STRUCTURAL STUDIES OF PROTEINS IN APOPTOSIS AND LIPID SIGNALING

Maria Dolores Hermán Moreno
Structural Studies of Proteins in Apoptosis and Lipid Signaling

Doctoral Thesis

Maria Dolores Hermán Moreno
Abstract

Signaling pathways control the fate of the cell. For example, they promote cell survival or commit the cell to death (apoptosis) in response to cell injury or developmental stimuli, decisions, which are vital for the proper development and functioning of metazoan. Tight control of such pathways is essential; dysregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death ending up in pathological processes, including cancer, autoimmunity diseases, inflammatory diseases, or degenerative disorders. We have used a structural genomic approach to study the structure and function of key proteins involved in apoptosis and lipid signaling: the anti-apoptotic Bcl-2 family member Bfl-1 in complex with a Bim peptide, the BIR domains of the Inhibitor of Apoptosis (IAP) family members, cIAP2 and NAIP and the lipid kinase YegS. The structural analysis of the apoptosis regulatory proteins has revealed important information on the structural determinants for recognition of interacting proteins, which can now assist in the development of therapeutic drugs for human diseases. The structural and complementing biochemical studies of the lipid kinase YegS have revealed the first detailed information on a lipid kinase and explained important aspects of its structure-function relationship.

Finally, one subject of this work aim to solve what is arguably the most challenging problem in structural projects – to obtain a high production level of proteins suitable for structural studies. We have developed a high-throughput protein solubility screening, the colony filtration (CoFi) blot, which allows soluble clones to be identified from large libraries of protein variants and now constitute a powerful tool for solving difficult protein production problems.
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II. The first crystal structure of BIR domains from Human NAIP and cIAP. Maria Dolores Herman, Martin Moche, Susanne Flodin, Martin Welin, Lionel Tresaugues, Ida Johansson, Martina Nilsson, Par Nordlund, Tomas Nyman. Submitted.


Additional Publications


### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2 family</td>
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<tr>
<td>BH</td>
<td>Bcl-2 Homology</td>
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<tr>
<td>BIR</td>
<td>Baculovirus IAP Repeats</td>
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<tr>
<td>CoFi</td>
<td>Colony Filtration</td>
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<tr>
<td>C-terminus</td>
<td>Carboxy-terminus</td>
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<tr>
<td>FiDo</td>
<td>Filtration Dot</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis Proteins</td>
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<tr>
<td>IBM</td>
<td>IAP Binding Motif</td>
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<tr>
<td>IMAC</td>
<td>Imobilized Metal Affinity Chromatography</td>
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<tr>
<td>MAD</td>
<td>Multiwavelength Analogous Dispersion</td>
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<tr>
<td>MOMP</td>
<td>Mitochondrial Outer Membrane Permeabilization</td>
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<tr>
<td>N-terminus</td>
<td>Amino-terminus</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
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<td>SAD</td>
<td>Single Wavelength Anomalous Dispersion</td>
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General Introduction

A living cell is a system in a dynamic interaction with its surrounding. Cell survival depends on the development of an efficient system to sense any change that can perturb homeostasis, and be able to adjust its own activity and composition accordingly. Unicellular organisms, such as bacteria, may be subjected to widely varying environmental conditions that require a sensitive system to sense these changes and launch an elaborate and rapid response from the cell.

In multicellular organisms, metazoan, this system is even more important and complicated, since the cells have to integrate the responses to different signals originated within the cell itself and as part of the organisms. Cells in metazoan communicate with each other by actively sending signals that can be sensed and interpreted, defining the concept of cell signaling. Signals used in cell signaling encompass molecules of very diverse nature – such as gases, nucleotides, lipids or proteins. Receptor proteins at the surface of the cell sense the extracellular stimuli and transfer the signal inside the cell using different signal transduction pathways, depending on the nature of the signal. Cell signaling often results in modification of gene expression that allows the cell to initiate a proper answer to the extracellular stimuli. The ability of cells to send and respond to signals is crucial to development, homeostasis and a wide variety of other fates of the cells. Disturbances in these signal transduction pathways can be responsible of developmental abnormalities, cancer, inflammation and/or other diseases.

Some of the proteins involved in regulation and execution of cell signaling pathways, have been identified but many still remains unknown. Genomes encode all the information to produce the proteins needed by an organism, to control biological structure and function at every level. Genomic projects have sequenced whole genomes of many different species (1, 2). In 2003, the Human Genome Project was finished, opening a door to systematic studies of the proteins of the cell, such as functional genomics, structural genomics and proteomics. The tentative functions of about half the proteins in humans and several others eukaryotic organisms have been predicted based on analy-
ses of genomic sequences (3). Such analyses have revealed that at least 10-15% of the proteins in eukaryotes function in cell signaling.

The human body may contain more than a million different proteins (Human Proteomic Initiative, HPI, Expasy) each having different functions, although, the human genome harbors only 20-25 000 genes. This discrepancy suggests that protein diversity cannot be explained from a simple gene-sequence point of view only. The protein diversity is thought to be due to alternative splicing and post-translational modification of proteins. Proteomics is a useful means for characterizing the role of this diversity in biological and cellular processes (4). Knowledge of the molecular structure and mechanism of proteins allow us to interpret complex cellular and organism data on protein function, which can be applied to explain the origins of pathological processes and diseases (5). Information on protein structures can be critical for the development of therapeutic drugs when potential drug binding sites and detailed binding modes of ligands can be revealed (6, 7).

Structural genomics (SG) is an international effort to streamline the path from gene to three-dimensional structure of proteins, by establishing high-throughput methods for protein production and structure determination (8, 9). The concept started simultaneously in several laboratories around the world in the end of the last century. Today the largest efforts are the Protein Structure Initiative (PSI) (10) in the US, the RIKEN initiative in Japan, and the Structural Genomic Consortium, an Anglo-Canadian-Swedish collaboration.

In my thesis work I have focused on the pathways that decide the fate of the cell, allowing cell survival or commit the cell to programmed death (apoptosis) in response to cell injury or developmental stimuli. We used a structural genomic approach to study the structure and function of key proteins involved in apoptosis and lipid signaling, which consequently are of great importance for the development of therapies against human diseases. One subject of this work aims to solve what is arguably the most challenging problem in structural projects – to obtain high-level production of proteins suitable for structural studies. We have developed a high-throughput protein solubility screening, the Colony Filtration (CoFi) blot, which allows soluble clones to be identified from large libraries of protein variants.

The Cell Cycle

The cell cycle defines the physiological process used by the eukaryotic cell to replicate, generating two new daughter cells. This process is an ordered
set of events, that can be divided into four well defined stages: stage GAP-1 (G1), Synthesis stage (S), GAP-2 stage (G2) and Mitosis stage (M). During stages G1, G2 and S, commonly known as interphase, the cell synthesizes most of the components needed for the new daughter cells, duplicates the chromosomes and augments in size (Figure 1). Mitosis is the final event of cell cycle where the cell literally divides the cytoplasm and chromosomes into two identical daughter cells. The cell cycle is tightly regulated by cyclin kinases, through protein phosphorylation.

Figure 1. The eukaryotic cell cycle. Divided into four stages, G1, G2, S and M. G0 is a regulation point, where cell decides to continue division or enter in a quiescent stage.

Cyclin kinases are heterodimers with a regulatory subunit cyclin, and catalytic subunit cyclin-dependent protein kinase (CDK). Animal cells have at least ten different cyclins and eight CDK, which can be combined depending of the response required. Cyclin kinases act by phosphorylating specific proteins at specific time points in the cell cycle to assure an orderly cell division process.

CDK activity is also regulated by phosphorylation and degradation or by extracellular signals such as growth factors and cytokines that act by controlling protein synthesis. Defects in the proper regulation of the cell cycle can cause abnormal cell growth and diseases, as will be discussed in a later section of this thesis (11).
Cell Death

Metazoans originate from a unique cell, which divides and differentiates into different cell types evolving into an organism. Cell death becomes very important in regulation of cellular homeostasis and during embryogenesis. Cell death can be achieved by necrosis, apoptosis or autophagy, depending on the stimuli that induces cell death and the basal energy state of the cell (12).

Cell death induced by a physical damage that results in a bioenergetic catastrophe with a total depletion of ATP, leads to necrosis (13). In this scenario, with no ATP supply, cell survival becomes impossible and the cell dies passively by breakdown of the plasma membrane and vacuolation of the cytoplasm. Necrosis triggers an inflammatory response in the organism.

Apoptosis, also known as cell suicide is a physiological process, a “programmed cell death” highly regulated by cell signaling pathways and needs energy input in the form of ATP (14, 15). Kerr, Willis and Currie discovered apoptosis in 1972 (16). Apoptosis is induced by a vast number of stimuli as growth factor deprivation, DNA damage, developmental signals, oncogene activation, cellular stress and anticancer drugs. Cells that undergo apoptosis present distinctive morphology characteristics, nuclear condensation and fragmentation, cleavage of chromosomal DNA into 200 bp fragments and packing of the cell into apoptotic bodies without plasma membrane fragmentation. Typically the apoptotic bodies are engulfed by nearby cells within 24 hrs and disappear without activation of an inflammatory response. Apoptosis is a highly conserved mechanism where cell death is executed as a result of cysteine proteases family (caspase) activation.

Autophagy is defined as programmed necrosis, where cell death is achieved in an apoptotic independent way, leading to necrotic death with the difference that necrosis is regulated by cellular signaling pathways rather than by accident (normal necrosis). It has been hypothesized that autophagy could be a survival strategy of the cell during periods of lean, and by degradation of cellular constituents the cell obtains the energy required for survival. In autophagic death, activation of caspases is not needed; instead a double-membrane vesicle that encapsulates whole organelles (autophagosome) is formed and fuses later on with the lysozome where the energy is obtained.

Autophagy is responsible for degradation/recycling process in eukaryotic cells, proceeding without an activation of inflammatory responses. Never the less, dysregulation of autophagy is often associated with an inflammatory response.
Apoptosis and Disease

Dysregulation of apoptosis by an excess or deficit in cell death, frequently lead to different types of human diseases (17). In cancer (18) and autoimmune diseases (19), cells escape the apoptotic program and promote cell survival, frequently by overexpressing anti-apoptotic proteins such as Bcl-2 anti-apoptotic family members or IAP proteins. Apoptosis is the death pathway used to remove autoreactive T-cells after an immune response - dysregulation of apoptosis can lead to the autoimmune lymphoproliferative syndrome (ALPS) (20). Anti-apoptotic proteins are also involved in tumor maintenance and resistance to chemotherapy treatments (21). Inhibition of these anti-apoptotic proteins is an attractive strategy for cancer therapy. There are more than 100 distinct types of cancer and subtypes of tumors that can be found within specific organs, the molecular differences that result in this high number of tumor types remains unknown.

Many viruses such as adenoviruses and some herpesviruses, have proteins homologous to anti-apoptotic Bcl-2 proteins, which they use in the strategy to evade host defense mechanisms that would otherwise trigger death of the infected cell (22). Inhibition of apoptosis in the infected cell will lead to persistence of the infection. Some of these viruses are responsible for generation of tumors.

Degenerative disorders such Alzheimer’s, Parkinson’s and Huntington’s diseases are characterized by the premature and excessive loss of neurons via apoptosis (23). Excessive apoptosis is also a hallmark of inflammatory diseases, spinal muscular atrophy, stroke and myocardial infarction (24).

It is therefore of great importance, for the future development of appropriate treatments, to understand the mechanism of regulation and structure of proteins involved in apoptotic signaling.

Apoptotic Pathways

Apoptosis is vital for the proper development and functioning of metazoan, by removing defected cells such as damaged, aged or infected cells (25). As was mentioned above, tight control is essential; dysregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death and could end up in a pathological process. Evasion of apoptosis has been recognized as one of the six essential alterations in cell physiology that indicate
malignant growth and is a hallmark of most, and maybe all types of cancer (26).

**Figure 2.** Schematic illustration of intrinsic and extrinsic apoptotic pathways in mammalian cells.

Multicellular organisms have developed two parallel apoptotic pathways to check the viability of the cell, the intrinsic and the extrinsic pathway (Figure 2) (27, 28). Both pathways are evolutionarily conserved and lead to apoptosis by triggering caspase activation (29). In the extrinsic pathway the death signal comes from outside of the cell, from the environment (30). It is detected by the death receptors on the surface of the cell that transmit the death stimuli into the cell activating caspase 8 (caspase 10 in humans) via adaptor proteins like Fas-associated death domain protein (FADD).
The second apoptotic pathway, the mitochondrial or intrinsic pathway senses the wellness of the cell itself, responds to stress and some developmental apoptotic stimuli that originates from the inside of the cell (31, 32).

The B-cell lymphoma 2 (Bcl-2) family proteins play a central role in the regulation of the mitochondrial apoptotic pathway (33, 34). Bcl-2 proteins propagate the death signal by inducing permeabilization of the mitochondrial membrane (MOMP), release of cytochrome c, that lead to activation of Apf-1 that in the presence of ATP forms the apoptosome. The apoptosome induces the proteolytic degradation of the cell by activation of caspases.

The activation by both pathways gives rise to the targeted degradation of both cytoplasmic and nuclear structures and the formation of apoptotic bodies that are rapidly engulfed and cleared by phagocytic scavenger cells (29).

Apoptosis can be stopped at the caspase level by the action of Inhibitor of Apoptosis Proteins family (IAP)(35-37). The IAP family interacts directly with caspases inhibiting their proteolytic function. Smac/DIABLO, another protein released together with cytochrome c from mitochondria during apoptosis, interacts with different IAP members regulating their activity (38).

In some cases, the intrinsic and the extrinsic pathways are cross-linked by the Bid protein, which can be activated by caspase 8 (or 10 in humans) in response to a death receptor signal and will deliver the death message to mitochondria inducing in this way the intrinsic pathway (39).

The Intrinsic Pathway

The Bcl-2 Family

Bcl-2 was discovered by Tsujimoto in 1984 in follicular lymphoma cells. Bcl-2 corresponded to a gene locus on chromosome 18 (band q21) that was not related to any other known oncogene and furthermore, in B cell lymphomas promotes cell survival (40). Since the discovery of Bcl-2, more than two dozens of members of this family have been identified. The Bcl-2 family is divided in three subclasses: the anti-apoptotic family members, pro-apoptotic and the sensor proteins BH3-only proteins (32, 33). The distinctive feature of the Bcl-2 is the presence of at least one and up to four, Bcl-2 homology motifs (BH1, BH2, BH3, and BH4) (Figure 3).
Bcl-2 family members that carry four BH motifs (BH1, BH2, BH3 and BH4) function as anti-apoptotic members. The mammalian anti-apoptotic family is composed of Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bfl-1 and Bcl-B in humans (41). The pro-apoptotic Bcl-2 members Bax, Bak, and Bok are the effectors of apoptosis, and contain only BH1, BH2 and BH3 motifs plus a well-defined trans-membrane C-terminal region. Bax, Bak and Bok upon apoptotic activation, oligomerize and interact with the mitochondrial outer membrane through the trans-membrane domain disturbing membrane integrity (31, 42). Permeabilization of the mitochondrial membrane results in the release into the cytoplasm, of cytochrome c and other proteins through the pores believed to be formed by Bax, Bak and Bok.

Bcl-2 members that contain only the BH3 motif are called BH3-only proteins, e.g. Bad, Bik, Bid, Bim, Hrk, Bmf, Noxa and Puma (43, 44). Considered also pro-apoptotic, they work as sentinels of the cellular well-being. BH3-only proteins are activated in response to a death signal, initiating in
this way apoptosis. Each mammalian BH3-only protein is coupled to distinct upstream controls, for example Puma responds to DNA damage, and by interacting with p53. Bim is crucial for eliminating lymphocytes that recognize self-antigens during development of T and B cells.

![Figure 4](image)

**Figure 4.** Binding profile of the different members of the BH3-only proteins towards the anti-apoptotic Bcl-2 family members.

BH3-only proteins interact with the other Bcl-2 family members via the BH3 motif, which is necessary and probably sufficient for their killing action (45, 46). Not all BH3-only proteins interact with the entire anti-apoptotic family. Profiling binding studies reveal that Bim, Bid and Puma can bind to all anti-apoptotic members, whereas Bad only interacts with Bcl-xl, Bcl-w, Bcl-2 and Noxa with Mcl-1, Bfl-1 (42, 47-50) (Figure 4).

Bcl-2 proteins are localized in different cellular compartments. They contain also a putative membrane anchoring sequence at their C-termini responsible for its interaction with biological membranes.

**Regulation of the Mitochondrial Pathway**

The mechanism for the regulation of apoptosis still remains controversial and is the focus of an ongoing discussion (17, 51). The balance between pro-apoptotic and anti-apoptotic Bcl-2 family members decides if the cell is going to live or die by a complicated set of protein-protein interactions, where the role of these interactions is not fully understood. It is known that after cell death signals are sensed by BH3-only proteins, they are activated and transduce the signal to other members of the family. The BH3-only proteins act upstream of Bak, and Bax, since it has been shown that apoptosis cannot
be induce in cells lacking these two proteins. This discovery established an essential role for Bak or Bax in apoptosis. However, our understanding of the dialog between pro-apoptotic and anti-apoptotic proteins, which decide the fate of the cell, is not conclusive.

There are different schools that propose different scenarios:

1. The direct activation model. In this model (Figure 5A), members of the BH3-only proteins are divided into two categories; activator and sensitizer (42, 49, 52). Activator BH3-only proteins (Bim, Bid and Puma) bind to Bax and Bak consequently inducing their activation and MOMP. Sensitizer BH-3-only proteins, bind only to different anti-apoptotic members, in this way they release Bim, Bid from the anti-apoptotic members, which will directly activate Bak and Bax.

2. The indirect activation model. This model (Figure 5B) suggests that BH3-only proteins only bind to their anti-apoptotic partners (50, 53). Bax and Bak, are constitutively active on the surface of mitochondria or inhibited by anti-apoptotic proteins in the cytosol. In response to a death stimuli BH3-only proteins bind to anti-apoptotic members releasing Bak and Bax, in other words inducing apoptosis indirectly. Bim, Bid and Puma have potent pro-apoptotic activity based on the fact that they can bind to all the anti-apoptotic family members. This model requires that BH3-only proteins must neutralize the entire set of anti-apoptotic member expressed in that cell.

3. The prime for death model is an adaptation of the indirect model (Figure 5C) (53). A healthy cell contains a small amount of Bak and Bax in a “prime” stage with the BH3 motif available and bound to anti-apoptotic members Mcl-1, Bcl-xl located on the mitochondrial surface. Additional Bak and Bax proteins that are not primed are found as monomers in the cytosol, until activation. Anti-apoptotic members in healthy cells are bound to activator BH3-only proteins. After cell death has been induced, sensitizer BH3-only proteins bind to anti-apoptotic members with higher affinity displacing the activator BH3-only proteins, and Bax now can oligomerize leading to MOMP and cell death.
Figure 5. Representation of the three proposed models of regulation for the apoptotic intrinsic pathway.

Caspases

Caspases are the final effectors of apoptosis acting downstream both of the intrinsic and extrinsic pathways (54, 55). In 1993, Yuang et al described the caspase family for the first time. Caspases is a family of proteases characterized by a cysteine residue at the active site, that recognize a tetra-peptide sequence where Asp is the last residue before the cutting site. There are known 14 different mammalian caspases that can be divided into two categories: initiator caspases, such as caspases -2, -8, -9, and effector caspases, such as caspases -3, -7.
Caspases are synthesized as zymogenes in the cell (56). They undergo activation in response to apoptotic stimuli by proteolytic cleavage at an internal Asp residue situated in the second loop. Initiator caspases catalyze their own auto-activation and the activation of effectors caspases in response to death signals.

**Figure 6.** Structure of a procaspase-7 zymogen, constituted by two monomers colored in yellow and in blue respectively.

From the tertiary structure point of view, initiator and effector caspases are quite conserved (Figure 6) – they are active as homodimers and each peptide has a small subunit linked to a large subunit. The main difference between initiator and effector caspases is found at the N-terminal region, which is longer and includes accessory domains in initiator caspases (> 90 residues) than in effector caspases (< 30 residues).

**Inhibitor of Apoptosis Proteins Family.**

Inhibitor of apoptosis proteins family (IAP) has a central role in inhibition of apoptosis downstream of MOMP activation and plays important roles in cell-cycle regulation, protein degradation and caspase-independent signal-transduction cascades (29, 37). Lois Miller and colleagues identified the first IAP in baculovirus in 1993. IAP protection against cellular death seems to be mediated by direct caspase inhibition and modulation of the transcription factor NF-kappaB.
The Human IAP family consists of eight members: NAIP, neuronal apoptosis inhibitory protein; XIAP (ILP-1, MIHA); ILP-2, (Ts-IAP); ML-IAP (Livin, KIAP); Survivin (TIAP); Apollon (Bruce); c-IAP1 (HIAP2, MIHB) and c-IAP2 (HIAP1, MIHC) (37, 57).

The specific feature that identifies the IAP family is the baculovirus IAP repeat (BIR) domain (58). BIR is a ~70-80 residues zinc-binding domain (Figure 7). Three cysteine and one histidine residues chelate zinc and form the characteristic globular structure of the BIR domain, that consists of four or five alpha helices and a variable number of anti-parallel β-pleated sheets. BIR is essential for the anti-apoptotic properties of the IAP, which seems to be related to its binding and inhibition of caspases (58, 59).

The RING (Really Interesting New Gene) domain is also characteristic of some members of the IAP family such as cIAPs, and XIAP. The RING domain is a zinc finger found at the C-termini of the protein that also needs a Zn atom for its function. RING-containing proteins can promote the degradation of both themselves (as XIAP) and selected target proteins (as c-IAP2) through ubiquitin ligase activity (E3 activity). Ubiquitylation can modify the biological activity or subcellular localization of proteins or lead to its degradation (29, 60).

c-IAP1 and c-IAP2 have an extra domain; a conserved CARD domain (caspases recruitment domain) with unknown function to the date.

XIAP is the best-characterized IAP, and a very potent suppressor of apoptosis (61). Today, structures of every known XIAP domain have been determined in addition to several structures in complex with caspses or small molecules that mimic caspase binding (62-66).
Figure 7. IAP in mammals. Proteins of the inhibitor of apoptosis (IAP) family include XIAP (X-linked IAP), c-IAP1, cIAP2, ILP2 (IAP-like protein-2), ML-IAP (melanoma IAP), NAIP (neuronal apoptosis-inhibitory protein), surviving and Bruce. Each member contains at least one BIR domain. RING, really interesting new gene. CARD, caspase-recruitment domain. NAIP has in addition, two domains involved in regulation LRR domain (Leucine-Rich Repeats) and the ATP-binding domain NOD (Nucleotide-Oligomerization Domain).

IAPs function is not restricted to apoptosis; they are also involved in other biological processes such as cell-cycle regulation, protein degradation and inflammatory response.

IAP Regulation

IAP activity can be regulated at the transcriptional, post-transcriptional and post-translational level (37). IAP activity is negatively regulated by IAP regulatory proteins (inhibitors of IAPs). Inhibitors of IAP bind to the BIR domains of IAP via N-terminal binding motifs (IBMs) (38, 64, 67). Members of the inhibitor of IAP family are Smac (second mitochondrial activator of caspases) in humans, DIABLO (direct IAP binding protein with low pl) in mice, other mammalian proteins such as HtrA2/Omi and GstPT/eRF3, etc (66, 68, 69).
During apoptosis, after MOMP induction, Smac/DIABLO is released together with cytochrome c. Smac/DIABLO binds to the BIR domain of IAP proteins (second and third BIR domains) in a manner that displaces caspases from binding to IAP proteins. In this way, Smac/DIABLO inhibit IAP activity, allowing caspases to act. IBM motifs have been defined as a tetrapeptide consensus sequence (NH2)P1P2P3P4, where P2 and P4 represent a hydrophobic amino acid and P1 is usually an Ala residue at the N-terminus (Figure 8). Recently, a selectivity study, using a randomized peptide library has identified the defining residues at the IBM groove, classifying the BIR domains in four groups (70). IAPs are functionally non-equivalent and regulate effectors caspases through distinct mechanisms (71). Every BIR-type needs special residues at the consensus tetra-peptide sequence and it is argued that the sequence correlates with their different functions.

Anti-apoptotic Proteins as Targets for Cancer Therapy

Structural studies of apoptotic proteins, like Bcl-2 or IAP families have provided insights into how these proteins interact with their molecular partners (72). Improved understanding of the structural basis for these protein-protein interactions has enabled strategies for the development of potential therapeutic treatments against apoptosis related diseases (17, 21, 35, 73-75).
Two different strategies are used for developing an effective therapeutic treatment against cancer; both of them aim at silencing the action of the anti-apoptotic proteins by blocking anti-apoptotic protein expression or protein-protein interactions. The first strategy involves the development of drugs that mimic the binding of regulatory proteins to anti-apoptotic proteins inhibiting their activity and therefore promoting cell death.

In the case of IAP inhibition, for example, drugs that can bind to the IAP, mimicking the IBM domain of Smac/DIABLO will result in the inhibition of IAP by blocking the binding of IAP with caspase (66, 76-78). As a consequence free caspases will turn on cell death. To date, there are such IAP-mimicking molecules that bind selectively to the BIR domains of XIAP, cIAP-1, cIAP-2, and ML-IAP, in preparation for phase I clinical trials.

In the case of the Bcl-2 family, low molecular mass drugs that mimic the binding of the regulatory proteins via the BH3 motif have been designed (27, 79-81). ABT-737 was designed based on the binding mode of BH3:Bad to Bcl-xl. ABT-737 has been demonstrated to have an effect in treatment of solid tumors and lymphoid malignancies, but not in other tumor types with high levels of Mcl-1 expression (82-85). ABT-737 binds with high affinity to Bcl-xl, Bcl-2 and Bcl-w, but does not show any effect over Mcl-1 or Bfl-1. ABT-737 exhibits poor solubility in aqueous solution that makes it a non-desirable candidate for clinical trials. The development of a second generation of drugs, has overcome some of the solubility problems of ABT-737, and has lead to a new candidate drug, ABT-263 (by Abbott laboratories), which is in phase I clinical trials (86). ABT-263 exhibits a similar binding profile as ABT-737 to the different anti-apoptotic Bcl-2 members, and does not block Bfl-1 and Mcl-1 activity. Therapeutic drugs, which hit all five proteins, might be most advantageous, and combinatorial therapy adding drugs with Mcl1-2 and Bfl-1 specificity, or alternatively developing single broader specificity inhibitors, could be a way forward to neutralize all five anti-apoptotic Bcl2 member. Therefore, understanding how the different Bcl-2 members regulate apoptosis and the “structural code” for the recognition between BH3-only proteins with anti-apoptotic and pro-apoptotic members is of great value.

The second strategy is to block the expression of anti-apoptotic proteins (76, 87). Inhibition of protein expression is achieved by the use of antisense oligonucleotides that down-regulate IAP and Bcl-2 anti-apoptotic protein levels. It has been shown that XIAP and Survivin antisense oligonucleotides can cause induction of apoptosis and if combined with irradiation and chemotherapy it can induce significant cell death in vitro and inhibit tumor growth in vivo. This strategy is currently used by a number of pharmaceutical companies, targeting XIAP (AEG-35156, Aegera Therapeutics, Inc.) and
Survivin (LY-2181308, ISIS Pharmaceuticals, Inc., and Eli Lilly & Company; refs. 78, 82). In addition, the compound YM-155 (Astellas Pharma, Inc.), an inhibitor that targets Survivin expression has entered phase I/II clinical trials in the United States and Europe. YM-155 inhibits Survivin expression by binding to mRNA (encoding Survivin) and inhibiting its translation to Survivin.
Structural Studies of the Bcl-2 Family

Until January 2008, the three dimensional structures of anti-apoptotic Bcl-2 (46), Bcl-w (88), Mcl-1 (89), effector Bak, Bax (90) and the BH3-only protein Bid (91) had been solved. Despite the sequence diversity among the family members and function (pro- and anti-apoptotic), they share the same fold that consists of two central, predominantly hydrophobic helices surrounded by six or seven amphipathic helices (72). A large loop between helices α1 and α2 is involved in activity regulation. The Bcl-2 canonic fold reminds of the overall fold of the pore-forming domain of bacterial toxins, in fact Bcl-xL, Bcl-2 and Bax can disturb membrane integrity.

A hydrophobic groove on the surface of the anti-apoptotic members is the binding site for the BH3 regions of pro-apoptotic Bcl2 family members. This binding site is composed of structures of the BH1, BH2 and BH3 sequence motifs. A conserved feature of the family is the presence of the conserved sequence NWGR at the beginning of the α5, which has been suggested to stabilize the structure.

In an attempt to understand the basis for the recognition between the Bcl-2 members and to identify the exact hierarchy of the interactions required in different cell contexts, several binding profiling studies have been performed followed by structural studies of protein complexes between anti-apoptotic and BH3-only members (92, 93) (Figure 9). The binding profile of anti-apoptotic proteins to BH3-only proteins reveals that the BH3-only proteins Bim, Bid and Puma can bind to all five anti-apoptotic proteins (47). For other BH3-only proteins, the anti-apoptotic family can be divided into two classes. Class I constituted by the anti-apoptotic members Bcl-2, Bcl-xL and Bcl-w, which have affinity for the BH3-only proteins, Bad but not to Noxa whereas class II (Mcl-1 and Bfl-1) bind to Noxa and show no affinity for Bad.

The protein complex structure for mouse Bcl-xL:Bim (94), mouse Mcl-1:NoxaB (95), human Bcl-xL:Bak (93), human Bcl-w:Bid (96) and human Mcl-1:Bim (95) have been determined. In particular the Bcl-xL:Bad structure
has assisted in the design of the inhibitors, ABT-737 and ABT-263. The structure of ABT-737 in complex with Bcl-xl has also been determined (97).

![Figure 9](image)

**Figure 9.** Structure representation of the anti-apoptotic Bcl-2 family members in complex with the BH3-only protein, Bim, **A**, structure of Bcl-xl (in green) in complex with Bim (red). **B**, structure of Mcl-1 (blue) in complex with Bim (bourgeon).

Through the present work, we have solved the crystal structure of the remaining unknown member of the mammalian Bcl-2 anti-apoptotic family, Bfl-1/A1 in complex with a Bim peptide corresponding to the BH3 motif (25-mers). Our structure allows, for first time, the direct comparison of the binding mode of the same BH3-only protein, Bim to two different human anti-apoptotic family members, Mcl-1 and Bfl-1.

### Completing the Family Portrait of the Anti-apoptotic Bcl-2 Proteins: Crystal Structure of Human Bfl-1 in Complex with Bim (Paper I)

**Bfl-1**

Bfl-1 is expressed preferentially in haematopoietic, endothelial cells, neurons and in many cells of the immune system, including myeloid progenitor cells, macrophages, neutrophils, thymocytes, T-lymphocytes, and B-lymphocytes (98, 99). It is expressed in respond to oxidative stress, is a direct transcriptional target of NF-kB, and is induced by the inflammatory cytokines tumor necrosis factor (TNF-α) and interleukin (IL)-1b (100-102). Bfl-1 also protects cells from apoptosis when treated with chemotherapeutic
drugs (103-105). Bfl-1 antagonizes Bax activation in response to extrinsic death signaling by TNFα (extrinsic pathway) by directly interacting with Bid and inhibiting its activity (106). Furthermore, Bfl-1 can sequester Bak in inactive complexes suppressing apoptosis, in a similar way to Mcl-1 (107).

Human Bfl-1 is a protein of 175 residues and has an alternative splice variant that lacks part of the C-terminal region, the 163 residues Bfl-1 that is localized in the nucleus (108). Bfl-1 shares the four BH motifs of the anti-apoptotic bcl-2 family, and has special characteristics that make it unique: all the other members of the anti-apoptotic bcl-2 family have a well defined C-terminal trans-membrane domain that can interact with biological membranes and is suggested to be responsible for its localization. The C-terminal domain of Bfl-1, however, contains three charged amino acids, which confers a hydrophilic characteristic to the predicted trans-membrane helix (109). It has been proposed that the special C terminus of Bfl-1 controls the short half-life of the protein (110, 111).

Bfl-1 can act as an anti- or pro-apoptotic member, depending of the cell context, like other members of the anti-apoptotic family (e.g. Bcl-xl). When the BH4 motif is removed these proteins become pro-apoptotic and induce cell death. In the case of Bfl-1, TNF receptor activation results in vivo proteolysis of Bfl-1 by the proteosome and calpain-like protease, thus converting Bfl-1 into a pro-apoptotic molecule (106, 110, 112). It has been hypothesized that the intrinsic pro-apoptotic function of Bfl-1 is normally inhibited by anti-apoptotic moieties to account for the anti-apoptotic nature of the full-length Bfl-1 protein. The trans-membrane domain of Bcl-w is accommodated in the binding cleft when it is not activated and perhaps the C-terminal of Bfl-1 shares a similar function (32).

BIM

Bim, Bid and Puma are very potent killers since they can bind to all the five anti-apoptotic family members, as was mentioned earlier.

Knockout studies in mice have revealed the important physiological and pathological functions of Bim. The BH3-only protein Bim has an important role in the development of cells in the immune system; it is responsible for terminating the T-cell immune response as well as promoting cell death in granulocytes, osteoclast, mast cells, and neurons in response to cytotoxic stimuli (113-116). Loss of Bim expression can lead to kidney and renal dysfunctions as well as degenerative and immune diseases.
Structural Results

Protein sequence analysis and alignment studies based on structures obtained for the other mammalian anti-apoptotic bcl-2 family members, led us to expect that Bfl-1 could be a difficult protein to express in a heterogeneous system. Bfl-1 was predicted to have several hydrophobic regions that could make its production and crystallization difficult. To overcome the suspected protein production problem, several constructs of Bfl-1 were prepared including the full-length protein as well as a N- and C-termini deletion library. A Bfl-1 variant lacking 25 residues in the C-terminal region corresponding to the predicted special TM domain was successfully expressed in *Escherichia coli* and purified. The protein obtained was suitable for structural studies. Extensive efforts were made to crystallize Bfl-1 but without any success. Subsequently an equimolar solution of Bfl-1 and the Bim: BH3 (25 residues) peptide was set up for crystallization trials. Diffracting crystals were obtained and the complex structure was determined at 2.2 Å resolution by MAD, using both inflection and peak data sets. No density was obtained for the residues 25-30 corresponding to a long unstructured loop connecting the first two α-helices.

Distinctive Features of Motif in Bfl-1

Despite the low protein sequence identity (Table 1) with the other members of the anti-apoptotic family, Bfl-1 shares the canonic Bcl-2 fold.

**Table 1.** Structural comparison of Bfl-1 with the different anti-apoptotic members of the Bcl-2 family.

<table>
<thead>
<tr>
<th>Bfl-1 with</th>
<th>rmsd (Å)</th>
<th>Identity (%)</th>
<th>Residues aligned</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>1,93</td>
<td>23,4</td>
<td>124</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>3,21</td>
<td>19,3</td>
<td>114</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>1,79</td>
<td>33,4</td>
<td>144</td>
</tr>
<tr>
<td>Mcl1</td>
<td>1,91</td>
<td>34,7</td>
<td>147</td>
</tr>
</tbody>
</table>
The Bim peptide is bound in a similar fashion to peptides in other anti-apoptotic protein-BH3 peptide complexes (72, 92, 95). The binding groove of Bfl-1 is formed by the BH1, BH2 and BH3 motifs, and has special features in comparison with other members of the family, that could explain the distinctive binding profile of Bfl-1 (Figure 10) for pro-apoptotic proteins/peptides:

- The central region of the pocket site exhibits a high negative charge.
- Amino acid insertion at the connecting loops between α3-α4, and
- α5-α6 make the pocket wider and more solvent exposed.
- The BH3 motif has the lower degree of conservation between the family members. The BH3 motif of Bfl-1 shares higher similarity to the Mcl-1 BH3 motif. These differences in sequences are translated into a longer α2 and shorter and well-defined α3.

How Can Bim Bind to All Five Anti-apoptotic Members?

The comparison with other anti-apoptotic:BH3 peptide complexes, suggests that the affinity of Bfl-1 for the BH3-only proteins is, as for the other anti-apoptotic members, driven by the conserved hydrophobic interactions established between four conserved residues of BH3 peptides and four conserved hydrophobic patches at the binding site of the anti-apoptotic proteins. The complex structure Bfl-1:Bim also reveals a new conserved hydrophobic interaction (h5), which has not been reported previously. A conserved salt-bridge Bim Asp157:Arg88 Bfl-1 also may play an important role by assisting in orientating the BH3 peptide in the binding pocket.
**Figure 10.** Structure of Bfl-1 in complex with BH3:Bim peptide. **A,** Comparison of the electrostatic potential of the solvent-exposed surface of the complexes, mBcl-xL:mBim, Bfl-1:Bim and Mcl-1:Bim. **B,** space-filling model of Bfl-1 with Bim colored by degree of conservation where less conserved residues are colored blue < green < yellow < orange < red is completely conserved. The sequences used in the alignment were from anti-apoptotic Bcl-2 members from: *Homo sapiens, Bos taurus, Canis familiaris, Sus scrofa, Gallus gallus, Rattus norvegicus, Cricetulus griseus, Mus musculus and Xenopus laevis.* **C,** ribbon representation for the superposition of the complex structures of mBcl-xL:Bim (colored green:sand), Mcl-1:Bim (blue: yellow) and Bfl-1:Bim (pink:red). **D,** closer look at the different conformation of some residues of Bim bound to mBcl-xL, Mcl-1 and Bfl-1 respectively (colored as in C).

Comparison of the Bfl-1:Bim complex with Mcl-1:Bim, reveals that Bim binds in a very similar overall peptide backbone conformation in both complexes but the interacting hydrophobic side chains adopt quite different conformations induced by the respective anti-apoptotic protein. The significant local plasticity of these interactions contributed by the Bim peptide could explain how Bim can bind to the entire set of anti-apoptotic Bcl-2 members.

The structure of the homologue Bfl-1 from mouse, A1 has recently been determined in complex with Bik, Bad, Bmf and Puma (117). A1 shares only 72% identity with Bfl-1 but the overall structures in both cases are very similar. The mouse A1 structure in complex with the different BH3 only proteins are also in agreement with our results, showing a certain plasticity in the binding of the different peptides. Helices α2 and α3 in this case also show slightly different conformations depending on the BH3 peptide bound (Figure 11A).
Furthermore, structural studies of a viral Bcl-2 homologue, M11 from murine γ-herpesvirus 68, lead to similar conclusions (118). The M11 is analogous to human anti-apoptotic protein Bcl-xl and can inhibit apoptosis and autophagy. M11 structure adopts a typical Bcl-2 fold (Figure 11B) and profiling binding studies have shown that bind to Bim, Beclin-1, Bak, Bid, Bmf, Puma and Noxa. The binding of M11 to Beclin-1 peptide is translated in a conformational change that involves the translocation of several residues of α2-α3 region (by a distance of 4-10Å). The mouse Bcl-xl:Bim structure shows certain flexibility at α3 (residues 104, 108 and 122), Bcl-xl suffers a conformational change displacing α3 and α4 upon binding of Bim (93).

Substitution studies of the key residues on the binding site have been performed with the aim to find the residues responsible of the binding selectivity (89, 94, 97, 119). The results suggest that the selectivity cannot be explained in terms of individual residues or hot spots but rather the collective effects of the hydrophobic and polar residues, differences in α2-α3 region, the helical propensities of the BH3 peptides, and specific differences in charge (such as the central negative charge in Bfl-1).

Modeling studies of Bad, which does not bind to Bfl-1 into our model, does not show any obvious steric hindrances or drastic charge repulsions, which cannot be explained by a single amino acid substitution. Modeling of ABT-737 based on its binding to Bcl-xl (97), exposes sterical hindrance at h2 and

Figure 11. Structure representation of anti-apoptotic Bcl-2 homologue from other species in complex with BH-3 only proteins. A, superposition of the structures of mouse A1 in complex with the different BH3-only proteins Bik, Bad, Bmf and Puma. BH3 peptides are colored in different tonalities of black, and the different conformations adopted by mouse A1 when is bound to Bik, Bad, Bmf and Puma is colored in yellow, blue, pink and violet respectively. B, viral M11 in complex with a peptide corresponding to the BH3 motif of beclin protein.
h4 hydrophobic residues that could explain the low affinity of this compound to Bfl-1.

Our results can now shed light on the structural basis for affinity and selectivity of Bfl-1 for BH3 only proteins. It also sheds light on the structural basis for the differences in selectivity between class I and class II anti-apoptotic proteins. For certain cells to be able to commit to apoptosis, the entire set of anti-apoptotic proteins must be silenced, therefore the Bfl-1:Bim structure can aid in the development of drugs targeting class II or the whole family of anti-apoptotic proteins.
XIAP is the best-characterized family member of the IAP family (61, 120). Crystal structures exist for every domain; in complex with IBM peptides, such as Smac (66, 69); and in complex with caspases (63). Structural studies have shown that XIAP binds and directly inhibits caspase 9, -3, and -7.

Although BIR domains share the same fold (Figure 12), their mechanisms for caspase inhibition and substrate recognition seem to vary (38, 54, 65, 71, 121). It has been shown by biochemical and structural studies that BIR3-XIAP binds and directly inhibits caspase 9 (57), whereas BIR2-XIAP inhibits caspase -3, and -7. cIAP1 and cIAP2 in some extent inhibit caspase 3 and caspase 7. Several IAPs have shown to bind to caspase 9, but is primarily inhibited by XIAP.

Figure 12. Cartoon representation of the conserved structure of the BIR domain. The bound zinc atom is shown as a pink sphere, and the four conserved Cys/His residues are labeled.

XIAP is the only member that directly can inhibit caspase enzymatic activity. Some members of the family can bind to caspases but inhibit their activity through alternative mechanisms (37, 70). It seems that the IAP family
members are functionally non-equivalent and BIR domains are not sufficient in some cases for caspase inhibition. BIR2-XIAP needs part of the linker region within BIR1 and BIR2 to bind to caspase 3 and caspase 7.

Understanding the basis of this recognition and how IAP activity is regulated is essential for drug-design projects. The result of our structural studies of the IAP family has led to the determination of the first structure of BIR domain for NAIP and cIAP2.

The First Crystal Structure of BIR Domains from Human NAIP and cIAP2 (Paper II)

cIAP2 and NAIP

In almost all human cancer malignancies, high levels of IAP expression e.g. XIAP, c-IAP1 and c-IAP2 have been found (24). A high level of IAP activity achieved by overexpression or overactivation, make cells immortal and no longer able to enter apoptosis. Different IAPs members regulate effector caspases through different and distinctive mechanisms.

Neuronal apoptosis inhibitory protein (NAIP) was the first mammalian IAP identified, as responsible for developing spinal muscular atrophy (122, 123). Later on c-IAP1 and c-IAP2 were discovered by homology screening and as part of TNFα-complex receptor 2, where they interact with TNFα-receptor-associated factors -1 and -2 (TRAFs) (124, 125). The TNFα receptor (TNFR) can mediate both survival and death signals. TNFα leads to survival by c-IAPs, which inhibits caspase 8 activation.

A genetic translocation of c-IAP2 fuses its BIR domains with MALT1 (mucosa-associated lymphoid tissue protein), which seems to correlate with resistance to apoptotic signals resulting in over-activation of anti-apoptotic and inflammatory pathways.

It has been suggested that c-IAP2 and NAIP do not directly inhibit caspase activity; instead c-IAP2 and NAIP may mediate indirectly caspase inhibition by binding to Smac, in this way XIAP is free from Smac, which can directly inhibit caspase activity (120, 126).
NAIP is unique among the IAPs (127). BIR3-NAIP binds to caspase 9 but not to Smac. It has been suggested that NAIP binding to caspase 9, is regulated by the LRR domain and ATP availability. In the absence of ATP the LRR (leucine-rich repeats) domain negatively regulates BIR3 activity, inhibiting its binding to caspase 9.

The availability of the different structures of IAP members could help to understand the structural basis for similarities and differences in their mechanisms and how they are regulated.

Structural Results

Following the same strategy used for Bfl-1, the expression screening of several constructs of the BIR domains was performed. A construct containing residues 141-244 corresponding to the BIR2-NAIP and one containing residues 238-330 corresponding to BIR3-cIAP2 were successfully expressed and protein suitable for structural studies was obtained.

Well-diffracting crystals generated a native dataset to 1.7 Å resolution for BIR3-cIAP2 and 1.8 Å for BIR2-NAIP. Structure determination in both cases was achieved by molecular replacement by using the BIR structure of ML-IAP.

Structures of BIR2-NAIP and BIR3-cIAP2 share the conserved BIR fold (Figure 13). The BIR2-NAIP structure is formed at the N-termini of three α-helices followed by three antiparallel β-strands and two α-helices at the C-termini. BIR domains usually exhibit three α-helices at the C-termini, where the third expected α-helix at the C-termini of BIR2-NAIP is unstructured and does not appear as a helix.

An unexpected result was that both structures appear to be stabilized by the binding of the N-termini into the hydrophobic IBM pocket, in a similar fashion to physiological substrate peptides found in other IAP complexes with IBM domains, such as Smac (Figure 14). For BIR2-NAIP, one molecule was found in the asymmetric unit, and the N-terminal sequence SMRV belongs to the linker region of the construct generated after proteolytic removal of the His-tag (TEV-cleavage). One consequence of this binding is the formation of an extra β-strand at the C-terminal, which binds across the peptide-harboring IBM binding groove, stabilizing the binding of the tetra-peptide backbone. The BIR3-cIAP2 structure is a dimer in the asymmetric unit, where the N-terminus of one molecule stabilizes the other through binding to the hydrophobic groove.
Figure 13. Schematic representation of the structures of cIAP2-BIR3 domain (A) and of the NAIP-BIR2 domain (B).

From the structural studies of BIR2-XIAP in complex with caspases 3, and -7, the mechanism of caspase inhibition by XIAP has been elucidated (62-64). A two-site binding mechanism is recognized to be necessary for binding and direct inhibition of effector caspases by XIAP. Caspases bind to BIR2-XIAP at the IBM interacting groove (exosite) through its N-terminal tetrapeptide sequence and a second interaction is required for inhibiting caspase activity by blocking the caspase active site. The linker region connecting BIR1 and BIR2 in XIAP mediates the second interaction, residues 140-156 form a peptide strand that binds to the catalytical active site of caspases in a “back to front” orientation.

The BIR2-NAIP structure accommodates a tetra-peptide in the IBM interacting groove, in a similar fashion to the binding of caspases -3, -7 to BIR2-XIAP. The consensus IBM sequence has been defined as (NH2) AφPφ, where φ stands for a hydrophobic residue. In the BIR2-NAIP structure the N-terminus of the construct is bound at the interacting groove resembling the binding of caspases to BIR2-XIAP, with the only difference that the first residues of the IBM tetra-peptide is a Ser residue instead of the conserved Ala. The BIR2 domains of NAIP, cIAP1, cIAP2 and XIAP have been reported to bind to caspase -3 and -7, and interestingly the activated form of theses caspases start with a Ser residue at the N-terminal generated after proteolytic cleavage by caspase 9. Also, comparing both structures we can determine the role of the conserved residues at the IBM binding groove.
Figure 14. Comparison of BIR domains. A, space-filling model of NAIP-BIR2 colored by degree of conservation where less conserved residues are colored blue < green < yellow < orange < red is completely conserved. The sequences used in the alignment were from human BIR domains. B, space-filling model of cIAP2-BIR3 colored by degree of conservation where less conserved residues are colored blue < green < yellow < orange < red is completely conserved. The sequences used in the alignment were from human BIR domains. C, Crystal structures of XIAP-BIR3 (magenta) and cIAP2-BIR3 (red), the residues involved in binding of tetra-peptide are labeled. D, Crystal structures of XIAP-BIR2 (yellow) and NAIP-BIR2 (cyan), the residues involved in caspase inhibition are labeled.

involved in binding to the IBM tetra-peptide. For the BIR2-NAIP interacting groove these residues are D210, E216 and W220 (Figure 14).

The C-terminus of the BIR2-NAIP is found in the crystal structure, to make an interesting crystallographic contact. Residues 238-244 of the construct, forms a β-strand, which interacts in a region at the BIR2 domain that overlaps with a dimerization interface. This interaction is intriguing since it resembles a typical β-strand protein-protein interaction also seen in other signaling processes.

Several aspects of caspase 9 inhibition still remains unclear but from the crystal structures of BIR3-XIAP in complex with caspase 9 (initiator caspase) it is believed that the mechanism of caspase inhibition in this case, is a slightly different two site-binding mechanism (65). The first interaction is made by the N-terminal IBM tetra-peptide of caspase 9 (generated after caspase 9 auto-activation) at the IBM interacting groove of BIR3-XIAP. The second interaction required for inhibition of caspases is executed by the C-terminal α-helix formed immediately after the BIR3 domain of XIAP. This helix blocks the dimer interface of caspase 9, which needs to form a dimer to be active.

The structure of BIR3-cIAP2 reveals a dimer where the N-terminus of each monomer binds and stabilizes the IBM interacting domain of the other monomer. The N-terminal tetra-peptide sequence of BIR3-cIAP2 is (NH2) SMRY, and a superposition of the BIR3-cIAP2 structure with the BIR3-XIAP:caspace 9 complex structure shows a high degree of conservation of the IBM binding groove between BIR3 domains. The backbone of the tetra-peptide overlaps with very little variation.

Recently, a peptide-binding study aimed to determine the specificity of BIR-IAP domains for caspases and IBM regulatory proteins, shows the possibility of binding a Ser residue at the first position (P1) of the tetra-peptide, and an Arg at P3. BIR domains are classified in this study into four different
classes, depending on the conserved residues in the IBM groove involved in the binding of the tetra-peptide (70, 128). The results of this study are in agreement with our conclusions from the study of both structures.

BIR2-NAIP and BIR3-cIAP2 are the first structures for the NAIP and cIAP2 members, and provide important structural information for the further characterization of the action of these proteins.
Structural Studies of Proteins in Lipid Signaling

Extracellular stimuli is sensed by receptor proteins at the surface of the cell and the signal is transferred to the inside of the cell by different signal transduction pathways depending on the nature of the signal (129). Disturbances in these signal transduction pathways that link extracellular stimuli to e.g. nuclear or metabolic events, and can be responsible for developmental abnormalities, cancer or inflammatory disease (130). The transfer of signals from the plasma membrane to the cell nucleus is a highly complicated process, which depends on specialized proteins and second messengers such as e.g. lipid signaling molecules.

The discovery in the 1980s, of diacylglycerol (DAG) and inositol(1,4,5) trisphosphate (Ins(1,4,5)P3) roles as second messengers constituted a very significant breakthrough in cell biology (131, 132). Activated Phospholipase C (PI-PLC) cleaves phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) into the two second messengers, diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (Ins(1,4,5)P3) (133). These second messengers are released into the cytosol, transferring the signal into the nucleus by signaling pathways involving a cascade of specific proteins. Nuclear responses could involve specific gene transcription, DNA replication, DNA repair, or DNA cleavage, resulting in cell differentiation, proliferation, or apoptosis depending on the required response.

Many proteins involved in these signaling cascades have been identified but still the understanding of the molecular details of many of these signaling events is rudimentary. Proteins involved in lipid phosphorylations, such as DAG-kinases, have been identified as key proteins in these processes (134-136). In paper III we present the study of a putative lipid kinase suggested to be involved in signaling pathways. YegS is a prokaryotic lipid kinase with homology to the mammalian lipid kinases.
Crystal Structure of YegS, a Homologue to the Mammalian Diacylglycerol Kinases, Reveals a Novel Regulatory Metal Binding Site (Paper III)

YegS

YegS is a soluble *E. coli* protein of 32 kDa with sequence similarity to mammalian lipid kinases such as diacylglycerol, ceramide, sphingosine kinase and the recently identified Multi-lipid kinase (MulK) (137-139). The function of YegS has been unknown but it was annotated as a putative diacylglycerol kinase (DGK) based on sequence similarity to the first reported mammalian DGK-α (140).

However, in *E. coli* another, membrane bound, and well characterized DGK is present (141, 142). This DGK is a homotrimeric integral membrane protein in which each subunit has three predicted transmembrane helices. At sequence level, YegS does not exhibit any similarity with this DGK, instead close relatives to YegS are found in human pathogens such as *Shigella flexneri, Salmonella typhimurium* LT2 and *Yersinia pestis*.

We have solved the crystal structure of YegS and tested its function using biochemical strategies. Our results show that YegS has lipid kinase activity but no detectable activity for DAG as substrate. Instead, it shows a high activity for phosphatidylglycerol. YegS activity is sensitive to the presence of Ca$^{2+}$ and Mg$^{2+}$. The structure of YegS reveals a two-domain protein with significant resemblances to the fold and active site of NAD-kinases – including six conserved aspartic acid residues likely to be important for kinase activity in these families. YegS does not contain accessory domains like, for example, the mammalian lipid kinases. The YegS structure reveals a novel metal binding site that might be a Ca$^{2+}$ regulatory site, with two of the Asp conserved residues involved in metal coordination.

Structural Results

The *E. coli* YegS gene was attributed to cloning and expression screening in the context of a high-throughput project. It was cloned into the Gateway expression vector PT73.3, and scale up purification was done by IMAC-affinity and gel filtration chromatography. A highly pure protein sample was subjected to crystallization trials. Crystal diffraction was to 1.9 Å and a native data set was collected. Using new crystals containing TEV-cleaved sele-
nomethionine labeled protein, the phasing problem could be solved using SAD methodologies. The YegS structure was also solved with ADP bound at the active site to a resolution of 2.5 Å.

The YegS structure has two domains as shown in Figure 15, an N-terminal domain containing a Rossmann like α/β-fold (143), and a C-terminal β-strand dominated domain. The cleft between the domains harbors the nucleotide-binding site as revealed by the ADP complex structure. YegS shares sequence and structure similarities with PFK and NAD kinase families at the N-terminal domain, including the GGDG motif, NXKS and LPLGTXNDXA motifs. However, the C-terminal domain is only related between NAD kinase and YegS.

The C-terminal domain is uniquely found in YegS, NAD kinases and in the Vp4 a sialic acid binding domain of Rhesus Rotavirus. On the other hand, since Vp4 (144) does not contain a Rossmann fold, we could conclude that the overall fold of YegS is uniquely shared with NAD kinases.

In a recent classification of kinases, NAD kinases and PFK structures were grouped into the group 2 Rossmann-like family and PFK-like subfamily, and 1998 sequences were predicted to be part of this subfamily. Our structural results suggest that a revision of the classification should be made, and we propose YegS and NAD kinases to be included into a distinctive subgroup of the Rossmann fold kinases along with the distantly related human lipid kinases, as well as several other hypothetical proteins with closer sequence similarity to YegS.

In the structure of YegS in complex with ADP, the nucleotide binding site, the binding site for the phosphate donor was identified but only electron density for the pyrophosphate moiety of ADP could be detected. This density is positioned in the cleft formed in between the N- and C-terminal domains, and in close proximity to the conserved GGDG motif. A glycine rich cluster, a feature known as the G-loop (145), is conserved in nucleotide binding proteins such as kinases. The G-loop is involved in the binding of the nucleotide in proximity to the pyrophosphate group. Based on structural comparisons of the monomeric structure of NAD kinases, PFK, and YegS, the GGDG motif seems to perform a similar role, since the position in relationship to the phosphate donors in these three proteins is equivalent.
**Figure 15.** Structural comparison of bacterial YegS and to human NAD kinase. A, structure of E. coli YegS. The pyrophosphate moiety (POP) of ADP molecule as well as key residues marking the four signature motifs of lipid kinases are shown. Mg²⁺ marks the novel metal site. The N-terminal domain is the nucleotide binding core of the structure. The two helices shown in green constitute the likely substrate binding region of YegS. B, the NAD kinase structure is shown in an equivalent orientation. The NAD substrate/product binding site is at the cleft between the N- and C-terminal domains. C, Space-filling model of YegS colored by degree of conservation where less conserved residues are colored blue < green < yellow < orange < red is completely conserved. The sequences used in the alignment were from: E. coli YegS, S. typhimurium YegS and human NAD kinase. D, superposition of YegS structures from E. coli (cyan) and S. typhimurium (hot pink). E, superposition of YegS structures from E. coli (cyan) and Staphylococcus aureus (light pink).

Phosphatidylglycerol (PG) is a membrane lipid, which means that in order for the reaction to take place, soluble YegS protein should bind transitionally to the membrane. However, it is not perceptible from the surface charge distribution which surface region might be involved in membrane interaction (146). Interestingly, the Membrane Protein Explorer (MPEx) server (147), proposed the region 157-175, including α6, which contains the conserved Tyr164, as a potential membrane interaction region, as shown in Figure 15 (148, 149). Tyr164 is conserved as a Tyr or Phe in most lipid kinases. It is well documented that tyrosine along with tryptophan residues are overrepresented at the membrane interface in transmembrane helices. Furthermore, the fact that the substrate-binding site in NAD kinases is found close to this region strengthens the possibility that this is in fact the region involved in membrane interaction (Figure 15).

**Function**

YegS function, as a DAG lipid kinase, was tested in vitro by a radioactive assay using labeled [γ-³²P] ATP and DAG as a lipid substrate. Surprisingly, the data indicated that YegS lacked activity towards DAG. To investigate the possibility that YegS could still be a lipid kinase we tested its activity towards an extract of E. coli lipids revealing a single band in a TLC corresponding to phosphatidylglycerol phosphate (PGP), leading to the conclusion that YegS indeed exhibits a lipid kinase activity. The use of an E. coli extract also gave strong support that this reaction can occur in vivo. Figure 16 shows the TLC analysis of the activity assay.
The crystal structure of YegS revealed a metal binding site. A putative magnesium ion was found coordinated by the carbonyl oxygens of Leu215 and Leu220, the side chain of Asp218, and three water molecules that complete the octahedral coordination. One of these water molecules was coordinated by the conserved Asp125. Mammalian kinases are also regulated by metals, and for example, MulK kinase shows high activity for different lipids and its activity is regulated by Ca$^{2+}$ and Mg$^{2+}$ (139). In an attempt to explore the relevance of this metal site and the effect of Mg$^{2+}$ and Ca$^{2+}$ on the activity of YegS, we performed a similar experiment to the one performed by Wagggoner for the MulK activity assay (139). YegS kinase activity showed strict metal dependence, to the point that no activity was detected in the absence of Mg$^{2+}$ or Ca$^{2+}$. In the presence of 1 mM Ca$^{2+}$, some activation could be detected. When the same amount of Mg$^{2+}$ was used, lower activity was detected. A boost in activity was observed with a higher concentration of Mg$^{2+}$ (5 mM) and such activation could be sustained at low Ca$^{2+}$ concentration (1 mM disappearing when the concentration was raised to 1 mM Ca$^{2+}$). Together these results point to a complex behavior of YegS towards the two metals and that human MulK kinase and YegS might be related enzymes sharing a similar mechanism for metal dependent regulation.
No structures for the mammalian lipid kinases exist to date. Recently two homologous of YegS in *S. typhimurium* (150) and *Staphylococcus aureus* have been determined (Figure 15). From a sequence point of view, YegS share higher similarity with the DGK in eukaryotes than within prokaryotes. The superposition of the YegS structures (Figure 15) reveals that the three structures shares the same 2-domain fold, where the N-terminus is more conserved and the largest structural differences are found at the C-termini of the YegS structures. YegS structure in *S. typhimurium* and *S. aureus* share a higher similarity at the C-terminal domain between them, than in comparison with *E. coli*.

YegS in *S. typhimurium* has two Ca$^{2+}$ bound, at Ca-binding site 1 and Ca-binding site 2. Ca-binding site 1 is localized at the same region where Mg$^{2+}$ is bound in YegS from *E. coli*, and *S. aureus*.

YegS from *S. typhimurium* did not show any activity toward DAG, which agrees well with our results on the *E. coli* YegS,

The eukaryotic Mulk has been suggested to be a signaling protein. However, it shares some important features with YegS, such as both are soluble proteins, share high similarity at the N-terminus (the DGK fold), high affinity toward other substrates than DAG and have a complex metal regulation. This could mean that YegS has a more important role in signaling pathways in prokaryotes. Several genes regulated by the same operons are found to be involved in stress response, indicating that YegS may be activated in response to stress signals.
**Protein Production for Structural Studies**

The rate-limiting step for any structural biology or structural genomics project is the efficient production of suitable proteins (151). *E. coli* is usually the preferred choice of expression host for a number of reasons; it allows high amount of biomass to be produced at low cost, it is a well characterized and versatile expression system and does not perform excessive post-translational modifications, allowing the production of large amounts of homogeneous protein sample at a low cost. The problem usually arises when trying to overexpress a eukaryotic protein in a prokaryotic host, since they exhibit a higher tendency to misfold and aggregate as inclusion bodies (152, 153). Eukaryotic proteins have a higher degree of complexity than prokaryotic proteins. They are by average larger, possess a higher number of domains, and they may need post-translations modifications for correct folding. To increase the success of protein expression one can vary different expression parameter such as host system, temperature and media or one can modify the target protein itself by *in vitro* evolution methods, generating libraries with thousands of variants of the target protein (154-158).

Modification of any of theses parameter will generate a large number of samples to be tested which adds cost and time. Therefore, a robust and generic method for screening the protein solubility of many samples in parallel is of great value. There are a number of different methods that can be used to screen the solubility of proteins expressed in *E. coli* in a high through put manner such as centrifugation, filtration and purification (159, 160). Inclusion bodies are larger in size than soluble proteins and centrifugation and filtration use this distinctive characteristic to separate inclusion bodies from soluble protein. Our lab has earlier developed a Filtration Dot blot (FiDo) method where soluble protein is filtrated through a multi-well filtration plate that retains the inclusion bodies, and soluble proteins are subsequently recovered on a blotting membrane (161). Protein detection is achieved by immunodetection. In generic purification methods, the target protein is expressed with a purification tag such as a 6xHis-tag that will facilitate subsequent purification. For the screening of large numbers of protein variants for solubility on e.g. colony level, the GFP method has been very popular. It has however also been demonstrated to have some problems with false positives, and low sensitivity (162). Recently, a new method that uses the reef coral
fluorescent protein (ZsGreen) as reporter, has been established (163). The methods decreased the problems of low sensitivity of GFP by using a variant with higher brighter fluorescent, the ZsGreen protein, which has a similar structure to GFP.

In a study during my thesis work (data not published) we found that GFP can interfere and effect folding of many proteins. By using construct libraries of 10 human proteins that were difficult to express, we found that correlation between positives selected clones from the CoFi blot and small scale purification was very good, 68%, while the correlation between GFP colony fluorescents and small scale purification was only 12%. Therefore, after expression in liquid culture, a large fraction of the construct identified with the GFP method was insoluble. In general, the ratio of soluble constructs per plate was higher when construct containing stop codons were used, suggesting that GFP decreases the solubility of the target protein.

After these experiments were performed, an alternative GFP based method for optimizing the solubility of the target protein was developed (164, 165). This method uses a split-GFP strategy where an essential peptide is fused to the target protein, and fluorescence is only seen when the target proteins has stayed soluble for a defined period of time until the core GFP protein is induced. The split-GFP method is likely to be a significant improvement to the traditional GFP method, when it is a better reporter of solubility. The method has been successfully used in combination with direct evolution techniques to increase the level of soluble protein expression for several difficult to express proteins.

The CoFi blot method however, offers significant advantages over the fusion-reporter method for studies of expression on the colony level, when the proteins selected for survive lysis and filtration. The selected protein can also be directly used for scale up studies without recloning.

Colony Filtration Blot: a New Screening Method for Soluble Protein Expression in *Escherichia coli* (Paper IV)

The Colony-Filtration (CoFi)-blot method was developed as an extension of the FiDo method but with the advantage of being useful for analyzing thousands of samples in parallel at low cost (161). It uses a filter sandwich process on the colony level and can be used as a detection step for selecting
clones expressing soluble proteins from large libraries of variants (e.g. random mutations or construct libraries).

The method is based on filtration of soluble proteins through a membrane filter with sub-micron pore size (Durapore membrane), which retain inclusion bodies but does allow soluble proteins to pass. Soluble protein is recovered on a nitrocellulose membrane that it is attributed to detection by classical immunodetection techniques.

The method is very simple to execute and requires no automation (166). After the target gene has been cloned into the E. coli strain of choice, bacteria are plated on an agarose plate and a replica of the colonies is made on the Durapore membrane. Thereafter, the membrane is placed on a new plate where protein expression is induced. After expression has been finished a lysis and detection sandwich is made as presented in figure 17. The lysis buffer, combined with freeze thawing, will break the cells, allowing the free soluble protein to pass through the Durapore filter to the nitrocellulose membrane. This processes is probably driven by capillary tension induced by the filters.

**Figure 17.** Schematic illustration of the CoFi blot procedure.

The target protein is expressed with an N-terminal His-tag. Immunodetection of the soluble protein is done by using a reactant that will exclusively react with our target protein. A chemiluminescent signal will help us identify the colony/colonies that express the target protein as a soluble form.
To verify that the method works, we made a comparison with the classical centrifugation technique combined with small-scale purification. We selected 32 eukaryotic proteins and 24 E. coli proteins cloned into two different expression vectors (constructs with either a N-terminal Flag or with a His-tag) making up to a total of 108 different constructs. Chemically competent E. coli was transformed with a corresponding plasmid and cells were plated and simultaneously grown in 1ml liquid culture. The colonies were screened for soluble expression using the CoFi blot method and the liquid culture by centrifugation and subsequent small-scale purification. An 84% correlation could be observed between both methods.

![Figure 18](image)

**Figure 18.** Example of CoFi blots for a recalcitrant protein. **A,** CoFi- blot for wild-type expression and **B,** represent the results of a CoFi-blot for the target protein after it had been submitted to random mutagenesis.

The method has now been used successfully for the expression of a large number of difficult proteins to express. Cornvik et al showed that the success rate of obtaining expressing constructs from human proteins was doubled (167) (Figure 18). A CoFi-Blot method for screening membrane proteins expression has also been implemented as an extension of the CoFi-Blot for soluble proteins. It has been used in combination with in vitro evolution (random mutations) to select membrane proteins, which express at higher levels (168). Both these strategies have produced proteins suitable for further crystallography studies and to date 6 structures have been determined that had been selected from libraries using the CoFi-blot.
Prospects

Structural studies of the anti-apoptotic family Bcl-2 and IAP, reveal important features related to the binding and specificity of Bfl-1, and BIR domains for its partners. The information from these studies could help in drug design targeting specifically these proteins. Structural studies of these anti-apoptotic protein in complex with other partners such as Puma, Bid in the case of Bfl-1, or complexes of IAP with caspases, would add insights into structural basis for the regulation of apoptosis. Furthermore, additional site directed mutagenesis studies of the key residues predicted to be responsible for the selectivity, coupled to isothermal titration calorimetry measurements, could help in understanding details of the recognition process. Together this provides information assisting in the development of proper treatment for diseases due to dysregulation of programmed cell death.

With respect to the lipid kinase YegS, we have discovered a novel metal binding site, opening for a possibility of an additional regulatory mechanism of members of the lipid kinase family. This also opens for the possibility that YegS is controlled by specific signaling mechanisms. Although we have identified distinctive features of YegS at the structural and biochemical level, which have increased the understanding of this protein family, a number of key questions still remain to be answered such as, how, is the regulation of the activity by metal binding executed, how is the substrate (and membrane) recognized and how do we get conclusive evidence for the substrate of YegS. Again, mutational studies of the key residues will be required to elucidate the mechanism of action of this enzyme. Further biochemical studies as well as cellular studies, can shed further light on the substrate specificity of the enzyme.

Finally, the efficient production of suitable protein for structural and biochemical studies is still a limiting step. We have shown the versatility of using the CoFi-blot combined with different library techniques, for improving not only the expression of difficult proteins in E. coli, but also the speed up of expression screening of complete ORFeomes and other expression libraries. In addition, we can use this technology in screening different physical variables for complete libraries such as additions of cofactors or ligands, induction time, temperatures, lysis buffers etc. Furthermore, an ex-
tended version of the CoFi-blot could be used for screening for stable protein complexes. Therefore, we anticipate that the CoFi-Blot will have a very large applicability in protein sciences in the future.
Acknowledgments

I would like to express my gratitude to all the people who have helped and supported me during my PhD studies, without them you will not be reading this thesis today. In particular, I would like to thanks:

Pär, my supervisor, for giving me the possibility of doing my thesis at his lab, which has been a great experience both, at scientific and personal level. I would like also to thank him, for his inspiring ideas and dedication that have made my thesis projects very fun and exciting!

Stefan Nordlund, who let me be a part of DBB, and has guided me during this time, always being there with the right solution and a positive attitude.

Anki, Ann, Kicki, Lotta and Maria, who have made so easy all the administrative work, fast and with a big smile!

Everybody in the group, for making the lab a great and fun place to work every day, thanks: Debora Berthold, Marina, Pelle, Said, Maria, Hanna, Marie, Elizabeth, Christine, Cédric, Christian, Andreas, Flo, Agnes, K-Magnus, Martin Hög bom, Pål, Heidi, Albert, Jessica, Martin Hällberg, Tove, Therese, Helena, Ken, Martin Moche, Amin, Susanne van den Berg, Ulrika, Anna, Anna-Karin, Herwig, Henrik, Karin, Daniel Martinez.

A big thanks to the computer team, Daniel and Damian who had a little extra-work with my computer! And always fixed it!!!

I would like to thank my collaborators for the successful projects presented in my thesis:

The CoFi team: Tobias, SueLi, Audur, Monica, Susanne and Victoria, whom became not only my colleagues but also very good friends during this time. With them I have shared very good moments at the lab, fika, conferences, weddings, dinners, parties, babies…..

Every body at SGC for welcoming me for the “Apoptosis collaboration”, and introduced me in structural genomics and crystallography, which I have
found fascinating and learned so much! With them I have shared also very good moments, my first crystal-protein, my first diffraction pattern (not salty…). The time that I had expended at SGC was very positive. I would like to thanks in particular, Team 1, Susanne Flodin, Martin Welin, Tomas, Lari, Lionel and Pär.

The Lipid team, for the YegS collaboration, Åke’s lab, that taught me a lot about how to work with lipids and make my understanding of lipid wider! Especially to Amelie Kelly who I worked very close with and has a very optimistic attitude!

Inger, for adopting me as her student, during the D-Kurs teaching, and let me stay at her lab. Thanks for helping me and teaching me how to teach!

I’m very grateful to SueLi, Helena, Pelle and Stefan who spent several hours and reading through and correcting my thesis.

Gustav Dallner and Pavel Sindelar groups, who invited me to come to Sweden for first time and expend a very special time at their lab as exchange student. Thanks: Gustav, Pavel, Jacob, Mike, Kajsa, Barbara, Magnus, Mats, Ulrika, Eva and Tadeus.

Finally, I would like to thank my family and friends in Spain, for supporting me and always being there for me, although we are physically separated a few miles!

Gracias a mi familia, la mejor del mundo, por vuestro apoyo, y por estar siempre cerca de mí, Papa, Mama, Rafi y Gustavo! Y a los mejores amigos que uno nunca pueda imaginar, Eva Moreno, Eva Cadiz, Mari Tere, Maria Jose, Mario, Lola, Maria Jesus y Victoria!

My friends and family in Sweden for making me feel at home!

And to my boyfriend that has been helping me and trying to make everything easier for me, my Daniel!
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