** and **Figure 4.2** were made with BioRender.
References


CHAPTER V:
CHEMOPROTEOMICS USING NUCLEOTIDE ACYL PHOSPHATES REVEALS AN ATP BINDING SITE AT THE DIMER INTERFACE OF PROCASPASE-6


Authors’ Contributions: K.B.D. prepared relevant DNA constructs, purified caspase variants, determined enzymatic activity (VEIDase assay) of WT caspase-6 and caspase-6 Y198A, performed caspase-6 VEIDase assay with varied concentrations of ATP, prepared figures, and assisted with manuscript preparation. I.S. developed the method for purifying N-terminally His-tagged caspase-6 – a mimic of catalytically active procaspase-6, performed procaspase-6 auto-activation assay in the presence of ATP in a time-dependent manner, determined IC₅₀ of procaspase-6 auto-activation by ATP, prepared figures, and assisted with manuscript preparation. J.A.H. obtained funding and directed work in the Hardy lab and manuscript preparation. S.N.S. performed modeling studies and assisted with manuscript preparation. A.A. performed caspase activation experiments and prepared probe-labeled samples. T.K.N. planned experiments and analyzed data for nucleotide competition experiments. J.L.G. performed cellular assays, analyzed data, and assisted with manuscript preparation. E.S.O. planned experiments, performed experiments, analyzed data, generated figures, and assisted with manuscript preparation. J.W.K. directed the experimental design and manuscript preparation.

Abstract

Acyl phosphates of ATP (ATPAc) and related nucleotides have proven to be useful for the interrogation of known nucleotide binding sites via specific acylation of conserved lysines (K). In addition, occasional K acylations are identified in proteins without such known sites. Here we
present a robust and specific acylation of procaspase-6 by ATPAc at K133 in Jurkat cell lysates. The K133 acylation is dependent on \(\pi-\pi\) stacking interactions between the adenine moiety of ATPAc and a conserved Y198–Y198 site formed at the homodimeric interface of procaspase-6. Significantly, the Y198A mutation in procaspase-6 abolishes K133 acylation but has no effect on the proteolytic activity of the mature, active caspase-6 Y198A variant. Additional in vitro studies show that ATP can inhibit the autoproteolytic activation of procaspase-6. These observations suggest that ATP, and possibly other nucleotides, may serve as the endogenous ligands for the allosteric site at the procaspase-6 dimer interface, a site that has persisted in its “orphan” status for more than a decade.

**Introduction**

Chemoproteomic analysis of nucleotide binding sites using acyl phosphates of nucleotide di- and triphosphates has become a broadly accepted method for identification of active-site lysines and profiling of inhibitors that compete with binding of the nucleotide probe (Figure 5.1.A).\(^1\)\(^–\)\(^3\) The ATPAc and ADPAc probes provide broad coverage of protein kinases and other ATPase families, such as the heat shock proteins (HSP90s and HSP70s) and the AAA+ families.\(^4\) Incorporation of biotin, or an analogue thereof, into the acyl side chain of the probe permits selective capture of peptides containing acylated lysines. Exposure of cellular lysates to the nucleotide acyl phosphate probe causes transfer of the acyl moiety to the \(\varepsilon\)-amino group of closely positioned lysines within nucleotide binding sites. Weaker, second-order acylations of surface lysines rarely interfere in the analysis due to the “wash out” effect over many thousands of random peptides derived from the bulk of the proteome. The reaction is complete in 5–10 min and results in a covalent \(\varepsilon\)-amide on the modified lysine residues that is stable to enzymatic proteolysis. Following proteolysis, biotinylated peptides are captured and enriched on streptavidin beads and subjected to mass spectrometry analysis for identification and relative quantitation. Hundreds of ATP binding proteins can be simultaneously analyzed from a single proteomic sample based on detection of probe-labeled peptides.\(^1\)\(^–\)\(^3\)
For protein kinases, which contain highly conserved active-site lysines, robust acylation is clearly observed and more than 200 protein kinases are routinely identified from a single proteomic sample. In addition, acylation of lysines not directly in ATP binding sites has been observed in some kinases and HSP90s. For example, probe labeling of HSP90s has been shown to result from protein conformations where apparently distal lysines are brought into the proximity of the ATP binding site. In another example, acylation of p38α at K15, external to the ATP binding site, occurs only in the presence of MAPKAPK-2 and/or MAPKAPK-3, known binding partners of p38α. Protein–protein interactions orient K15 from p38α into the ATP binding site of the respective MAPKAPK binding partner, resulting in transacylation of K15. With few exceptions, nucleotide acyl phosphates affect acylation of lysines in a proximity-driven manner that requires nucleotide

Figure 5.1. Chemoproteomic identification of the acylated K133 peptide from procaspase-6. (A) ATPAc (Ac = desthiobiotinyl) along with the corresponding ADPAc, GTPAc, and GDPAc probes used in this study. Desthiobiotin shows superior properties for capture by and release from streptavidin beads compared to those of biotin on our chemoproteomic platform. For a full description of the platform, see Methods. (B) Identification of the acylated K133 peptide by LC–MS/MS spectra from a proteomic lysate of Jurkat cells treated with ATPAc. Peaks including the site of acylation (asterisk) are matched in agreement with a high cross-correlation score (Xcorr > 5.0). Translation of amino acid sequence using genomic database sequences confirmed the peptide was derived from CASP6. (C) Relative amounts of streptavidin-captured, acylated K133 peptide determined by LC–MS/MS using four related acyl phosphate probes. Percent are based on ATPAc capture defined as 100% under these experimental conditions.
binding of the probe for efficient delivery of the acyl group. In this work, we present an example of an acylated lysine in procaspase-6, a protein not previously known to bind nucleotides.

Results

Identification of procaspase-6 as an ATP binder in a native screen of human cell lysates.

During proteomic analyses of Jurkat cell lysates with the ATPAc probe, we were struck by the robust acylation of a 15-amino acid peptide identified by LC−MS/MS after Asp-N proteolysis as DAK*IEIQTLGLFKG (the asterisk indicates the site of acylation), corresponding to amino acids 131−145 from the CASP6 gene (Figure 5.1.B). Acylation of this peptide at K133 was inhibited by addition of ATP to the cellular lysate, suggesting that the acylation was not due to the second-order reaction of a surface lysine but rather was dependent on the ATP moiety in the probe.

Two other probe-related observations were intriguing. GTPAc was as effective as ATPAc in K133 acylation, and both ADPAc and GDPAc were significantly less effective in the acylation of K133 than the corresponding triphosphate probes (Figure 5.1.C).

The immediate protein biosynthesized from the gene, CASP6, is procaspase-6, the inactive dimeric zymogen of active caspase-6. This homodimer is composed of a prodomain (residues 1–23), a large subunit (residues 24–179), an intersubunit linker (residues 180–193), and a small subunit (residues 194–293) for each monomer. The zymogen dimer requires activation by cleavage of the pro-domain at D23 and of a linker region at D179–D193, yielding a dimer of heterodimers that constitutes active caspase-6. This maturation of procaspase-6, removal of the active prodomain and the intersubunit linker, results in structural−dynamic changes.6,7 Because both procaspase-6 and active caspase-6 contain amino acids 131−145, the zymogen/enzyme selectivity of ATPAc for acylation of K133 remained to be determined. Moreover, the mechanism and specificity for both ATPAc recognition and acylation were unresolved because no ATP binding site had been reported for CASP6. With these questions in hand, we investigated further.
**Procaspase-6 is the target of K133 acylation.**

To determine whether procaspase-6 and/or active caspase-6 generates the observed probe-labeled peptide, we evaluated cellular lysates from resting cells and apoptotic cells induced by anti-Fas antibody 4C3. Nearly complete apoptosis-induced cleavage of procaspase-6 to active caspase-6 was confirmed by immunoblotting (Figure 5.2A). When these samples were analyzed for K133 acylation, the amount was proportional to the amount of procaspase-6 in the lysates (Figure 5.2B), suggesting that ATPAc exclusively recognizes procaspase-6 for acylation of K133.

**Inhibition of K133 acylation by nucleotides.**

Addition of exogenous nucleotides to cellular lysates has been shown to inhibit ATPAc acylation of active-site lysines in a variety of protein kinases and ATPases and serves as a corroborating test for binding specificity. Thus, several nucleotides were tested for their inhibitory effect on the K133 acylation of procaspase-6 in Jurkat lysates (Figure 5.2C). As expected, ATP and GTP inhibited K133 acylation by the corresponding acyl phosphates. The IC$_{50}$ for inhibition by ATP was determined by Western blotting and by LC–MS/MS. The results of both procedures were similar and in the range of 1–3 mM in cell lysates. Both ADP and GDP showed similar IC$_{50}$ values for inhibition of K133 acylation, suggesting that the corresponding ADPAc and
GDPAc probes should be capable of binding to procaspase-6. Thus, the reduced level of acylation observed with diphosphate probes (Figure 5.1.C) is possibly due to the shortened “reach” of the terminal acyl β-phosphate compared to the corresponding terminal acyl γ-phosphate of the triphosphate probes, preventing efficient transfer of an acyl group to K133. The observation that multiple nucleotides inhibited transfer of an acyl group to K133 with roughly similar potency suggested a relative lack of nucleic acid base specificity in the binding interaction (Figure 5.2.C).

A Role for Y198 in nucleotide binding.

The recent identification of a small molecule binding site at the procaspase-6 dimer interface provided important clues for elucidating the mechanism of K133 acylation by ATPAc. Crystallographic studies determined that a hydrophobic crevice extends along the dimer interface of procaspase-6 (Figure 5.3.A), similar to those observed in the other executioner caspase-3 and -7. Upon closer inspection of the dimer interface, the K133 pair is found to be symmetrically positioned near the edge of the crevice, close (∼14 Å) to a π−π stacking motif formed by the Y198 pair at the hydrophobic interface (Figure 5.3.A, inset). The Y198 pair in procaspase-6 was identified recently as the binding site for aromatic heterocyclic compounds using surface plasmon resonance (SPR). X-ray crystallography established that compound 3 (K_D = 1.8 μM), an isoquinolininone with an exo-amino group (3-aminoisoquinolin-1-ol), was sandwiched between both faces by π−π interactions with the Y198 pair (Figure 5.3.B). To date, no endogenous allosteric molecules that access the Y198 pair have been identified.

Molecular modeling.

The position of the K133 pair relative to the Y198 stack suggested that the stacking-capable, electron-deficient adenine ring of ATPAc could bind in the Y198 interface forming a π−π interaction sandwich similar to that reported with compound 3. Extension of the triphosphate linker could favorably position the acyl phosphate moiety in the proximity of the ε-amino group of K133 for efficient acyl transfer (Figure 5.3.C). In fact, the fully extended form of ATP presents
the acyl carbonyl up to 14 Å from the anomeric carbon (C-1') of the pentose, which matches well the distance between the α-carbons of Y198 and K133.

Docking studies of the acyl phosphate probes using the crystal structure of procaspase-6 in a complex with compound 3 (PDB: 4N6G) supported this hypothesis and provided an explanation for the apparent preference for ATP over ADP in the acyl phosphate reaction. In the optimal docking arrangements, the adenine heterocycle forms a π−π−π sandwich with the Y198 pair that is stabilized by H-bonding between the proximal (to the acyl group) Y198 hydroxyl and the

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**Figure 5.3 Structural models of the ATP binding site at the dimer interface of procaspase-6.**

(A) Structure of the procaspase-6 dimer highlighting the hydrophobic cavity (yellow) at the dimer interface. This cavity is observed in the executioner caspase-3, -6, and -7, as well as caspase-1. Tyrosine 198 (yellow sticks) has been identified as a site that is critical for ATP binding (inset). (B) The adenine ring in the acyl phosphate probe (green sticks) is predicted to bind between the two Y198 residues at the dimer interface. This allows covalent modification of K133, which is located at the edge of the cavity ~18 Å from the adenine-Y198 pair. (C) The crystal structure of compound 3 (brown sticks), identified by SPR as a caspase-6 inhibitor, can be similarly observed sandwiched between the Y198 (yellow) pair of the procaspase-6 dimer. (D) The modeled interaction between the acyl phosphate probe and procaspase-6 shows excellent shape complementarity.

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endocyclic oxygen of the ribose ring. Importantly, H-bonding is also predicted to occur between the distal Y198 hydroxyl and the 2’-OH of ribose (Figure 5.3.C, inset). This arrangement also features stacking of both Y198 hydroxyphenyls with the pyrimidine portion of the purine system. The phosphates projecting from the ribose are guided via a network of H-bond donors (N125, G124, and H126) to project the acyl group toward K133.

**Chemoproteomic analysis of pro-caspase-6 variants.**

To evaluate the role of Y198 in nucleotide binding and zymogen-related processes, several mutants of full-length pro-caspase-6 were constructed, expressed in *E. coli*, and analyzed for K133 acylation (Figure 5.4). Under the expression conditions tested, wild-type pro-caspase-6 was fully processed to mature active caspase-6 through self-activation prior to analysis, resulting in little acylation of K133 (0.9%, data not shown). In contrast, the active-site knockout C163S variant, which is enzymatically incompetent for self-activation due to replacement of the catalytic cysteine, persisted as the full-length zymogen and demonstrated robust acylation of K133 (defined as 100%). The absence of wild-type zymogen and the persistence of the corresponding C163S variant are consistent with earlier observations of pro-caspase-6 self-activation, a noncanonical route for caspase activation.\(^{16,17}\) Because pro-caspase-6 has a loop 2 (L2) that is longer than those of pro-caspase-3 and -7, the scissile peptide bond between Asp-193 and Ala-194 within the intersubunit linker, a contiguous part of L2, sits close to the active-site Cys-163 and can then be primed for hydrolysis by intramolecular self-cleavage. In *E. coli* lysates, the inability to isolate wild-type pro-caspase-6 is consistent with this self-activation, while the C163S pro-caspase-6

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**Figure 5.4. ATPAc labeling of pro-caspase-6 variants.**
The amount of intact pro-caspase-6 was determined by immunoblotting and compared to the amount of ATPAc probe labeling detected. Strong probe labeling was observed for the C163S variant compared to that of the C163S/Y198A variant, even though the proenzyme was present in similar amounts for both variants.
stability confirms that there is no caspase-like activity in the *E. coli* lysate to efficiently process the variant zymogen. Unlike *E. coli*, mammalian cells such as Jurkat and HEK293 produce stable wild-type procaspase-6, suggesting the presence of a mammalian factor(s) that prevents autoactivation, such as the presence of zinc$^{18}$ or endogenous inhibitors of apoptosis.$^{17,19}$

Several Y198A variants were generated to directly assess the effect of Y198 on ATPAc. The Y198A variant was weakly acylated by ATPAc (1.9%). However, we observed that the Y198A zymogen was also processed to the mature Y198A caspase-6 via the self-activation pathway (confirmed by immunoblotting), suggesting that loss of acylation might be due to the loss of procaspase-6 protein. In fact, isolation of Y198A procaspase-6 with the active-site cysteine intact has proven to be intractable in *E. coli*. Removal of the Y198 pair has no apparent deleterious effect on self-activation, and this variant is as active as WT caspase-6 in substrate-based assays (Table 5.1). In contrast, the Y198A/C163S variant yielded full-length procaspase-6 with a dramatically reduced capacity for K133 acylation by ATPAc (1.7%). These findings strongly suggest that Y198 π–π stacking in procaspase-6 is the primary factor for ATPAc binding to procaspase-6 and, thereby, K133 acylation.

**Procaspe-6 self-processing in vitro is inhibited by ATP.**

Given that ATPAc robustly labels procaspase-6 and the site of binding has been identified, it seemed plausible that ATP would impact some aspect of procaspase-6 function or self-processing, considering the well-characterized changes in intracellular ATP concentration during apoptosis.$^{20-25}$ While we routinely use a C-terminal His$_6$ tag to isolate active caspase-6, this construct was not appropriate for isolating procapase-6 because it repeatedly resulted in

<table>
<thead>
<tr>
<th>caspase-6 variant</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>56.1 ± 6.2</td>
<td>2.98 ± 0.10</td>
<td>53,202</td>
</tr>
<tr>
<td>Y198A</td>
<td>64.4 ± 3.5</td>
<td>2.84 ± 0.04</td>
<td>44,064</td>
</tr>
</tbody>
</table>
predominantly active (cleaved) caspase-6. Using a new N-terminal His$_6$ construct, we were able to isolate a nearly homogeneous preparation of procaspase-6 that enabled subsequent analyses regarding the impact of ATP on procaspase-6 self-processing in vitro. Procaspase-6 was incubated for ≤8 h in the presence or absence of 20 mM ATP. At this concentration of ATP, cleavage of procaspase-6 was significantly impeded (Figure 5.5.A). Dose–response studies (Figure 5.5.B) demonstrated an IC$_{50}$ of 19 ± 0.8 mM for ATP-dependent inhibition of procaspase-6 self-processing (Figure 5.5.C). Conversely, the activity of mature caspase-6 is not affected by increasing concentrations of ATP (Figure 5.5.D). From these analyses, it is clear that procaspase-6 is sensitive to the presence of ATP, which inhibits self-processing, but that the activity of mature caspase-6 is not sensitive to ATP.

**Discussion**

The dimer interfaces of the executioner caspase-3, -6, and -7 have received attention as possible sites of allosteric regulation and activation of these key apoptotic enzymes. The discovery of the hydrophobic cavity at the interface of caspase-3 and -7 led to the
identification of small drug-like aromatic hetero- cycles by screening a library of 10000 thiol-containing compounds against accessible cysteines in the cavity.\textsuperscript{11,12} The compounds inhibited native caspase activity by reorienting a side chain at the dimer interface, which blocked the correct conformation of the active-site loops, thus preventing substrate binding at the active site. These compounds appeared to function by trapping the active caspase into a zymogen-like and, therefore, inactive structure. The identification of allosteric inhibitors of procaspase-6 using SPR marked a significant departure from the caspase-3 and -7 studies in that it focused on compounds that could specifically prevent zymogen activation.\textsuperscript{10} The choice of SPR as the binding readout was necessary because it provided a direct measurement of the binding to the inactive C163S zymogen in place of an activity assay. However, the \textit{in vitro} conditions required to measure binding by SPR may not translate to cellular conditions. For instance, the K\textsubscript{D} for compound 3 in the SPR assay (\(~1.8 \mu M\)) was not predictive of its binding (IC\textsubscript{50} >1 mM) observed in cellular lysates using ATPAc. This is not surprising because the relatively pristine, non-physiological conditions of the SPR assay do not reflect the myriad of factors that might attenuate binding of compound 3 in a whole cellular lysate.

The Y198 pair appears to be structurally unique to procaspase-6. The Y223 pair in caspase-7 was shown to interact with the inhibitors mentioned above in an extended arrangement not comparable to the π−π stack reported here. The fact that Y198 is needed for ATP binding supports the notion that ATP and perhaps other nucleotides or aromatic intracellular compounds bind at the dimer interface allosteric site and modulate caspase-6 function.

The key features mediating the ATP−procaspase-6 interaction, Y198, K133, and associated protein motifs, are completely conserved across all primates and highly conserved across all mammalian orthologs with few exceptions, the most notable being a Y198H single-point mutation in a number of aquatic and southeast Asian mammals and, remarkably, a Y198S single-point mutation found in virtually all members of the cat family (Felidae). Caspase-6 orthologs identified in birds, lizards, modern amphibians, and teleosts also show high levels of conservation for Y198 and K133.
Our suggestion that ATP is an endogenous ligand for dimeric procaspase-6 is strengthened by the generally accepted range of intracellular ATP concentrations of roughly 1–10 mM (average of ~3.2 mM), by far the most abundant nucleotide.\textsuperscript{27} The use of low levels (micromolar) of the irreversible acyl phosphate probe and short reaction times (minutes) in our chemoproteomic platform results in a low extent of acylation across the hundreds of susceptible active-site lysines in the whole cell lysate. This yields IC\textsubscript{50}’s for competitive inhibition of acylation by nucleotides that are close approximations of the K\textsubscript{i}’s under these conditions.\textsuperscript{1,2} Thus, ATP appears to be unique among the nucleotides tested in that its binding constant (~2 mM) with procaspase-6 lies within range of its intracellular concentration.

\textit{In vitro} inhibition of procaspase-6 self-activation by ATP (Figure 5.5) is complicated by intervening enzymatic processing, relative enzyme concentrations, and modifications in procaspase-6 that alter autoactivation. The relationship between the K\textsubscript{i} for binding of ATP to the dimer interface and the IC\textsubscript{50} for the inhibition of procaspase-6 self-processing is, therefore, correspondingly complex, leading to IC\textsubscript{50}’s that could be considerably larger than the K\textsubscript{i} for ATP binding. However, the 19 mM ATP IC\textsubscript{50} for procaspase-6 self-processing is still reasonably close to the physiological ATP concentration range. We are exploring other \textit{in vitro} conditions to better define the mechanisms for this discrepancy.

In summary, our chemoproteomic analysis and supporting studies presented here suggest that ATP is a bonafide endogenous procaspase-6 ligand and potentially an \textit{in vivo} inhibitor of self-processing at concentrations approximating cellular levels. Our findings should prompt further investigations to identify additional native ligands for the procaspase-6 dimer interface, the biological outcomes of ligand binding, and the regulation of other procaspases and active caspases through similar mechanisms.
Materials and Methods

ATPAc-labeled Sample Preparation.

ATPAc-labeled Sample Preparation: Cell lysate were prepared by sonicating cell pellets in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, phosphatase inhibitors [Cocktail II AG Scientific #P-1518]). Lysates were cleared by centrifugation and MnCl₂ was added to final concentration of 20 mM. For small molecule studies, 5 μL of 100X compound or DMSO was added to 445 μL of lysate; all samples were prepared in duplicate. Small molecules were incubated for 15 minutes prior to addition of 50 μL of a 10X desthiobiotin-nucleotide-acyl phosphate probe (ADP or ATP probe) for a final probe concentration of 20 μM. Probe labeling reactions were allowed to proceed for 15 minutes prior to further processing.

Targeted LC-MS/MS analysis.

Samples were prepared for MS analysis as described previously² with the only exception that Asp-N was used, in place of trypsin, to cleave intact proteins into the corresponding peptide complement. Briefly, probe-labeled lysates were denatured and reduced (6 M urea, 10 mM DTT, 65°C, 15 min), alkylated (40 mM iodoacetamide, 37°C, 15 min), and gel filtered (Bio-Rad EconoPac 10G) into 2 M urea, 5 mM methionine, 1M Tris. The desalted protein mixture was digested with Asp-N (1 μg/mL) for 24 h at 37°C and desthiobiotinylated peptides were captured using 12.5 μL high-capacity streptavidin resin (Thermo Scientific). Bead-bound peptides washed extensively, and probe-labeled peptides were eluted with two 35 μL washes of 50% CH3CN/water with 0.1% TFA.

Whole protein-Western blot analysis.

Probe labeling of intact caspase-6 was evaluated using a western blot coupled probe-based assay, as described previously.²⁸ After the probe-labeling reaction, samples were denatured, reduced, alkylated, and gel-filtered as described above. However, samples were directly incubated with streptavidin beads to capture probe-labeled, intact proteins (with overnight incubation). Samples were washed extensively with PBS to remove non-specifically bound proteins. Probe-
labeled proteins were then eluted with two 50 μL washes of reducing gel-sample buffer, separated with SDS-PAGE, and detected by western blotting for the target of interest.

**Western blotting.**

Proteins were transferred to PVDF membranes after SDS-PAGE using a Bio-Rad transblot turbo system. Membranes were blocked with 5% nonfat dry milk in TBS, 0.1% Tween for 1 hr. prior to incubating with appropriate caspase-6 antibodies (Cell Signaling, #9761 and #9762). Primary antibodies were utilized at dilutions of 1:1,000. Secondary antibodies obtained from LiCor were used at dilutions of 1:20,000 (IRDye IgG antibodies, #5470S, 5151S, 5257S, and 5366S). Images were both collected and analyzed using a LiCor Odyssey classic imaging system.

**LC-MS/MS.**

Samples were analyzed by LC-MS/MS as described previously. Agilent 1100 series micro-HPLC systems with custom packed C18 separation columns were coupled to Thermo LTQ ion trap mass spectrometers. Data were collected using custom target lists including the m/z value for the probe-labeled pro-caspase-6 peptide. Extracted ion chromatograms were generated using MS2 fragments resulting from the probe-labeled caspase-6 peptide and integrated peak areas were used to determine relative signal between treated samples and controls.

**Generation of Caspase Variants.**

The full-length wild-type (FL WT) caspase-6 used in this study was derived from a synthetic, *E. coli* codon-optimized (His)_6 C-terminally tagged caspase-6 gene (Celtek Bioscience) that was ligated into the Ndel/BamHI sites of pET11a vector. Caspase-6 variants (C163S, Y198A, C163S/Y198A) as well as the N-terminally (His)_6 tagged FL casp-6 were generated using Phusion® site-directed mutagenesis (Thermo Scientific™) in the FL WT caspase-6 construct. Fully cleaved and active caspase-6 was also used in a form of a constitutive two-chain (CT), which was designed to independently express the large and the small subunits of caspase-6 with the prodomain (residues 1-23) and linker (residues 180-193) removed.
Caspase Protein Expression and Purification.

All caspase-6 constructs used in this study were transformed into the BL21(DE3) T7 express strain of E. coli (NEB). Overnight seed cultures were initially grown in 2×YT media supplemented with 0.1 mg/mL ampicillin (Sigma) at 37 °C. Dense cultures were then diluted 1000-fold with 2×YT media containing 0.1 mg/mL ampicillin at 37 °C until Abs600 reached 0.6. For the following caspase-6 variants: FL procaspase-6 C163S (active-site knockout), FL procaspase-6 C163S/Y198A, FL Y198A, protein expressions were induced by addition of 1 mM IPTG (GoldBio) at 20 °C for 18 h. Caspase-6 FL N-terminally (His)$_n$ was induced by addition of 1mM IPTG (GoldBio) at 37 °C for 10 minutes. Cells were centrifuged at 4,700 x g for 10 min at 4 °C and stored at -80 °C until use. Freeze-thawed cells were lysed using a microfluidizer (Microfluidics, Inc.) in ice-cold lysis buffer (50 mM Tris, pH 8.5, 300 mM NaCl, 5% glycerol, and 50 mM imidazole) and centrifuged at 30,600 x g for 1 hour at 4 °C. The supernatant was loaded into a 5-mL HiTrap nickel-affinity column (GE Healthcare) and washed with lysis buffer until the absorbance returned to baseline. The protein was eluted with elution buffer (50 mM Tris, pH 8.5, 300 mM NaCl, 5% glycerol, and 250 mM imidazole) and diluted 5-fold with buffer A (20 mM Tris, pH 8.5, and 2 mM DTT) to reduce the salt concentration. This protein sample was then loaded into a 5-mL HiTrap Q HP column (GE Healthcare). The column was developed with a linear NaCl gradient, and the protein was eluted in 20 mM Tris, pH 8.5, 200 mM NaCl, 2 mM DTT. This eluted protein was stored at -80 °C until use. The purified caspases were analyzed by SDS-PAGE to confirm identity and purity.

Caspase Activity Assays.

To measure caspase activity, 100 nM purified caspase-6 variants were assayed over 7 min at 37 °C in casp-6 activity assay buffer (100 mM HEPES, 120 mM NaCl, 0.1% CHAPS, 10% sucrose, 5 mM DTT). For substrate titration, a range of 0-500 μM fluorogenic substrate VEID-AMC [N-acetyl-Val-Glu-Ile-Asp- (7-amino-4-methyl-coumarin), Enzo Life Sciences Inc.] was used for caspase-6. Fluorescence kinetic measurements ($\lambda_{ex}/\lambda_{em}$: 365 nm/495 nm) were performed
in three independent trials on three different days in 100-μL reactions in a 96-well format using a microplate reader (SpectraMax M5, Molecular Devices). Initial velocities versus substrate concentration were fit to a rectangular hyperbola using GraphPad Prism (GraphPad Software, San Diego, USA) to determine the kinetic parameters $K_m$ and $k_{cat}$. Enzyme concentrations were determined by active-site titration with the quantitative covalent inhibitor VEID-cho (N-Acetyl-Val-Glu-Ile-Asp-aldehyde; Enzo Life Sciences Inc.) for caspase-6. Protein was added to inhibitor solvated in DMSO in 96-wells V-bottom plates at room temperature for 1.5 hours in caspase activity assay buffer. Aliquots (90 μL) were transferred in duplicate to black-well plates and assayed with 50-fold molar excess of substrate. The protein concentration was determined to be the lowest concentration at which full inhibition was observed and was thus used to calculate $k_{cat}$.

**Effect of ATP on the activity of cleaved, active caspase-6.**

Cleaved and active caspase-6 ΔN D179CT (20 nM) was incubated with increasing concentration of ATP (Fisher Scientific, S25123) (0–40 mM, 1.5-fold dilution) in caspase-6 activity assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 120 mM NaCl, and 5 mM DTT) at 37°C for 1.5 h. VEID-amc fluorogenic substrate (Enzo Life Sciences Inc.) was then added to a final concentration of 60 μM. The final volume was 30 μL in a 384-well black flat bottom plate (BD Falcon™). Fluorescent kinetic measurements ($\lambda_{ex}/\lambda_{em}$: 365 nm/495 nm) were performed over 7-min and the initial velocities were calculated. The data was presented as percent activity of caspase-6 setting the buffer-only control as 100%. The data represent an average of two independent experiments (each run in duplicate) and the reported errors represent S.E.M.

**Effect of ATP on the self-activation of procaspase-6.**

Catalytically competent full-length version of procaspase-6, Caspase-6 FL N-terminally (His)$_6$ (3 μM) was incubated with varying concentrations of ATP (0-60 mM) in caspase-6 assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 120 mM NaCl, and 5 mM DTT) at 37°C for 6h. SDS loading buffer were added to the sample and boiled for 10 mins and analyzed by 16% SDS-PAGE. The gels were imaged using ChemiDoc™ MP imaging system (Bio-Rad) and
band intensities were quantified using Image Lab software (Bio-Rad). The intensities of full-length procaspase-6 was set at 100% and extent of cleavage with varying ATP concentration was reported as percent procaspase-6 remaining. The IC$_{50}$ values were determined using nonlinear curve-fitting algorithm (four-parameter equation) in GraphPad Prism (GraphPad Software, San Diego, USA). A time-point experiment (hrs: 0, 0.25, 1, 2, 4, 6, 8) was also performed to assess the extent of procaspase-6 activation in the presence or absence of 20 mM ATP.

**Molecular Modeling.**

All computational procedures were carried out using Schrödinger’s Small-Molecule Drug Discovery suite (v. 2016-1, Schrodinger, LLC, New York, NY, USA), using the following applications: Maestro, Protein Preparation Wizard, Epik, Glide, Prime and Desmond. An all-atom model for docking and molecular dynamics studies was generated using a crystal structure of procaspase-6 liganded at the homodimeric interface (PDB ID: 4N6G) via a protonation state assignment (Epik), missing atom/loop reconstitution (Prime, OPLS3 force field) and constrained minimization (Prime, force field: OPLS3) sequence within Maestro’s Protein Preparation Wizard tool. Docking (Glide) was initiated by centering the interfacial cavity into a $36 \times 36 \times 36$ Å³ grid box and implementing a high-precision (XP) algorithm without any constraints with 3D models of nucleotide conjugates in physiologically relevant protonation states. The resulting structures were further refined through restrained minimization (Prime, force field: OPLS3). The best scoring models served as starting points for Molecular Dynamics (MD) simulations, which were set up as NPT ensembles using neutralized systems (Na$^+$-ions) with explicit water (SPC solvent model) within 10Å buffer box and NaCl or MgCl$_2$ 200 mM buffer salts (Desmond). The resulting ensembles were then subjected to unconstrained 1.2 ns-long MD simulations at 300 K and 1 atm. The quality of the simulation and validity of the complex was evaluated by the Root Mean Square Deviation of the interacting proteins throughout the simulation. The resulting trajectories were analyzed using Simulation Interaction Diagram tool within Desmond to reveal relative persistence of molecular contacts.
Accession Codes

UniprotKB: CASP6, P55212; CASP7, P55210; CASP3, P42574; p38-α, Q16539; MAPKAPK2, P49137; MAPKAPK3, Q16644.

Acknowledgements

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References


CHAPTER VI:
CONCLUSIONS

The studies described in this dissertation have provided a number of key insights into the structures, functions and mechanisms of the caspases that were investigated. The goal of this chapter is to identify additional questions about caspase structure and function that remain and to attempt to put into context how the work performed here provides important groundwork for additional studies in these key research areas.

Toward the Goal of Determining Caspase-9 Interactions with Its Protein Substrates by Hydrogen/Deuterium Exchange - Mass Spectrometry (H/DX-MS)

Caspase-9 is classified as an apoptotic initiator. Lack in caspase-9 functionality contributes to multiple diseases including acute and chronic neurodegeneration, retinal neuropathy, slow-channel myasthenic syndrome, lumbar disc disease, cardiomyopathies, atherosclerosis and autoimmune disease (see review¹). Therefore, studying caspase-9 structure is important. We performed H/DX-MS on several constructs of caspase-9 (Chapter II). There was no structural/dynamics information on the full-length caspase-9 consisting of both CARD and catalytic core domains. Our H/DX-MS results revealed the dynamics of full-length monomeric caspase-9 (both uncleaved zymogen as well as cleaved at intersubunit linker, ISL, form). Our data also included the dynamics information on the CARD domain linker (CDL) and ISL which were missing in the structures determined by employing X-ray crystallography and cryogenic-electron microscopy (PDB: 3GYS, 1JXQ, and 5WVE). We understand the structural details of dimeric caspase-9 without the CARD domain in which the dimerization was induced by an active site inhibitor, z-EVD-debmk (a substrate mimic) (PDB: 1JXQ). However, we did not know how caspase-9 dynamics are changed upon substrate-induced dimerization. Our H/DX-MS results revealed some details about the dynamics of caspase-9 dimerization. From these results, we identified the regions which undergo huge confirmational changes upon substrate-induced
dimerization (e.g., the dimer interface and the 130’s helix: strand region). Our H/DX-MS results also for the first time revealed the putative regions (e.g., CDL and loops surrounded to the caspase-9 active site including ISL) involved in the interactions between the CARD and the catalytic core domain.

There is not enough structural information on how caspases (including caspase-9) interact with their protein substrates. For example, in the protein databank (PDB), there are no structures of a complex of caspase-9 with its protein substrate such as procaspase-3 and -7. Using the knowledge of our H/DX-MS data, we can perform experiments to determine interactions between caspase-9 and its protein substrates. In addition, we recently deorphanized 124 putative protein substrates of caspase-9 demonstrating caspase-9 is not just an apoptotic initiator (see Chapter IV). From this list of caspase-9 substrates, we can perform H/DX-MS on caspase-9, its protein substrates, and the complexes. To begin with, we can perform biophysical studies such as fluorescence polarization (FP) and size exclusion chromatography (SEC) to determine the binding affinities of caspase-9 to its protein substrates. We can then select caspase-9 substrates with the strongest binding affinities to perform H/DX-MS. From the H/DX-MS data, we can identify the regions of caspase-9 undergoing dynamics changes upon its substrate binding. Similarly, regions of caspase-9 substrate/s involved in the binding can be identified. Results from these experiments will be beneficial in 1) determining the mechanism of caspase-9 binding to its substrate/s and 2) identifying allostERIC sites and exosites of caspase-9 and its substrate/s to design therapeutics of diseases caused by caspase-9 dysfunctional.

To Interrogate Phosphorylation of Caspase-9 by H/DX-MS

In Chapter II, H/DX-MS was employed to determine the structural-dynamic information of difference states of caspase-9. We examined seven different constructs of caspase-9, and each of them had high peptide coverage (>90 %) with > 2 redundancy. Thus, majority of caspase-9 regions can be detected by employing H/DX-MS. This can be used as an advantage to study the structures and dynamics of other forms of caspase-9. Caspase-9 is highly phosphorylated at
different sites by various kinases (Figure 6.1). Phosphorylation of caspases has been shown to regulate their functionality.\textsuperscript{2-6} There are structural details on the phosphorylation of caspase-3, -6, and -7.\textsuperscript{3,4,6} Although many molecular level studies have been conducted to determine how phosphorylation impacts caspase-9 regulation,\textsuperscript{2,5} there is not enough information on the structure and the dynamics. Using site-directed mutagenesis and H/DX-MS, structural-dynamics information on caspase-9 phosphorylation can be determined. For example, caspase-9 phosphorylation by protein kinase A (PKA) can be investigated. PKA phosphorylates caspase-9 at three distinct sites, S99, S183, and S195.\textsuperscript{5,7} From the observed activities of phosphomimetics (substituting Glu with Ser), phosphorylation at S99, S183, and S195 inhibits caspase-9 by 76\%, >99.9\%, and 37\%, respectively.\textsuperscript{5} It was demonstrated that caspase-9 that is cleaved at the intersubunit linker (ISL) aggregates upon phosphorylation at S183; nevertheless, there are no structural details on the uncleaved caspase-9 phosphorylated at S183.\textsuperscript{5} We can generate phosphomimetics with single and multiple substitutions at S99, S183, and S195, and perform H/DX-MS on the uncleaved caspase-9 constructs. The structural/dynamics information on these constructs will be insightful in identifying the caspase-9 regions impacted upon phosphorylation by PKA. The H/DX-MS results of phosphorylated caspase-9 can be compared with the different states of caspase-9 (e.g., zymogen and ISL cleaved) to determine the similarity as well as uniqueness of the caspase-9 structure upon phosphorylation. Similarly, H/DX-MS can be employed to interrogate caspase-9 phosphorylation other kinases (Figure 6.1) which phosphorylate at different sites of caspase-9. We anticipate that structural/dynamics information gathered from the H/DX-MS of phosphomimetics will guide in the ongoing research of targeting caspase-9 (e.g.,
identify allosteric sites and then perform allosteric activation/inhibition) in designing therapeutics to treat diseases caused by caspase-9 dysfunction.

**To Determine the Intracellular Cleavage Preference of Caspase-9**

In the intrinsic apoptotic pathway, caspase-9 activates executioners, procaspase-3 and -7 by cleaving their intersubunit linkers (ISL). Consequently, activated caspase-3 (and caspase-7 in the absence of caspase-3) activates procaspase-6 by cleaving its ISL cleavage sites. Both in vitro as well as cell-based studies have demonstrated that caspase-9 does not directly activate procaspase-6.8–10 In **Chapter III**, we employed site-directed mutagenesis to engineer constructs of procaspase-3 and -6. Using substrate-digestion assays on these constructs, we showed that caspase-9 does not cleave procaspase-6 cleavage site 1 (176DVVD↓N) because of the sequence and cleavage site 2 (190TEVD↓A) because of the local context.

Studies conducted in **Chapter III** were performed in an *in vitro* setup by employing recombinant proteins. Using the outcomes of these studies, the role of caspase-9 as a protease has become clearer. As a further investigation, the same types of studies can be done in cells to determine the cleavage preference of caspase-9 intracellularly. For that caspase-3/-7/-6/-/ knock out cells can be generated using a multicolor LentiCRISPR system.10 This is feasible since LentiCRISPR system was employed before in multiple human cell lines, NALM 6, 658w, and Jurkat, to generate single and multiple caspase knock outs including caspase-3/-7/-6/-/ (**Figure 6.2**).10 Using site-directed mutagenesis, procaspase-3 and -6 constructs described in **Chapter III**

![Figure 6.2. Single and multiple caspase knockout using LentiCRISPR method.](image-url)

Using LentiCRISPR method, single and multiple effector caspases (caspase-3, -6, and -7) knockouts of (A) NALM 6, (B) 658w, and (C) Jurkat cells were generated. This figure was copied from10.
can be engineered in mammalian vectors. These engineered constructs along with procaspase-3 and -6 (as controls) can be transfected in caspase-3/-7/-6−/− cells. Transfected cells then can be treated with an antiapoptotic BCL2 family inhibitor, ABT263. This inhibitor will trigger the intrinsic apoptotic pathway and activate caspase-9. Ultimately, cleavage of procaspase-3 and -6 constructs by endogenous caspase-9 can be monitored by immunoblotting. Results from these experiments will be beneficial in understanding how caspase-9 selectively proteolyzes executioner procaspase-3 but not -6 in cells.

**To Determine the Cleavage Preference of Caspase-7 to Proteolyze Procaspase-6**

Due to its involvement in neurodegenerative pathways, caspase-6 is considered as an excellent target in many neurodegenerative diseases including Alzheimer’s Disease, Huntington’s Disease, and Parkinson’s Disease. To target caspase-6, studying its activation in different pathways is crucial. In an intrinsic apoptotic pathway, caspase-3 activates procaspase-6 by cleaving the ISL. Procaspase-6 activation by proteases, caspase-3, caspase-6, and caspase-9 have been studied at molecular level (Table 6.1). Caspase-3 protease activates procaspase-6 by first cleaving the ISL site 1 (176DVVD↓N) and only then ISL site 2 (190TEVD↓A). In contrast, via intermolecular interactions, caspase-6 protease activates procaspase-6 by first cleaving at the ISL cleavage site 2 (190TEVD↓A) and only then cleavage site 1 (176DVVD↓N). Caspase-9 does not cleave procaspase-6 ISL site 1 (176DVVD↓N) and ISL site 2 (190TEVD↓A) because of the sequence and local context, respectively. In the absence of caspase-3, caspase-7 can activate procaspase-6 via ISL cleavage. However, a molecular level investigation (as performed in Chapter III) is needed

<table>
<thead>
<tr>
<th>Caspase</th>
<th>ISL Site 1 (176DVVD↓N)</th>
<th>ISL Site 2 (190TEVD↓A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>Yes (cleaved first)</td>
<td>Yes (cleaved only after ISL Site 1 cleavage)</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Yes (cleaved only after ISL Site 2 cleavage)</td>
<td>Yes (cleaved first)</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Not cleaved due to the sequence</td>
<td>Not cleaved due to the local context</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

The data of this table were obtained from9,11,12.
in order to understand how caspase-7 activates procaspase-6. This investigation will be able to provide answers on 1) which factors (sequence and/or local contexts) are important in facilitating procaspase-6 ISL cleavage by caspase-7, 2) how procaspase-6 behaves as a substrate, and 3) how caspase-7 acts as a protease.

To Categorize the Deorphanized Caspase-3 and -9 Substrates

By Taking into Account of Their Cellular Roles

Identifying the substrates of an individual caspase is crucial to determine if that caspase is involved in cellular processes other than apoptosis (for apoptotic caspases) and pyroptosis (for inflammatory caspases). One proteomics method of determining the substrates of an individual caspase is reverse N-terminomics, in which a recombinant form of caspase can be incubated in cell lysate to perform proteolysis, and then cleaved substrates bound to a biotinylated ester-tag can be detected by mass spectrometry. Using reverse N-terminomics on caspases, caspase-9 and -3, we identified 124 and 906 substrates, respectively (Chapter IV).14

Of 124 substrates of caspase-9, 20 substrates were not reported in the Degrabase. Of 906 substrates of caspase-3, 257 substrates were not reported in the Degrabase.15 Thus, the caspase-3 and -9 substrates which were not reported in the Degrabase could, in principal, either be apoptotic or nonapoptotic substrates. For example, caspase-9 substrates, RECQL5, NUP43, and RNF126 were not reported in the Degrabase (Chapter IV). From the immunoblotting analysis of Jurkat cells treated with staurosporine (STS), we found that RECQL5 is proteolyzed during apoptosis. However, NUP43 and RNF126 are not proteolyzed in STS-induced apoptosis. Thus, we identified two potential nonapoptotic caspase-9 substrates. Using the same approach as described in Chapter IV, we can perform immunoblotting on STS treated cell lysates to determine whether the substrates that are nonreported in the Degrabase are apoptotic or nonapoptotic substrates. Thus, this approach will be beneficial in identifying nonapoptotic substrates of caspase-3 and -9. From this knowledge, we can also determine the involvement of caspase-3 and -9 in the cellular pathways other than apoptosis, which would be a substantial contribution to the field.
To Identify Common Substrates of Apoptotic Caspases

From our N-terminomics results as described in the Chapter IV, we identified 50 and 832 substrates that are unique for caspase-9 and -3, respectively. From the same analyses, we found 74 common substrates of caspase-3 and -9. Identifying common substrates of apoptotic caspases specifically with the same cleavage site can be beneficial in determining the significance of the proteolytic redundancy. For example, PARN is cleaved by both caspases, caspase-3 and -9, at D595. Thus, PARN cleavage at D595 by two caspases is therefore highly likely to be an important and perhaps an indispensable event during apoptosis. To further investigate, an in vitro analysis, as shown in Figure 6.3\textsuperscript{16} can be performed for apoptotic caspases, caspase-3, -6, -7, -8, -9, and -10. Cell lysates separately incubated with these apoptotic caspases can be analyzed via immunoblotting to detect the 74 common substrates of caspase-3 and -9. From these data, we can identify common substrates of apoptotic caspases.

![Figure 6.3](image)

**Figure 6.3. Immunoblotting was employed to derive which caspases cleave SNX1.**
HeLa cell lysates were incubated with recombinant caspase-2, -3, -6, -7, -8, -9, and -10. SNX1 is cleaved by caspase -8, -9, and -10 but not by caspase-2, -3, -6, -7, and 10. Thus, SNX1 is a common substrate of caspase-8, -9, and -10. The figure was adapted\textsuperscript{16}.

To Derive the Catalytic Efficiencies of Caspase-3 and -9 to Cleave Their Protein Substrates

Deriving the catalytic efficiency (or cleavage rate) of any caspase to proteolyze its substrates is necessary to identify most preferred substrates of that particular caspase. Based on the list of the most preferred substrates, the major cellular roles of a caspase can be categorized (e.g.,
prefers to cleave RNA binding proteins, or prefers to cleave mitochondrial proteins, or prefers to cleave ATPases). Our N-terminomics results identified 124 and 906 substrates of caspase-9 and -3, respectively. Kinetics studies as shown in Figure 6.4\(^\text{16}\) can be employed on the substrates of caspase-3 and -9. To begin with, a few substrates that belong to an individual protein family (e.g., GTPases, RNA helicases, and E3 ligases) can be assigned as representatives. We can incubate cell lysates with varying concentrations of recombinant caspase-3 or -9. Substrates (and therefore the protein family which they represent) with the higher cleavage rates can be correlated as the most preferred substrates of caspase-3 or -9. Consequently, these results will be helpful in learning the predominant proteolytic role/s of caspase-3 and -9.

**To Determine Cleavage Preference of Caspase-9 for Other Substrates**

As described in Chapter IV, using reverse N-terminomics, we deorphanized 124 and 906 substrates of caspase-9 and -3, respectively. These experiments were performed in duplicate using similar approach with the only change of incubating cell lysates with respective caspase (caspase-3 or -9). Thus, caspase-3 has ~8 times more substrates than caspase-9. One reason of this can be that caspase-3 is much more active ($k_{cat}/K_m = 7.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) than caspase-9 ($k_{cat}/K_m = 3.3 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$).\(^\text{17,18}\) However, kinetic rates determined by peptide substrates cannot be generalized for all protein substrates. For example, caspase-3 is more active than caspase-7; nevertheless, using its exosites, caspase-7 proteolyses PARP and p23 more efficiently than caspase-3 does.\(^\text{19}\) From the Chapter III, we also found the factors such as the sequence and the local context of the substrate cleavage site play major role in a successful proteolysis. Therefore, to understand why caspase-9 has almost 8 times fewer substrates than caspase-3, a molecular level investigation (such as shown in Chapter III) on a caspase-3 substrate that is not cleaved by caspase-9 can be done (e.g., GSDMD...
and PAK2). GSDMD is cleaved at DAMD\(^{87}\)G by caspase-3 but not by caspase-9. The caspase-3 cleavage site of GSDMD (DAMD\(^{87}\)G) can be replaced to caspase-9 canonical sequence, LEHDS.\(^{20}\) Substrate-digestion assays on these engineered constructs will show whether GSDMD protects its cleavage by caspase-9 due to the sequence or the local context or exosites. From prior work, it was demonstrated that cleavage of GSDMD at D87 by caspase-3/7 is critical for faithful execution of apoptosis, as it is sufficient to prevent pyroptosis.\(^{21}\) GSDMD is a potential therapeutic target in multiple inflammatory diseases.\(^{22,23}\) Therefore, investigating GSDMD cleavage by different caspases at molecular level will be helpful in targeting GSDMD.

**To Determine the Mechanism of Procaspase-6 ATP Binding**

Because of its involvement in neurodegenerative pathways, caspase-6 is a potential therapeutic target in the treatment of neurodegenerative diseases. In the efforts of designing therapeutics of caspase-6, studying its cellular functions is vital. One such type of function is ATP-binding to procaspase-6 as described in the *Chapter V*.\(^{24}\) By employing an ATP-based acyl phosphate probe, we demonstrated that ATP binds to the inactive procaspase-6 but not mature caspase-6. By employing the same chemoproteomics approach, we confirmed that the dimer interface of procaspase-6 consisting of Y198 is a putative ATP binding site. We also showed that ATP attenuates the auto-activation of procaspase-6 with an IC\(_{50}\) of 19 mM.

Although by employing an ATP-probe, we confirmed that Y198 of procaspase-6 is the ATP-binding site, the binding mechanism remains to be determined. To further investigate, we can identify other key residues of procaspase-6 involved in ATP-binding. For that, we can first test the ATP-binding impact on the residues that bind to known inhibitors at dimer interface of procaspase-6.\(^{25}\) Residues are E214, T199, N125, and Q137 at the dimer interface of procaspase-6 are putative nucleotide-binding site at dimer interface (*Figure 6.5*). All these residues can be substituted with alanine via site-directed mutagenesis. The resulting constructs can be tested against the ATP-probe as Y198A was described in *Chapter V*. We anticipate lower probe labeling of ATP-probe for the constructs lacking residue/s facilitating ATP-binding. As an alternative approach, the self-
proteolysis rate of procaspase-6 constructs can be derived in the presence and absence of ATP as performed in Chapter V. We can expect poor IC\textsubscript{50} values for the constructs lacking facilities of ATP binding.

To determine the mechanism of ATP binding to procaspase-6, X-ray crystallography approach can be employed. For that, we can use known and novel crystallizing conditions of caspase-6 and procaspase-6 with two different approaches: co-crystallization method and the ligand soaking method. Murray et. al\textsuperscript{25} used soaking approach to solve the structure of procaspase-6 with its known inhibitors that bind at the dimer interface. Therefore, we can start with crystallizing unliganded procaspase-6, full-length caspase-6 C163S, using condition: 45% (w/v) 2-Methyl-2,4-pentanediol, 0.3 M Ammonium Phosphate monobasic, and 0.1 M Tris pH 8.5. Grown crystals can be soaked in a solution containing ATP. Soaked crystals can be subjected to X-ray diffraction analysis to gather crystallography data. From the crystal structure, we can anticipate that ATP will

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**Figure 6.5. Putative ATP-binding residues at the dimer interface of procaspase-6.**

Crystal structure of procaspase-6 is shown (PDB: 3NR2). The active site C163 residues are shown as red spheres. The solvent exposed residues and K133 (known to bind ATP-probe, colored yellow) at dimer interface are shown as sticks where Y198, the major binding site, is shown as cyan. The orange-colored residues are from chain A and green colored residues are from chain B. Known chemically synthesized inhibitors interact with Y198 by pi-pi stacking while the other residues by H-bonding.
be bound to the dimer interface of procaspase-6. At last, the mechanism of ATP-binding can be
determined by comparing the structure of ATP-bound procaspase-6 with the unliganded
procaspase-6. This knowledge of ATP binding mechanism will be very helpful in the ongoing
research of synthesizing potent and selective inhibitors of caspase-6 for neurodegenerative
diseases.

**Studying Caspases at Molecular Level Is Crucial**

As we described throughout in this dissertation that caspases are centric in various cellular
pathways. Therefore, understanding their functions and structures is necessary. In this dissertation,
we thoroughly investigated key apoptotic caspases especially caspase-9 and -6. We employed
H/DX-MS technique and determined the dynamics of different states of caspase-9. We also
interrogated caspase-9 substrate cleavage preference by performing substrate-digestion assays on
engineered procaspase-3 and -6 constructs. This study demonstrated that both the sequence as well
as the local context of the substrate cleave site play important role in proteolysis. We employed N-
terminomics and deorphanized 124 and 906 substrates of caspase-9 and -3, respectively. Also, from
immunoblotting on STS treated c
ells, we classified some apoptotic and nonapoptotic substrates of
caspase-9 and -3. At last, using ATP acyl probe, we found the dimer interface is a putative
procaspase-6 ATP-binding site; moreover, ATP inhibits self-proteolysis of procaspase-6 with an
IC$_{50}$ of 19 mM. We are optimistic that in addition to these findings, the future directions mentioned
in this chapter (**Chapter VI**) will be valuable in the ongoing research of apoptotic caspases
(specifically caspase-3, -6, and -9) and their involvement in various cellular pathways.
References


APPENDIX I:

THERMAL STABILITY OF CASPASE-6 SNPS
(SINGLE NUCLEOTIDE POLYMORPHISMS): G66R AND R65W

This appendix section is my contribution in the Results and Materials and Methods of the publication: Tubeleviciute-Aydin, A.; Zhou, L.; Sharma, G.; Soni, I. V.; Savinov, S. N.; Hardy, J. A.; and LeBlanc, A. C. Rare human Caspase-6-R65W and Caspase-6-G66R variants identify a novel regulatory region of Caspase-6 activity. Sci Rep 8, 1-14 (2018).

Note: Please see above publication for full manuscript.

Results

The thermal melting curves of wild-type (WT) caspase-6, caspase-6 R65W and caspase-6 G66R with and without a substrate mimic, Ac-VEID-cho.

Thermal shift assays were performed in the absence of an active site ligand to assess the intrinsic stability of wild-type (WT) caspase-6 and single nucleotide polymorphisms (SNPs): caspase-6 R65W and caspase-6 G66R, and their melting temperatures (T_m) were compared. The results revealed that the intrinsic thermal stability of caspase-6 R65W was essentially unchanged relative to WT caspase-6, and the stability of caspase-6 G66R was decreased by ~4 °C (Figure A.1.1), suggesting that the changes in the activities of these variants was not due to global unfolding or misfolding of the proteins. In the presence of an excess amount of active site inhibitor, Ac-VEID-cho substrate, a 7.3 °C stabilization of WT caspase-6 was observed. The increase in stability upon incubation with Ac-VEID-cho was lower for caspase-6 R65W (4.6 °C) and absent for caspase-6 G66R. This suggests that caspase-6 R65W does bind substrate, albeit much less efficiently than WT caspase-6 and that caspase-6 G66R does not bind substrate to any appreciable extent. These results were consistent with the catalytic parameters of these variants.¹
Materials and Methods

Thermal shift assays.

Caspase-6 ΔN D179CT (a WT mimic of caspase-6), caspase-6 R65W large and small subunits, and prodomain lacking ΔN caspase-6 G66R were expressed and purified as described.  
These caspase-6 variants (10 μM) were incubated in 20 mM Tris, pH 8.5 and 5 mM DTT with or without an active-site inhibitor Ac-VEID-cho (50 μM; Enzo Life Sciences) and 5×SYPRO® Orange dye (Thermo Fisher Scientific) in a 60 μL reaction. Fluorescence (ex/em: 490/575 nm) was measured in a 96-well plate using a CFX Connect Real-Time PCR instrument (BioRad). RFU recorded from 25 to 95 °C with 0.5°C/ 3 second intervals were normalized to the highest observed intensity which was set to 1. The normalized fluorescence was fit to a Boltzmann sigmoidal curve using Prism (GraphPad) software. Melting temperature ($T_m$) was found to be the temperature at the midpoint of the denaturation curve.

Figure A.1.1. The thermal melting curves of WT caspase-6, caspase-6 R65W and caspase-6 G66R with and without Ac-VEID-cho.

The thermal melting temperatures ($T_m$) defined as the temperature at the midpoint of the unfolding. The difference between the melting temperatures of the unliganded and Ac-VEID-cho bound protein were calculated as $\Delta T_m = T_m$ (with Ac-VEID-cho) − $T_m$ (without Ac-VEID-cho). Each experiment was done in triplicates using three different aliquots of caspase-6 variants on three separate days.
References


APPENDIX II:

THERMAL STABILITY OF CASPASE-2, -3, -6, -7, -8, AND -9

IN THE PRESENCE OF COMPOUND A

This appendix section is my contribution in the Results and Materials and Methods of the manuscript being prepared as: Narasimharao, MP, Dagbay, K. B.; MacPherson, D. J., Pei, Y., Velazquez-Delgado, E. M., Soni, I. V., and Hardy, J. A. Discovery of Potent and Selective Inhibitors of Caspase-6. *manuscript in preparation*

**Note:** Data of other authors mentioned above are available in the dissertation of Derek J. MacPherson. Present members of the Hardy lab, Andrew J. Smith and Ma. Irina Sagarbarria, and the collaborators are currently working on the other aspects of this project.

**Results**

**Compound A selectively binds to caspase-6 over other caspases, caspase-2, -3, -7, -8, and -9.**

We performed thermal shift assays on unliganded caspase-6, caspase-6 with Compound A, and caspase-6 with an active site inhibitor, Ac-VEID-cho (Figure A.2.1.A). A shift in melting curve was observed for caspase-6 in the presence of Compound A and Ac-VEID-cho. Thus, Compound A binds and stabilizes caspase-6. We also performed thermal shift assays on other caspases, caspase-2, -3, -7, -8, and -9 with and without Compound A (Figure A.2.1.B-F). We did not observe a significant change in the melting curves for these caspases (Figure A.2.1.B-F) as we observed for caspase-6 (Figure A.2.1.A). The melting temperatures ($T_m$) derived from the melting curves are reported (Figure A.2.1.G). Ac-VEID-cho is a known inhibitor of caspase-6, and it stabilizes caspase-6 upon binding to its active site.\(^1\)\(^2\) Upon Ac-VEID-cho binding, the thermal stability of caspase-6 increases by ~ 8°C (Figure A.2.1.G), consistent with what has been reported earlier.\(^2\) We observed ~ 3°C increase in the thermal stability of caspase-6 in the presence of Compound A (Figure A.2.1.G). The thermal stabilities of other caspases, caspase-2, -3, -7, -8, and -9, in the presence of Compound A did not change significantly, indicating that Compound A
Figure A.2.1. Compound A selectively binds to caspase-6 over other caspases, caspase-2, -3, -7, -8, and -9.

(A) Thermal melting curves of caspase-6 unliganded, with Compound A and with an active site inhibitor, Ac-VEID-cho. (B-F) Thermal melting curves of caspase-2, -3, -7, -8, and -9 unliganded and with Compound A. (G) Melting temperatures ($T_m$) of the curves in (A-F). $T_m$ is defined as the temperature at the midpoint of the unfolding. The difference between the melting temperatures of the unliganded and Compound A bound caspase were calculated as $\Delta T_m = T_m$ (+ Compound A) $- T_m$ (unliganded). Similarly, difference between the melting temperatures of the unliganded and Ac-VEID-cho bound caspase-6 was calculated as $\Delta T_m = T_m$ (+ Ac-VEID-cho) $- T_m$ (unliganded). Each experiment was done in duplicates using two different aliquots of caspases on two separate days.
does not stabilize these caspases as it does caspase-6 (Figure A.2.1.G). These results complemented our counterscreen and IC$_{50}$ determination data demonstrating why Compound A is highly selective towards caspase-6 over other caspases.

**Materials and Methods**

**Expression and purification of caspases.**

Wild-type (WT) caspase-3 (which self-activates upon bacterial overexpression), caspase-6 ΔN D179CT (a WT mimic of active caspase-6), caspase-7 D198CT (a WT mimic of active caspase-7), caspase-8 ΔDED (which self-activates upon bacterial overexpression), and WT caspase-9 (which self-activates upon bacterial overexpression) were expressed and purified as described. Caspase-2 ΔCARD (Addgene plasmid: 11810; which self-activates upon bacterial overexpression) was expressed and purified as previously described.

**Thermal shift assays.**

We followed previously described method to follow thermal melting curves using the Sypro orange assay. Caspases, caspase-2, -3, -6, -7, -8, and -9 (10 μM) were incubated for 1.5 hours in 20 mM Tris, pH 8.5 and 5 mM DTT with or without Compound A (50 μM), and 5×SYPRO® Orange dye (Thermo Fisher Scientific). Similarly, caspase-6 (10 μM) was separately incubated for 1.5 hours in 20 mM Tris, pH 8.5 and 5 mM DTT with an active-site inhibitor, Ac-VEID-cho (50 μM; Enzo Life Sciences). Fluorescence (ex/em: 490/575 nm) was measured in a 96-well plate using a CFX Connect Real-Time PCR instrument (BioRad). Relative fluorescence (RFU) was recorded from 25 to 94°C with 0.5°C/ 3 second intervals and were normalized to the highest observed intensity, which was set to 1. The normalized fluorescence was fit to a Boltzmann sigmoidal curve using Prism (GraphPad) software. The melting temperature (T$_{m}$) was found to be the temperature at the midpoint of the denaturation curve.
References


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