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Marek Los and Henning Walczak

Caspases— Their Role in Cell Death and Cell Survival

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Dedication

To our mentors & beloved ones.

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PREFACE

The understanding of basic biological processes and the pathophysiology of human disease critically depends on our knowledge of intracellular signaling pathways. Programmed cell death is indispensable for development and homeostasis of multicellular organisms and it has been shown to be involved in the pathogenesis of an ever increasing number of diseases. The name that was coined for the process by which programmed cell death occurs is *apoptosis*. Although the morphological features of programmed cell death by apoptosis had been known for quite some time, the first molecules responsible for initiation and execution of this process were only identified during the last decade. The key developments in the field were the discovery of membrane receptors (the so-called *death* receptors) that when triggered by agonistic antibodies or their natural ligands induce death of the cell and the subsequent identification of a family of proteases, namely cysteine-dependent aspartases (*caspases*), as important downstream signaling molecules in this process. Both findings helped to answer eminent questions in various areas of biology and medicine including morphogenesis, organogenesis and the regulation of the immune system as well as the pathogenesis of a number of diseases. The proteases of the caspase family play the key role in the initiation and execution of apoptosis. Although the prototypic member of this protease family, the Interleukin-1β converting enzyme (ICE), now termed caspase-1, is in fact involved in cytokine maturation and, thus, inflammation, most other members of the caspase protease family are primarily involved in apoptosis. In the meantime twelve caspase family members have been cloned and characterized.

The rapid development of the field has prompted us to prepare a book, which provides in a compact form insight into the key research topics that involve caspases. To achieve this objective we invited leading experts from various areas of biology and medicine to contribute to this book. All of the contributors have conducted excellent research on apoptosis and caspases over the last decade. The book is constructed in a way that readers who do not (yet) work on programmed cell death including students, as well as those with proficiency in selected subjects of apoptosis research will be able to quickly find the information they are looking for. The first chapter serves as an introduction and is, therefore, significantly longer than the remaining ones. This chapter touches upon most areas of caspase-related research and thereby provides the reader with a solid basis before proceeding further into the book. Therefore, even readers who are unfamiliar with the subject will become familiar with the basic terms used in the further parts of the book. The following chapters describe in more detail the key fields of caspase-related apoptosis research, characterizing death receptor- and mitochondria-dependent death pathways which both involve proteolytic caspase signaling cascades. This is followed by chapters that focus on the physiological and pathological

roles of caspases. In the next chapters the authors characterize in greater detail the role of caspases in research areas that are not directly related to the apoptosis field, particularly their regulatory role in the immune system. Credit is given to experimental work that through targeted disruption of caspase genes revealed unique and indispensable functions of selected caspase family members. Chapters 11 to 14 focus on more applied aspects of caspase research. Here, the role of caspases as executioners of cancer therapy-induced death and as potential targets for drug development that may help to treat cancer, autoimmunity, degenerative disorders and stroke is described. Two chapters then focus on various methods to directly and indirectly detect caspase activity in vitro and in vivo. The last chapter serves to give an overview of the cell death mechanisms that do not involve caspases and thereby provide an interesting outlook.

We hope that this compact compendium on caspases will be of use to many researchers from various fields of biology and medicine by both, making the ones who are new to the apoptosis field familiar with it and by broadening the knowledge of the researchers who already work in the field. While preparing this book we surely experienced the broadening of our knowledge of the field, and at this point we would like to thank the authors of all chapters for their excellent contributions, for their creative involvement and also for their patience while handling our queries and emendations.

Last but not least we would like to thank the publishers and their teams for the professional and efficient handling of all contributions without which the rapid publication of this book would not have been possible.

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ABBREVIATIONS

7-AAD	7-aminoactinomycin D
Ac	Acetyl
Acinus	Apoptotic chromation condensation inducer in the nucleus
AFC	7-amino-4-trifluoromethylcoumarin
AI	Apoptotic index
AIF	Apoptosis inducing factor
Akt	Ser / Thr kinase; cellular homologue of v-Akt (protein kinase B)
ALEC	Affinity labeling enzyme centers
AMC	7-amino-4-methylcoumarin
AP-1	Activating protein-1
AP-2α	Activating protein-2α
Apaf-1	Apoptotic protease-activating factor-1
APP	β-amyloid precursor protein
ARC	Apoptosis Repressor with caspase Recruitment Domain
AS	Antisense
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
Bap31	B cell antigen receptor-associated protein 31
Bcl-2	B-cell lymphoma gene 2
BH3	Bcl-2 homology domain 3
BIR	Baculovirus inhibition of apoptosis protein repeat
Boc-D-fmk	t-Butyloxycarbonyl-aspartate-fluoromethylketone
C57BL/6	C67-black 6, murine strain
CAD	Caspase-activated DNase
CAI	Cumulative apoptotic index
CARD	Caspase activating recruitment domain
CARDIAK	CARD-containing ICE-associated Ser / Thr kinase (RICK / RIP-2)
Caspase	Cysteinyl aspartate-specific protease
CD	Cluster of differentiation
CD95	Cluster of differentiation antigen 95 (APO-1/Fas)
Cdc	Cyclin-dependent kinase
CDK	Cyclin-dependent kinase
Ced-3	Cell death defective-3
Ced-4	Cell death defective-4
Ced-9	Cell death defective-9
C.elegans	*Caenorhabditis elegans*
CHO	Aldehyde

c-IAP1	Cellular Inhibition of apoptosis protein 1
c-IAP2	Cellular Inhibition of apoptosis protein 2
CLARP	Caspase-like apoptosis-regulatory protein
CMH-1	CPP32/Mch2 homolog-1
CNS	Central nerve system
COP	Caspase activating recruitment domain Only Protein
CPP32	Cysteine protease protein of 32 kD
CPT	Camptothecin
CrmA	Cytokine response modifier A (SPI-2)
CSP-1	Caspase homolog-1
CSP-2	Caspase homolog-2
CPAN	Caspase-activated nuclease
CTL	Cytotoxic lymphocyte
D_2Rhodamine	$(Aspartyl)_2$-Rhodamine 110
DAMM	Death-associated molecule related to Mch2
Dapaf-1	*Drosophila* apoptotic protease-activating factor-1
DAP kinase	Death-associated protein kinase
DARK	*Drosophila* Apaf-1-related killer
dATP	Deoxyadenosine triphosphate
DCP-1	*Drosophila* caspase-1
DD	Death domain
DECAY	*Drosophila* executioner caspase related to Apopain/Yama
DED	Death effector domain
DEDD	Death effector domain containing DNA-binding protein
DEFCAP	Death Effector Filament-forming Ced-4-like Apoptosis Protein
DFF	DNA fragmentation factor; inhibitor of CPAN (ICAD)
DFF-45	DNA fragmentation factor 45 kD subunit
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signaling complex
DN	Dominant-negative (inhibitory)
DNA	Deoxyribonucleic acid
DR	Death receptor
DREDD	Death-related *ce*d-3/*Ned*d2-like gene
DrICE	*Drosophila* interleukin-1β converting enzyme
DRONC	*Drosophila* Nedd2-like caspase
DTIC	Dacarbazine
DTT	Dithiothreitol
EAE	Experimental allergic encephalomyelitis
EGF	Epidermal growth factor
eIF2-α	Eukaryotic initiation factor 2-α

eIF-3	Eukaryotic initiation factor 3
eIF4-G	Eukaryotic initiation factor 4-G
EMAP-II	Endothelial-monocyte-activating polypeptide II
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tag
ET	Eppendorf tube
FADD	Fas receptor associated death domain
FAK	Focal adhesion kinase
Fam	Carboxyfluorescein
Fas	FS7-associated surface antigen (apo-1)
FasL	Fas ligand
Fkbp	FK506-binding protein
FLICA	Fluorochrome-labeled inhibitor(s) of caspases
FLICE	FADD-like Interleukin-1β converting enzyme
FLIP	FLICE-inhibitory protein
fmk	Fluoromethylketone
Gads	Grb-2-related adaptor downstream of Shc
GAS2	Growth-arrest-specific 2
GATA-1	GATA-binding transcription factor-1
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HAC-1	Homolog of *Apaf-1* and *ced-4*
HEF-1	Human enhancer of filamentation
HSF	Heat shock factor
HSP	Heat shock protein
IAP	Inhibition of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
ICE	Interleukin-1β converting enzyme
ICEBERG	Caspase-1-(ICE)-inhibitory molecule
ICE-LAP3	Interleukin-1β converting enzyme -like apoptotic protease 3
ICE-LAP6	Interleukin-1β converting enzyme -like apoptotic protease 6
$ICE_{rel}II$	Interleukin-1β converting enzyme related gene II
$ICE_{rel}III$	Interleukin-1β converting enzyme related gene III
Ich-1L	Interleukin-1β converting enzyme homolog 1 Large
Ich-2	Interleukin-1β converting enzyme homolog 2
Ich-3	Interleukin-1β converting enzyme homolog 3
IFN	Interferon
IGF	Insulin-like growth factor
I-κB	NF-κB repressor (inhibitor) protein
IL	Interleukin

IL-1β	Interleukin-1β
IL-1-Ra	IL-1 receptor antagonist
ILP	IAP-like protein (XIAP)
INCENP	Inner centromere protein
(Ipa)B	Shigella invasion plasmid antigen B
Ipaf	ICE-protease-activating factor
kD	Kilodalton
KIP-1	Cyclin-dependent kinase inhibitor p27
LAT	Linker of activation
KO	Knock-out (targeted disruption)
LPS	Lipopolysaccharide
LR	Linker region
LSC	Laser scanning cytometer
MACH	Mort1-associated Ced-3 homolog
MAPK	Mitogen-activated protein kinase
Mch2	Mammalian ced-3 homolog 2
Mch3	Mammalian ced-3 homolog 3
Mch4	Mammalian ced-3 homolog 4
Mch5	Mammalian ced-3 homolog 5
Mch6	Mammalian ced-3 homolog 6
MCM-3	Minichromosome maintenance protein
MDa	Mega Dalton
MEK-1	Mitogen-activated protein kinase / ERK kinase 1
MEKK	Mitogen-activated protein / ERK kinase kinase
MICE	Mini-Interleukin-1β converting enzyme
MLCK	Myosin light chain kinase
MNC	Peripheral mononuclear cells
MORT	Mediator of receptor-induced toxicity (FADD)
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NAD^+	Nicotine amide dinucleotide
NAIP	Neuronal IAP
NDED	NF-κB-inducible death effector domain
Nedd	NPC-expressed developmentally downregulated genes
NEDD2	Neural precursor cell expressed, developmentally down-regulated2
NF-κB	Nuclear factor κB
NF-κBp65	Nuclear factor κB of 65 kD
NGF	Nerve growth factor
NHL	Non-Hodgkin's-lymphoma
NK	Natural killer

nM	Nanomolar
NO	Nitric oxide
NOD1	Nucleotide-binding oligomerization domain protein 1
NPC	Neural precursor cell
NSCLC	Non-small cell lung cancer
PI3-K	Phosphoinositide 3-kinase
PACAP	Proapoptotic caspase adaptor protein
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PC12	Pheochromocytoma cell line
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium iodide
PI9	Proteinase inhibitor 9
PIP2	Phosphatidylinositol 4,5-bisphosphate
PITSLRE	Family of $p34^{cdc2}$-related kinases
PKC	Protein kinase C
PKCδ	Protein kinase Cδ
PKB	Protein kinase B
PKR	DsRNA-activated protein kinase
pM	Picomolar
PMN	Polymorphonuclear granulocyte
pNA	Para-nitroanilide
polyQ	Poly-glutamine repeats
PP2a	Protein phosphatase 2a
ProEMAP-II	Pro-endothelial-monocyte-activating polypeptide II
ProIL-16	Pro-interleukin-16
ProIL-18	Pro-interleukin-18
ProIL-1β	Pro-interleukin-1β
RAIDD	RIP associated Ich-1/CED homologous protein with death domain
Rb	Retinoblastoma
RICK	CARD-containing Ser / Thr kinase (CARDIAK / RIP-2)
RCL	Reactive center loop
RING	Really interesting new gene
RIP	Receptor Interacting Protein
RIP-2	Receptor Interacting Protein 2
ROCK-1	Rho-associated kinase-1
ROS	Reactive oxygen species

RT	Room temperature
SATB-1	Special A-T-rich binding protein (MAR DNAbinding protein)
SCC	Squamous cell carcinoma
Serpin	Serine protease inhibitor
SipB	Salmonella invasion protein B
SLK	Ste20-related kinase
SLP-76	SH-2-containing leukocyte protein of 76 kD
SMA	Spinal muscular atrophy
Smac/DIABLO	Second mitochondria-derived activator of caspases
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SP1	Transcription factor
SPI-2	Serine proteinase inhibitor–2 (CrmA)
Sr	Sulforhodamine
SREBP	Sterol regulatory element binding protein
STAT	Signal transducer and activator of transcription
tBid	Truncated Bid
TCR	T-cell receptor
t-butyl ester	Tertiair butyl ester
TGF	Transforming growth factor
TLR	Toll receptor
TLR4	Toll-like receptor-4
TNF	Tumour necrosis factor-α
TNF-R	TNF receptor
TRADD	Tumor necrosis factor-receptor associated death domain
TRAF	TNF-receptor-associated factors
TRAIL	TNF-related apoptosis-inducing ligand
TUCAN	Tumor-up-regulated CARD-containing antagonist of caspase nine
UBC	Ubiquitin-conjugating domain
UBF	Upstream binding factor
UV	Ultraviolet
VEGF	Vascular endothelial cell growth factor
vIAP	Viral IAP
XAF1	XIAP-associated factor 1
XIAP	X-linked inhibitor of apoptosis protein
z	Benzyloxycarbonyl
zVAD-fmk	Benzyl-oxyl-carbonyl-Val-Ala-Asp-fluoro-methyl-keton

CHAPTER 1

The Caspase Family

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Abstract

Caspases, a family of cysteinyl aspartate-specific proteases, are central mediators of apoptotic and inflammatory pathways. Caspases are synthesized as zymogens with a prodomain of variable length followed by a large subunit (p20) and small subunit (p10). The large prodomains contain protein recruitment motifs that consist of six or seven antiparallel amphipathic α-helices. These prodomains allow recruitment and proximity-induced activation in protein complexes. The caspases are activated through proteolysis at specific Asp residues residing between the prodomain, p20 and p10 subunits. This results in the generation of mature tetrameric caspases consisting of two p20/p10 heterodimers aligning in a head-to-tail configuration, thereby positioning the two active sites at opposite ends of the molecule. The catalytic mechanism is exerted by a Cys-His catalytic diad. To date 10 murine and 11 human caspases have been reported. Caspase activation and processing is further modulated by the existence of numerous caspase splice variants and cellular inhibitors such as IAPs. The final outcome of these proteolytic cascades is the specific proteolysis of a wide variety of substrates that are implicated in apoptosis and inflammation. Especially the role in apoptosis and inflammation promoted caspases as potentially interesting targets for the development of synthetic inhibitors. A phylogenetic analysis of caspases devoided of their prodomain reveals a segregation in three groups that fit remarkably well with their function and substrate specificity. During the evolution of caspases, caspases seem to have deviated from their initial apoptotic role to more diverse functions in higher animals. Caspases have been classified to a superfamily of cysteine proteases, which includes bacterial and eukaryotic proteases, suggesting a shared ancestry.

Introduction

Programmed cell death (PCD) is crucial for the development and homeostasis of multicellular organisms. Apoptosis is the form of cell death that is most extensively used for the execution of PCD and is characterized by morphological and biochemical parameters such as chromatin and cytoplasmic condensation, membrane blebbing, the formation of apoptotic bodies, chromatin fragmentation and phosphatidylserine exposure.[1,2] In fact, William Councilman described acidophilic bodies in liver tissue from yellow fever patients as early as 1890, without realizing these were apoptotic cells.[3] Although the concept of apoptosis has been introduced 30 years ago, the death pathways that regulate apoptosis have remained elusive until the last decade.[1] In the past years it has become clear that a group of cysteinyl aspartate-specific proteases, named caspases, are central regulators and executioners of apoptosis. The prototype caspase is interleukin-1β converting enzyme (ICE, caspase-1) that was initially identified as the protease required for the maturation of proIL-1β to its 17 kD biologically active form.[4] In the same period it became clear that the developmental cell death pathway in the nematode *Caenorhabditis elegans* is genetically defined.[5] In *C. elegans*, *ced-3* (cell death defective 3) and

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ced-4 are essential for the 131 developmental cell deaths. CED-3 was found to be an orthologue of human ICE/caspase-1.[6] CED-4 is the orthologue of the mammalian Apaf-1 (apoptotic protease-activating factor-1), which is the central protein of the apoptosome involved in the execution of the mitochondrial caspase-dependent cell death pathway (see chapter 4). *Ced-9*, another *C. elegans* gene, protects cells from undergoing programmed cell death and is a functional orthologue of the mammalian *bcl-2* gene. This caspase/Apaf-1/Bcl-2 apoptotic triad seems to be conserved in higher eukaryotes. The *bcl-2/ced-9* gene family constitutes a large family of pro-apoptotic and anti-apoptotic proteins involved in the control of apoptosis.[7] The fact that caspases contain evolutionary conserved peptide sequences made it possible to clone several other CED-3/caspase-1-related proteins by means of family PCR using degenerate primers.[8,9] This fact together with the rapid accumulation of EST (expressed sequence tags) and genome sequence information during the past years led to the identification of many more caspases in different species from Cnidaria such as *Hydra vulgaris*[10] to man (Fig. 1). To date the human and mouse caspase family comprises 13 members (Table 1).

Extensive studies on caspases carried out in the last 10 years made it clear that they are involved in many cellular processes. The knowledge we have today points to a role mainly in apoptosis and inflammation. In addition, caspases are probably involved in regulating differentiation of erythrocytes, thrombocytes and epidermal keratinocytes, and proliferation of T cells.[11-18] The crucial role of caspases in inflammation has been validated by experiments in caspase-1 or -11 deficient mice (see chapter 10).[19-21] However, most caspases are involved in the initiation and execution of apoptosis. Once activated, caspases can cleave other caspases thereby generating an intracellular protease cascade leading to cellular demise. Caspases also cleave a variety of substrates involved in activities that lead to dismantling of the cell such as disruption of organelle function and cytoskeletal and nuclear disassembly, resulting in the typical hallmark features of apoptotic cell death. Of course, one should also be aware that non-apoptotic and caspase-independent ways of cell death exist.[22-24]

Primary Structure of Caspases

Caspases are synthesized as zymogens consisting of an N-terminal prodomain followed by a large subunit of about 20 kD, named p20, and a small subunit of about 10 kD, named p10 (Fig. 1 and 2). In a number of procaspases the p20 and p10 subunits are separated by a small linker sequence. Caspase prodomains range in length from 5 amino acids for murine caspase-6 to 219 amino acids for caspase-10. The large N-terminal prodomains of mammalian caspases generally encompass protein-protein interaction modules such as the caspase recruitment domain (CARD) and death effector domain (DED). The prodomains of zebrafish caspy and caspy2 contain a PYRIN motif, originally found in PYRIN.[25] CSP-1 and -2 in *C. elegans* and STRICA in *Drosophila* also have large prodomains. However, their role in protein-protein interactions is still unclear and no known motifs were identified in these domains so far. Modules such as CARD, DED and probably PYRIN allow caspases to be recruited in protein complexes resulting in proximity-induced proteolytic activation, thereby initiating the caspase-dependent apoptotic and inflammatory pathways.[26-29] The DED motif is present in the prodomains of caspase-8 and -10, while the CARD motif can be found in the prodomains of caspase-1, -2, -4, -5, -9, -11 and -12 (Fig. 1). CARD, DED and PYRIN motifs structurally resemble the death domain (DD) and all four are tightly packed bundles of six or seven α–helices.[30-32] Alignment of the 11 human and the 10 murine procaspases described to date shows that 16 residues are identical and 22 others are conserved in all human and murine family members (Fig. 2). Seven of these residues (Arg179, His237, Gln283, Cys285, Arg341 and Ser347 for human caspase-1) are involved in the P_1 substrate aspartate recognition and catalysis, whereas most other conserved residues are hydrophobic and are likely to be involved in maintenance of the overall structure of the native enzyme.

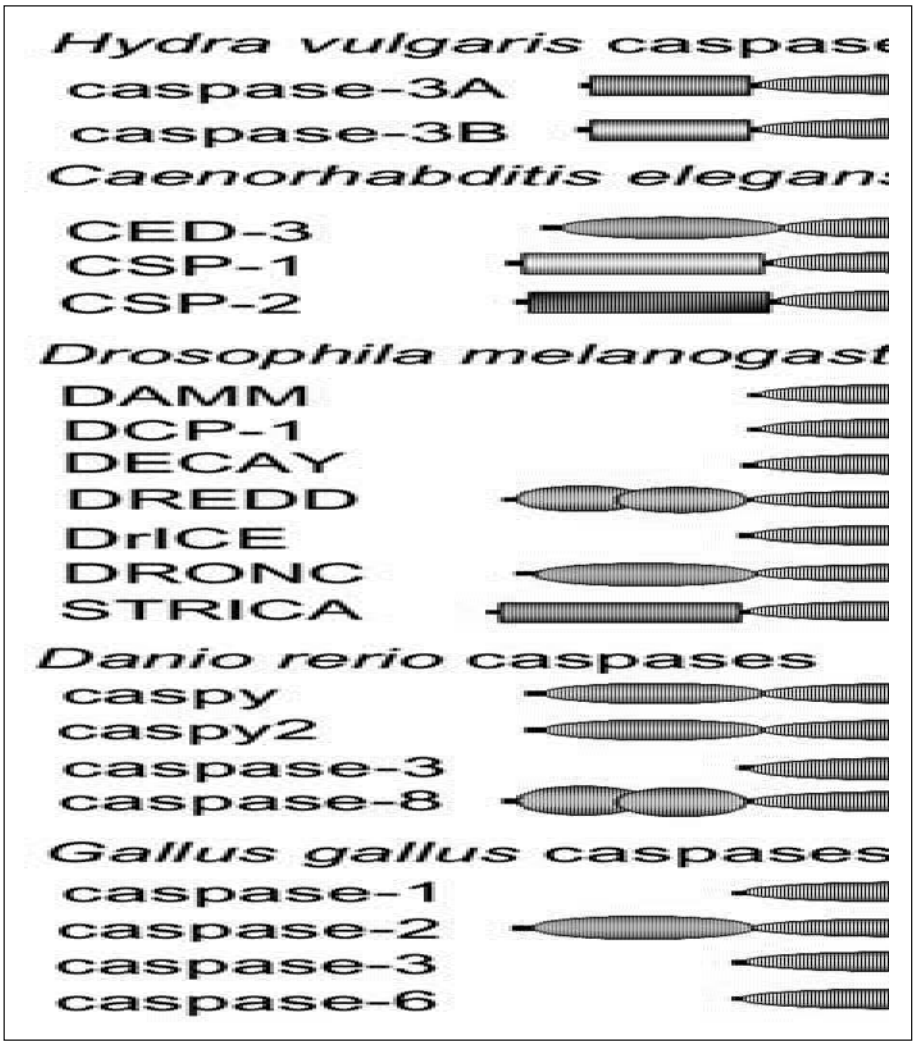


Figure 1. Schematic representation of caspase domain architecture from different species. Caspases from *Hydra vulgaris*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, *Gallus gallus*, *Xenopus leavis*, *Mus musculus* and *Homo sapiens*; the prodomain, the large (p20) and small (p10) subunits are indicated.

Pro Caspase Maturation

The catalytically dormant caspase zymogens are cleaved at specific Asp-X bonds (Fig. 2 and 3). This promotes the tetrameric assembly of two p20 and two p10 subunits and the release of the prodomain. This proteolytic maturation occurs either by interaction with other proteases or by adaptor-dependent homodimerization resulting in transproteolytic processing. Forced oligomerization of short prodomain caspases also results in proteolytic maturation, suggesting that all caspases irrespective of whether they have a long prodomain or not, carry some intrinsic protease activity.[33,34] Biochemical data indicate that the caspase-8 zymogen possesses about 1% of the mature caspase-8 activity.[27] This activity is sufficient to enable proximity-induced

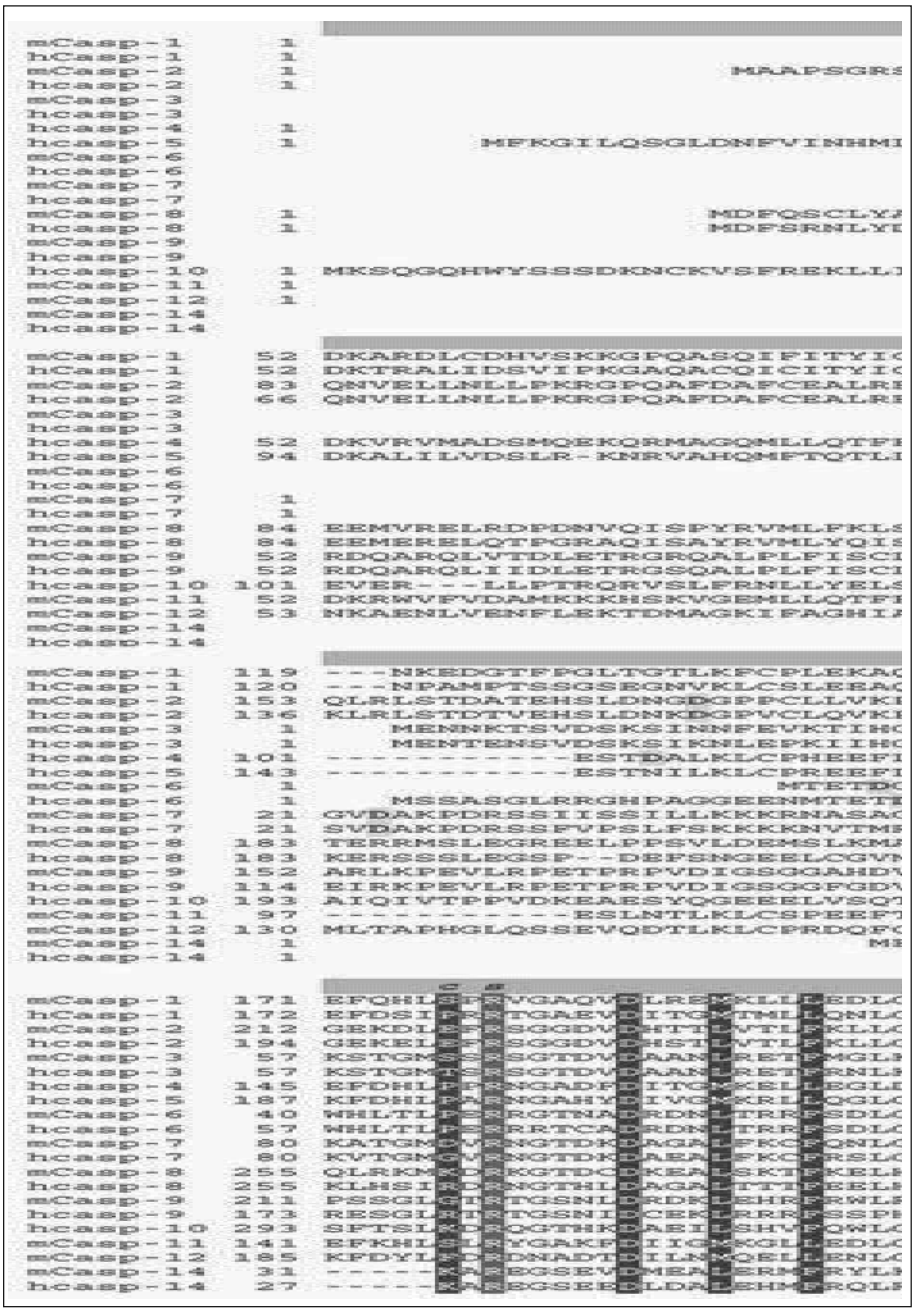


Figure 2. Continued on next page.

maturation of the precursor. The role of adapter molecules is to bring the caspase zymogens together in the correct way, enabling them to undergo autocatalytic activation. In vitro studies

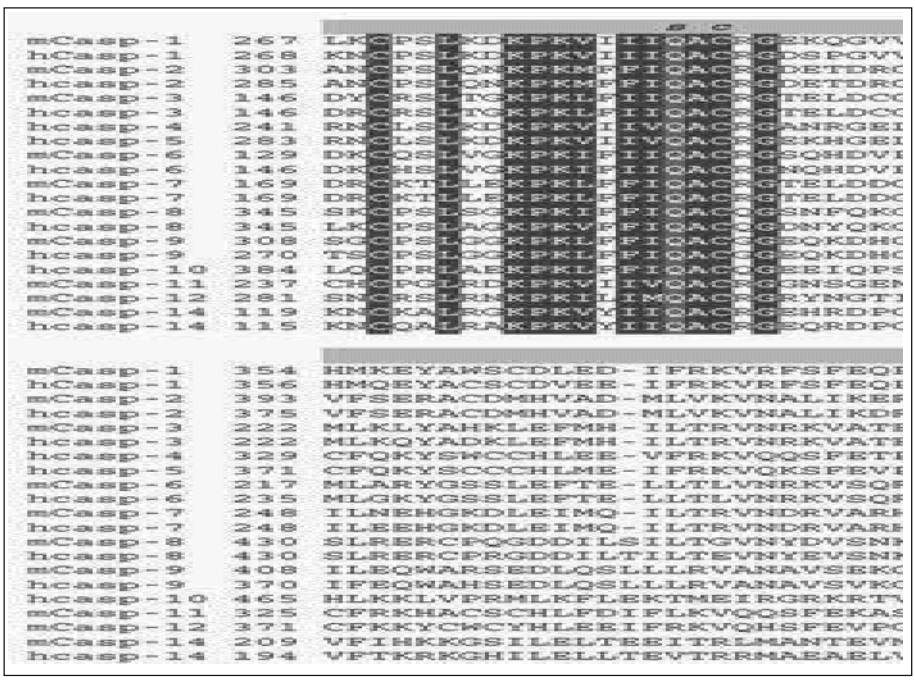


Figure 2. Sequence alignment of the human and murine caspases. Polypeptide sequences for the human and murine caspases were aligned using Clustal X.[314,315] mCasp-x: murine caspase-x; hCasp-x: human caspase-x; blue background and indicated with c on top of the alignment: the catalytic residues His237, Cys285 and Pro177 of human caspase-1; red background and indicated with c on top of the alignment: residues involved in coordinating the P_1 aspartate; black background: residues probably involved in maintaining the native enzyme structure; yellow background: Asp residue after which the peptide bond is cleaved during maturation of the procaspase. Cleavage sites have been determined by amino acid sequencing or analysis of site-directed mutants for the human caspases and caspase-12.[47,65,76,231,316-321] Other cleavage sites, like for most of the murine caspases, are based on theoretical prediction. DDD: acidic safety catch present in caspase-3[36] and the surmised safety catch for the other caspases. Except for caspase-1, -3 and -7 the cleavage site is part of the potential acidic safety catch. To view the color version of this Figure please visit http://www.eurekah.com/chapter.php?chapid=717&bookid=61&catid=69.

with recombinant caspase-9 demonstrated that the precursor form displays 10% of the activity of the recombinant processed form.[35] However, the catalytic activity of the processed form is increased 2000-fold by its integration in the apoptosome complex, showing that cytosolic factors can influence caspase activity dramatically.[35] An alternative, pH-dependent zymogen activation mechanism has been suggested for caspase-3.[36] The three adjacent aspartate residues located in the beginning of the p10 domain are functioning as a pH-sensitive safety catch allowing autoactivation in a slightly acidic environment, as in the cytosol of apoptotic cells.[36] The alignment in Figure 2 indicates that caspase-1, -2, -4, -5, -6 (human), -7, -8, -11, -12 and -14 (murine) contain an acidic D/EXD/E motif that may function as a pH-sensitive safety catch. Caspases are also activated by other proteases. The serine protease granzyme B, released by cytotoxic T lymphocytes in their target cells, can activate several caspase family members in vitro and in cells.[37-41] Furthermore, proteases such as subtilisin, cathepsin B and G may be involved in the activation of some caspases.[42-44] However, a recent study argues against a direct activating proteolysis of caspases by cathepsins.[45] Finally, it has been proposed that calpains can proteolytically activate caspase-7 and -12.[46,47]

Table 1. Synonyms, chromosomal localization, substrate specificity, inhibitors and adaptors of human and murine caspases

Caspase	Synonyms	Human / Murine Chromosomal Locus[a]	Specificity[b]	Natural Inhibitors	Adaptors
Caspase-1	ICE	11q22 / 9	YEVD WEHD	CrmA[163,165,166], p35[210,211]	RIP2[212], IPAF[196], CARD12[213]
Caspase-2	Ich-IL, NEDD2	7q35 / 6	VDVAD DEHD		RAIDD[208], DEFCAP[71], PACAP[207]
Caspase-3	CPP32, Apopain, Yama, Lice	4q34 / 8	DMQD DEVD	p35[210], c-IAP1[182], c-IAP2[182], XIAP[181,214], Survivin[184], Livin[215]	
Caspase-4	ICEreIII, Tx, Ich-2, Mih1	11q22 / unknown	LEVD (W/L)EHD	CrmA[165]	
Caspase-5	ICEreIIII, Ty	11q22 / unknown	(W/L)EHD	CrmA[165]	
Caspase-6	Mch2	4q25 / unknown	VEID VEHD	p35[210]	
Caspase-7	Mch3, CMH-1, ICE-LAP3, Lice2	10q25 / 19	DEVD	p35[210], c-IAP1[182], c-IAP2[182], XIAP[181,214], Survivin[184],Livin [215]	
Caspase-8	Mch5, FLICE, MACH	2q33 / 1	IETD LETD	CrmA[165,166], p35[210]	FADD [216, 217]
Caspase-9	Mch6, ICE-LAP6	1p34 / unknown	LEHD	c-IAP1[183], c-IAP2[183], XIAP[183]	Apaf-1[218], DEFCAP[71], NOD1[219], PACAP[207]
Caspase-10	Mch4, FLICE-2	2q33 / unknown	IEAD	p35[210]	FADD[220]
Caspase-11	Ich-3	/ unknown			
Caspase-12		/ unknown			TRAF2[221]
Caspase-14	MICE	19p13 / unknown			

[a] The chromosomal localization was determined using the OMIM database at http://www.ncbi.nlm.nih.gov/Omim/ and by blast searching the Human genome at http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html.

[b] adapted from refs. 38,83,136,137

Three-Dimensional Structure of Mature Caspases

X-ray crystal structures have been determined for mature caspase-1,[48,49] caspase-3,[50-52] caspase-7,[53-55] caspase-8[56-58] and caspase-9,[59] all bound to either synthetic substrate-based peptide inhibitors or natural inhibitors. The overall architecture of caspase-1, -3, -7, -8 and -9 is similar and consists of two heterodimers composed of a large and small subunit. The subunits

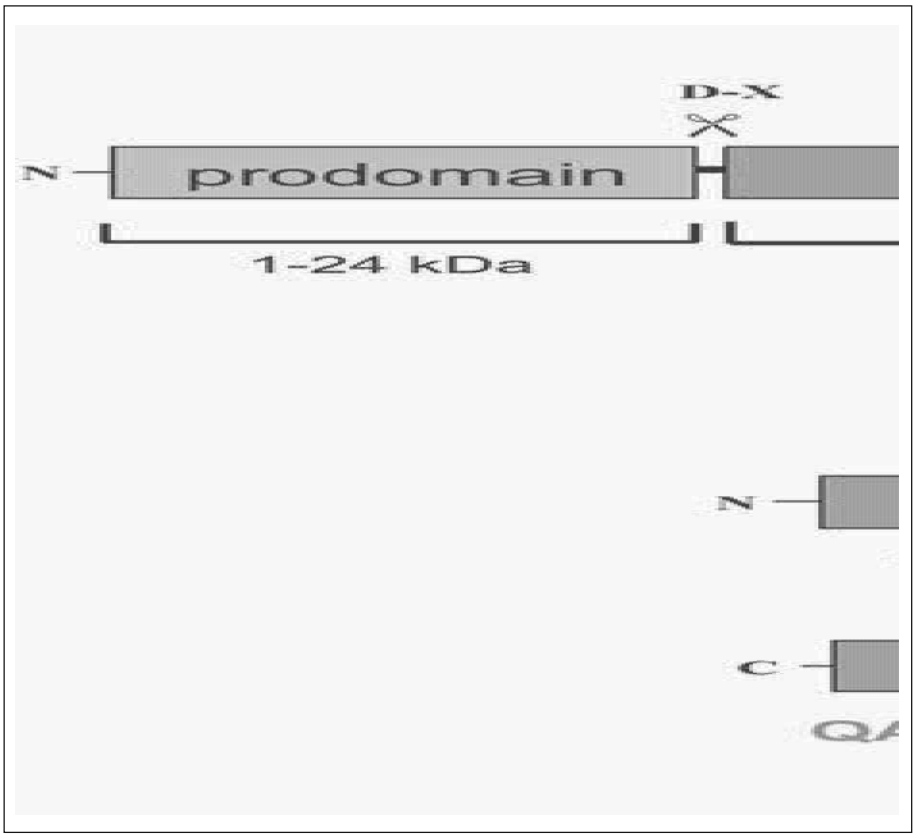


Figure 3. Overview of procaspase activation. A caspase zymogen exists of a prodomain of variable length (5 to 219 amino acids) followed by a p20 and a p10 domain. (*) A peptide linker separates the latter two domains in caspase-1, -2, -4, -5, -6, -8 and -9. The diagram depicts the position of the catalytic cysteine and histidine residues in the p20 domain and the aspartate-X bonds that are cleaved during the proteolytic maturation of the enzyme. Mature caspase is a heterotetramer, with each heterodimer consisting of a p20 and a p10 subunit. The consensus QACXG active site pentapeptide is partially conserved in *C. elegans* CSP-1 (SACRG) and CSP-2 (VCCRG), in *Drosophila melanogaster* DREDD (QACQE), DRONC (PFCRG), STRICA and DAMM (QACKG) and in *Gallus gallus* caspase-1 (QCCRG).

of each heterodimer are folded into a compact cylinder that is dominated by a central six-stranded β-sheet and five (α-helices that are distributed on opposing sides of the plane that is formed by the β-sheet. This so-called caspase-fold is a unique quaternary structure among proteases and has only been described for caspases and for gingipain R, arguing for an ancient origin for this family of cysteine proteases or an example of convergent evolution.[60] Gingipain R is a secreted cysteine protease from the bacterium *Porphyromonas gingivalis* (Protein Data Bank ID code 1CVR), which has no significant homology with caspases on the primary sequence. In this view, the caspases have been classified to a clan or superfamily of cysteine proteases, designated clan CD.[61] This clan also includes the evolutionary related families of the gingipains, legumains, clostripains and separin.[61] Each of these families is thought to share a catalytic-site motif and a common scaffold to their catalytic domains, implying shared ancestry.[62] All members of clan CD have specificities dominated by the interactions of the S_1 pocket.[61] Furthermore, two distinct families of caspase-like proteins, the paracaspases (found in metazoans and *Dictyostelium*) and metacaspases (found in plants, fungi and protozoa), have been identified.[63]

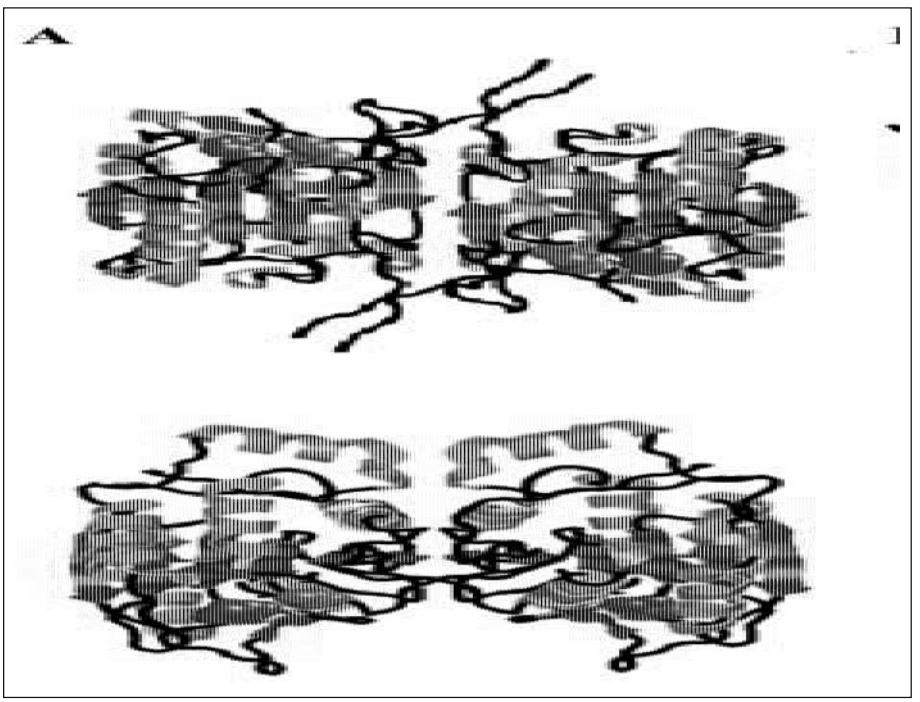


Figure 4. Structural comparison between caspase-1 and gingipain. Typical caspase fold of caspase-1 (A, C) and gingipain R (B). The upper and lower panels represent a 90° rotation over the horizontal axis. (A) The three-dimensional structure of tetrameric caspase-1 (Protein Data Bank ID code 1BMQ) has the typical caspase fold existing of a central 12-stranded β-sheet surrounded by 5 α-helices on each side. Coloring indicates the secondary structure: α-helices in yellow and β-strands in red. The catalytic residues His237 (blue) and Cys285 (green) are represented in space fill. (B) Gingipain R (Protein Data Bank ID code 1CVR) shares the caspase fold, arguing a common ancestral origin between this bacterial protease and caspases. The caspase fold of the monomeric gingipain R contains one catalytic site consisting of His (blue) and Cys (green), in contrast to the heterotetrameric caspase-1. (C) caspase-1 consists of two heterodimers that are oriented in an antiparallel fashion. The subunits of the left heterodimer are colored yellow (p20) and blue (p10). The subunits of the right heterodimer are colored green (p20) and red (p10). The catalytic residues His237 (orange) and Cys285 (black) are shown in space fill. These figures have been made using Rasmol software.

In the caspase tetramer, the two heterodimers align in a head-to-tail configuration, thereby positioning the two active sites at opposite ends of the molecule (Fig. 4). The C-terminus of the p20 subunit is topologically distant from the N-terminus of the p10 subunit in the same heterodimer, but is closer in space to the N-terminus of the p10 subunit in the adjacent heterodimer (Fig. 4). This orientation suggests that proenzymes interact in an anti-parallel way before processing. Since the X-ray structures of the mature caspases indicate that they function as $(p20/p10)_2$ heterotetramers, two mechanisms can be proposed for their proximity-induced autoactivation (Fig. 5). Each proenzyme could be capable of processing itself or a nearby zymogen. During maturation the two subunits of the mature p20/p10 heterodimer can be derived from the same precursor molecule (intramolecular assembly model). Alternatively, the subunits of two adjacent proenzymes could complement each other (intermolecular assembly model). Here, the p10 subunit of the mature heterodimer is derived from the first precursor molecule while the p20 subunit comes from the second procaspase. The recently solved X-ray structure of procaspase-7[64] and in vitro data on maturation of procaspase-9[65] strongly support

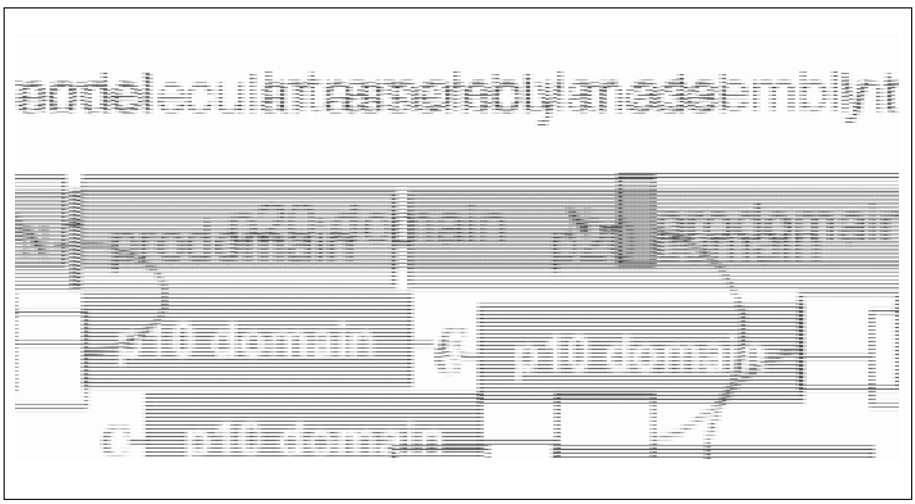


Figure 5. Two models for the assembly of mature caspases. In the *intramolecular assembly* model the two subunits of the mature heterodimer are derived from the same precursor molecule. Alternatively, in the *intermolecular assembly* model both p20 and p10 subunits of the heterodimer originate from two different precursors. Complementation studies using mutant variants of procaspase-9[65] and the X-ray structure of procaspase-7[64] strongly support the intramolecular assembly model.

the intramolecular assembly model. Despite the presence of two active sites, there is no evidence for cooperativity or allosteric modulation between the two sites. The active site spans both the p20 and p10 subunits, thus explaining the requirement of both domains for activity. His237 and Cys285 (numbering in human procaspase-1) form a catalytic diad in the active site of the caspases. The active site pentapeptide QACXG is always part of the p20 in mammalian caspases. In other organisms the active site pentapeptide can differ from this consensus (Fig. 3). Amino acid residues involved in forming the P_1 Asp pocket of the enzyme (S_1) include Arg179, Gln283, Arg341 and Ser347 (numbering in human procaspase-1). Only the first two residues are delivered by the p20 subunit. A remarkable property of the caspase-9 structure is that two conformations of the catalytic site are present in one heterotetramer. One active site is in a catalytically competent conformation while the other is catalytically incompetent.[59]

Catalytic Mechanism

Like other proteases, caspases break peptide bonds by forcing the trigonal planar peptide bond into a tetrahedral geometry as a prerequisite for hydrolysis. Thus, the majority of the available binding energy is used for stabilizing this tetrahedral intermediate rather than merely forming an enzyme-substrate complex. During caspase-substrate interaction the catalytic Cys285 residue promotes the formation of this tetrahedral intermediate. Like other cysteine proteases, caspases require a reducing environment for enzymatic activity. The catalytic mechanism occurs in steps (Fig. 6). First, His237 in the catalytic diad acts as the general base attracting a proton from Cys285 and thus enhances the nucleophilic property of the latter (Fig. 6A). Then, Cys285 attacks the P_1 amide bond of the substrate thereby inducing formation of the tetrahedral intermediate. At this stage the substrate is covalently attached to the catalytic cysteine that has lost its nucleophilic property (Fig. 6B). Next, the C-terminal peptide of the substrate is released from the complex, while the peptide N-terminal of the scissile bond and the enzyme form an acyl-enzyme complex (Fig. 6C). The oxygen pole of a water molecule, acting as a nucleophile, attacks the carbonyl group of the acyl-enzyme complex and induces a second tetrahedral intermediate (Fig. 6D). The mechanism of action of some natural caspase inhibitors

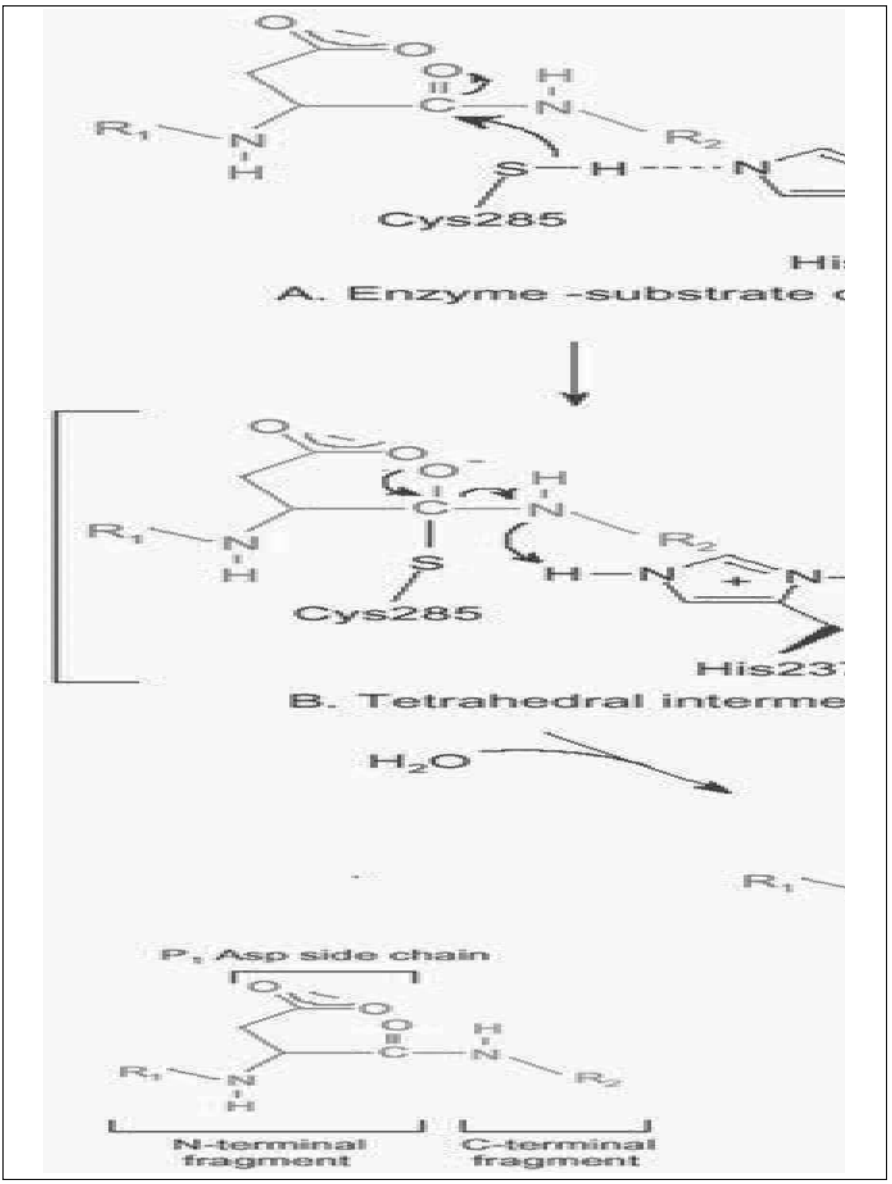


Figure 6. Catalytic mechanism of caspases. The numbering of the catalytic residues refers to human caspase-1. The enzymatic reaction is represented in five steps (adapted from ref. 49). (A) First, His237 attracts a proton from Cys285 by which the latter becomes more nucleophylic. Cys285 then attacks the P_1 amide bond of the substrate, inducing the formation of a tetrahedral intermediate in which enzyme and substrate are covalently bound (B). Next, the C-terminal fragment of the substrate (R_2, green) is released and an acyl-enzyme complex is formed (C). The oxygen pole of an entering water molecule attacks the acyl-enzyme complex and induces a second tetrahedral intermediate in which the enzyme and N-terminal fragment (R_1, red) are covalently bound (D). Finally, electron relocalizations in the tetrahedral intermediate lead to the release of the N-terminal fragment. The enzyme is restored in its initial state (E). The backbone amide of Pro177 may be involved in catalysis, beside His237 and Cys285 (see text). To view color version of the Figure please visit http://www.eurekah.com/chapter.php?chapid=717&bookid=61&catid=69.

such as CrmA and p35, which initially act as substrates, is prevention of the entry of the water molecule, allowing them to remain covalently attached to the catalytic cysteine. Finally, electron relocalizations in the tetrahedral intermediate lead to the release of the N-terminal fragment of the substrate and restore the enzyme to its initial state (Fig. 6E). In many proteases a side-chain of a third residue is implicated in the catalysis. The catalytic diad in caspases may be assisted by a third component delivered by the backbone carbonyl of residue 177 (proline in caspase-1, threonine in caspase-3, glycine in caspase-7, arginine in caspase-8) (Fig. 2 and 6).

The transition state is stabilized by additional amino acid residues in the binding site that secure proper positioning of the substrate. The binding site of the enzyme is divided into a number of sub-sites (S_x with x counting from the scissile bond in the bound substrate), each securing the corresponding P_x amino acid residue in the substrate by multiple interactions. In addition to interactions with specific side-chains, binding of the substrate peptidyl backbone also plays an important role in catalysis.

The structures of caspase-1, -3, -7 and -8 reveal that the details of the interactions with different tetrapeptide inhibitors are very similar in the four proteins with the exception of the S_4 subsite.[48-58] The P_1 aspartyl side chain of the co-cristallized inhibitor is involved in ionic interactions with the side-chains of Arg179, Gln283 and Arg341 (numbering in human procaspase-1), which are highly conserved (Table 2). Furthermore, three hydrogen bonds are formed between the conserved residues Ser339 and Arg341 of the enzyme and the inhibitor backbone. Compared to caspase-1, caspase-3 and -7 possess an extra residue (phenylalanine), which contributes to the binding of the inhibitor in the S_2 site. Caspase-8 has a tyrosine (Tyr365) at the same spatial position, but this residue is not involved in hydrogen bonds with the inhibitor. A conserved arginine, Arg341 for caspase-1, in the S_3 pocket of the caspases is involved in hydrogen interactions with the P_3 backbone.[49,53] Interactions at the S_4 subsite determine largely the differences in the specificity of the caspases. The residues in the S_4 subsite of caspase-1 form a large and shallow hydrophobic depression that easily accommodates a tyrosyl side chain, while the corresponding site in caspase-3 and -7 is a narrow pocket that closely envelops a P_4 Asp side chain.[50] In caspase-8, the S_4 Tyr412 replaces the more bulky S_4 tryptophan present in caspase-1, -3 and -7 (Table 2). Due to the spatial organization of the S_4 site of caspase-8, it is impossible to form hydrogen bond interactions equivalent to that observed between the Phe250 residue of caspase-3 and the P_4 side chain. In addition, a negative charge present in the S4 pocket of caspase-8 is unfavorable for a P_4 aspartic acid. Therefore, the caspase-8 S_4 pocket is wide but preferentially accommodates aliphatic residues at the P_4 position, though other residues are also tolerated.

The structures of an unprocessed caspase-7 zymogen and of mature caspase-7 either with or without bound inhibitor demonstrated that during proteolytic activation large conformational changes occur in loops containing the S_2, S_3 and S_4 subsites.[53-55,64] Induced fitting of the substrate or inhibitor further completes the conformational changes required for catalysis or inhibition.[64]

Caspase Prodomains

Large prodomains of mammalian caspases contain structural motifs that belong to the so-called 'death domain superfamily.' These structural motifs have emerged as the prime mediators of the interactions necessary for transducing inflammatory and death signals and can be found in a growing number of proteins involved in apoptosis and inflammation. This superfamily consists of the death domain (DD), the DED, the CARD[66] and the recently identified PYRIN domain.[30-32] Each of these motifs interacts with other proteins through homotypic interactions. Among the four families (DD, DED, CARD, PYRIN), DDs are commonly found in upstream components of the apoptotic pathways, such as death receptors (e.g., CD95, TNF-R1) and the adaptor molecules that are recruited to these receptors (e.g., FADD, TRADD, RAIDD and RIP).[67] On the other hand, DEDs and CARDs are generally responsible for recruiting the initiator procaspases to death- or inflammation-inducing complexes through specific adaptor molecules.[67-69] The PYRIN domain has only been found in zebrafish caspases[70]

Table 2. Comparison of residues in caspase-1, -3, -7 and -8 involved in interactions with the reversible tetrapeptide inhibitor Ac-DEVD-CHO

	Caspase-1	Caspase-3	Caspase-7	Caspase-8
S_1	Arg179	Arg64	Arg87	Arg260
	Arg341	Arg207	Arg233	Arg413
	Gln283	Gln161	Gln184	Gln358
	Ser339	Ser205	Ser231	Ser411
	Gly238	Gly122	Gly145	Gly318
S_2	Trp340	Trp206	Trp232	Tyr412
		Phe256	Phe282	Tyr365
	Val338	Tyr204	Tyr230	Val410
S_3-S_4	Trp340	Trp206	Trp232	Tyr412
	Arg341	Arg207	Arg233	Arg413
	Pro343	Trp214	Pro235	Pro415
	Arg383	Phe250	Gln276	Asn414
				Trp420
	His342	Ser209	Asp278	Asn414

The binding site of the enzyme is divided into a number of sub-sites indicated as S_x with x counting from the scissile bond in the bound inhibitor. Each subsite secures the corresponding P_x amino acid residue in the inhibitor by multiple interactions. (adapted from ref. 53)

and in the N-terminus of several proteins thought to function in apoptotic and inflammatory signaling pathways, such as the recently identified human CED-4 family members DEFCAP[71] and ASC.[72] The different superfamily members have only weak, but relevant, sequence homology to each other. The structures of different DD, CARD and DED domains made it clear that they belong to the same superfamily.[66] Using secondary structure prediction and threading methods the PYRIN domain is proposed to be a new member of the DD superfamily.[30-32] All members of the DD superfamily possess similar structures comprising six or seven antiparallel amphipathic (α-helices (Fig. 7A). However, there are significant differences in the orientation of the helices and the nature of the homophylic interactions between the four domain families. Members of the CARD family tend to contain six helices aligned almost parallel to each other, whereas those of the DD family can be described as two mutually perpendicular three-helix bundles.[66,73] Electrostatic interactions mediate homotypic interactions in DDs[74] and CARDs.[73] This is reflected by the presence of a large positive electrostatic patch at one side and a negative one at the opposing side of the CARD and DD surfaces (Fig. 7B). DED surfaces lack the large positively charged surface regions and hence mainly use hydrophobic interactions.[75]

Caspase Splice Variants

A possible way of regulating caspase function is by alternative splicing and to date many caspase mRNA splice variants have been described (Table 3). Some of these splice variants are expressed in a tissue- or cell line specific way. On a structural basis one could consider different functional forms of caspases as a result of alternative splicing. First, the prototype caspases are generally spoken expressed most abundantly and possess full enzymatic activity upon activation. Second, there are caspase forms that lack essential residues for enzymatic activity but still contain a large prodomain. These may function as dominant negative molecules in caspase recruitment. Third, some of the large-prodomain-containing caspase splice variants lack a functional prodomain and thus rely on upstream caspase activity for their activation. Last, splice

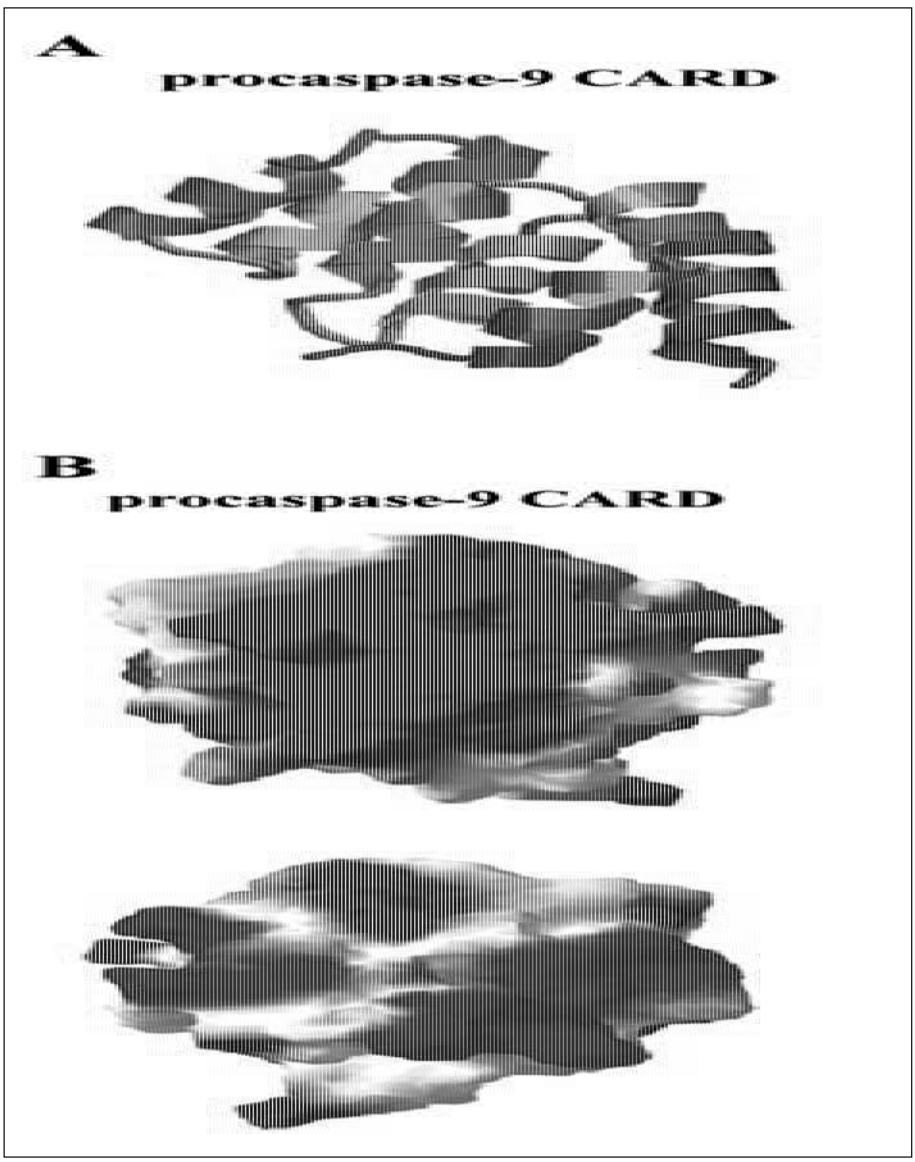


Figure 7. Structural similarities between CARD, DD and DED of procaspase-9 and FADD. (A) Tertiary structure of the caspase recruitment domain (CARD) of caspases-9, the death domain (DD) of FADD and the death effector domain (DED) of FADD. All three are members of the DD superfamily and possess similar structures comprising six (FADD DD and DED) or seven (procaspase-9 CARD) antiparallel amphipathic α-helices. (B) Electrostatic surface potential of the procaspases-9 CARD, DD-FADD and DED-FADD. The upper and lower panels represent a 180^{o} rotation over the vertical axis. The presence of large patches of positive (> 8 k_{BT}) (blue, upper panel) and negative (< -8 k_{BT}) (red, lower panel) charges on opposite sides of the CARD and DD surfaces allow homotypic electrostatic interactions.[73,74] The DED surface is negatively charged on one side (upper panel) and lacks large positively charges on the opposing side (lower panel). Therefore homotypic DED interactions are mainly of hydrophobic nature.[75] These figures have been made using the Rasmol and Swiss PDB viewer software.[322]

Table 3. Caspase isoforms in human and mouse (to view color version of Table please visit http://www.eurekah.com/chapter.php?chapid=717&bookid=61&catid=69)

Organism	Caspase
Homo sapiens	Caspase-1
	Caspase-2
	Caspase-3
	Caspase-4
	Caspase-5
	Caspase-6
	Caspase-7

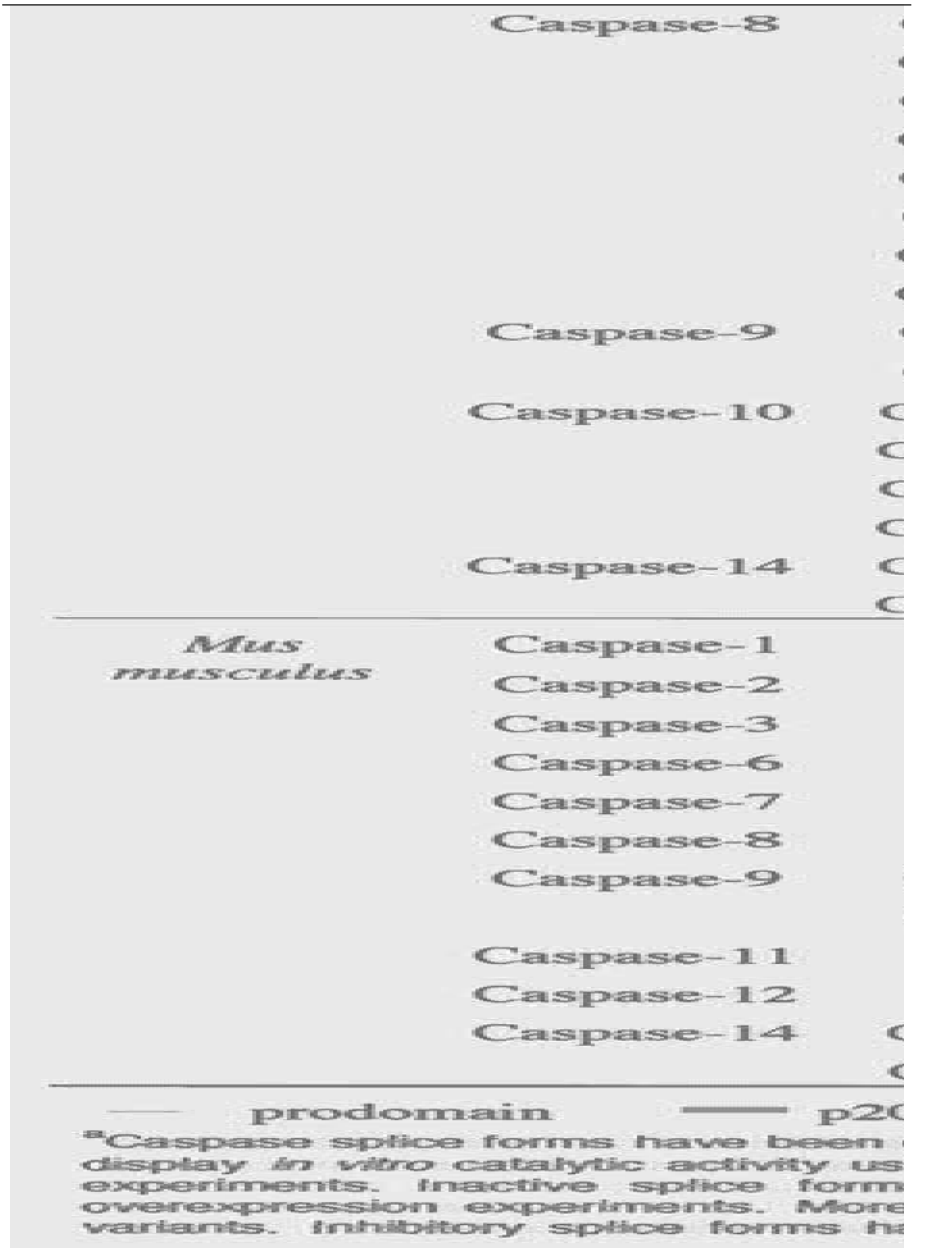
Continued on next page

variants have been reported that lack both enzymatically important residues and recruitment motifs resulting in proteins without obvious function. They may just contribute to a reduction of the mRNA pool coding for the active caspase. Enzymatically impaired splice variants of short prodomain caspases also belong to this category. However one should keep in mind that the physiological function of these splice variants has not been studied in detail and most data are based on overexpression experiments.

Caspase Substrates

The list of publications describing new cellular caspase substrates is growing rapidly. Cleavage of a substrate by caspases may have different effects on the protein and its function. For

Table 3. Continued



certain proteins, cleavage by caspases will lead to their subcellular relocalisation, to their inactivation or to activation of a function contributing to apoptosis or inflammation. A restricted list of examples of cellular caspase substrates, demonstrating different effects of the action of caspases is presented in Table 4. A selection of substrates is discussed next.

Table 4. Limited selection of cellular caspase substrates classified according to their function in apoptosis

Effect on the Cell	Substrate	Cleavage Site	Caspase	Effect On the Protein	References
I. Cellular Morphology					
Disassembly of the cytoskeleton, loss of cell to cell contact, desintegration and fragmentation of the cell	Actin	ELPD*G	8	Inactivated	252-254
	Plectin	ILRD*K	8		255
	α-Adducin	DDSD*A	3		256
	β-Catenin	DLMD*G YPVD*G SYLD*S ADID*G TQFD*G	3, 7, 8		257,258
	E-cadherin	DTRD*N	3, 7		132,134
	Desmoglein-3	DYAD*G	3, 7 ?		133
	Vimentin	IDVD*V DSVD*F	3, 6, 7, 9		259,260
	Cytokeratin-18	VEVD*A	3, 6, 7		261,262
	FAK	DQTD*S	3,7		
		VSWD*S	6		263,264
	Fodrin/ αII-Spectrin	DETD*S DSLD*S	3		265
	βII-spectrin	DEVD*S ETVD*S	3		265
	Gelsolin	DQTD*G	3	Activated	266,267
	SLK	DTQD*Q	3		268
	Gas2	SRVD*G	3, 7		269
Blebbing of the membrane	ROCK-I	DETD*G	3	Activated	85,86
II. Organelles					
Nuclear breakdown	Lamin A	VEID*N	6	Inactivated	270
	Lamin B	VEVD*S	3		76,270
Chromatin condensation	Acinus	DELD*Y	3	Activated	271
DNA degradation	ICAD/DFF-45	DEPD*S DAVD*T	3	Inactivated	87,88
Loss of DNA repair	PARP	DEVD*G	3, 7, 9	Inactivated	92,94,96
Inhibition of DNA replication	DNA-RC C140	DEVD*G	3	Inactivated	272
	Topoisomerase	DDVD*Y	3	Inactivated	273
		EEED*G	6		
Desintegration of the Golgi complex	Golgin-160	ESPD*G	2	Inactivated	99
		CSTD*S	3		
		SEVD*G	2, 3, 7		
Inhibition of the transport from the ER to the Golgi	Bap31	AAVD*G AAVD*G	3,8		97,100,274

continued on next page

Table 4. Continued

Effect on the Cell	Substrate	Cleavage Site	Caspase	Effect On the Protein	References
II. Organelles (Cont'd)					
Disruption of the mitochondria and amplification of the apoptotic signal	Bid	LQTD*G	3, 8	Activated	101,102,275
	BAX	GIQD*R	3		276,277
	Bcl-2	DAGD*V	3	Inactivated	115-118
	Bcl-X_L	HLAD*S	1, 3		114,276
III. Cellular Functions					
Cell cycle arrest	Rb	DEVD*G	3	Inactivated	278-281
	$p27^{kip1}$	DPSD*S	3		282-284
	$p21^{cip1/waf1}$	DHVD*L	3		282,285,286
	PKCδ	DMQD*M	3	Activated	287-291
Inhibition of mRNA transcription	RIP	LQLD*C	8	Inactivated	79,292-294
	NFκBp65	DCRD*G	3		125,295,296
	STAT-1	MELD*G	3		297
Inhibition of mRNA splicing	U1-70KsnRNP	DGPD*G	3	Inactivated	168
Inhibition of de novo protein synthesis	PKR	DPLD*M	3, 7, 8	Activated	126
	eIF4-G	DLLD*A DRLD*R	3	Inactivated	298-301
	eIF2-α	AEVD*G	3, 7, 8		302,303
	ProEMAP-II/ p43 Met-tRNA systhetase	ASTD*S	7		128-130
IV. Inflammation and Disease					
Inflammation	ProIL-1β	YVHD*A FEAD*G	1	Activated	4,138, 304,305
	ProIL-16	SSTD*S	3		306
	ProIL-18	LESD*N	1		127,307
	ProEMAP-II/ p43 Met-tRNA synthetase	ASTD*S	7		128-130
Disease	Huntingtin	DSVD*L	3, 7	Inactivated	308-310
	Presenilin-1	ARQD*S	3		311-313
	Presenilin-2	DSYD*S	3		311,312

In apoptosis a cascade of caspase cleavage events initiates the cell death process. Caspases turn off cell protective mechanisms and activate pathways that lead to cell destruction (Table 4). Caspases can cleave and activate other caspases. Caspase-cascades were studied mainly in vitro by assessing the processing of procaspases by their enzymatically active counterparts or by depletion of different caspases from cell lysates followed by caspase activation with cytochrome

c and ATP.[76-79] caspase cascades were also studied by co-expression of different caspases in the yeast *Saccharomyces cerevisiae*, which is devoid of endogenous caspases.[80] These studies suggest the existence of a hierarchical proteolytic procaspase activation network with apical long-prodomain-caspases that proteolyze and activate other caspases. The former are often referred to as initiators (caspase -8, -9 and -10) whereas the latter, mostly the short-prodomain caspases (caspase-3, -6 and -7), are considered as executioners. The apoptotic signal is further amplified by positive feedback loops in which the executioner caspases process and activate other caspases, including those containing long prodomains. Some caspases, such as caspase-8, can process all procaspases whereas others, such as caspases-2, only seem to activate their own precursor.[78] Although caspase-1 and -11 are needed mainly for induction of inflammation,[20,81] they were shown to process the apoptotic effector caspases -3, -7 and -6 in vitro and may initiate apoptosis in vivo.[78,82-84] However, none of the apoptotic effector caspases activates caspase-1 and -11 excluding such a link between apoptosis and inflammation.[78]

In addition to participation in the caspase cascade, caspases are involved in many of the key events occurring during the execution of apoptosis (Table 4). Caspases cleave and dismantle many of the components of the cytoskeleton, thus causing loss of cellular contacts, fragmentation of the cell and the appearance of the typical apoptotic morphology (Table 4, Substrates I). Examples of such substrates are β-catenin, E-cadherin, actin, vimentin, GAS2, α-adducin, SLK, gelsolin and cytokeratin-18. Caspases also cleave and activate Rho-associated kinase-1 (ROCK-1) leading to the phosphorylation of myosin light chains, resulting in membrane blebbing.[85,86]

Caspases initiate the destruction of the nucleus (Table 4, Substrates II). Nuclear destruction involves proteolysis of structural proteins, such as lamin A and B as well as DNA degradation. Proteolysis of ICAD, the inhibitor of the caspase activated DNase (CAD), leads to the activation of the nuclease and thus to DNA degradation[87,88] whereas cleavage of poly(ADP-ribose)polymerase-1 (PARP-1) and poly(ADP-ribose)glycohydrolase (PARG) is thought to prevent the repair of DNA breaks.[89,90] PARP-1, one of the first caspase substrates discovered, is readily cleaved by caspases into a p85 and a p25 fragment during apoptosis.[91-93] The p25 PARP-1 fragment retains the DNA binding function and acts as a dominant negative PARP-1.[94] DNA breakage induces rapid activation of PARP-1. PARP-1 in turn depletes the intracellular concentration of its substrate, NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation. Thus, cleavage of PARP-1 prevents depletion of the cellular energy needed for apoptosis and avoids counteraction of DNA degrading processes.[95] Moreover, cleavage of PARP-1 was suggested to allow the activation of the endonuclease DNAS1L3.[96]

Apoptosis is further characterized by disruption of the endoplasmatic reticulum and Golgi apparatus. Cleavage of golgin was suggested to affect the integrity of the Golgi and proteolysis of Bap31 disrupts the transport between the ER and the Golgi.[97-100] Damage to the mitochondria can be mediated for example by cleavage of Bid, a cytoplasmic BH3-only member of the Bcl-2 protein family.[101,102] caspase-cleaved Bid, known as tBid, translocates from the cytosol to the mitochondria where it promotes the release of cytochrome c and other proapoptotic mitochondrial intermembrane proteins.[103-113] In certain cells Bcl-2 and Bcl-X_L that normally protect the mitochondria from damage,[7] including that done by tBid, are cleaved by caspases and inactivated. Moreover, some reports suggest that the cleaved forms of Bcl-2 and Bcl-X_L are proapoptotic.[114-119]

Caspases cleave proteins involved in different signaling pathways. They activate kinases and phosphatases, inactivate several transcription activators, inhibit translation and induce cell cycle arrest (Table 4, Substrates III). Several proteins involved in the control of different cellular functions were shown to be caspase substrates. For some of them cleavage by caspases results in inactivation (e.g., STAT-1, NF-κBp65, RIP, FAK, eIF4G, eIF2a, eIF3) (Table 4). However for many kinases the effect of caspase-mediated proteolysis is activation (e.g., ROCK-1, SLK, PKCδ and PKR) (Table 4). A major cellular protein phosphatase, PP2a, can also be activated by caspase cleavage.[120] In addition, caspases can indirectly activate proteases such as calpain by cleavage and inactivation of its inhibitor calpastatin.[121-124]

Inhibition of transcription and of de novo protein production can prevent the production of proteins that antagonize the death process. For example, caspase cleaved NF-κBp65 loses its transcriptional activity and potentiates apoptosis by the negative regulation of cell survival signals.[125] A similar effect is obtained by caspase-cleaved PKR, which leads to eIF2-α phosphorylation and thereby contributes to inhibition of translation initiation.[126]

Caspases are also involved in the induction of inflammation and disease (Table 4, Substrates. IV). Caspase-1 processes and activates proIL-1β and proIL-18.[20,127] caspase-7 can cleave the auxiliary p43 component of the aminoacyl-tRNA synthetase complex resulting in release of the leukocyte recruiting EMAP-II cytokine.[128-130] Caspases were also suggested to be involved in the onset of several diseases due to the aberrant cleavage of several substrates such as huntingtin and presenilin 1 and 2 (Table 4, Substrates IV).

In some substrates a form of cooperativity regarding the sensitivity to proteases exists. Cleavage at one site may allow the exposure and cleavage of another. This occurs, for example, during processing of proIL-1β by caspase-1.[131] Golgin is cleaved first by caspase-2 and only then falls prey to other caspases.[99] Cleavage by caspases may also render certain proteins more susceptible to other proteases. For example, cleavage of E-cadherin and desmoglein-3 occurs first in the cytoplasmic region by caspases and then in the extracellular part by metalloproteinases thus allowing the shedding of the proteins from the cell surface.[132-134] Protein phosphorylation can protect caspase substrates from cleavage as was observed with Bid phosphorylation by casein kinase I and II.[35]

Caspase activation does not always lead to cell death. Activation of the apoptosis-related caspases 3, 6 and 8 is required for T-cell proliferation and activation.[11-13] In such cells several typical caspase substrates are cleaved (e.g., PARP-1, lamin B and WEE1 kinase) whereas others are protected (e.g., ICAD and DNA-RC C140).[12]

Synthetic Peptide Substrates and Site Preferences of Human Caspases

The first synthetic substrates designed and used to measure and analyze caspase activities were based on the cleavage sites of the initial cellular protein substrates that were identified. These were usually a tetrapeptide with aspartate at the P_1 position (XXXD) conjugated to a fluorogenic [7-amino-4-trifluoromethylcoumarin (AFC) or 7-amino-4-methylcoumarin (AMC)] or colorimetric moiety [p-nitroanilide (pNA)] (Tables 1 and 5). The use of combinatorial peptide libraries allowed the definition of individual tetrapeptide specificities and preferences of several human caspases (Table 5).[38,83,136,137] The results indicate that caspases can be divided into three major groups. Caspase-1, -4, and -5 (Group I) prefer the tetrapeptide sequence WEHD. Caspase-2, -3, -7 and the *C. elegans* caspase CED-3 (Group II) prefer DEXD, whereas caspase-6, -8, -9 and -11 (Group III) prefer the sequence (L/V)EXD. The study demonstrated that beside their stringent requirement for Asp in P_1, the most critical determinant of specificity distinguishing between the different caspases is P_4. Members of Group I can handle large aromatic or hydrophobic amino acids in the P_4 position, whereas Group II caspases require Asp for efficient proteolysis. Group III caspases can tolerate different amino acids in P_4 but prefer large aliphatic side chains. All of the caspases tested prefer, to varying degrees, glutamate in position P_3. Most of the caspases tolerate any amino acid in P_2 with the exception of caspase-9, which requires histidine in this position. Comparison of the caspase cleavage site in cellular protein substrates and experimental results using internally quenched fluorescent peptide substrates suggest a preference for a small uncharged residue such as Ser, Gly and Ala at position P_1' immediately C-terminal of the P_1 Asp (Table 5).[137] The results described in Table 5 present the preferences of the different caspases in vitro. However, a certain degree of tolerance can be observed for most caspases. Therefore substrates can contain less optimal tetrapeptide cleavage sites (Table 4).

Interestingly, caspases with long prodomains (caspase-1, -2, -4, -5, -8, -9 and CED-3) have substrate specificities that resemble their own activation sequence, suggesting that these enzymes may employ an autocatalytic mechanism during proximity-induced activation (Fig. 5

Table 5. Caspase proteolytic specificities determined by the use of combinatorial tetrapeptide libraries

Group	Caspase	P_4	P_3	P_2	P_1	P_1'
I	1	W, Y, F	E	H	D	G, S, A
	4	W, L				
	5	W, L, F				
II	2	D	E	H	D	G, S, A
	3			V, I		
	7			V, I		
	CED-3			T, V, I		
III	6	T, V, I	E	H, V, I	D	G, S, A
	8	L, V, D		T, V, I		
	9	L, V, I		H		
	11	V, I, P, L		H		

The tetrapeptide caspase substrate is divided in P_X amino acid residues with x counting from the scissile bond. The residue after the scissile bond is indicated as P_1'. In general, caspases can be divided in three groups according to their tetrapeptide substrate specificities. Enzymes from group I (caspase-1, -4 and –5) prefer the sequence WEHD. Group II caspases (caspase-2, -3, -7 and CED-3) prefer DEXD and Group III enzymes (caspase-6, -8, -9 and –11) prefer the tetrapeptide (I/L/V)EXD as a substrate. (adapted from refs. 38,83,136,137)

and Table 5). The optimal recognition motif for group III caspases is also similar to activation sites within some of the executioner caspase proenzymes, such as caspase-3 and -7 (Fig. 2), suggesting that they operate in a hierarchical proteolytic cascade.[38,77,78] caspase-3 and caspase-7 have similar specificity profiles.[38] However, their function and substrate repertoire in cells seems to be distinct and not redundant.[79] Most of the cellular caspase substrates reported were identified to be caspase-3 substrates using in vitro cleavage assays (Table 4). This may be somewhat biased since active recombinant caspase-3 is and was relatively easy to obtain. Still, caspase depletion studies have shown that this enzyme is also the main effector caspase in apoptotic cells needed for the proteolysis of most of the caspase substrates tested, whereas caspase-6 or -7 show a limited substrate repertoire.[79]

Caspase Inhibitors

Possible ways to hinder caspase function, apart from controlling caspase activation, are blocking the substrate binding pocket, oxidizing the catalytic cysteine thiol group or targetting the protease for degradation. Natural and synthetic caspase inhibitors have been described. Although vast efforts were made to develop artificial caspase inhibitors for research and therapeutic use, the design of selective inhibitors is still a major challenge.

Thiol alkylating reagents such as iodoacetamide and N-ethylmaleimide[4,138] and oxidizing agents, like oxidized glutathion, selenium and NO can efficiently block caspase activity, although they lack specificity for caspases and inhibit other cysteine proteases.[4,139,140] Oxidized glutathion is often used to avoid unintentional caspase activation during cell lysis or caspase purification.[141] Nitrosylation of the active site can also be part of the cellular repertoire to control caspase activity.[142] The phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP2) has also been reported to directly block caspase-3, -8 and -9 in vitro, although the mechanism is unclear.[143] Since phosphoinositides can also activate the survival kinase Akt that in turn can inactivate pro-apoptotic molecules such as Bad, caspase inactivation by PIP_2 may represent an extra restraint on the activation of the apoptotic pathway. Most synthetic caspase inhibitors used to date are peptide derivatives that were developed based on the strict requirement for an

aspartic acid residue in the P_1 position of the tetrapeptide motif recognized in the substrate. These inhibitors are thus competitive inhibitors. The number and the nature of the amino acid residues upstream of the P_1 aspartate determine their specificity (Tables 1 and 5). These peptide inhibitor sequences are often identical to the one used for synthetic caspase peptide substrates. A reactive electrophile linked to the carboxyl end of the peptide moiety, determines whether the inhibition of the active site thiol is reversible or not (Fig. 8). Aldehyde, ketone or nitrile groups react reversibly with the active site cysteine, forming a thiohemiacetal with its oxyanion stabilized by His237.[49,143] Irreversible competitive inhibitors have a substituted methylketone group attached to the P_1 position. Either chloro-, fluoro-, diazo- or acyloxymethylketone derivatives can be used.[145] Oligopeptide-halomethylketones such as t-butoxycarbonyl (Boc)-D-fmk, benzyloxycarbonyl (z)-VAD-fmk, z-YVAD-fmk/chloromethylketone (CMK) or z-DEVD-fml are popular tools to study the function of caspases.[145,146] In general, peptides have poor membrane permeability and limited half-life in circulation. Moreover, halomethylketone peptide inhibitors exhibit additional drawbacks. First, the free carboxyl group of the P_1 aspartic acid can react with the fmk group thereby debilitating the inhibitor. Esterification of this P_1 carboxyl function prevents this reaction and improves the cell membrane permeability of the peptide inhibitors (Fig. 8, inset 2). In particular benzyl or t-butyl esters improve the transport across the cell membrane and they are also more resistant against esterase activity in vivo than methyl or ethyl blocks.[148] Second, in cells the fmk group can be converted into fluoroacetate, a highly toxic compound that inhibits the citric acid cycle enzyme aconitase and damages the mitochondria.[148,149] The selective disappearance of mitchondria in cells treated with apoptotic stimuli and the pan-caspase inhibitor Boc-D-fmk might be related to metabolic fluoroacetate generation.[151] The effective peptide-fmk dosage applied in animal models of apoptosis usually ranges from 1-10 μM/kg, corresponding roughly to an initial concentration in circulation of 10-100 μM, if administered intravenously. This concentration would accord with working concentrations often used in cell culture experiments in order to prevent caspase activity, but is 100-1000 fold higher compared to the effective in vitro concentrations of some of the tetrapeptide FMK inhibitors (Table 6). Variation in inhibitor uptake between different cell types may therefore contribute to diminished selectivity for a given caspase and enhance targeting of other cysteine proteases such as cathepsins.[152] In addition, these concentrations augment the risk of generating toxic metabolic byproducts. Despite these considerations, examples of successful treatment with peptide-based caspase inhibitors in animal models of pathological conditions characterized by massive apoptotic cell death or caspase-dependent inflammation have been described.[153-156]

Various pharmaceutical companies are developing new generation caspase inhibitors with improved selectivity, membrane permeability and stability compared to the peptide-based inhibitors described above. Recently, peptidomimetic caspase inhibitors have been developed such as N-indolyl-XD-fmk. The specificity of these irreversible caspase inhibitors is determined both by substitutions in the indolyl moiety and the nature of their P_2 amino acid. Some of these inhibitors exhibit inhibition constants as low as 15 nM for a particular caspase and can overcome lethality in a mouse model of fulminant hepatitis and septic shock.[157-159]

More recently, potent and selective non-peptide isatin sulfonamide caspase-3 and -7 inhibitors have been developed.[160] Interestingly, the selectivity of these non-peptide inhibitors is determined primarily by interactions with the hydrophobic S_2 substrate binding pocket in caspase-3 and -7. In cellular models of osteoarthritis and neuronal cell death these isatins could prevent both cell death and DEVD-ase activity at low micromolar concentrations whereas z-DEVD-fmk could only overcome DEVD-ase activity.[160,161]

Several cellular and viral encoded caspase inhibitors have been discovered. Orthopoxviruses (e.g., vaccinia virus) encode the caspase inhibitor CrmA (cytokine response modifier A) that structurally belongs to the serpin (serine protease inhibitor) type of protease inhibitors. Although CrmA can inhibit the serine protease granzyme B, involved in cytotoxic T cell killing,[162] its principal host cell targets are caspases and therefore CrmA is a ‚cross-class inhibitor'.[163,164] Like serpins, CrmA acts as a pseudosubstrate with the caspase recognition site LVAD,

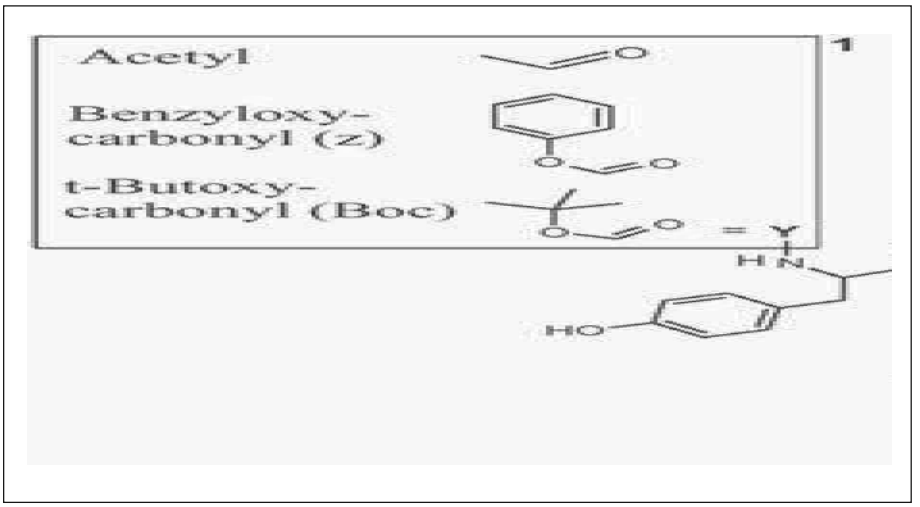


Figure 8. Schematic representation of the different functional groups in peptide-based caspase inhibitors. As an example the tetrapeptide YVAD is depicted. Inset 1: N-protective group incorporated for organic synthesis of the peptide core, usually benzyloxy-carbonyl (z), t-butyloxycarbonyl (Boc) or acetyl. These have also been selected to improve cell membrane penetration. Inset 2: esterification of the carboxyl function in the P_1 aspartyl resulting in the addition of a methyl, ethyl, t-butyl or benzyl group. Inset 3: protease inhibitory function that interacts with the catalytic cysteine such as aldehyde, halomethylketone, diazomethylketone or acyloxymethylketone. The aldehyde group allows reversible inhibition of proteases. The substituted ketone function irreversibly binds the catalytic cysteine of the caspase in a carbon-sulfur bond.

and following cleavage becomes covalently linked to the active site cysteine (see also: The catalytic mechanism). In vitro caspase inhibition studies demonstrated that CrmA has the highest selectivity for caspase-1 acting in the 10 pM range, followed by caspase-5 (K_i = 100 pM), caspase-8 (K_i = 300 pM), caspase-9 (K_i <2.3 nM) and caspase-10 (K_i = 17 nM). CrmA poorly inhibits caspase-2, -6 and group II caspases (Table 7).[165,166] By replacing the tetrapeptide pseudosubstrate LVAD in CrmA with the substrate recognition site DQMD from the promiscuous caspase inhibitor baculovirus p35 (see below), it became clear that this tetrapeptide, by large, determines the specificity of CrmA for its target caspase.[146,162] In vivo, CrmA overexpression protects cells from death receptor induced apoptosis and suppresses the release of IL-1β.[167-169] Interfering with both apoptosis and inflammation can be considered beneficial for the virus. PI-9 is a serpin found in humans that shares 54% identity with CrmA in the reactive loop.[170] This inhibitor has two close homologues in mice.[171] Though the PI-9 reactive loop contains a glutamate instead of an aspartate, PI-9 is capable of inhibiting caspases[172,173] and PI-9 blocks the caspase-mediated processing of IL-1β and IL-18 in vascular smooth muscle cells.[174] This serpin is also a granzyme B inhibitor.[175]

The baculovirus *Autographa californica nucleopolyhedrovirus* encoded p35 prevents apoptosis during infection of the lepidopteran host cell.[176] The activity of most caspases, including mammalian caspases and CED-3, can be blocked by p35 with K_i values as low as 100 pM (Table 7). Its mechanism of action is similar to the one of CrmA and has been described as covalent suicide inhibition: p35 acts as a substrate whereby the caspase cleaves after the DQMD motif and the Asp87 residue becomes covalently linked to the active site Cys by the formation of a thio-ester.[58] Additionally, upon cleavage the amino terminus of p35 repositions into the active site of the caspase, preventing solvent accessibility to the catalytic site thereby contributing to the stabilization of this thio-ester bond. Remarkably, p35 also potently blocks the non-caspase

Table 6. Inhibitor binding affinity constants (K_i) of reversible peptide inhibitors and inactivation rate constant of the irreversible inhibitor z-VAD-fmk

	z-VAD-fmk $t_{1/2}$ (sec) at 1 μM	Ac-DEVD-CHO K_i in nM)	Ac-WEHD-CHO (K_i in nM)	Ac-YVAD-CHO (K_i in nM)	Boc-IETD-CHO (K_i in nM)	Boc-AEVD-CHO (K_i in nM)
Caspase-1	2.5	15-18	0.056	0.76	<6	<12
Caspase-2	2400	1710	> 10 000	> 10 000	9400	> 10 000
Caspase-3	43	0.23-2.2	1960	> 10 000	195	42
Caspase-4	130	132	97	362	400	375
Caspase-5	5.3	205	43	163	223	438
Caspase-6	98	31	3090	> 10 000	5.6	52
Caspase-7	39	1.6	> 10 000	> 10 000	3280	425
Caspase-8	2.5	0.92	21.1	352	1.05	1.6
Caspase-9	3.9	60	508	970	108	48
Caspase-10	unknown	12	330	408	27	320

The presented data have been determined by using fluorescentic substrate conversion assays and recombinant mammalian caspases. (adapted from ref. 165)

bacterial cysteine protease gingipain K (K_i < 200 pM) but the cleavage site is shifted by 7 amino acids towards the carboxy-terminus at Lys94.[177] No mammalian homologues of p35 have been found.

Inhibition of apoptosis proteins (IAPs) were first identified by complementation experiments with p35 deficient baculoviruses.[178] Subsequently, IAPs were identified in many eukaryotes (reviewed in refs. 179,180). IAPs possess 1 to 3 baculovirus IAP repeat (BIR) motifs. Overexpression of most IAPs suppresses apoptosis by intrinsic and extrinsic apoptotic stimuli.[179] At least part of their anti-apoptotic function has been explained by their ability to directly inhibit active caspases and IAPs therefore may represent one of the last remaining hurdles before the cell is committed to die.[181,182] XIAP, with K_i < 1 nM, cIAP-1 and -2, with K_i values < 100 nM and survivin, with K_i values of 15-36 nM, specifically bind and block caspases-3 and -7.[181-185] In addition XIAP, cIAP-1 and -2 bind to pro-caspase-9 and prevent cytochrome c-induced caspase-9 processing in cytosolic extracts. At least XIAP binds to and inhibits mature caspase-9.[183] XIAP comprises 3 BIR motifs followed by a RING finger motif. The latter domain may act as an ubiquitin ligase that may target XIAP as well as the bound caspase for degradation.[186] Interestingly, during apoptosis caspases may sever XIAP between the second and the third BIR motif.[187] The amino-terminal part specifically blocks caspases-3 and -7 in vitro but is rapidly degraded in the dying cell. The C-terminal fragment, containing the BIR3 and the RING-finger, specifically restrains caspase-9 activity. Cleavage at Asp315 in caspase-9 liberates the ATPR sequence present in the linker between the large and small subunit that is recognized by the BIR3 motif in XIAP. Hence, XIAP can only hinder caspase-9 that has been activated in the apoptosome complex.[188] The pro-apoptotic activity of the mitochondrial protein Smac/DIABLO is surmised to be conducted through its affinity for IAPs. The amino-terminal AVPI motif in Smac/DIABLO competes for binding to the BIR3 of XIAP and can liberate bound caspase-9.[189,190] However, a recent study suggested the existence of an alternative apoptosis promoting mechanism for Smac/DIABLO, independent of its XIAP interaction domain.[191] Co-crystallization of caspase-3 and -7 with XIAP fragments containing BIR2 and flanking sequences revealed that the linker between the BIR1 and -2 domains of XIAP is tightly associated with the substrate binding groove in the enzyme, but in reverse orientation compared to substrates.[52,54,55] This finding may lead to the development of new synthetic caspase inhibitors.

Table 7. Inhibition of mammalian caspases by the viral inhibitors CrmA and p35

	CrmA (K_i in nM)	p35 (K_i in nM)
Caspase-1	<0.01-0.01	9.0
Caspase-2	>10 000	unknown
Caspase-3	500-1600	0.1
Caspase-4	1.1	unknown
Caspase-5	<0.1	unknown
Caspase-6	110-1300	0.4
Caspase-7	>10 000	2.0
Caspase-8	<0.3-0.95	0.5
Caspase-9	<2.3	unknown
Caspase-10	17	7.0

The dissociation constants (K_i) of the viral inhibitors CrmA and p35 for a given caspase have been determined using recombinant inhibitors and caspases in fluorometric assays. (adapted from refs. 165,166,210).

Phylogenetic Analysis of the Caspases

Alignment of different proteins of the same family can provide information on their phylogenetic relationship and in some cases indicate which family members execute similar functions. The caspase phylogenetic tree we present in Figure 9 is based on the amino acid sequence containing the p20 and p10 units and, when present, the short interdomain between them. This is referred to as p30 caspase. We have omitted the prodomains because these contain functional domains unrelated to the catalytic properties of caspases and these motifs occur in other proteins as well. A separate phylogenetic analysis of the caspase prodomains is presented in Figure 10. Only the long prodomain caspases have been included in this analysis since short prodomains do not give reliable results.

In Figure 9 a phylogenetic analysis of the enzymatic relevant domains of the known *Hydra vulgaris, Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevis, Danio rerio, Gallus gallus, Mus musculus* and *Homo sapiens* caspases shows divergence in three main clusters. It is immediately apparent that all three clusters contain functionally related members. Cluster I contains vertebrate caspases that are not part of the general apoptotic machinery though some of them might be involved in apoptosis or other forms of programmed cell death in specific tissues[15,16,192] or under pathological conditions.[82-84] All *C. elegans* caspases gather in cluster I. All vertebrate and insect apoptosis-related executioner caspases belong to cluster II. These executioner caspases have a short prodomain, with the exception of *Drosophila* STRICA. The main apoptotic initiator caspases, with a large prodomain, gather in cluster III.

The first cluster contains two branches: Iα comprising solely the three *C. elegans* caspases and Iβ consisting of caspase-1, -2, -4, -5, -11, -12 and -14, found only in vertebrates so far. The *C. elegans* CED-3 was the first gene product identified to be essential for programmed cell death.[5,6] CSP-1 and CSP-2 have a long prodomain without obvious functional motifs. In the nematode the apparent lack of short prodomain caspases is bypassed by alternative splicing of CSP-1 and -2. Protease activity has only been reported for CED-3 and CSP-1 and little is known about their possible involvement in proteolytic cascades.[193] The genome sequences of the fly and the nematode did not reveal caspase orthologues belonging to branch Iβ. Thus this group of caspases, often referred to as inflammatory caspases, may have evolved together with a complex haematopoietic system implicated in inflammatory and immune responses. The prototype of this group is caspase-1, which is mainly implicated in the processing of inflammatory cytokines such as proIL-1β and proIL-18.[20,127] It is not yet clear if this function exists in *Xenopus*, as its proIL-1β orthologue seems to lack a clear caspase-sensitive cleavage site at the

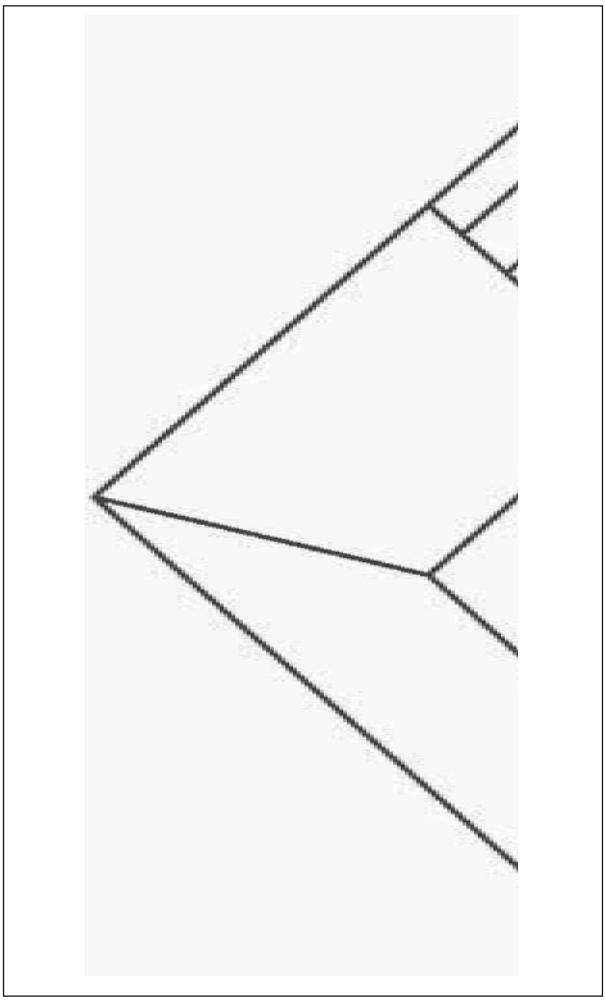


Figure 9. Phylogenetic relationship of the caspases based on their p20 and p10 domains. The inflammatory caspases (cluster I), the apoptotic executioner caspases (cluster II) and the apoptotic initiator caspases (cluster III) evolved as separate groups. The sequences were aligned using the CLUSTAL X software (gap weight = 10.00; gap length weight = 0.20) and trees visualized in TreeView.[314,315] *Hydra vulgaris* (hy), *Danio rerio* (z), *Xenopus laevis* (x), *Gallus gallus* (g), *Mus musculus* (m), *Homo sapiens* (h).

appropriate position.[194] The involvement of other group members in inflammation is supported by the inability of LPS to induce endotoxemia in both caspase-1- and caspase-11-deficient mice.[20,21,127] caspase-11 is most likely an upstream activator of caspase-1. Caspase-1 can also be activated in response to binding of bacterial compounds to Toll-like receptors, suggesting a link between the Toll receptor system and the activation of inflammatory caspases.[195] Similar to CED-3 recruitment by CED-4, the CED-4/Apaf-1-like factor Ipaf recruits and activates procaspase-1.[196] caspase-12 has been reported to be an ER stress-sensing protease.[197] Currently no human orthologue of caspase-12 has been reported. It can be argued that human caspase-4 and -5 are duplicated counterparts of murine caspase-11, especially when considering the full-length proteins including the CARD domain (see also Fig. 9). When comparing

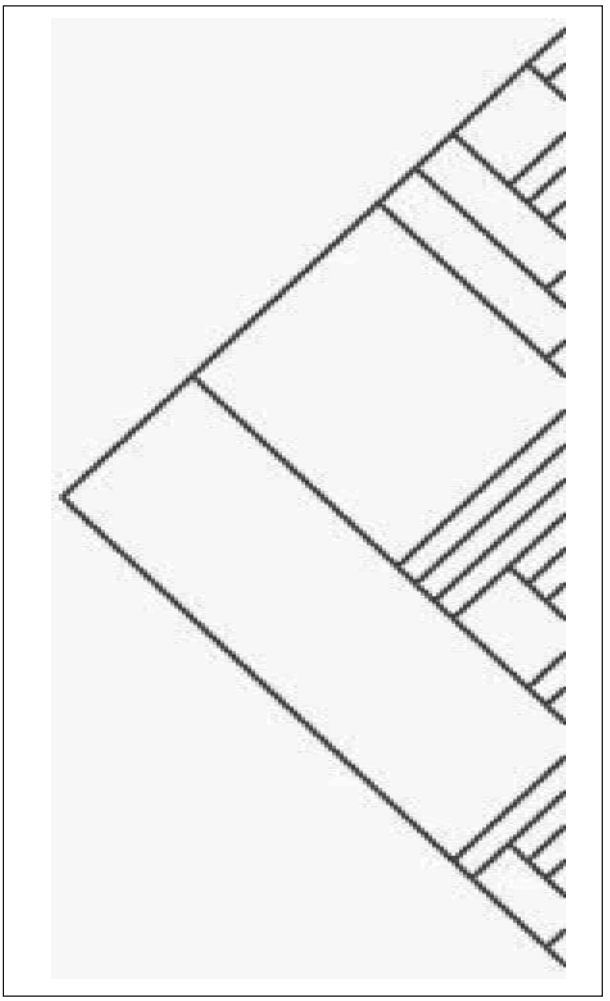


Figure 10. Phylogenetic relationship of the prodomains of caspases.This analysis reveals two separate clusters: CARD and PYRIN containing prodomains (groups A1 and A2) and the DED-containing prodomains (group B). The group A1 CARD prodomains are part of the inflammatory caspases, while group A2 CARD prodomains belong to apoptotic caspases. The sequences were aligned using the CLUSTAL X software (gap weight = 20.00; gap length weight = 0.10) and trees visualized in TreeView.[314,315] *Hydra vulgaris* (hy), *Danio rerio* (z), *Xenopus laevis* (x), *Gallus gallus* (g), *Mus musculus* (m), *Homo sapiens* (h).

human procaspases, procaspase-4 and -5 have an amino acid sequence identity of 77%, the next highest identity score being 55% (between procaspase-1 and -4). Procaspase-4 and -5 amino acid sequences are 59% and 54% identical to procaspase-11, respectively. They are only 48% and 45% identical to procaspase-12, respectively. Furthermore, caspase-4 and -11 mRNA have similar tissue distribution patterns[9] and both caspase-5 and -11 are LPS- or IFN-γ-inducible.[198] Most probably caspase-13 is the bovine orthologue of caspase-4,[199] although it was first described as a human caspase. Caspase-2 has deviated from the main branch leading to the caspase-1-likes and is implicated in neuronal cell death, with evidence for an apoptotic initiator function for this caspase.[200] Like caspase-1, caspase-2 can also mediate apoptosis of

macrophages infected with Salmonella.[201] Zebrafish caspy and caspy2 are the orthologues of mammalian caspase-1 and -2, respectively, though their prodomains contain a PYRIN motif instead of a CARD.[70] The only short prodomain caspase of this cluster is caspase-14. This caspase is mainly expressed in differentiating keratinocytes of the skin and processed in later stages of epidermal differentiation.[15,16] It might therefore contribute to the terminal differentiation of skin cells especially since the classical apoptotic cell death cascade is not activated in this form of programmed cell death.[16]

The caspases gathered in cluster II have in common that they all are activated during the execution fase of apoptosis. In a separate branch (IIα) are the vertebrate caspases-3 and -7. The latter has only been found in frog, mouse and man. In mammalians these two proteases share large substrate specificity but they are not completely redundant and it is still unclear whether both caspases are always activated under the same circumstances. This functional difference is most apparent from the difference in phenotype in the knockout animals (see Chapter 10). *Hydra* caspase-3B shares more homology with caspase-3 and -7 than with any other mammalian caspase.[10] caspase-6, found in all tetrapods represented here, belongs to the (branch of cluster II together with *Hydra ca*spase-3A and the *Drosophila* caspases DCP-1, DRICE, DECAY, DAMM and STRICA that are considered to be downstream executioner caspases in the fly based on structural and enzymatic properties. DAMM and STRICA are closely related to each other if only their p30 domain is considered. There is nevertheless a striking difference in the prodomain. DAMM has a short prodomain like all members of the cluster, whereas STRICA has a large prodomain (Fig. 1 and 10). Remarkably, *Hydra* possesses caspases that belong to both branches of cluster II, while *C. elegans* caspases gather in cluster Iα.

Cluster III, which contains all upstream initiator caspases separates into two main branches. Branch IIIα contains proteins closely related to caspase-8, in mammalians characterized by their activation in the death receptor complex, and branch IIIβ contains caspase-9 that is involved in the initiation of the intrinsic mitochondrial death pathway. Even though prodomains were not included in the alignment, caspases in this cluster are divided over two branches according to the structural domains in their prodomain (Fig. 1). All caspases in branch IIIα have two DED domains while those in branch IIIα have a single CARD. As mentioned, some caspases in cluster I also contain a CARD. Branch III(includes the upstream initiator caspase-8 in *M. musculus*, caspase-8 and caspase-10 in *X. laevis* and *H. sapiens*, and DREDD in *D. melanogaster*. DREDD seems to play a role in the innate immunity signalling pathway of the Toll receptor by proteolytically processing Relish, an NF-κB-like transcription factor.[202] In mammalians, a link between the Toll-like receptor 2 and caspase-8 through the MyD88-dependent recruitment of FADD has been proposed.[195] Since both caspase-8 and -10 have been identified in *Xenopus* and man, the apparent absence of caspase-10 in mice is remarkable. Branch IIIβ contains caspase-9 orthologues including DRONC that have conserved their function and activation mechanism during the separation of vertebrates and insects. In mammalians procaspase-9 is bound and activated by Apaf-1 while the *Drosophila* orthologue that activates DRONC is called DARK/Dapaf-1/HAC-1.[203-205] In both cases binding and activation is dependent on the presence of dATP and cytochrome c. This mechanism is different from the apoptosome complex in *C. elegans*, since Ced-4 does not contain the WD-40 repeats required for cytochrome c binding.[206]

As mentioned above human caspases and Ced-3 have also been classified in three groups based on screening of a combinatorial tetrapeptide substrate library.[38] In general this classification fits the phylogenetic relationships presented here, with the exception of caspase-2, -6, -11 and Ced-3. In other words, evolutionary related caspases usually have related substrate specificities suggesting evolutionary constraints both on the enzymes and their substrates.

The second phylogenetic tree (Fig. 10) we present here illustrates the relationship between the large prodomains found in different caspases. This tree also segregates into three groups, named A1, A2 and B. The first two groups, consisting of CARD-like prodomains, are more related to each other than to the third group, containing the DED-motif prodomains. There is

a remarkable resemblance between the phylogenetic analysis of the prodomains and that of the p30 caspases (Fig. 9 and 10). As mentioned before short prodomain caspases, except caspase-14, cluster in one group based on their p30 homology. Both phylogenetic analyses indicate a strong coevolution between the prodomains and the enzymatic part of caspases. Phylogenetic analysis of the prodomains of intermediate length of the two reported *Hydra* caspases[10] did not allow reliable classification in the three clusters.

Group A1 includes the CARD-containing prodomains of the inflammation-related caspases. Furthermore, this group harbours the PYRIN-containing prodomains of zebrafish caspy and caspy2 and the prodomain of STRICA that is related to the prodomain of *C. elegans* CSP-1. Group A2 holds the CARD-containing prodomains of the apoptosis-related caspases together with the prodomain of CSP-2. The phylogenetic positioning of the zebrafish PYRIN motifs within group A1 suggests a close relationship between PYRIN and CARD motifs both belonging to the DD superfamily. The same may hold for the prodomains of CSP-1, -2 and STRICA. The resemblance of the CARD domains of procaspase-2 an -9 seems to have functional consequences since both specifically interact with the Apaf-1/Ced-4-like proapoptotic caspase adaptor PACAP, a protein that promotes the proteolytic activation of these caspases.[207] In addition, the CARD domain of caspase-2 binds the CARD and DD-domain-containing adaptor RAIDD and could be recruited in the TNF-R1 complex.[208] However, a precise function for this interaction has not yet been found. It is remarkable that the three *Caenorhabditis* caspases, which are closely related according to their enzymatic entity (Fig. 9), have highly diverged prodomains. Group B gathers all DED-containing prodomains from caspase-8, -10 and DREDD. DED motifs are used for protein recruitment in the extrinsic cell death pathway initiated with the formation of a DISC complex.[67] Interestingly, no DED-containing caspases are found in *C. elegans*.

The sequence relationship between caspases allows us to speculate about the functional evolution caspases underwent during the separation of the different animal groups. In nematodes all described functions of the caspases are related to the apoptotic mechanisms in the worm. The two known *Hydra* caspases are probably also involved in apoptotic programs.[10] In more complex organisms caspases acquired more diverse functions. In the fly, like in vertebrates, there is a clear distinction between initiator (cluster III) and executioner caspases (cluster II). However, most caspases still seem to be involved in apoptosis, although DREDD acquired a role in innate immunity.[202] In vertebrates many caspases have a function outside the classical apoptotic machinery, such as the maturation of cytokines. These all belong to branch Iβ, indicating a common origin. Some of these caspases can play a role in specialized forms of programmed cell death, however only in certain tissues[16,192,209] or pathological conditions.[82-84]

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CHAPTER 2

Caspase Cascades in Apoptosis

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Summary

Apoptosis can be thought of as a controlled demolition process that ensures the safe dismantling of cellular structures and removal of the resulting debris such that collateral damage to surrounding tissue is minimized. To achieve this aim, the agents of destruction must be well managed in order to ensure that they are not deployed under the wrong circumstances, or in the wrong order. As with any complex and potentially hazardous task, cellular demolition is best left in the hands of specialists that will coordinate the process and minimise the likelihood that things will happen in an unpredictable and chaotic way.

Caspases: Molecular Wrecking Balls

In apoptosis, the family of aspartic acid-specific proteases known as caspases appear to be the demolition experts that are called upon to coordinate as well as execute the process.[1-4] Because of the potentially lethal nature of these enzymes, caspases are synthesised as inactive precursors (zymogens) that require limited proteolysis at internal aspartic acid residues to become fully active.[1-4] This strategy provides a very important means of keeping caspase activities in check and minimises the likelihood that cells will be inadvertently killed. However, this also presents us with a chicken and egg conundrum. Caspases have a rare substrate specificity for aspartic acid residues and also require limited processing at this very same residue in order to become activated.[1-4] While it is therefore easy to conceive how active caspases may process and activate other caspases further downstream, what activates the first (or apical) caspase in the series? As we shall discuss in more detail below, several variations on a similar theme are used to activate apical caspases in different contexts.

The common strategy used to achieve apical caspase activation appears to be the formation of aggregates containing several caspase zymogens. This is achieved by recruitment of apical caspase zymogens into complexes by specific adaptor proteins.[5-6] This appears to facilitate activation of the apical caspase because 'inactive' caspase zymogens possess low but detectable catalytic activity that is sufficient to process other caspases in circumstances where sustained close proximity between the zymogens is achieved.[5,7-9]

Although we still have a long way to go before we understand precisely how active caspases coordinate all of the events that take place in apoptosis, we do have a reasonably good understanding of how caspases become activated in many instances and some of the events that follow on from there. Here, we will briefly discuss our current understanding of how caspases become activated during divergent forms of apoptosis, how activation of a specific caspase can result in a cascade of additional caspase activation events, and how active caspases may be regulated downstream.

Caspases—Their Role in Cell Death and Cell Survival, edited by Marek Los & Henning Walczak.

Caspase Activation Pathways: Getting the Wrecking Ball Rolling

There are many contexts in which cells die by apoptosis and it is still unclear as to how caspases become activated in all of these situations. However, many stimuli that promote apoptosis appear to engage the caspases in one of three main ways, which we will now briefly discuss.

Mitochondrial Stress: The Apoptosome Pathway

A large body of evidence suggests that mitochondria act as important conduits for signals associated with cell damage and that many key regulators of apoptosis promote or inhibit the loss of mitochondrial integrity.[10-12] In this pathway, divergent cellular stresses such as DNA damage, heat shock, oxidative stress and many other forms of cellular damage, result in caspase activation through the release of cytochrome c from the mitochondrial intermembrane space, into the cytoplasm.[10-12] Efflux of cytochrome c from mitochondria drives the assembly of a high molecular weight caspase-activating complex in the cytoplasm—termed the mitochondrial apoptosome.[10-14] In the presence of cytochrome c and dATP, Apaf-1, a major component of the apoptosome, recruits and activates caspase-9 which then propagates a cascade of further caspase activation events downstream.[10-16] In this context, caspase-9 is thought to become activated due to the increase in local concentration of caspase-9 zymogens through recruitment into the apoptosome, and also due to the fact that association of caspase-9 with Apaf-1 may force the caspase active site into an active configuration.[17,18]

Death Receptor Engagement

Certain members of the TNF receptor superfamily share a distinct domain within their cytoplasmic tails that has been termed the 'death domain'.[6] Upon engagement of these receptors with their extracellular ligands (such as FasL/CD95L, TNF, TRAIL), the receptor death domains recruit adaptor proteins (such as FADD and TRADD) that can directly recruit caspases into the receptor complex.[6] In most cases, the caspase that is recruited into death receptor signalling complexes appears to be caspase-8, however a close relative of the latter (caspase-10) may be able to substitute in certain instances.[19,20] As in the apoptosome context, recruitment of caspase-8 into death receptor complexes is thought to drive caspase activation through increasing the proximity of partially active caspase-8 zymogens, which facilitates complete processing of these proteases.[21,22]

Granzyme B-Induced Caspase Activation

The third major pathway to caspase activation that has been established to date is initiated by the constituents of cytotoxic granules that are released by cytotoxic T cells (CTL) and Natural Killer (NK) cells upon encounter with transformed or virally-infected target cells.[23] Although CTL and NK granules contain a rich mixture of potentially cytotoxic enzymes, granzyme B seems to be a particularly important component of these granules.[23] Granzyme B is a serine protease that, in common with caspases, shares a similar preference for aspartic acid residues within its target substrates.[24-26] Thus, one way in which granzyme B can engage the demolition machinery upon entry into the target cell, is through direct proteolysis of certain caspase zymogens such as caspase–3 and –8.[24-26]

Caspase Activation Cascades: A Trickle Becomes a Flood

We have briefly described the major pathways to apical caspase activation that are currently well defined. Undoubtedly, there are likely to be other routes to apical caspase activation that remain to be discovered. Some additional pathways have been proposed that have yet to be fully substantiated or defined—p28Bap31-mediated activation of caspase-8 at the endoplasmic reticulum, or activation of caspase-12 within the context of ER-associated stress for example.[27-29] However, for the remainder of this discussion we will confine our comments to the three major pathways described above.

Upon activation of the apical caspases in the mitochondrial, death receptor or granzyme B pathways, what exactly happens next? In order to demolish the cell and achieve all of the desirable endpoints of apoptosis, several caspases become activated—presumably to deal with specific aspects of the demolition job. It is often speculated that caspase–3, -6 and –7 are the central effector (or downstream) caspases that perform most, if not all, of the proteolytic degradation that produces the apoptotic phenotype. However, the latter view remains unproven, as it is currently unclear as to the relative contributions that each caspase makes to cellular demolition. Moreover, it is also frequently assumed that caspase–3, -6 and –7 are somehow functionally equivalent and that loss of one can be compensated for by the others. In contradiction of this view, murine knockout studies actually suggest that significant redundancy does not exist between the mammalian caspases.[30] Mice deficient in either caspase-3, -7, -8 or -9 are all severely compromised in apoptosis regulation at various stages of development and die prematurely as a result.[30-34] Interestingly, the primary exception to this rule may be caspase-6 null mice, as these have reportedly few, if any, defects in apoptosis regulation and are viable.[30] However, it is also rather surprising how relatively subtle that some of the other caspase knockout phenotypes are. Irrespective of the relative contributions that effector caspases make to the demolition of the cell, how are these caspases activated by the apical caspases to ensure that the correct repertoire of caspases is activated downstream?

To answer this question requires a fairly painstaking analysis of systems that are lacking in one or more of the caspases to determine the precise sequence of events involved. This has been achieved largely through analysis of cells deficient in a particular caspase, or cell-free extracts depleted of specific caspases.[16,30,35] Use of recombinant caspases to explore the range of other caspases that an individual caspase can process has also been useful.[36,37] However, the latter approach can be somewhat misleading due to the use of recombinant caspases at concentrations that are not physiologically relevant. Moreover, due to the lack of caspase inhibitors of a suitably narrow specificity, pharmacological-based approaches to unravelling caspases activation cascades have proved largely equivocal. However, through a combination of these approaches, a reasonably detailed picture has emerged of the order of caspase activation events in each of the three pathways described above.

Coordinating the Demolition: Foremen and Laborers

Examination of the order of caspase activation events during various routes to apoptosis have yielded caspase activation cascades that sit somewhat uncomfortably with some of the dogma in the field that tends to have its roots more in prediction than experiment. For example, before much was known concerning the role of the various caspases in apoptosis, predictions were made that apical caspases would be those that possessed long prodomains.[4,38] The logic behind this prediction was that long caspase prodomains would be required to enable recruitment of apical caspases into activation complexes as described above.[4,38] Following a similar line of reasoning, it was also proposed that downstream caspases were likely to be those with short or absent prodomains and that these would play a role in substrate proteolysis rather than in initiation or propagation of caspase cascades. On this basis, the notion that caspase-2, -8, -9 and –10 were apical caspases and that caspase–3, -6 and –7 were effector caspases was firmly established. Other criteria, such as caspase preferences for synthetic substrates, can also be used to support this model but some difficulties do arise immediately (e.g., caspase-2 shares a similar substrate preference with caspase–3 and –7 and caspase-6 shares a similar substrate preference with caspase-8).[2,39] While there is no doubt that this model is a very useful starting point, it is also clear that it is a crude approximation of the real situation and that caspases cannot be slotted neatly into either category (i.e. exclusively apical or effector). As will be seen, it is now clear that certain caspases may play dual roles—as initiators or effectors—with others playing more dominant roles in either phase of the cell death programme.

The Cytochrome c / Apaf-1-Initiated Caspase Cascade

It is now apparent from several lines of evidence, both from in vitro and murine knockout experiments, that caspase-9 is the sole apical protease in the Apaf-1 pathway. Indeed, the absence of caspase-9 abrogates the processing of all other caspases in the cytochrome c-inducible caspase activation system.[13,16,33,34] It is also clear from many studies that multiple caspases, including caspase-2, -3, -6, -7, -8 and -10, become activated in response to stimuli that engage the apoptosome pathway.[13,15,16,40-43] These data strongly support the notion that complex caspase activation cascades are harnessed in apoptotic cells, possibly to coordinate and promote the timely completion of the death program.

Evidence from several groups suggests that there are several tiers of caspase activation events (see figure 1) resulting from caspase-9 activation within the apoptosome complex.[16,42,43] Using cell-free extracts devoid of specific caspases, work performed in our laboratory has revealed that caspase-9 is directly responsible for the activation of caspase-3 and –7 downstream (Fig. 1).[16] This is consistent with gel filtration chromatography experiments that demonstrate the co-elution of these enzymes, particularly caspase-3, with the apoptosome complex.[44-45] Interestingly, in contrast to predictions from studies that have employed recombinant active caspase-7, immunodepletion of the latter had no effect on the activation of caspase-2, -3, -6, -8 and -10 at endogenous levels.[16,37] However, caspase-3 depletion halted the apoptosome-induced processing of caspase–2, –6, -8 and –10, suggesting that caspase-3 is directly or indirectly responsible for the activation of these enzymes in this context.[16-42] Strikingly, caspase–8 and –10 fail to become activated in the apoptosome pathway in the absence of caspase-6, suggesting that the latter short prodomain enzyme is actually responsible for processing these long prodomain caspases in the cascade.[16] In summary, as indicated in the scheme in figure 1, caspase-9 initiates the proteolytic cascade by simultaneously activating caspase-3 and -7. While caspase-7 does not appear to be required for any further caspase activation event thusfar identified, caspase-3 then further propagates the cascade by activating caspase-2 and -6.[16] In addition, several groups have shown that caspase-3 participates in a positive feedback amplification loop to promote further processing of caspase-9.[15-16] In the final stage of this cascade, caspase-6 catalyzes the activation of caspase-8 and -10 (Fig. 1).[16]

Initiator and Executioner Caspases: Blurring the Distinctions

These data are illuminating from several perspectives. Firstly, contrary to expectations, it is apparent that these caspases are not functionally redundant, at least with respect to amplification of the Apaf-1-initiated caspase cascade. This is demonstrated, for example, by the inability of caspase-7 to activate the caspases that would normally be cleaved by caspase-3, or indeed because neither caspase-3 nor caspase-7 can substitute for the loss of caspase-6, to facilitate the activation of caspases-8 and -10.[16] Furthermore, studies that have examined the ability of endogenous levels of executioner caspases to cleave a repertoire of substrates revealed that caspase-3 is the major executioner enzyme, with caspase-6 and -7 being largely incapable of substituting for the loss of caspase-3, at least with respect to the range of substrates examined.[35,46] Moreover, the brain abnormalities associated with decreased apoptosis in caspase-3-null mice again support the notion of a lack of redundancy amongst the short prodomain caspases, at least within certain tissues.[30,31]

Secondly, these data further challenge the notion that caspases can be subdivided into initiator and effector enzymes, based purely on their prodomain lengths, or their preference for synthetic tetrapeptide-based substrates.[2,4,38,39] One possibility is that, dependent on the context, initiator and effector enzymes can trade places, for example, with the so-called effector enzymes (such as caspase-3 and -6) calling the shots by activating the long prodomain caspases -2, -8 and –10 within the apoptosome pathway.[16,42] As another example of this, caspase-3 plays the role of an amplifying enzyme in the apoptosome cascade via a feedback mechanism, either by directly cleaving caspase-9 at aspartate 330, or by inducing further release of cytochrome c via caspase-3-catalyzed Bid cleavage.[15,42,47,48] It is thus tempting to speculate that caspases can

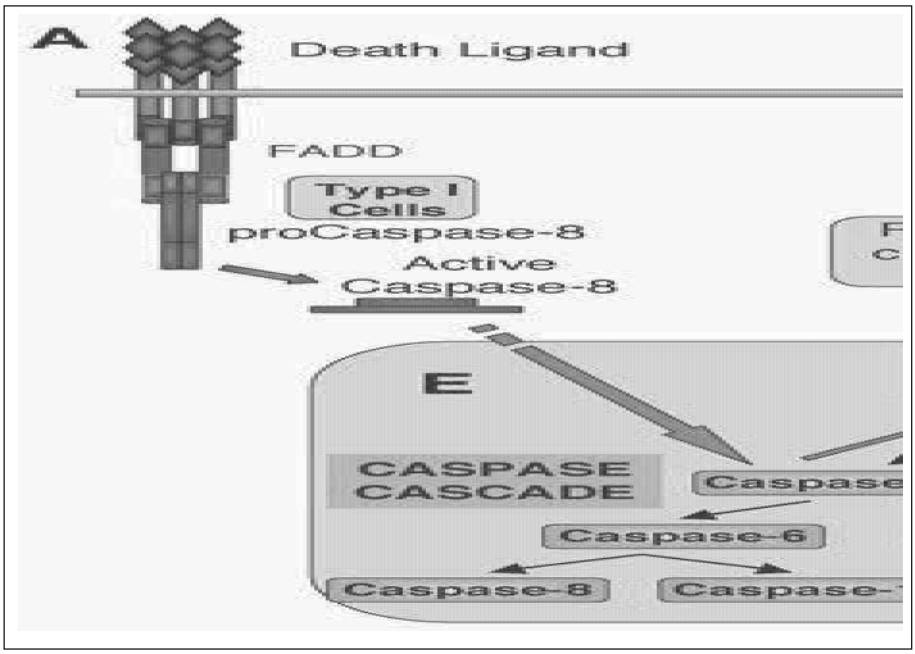

Figure 1. Diagram illustrating how several pro-apoptotic pathways integrate into the caspase cascade. (A) In type I cells illustrated on the left hand side, death receptor ligation promotes the recruitment and activation of caspase-8 within the DISC complex. Sufficient active caspase-8 is generated to enable direct access to the caspase cascade, via activation of caspase-3. Active caspase-3 further contributes to the cascade by processing caspases-9, -2 and –6. (B) Alternatively, in type II cells, access to the caspase cascade is achieved via cleavage of Bid by caspase-8. The c-terminus of Bid (tBid) acts as a mitochondrial death ligand, facilitating the efflux of cytochrome c from the intermembrane space. (C) The presence of cytochrome c within the cytoplasm provokes the assembly of the Apaf-1 apoptosome complex, leading to the activation of caspase-9. (D) The release of CTL granules by a cytotoxic lymphocyte (CTL) facilitates access of granzyme B to the cytoplasm. Within the cytoplasm, Granzyme B processes Bid into a c-terminal truncation called gtBid that provokes the release of cytochrome c, thereby engaging the apoptosome pathway. Alternatively, granzyme B can directly access the caspase cascade by processing caspases-3 and –8. (E) Schematic representation of the hierarchical activation of caspases within the cascade. Active caspase-9 directly processes caspases-3 and –7. In turn, caspase-3 provides a caspase-9-activating amplification loop. Active caspase-3 also processes caspases-2 and caspase-6. Finally, active caspase-6 processes caspases –8 and –10.

serve as both proximal or distal components within the cascade, depending on the origin and nature of the stimulus.

However, it is important to point out the caveat that the role of the so-called initiator caspases downstream in the apoptosome cascade, has yet to be ascertained. Moreover, caspase-2 null mice are essentially phenotypically normal with respect to stimuli that engage the apoptosome pathway, and a study has reported that caspase-10 is not activated in cells undergoing apoptosome-dependent apoptosis.[49,50] Although numerous studies have reported the activation of caspase-8 in death receptor-independent settings, it is currently unclear whether this represents an essential component (for example, responsible for accelerating the execution phase) or simply constitutes a late bystander event within the apoptosome pathway.[16,42,50,51] Indeed, embryonic fibroblasts from caspase-8-null mice can still die in response to stimuli that engage the apoptosome pathway.[32] Notwithstanding these caveats, caspase-8 processing is clearly impaired in UV-irradiated caspase-$9^{-/-}$ embryonic stem cells as well as in caspase-$9^{-/-}$ thymocytes,

thus demonstrating that the activation of caspase-8 is a genuine feature of the apoptosome cascade, as provoked by physiological stimuli.[34]

The Roads to Ruin Beyond the DISC

As discussed previously, the activation of caspase-8 in death receptor pathways is achieved by enforced aggregation of the zymogen form of the enzyme.[21,22] In this scenario, the pro-apoptotic stimulus (in the form of extracellular death ligands) is transduced by a bipartite caspase adaptor protein called FADD, that acts as a caspase-8-aggregating scaffold within intracellular death receptor complexes.[6] Downstream of caspase-8 activation within the DISC complex, there are potentially two alternative pathways to destruction that a cell may embark upon.[52] Both pathways ultimately make use of the same repertoire of caspases to trigger the inexorable demolition of the cell. Critically however, the ability of caspase-8 to generate sufficient quantities of active caspase-3 to propagate the apoptotic stimulus determines the route to death that the cell will embark upon.

Type I Cells: By-Passing the Apoptosome

In many cell types, stimulation of death receptors results in the activation of sufficient caspase-8 to propagate the death signal without requiring mitochondrial participation.[52,53] In these cells, death receptor-induced death cannot be blocked by the anti-apoptotic molecule Bcl-2.[53] As outlined in figure 1, in this scenario, mature caspase-8 cleaves sufficient caspase-3 to enable the apoptotic signal to access the caspase cascade directly. Active caspase-3 can potentially access—and thus activate—the cascade at several points. Starting at the apex of the cascade, caspase-3 can cleave caspase-9 (at aspartate 330 between the large and small subunits), thus setting the demolition ball in motion.[15] Alternatively, caspase-3 can also feed into the cascade further downstream by directly processing caspases –2 and –6, again starting the demolition process.[16]

Type II Cells: Death by Igniting the Apoptosome

In contrast to the situation outlined above, certain cell types including hepatocytes fail to activate sufficient amounts of caspase-8 within the DISC to properly guarantee the destruction of the cell.[52-54] In these cell types, the pro-apoptotic signal must be boosted via engagement of the cytochrome c/Apaf-1 pathway, with the result that death receptor-dependent death is Bcl-2-sensitive in these cells.[53] As illustrated in figure 1, cells that use the apoptosome as a means of enhancing the death signal ultilize Bid as a cytochrome c and Smac/DIABLO-releasing factor.[55-58] In this pathway, the reduced level of caspase-8 activated by the DISC is nonetheless sufficient to fuel the processing of the caspase-8 substrate Bid.[55,56] Upon cleavage, the active ~15 kD C-terminal fragment of Bid, tBid behaves as a death ligand for molecules such as Bax or Bak, that in turn, provoke the efflux of cytochrome c from mitochondria (Fig. 1).[55,56,59] This triggers apoptosome assembly and enables the amplification of caspase activity via the caspase-9 cascade (Fig. 1). Additionally, the release of Smac/DIABLO from mitochondria further boosts the strength of the demolition signal, by overcoming the ability of XIAP and other IAP molecules to inhibit active caspases.[11,57,58,60]

The Granzyme B-Initiated Caspase Cascade

As mentioned earlier, CTL and NK cells induce apoptosis in virally-infected and tumour cell targets through the concerted action of effector molecules contained in cytolytic granules that engage the death pathway.[23] These granules contain components such as perforin, a pore-forming protein that is likely to facilitate the entry of the other granule components into target cells, and granzyme B, a serine protease that cleaves following aspartate residues, suggesting a mechanism for cytotoxic lymphocyte-initiated caspase activation.[24,26] The first substrate identified for granzyme B was found to be caspase-3.[24,26] Several additional caspases have now been identified to serve as granzyme B substrates in vitro, suggesting that granzyme B induces

apoptosis by triggering the activation of multiple caspases.[61] However, only caspases-3 and –8 have been demonstrated to be direct substrates for granzyme B in intact cells to date, indicating that the activation of these caspases are critical events during CTL/NK granule-mediated killing.[62,63]

Many viruses encode proteins that specifically interfere with caspases, such as the poxvirus inhibitor CrmA, which has been used extensively for elucidating apoptosis pathways.[64] It is understandable therefore, that redundancy is built into the CTL/NK granule-induced cell death pathway as a counter-measure to viral caspase inhibitors. As evidence of this, granzyme B has also been demonstrated to cleave Bid into an active product, gtBid, which translocates to mitochondria and provokes cytochrome c release.[65-67]

Moreover, a study by Green and colleagues recently determined the necessity for cytochrome c release during CTL-granule mediated killing by demonstrating that Bcl-2 significantly inhibits granzyme B-induced apoptosis.[68] Kinetic and rate-limiting comparisons of granzyme-B mediated proteolysis of Bid, caspase-8 and -3 in vitro suggest that Bid is the preferred granzyme B substrate.[68] It appears therefore that, although granzyme B can activate caspases directly, the primary route to apoptosis under limiting conditions may be via Bid cleavage.[68] The resulting Bid-induced cytochrome c release from mitochondria could then trigger apoptosome formation and the caspase-9-induced caspase cascade (Fig. 1). Alternatively, if caspases are inhibited downstream through virally encoded caspase inhibitors, death through mitochondrial dysfunction, resulting in a necrotic phenotype, may occur.

Lonely Caspase Seeks Others for Meaningful Cascade

Besides the caspase activation cascades discussed thus far, additional caspase activation cascades are likely to exist. One of the areas in which our knowledge concerning the route to caspase activation is glaringly patchy concerns the first mammalian caspase to be identified, caspase-1. Caspase-1 is well established to play a required role in IL-1β and IL-18 processing in the context of inflammation. *CASP-1* null mice fail to produce either IL-1α or IL-1β upon challenge with lipopolysaccharide (LPS).[69] LPS is a component of gram-negative bacteria that plays an important role in instigating innate immune responses through binding to the Toll-like receptor molecule, TLR4.[70] However, although certain components of the TLR4 signaling pathway have been elucidated, it remains entirely unclear as to how caspase-1 activation is achieved in this context. It is possible that caspase-1 is both initiator and effector caspase in this pathway and that no other caspase is required for caspase-1 activation. However, this still leaves the problem of how caspase-1 becomes activated upon LPS-stimulation unresolved. Furthermore, caspase-11 null mice share a very similar defect in IL-1 processing/production with caspase-1 null mice, suggesting that caspase-11 may act upstream of caspase-1 in this context (since caspase-11 cannot process IL-1β directly).[71] A further complication arises due to the fact that proteolytically processed caspase-1 has not been observed in cells that are actively producing IL-1β.[72] This suggests that caspase-1 may not require proteolytic processing in order to become activated, or that caspase-1 activation is stringently regulated such that concentrations of processed caspase-1 enzyme are produced that are below the detection limits of conventional detection methods.

Human caspases –4 and –5 have also been implicated in inflammatory pathways, largely through their sequence similarity to caspase-1. However, other than the fact that caspase-5 is upregulated upon LPS-treatment, little is known concerning their role in apoptosis or inflammation, or indeed, what their natural substrates are.[73]

Another long prodomain caspase that remains enigmatic is caspase-2. Caspase-2 is highly expressed during embryogenesis when extensive cell death occurs, suggesting an important role for this caspase during development.[74] caspase-2 interacts with RAIDD, an adaptor protein containing a death domain and a caspase recruitment domain (CARD).[75,76] The death domain of RAIDD can bind to a homologous region within RIP, a serine/threonine kinase that acts as a signaling component of the TNF receptor pathway.[77] While these data suggest a role for

caspase-2/RAIDD as signaling components in the TNF pathway, it is important to note that caspase-2 null embryonic fibroblasts remain sensitive to TNFα-associated apoptosis.[49] Interestingly, studies using caspase-$2^{-/-}$ mice suggest that this caspase may be important in regulating germ-cell apoptosis in the ovary.[49] However, these mice develop normally and are devoid of obvious phenotypic abnormalities.[49] Thus, while caspase-2 is unlikely to play a global role in regulating apoptosis, the specific role of this caspase in certain tissues remains to be fully elucidated.

Concluding Remarks

It is now apparent from emerging data that it is no longer feasible simply to subdivide caspases into either initiator or effector caspases on the basis of caspase domain structure, or from combinatorial peptide screening data alone. Rather, as has been illustrated, it appears that the roles of initiator and effector caspases within signaling cascades are somewhat interchangeable, dependent on the context and origin of the pro-apoptotic stimulus. As further data accumulates regarding the role that individual caspases play downstream in the demolition program, it will be interesting to determine whether the majority of caspase substrates are the preserve of the traditional effector enzymes, or whether the so-called initiator enzymes are also entrusted with cleaving a critical subset of protein targets.

Nonetheless, the original dogma—that the long prodomain caspases act at the apex of a signaling cascade, whereas the short prodomain enzymes function as primarily as effector caspases—still remains an invaluable hypothesis when attempting to implicate hitherto uncharacterized caspase molecules within their respective signalling pathway(s). Thus, based on the prediction that the long prodomain caspases (such as caspase-8 and –9) are activated by recruitment into oligomeric complexes by specific adaptor molecules, it will be interesting to determine whether CARD-containing molecules such as caspases –1, -4 and –5 also possess binding partners that recruit and activate them. Within this context, it will be interesting to explore the contribution that hitherto unidentified caspase signalling cascades make to the regulation of apoptosis and indeed, to the modulation of pro-inflammatory responses.

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CHAPTER 3

Caspase Activation at the TNF-R Family Members Death Inducing Signaling Complexes (DISCs)

Martin R. Sprick and Henning Walczak

During the life span of a multicellular organism most cells die at a certain point. The decision to die serves the common purpose of all cells in such organisms which is self propagation. Multicellular organisms have evolved a system where a single cell either by itself decides to die or where specialized other cells, so-called effector cells, make this decision for a certain cell. They do so when the cell in question is recognized as one that could potentially interfere with the above mentioned common goal. Examples for such potentially dangerous cells are plentiful and include e.g., virally or oncogenically transformed cells. These cells are recognized as such and are therefore killed by the effector cells of immune system. For many years it was unknown how these cells kill their targets and it was only after decades of research that we finally got a glimpse. We now know that—besides other mechanisms— programmed cell death induced by certain members of the tumor necrosis factor (TNF) family is crucial for these targeted cell deletion mechanisms.

Identification of "Tumor Necrosis Factor"

The first description of an activity which was much later attributed to a molecule named TNF for "Tumor Necrosis Factor" dates back to the 19th century. In 1868 Brunes observed the spontaneous regression of well-established tumors in some of his cancer patients when they suffered acute bacterial infection. A quarter of a century later it was shown that life bacteria were in fact not needed for this anti-tumor effect to occur but it was not until the middle of the 20th century that a substance capable of inducing hemorrhagic tumor necrosis in tumor-bearing mice was isolated from cell-free extracts obtained from gram-negative bacteria. This substance turned out to be lipopolysaccharide (LPS), a major constituent of the cell wall of gram-negative bacteria. However, LPS (also called endotoxin) did not kill the cells but rather acted as the inducer of a factor that was produced by and subsequently found in the serum of LPS-treated animals. This factor was then named tumor necrosis factor (TNF). The race for the identification and cloning of TNF ended with a surprise. LPS injection into animals had been shown to induce wasting syndrome (also called cachexia), a disease highly similar to the wasting that is often observed in terminally ill cancer patient. The cloning of a factor that had been shown to induce lymphocyte killing (coined lymphotoxin, LT), of TNF itself and of the cachexia-inducing factor (cachectin) resulted in the almost simultaneous identification and cloning of LT (for the LPS-induced lymphocyte-derived cell death-inducing factor),[1] as well as human TNF (for the LPS-induced macrophage-derived tumor necrosis),[2] and murine TNF (for the LPS-induced cachexia-inducing factor in mice).[3] Surprisingly, LT and TNF shared considerable sequence

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homology, thus, they were related proteins. Therefore, two activities namely hemorrhagic tumor necrosis and cachexia, turned out to be mediated by only one molecule, by TNF. In addition, not only one death-inducing factor but with TNF (then named TNF-α) and LT (then called TNF-β) in fact two different but related ones were identified. With the identification of these two non-identical twins we got a first glimpse at what later turned out to become a complex network of interacting ligand members of the TNF super family (TNF-SF) andreceptors that are all members of the TNF receptor super family (TNFR-SF).

The Death Ligands

Most members of the TNF-SF adopt a typical "jellyroll" sandwich topology. TNF family ligands are mainly expressed as transmembrane proteins and in some cases soluble ligands are generated by proteolytic cleavage of the transmembrane precursor. The crystal structures of the two TNF family members in complex with soluble receptor constructs led to the elucidation of a trimeric structure. Three ligand monomers forms a peculiar structure that, when bound to its receptors on the cell surface, looks like a pyramid that stands on its tip. When this ligand trimer binds its receptors on the cell surface three receptors are cross-linked and their intracellular domains adopt a conformation that allows for the recruitment of intracellular adapters and effectors which fulfill the dead. In addition other biochemical evidence like gel filtration experiments support a trimeric structure of TNF family ligands in solution.

However, additional molecular assemblies have been reported. Soluble TRAIL has been reported to exist in a stable dimeric form that is inactive. This form is the result of interchain disulfide bridge formation which probably occurs under expression conditions favoring disulfide bond formation between the free cysteine observed, in the TRAIL monomer instead of the chelation/coordination of a zinc atom by all three TRAIL monomers in the complex. So far, TRAIL is the only known TNF family ligand which coordinates a metal ion, necessary for its complete activity. Dependent on pH, crystals of TNF contained dimers which oriented head to tail. TALL, a ligand for BAFF-R, a non death-receptor member of the TNFR family, has been reported to form a large virus like assembly consisting of multiple trimers at pH 7.5. At lower pH values, only single trimers were observed.[4]

The Death Receptors

The TNFR-SF consists of a large group of mostly membrane-associated receptors with 29 family members. The extracellular domains of these receptors are characterized by the presence of up to 6 copies of a typical fold, the cysteine rich domain (CRD), in the extracellular part.[5] These receptors regulate diverse functions in development, tissue homeostasis and responses of the immune responce.[6] A subgroup of these receptors, the death receptors, can trigger cell death when engaged by their respective ligand. This ability has first been discovered for TNF-R1 and CD95 (APO-1/Fas). These two receptors share an intracellular motif, the death domain (DD).[7] Soon after the recognition of the death domain, it was established that the ability to transmit cell death relies on the presence of an intact DD. This was shown by studies employing site-directed mutagenesis[7] and by the identification of naturally occurring mutations in this domain.[8,9] Later, additional TNF-R family members which contain a DD were identified. These are TRAIL-R1 (DR4), TRAIL-R2 (APO-2, DR5, KILLER, TRICK), TRAMP (APO-3, DR3, Wsl1, LARD, TR3, TNFRSF12), DR6, the p75 NGFR and EDAR. However, not all of these DD-containing receptors when triggered, seem capable of efficiently signaling cell death. Of the above mentioned receptors, the ability to induce apoptosis is well established for CD95, TNF-R1, TRAIL-R1 and TRAIL-R2. The remaining DD—containing members seem to be primarily involved in activating other signaling pathways like the NF-κB, JNK or MAPK pathways when triggered.

Biochemistry of TNF and TNFR Interactions

TNF can bind two receptors, TNF-R1 (p55TNF-R) and TNF-R2 (p75 TNF-R). Only TNF-R1 contains a death domain and is capable of potently inducing cell death when triggered, although the ability to induce cell death has also been described for TNF-R2.[10-13] The protein complex involved in TNF-R2-mediated apoptosis has been proposed to involve TRAF-2, RIP and FADD.[13] In addition to their principal ligand TNF, TNF-R1 and TNF-R2 can also be triggered by LTα which consists of homotrimeric LT (LTα3). Additional complexity arises from the fact that a second membrane bound ligand, LTβ can form a heteromeric complex with LTα, yielding $LT\alpha_1\beta_2$ which neither binds to TNF-R1 nor to TNF-R2 but instead to a third receptor named LTβ-R. A complex interplay between these three different receptors and ligands seems to play an important role in the development of secondary lymphoid tissues.[14]

Identification of CD95 (APO-1/Fas) Apoptosis System

In 1976 Murphy and Roths described a novel single gene-mutated mouse model for massive "lymphoproliferation" (*lpr*) with immune complex disease.[15] Eight years later the same group discovered another mutation that caused a virtually identical phenotype. This mutant strain of mice was named *gld* for "generalized lymphoproliferative disease."[16] Like *lpr*, also gld is autosomal and recessive, resulting in autoimmunity and lympho-"proliferation." The phenotype of *lpr* and *gld* mice was virtually indistinguishable when they were compared on the same genetic background. These observations suggested that the underlying mutations affect genes that encode proteins which act in a pathway that seemed to be important for inhibition of lymphoproliferation. Interestingly, *lpr* and *gld* were shown to be non-allelic and did not complement each other in double heterozygotes.[16] In 1990 a newly identified mutant with the same phenotype as *lpr* mice was described.[17] The newly identified gene turned out to be allelic with lpr and was named lpr^{cg} for "complementing *gld*" since double heterozygous (i.e., lpr^{cg}/+, gld/+) mice also showed the "*lpr*" phenotype whereas the non-complementing "normal *lpr*" in *lpr*/+, *gld*/+ mice did not.[17] The phenotype of these different mutant mouse strains (*lpr, gld,* lpr^{cg}, and the lpr^{cg}/+, *gld*/+ double heterozygotes) was indeed striking.[18] On the genetic background of e.g., the inbred MRL mouse strain a 200-fold increase in cell number compared to wild type MRL controls is commonly observed in MRL *lpr*. The mice have massive autoimmunity and 50 % of the animals die at 5 months of age due to glomerulonephritis.[19] On the C57BL/6 background, however, the disease is much milder and only an indolent autoimmune disease without arthritis develops. Surprisingly, the vast majority of the accumulated cells were not cycling, despite the name "lymphoproliferation". Thus it seemed that "lpr" is in fact a lympho-accumulative disorder. However, the reason for this accumulation of mainly aberrant T cells remained elusive.

In 1989 two groups reported on the identification of monoclonal antibodies which actively induced apoptosis in target cells. One of the antibodies supposedly bound to a cell surface protein of about 200 kD and was shown to be co-downregulated with TNF-R1[20] while the other antibody was shown to bind to a receptor with a molecular weight of about 50 kD that was thought to induce apoptosis upon crosslinking.[21] Moreso, in the second paper it was shown for the first time that the concept of direct apoptosis induction in cancer cells in order to delete a tumor was feasible. Given the mentioned initial biochemical characterizations it was surprising to find out upon cloning of the respective antigens that both antibodies reacted with the very same receptor on the cell surface, namely the 48 kD transmembrane receptor which was initially called Fas [22] and APO-1 [23] and is now called CD95. Shortly after the discovery of the human CD95 antigen it was shown in a landmark paper that the murine gene that encoded this receptor was in fact mutated in *lpr* mice.[24] At that time Allen et al.[25] had suggested in a very elegant study employing bone marrow transplantations that *lpr* and *gld* are mutations that

encode an interacting pair of molecules where each one of these molecules is expressed on a different cell population. After the cloning of murine CD95 and the exciting finding that the *lpr* mutation affected the gene encoding this novel member of the TNFR-SF it was very likely that the ligand for CD95 would be an apoptosis-inducing member of the TNF family of ligands and that a mutation in the gene that encodes this ligand was causative for *gld*. And in fact following the identification of a CTL hybridoma which would be capable of killing CD95-positive but not CD95-negative cells [26] the ligand for CD95 was cloned from this cell line.[27] After the identification of the molecular components, namely CD95 and CD95L (FasL/APO-1L) it was clear that we already knew quite a bit about the biology of these molecules due to about a decade of intensive research on *lpr* and *gld* mice. However, the physiological function of the proteins encoded by the *lpr* and *gld* genes had remained elusive. With the finding that an apoptosis-inducing receptor/ligand system was affected the pathology that was manifested in this massive lympho-accumulation became explainable and it was finally shown that autocrine T cell suicide upon T cell activation, also called activation-induced cell death (AICD), was due to the interaction of CD95 with its ligand.[28-31] Shortly after that, another important physiological role for the CD95 system was discovered when it was shown that immune privilege in eye and testis was due to CD95L expression.[32,33] In addition, a pathological role was then attributed to CD95L expression on certain tumors which had been shown to be immune privileged as well.[34] In the following years the CD95 system has been shown to be involved in a plethora of physiological and patho-physiological situations. However, the desired therapeutic activity, namely the induction of apoptosis in tumor cells, longed for since the identification of the tumor-necrotizing properties of the bacterial extracts discovered by Brunes in 1868, was again hindered by the finding that agonists of the CD95 receptor, both antibodies and CD95L, were highly toxic upon systemic administration.

Identification of the TRAIL (APO-2L) Apoptosis System

In 1995 and 1996 Wiley et al and Ashkenazi et al independently identified a novel member of the TNF-SF solely due to its homology to the CD95 ligand.[35,36] In fact the expressed sequence tag (EST) that resulted in the identification of this novel ligand had already been labeled as "homologous to Fas ligand". Therefore it was not surprising to see that this new protein which was named TRAIL for "TNF-related apoptosis inducing ligand" and APO-2L for its close homology to APO-1L (CD95L/FasL), respectively, was capable of inducing apoptosis. Interestingly, however, TRAIL induced apoptosis in many different tumor cell lines but not in the majority of the normal cell types that were tested. This property of TRAIL prompted the testing of its antitumor potential in vivo. It was shown that TRAIL (APO-2L) was capable of inhibiting tumor growth in vivo and that it acted synergistically with standard chemotherapeutics and thereby achieved even more striking anti-tumor effects.[37-42] This combinatorial effect can best be explained when one considers that chemo- and/or radio-therapy mainly engages the mitochondrial apoptosis pathways whereas TRAIL engages the death receptor-induced pathway. By hitting the tumor from these two different angles such a combinatorial treatment may result in less chances of the tumor to develop a therapy-resistant mutant. With the identification of the tumor-specific apoptosis-inducing capacity of TRAIL/APO-2L, the concept of direct induction of apoptosis in tumor cells through the engagement of cellular surface-expressed death receptors, first proposed upon the identification of the anti-APO-1 antibody, has finally become feasible and is now at the doorstep of the clinic.

At the biochemical level it was of course interesting to identify the receptor for TRAIL which was responsible for the induction of cell death on the surface of the target cell. The outcome of the resulting cloning race was surprising. With many different receptors at its disposal TRAIL emerged as the most promiscuous of all cytokines known.[43,44] TRAIL can bind two apoptosis-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (Killer, DR5,

TRICK2), two additional cell-bound receptors incapable of transmitting an apoptotic signal, TRAIL-R3 (LIT, DcR1) and TRAIL-R4 (TRUNDD, DcR2), and, lastly, a soluble receptor called osteoprotegerin (OPG). OPG does not only bind to TRAIL but also to another member of the TNF family, the osteoclast differentiation factor (ODF, OPGL, RANKL). At first it seemed as if the existence of these functionally distinct receptors might provide an answer to the differential sensitivity to TRAIL observed between normal and transformed cells. The initial findings indicated that TRAIL-R3 and TRAIL-R4 may act as so-called "decoy receptors" by competing with TRAIL-R1 and TRAIL-R2 for binding of TRAIL. However, these results were obtained solely by relying on overexpression data. By using monoclonal antibodies specific for each one of the individual surface-bound TRAIL receptors it was recently shown for various cellular systems that TRAIL resistance is controlled intracellularly rather than at the level of TRAIL-R3 and/or TRAIL–R4, the putative ”decoy receptors”. Defining the intricate differences between TRAIL-sensitive and -resistant cells—and i.e., in both normal and transformed cells—will be central to further our efforts in defining possible tumor entities that can be targeted clinically by TRAIL therapy.

Initializing Death: Formation of the Death Inducing Signaling Complex (DISC)

The initial discovery of receptors that are capable of inducing cell death when triggered was surprising and exciting enough. However, the mechanism by which the ligand-induced crosslinking of these receptors was connected to downstream signals that would finally result in death of the cell remained enigmatic for quite some time. It was known at that time that proteases identical or homologous to the interleukin-1 converting enzyme (ICE, now also known as caspase-1) played a key role in CD95- and TNF-induced apoptosis. Yet, the link between activation of death receptors and the activation of cellular caspases was unknown. The first hint to a possible signal transduction pathway came from the observation that, after stimulation, several proteins, termed CAPs (for cytotoxicity-dependent APO-1 associated proteins) are recruited to the CD95 receptor. This protein complex has been termed ‘DISC’, for death-inducing signaling complex.[45] The first clues as to the nature of these proteins came in the same year with the identification of FADD/MORT1, a protein that bound to the intracellular domain of CD95.[46,47] It was found that this protein when overexpressed induced cell death which could be blocked by caspase inhibitors. Still, it remained unclear how the DISC recruitment of this protein which lacks catalytic activity could lead to activation of caspases. However, only one year later, two groups provided the unexpected and exciting solution to this problem. Two complementary cloning approaches led to the identification of a protease of the caspase family (now know as caspase-8), which is recruited to CD95 and TNF-R1 after ligand stimulation.[48,49] Further, it was found that binding of this caspase to the receptor complex leads to activation of its proteolytic activity.[50] This milestone discovery, the identification of a caspase as an integral component of the DISC, provided the missing link that coupled death receptor ligation to activation of the proapoptotic caspases.

Later, FADD and caspases-8 were also found in the signaling complexes of other death receptors, like e.g., the TRAIL death receptors TRAIL-R1 and TRAIL-R2. This provided the framework for a general model of how these receptors transmit their downstream signal: By a series of protein-protein interactions finally resulting in the deadly activation of caspases directly at the receptor complex and their subsequent release into the cytosol. In the years after the initial discovery of the DISC complex, a plethora of proteins have been proposed to interact wiht the different death receptors. Yet, reliable data showing an unambiguous involvement in death receptor-triggered signaling events exist only for a handful of proteins. The following sections describe these DISC components in more detail and highlight our current knowledge of how these proteins interact and their known functions.

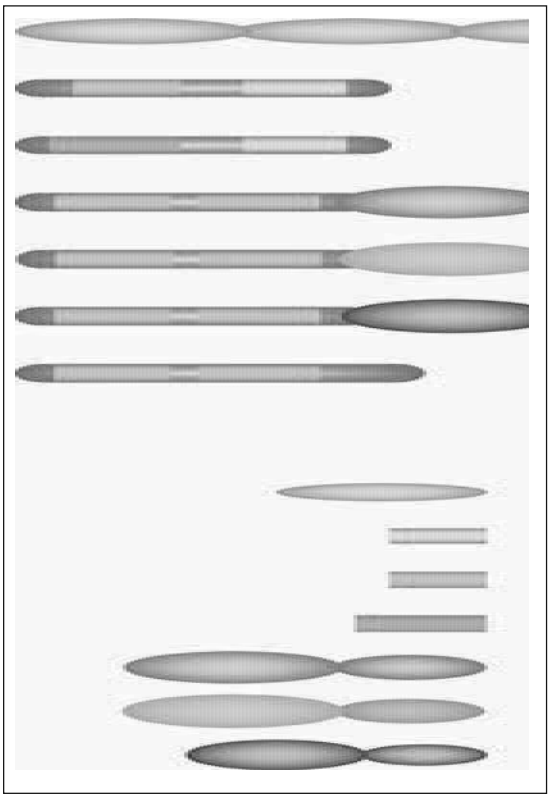

Figure 1A. Overview of the domain structure of the proteins involved in initiating apoptosis at the DISC

DISC Components and Mode of DISC Assembly

Interaction Domains

The interactions between the DISC components is mediated by several conserved protein motifs, which interact in a homotypic mode. Two prominent domains involved in these interactions are the death domain (DD) and the death effector domain (DED) (Figure 1A). Structural studies showed that both domains adopt a fold consisting of six antiparallel helices. This evolutionarily conserved motif is also present in a third protein domain which mediates protein-protein interactions, the CARD domain.[51-58] Protein-protein interactions between these protein folds occur in a homotypic manner between DD and DEDs respectively. While the interactions between DDs are mainly mediated by electrostatic interactions, DED domain interactions preferrently seem to utilize hydrophobic interactions as has been elucidated by mutagenesis studies of the interaction patch.[51]

FADD/MORT1

FADD/MORT1 has been originally identified in a yeast two hybrid screen with the CD95 death domain as a bait.[46,47] It contains both a DD and a DED and acts as an adapter between DD—containing receptors and the DED domain—containing caspases, namely caspase-8 and caspase-10. It is generally believed, that FADD binds directly to the death domains of CD95, TRAIL-R1 and TRAIL-R2 by interaction of its death domain with the death domains of the receptors. This model is supported by in vitro experiments which show that FADD and the death domains of the receptors interact. The central role of FADD as an adaptor linking death receptors to caspase activation, is further supported by experiments using cell lines or mouse

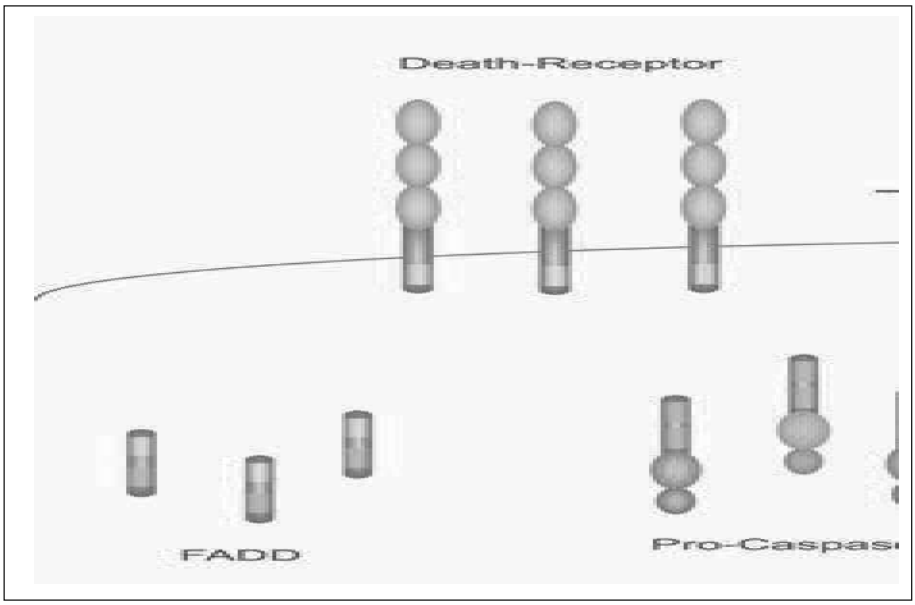

Figure 1B. Stimulator-dependent formation of the DISC results in caspase activation emanating from the activated DISC.

embryonic fibroblasts (MEFs) deficient in FADD. In these systems, FADD has been shown to be essential for recruitment of caspase-8 and caspase-10 not only to the CD95 DISC, but also to the TRAIL-R1 and TRAIL-R2 DISCs.[59-61] In addition, cells lacking FADD are resistant to CD95-, TNF- and TRAIL-mediated apoptosis.[62-65] Blockage of CD95- and TNF-mediated killing could also be observed when a dominant negative version of FADD was overexpressed.[66] However, cell death after overexpression of TNF-R1 in MEFs derived from FADD-deficient mice was only partially blocked, opening the possibility for a FADD-independent death pathway, at least for TNF-R1.

Amongst the death receptors, TNF-R1 differs from CD95 and TRAIL-R1 and -R2. Recruitment of FADD to TNF-R1 does not seem to be mediated by direct interaction of the DD of FADD with the DD of TNF-R1. Instead, it has been proposed that an additional molecule, the TNF-R-associated DD-containing protein (TRADD) acts as a bridge between TNF-R1 and FADD. However, this model leaves some questions unanswered (as discussed later).

In addition to its function in pro-apoptotic pathways, another possible function for FADD was determined by the analysis of mice that either lack FADD in their T cell compartment or overexpress a dominant negative version of FADD under a T cell specific promoter.[64,67-69]

Interestingly, lymphocytes derived from these mice are not only resistant to death receptor mediated apoptosis, but also show a defect in lymphocyte proliferation. In line with these results, FADD has been shown to be phosphorylated by a cell cycle regulated kinase also suggesting a connection between FADD and the regulation of cell proliferation.[70]

The Initiator Caspases

Caspase-8

Members of the caspase family are divided into three distinct groups according to their substrate specificity and main function (see chapter 1 for an in-depth analysis). The initiator caspases, which are characterized by a long N-terminal prodomain, are usually the apical

enzymes activated in the caspase cascade. The executioner caspases which are activated by the initiator caspases are the downstream workhorses, that degrade a variety of cellular substrates during apoptosis.

The discovery of caspase-8 (FLICE, Mach, Mch5) as a component of the CD95 DISC yielded the missing link between the activation of death receptors and cell death induced by activation of caspases.[46,47,71] Among the initiator caspases, only caspase-8 and its close homologue, caspase-10, contain two DEDs in their prodomain. These death effector domains have been shown to mediate the interaction between the adapter protein FADD and each one of these caspases.

Caspases are synthesized as inactive proenzymes. Maturation of caspases to the active enzyme occurs by proteolytic separation of the large and small catalytic subunit from the pro-enzyme. Once liberated, two large and two small subunits form the active tetrameric enzyme.[72,73] caspase-8 is expressed in at least three splice variants of which two, caspase-8a and caspase-8b, are catalytically active.[74] The third expressed variant, termed CAP3, represents a truncated form that lacks catalytic activity.

Experiments using cell lines or mouse cells deficient in caspase-8 suggest that caspase-8 is the major caspase necessary for death receptor-mediated apoptosis. In cell lines deficient in caspase-8, death receptor-mediated apoptosis is strongly suppressed.[60,61,75] However, residual cell death can be observed after longer stimulation and/or stronger stimuli also in the absence of caspase-8, suggesting the existence of an alternative pathway which does not rely on caspase-8.[59,76,77] Homozygous deletion of the caspase-8 locus in mice leads to embryonic lethality, and murine embryonic fibroblasts (MEF) derived from these mice are resistant to CD95-, TRAMP- and TNF-induced cell death providing evidence for a non-redundant role of caspase-8 in the embryonic development in mice.

Caspase-10

Caspase-10 is, in addition to caspase-8, the only caspase containing two death effector domains in its prodomain.[78-80] Like caspase-8, caspase-10 has also been shown to be recruited to the CD95 and TRAIL-R DISCs, where it is activated with kinetics comparable to caspase-8.[59,76] Due to its high homology to caspase-8 and obviously identical activation pattern, it is debated whether caspase-10 can also mediate TRAIL- and CD95-induced apoptosis in the absence of caspase-8. In some cases it seems to do so, although at a much lower efficiency than caspase-8, making it unlikely that caspase-10 is a backup caspase for receptor-mediated apoptosis. Yet, it might serve to transmit signals other than cell death emanating from stimulated death receptors. In addition, it might play a more prominent role in apoptosis induced by other receptors of the TNF family. Interestingly, in a number of tumor cell lines analyzed caspase-10 is downregulated at the protein level by a posttranscriptional mechanism, pointing towards a possible role of this caspase in tumor suppression.

While both, a caspase-8 and a caspase-10 homologue have been identified in Xenopus laevis,[81] to date no mouse caspase-10 gene has been identified and might be absent in this species, at least in the laboratory strains analyzed thus far.

cFLIP

The first flice inhibitory protein (FLIP, also called Casper, CASH, MRIT, FLAME-1, I-FLICE, CLARP and Usurpin, for a recent review see ref. 82) that was discovered was a viral protein (v-FLIP) capable of inhibiting CD95-, TRAIL- and TRAMP- mediated apoptosis.[83-85] Later two cellular homologues, $cFLIP_L$ and $cFLIP_S$ were identified.[86-93] Of the multiple splice variants that were originally reported only two forms, $cFLIP_L$ and $cFLIP_S$ have been shown to exist on the protein level.[74] The primary structure of cFLIP resembles that of caspase-8 and caspase-10. Both variants contain the tandem DED, enabling this protein to interact with FADD and thus to be recruited to the DISC complexes. The longer variant in addition contains domains which are similar to the large and small subunit of the initiator caspases. Yet,

despite this high homology, $cFLIP_L$ does not contain any catalytic activity, because residues critical in forming the active site, which are conserved in all caspases are missing in this protein. Therefore $cFLIP_L$ can interact with initiator caspases but $cFLIP_L$ itself is not a caspase. $cFLIP_L$ can also be cleaved by caspase-8 between the region homologous to the large and the small subunit. When present in high amounts, $cFLIP_L$ and $cFLIP_S$ have been shown to block death receptor-mediated cell death by interfering with caspase activation at the DISC.[94]

TRADD

TRADD, for TNF-R1-associated DD-containing protein.[95] has been found in a yeast two hybrid screen to bind to the DD of TNF-R1. TRADD contains a C-terminal DD and an N-terminal domain capable of interacting with TRAF2. TRADD has been found to bind to the death domain of TNF-R1 via its death domain. The presence of TRADD in the TNF-R1 signaling complex has also been confirmed to occur after stimulation with TNF-α under native conditions.[95-97] The proposed role of TRADD in this complex is to form a docking platform for recruitment of FADD, which subsequently leads to activation of caspase-8, analogous to the CD95 and TRAIL-R DISCs.

Mechanism of Caspase Activation at the DISC

Binding of the Ligand and Receptor Multimerization

The crystal structure of the extracellular domain of death receptors in complex with their respective soluble ligands yielded important first insights into the mechanism of DISC formation. Both complexes crystallized so far, TNF-R1 and TRAIL-R2 in complex with their ligands TNF-α and TRAIL respectively, show a trimeric ligand binding to a receptor trimer. These data suggested that the minimum requirement for the active complex formed on the cell surface is a trimer as well. The model of receptor activation deduced from these data led to the following model: Monomeric death receptors become trimerized by their ligands leading to intracellular crowding of their death domains. Alternatively, a conformational change in receptor structure upon ligand binding could also be involved. The intracellular assembly of death domains creates a binding surface for the adapter molecule FADD which in turn recruits caspase-8 and caspase-10 which leads to their activation (see Figure 1B).

Recent genetic and biochemical evidence however, revealed the existence of a domain called PLAD (for pre-ligand assembly domain) in several members of the death receptor family.[98,99] It has been shown that ligand-independent receptor dimerization or possibly multimerization is mediated in some death receptor family members by a domain located in the most N-terminal CRD. This domain itself does not take part in ligand binding. The analysis of naturally occurring mutations as well as mutagenesis studies indicated that this ligand-independent receptor-receptor interaction is essential for their function in apoptosis induction. Further, receptors bearing a mutated PLAD can act as dominant inhibitors of receptor function. The authors of these studies suggested the following model for death receptor activation: A stable trimer of receptors is pre-formed at the cell surface but inactive. Upon ligand binding, a conformational change in the receptor occurs allowing the death domains to come into close proximity and thereby enabling the recruitment of FADD.

Interestingly, the PLAD domain is located opposite of the receptors' ligand binding site. Thus the formation of stable trimers as proposed would obscure the receptors' ligand binding site. An alternative model which is consistent with the reported observations was also proposed. Here, receptor dimers or trimers are formed through PLAD domain interactions, leaving the ligand binding site accessible. Addition of the ligand could then lead to supra molecular cluster formation where these pre-associated receptor dimers or trimers are crosslinked by the ligand trimers.

Receptors for which a PLAD domain has been reported to date are CD95, TNF-R1, CD40, TRAIL-R1 and TRAIL-R2. Given the general similarities in the members of the TNF- and

TNF-R superfamilies it is quite likely that this phenomenon is not restricted to the above mentioned receptors but is of more general importance in this family of proteins.

Binding of FADD

The clustering of the receptors and/or a conformational change induced by ligand binding leads to intracellular binding of the adapter molecule FADD to the receptors. This binding is rapid and is detectable within seconds after receptor triggering. Both the phosphorylated and the non-phosphorylated forms of FADD are equally well recruited to the CD95 and TRAIL DISC. Importantly, binding of FADD to the death receptors is only observed after receptor stimulation. Immunoprecipitations of unstimulated death receptors do not contain co-precipitated FADD. Thus it is the ligand-induced crosslinking that creates the binding surface on the receptors' DD for the DD of FADD.

Activation of the Initiator Caspases 8 and 10

Recruitment of Procaspase to the DISC

The FADD molecules bound to the oligomerized receptors are thought to form a binding platform for recruitment of procaspase-8 and -10 to the DISC complexes. This recruitment is mediated via homotypic interactions between the FADD DED and the procaspase-8 and -10 DED, respectively. The recruitment of caspase-8 and caspase-10 occurs simultaneously with FADD binding. As in the case of FADD, the association of these two caspases with the death receptors can only be detected after receptor triggering. This is not surprinsing since FADD binding to the receptor is required for the binding of caspase-8 and –10. Cytoplasmic complexes of caspase-8/10 and FADD can also not be detected, highlighting the function of stimulated death receptors as a platform mediating interactions of the proteins necessary to initiate the caspase cascade. This tight regulation makes sense because a pre-associated FADD/initiator-caspase complex could be of potential danger to the cell.

The Activation Cycle

The main function of the DISC complex obviously is to provide a platform for activation of caspase-8 and –10. As the procaspases do not contain significant proteolytic activity in solution at low concentrations, it initially remained unclear how caspases are activated at the DISC complex. Possible models involved other DISC-associated proteases, conformational changes of the procaspases or autoproteolytic activation. Soon, several experimental observations led to a possible model explaining the activation of caspases in the DISC.

First, it was observed that procaspase-8 can undergo proteolytic maturation when expressed in *E. coli*. Second, artificially induced dimerization of caspase-8 in cells leads to rapid activation of caspase-8 and subsequent apoptosis without the necessity for caspase-8 recruitment to the DISC.

These observations prompted the hypothesis of "induced proximity" which describes the mode of caspase activation by an increase in local concentration.[100-102] In the case of death receptor signaling, this increase is mediated by FADD-dependent recruitment of procaspase-8 and -10 to the oligomerized receptors. Probably the receptor-recruited caspases form a complex which resembles the active tetrameric complex formed in solution by activated caspases. A conformational change in the structure upon interaction with FADD in the DISC could also play a role in activation of the enzymatic activity. Thus, with respect to caspase activation the DISC provides the stage for proper alignment of sufficient procaspase molecules in a manner that promotes their auto activation.

After recruitment of the procaspases to the DISC, proteolytic cleavage between the large and the small subunits occurs. The intermediate products are still bound to the DISC complex. Subsequently a second cleavage event between the prodomain and the large subunit occurs, thereby liberating the large subunit from the prodomain. The processed subunits now form the

active caspase enzyme, consisting of two large and two small subunits. At this stage, the remaining prodomain detaches from the DISC and allows for a new proenzyme to be recruited to and activated at this complex. How this exchange of the remaining prodomain for a new caspase-8/10 proenzyme takes place is elusive but probably the affinity of the proenzyme for the DISC is higher than that of the remaining prodomain.

Caspase Inhibition by cFLIP

How do $cFLIP_L$ and $cFLIP_S$ interfere with caspase activation at the DISC? Both cFLIP splice variants are recruited to the CD95 and the TRAIL DISC complexes along with caspase-8 and caspase-10. It has recently been shown that, although both molecules can efficiently inhibit caspase activation at the DISC when overexpressed, they differ in their mode of inhibition.[94] If $cFLIP_L$ is present in the DISC, the initial cleavage between the large and the small subunits of the initiator caspases is observed. Concomitantly, $cFLIP_L$ is also cleaved leading to the appearance of a $cFLIP_L$ fragment which stays bound to the DISC.

The presence of high amounts of $cFLIP_S$ in the DISC in contrast seems to block at the initial caspase-8 cleavage step as no intermediate caspase-products are observed and only full length caspase-8 can be found in the DISC.

In addition to its observed function in suppression of death receptor mediated caspase-activation, other functions have been proposed for cFLIP. It was reported that cFLIP can activate NF-κB, JNK and ERK pathways in response to death receptor stimulation, however, the relevancy of these pathways in a true physiological setting remains to be determined, as all experiments to date used overexpression of cFLIP at levels that were several fold higher than naturally occuring levels.

Spreading the Fire

Activated caspase-8 and caspase-10 alone are not sufficient to commit a cell to a regulated death program owing to their limited number of substrates which they are able to process when compared to other caspases. Thus, for a cell to die activation of the effector caspases 3 and/or 7 is needed which in contrast to the initiator caspases cleave a multitude of cellular substrates. It is the cleavage of these targets which leads the way to the typical hallmarks of apoptosis like membrane blebbing, nuclear fragmentation and phosphatidylserine exposure. For caspase-8 and caspase-10 it has been shown that both can cleave caspase-3 and -7. This first cleavage step is required for maturation and, thus, activation of these caspases. However, while this initial cleavage step is a prerequisite, it is not sufficient for caspase-3 activation. An additional autocatalytic cleavage step of caspase-3 is required for this enzyme to become active. This maturation step can be inhibited by the so called inhibitors of apoptosis proteins, the IAPs which interact with caspases in a manner that inhibits the final autocatalytic step of caspase activation.

Amplification Loops, the Role of Mitochondria

Activation of the initiator caspases at the DISC does not necessarily lead to immediate and rapid cell death by activation of the downstream caspases (Figure 2). In some settings, a cell can tolerate remarkable amounts of DISC-activated caspase-8 without committing itself to apoptosis. The obvious explanation for this phenomenon would be that the activation and/or activity of the downstream caspases may be inhibited. This idea was supported by the recent identification of several proteins involved in the regulation of effector caspase activation. On the on side, there are the IAPs as just mentioned. These proteins were first discovered as baculoviral proteins, the vIAPs, which were able to inhibit apoptosis when overexpressed. Subsequently, the cellular counterparts, the cIAPs were identified (reviewed in ref. 103). The human IAP family currently consists of eight members. These IAPs are able to inhibit apoptosis by blocking caspase-3, -7 and –9 by distinct mechanisms, with XIAP being the most potent.[103] Inhibition occurs by interference with the maturation of these enzymes to the fully active enzyme. This observed

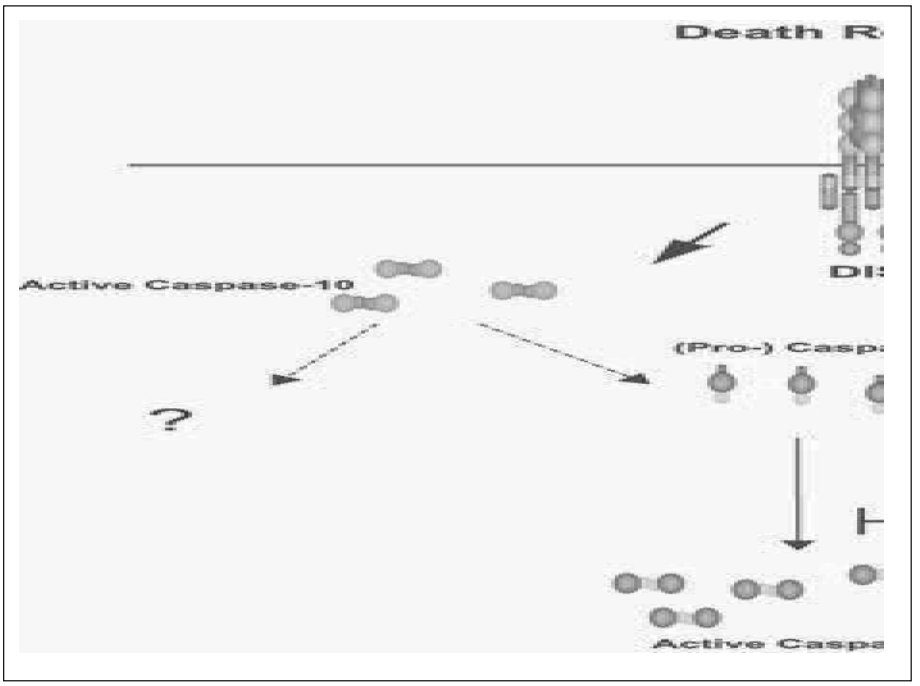

Figure 2. DISC-induced caspase activation and apoptosis initiation. Caspase-8 is activated at the DISC and cleaves Bid and caspase-3. Cleaved Bid acts on mitichondria and thereby induces the release of cytochrome c and Smac/DIABLO. Cytochrome c release allows for apoptosome formation. Smac/DIABLO release results in the inactivation of XIAP's caspase-3-inhibitory function, thereby allowing for caspase-3 activation and apoptosis. Caspase-10 is also activeted at the DISC but cannot substitute for caspase-8 in apoptosis induction. The function for caspase-10 remains to be determined.

inhibition can take place even if the first cleavage step, mediated by activated initiator caspases, has already occurred. Recent experiments suggested that XIAP inhibits the second, autocatalytic cleavage step, necessary for caspase-3 and –7 maturation. These findings explain why some cells are able to tolerate active initiator caspases without dying. In this setting, the initial processing of caspase-3 is readily observed, yet the fully matured enzyme can not be detected.

This linear pathway of caspase activation, however, is not the whole story. It was observed that in some situations the death-receptor mediated pathway can be modulated in part by the expression of antiapoptotic molecules of the Bcl-2 family. This led to the classification of two cell types with regard to CD95-induced apoptosis.[104] Type I cells formed a strong DISC after stimulation and were independent of mitochondrial pathways in their commitment to cell death. Type II cells in contrast formed only little DISC and depended on the mitochondrial release of pro-apoptotic molecules (with cytochrome c being the only one known at that time). However, it is becoming clear that the mitochondria act as integrators of a plethora of signals governing cellular integrity. This is achieved by influencing the balance between pro- and antiapoptotic Bcl-2 family members.[105]

At first, it remained unclear how mitochondria can be involved in death-receptor mediated pathways. A solution to this puzzling finding was provided by the identification of Bid, a protein which provided the link between death receptors and mitochondria. Bid can be cleaved by activated caspases to its truncated form tBid, which then in turn can act at the mitochondrial membrane to promote the release of proapoptotic molecules. The best defined molecule

known so far to be released from the mitochondria is cytochrome c. Upon its release from mitochondria, cytochrome c promotes the formation of the apoptosome consisting of Apaf-1, caspase-9 and cytochrome c.

Once formed, the apoptosome mediates the activation of caspase-9 and subsequent cleavage of effector caspases within the apoptosome, thus providing additional activated caspase-3 molecules. Another important molecule released from mitochondria upon their apoptotic activation is the recently discovered Smac (Second mitochondria derived activator of caspases)[106] in humans and its mouse homologue DIABLO (direct IAP binding protein with low pI)[107] Interestingly, Smac/DIABLO has been shown to inhibit XIAP by direct interaction, thereby interfering with its binding to caspase-3 and -7. Thus, upon Smac/DIABLO release from mitochondria, the inhibition of effector caspase maturation is relieved, allowing for full activation of caspase-3 and/or –7 and, thus execution of cell death. Recently, another molecule named HtrA2/Omi, also capable of interfering with XIAP's inhibitory action was identified, adding additional complexity to the inhibitory machinery.[103]

One interesting consequence of this finding is that most likely it is not only the efficiency of DISC formation that decides whether a cell is a type I or type II cell regarding the necessity of mitochondrial apoptotic activation but that it is also, if not more importantly, the presence or absence of Smac/DIABLO-inhibitable blockers of full effector caspase maturation (i.e., IAPs) that is decisive for the degree of dependency of death receptor-induced apoptosis on mitochondrial activation.

The Step-Sisters of the DISC

The interactions with and the function of the above described proteins within the DISC has been confirmed by a number of laboratories using complementing biochemical and genetic approaches. In addition to these proteins, a plethora of other molecules has been proposed to take part in death receptor signaling, with many controversies still unresolved. Interactions found in one laboratory or experimental setting could sometimes not be reproduced by other researchers and laboratories. To evaluate these discrepancies, one has to take a critical look at the experiments performed.

Most of the conflicting results stemmed from the fact that many of the putative interacting proteins have been found by yeast two hybrid screens or homology searches in EST or genome databases. Subsequent experiments showing the association with specific signaling complexes almost always involved overexpression of at least one of the putative interacting proteins. While these methods are very powerful and resulted in the identification of important mediators of death receptor signaling, caution should be applied when interpreting these results. Specificity of the proposed interaction needs confirmation in a system where the proteins are expressed at native levels. For several reasons, overexpression can lead to false-positive or -negative results. First, protein overexpression favours the occurence of partially misfolded and unfolded proteins. These species naturally tend to aggregate yielding positive results in interaction assays. In addition, the subcellular localization/compartimentalization of the expressed proteins may not reflect the naturally occuring situation, thus allowing proteins to interact which are separated from each other under native conditions. Also, many investigators utilize tagged versions of the proteins. It is possible that under certain circumstances these tags interfere with the assembly of protein complexes. Unfortunately, it is still common practice to investigate protein-protein interactions solely by transient transfection/overexpression systems or pull-down assays with the purified proteins only. One example are the proteins DAXX, RIP and RAIDD, which have been proposed to take part in CD95-mediated cell death, yet a recent publication shows that these proteins are dispensable for this apoptosis pathway.[108]

TRADD and TNF-R DISC Assembly

Amongst the death receptors, TNF-R1 differs in the mode of DISC assembly. In contrast to CD95 and TRAIL-R1/-R2, TNFR-1 does not bind FADD directly via its death domain. Rather

it is proposed that FADD binding to TNF-R1 occurs via the adapter protein TRADD. TRADD is suggested to bind to the TNF-R1 death domain via homotypic interactions with its death domain. Then, TNF-R1-bound TRADD is able to recruit FADD to the TNF-R1 DISC which leads to recruitment and subsequent activation of caspase-8. This proposed model, however leaves several questions unanswered.

Association of FADD with TNF-R1 has only been observed after simultaneous overexpression of FADD, TRADD and TNF-R1. And even in this experiment, the interaction of FADD with the TNF-R1/TRADD complex was weak. Recruitment of FADD to the native TNF-R1 DISC has never been reported so far.

This weak interaction might explain why many cells are not highly sensitive to TNF-mediated apoptosis without addition of protein synthesis blockers. Blockage of protein synthesis might lower the cellular levels of a putative inhibitor which might prevent efficient recruitment of FADD to the TNF-R1 complex. In addition blockage of protein synthesis could lower the levels of cellular inhibitory proteins, thereby allowing for cell death to occur upon weak initiator caspase activation. Alternatively, a different adapter or stabilizing protein might be needed to strengthen the interaction between the TNF-R1/TRADD/FADD complex. The precise composition of the TNF-R1 complex which leads to activation of caspases is in fact still elusive. Nevertheless the results from knockout animals and cell lines as well as biochemical evidence from mutagenesis studies imply an important role for FADD as the central adapter for coupling death receptors including TNF-R1 to caspase activation and cell death.

One peculiarity of the TNF-R1 DISC concerns the proposed interactions in this complex. TRADD has been suggested to bind RIP and FADD via a homotypic interaction between the DD of TRADD and the DD of RIP or TRADD. It is currently unknown how this dual binding is achieved as both, the death domains of FADD and TNF-R1 would occupy the same binding patch TRADD is suggested to use for binding to the TNF-R1 DD. A recently proposed alternative model, which could explain this dichotomy proposes that the interactions between DDs (and DEDs) in the DISC lead to the formation of another interaction motif, providing more than one binding site per protein.[109]

Other DD-Containing Receptors

Apart from the well characterized death receptors CD95, TNF-R1, TRAIL-R1 and TRAIL-R2 several other DD-containing receptors have been identified in recent years. However, they differ widely in their ability to trigger cell death and much less is known about the pathways these receptors utilize to transmit their downstream signals. From the data available it seems as if these receptors' main function is not to induce apoptosis, but rather to transmit signals utilized during adaption of the immune response or regulating developmental processes. For a description of the biology of these receptors like TRAMP (DR3, Wsl1, Apo-3, LARD, TR3, TNFRSF12),[110-115] DR6[116] and EDAR we refer the interested reader to an excellent review.[6]

Final Words and Reflections

The outcome of death receptor triggering is regulated at several steps. The first step is binding of the ligand to its receptors, a step which might be regulated by the presence of soluble or membrane bound decoy receptors. Second, the amount of DISC complexes formed is naturally dependent on the amount of receptors that are expressed at a cell's surface. Third, the ability of death receptors to cluster and inititiate signaling might be regulated as exemplified by the type I/type II dichotomy. Third, the activation of initiator caspases at the DISC can be blocked by the simultaneous presence of FLIP proteins or other elusive inhibitory proteins in the DISC, inhibiting the first activation step of the caspase cascade. Once activated, initiator caspases activate two principal downstream pathways. The first one aims at the initial cleavage step that is necessary for activation of executioner caspases. The maturation of these 'primed' executioners

might be blocked by high levels of IAPs. However, high levels of primed caspase-3 might overcome cellular levels of IAPs and thus circumvent the need for removal of this inhibition. Thus if the ratio of caspase-activation at the DISC to XIAP or other inhibitory molecules is high enough, no amplification-loop might be needed. Alternatively expression of IAPs might be low or absent.

In case further caspase-3 maturation is inhibited, this blockage can be overcome by the Bid mediated release of mitochondrial Smac/DIABLO, relieving the XIAP inhibition. Cytochrome c released from the mitochondria can also provide additional primed caspase-3 molecules via activation of caspase-9 by the apoptosome. As the mitochondrial integrity is an important factor in the commitment of a cell to die, it is also tightly regulated by the balance of pro and anti-apoptotic proteins.

Thus, the outcome of death receptor triggering is not necessarily cell death, and it becomes clear that the decision whether a cell is destined to die or not is regulated at many steps, which might themselves be subject to regulation by intra- and extracellular-stimuli. It makes sense that a decision about life and death of a single cell has to be interweaved in the intricate signaling network that evolved in multicellular organisms. This is most dramatically exemplified by the consequences that dysregulation of apoptosis has in many diseases as e.g., in cancer and autoimmunity.

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CHAPTER 4

Mitochondrial/Apoptosome Dependent Activation of Caspases

Kelvin Cain

Abstract

Many key biological processes, including caspase activation during apoptotic cell death are executed by large multi-protein complexes. Apoptosis can be initiated via death receptors or by perturbation of the mitochondria, which results in the release of apoptogenic proteins. These initiate and promote the activation of the caspases, which produce the biochemical and morphological changes that are characteristic of apoptotic cell death. Caspases are normally present as inactive zymogens, and require specific proteolytic cleavage for activity. This activation process occurs in large specialised protein complexes known as the DISC (receptor mediated cell death) and the apoptosome (mitochondrial mediated cell death) that are assembled de nouveau when apoptosis is initiated. In mammals the central scaffold protein of the apoptosome is an ~130 kD protein known as Apaf-1 which is a homologue of CED-4, a key protein involved in programmed cell death in the nematode *C.elegans*. Apaf-1 in the presence of cytochrome c and dATP oligomerizes to form a very large (~700 to 1400 kD) apoptosome complex, which recruits and activates/processes caspase-9 to form the active caspase processing holoenzyme complex that subsequently recruits and activates the effector caspases. The apoptosome has been described in cells undergoing apoptosis, in dATP activated cell lysates and in reconstitution studies with recombinant proteins. Recent studies show that formation and function of the apoptosome is tightly regulated by intracellular levels of K^+, inhibitor of apoptosis proteins (IAPs), heat shock proteins and at least two mitochondrial-released proteins, Smac/Diablo and Omi/Htra 2 a serine protease. Thus, a variety of factors ensure that the apoptosome complex is only fully assembled and functional when the cell is irrevocably destined to die.

Introduction

The morphological and biochemical changes of apoptotic cell death largely result from the activation of a group of cysteine aspartic acid-specific proteases known as caspases (for review see refs. 1-5). The activation of caspases is a central feature of apoptosis and key components of this mechanism are highly conserved throughout evolution from *Caenorhabdtitis elegans* to *Drosophila melanogaster* and ultimately to mammals. In *C. elegans* there are at least four genes, *ced-3, ced-4, ced-9* and *egl-1*,[6,7] which are critical for the the execution of apoptotic cell death. Ced-3 encodes for a cysteine protease which is homologous to interleukin (IL)-1β converting enzyme or ICE[8] which is now known as caspase-1. Thirteen further caspases have now been identified in mammals, some of which are involved in cell death (CED-3 sub-family), whilst others are involved in inflammation (ICE sub-family). Caspases are synthesized as pro-enzymes or zymogens, which usually (see later) are activated by proteolytic cleavage.

Caspases—Their Role in Cell Death and Cell Survival, edited by Marek Los & Henning Walczak.

Current dogma states that caspases are activated via a cascade mechanism in which an initiator or activating caspase, cleaves/activates a downstream caspase, which in turn activates the next caspase and so forth (see Chapter 2 for further discussion). The execution of the caspase cascade requires an independent, controllable mechanism for activating the proximal or initiator caspase. Evolution has produced at least two primary pathways for inducing apoptosis involving either stimulation of cell surface death receptors or perturbation of mitochondria. However, in both pathways constitutive molecules are assembled into large protein complexes, which then recruit and process the initiator caspases (for review see ref. 9).

In death receptor-mediated apoptosis, ligands such as CD95/Fas/APO-1 and TRAIL induce receptor trimerization and formation of a death inducing signalling complex (DISC).[10,11] This complex, located at the cell membrane, then recruits procaspase-8, via the FADD/MORT1 adaptor molecule[12,13] and induces a conformational change in procaspase-8, which results in caspase-8 cleavage and activation.[10,12-14] caspase-8, as an initiator caspase, can then activate directly or indirectly all other caspases (see also Chapter 3). In many respects the DISC can be regarded as the membrane bound equivalent of the apoptosome.

Many apoptogenic agents induce cell death by disrupting the membrane integrity of mitochondria, which release proteins that initiate and promote caspase activation catalysed by the apoptosome/aposome complex.[15-18] Release of cytochrome c from the mitochondria provides the signal for the initiation of the assembly of the large multi-subunit apoptosome complex. Once assembled the apoptosome recruits, processes and activates caspase-9 which remains bound to Apaf-1 to form a holo-enzyme complex that activates downstream effector caspases.[19] This chapter reviews the current evidence on the assembly, regulation and function of the apoptosome.

Mechanisms of Caspase Activation

In order to understand the role of the apoptosome in cell death, it is important to determine how caspases are activated and why, in the case of the initiator caspases, this requires large complexes. For the purpose of this chapter I will concentrate primarily on caspases-9, -3 and -7, which are members of the CED-3 group of caspases and are key players in the apoptosome complex. Caspases are synthesised as proenzymes and after activation exhibit considerable substrate specificity, particularly in relation to down stream procaspases (Fig.1, and Chapter 2). Caspases-3 and -7 are effector caspases, which preferentially cleave at DExD motifs, whereas caspase-9 is an initiator caspase, which on the basis of recombinant studies prefers an (I,L,V)ExD motif.[20] Caspases can be further distinguished by their characteristic domain structure, comprising an N-terminal prodomain, a large (~p20) and a small (~p10) subunit. Initiator caspases (caspase -8, -9 and -10) have long prodomains and the effector caspases have short prodomains. However, the length of the prodomain is not necessarily an indicator of function. For example caspase-6 has a short prodomain and cleaves lamins,[21] but could be classified as an initiator caspase because of its synthetic peptide substrate specificity (VEID).[3] caspase-2 has a long prodomain but whether it is an initiator or effector caspase is still controversial.

caspase activation usually requires cleavage at a specific motif between the large and small subunits, whereas most non-caspase proteases are activated by removal of an inhibitory prodomain.[22] Removal of the N-terminal prodomain of caspases is usually a secondary autocatalytic event and in the case of caspase-3 does not effect the enzymic activity of the processed enzyme.[23] Unusually, after caspase-9 is cleaved at Asp^{315} to yield a p35 large subunit, it is not further degraded during activation.[24-26] However, a secondary p37 subunit can be subsequently generated by caspase-3 mediated cleavage of the zymogen at Asp^{330} (Fig. 1).

The mechanism of caspase activation has not been fully elucidated, although initial X-ray crystallographic studies of active caspase-1, -3, -7 and -8 complexed with peptidyl inhibitors have shown that the active enzyme is a heterotetramer composed of two p20/p10 dimers.[28-32] The active site contains the catalytic cysteine (Cys^{285}, caspase-1 numbering system), which is conserved in a pentapeptide (QACRG) motif and forms a catalytic dyad with His^{237}.[26] However, other residues, including Arg^{179}, Gln^{283}, Arg^{341} and Ser^{347} are required to form the P_1

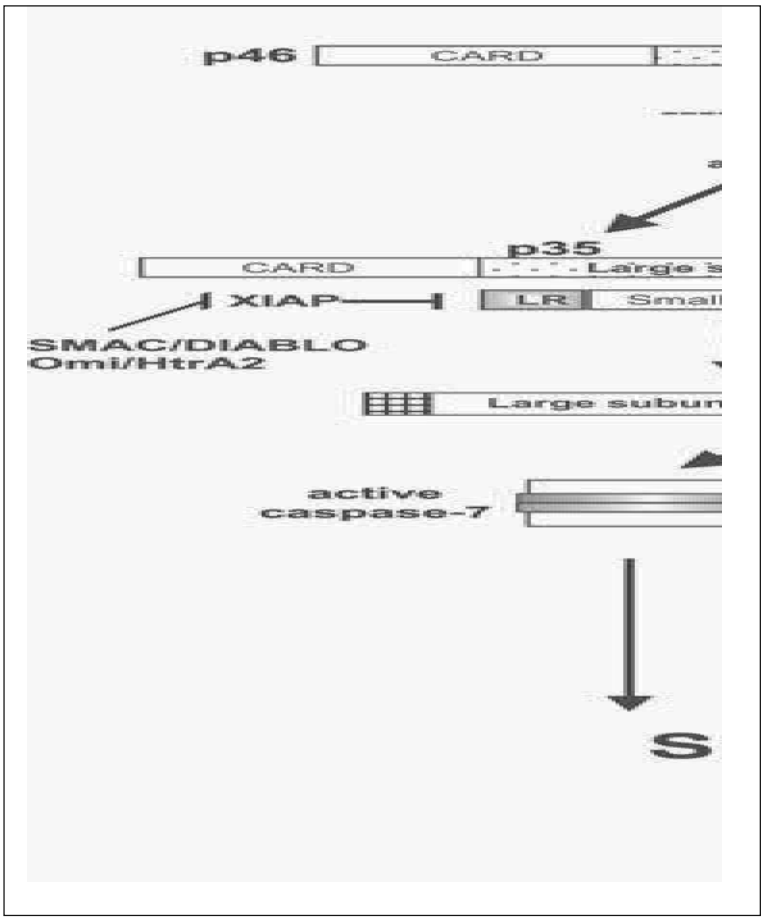

Figure 1. Processing and activation of caspase-9 initiates the caspase cascade. Procaspase-9 consists of a CARD domain, a large and small subunit, are connected via a linker region (LR). The zymogen form of caspase-9 has intrinsic activity, that is markedly increased after binding to Apaf-1 in the apoptosome complex (see text). This also facilitates the autocatalytic cleavage at D315*, producing p35 (CARD domain plus large subunit) and p12 (LR plus small subunit) subunits. . The linker region has an exposed ATPF motif that binds to XIAP thereby inhibiting the processed caspase-9 activity. Pro-apoptogenic proteins released from the mitochondrion such as SMAC and Omi can antagonize this inhibition. Once activated within the apoptosome, caspase-9 recruits, cleaves and activates caspase-3 and -7, thereby initiating the caspase-cascade as described in chapter 2. Caspase-3, amplifies the proteolytic processing potential of caspase-9 by cleaving the proform at D330* to form the p37 and p10 subunit form of the enzyme, which lacks the ATPF motif and cannot be inhibited by XIAP. Scheme adapted from Cain et al.[106]

pocket, which accommodates the Asp residue of the substrate.[28] These residues are located on both the p20 and p10 subunit domains and the first structural studies suggested that the relative shortness of the linker regions in caspase-3 and -7 did not allow the peptide chain sufficient flexibility to form the catalytic site. Proteolytic cleavage between the large and small subunits would allow rearrangement of the subunits to form the active site, possibly in an heterologous fashion with the p20 and p10 subunits derived from separate zymogens.[28] This mechanism would explain why the procaspases (with the notable exceptions of caspases-8 and -9) have little or no proteolytic activity.

However, very recent studies with recombinant C186A (or C285A, using caspase-1 numbering convention) caspase-7 zymogen mutants have suggested a slightly different mechanism for effector caspase activation.[33,34] These studies show that procaspase-7 at normal physiological concentrations is a homodimer in which each monomer contains a central 6-stranded β sheet and five α-helices. Four surface loops (designated L1-L4 and L15-L45, respectively for the left and right monomers) protrude from these core elements, to potentially form the catalytic sites (Fig. 2A). Structurally, the central core elements are essentially the same as in the active heterotetramer, however the surface loops 2-4 adopt dramatically different conformations with only L1 retaining its active site configuration.[33] In the inactive zymogen the active site pocket is effectively flattened and distorted with the L2 loop containing the catalytic cysteine inaccessible to solvent. In contrast, in the inhibitor-active enzyme complex the opposing heterodimer loop L25 is cleaved and the resultant C-terminal segment rearranges with L4 and the N-terminal segment of the L2 loop to form the so-called active site 'loop-bundle'. This rearrangement is blocked in the zymogen as the C-terminal portion of L25 is still attached to its N-terminal peptide chain at the opposite end of the molecule and thus is physically restrained from forming the loop bundle. Both the L2 and L25 are accessible to proteolytic cleavage, upon which they assume an open confirmation, which primes the formation of the active site to allow substrate/inhibitor binding and a further conformational change, which promotes catalysis or inhibition (Fig. 2A).

Whilst the mechanism for activating the effector caspases has now been elucidated, it is clear that the activation of caspase-9, the initiator caspase occurs by a different mechanism. Caspase-9 is unusual in that the zymogen is proteolytically active in contrast to caspase-3 and -7. This difference is illustrated by the zymogenicity ratio (processed divided by the unprocessed caspase activity) of caspase-9 and -3 which is 10 and >10000 respectively.[35] The linker region between the large and small subunits of procaspase-9 is much longer that those of caspase-3 and caspase-7[33] and presumably is flexible enough to allow formation of the active site within the zymogen. However, the cleavage activity of recombinant pro and fully processed (p35) is relatively low when measured with the synthetic peptide Ac-LEHD.AFC or recombinant procaspase-3.[26] Significantly, the ability of recombinant caspase-9 to process procaspase-3 is enhanced 2000-fold in the presence of cytosol, dATP and cytochrome c.[26] Furthermore, the non-cleavable D315A, D330A and D315A/D330A mutants can also process caspase-3 when incubated with cytosol, dATP and cytochrome c.[26,36] These increases in caspase-9 activity in the presence of cytosolic factors are due to interactions with Apaf-1 in the apoptosome complex and suggest that CARD : CARD interactions with Apaf-1 are required for the active site to assume its optimal catalytic conformation.

The structural basis for this mechanism remains to be elucidated, as crystals of a full-length caspase-9/Apaf-1/apoptosome complex have as yet not been isolated. However, a very recent X-ray crystallography study with a recombinant ΔCARD non-cleavable caspase-9 mutant provides a possible mechanism[37] and shows that in contrast to the effector caspases, procaspase-9 is an inactive monomer at normal physiological concentrations. However, at the high concentrations used in crystal formation the ΔCARD caspase-9 mutant is an active dimer containing two markedly different active-site conformations. One site is very similar to the catalytically active sites, which have been described for caspase-3 and caspase-7, and is catalytically competent and reacts with a tri-peptidyl inhibitor. The other site is catalytically inactive as the dimer conformation dislocates the 'activation loop' rendering it catalytically inactive and unable to react with tripeptidyl inhibitors (Fig. 2B). Thus, dimerization forces an allosteric rearrangement of the active site of least one monomer in the dimer to produce an active enzyme. This suggests a possible mechanism for Apaf-1 enhancement of capase-9 activity, whereby Apaf-1 recruitment recruits and concentrates caspase-9, induces dimerization and activation. Alternatively, Apaf-1 by binding to caspase-9 through its very tight CARD : CARD interactions may cause an allosteric rearrangement of the active site, switching it from the inactive to the active conformation. Further studies will be required to answer these questions, but clearly the

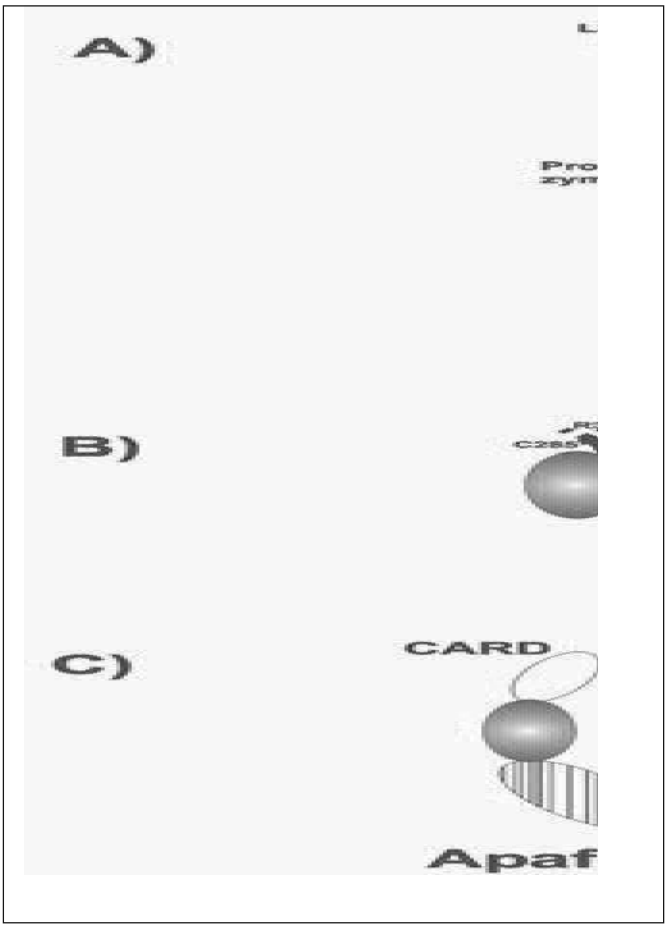

Figure 2. Structural basis for the activation of initiator and effector caspases. Two schematic diagrams are shown for the activation of caspase-7 (A) and caspase-9 (B). The scheme for caspase-7 is adapted from ref. 33 and show that the caspase -7 is normally present in the cell as a dimer, but the active site loops (L2-L4) are conformationally distorted and do not form a catalytically competent active site. The uncleaved interdomain loop L2 containing the catalytic cysteine (Cys 186) is locked into a closed conformation that prevents the necessary conformational changes required for formation of fully functioning active site. Cleavage at Asp198 allows the active site loops to rearrange to form a 'primed' active site that can bind substrate or inhibitor. The newly formed N-terminini of the small subunit can then rearrange to form the fully functional active site. In caspase-7 and -3 the active enzyme is a heterotetramer with two identical active sites, whereas in caspase-9 (B) the enzyme appears to have only one functional catalytic site ref. 37. In this scheme derived from studies on ΔCARD caspase-9 mutant, caspase-9 is believed to exist as a folded monomer with the active sites catalytically inactive. Enforced dimerization and/or possibly binding to Apaf-1 causes the surface loops of only one active site to assume the correct conformation for catalysis in a manner analogous to caspase-7. The long interdomain loop of caspase-9 allows formation of the active site without cleavage and shows how the zymogen can be active.

fundamental difference in the initial activation of caspase-9, compared to caspases-3 or -7, is the requirement for Apaf-1 and the apoptosome complex.

Primitive Apoptosomes

The earliest evolutionary example of an apoptosome is observed in the nematode *C. elegans* (for review see ref 38). In *C. elegans*, pro-CED-3 is essentially inactive and requires activation by interaction with CED-4, which is normally inactive as it is bound to the mitochondrial/cell membranes by CED-9.[7,39] During cell death, EGL-1 another pro-death protein is up-regulated and binds to CED-9, thereby releasing CED-4, which then translocates to the perinuclear membrane, where it oligomerizes and activates pro-CED-3.[7] Oligomerization of CED-4 is believed to bring CED-3 molecules into close proximity leading to intermolecular autocatalytic cleavage and activation of adjacent CED-3 molecules.[40,41] Cell death is abolished when key residues in the oligomerization domain of CED-4 are mutated.[42] Furthermore, aggregation can be induced artificially by using fusion proteins of the CED-3 protease domain and the FK506-binding protein (Fkbp). The Fkbp dimeric binding ligand, AP1510 cross-links the CED-3 fusion protein which activates the caspase activity of the CED-3.[42] Similar studies with Fkbp-procaspase-8[43] or Fkbp-Fas also stimulate oligomerization and proximity induced activation of procaspase-8.[44] These studies suggest that the intrinsic caspase activity of initiator zymogens is sufficient to facilitate autocatalytic processing when the caspases are in close proximity to one another. In *C. elegans*, the interactions of CED-9 with CED-4, prevent oligomerization and hence keeps CED-3 in its inactive monomeric state.

The next evolutionary development of the apoptosome can be observed in *Drosphila melanogaster* which also contains a caspase-activating complex, which is formally similar but has not been so well characterised as the mammalian apoptosome (see below). After Apaf-1 was identified, database searching produced the fly homologue, Dapaf-1/DARK/HAC-1, which is similar to Apaf-1, but unlike CED-4, in that Dapaf-1[1] contains C-terminal WD40 repeats.[45-47] Dapaf-1 is alternatively spliced to form both full length (L) and truncated (S) forms of the protein, which may be caspase specific.[45] However, the identity of the initiator caspase which interacts with Dapaf-1-in the apoptosome has not been resolved. Both Dredd and Dronc interact with Dapaf-1 in overexpression systems, and catalytically inactive mutants of both caspases are reported to inhibit Dapaf-1-induced cell death.[45-47] The specificity and high affinity of the Apaf-1/caspase-9 (CARD : CARD) interaction (see later) suggest that Dronc is probably the initiator caspase involved in the *Drosophila* apoptosome.

The involvement of cytochrome c in formation of the *Drosophila* apoptosome is unclear, although cytochrome c associates with Dapaf-1 in SL2 cells stably transfected with Dapaf-1 it does not do so in cells transiently transfected with Dapaf-1.[46] Moreover, while addition of cytochrome c and dATP to SL2 lysates does not lead to activation of caspases,[45,48] increased DEVDase activity, though very modest, can be observed in lysates obtained from wild-type but not Dapaf-1 null dpf^{K1}/dpf^{K1} *Drosophila* embryos.[45] Cytochrome c has been detected in digitonin-extracted lysates obtained from SL2 cells treated with staurosporine or cycloheximide.[45] However, in another study, cytochrome c appeared to undergo a conformational change during apoptosis, but remained within the mitochondria.[48]

The studies in *C.elegans* and *Drosphila* indicate a common evolutionary mechanism by which initiator caspases are recruited into an apoptosome complex and activated. However, both the CED-4/CED-3 and the Dapaf-1/Dronc complexes have not been characterized biochemically or physically, nor reconstituted from recombinant proteins. In this respect the mammalian apoptosome complex has been studied in far greater detail and clearly similar studies are needed on the primitive apoptosomes.

The Mammalian Apoptosome

In the last ten years the mammalian homologues of CED-3, CED-9 and EGL-1 were identified by conventional cloning strategies, but finding a CED-4 homologue proved to be more

difficult and the identification of Apaf-1 as a CED-4 homologue required a classical biochemical approach. Xiaodong Wang and colleagues showed that dATP, induced caspase activation in cell cytosols[49] by a mechanism which involved three apoptotic protease-activating factors (Apaf-1-3). These were ultimately isolated and identified as a CED-4 homologue (Apaf-1), cytochrome c (Apaf-2) and caspase-9 (Apaf-3), and are now known to be the core components of the mitochondrially-mediated cell death pathway.[49-51] The physiological importance of these three molecules has been emphatically demonstrated in gene knockout studies. Thus, Apaf-1$^{-/-}$ mice exhibit marked embryonic lethality with striking craniofacial lesions and brain abnormalities,[52,53] embryonic thymocytes and stem cells from Apaf-1$^{-/-}$ mice which are sensitive to Fas-induced cell death are resistant to non-receptor mediated cell killing even though cytochrome c is still released. Caspase-9$^{-/-}$ mice exhibit defective brain development, marked embryonic lethality, and embryonic stem cells and fibroblasts are resistant to various mitochondrially mediated apoptotic stimuli[54,55] Finally, cells from cytochrome-c-deficient mouse embryos which survive up to day 8.5 are insensitive to a variety of apoptotic stimuli.[56]

Functional Structure of Apaf-1 Domains

In trying to understand the apoptosome it is necessary to consider the structure and function of Apaf-1, which is the backbone of the complex. Apaf-1 is an ~ 130 kD protein, with an N-terminal CARD domain, a region homologous to CED-4 and a C-terminal domain containing multiple WD-40 repeats. Four isoforms have been cloned/characterized which differ by insertion of an extra 43 amino acid long WD-40 repeat, and/or the insertion of 11-amino acids after the CARD domain (Fig. 3). These are known as Apaf-1 /Apaf-1S, Apaf-1XL/ Apaf-1L-WD13, Apaf-1LN and Apaf-1LC/ Apaf-1L.[18,50,57-59]

All the Apaf-1 isoforms contain an N-terminal (1-97 amino acids) caspase recruitment domain (CARD) which is a protein fold consisting of a six membered α-helical bundle initially identified in some caspases, including CED-3, and adaptor proteins.[60] The CARD domain acts as a docking region, allowing initiator caspases to be aggregated and activated by adaptor proteins and is not normally caspase-9 unless Apaf-1 is activated by dATP and cytochrome c.[51] Truncated Apaf-1 mutants with a deleted CARD region do not activate caspase-9[27] whilst deletion of the WD repeats renders Apaf-1 constitutively active, allowing caspase-9 to be processed in the absence of dATP and cytochrome c.[27,61] This implies that access and binding to the CARD domain is normally blocked by the WD repeats and that dATP and cytochrome c induces conformational changes in Apaf-1, which unmask the CARD and allow recruitment of caspase-9 (see below).

All the Apaf-1 isoforms contain a CED-4 domain, but only Apaf-1XL/Apaf-1L-WD13 and Apaf-1LN/Apaf-1L have an additional 11- amino-acid insertion of the between the CARD and CED-4 domains.[60] The functional importance of this in these isoforms of Apaf-1 is unclear. It does not seem to be involved in cytochrome c binding or caspase activation as the Apaf-1LC/Apaf-1L is competent in both these respects.[17,59] The central CED-4 domain (98-412) includes conserved Walker's A (P-loop) and B boxes forming nucleotide binding sites which constitutes a putative ATPase domain.[51,62] The role of the nucleotide-binding sites in Apaf-1 is controversial as in one study, a P-loop (Walker A box) mutant Apaf-1 K160R did not bind to Apaf-1 and also inhibited/ recruitment and processing of caspase-9.[58] Conversely, other studies reported that this mutation did not markedly affect the ability of Apaf-1 to process caspase-9.[27] Furthermore, studies with [α-^{32}P] dATP showed that nucleotides bound to Apaf-1 were hydrolysed[17] and that the non-hydrolyzable ATP-γS analogue strongly inhibited caspase-3 activation and did not support Apaf-1 self-association or binding to procaspase-9.[58] Similar results were also reported by other groups and appeared to confirm that dATP/ATP binding to Apaf-1 was accompanied by hydrolysis.[18] However, a very recent study has shown that dATP is not hydrolysed on binding to Apaf-1 and furthermore another non-hydrolyzable analogue b,g-methylene adenosine 5'-thiotriphosphate (ADPCP), unlike ATP-γS, will activate Apaf-1.[63] These contradictory results are explained on the basis that in the earlier studies the

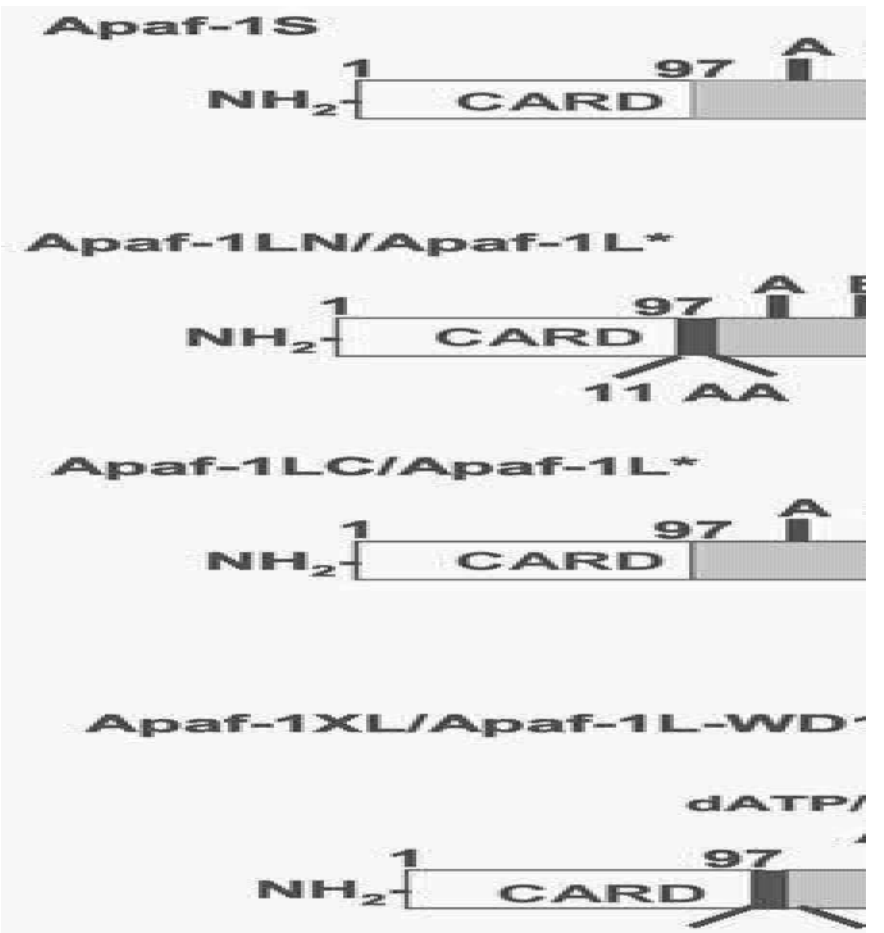

Figure 3. Domain structure of the four isoforms of Apaf-1. In the scheme The CARD, Walker nucleotide binding domains (A and B) and WD-40 repeats are shown for the various isoforms. The presence of the 11 amino acid insert following the CARD domain and the 43 amino acid extra WD repeat insert between the fifth and sixth WD repeats are also shown. Two different Apaf-1L have been described in the literature, [17,18] but unitl a standard nomenclature has been agreed, this chapter use the nomenclature used in the paper that characterised all four isoforms.[59] In the figure this nomenclature is shown first, followed by its synonym.

recombinant Apaf-1 was contaminated with other proteins, which could hydrolyse dATP/ATP. As yet there is no direct evidence whether it is dATP or ATP, which is required for efficient formation of the apoptosome in cells undergoing apoptosis. In the cell, dATP and ATP are present at about 10-20 mM and 0.2-10 mM respectively[64] suggesting that both nucleotides could contribute to cytochrome c dependent oligomerization of Apaf-1. Interestingly, in many in vitro cell free model systems, dATP is routinely used at between 0.1 to 1 mM, even though

the K_d for dATP binding to Apaf-1 is reported to be only 1.7 mM.[63] Significantly, the ATP concentration in the cell is a critical determinant of whether or not a cell dies by necrosis or apoptosis.[65]

Interactions of the CED-4 domain have also been investigated using truncated Apaf-1 (1-530 and 1-570) mutants, which oligomerize and recruit and process procaspase-9 in a cytochrome c and dATP-independent mechanism.[27,58] These mutants do not require dATP for activity and this suggests that the nucleotide binding sites are involved in removing the inhibitory effect of the WD domain, which regulates the ability of Apaf-1 to self associate through its CED-4 domain,[66] Interestingly, truncated apoptosome complexes do not recruit and process caspase-3, demonstrating that the WD repeat domain may also play an important role in recruiting pro-caspase-3 to the complex. It appears that in the absence of cytochrome c and dATP, the WD repeat domain is folded over and masks the CED-4 domain interaction surfaces, thereby preventing oligomerization. Interestingly, an Apaf-1 (M368L) mutant, which shows decreased binding to the WD repeat region of Apaf-1, processes and activates caspase-9 in the absence of dATP and cytochrome c.[58] Thus the M368 residue may serve to lock the WD domain over the CED-4 domain. Deletion of residues 456-559 of the CED-4 domain also abolishes the ability of Apaf-1 to activate procaspase-9.[61] Clearly, further studies are needed to fully understand the interactions of dATP with the CED-4 region of Apaf-1.

The functional significance of the WD-40 repeats in Apaf-1 is still poorly understood. It is clearly not required for oligomerization as CED-4, which does not have a similar region can readily self-associate. However, the original Apaf-1 (S) clone (1194 amino acids), contained neither the 11 nor 43 (WD-40) amino acid inserts and does not form a functional apoptosome.[17,18,59] However, the Apaf-1XL/Apaf-1L-WD13 and Apaf-1LC/Apaf-1L isoforms which contain the additional WD-40 repeat are all capable of supporting caspase activation.[59] Although, the exact function of the WD-40 repeats is unclear, they do seem to be required, although not exclusively for cytochrome c binding. Thus, neither the N-terminal deletion mutant of Apaf-1S (468-1194) nor the C-terminal deletion mutants of Apaf-1XL (1-570) bind cytochrome c.[58] Typically, WD repeats comprise a 44-60 residue sequence containing a GH dipeptide, 11-24 residues from the N-terminus and separated by a conserved core sequence from the C-terminal WD dipeptide (see for review see ref. 67). A well-characterized example of a WD-repeat protein is the Gβ subunit of heterotrimeric G proteins. This is a seven bladed β-propeller structure, in which each propeller blade is a four-stranded anti-parallel β sheet, composed of 3 strands from one WD repeat and the remaining strand from the next repeat. The commonest β-propeller structures contain 4-8 blades, although some proteins have as many as 16 WD repeats, although it is unclear to whether this results in one large 16- or two smaller 8-bladed propellers. If the latter structure is favoured then Apaf-1 may contain two asymmetric (7- and 6- bladed) β-propellers. The extremely rigid closed circular structure of β-propellers does not readily undergo conformational changes. Other than protein-protein interactions with other molecules, WD-40 repeats have no obvious function or enzymic activity. Interestingly cytochrome oxidase cd1 also contains a rigid 8-bladed propeller in which the cytochrome c moiety is located at and above the axis of the propeller[68] and perhaps a similar structure exists in Apaf-1.

Correct Oligomerization of Apaf-1 is a Critical Step for Forming an Active Apoptosome

In the absence of cytochrome c and dATP, both native (and recombinant Apaf-1 elute on gel-filtration columns with a M_r of between 130 to 300 kD, indicating either a monomeric or perhaps dimeric structure.[16,69] However, after dATP/cytochrome c activation, Apaf-1 undergoes oligomerization and elutes as a very large caspase-activating complex with M_r between ~700 to 1.4 MDa.[16-18,69] The apoptosome complex reconstituted from recombinant Apaf-1, procaspase-9 and cytochrome has been estimated to be ~1.4 MDa and suggested to contain eight Apaf-1 subunits in a 1:1 stoichiometry with caspase-9.[17] However, in dATP activated

THP.1 cell lysates, we initially isolated an ~700 kD Apaf-1 apoptosome (aposome) complex containing activated caspases-9, -3 and -7 (16). Using Superose-6 gel filtration chromatography, we showed that this could be separated into ~700 and ~1.4 MDa apoptosome complexes both of which contained Apaf-1 and processed caspase-9.[69] The ~700 kD complex, was the most active at processing exogenous procaspases-9 and -3.

Furthermore, only the ~700 kD apoptosome complex which processed effector caspases, was found in human tumor monocytic THP.1 cells after induction of apoptosis with either etoposide, a DNA topoisomerase II inhibitor, or N-tosyl-l-phenylalanyl chloromethyl ketone.[69] Similarly, in B-chronic lymphocytic leukemic cells (B-CLL) obtained from patients, induction of apoptosis was accompanied by formation of the ~700 kD Apaf-1 containing apoptosome complex.[70] Thus in cells induced to undergo apoptosis, the ~700 kD Apaf-1 containing apoptosome complex appears to be the predominant and biologically active apoptosome complex formed. Interestingly in the above studies on the formation of the apoptosome in apoptotic cells, as well as in apoptotic FaO hepatoma cells,[16,71] only a very small proportion of the Apaf-1 is oligomerized to form the apoptosome complex. This is marked contrast to in vitro studies with dATP-activated cell lysates where all the Apaf-1 can be oligomerized. This suggests that in the apoptotic cell only a small number of apoptosome complexes need to be formed to trigger the caspase cascade.

The larger and relatively inactive ~1.4 MDa apoptosome complex appears to be more readily formed in dATP-activated lysates, although small amounts of this complex can be detected in some cells exposed to apoptotic and necrotic stimuli (unpublished data). The reasons why the ~1.4 MDa complex is relatively inactive are not clear, although there is evidence from protease digestion experiments that the Apaf-1 in the ~1.4 MDa complex is in a different conformation.[36]

The actual size of the apoptosome complex has not been unequivocally determined. Whilst gel filtration chromatography has been enormously helpful in isolating the apoptosome it has limitations in that proteins and complexes elute in fairly broad peaks. The ~700 kD apoptosome complex we have observed in cell lysates essentially spans a M_r range from around 600-900 kD, with the apex of the peak eluting slightly earlier than a thyroglobulin (669 kD) protein marker. This suggested that the most active apoptosome complex is ~700 kD. Interestingly the 20 S (~700 kD) proteasome, which is not globular, also co-migrates with the apoptosome peak (data not shown) following Superose 6 chromatography and also in sucrose density gradients.[16] However, it should be stressed that estimations of the size of large complexes by gel filtration chromatography are affected by the gel filtration media used and also assume that the assembled complex and calibration standards are globular. The latter assumption may be the most critical as we have no idea of the real configuration of the complex and further work is required to characterize the stoichiometry and structure of the apoptosome complex.

The Role of Cytochrome c and dATP in Apoptosome Formation

Although, the binding site for cytochrome c on Apaf-1 has not been characterised, fluorescence polarization studies have determined that cytochrome c binds to recombinant Apaf-1 in a 2:1 stoichiometry with high affinity ($K_a = 10^{11}\ M^{-1}$,[72]). In the presence of the normal intracellular K^+ ($[K^+]_i$) concentrations the affinity of cytochrome c for Apaf-1 is markedly reduced ($K_a = 4 \times 10^7\ M^{-1}$,[73]). Interestingly, normal intracellular K^+ concentrations suppress caspase activation by inhibiting the formation of the ~700 kD complex.[74,75] However, the inhibitory effects of $[K^+]_i$ can be overcome by high concentrations of cytochrome c and this suggests that the intracellular $[K^+]_i$ acts to safeguard the cell against inappropriate caspase activation which might be caused by the accidental release of small quantities of cytochrome c. As normal intracellular $[K^+]_i$ antagonizes cytochrome c binding to Apaf-1, it is possible that it is only when cytochrome c reaches a critical threshold level (during apoptosis) that this inhibition is overcome and Apaf-1 is initiated (Fig. 4).

As discussed earlier it is tempting to suggest that the WD-40 domain consists of two propellor structures, each of which can bind a cytochrome c molecule and mutational epitope studies

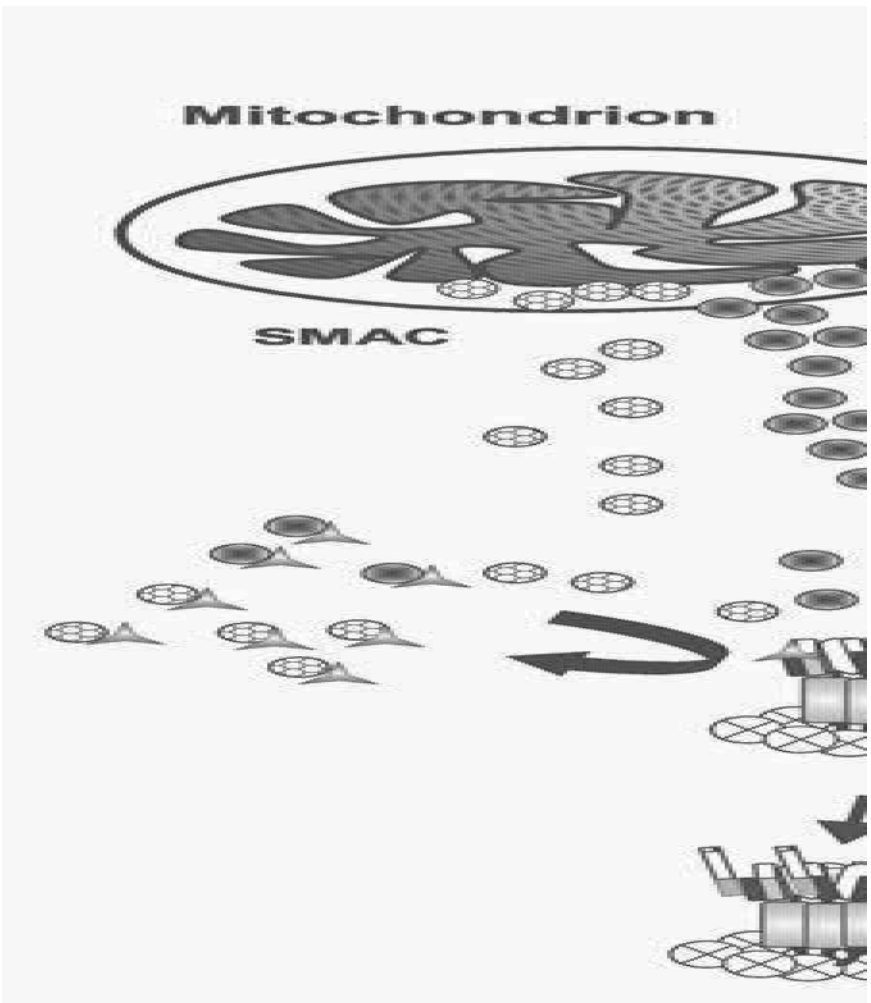

Figure 4. Assembly and regulation of the apoptosome complex. In this scheme, which is adapted from a recent review [106] Apaf-1 is depicted with the CARD domain and the WD-40 domains (shown as two separate propeller structures) folded over and masking the CED-4 oligomerization and nucleotide binding domains. Released cytochrome c binds (possibly) to the WD-40 repeats, which unfold and unmask the nucleotide binding sites, which can now bind dATP/ATP. This induces more conformational changes in Apaf-1, allowing rearrangement of the CARD domains, which can now recruit and allosterically activate caspase-9. One procaspase-9 is activated and autocatalytically cleaves itself to produce the p35/p12 form of the enzyme, which exposes the ATPF motif that bind XIAP. SMAC and Omi , which are also released from the mitochondrion and bind very tightly to XIAP and antagonise its effect on caspase-9. The uninhibited caspase-9 can then recruit and process the effectors caspases and intitiate the caspase-cascade as described in Figure. 1. Various modulators of the process, such as ionic strength, and heat shock proteins are also shown (see main text for more details).

show that the cytochrome c binding site on Apaf-1 is different from that observed in usual electron transfer proteins.[76] In these proteins, the lysine rich interface close to the heme pocket is the main determinant for binding, whereas cytochrome c binding to Apaf-1 apparently not only involves this interface but also requires an opposite surface of the molecule. This suggests that Apaf-1 essentially wraps around cytochrome c and this large interface explains the high binding affinity between cytochrome c and Apaf-1. Cytochrome c also facilitates dATP binding to Apaf-1, possibly by conformational changes which expose the dATP binding sites.[63] However, both cytochrome c and dATP seem to be only necessary for the oligomerization of Apaf-1 to the form the apoptosome complex, because once assembled it is unaffected by further addition of these co-factors.[17,77]

The Apoptosome Complex Recruits and Processes Caspase-9

Oligomerization of Apaf-1 to form the ~700 kD apoptosome complex is a rapid process and is maximal within 5-10 min of initiating dATP activation and once assembled the apoptosome is relatively stable as shown by its processing of exogenous caspases.[69] caspase-9 is recruited and rapidly processed can be detected as the free form as well as bound to apoptosome complexes.[17,18,69] But it is now evident that caspase-9 is only really active when it is bound to Apaf-1.[69,77] Procaspase-9 is not normally detected in the apoptosome complex, so presumably it is rapidly processed on recruitment and significantly the non-cleavable D315/330A mutant of caspase-9 associates normally with oligomerized Apaf-1 and rapidly recruits and activates caspase-3.[19] In addition wild type fully processed p35 can bind to oligomerized Apaf-1.[19] Interestingly, in the presence of z-VAD-fmk caspase-9 processing is blocked and procaspase-9 is detected in the~700 kD complex (K.Cain and C.Langlais, unpublished results). This suggests that procaspase-9 is activated directly within the apoptosome and requires autocatalytic processing for its release.

The stoichiometry of Apaf-1 and caspase-9 binding is reported to be 1:1[17] as predicted from the high affinity of the CARD-CARD interactions between caspase-9 and Apaf-1.[78,79] The CARD domain of caspase-9 binds to the Apaf-1 CARD though a mixture of homophilic and hydrophobic interactions and as a result this interaction is very stable in high ionic strength.[78] It is possible that the caspase-9 zymogens bind to Apaf-1 molecules in such close association that the dimeric activation complex is formed in analogous manner to that described for recombinant caspase-9.[37] Alternatively, the binding of procaspase-9 to Apaf-1 may be sufficient to produce the necessary rearrangement of the 'activation loop' for forming the active site (Fig. 3B). Once activated procaspase-9 could autocatalytically cleave other neighbouring or incoming zymogen molecules, a conclusion supported by experiments with Apaf-1 (1-530) which in the presence of WT procaspase-9 catalyses auto-processing of a C287A procaspase-9 mutant.[27] The processed p35 subunits could then bind to free Apaf-1molecules within the complex. Interestingly, although processed caspase-9 reaches a maximum in the ~700 kD complex within 5-10 min, it rapidly declines and after 30-60 min only small amounts remain associated with the complex (K.Cain and C.Langlais, unpublished data). The mechanism of caspase-9 is release from the apoptosome complex is unclear, but it is possible scenario that caspase-3 is responsible as it cleaves Apaf-1 within the CED-4 domain at SVTD271↓ S to give a 30 kD fragment, which binds preferentially to the ~1.4 MDa apoptosome complex and has been detected in apoptotic cells.[36] Other studies have also shown Apaf-1 is cleaved at the N-terminus, removing the CARD H1 helix to give a p84 subunit, although they did not identify the cleavage site.[80]

Neither the p30 fragment nor XIAP are responsible for the inactivity of ~1.4 MDa which seems to be formed by inappropriate oligomerization of Apaf-1.[36] In support of this, we have found that simply incubating cell lysates at 37°C in the absence of dATP and cytochrome c results in the incorrect oligomerization of Apaf-1to form the inactive ~1.4 MDa complex (unpublished results). This appears to be an inherent property of Apaf-1, as recombinant Apaf-1 also readily oligomerizes into a variety of different-sized inactive complexes but in the presence

of dATP/cytochrome c favours the formation of the active ~700 kD complex.[75] Correct assembly of the apoptosome may also require the presence of other cytosolic proteins, such as chaperones.

Regulation and Modulation of the Apoptosome

The core components the apoptosome complex Apaf-1 and caspase-9, but it is clear that other cellular proteins and factors modulate the formation or activity of the complex (Fig. 4). Inititial studies on the characterization of the apoptosome in human THP.1 monocytic tumour cells demonstrated the presence of caspases-3 and -7 in the apoptosome complex.[16] Using various mutants of caspases-3 and -9 in this cell-free system, it was subsequently demonstrated that catalytically active processed or unprocessed caspase-9 initially binds to the Apaf-1 apoptosome and consequently recruits caspase-3 though an interaction of the active site cysteine (C287) of caspase-9 and the critical aspartate (D175) between the large and small subunits of caspase-3.[19] However, the binding of caspase-3 and -7 to the apoptosome is not as strong as the CARD: CARD interactions because the effector caspases are dissociated from the complex by 50 mM NaCl.[69] However, the binding of caspase-3 and -7 to the apoptosome may also involve other proteins or even be context dependent as suggested by experiments in FaO hepatoma cells where caspase-3 elutes as the free caspase form and only caspase-7 is found in the complex.[71]

XIAP, an inhibitor of apoptosis protein (IAP), is normally present in large molecular weight complexes in unactivated cell lysates, but directly interacts with the apoptosome in cytochrome c/dATP-activated lysates.[19] IAPs were first described in baculoviruses, where they block apoptosis as a response to viral infection. Currently seven mammalian IAPs have been described and they are characterized by the presence of one or more ~70 amino-acid zinc-finger motifs known as BIR (baculoviral IAP repeat) domains. IAPs, in particular XIAP, inhibit apoptosis primarily by direct inhibition of distinct caspases, although other mechanisms may be involved (see refs. 81,82,83 for review, and Chapter 5). Interestingly, XIAP inhibits active caspases-3 and -9 through distinct domains within the protein. The BIR-2 domain, together with a few critical residues in the linker region between the BIR-1 and BIR-2 domains, is sufficient for inhibition of caspase-3, whereas the BIR-3 domain inhibits caspase-9.[84,85] Interestingly in our studies, XIAP associates with oligomerized Apaf-1 and/or processed caspase-9 and influences the activation of caspase-3, but importantly also binds activated caspase-3 produced within the apoptosome and sequesters it within the complex. Thus, XIAP may regulate cell death by inhibiting the activation of caspase-3 within the apoptosome and by preventing release of active caspase-3 from the complex[36] It is interesting to speculate why XIAP should be present in the apoptosome. Following a modest stress in which only a small portion of caspases are activated, XIAP would likely prevent apoptosis, whereas following a major insult, the activation of too many apoptosome complexes and active caspases would overcome the protective effect of XIAP or other IAPs, resulting in cell death.

Interaction of XIAP with a recombinant Apaf-1 apoptosome complex requires cleavage of caspase-9 at D315 to expose an ATPF leader sequence in the linker region of the small subunit, which binds to the BIR3 domain of XIAP (Fig. 1 and 4).[86] Removal of the linker region or mutation of the crucial residues in the leader sequence removes the ability of XIAP to inhibit caspase-9. The selectivity of XIAP for the processed form of the Apaf-1/caspase-9 holoenzyme may provide a safety switch mechanism on the formation of the apoptosome complex. Thus, when Apaf-1 oligomerizes and recruits procaspases-9, it immediately autocatalytically produces the p35 form, which is inhibited by XIAP, so limiting any further activation of other caspases.

Interestingly the cell seems to have evolved compensatory mechanisms to negate the inhibitory effects of XIAP. Recently, a novel protein Smac (Second Mitochondria-derived Activator of Caspases), and its murine homologue DIABLO, have been described which promote caspase activation by eliminating IAP inhibition of caspases.[87,88] Smac is synthesized as a 239 amino acid precursor protein and after entering the mitochondrion the N-terminal 55 amino

mitochondrial targeting signal is proteolytically removed to produce the mature Smac. During apoptosis, Smac is released into the cytoplasm, where it binds IAPs and relieves the inhibition of caspases. This interaction has been mapped to the N-terminal 20 amino acids of the mature Smac protein which contains an N-terminal AVPI motif that binds the BIR3 domain of XIAP displacing caspase-9.[89-91] Additionally, to its ability to relieve IAP inhibition of caspases, Smac/DIABLO may also be pro-apoptotic by a different mechanism possibly involving the apoptosome.[92] As Smac prevents IAP activity, it has been proposed to be a human equivalent of the pro-apoptotic Drosophila proteins Reaper, Grim and Hid.[87,93] Interestingly the N-terminal four residues in Smac, Reaper/Hid/Grim and the linker region of caspase-9 all share significant homology defining a conserved class of IAP-binding motifs.[86]

Interestingly, another mitochondrial protein known as Omi/HtrA2 has been identified which can bind XIAP.[94-96,97] Omi is a serine protease whose mitochondrial targeting signal is proteolytically removed when it is imported into the mitochondrion to reveal an N-terminus conserved IAP (AVPS) binding site. During apoptosis Omi is released from the mitochondrion and inhibits the function of XIAP in analogous manner to Smac. Binding of Smac/DIABLO, Omi/HtrA2 and perhaps other as yet unidentified proteins can antagonise the binding of XIAP to caspase-9 and thereby modulate the caspase cleavage activity of the apoptosome. The magnitude of the apoptotic stimulus as well as cellular levels of Smac, Omi, XIAP and other as yet unidentified proteins may all contribute to the sensitivity of a particular cell type to apoptosis.

In addition to the important roles of IAPs and Smac and DIABLO in modulating apoptosome activity after it is assembled, other molecules could also be of importance. By analogy with CED-4 and CED-9, Bcl-2 family members might be involved. Initial reports suggested that Bcl-XL interacted directly with Apaf-1[98] but later studies showed that Bcl-2 homologues did not bind to Apaf-1.[99] In addition, in C.elegans, CED-4 and CED-9 share a common intracellular distribution, whereas Apaf-1 appears to be entirely cytosolic and distinct from Bcl-2 or Bcl-XL.[100] The heat shock proteins (Hsps) also block apoptosis (see for review ref. 101). Studies with Hsp70 suggest that it may bind to the CARD domain of Apaf-1 and Apaf-1 or the recruitment of caspase-9.[102,103] Hsp-90 has also been reported to block Apaf-1[104] and Hsp-27 has been reported to block Apaf-1 by binding to cytochrome c.[105] However, the interactions of Hsp proteins with the apoptosome are complex and the relative importance of their role still remains to be determined.

In conclusion, considerable progress has been made in understanding the apoptosome but clearly further studies are required to fully understand the workings of this remarkable complex. The identification of other cellular factors responsible for controlling Apaf-1 formation and function in cells undergoing apoptosis may offer new insights in devising novel treatments for various human diseases and disorders.

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CHAPTER 5

Modulation of Caspase Activity by Cellular Inhibitors

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Summary

Caspases are key effectors of the apoptosis process, therefore it is not surprising that mammals, as well as other species, evolved molecules that regulate caspases by directly binding and inhibiting them. Yet the IAPs are the only endogenous cellular caspase inhibitors identified to date. IAP-BIR domains have remained highly conserved through animal evolution and in many instances they suppress apoptosis across species barriers. Although it is clear that many of the cellular IAPs regulate apoptosis, their roles during development, differentiation and other aspects of cellular physiological remain to be determined. At minimum, it appears the IAPs set thresholds and are modulators for the amount of caspase activation required for particular cellular events—specifically apoptosis. However, it is possible that IAPs participate in cross talk between apoptotic pathways and other cellular pathways such as differentiation, maturation, proliferation and inflammation. How important are the caspases and IAP regulation of their activities for coordinating these cellular events and the cell life and death decision? The answer to this question is likely complex and dependent on the cell type under investigation and the specific stimulus involved. However, emerging evidence indicates that dysregulation of IAPs, and therefore caspase activity, contributes to various diseases characterized by excessive (ischemia, HIV infection, SMA) or inadequate (cancer, autoimmunity) cell death. Future studies on caspase regulatory mechanisms, IAP function and regulation in human disorders, will undoubtedly lead to new therapeutic approaches including more specific drug discovery programs for the treatment of human disease.

Introduction

Apoptosis is a physiological form of cell death (= programmed cell death) that is necessary for the development and homeostasis of multicellular organisms.[1] Programmed cell death is critical for successful development, cell differentiation, formation of digits and canals and the maintenance of these tissues once they are established. Response to disease and pathogen invasion, as well as immune system function, also depends upon cell death programs. Although cell death has been defined and re-defined, classical apoptosis is characterized by a stereotypical series of biochemical features including the activation of caspases.[2] The apoptotic morphology of condensed chromatin, cytoplasmic shrinkage, display of phagocytosis markers on the cell surface and formation of apoptotic bodies depend upon caspase activities.[3] Specifically, caspases cleave cytoskeletal and nuclear matrix-associated proteins that are required for cellular integrity such as lamins, inhibitors of DNA degradation enzymes (e.g., ICAD) and DNA repair enzymes like DNA-PK and PARP-1, to name only a few of the known caspase substrates. Thus, caspases execute the ordered dismantling of the cell and the irreversible destruction of its

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genome, with the resulting constituents packaged, presented and cleared by phagocytes without insult to surrounding tissues. Evidence for dysregulation of apoptosis contributing to the pathogenesis of human diseases has accumulated and not only increased our interest in caspase biology but also exposed these enzymes, and their cellular inhibitors, as possible therapeutic targets.[4,5]

Dysregulation of apoptosis in human diseases includes both extremes, too much cell death, as exemplified in neurodegenerative disorders, ischemia (e.g., stroke, myocard infarct), viral infections and immunodeficiency diseases, and too little cell death in oncogenesis, cancer therapy resistance and some autoimmune diseases. Because caspases are not only involved in apoptosis initiation, but are also the key effector proteases of the cell death machinery, their negative regulation by endogenous cellular inhibitors has generated clinical interest. Much of our understanding of caspase inhibitors originated from the identification of viral genes necessary for evading the apoptotic host cell suicide in response to viral invasion such as the cowpox virus protein CrmA (Cytokine Response Modifier A) and the baculovirus protein p35, both of which specifically target caspases.[6,7] CrmA and p35, which utilize a serpin-like mechanism for inhibiting caspases, are cleaved by the target caspase and remain tightly bound to the catalytic caspase pocket. Therefore p35 and CrmA are believed to inhibit caspases by a suicide substrate mechanism[8,9] (for more details see chapter VI:virus encoded caspase inhibitors).

Following up on the baculovirus encoded cell death suppressor p35, Miller and co-workers first identified the inhibitor of apoptosis (IAP) genes Cp-IAP (Cydia pomonella) and Op-IAP (Orgyia pseudotsugata) as viral anti-apoptotic genes, which rescued cells infected with a p35-deleted baculovirus strain.[10] IAPs exhibit no similarity to p35, but contain highly conserved motifs termed the baculoviral inhibitory repeat (BIR) domains. In addition to the lack of sequence homology between p35 and the viral IAPs, several studies suggested that IAPs blocked apoptosis by a mechanism distinct from p35.[11,12] However similar to p35, ectopic expression of baculovirus IAPs suppressed apoptosis in mammalian cells, suggesting conservation of the cell death programs among diverse species and commonalities in the mechanisms used by IAPs to inhibit apoptosis.[13]

Since the original identification of baculoviral IAPs, a number of cellular BIR domain containing proteins have been identified in yeast (*Schizosaccharomyces pombe, Saccharomyces cerevisiae*), fly (*Drosophila melanogaster*), worm (*Caenorhabditis Elegans*) and mammalian species, including humans, mice, rats, chickens and pigs.[14-16] While several of these BIR domain containing proteins appear to play roles other than regulation of apoptosis, and the function of others remains to be elucidated, the anti-apoptotic mechanism of at least some IAPs can be attributed to direct binding and inhibition of distinct caspases. The initial differences observed between p35 and the IAPs can now be explained, at least in part, by the distinct caspases specificities of the IAPs verses broad spectrum caspase inhibition by p35—as discussed in this chapter.

Although technically not caspase inhibitors, cellular FLIPs (c-FLIP= cellular FLICE (caspase-8) inhibitory proteins) interfere with receptor-mediated activation of the apical caspase-8. They are specific for TNF death receptor family initiated apoptosis due to their death effector domain (DED), which binds to FADD, caspase-8 or caspase–10 through DED-DED interactions. Their structure and role in inhibition of death receptor mediated apoptosis will be briefly discussed in this chapter.

The Inhibitor of Apoptosis (IAP) Protein Family

Structure and Function of IAPs

The IAPs are defined by a novel zinc-finger motif called the Baculoviral Inhibitory Repeat (BIR), the name of which derives from the initial discovery in baculoviruses and the ability to inhibit apoptosis.[17,18] The number of BIR domains within IAPs varies between one and three. BIR domains are typically about ~70 amino acids long and are characterized by a number of

invariant amino acids including three conserved cysteines and one conserved histidine residue within the sequence $CX_2CX_{16}HX_{6-8}C$. The protein structures of XIAP BIR2, XIAP BIR3 and cIAP-1 BIR3 are very similar and indicate that BIR domains typically comprise a series of four to five α-helices and three-stranded β-sheet with a single zinc ion coordinated by the conserved cysteine and histidine residues.[19-23]

Proteins containing BIR domains have been found in a wide range of species, including yeast (*Schizosaccharomyces pombe, Saccharomyces cerevisiae*), *Drosophila melanogaster*, viruses, the nematode *Caenorhabditis elegans* and mammalian species (humans, mice, rats, chicken and pigs). Although many of these BIR domain-containing proteins have been shown to suppress apoptosis, yeast and worm BIR proteins do not, but rather play essential roles in cytokinesis.[15] Interestingly, the mammalian survivin BIR appears to represent an evolutionary bridge between the yeast and worm BIRs, to those found in virus and mammalian IAPs, since survivin is reported to function both in suppressing apoptosis and regulating cell division.[24-26]

As pictured in Figure 1., eight IAP relatives have been identified in humans to date:NAIP (BIRC1), cIAP-1 (HIAP-2, BIRC2), cIAP-2 (HIAP-1, BIRC3), XIAP (hILP, BIRC4), Survivin (BIRC5), BRUCE (Apollon, BIRC6) and ML-IAP (Livin, cIAP-7, KIAP, BIRC7) and ILP2 (BIRC8).[26-33] Most of these human IAPs have mouse orthologs, implying conservation of the IAP gene family in mammals—the exceptions appear to be ML-IAP and ILP2.

Much of our knowledge about the function of IAPs originated from studies investigating how IAPs block distinct cell death programs (Fig. 2). These data demonstrated that human IAPs (XIAP, cIAP-1 and cIAP-2) directly interact with and inhibit active caspases.[34,35] XIAP, cIAP-1 and cIAP-2 were shown to bind and potently block caspases-3, -7 and -9, but not caspases-1, -6, -8 and -10 or CED3. In the case of XIAP, the ability to inhibit distinct caspases was subsequently localized the BIR2 (specific for caspases-3 and –7) and BIR3 domains (specific for caspase-9).[20,36,37] The preference of different BIR regions for their target caspases is more than 1000 fold and to date they are the most specific and potent known caspase inhibitors. Although the IAP-BIR domains reportedly exhibit strong interactions with their respective caspase targets, as reflected by estimated inhibitory constants in the low nM to pM range, their interaction was reported to be non-covalent, fully reversible and did not require IAP cleavage—thereby distinguishing the IAP and p35 caspase inhibitory mechanisms.[34,35]

Among the other human BIR domain containing proteins, NAIP has been reported to suppress apoptosis, however, evidence for direct inhibition of caspases by NAIP is lacking.[35] BRUCE has not been shown to block cell death or inhibit caspase activities—BRUCE functions have yet to be elucidated.[31] Survivin was reported to suppress caspase activation and cell death as well as associate with specific caspases,[38,39] however, direct inhibition of caspases has not been reproducibly documented. Furthermore, evidence is emerging that survivin function as a "spindle-checkpoint" monitor.[40-42] Thus further studies are necessary to determine survivin mechanisms that link its involvement in apoptosis and cell cycle regulation. ML-IAP has one BIR domain followed by a RING domain and was reported to inhibit the effector caspases-3 as well as the initiator caspase-9.[32,43,44] In these studies, ML-IAP was shown to suppress cell death pathways induced by Bax, death receptors or cytotoxic drugs. However, direct inhibition of both caspase-3 and caspase-9 by ML-IAP, a single BIR domain containing protein, is surprising given the distinct preferences between BIR domains for caspase-3 verses caspase-9. The most recent IAP to be identified, ILP-2, is very much like the BIR3-RING domain from XIAP, and similar to BIR3-RING, ILP-2 appears specific for caspase-9 and inhibits this protease in cell-based and cell-free systems.[33]

Recently solved co-crystal structures of the XIAP-BIR2 domain (amino acid residues 124-240) in complex with caspase-3 or -7 have provided the molecular details of IAP-mediated caspase inhibition.[21-23] BIR2 and its N-terminal upstream extension contact the caspase surface (Fig. 3). The N-terminal extension of BIR2, also called linker or hook/line/sinker directly binds to the catalytic site of the caspase, thereby blocking its enzymatic activity.[23] Interestingly, the hook/line/sinker lies across the substrate binding cleft in reverse orientation compared to

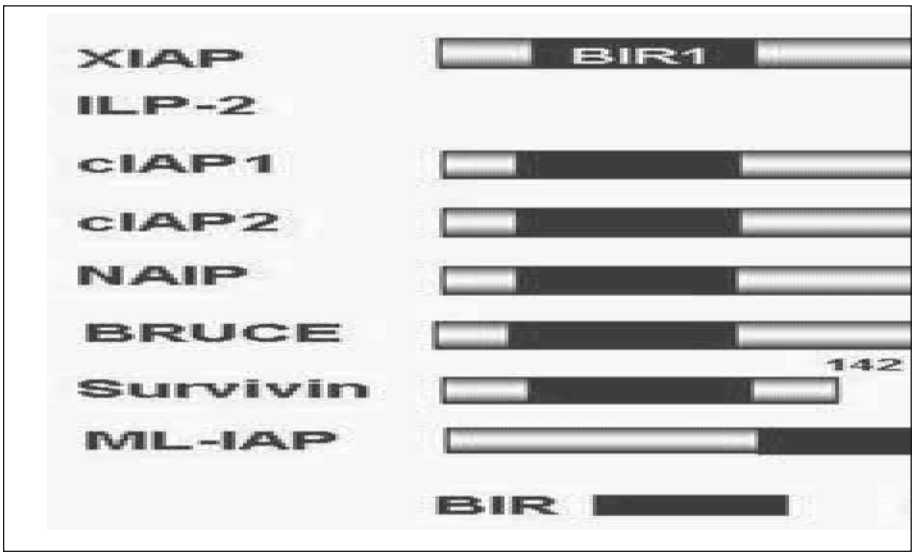

Figure 1. Human Inhibitor of Apoptosis Protein family. IAPs are characterized by a highly conserved Baculoviral Inhibitory Repeat (BIR) represented at least once and as many as three times in some IAPs. Of the human BIR domain containing proteins, all have been reported to suppress apoptosis, except BRUCE. Other motifs present in some IAPs include the Really Interesting New Gene (RING), caspase Activation Recruitment Domain (CARD), and the Ubiquitin Conjugation (UBC) domain.

the substrate peptide sequence. However, a synthetic peptide of the hook/line/sinker substructure is not sufficient for inhibition, suggesting the necessity of the interaction of the BIR2 domain and the caspase surface.[23]

Structure-function studies of IAP family proteins have uniformly demonstrated the requirement of at least one BIR domain for suppression of apoptosis, although the BIR domains can be found linked with a variety of other motifs. These non-BIR motifs presumably diversify the functions of IAPs or provide ways of regulating individual IAP family members. For example several of the mammalian, fly and viral IAPs have a C-terminal RING (really interesting new gene) domain. Some reports have indicated that the baculoviral IAPs require both amino terminal BIR domains and the carboxyl terminal RING domain for their anti-apoptotic function in insect cells.[46] However BIR domains of baculovirus Op-IAP, Drosophila D-IAP1, D-IAP2 and human XIAP, cIAP-1 and cIAP-2 were found to be sufficient for apoptosis inhibition in the absence of their carboxy-terminal RING domains.[34-36,47,48] Thus, the necessity of the RING domain for suppression of apoptosis appears to depend on the cellular context. Moreover, RING domains, as well as other BIR-associated domains, may facilitate BIR involvement in facets of cell death programs or other aspects of cellular functions not readily appreciated in in vitro or cell-based model systems.

IAP RING domains may function by promoting ubiquitination of caspases—thereby modifying caspase activities or targeting them for degradation by the 26S proteasome. In vitro ubiquitination assays revealed that cIAP-2 could function as an ubiquitin-protein ligase (E3) for caspase-3 and -7.[49] The E3-ligase activity of the IAPs was shown to depend upon their C-terminal RING domains, which has been recently recognized to be a general feature of RING domains.[50] These and similar observations inspired further studies demonstrating that the XIAP-RING domain promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death.[51]

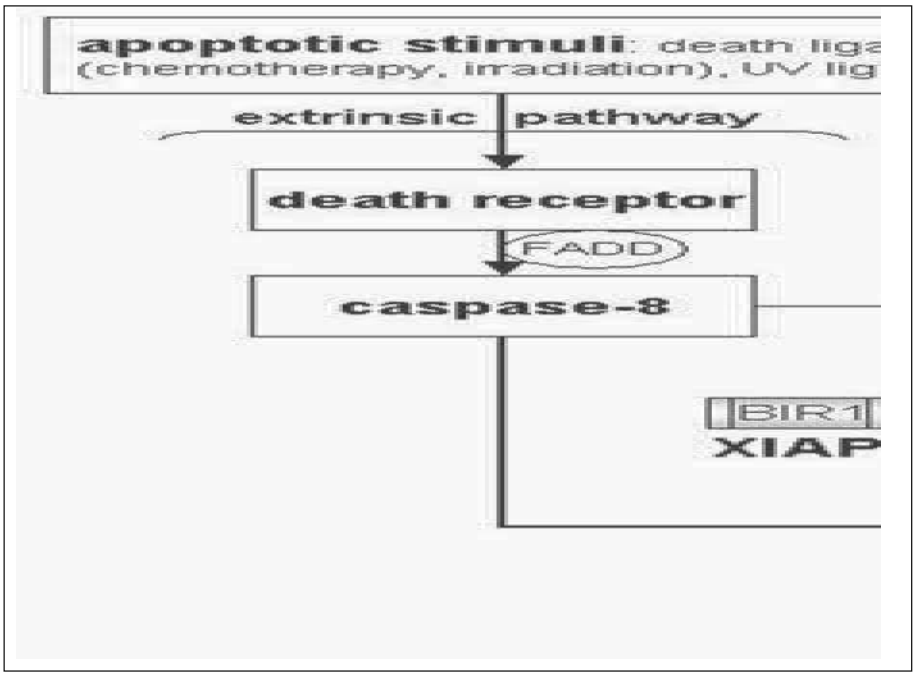

Figure 2. Basic apoptosis pathways and their inhibition by different XIAP domains. Extrinsic cell death programs, exemplified by TNF receptor/ligand family members in Type-I-cells, do not require participation of the mitochondria and cyctochrome c release (intrinsic pathways). Instead receptor ligation activates the apical caspase-8, which then activates other caspases leading to cell death. In Type-II-cells, amplification of receptor-mediated apoptosis employs the intrinsic pathway through cleavage of Bid and possibly other molecules that then target the mitochondria promoting cytochrome-c release and subsequent activation of caspase-9 and other caspases. Drugs or other apoptosis-inducing agents can initiate apoptosis through incorporation of various combinations of these two prototypical pathways depending upon cellular context. Because of its ability to inhibit caspases-3, -7 and –9, XIAP is able to block extrinsic and intrinsic pathways. These activities localize to the BIR2 domain, specific for caspase-3 and caspase-7, and to the BIR3 domain, specific for caspase-9. Thus, XIAP as well as other IAPs, have evolved to be versatile inhibitors of cell death programs and are also regulated by cellular binding partners. For example, mitochondrial released Smac protein binds to and prevents IAP inhibition of activated caspases—thereby promoting intrinsic apoptotic programs and representing another layer of cell death regulation.

The human cIAP-1 and cIAP-2 proteins contain caspase Activation Recruitment Domains (CARDs) between their third BIR and RING domain. The name relates to the ability of CARD domains to facilitate protein-protein interactions. For example, the CARD domain in the Apoptosis Protease Activating Factor (Apaf-1) facilitates its interaction with a caspase-9 CARD domain, forming a cytochrome-c/Apaf-1/caspase-9 complex, which results in caspase-9 activation. However, the function of this protein-protein interaction domain in IAPs is largely unknown and amino terminal cIAP-1 or cIAP-2 constructs lacking the CARD domain are sufficient to block programmed cell death processes.[35] So far no IAP CARD interactions with other CARD containing proteins or self-association through the CARD domain have been described.

Another domain of interest in the IAP family member BRUCE is the ubiquitin-conjugating domain (UBC). This very large (~528 kD) protein contains a N-terminal BIR domain and a C-terminal UBC domain.[31] UBC domains function as E2 enzymes that in combination with E3 ligases facilitate covalent attachment of ubiquitin moieties to specific proteins—thus altering

Figure 3. Co-crystal structure of the BIR2-linker region of XIAP bound to caspase-3.[23] The BIR2 inhibitor makes contacts with the caspase surface through its BIR domain, however, most of the caspase-3 contacts occur through the BIR2 linker region. This linker region lies across the substrate-binding cleft of the caspase—sterically preventing substrate binding. The Smac peptide inhibitor in complex with the BIR3 domain of XIAP revealed a "Smac pocket"[45] (see discussion in IAP post-translational regulation). The location of the analogous Smac binding pocket in the BIR2 domain shown here was revealed by superimposing BIR2 and BIR3 domains of XIAP. The modeling and image generation was generated with the ICM software package from Molsoft, LLC.

their function, localization or targeting them for degradation by the 26S proteasome. Possibly the BRUCE-UBC domain could facilitate ubiquitination of BRUCE-BIR-binding partners—thereby regulating the activities of these proteins by altering their stabilities. However the function of BRUCE, as well as its interacting partners, awaits discovery.

Expression of IAPs

The human cIAP-1 and cIAP-2 proteins appear to be widely expressed in all adult tissues.[30] Especially high levels of cIAP-2 were detected in thymus, spleen and ovary. Additionally, IAP expression is reportedly increased following activation of the NF-κB transcription factor[52,53] (see fig. 2 IAP involvement in signal transduction). NAIP mRNA appears to be expressed at low but detectable levels in adult liver and placenta and can also be detected in spinal cord and brain by RT-PCR.[28,54] XIAP exhibits a cytoplasmic location and inhibits cell death in response to a variety of apoptotic stimuli including UV-light exposure, TNF, Fas-L, staurosporin, growth factor withdrawal and a number of cytotoxic drugs. Western blot expression analysis in the National Cancer Institute 60 tumor cell line panel revealed that XIAP is also expressed in most

human cancer cell lines.[55] XIAP expression is reportedly influenced by radiation exposure, due to a radiation response element present in the promoter region of XIAP[56] (also see IAPs in disease).

Survivin exhibits very restricted expression—being absent in most normal adult tissues but abundant in dividing cells, like embryonic tissues, tumors and transformed cell lines suggesting a cell-cycle-dependent expression.[26,40,57,58] In this regard, reporter gene assays revealed the survivin promoter showed typical M phase inducible transactivation.[40] Another interesting feature of survivin is its gene structure. The coding strand of the survivin gene is entirely complementary and therefore antisense to the EPR-1 gene (effector cell protease receptor-1) and separate promoters control their expression in an exclusionary fashion. Transcripts produced from one of these genes appears to inhibit the expression of the other by an antisense RNA based mechanism.[59]

Based upon mRNA levels, the IAP BRUCE is expressed in most adult tissues with highest levels in brain and kidney.[31] The recently described seventh mammalian IAP (ML-IAP, Livin or BIRC-7) is detectable in embryonic tissue, selected adult tissues and in melanoma cell lines.[32,44,60] The ILP-2 gene, which lacks introns, appears exclusively expressed in the testis.[33]

IAP Involvement in Signal Transduction

The human cIAP-1 and cIAP-2 proteins were first discovered by virtue of their association with TNF receptor 2 complexes.[30] cIAP-1 and cIAP-2 do not directly contact TNF-R2, but are recruited to the receptor by binding to TRAF1/TRAF2 (TNF-receptor associated factors) heterocomplexes.[30] The N-terminal BIR-containing region of these IAPs is required for interactions with TRAFs. The interaction of cIAP-1 and cIAP-2 with TRAF1 and TRAF2 appears to be specific, in that these IAPs do not bind to TRAF 3, 4, 5, or 6 and other IAPs (XIAP, NAIP) reportedly fail to bind TRAFs altogether.[35] Thus, TRAF binding is not a universal feature and the significance of the IAP-TRAF association has not been solved.

Ligation of TNF receptors not only initiates apoptotic programs but also signal transduction pathways leading to NF-κB activation, which represents another layer of cell death and caspase regulation with relationship to inflammatory responses.[61,62] TNF-R associated adaptor proteins appear to play critical roles in these signaling events.[63,64] In this regard, IAP expression has been linked to NF-κB activation and NF-κB activation has been linked to IAP expression. TNFα was shown to induce expression of cIAP-2 through stimulation of NF-κB and over-expression of cIAP-2, as well as XIAP, reportedly can lead to NF-κB activation.[52,65] cIAP-2 expression was also reported to suppress cell death induced by TNFα through the receptor TNF-R1.[65] These cIAP-2 activities were blocked in cells by co-expression of a dominant form of I-κB that is resistant to TNF-induced degradation, implying that cIAP-2 participates in a positive feedback mechanism regulating NF-κB activation by targeting I-κB degradation. Moreover, a mutant of cIAP-2 lacking the C-terminal RING domain inhibited NF-κB induction by TNF and enhanced TNF killing. Based upon these findings the authors suggested that cIAP-2 is critically involved in TNF signaling events that induce NF-κB and that are required for suppression of TNF-induced apoptosis.[65]

In other reports, NF-κB was found to block TNFα-induced activation of procaspase-8.[53] Under conditions where NF-κB activation was prevented with dominant-negative I-κB, ectopic expression of the combination of TRAF1, TRAF2, cIAP-1 and cIAP-2 was needed to substitute for NF-κB and fully suppress TNFα-induced apoptosis. In the same cells, however, either cIAP-1 or cIAP-2 alone were sufficient to suppress apoptosis induced by the chemotherapeutic drug etoposide—a stimulus that appears to enter the apoptosis pathway primarily at the level of mitochondria.[53] The implication is that cIAP-1 and cIAP-2 require TRAF1 and TRAF2 to interfere with the upstream cell death protease, caspase-8, but not for inhibiting caspases that operate downstream of mitochondria. These and other data suggest that the IAPs have the ability to regulate different apoptotic pathways or distinct steps within the same apoptotic pathway, depending on IAP binding partners, such as TRAFs.

IAP Posttranslational Regulation

Posttranslationally, there have been two main mechanisms for IAP regulation that have been reported, the first involves the binding of antagonizing proteins and the second the ubiquitin-proteasome protein degradation pathway. The second mitochondrial activator of caspases (Smac) is a recently described cellular inhibitor of XIAP that is released from its mitochondrial localization concurrently with cytochrome c following appropriate apoptotic stimuli.[66-69] Smac binds to XIAP—thereby preventing IAP interaction and inhibition of caspases, which are activated by mitochondrial release of cytochrome c. The basis for Smac-IAP interaction appears to reside within the newly generated N-terminus of Smac, which is exposed following processing and release from the mitochondria. This motif is similar to the Drosophila IAP interacting and inhibiting proteins HID, GRIM and REAPER, as well as the p10 subunit of active caspase-9[70] (Table 1). Missense mutations in this sequence significantly compromised the function of Smac as in the homolog Drosophila proteins REAPER, GRIM and HID.

Intriguingly, peptides mimicking this N-terminal Smac motif were shown to be sufficient for XIAP inhibition.[69] To understand the structural basis for the interaction of Smac and the IAPs, crystal structures of the BIR3 domain of XIAP complexed with a functionally active nine-residue peptide from the N-terminus of Smac were generated.[45,67,68] These structures reveal that the Smac peptide binds across the third beta-strand of the BIR3 domain with only the first four residues contacting BIR3. Combined, these data have inspired the notion of therapeutic development of small molecules that mimic the Smac peptide.[5] It is interesting to note, however, that the Smac-peptide binding pocket, revealed by the solution co-structure of the Smac peptide bound to BIR3, would not be predicted to disrupt the BIR2 interaction with caspase-3 or -7 based upon the co-crystal structures of BIR2 bound to caspase-3 (Fig. 3). Thus, therapeutic peptides mimicking Smac might not be effective inhibitors of XIAP since BIR2-mediated inhibition of caspases-3 and -7 might still be sufficient to suppress apoptosis.

In addition to Smac, several other proteins have been reported to influence IAP function. A recently reported serine protease called HtrA2/Omi, which is also released from mitochondria, was shown to directly bind XIAP and prevent its interaction with caspases—thereby inhibiting XIAP function and facilitating apoptosis.[71,72] The cellular protein XAF1 (XIAP-associated factor 1) was isolated on the basis of its ability to bind and antagonize XIAP.[73] Expression of XAF1 triggers the redistribution of XIAP from the cytosol to the nucleus and it is ubiquitously expressed in normal tissues, but is present at low or undetectable levels in many different cancer cells.[74] Therefore XAF1 may also be important in mediating apoptosis resistance of cancer cells.

Posttranslational regulation of IAPs may also involve their ubiquitination and enhanced turnover by the 26S proteasome. IAPs were reported to catalyze their own ubiquitination in vitro, which was dependent on their RING domain.[75] These studies reported that over-expressed wild-type cIAP-1 was spontaneously ubiquitinated and degraded, and that stably expressed XIAP, lacking the RING domain or a cIAP-1- RING mutant, were relatively resistant to their apoptosis-induced degradation. Thus, auto-ubiquitination and enhanced degradation of IAPs may be a key factor of their regulation, although further studies have yet to support this notion.These processes as well as other aspects of IAP biology may be influenced by additional modifications such as phosphorylation. Although this type of IAP modification has not been reported so far, some IAPs do contain phosphorylation consensus sequences.

IAPs and Human Disease

IAPs and Neuronal Cell Death

The NAIP gene was first identified as a candidate gene defective in spinal muscular atrophy (SMA).[54] This is a hereditary autosomal recessive neurodegenerative disorder with spinal cord motor neuron depletion and one model of SMA pathogenesis invokes an inappropriate persistence of normally occurring motor neuron apoptosis. Although the primary genetic defect in SMA has been ascribed to a deletion of an adjacent gene SMN (survival motor neuron), the

Table 1. Sequence alignment of the IAP interacting motif of Smac, HID, GRIM, REAPER and the small subunit of human caspase-9

Smac	**A**	**V**	**P**	**I**	**A**	**Q**	**K**
REAPER	**A**	**V**	**A**	**F**	**Y**	**I**	**P**
Grim	**A**	**I**	**A**	**Y**	**F**	**I**	**P**
Hid	**A**	**V**	**P**	**F**	**Y**	**L**	**P**
Human Caspase-9 p12	**A**	**T**	**P**	**F**	**Q**	**E**	G

loss of functional NAIP may contribute to the severity of the disease.[76] The SMN protein has been reported to bind bcl-2 and enhance bcl-2 mediated protection from apoptosis raising the possibility that two survival genes may be lost in more severe cases.[77]

NAIP may also be involved in adaptive responses to brain ischemia. Up-regulation of endogenous NAIP, or intracerebral injection of NAIP encoding adenoviruses, reduced ischemic neuron loss in rat hippocampus suggesting that NAIP may play a role in neuronal cell death protection.[78] In similar studies, over-expression of XIAP also proved to prevent or at least delay ischemic neuronal cell damage.[79]

Human IAPs and Cancer

Tumorigenesis in humans is a multi-step process, which has been proposed to reflect four to seven rate limiting, stochastic gene alterations that drive the transformation of normal cells into malignant derivatives.[80] Hanahan and Weinberg further suggest that the vast differences of cancer genotypes are, in principal, manifestations of six common essential hallmarks of cancer cells:1. self-sufficiency in growth signals, 2. insensitivity to growth-inhibitory signals, 3. limitless replicative potential, 4. sustained angiogenesis, 5. tissue invasion and metastasis and 6. evasion of programmed cell death.[81] Thus resistance towards apoptosis is an acquired capability of most and possibly all cancers and lymphomas.

Because the apoptotic machinery can be broadly divided into two components, pro- and anti-apoptotic, there are two basic mechanisms of apoptosis resistance first the down-regulation of pro-apoptotic molecules by mutation or posttranslational modification and second the up-regulation of anti-apoptotic proteins. These anti-apoptotic mechanisms are not only involved in tumorigenesis but also in resistance to chemo- and radiotherapy, which induce caspase dependent cell death.[82]

Based, in part, upon their prominent function as caspase inhibitors and potent suppression of many apoptotic programs, one might suspect that dysregulation of IAP family members contribute to carcinogenesis and therapy resistance in some tumor types. In this regard, most is known about the expression of the IAP survivin in cancer.[26,57,59,83] Undetectable in normal terminally differentiated tissues, survivin is over-expressed in various human cancers, and its expression levels correlate with an unfavorable prognosis. In squamous cell carcinoma (SCC) survivin expression significantly segregated with high-grade and undifferentiated tumors and was associated with higher frequency of lymph node metastasis.[84] These data indicate that survivin expression may identify cases of SCC with more aggressive and invasive clinical phenotype. In human colorectal tumors survivin expression significantly increased in the transition from adenoma with low dysplasia to high dysplasia/carcinoma suggesting a role in colorectal cancer development.[85] Moreover immunhistochemical assessment of survivin expression in

esophageal cancer revealed a positive correlation with poor prognosis and response to chemotherapy.[86] Survivin expression in esophageal cancer patients, who achieved a partial response (PR), was lower than that in patients with no change (NC) or progressive disease (PD) and survivin expression was one of the significant predictors of survival on univariate analysis.[86]

Although survivin becomes prominently expressed in transformed cell lines, the most common human cancers (lung, colon, pancreas, prostate and breast) and in approximately 50% of high-grade non-Hodgkin's-lymphomas (NHL), it was not found in all tumor types. For example, low grade NHLs, which are known for their activation of other anti-apoptotic genes (e.g., bcl-2 by the translocation t(14/18) in follicular lymphoma), rarely express survivin. These low-grade lymphomas are also tumors with a very low growth fraction, a characteristic that could have bearing on the apparent cell cycle dependent expression of survivin.

Further studies, in which the anti-apoptotic pathway maintained by survivin was suppressed by dominant negative survivin mutants or antisense approaches, provided evidence that down regulation of this IAP enhanced chemotherapy-induced apoptosis and therefore may be beneficial for cancer therapy.[87] In these studies, infection with adenoviruses containing the dominant negative survivin mutant Thr34Ala, selectively induced apoptosis in breast, cervical, prostate, lung and colorectal cancer cell lines. In contrast the cell viability of proliferating normal human cells, including fibroblasts, endothelium or smooth muscle cells was not affected by the adenovirus. When expressed in established melanoma tumors in vivo, survivin Thr(34)Ala inhibited tumor growth by 60-70% and caused increased apoptosis and reduced proliferation of the melanoma cells.[88] Likewise down-regulation of survivin by antisense oligonucleotides in lung cancer cells induced apoptosis and sensitized the tumor cells to chemotherapy.[89] Thus, at least in survivin expressing tumor cell models, interference with survivin expression or function appears a viable therapeutic approach.

XIAP expression has been reported to correlate in a subtype of human leukemias with clinical outcome as much as survivin levels in selected solid tumors. AML patients with lower protein levels of XIAP had significantly longer survival times and a tendency toward longer remission duration than those with higher levels of XIAP.[55] On the other hand, XIAP expression analysis by immunohistochemistry on tumors from early-stage non-small cell lung cancer (NSCLC) revealed an unexpected inverse correlation of XIAP with survival time[90] and expression of cIAP-1, cIAP-2 and XIAP did not predict the response to chemotherapy in patients with advanced NSCLC.[91] In regard to therapeutic resistance mechanisms, a radiation response element has been reported in the promoter region of XIAP and is proposed to play a role in radiation resistance.[56]

cIAP-1 and cIAP-2 have been implicated in cancer initiation and progression. cIAP-1 was identified as a candidate target gene within an amplicon at chromosome location 11q22 in esophageal squamous cell carcinomas (ESC).[92] Cell lines derived from ESC containing the cIAP-1 amplification were also more resistant to apoptosis induced by chemotherapeutic reagents. Based upon these observations the authors suggest that cIAP-1 may be involved in the progression of ESC.[92]

The cIAP-2 gene is rearranged in approximately 50% of cytogenetically abnormal low-grade MALT lymphomas.[93] The translocation t(11;18)(q21;q21), which was suggested to be the key genetic lesion, results in fusion between the apoptosis inhibitor gene cIAP-2 and a novel 18q paracaspase. Since the function of the paracaspase protein has yet to be elucidated, the significance of its fusion to cIAP-2 is unclear. However, this IAP-paracaspase fusion protein may have increased stability or, due to the truncation of the potentially negative regulatory CARD and RING domains, posses enhanced anti-apoptotic function.[93]

Cellular FLIP

The second class of cellular caspase modulators are the FLICE (caspase-8) Inhibitory Proteins (FLIPs), which block death receptor signaling upstream of caspase-3. FLIPs have been characterized in herpesviruses (e.g., human herpesvirus 8) poxviruses (e.g., molluscum

contagiosum virus) and mammals FLIPs (FLIP-$_L$ and FLIP–$_S$).[94-96] The cellular full-length 55 kD long form of FLIP (FLIP-$_L$) exhibits high structural homology to caspase-8, containing two death effector domains (DED) and a nonfunctional caspase like domain. The alternatively spliced short form of cellular FLIP (FLIP–$_S$) contains only the two DEDs, that interact with either the adaptor molecule FADD or the initiator caspase-8 and –10, and displays reduced anti-apoptotic capacity. Through their binding to FADD and/or caspase-8, FLIPs inhibit autocatalytic activation of pro-caspase-8 in the DISC (death inducing signaling complex) complex and disconnect the receptor signal from the death machinery (for more details see also chapter III). Thus technically FLIPS are not direct caspase inhibitors but dominant negative regulators of caspase-8 activation. So far the function of numerous death receptors such as Fas (CD95, Apo-1), TRAIL (DR4 and DR5), TNFR1 and DR3 (TRAMP) was reportedly blocked by the FLIPs.

Like several of the IAPs, c-FLIPs are reportedly regulated by NF-κB, which is a major effector of the inducible resistance to death receptor mediated apoptosis,[97] and by the PI-3-kinase/Akt pathway in tumor cells, where constitutively active Akt increased FLIP expression.[98] Posttranslationally, c-FLIP was reportedly regulated by MEK1 in activated T-cells and via a p53 dependent ubiquitin proteasome pathway.[99]

Because many tumors and activated T lymphocytes express Fas and TRAIL receptors, but are resistant to apoptosis induced by exposure to Fas or TRAIL ligand, it has been proposed that FLIPs could be involved in this resistance mechanism.[100-102] In this regard, high levels of FLIP-$_L$ protein were reported in malignant melanoma cell lines and tumors.[96] Thus FLIP may contribute to cancer development and drug (e.g., chemotherapy, TRAIL ligand/intrinsic antibodies) resistance mechanisms.

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CHAPTER 6

Virus-Encoded Caspase Inhibitors

Grant McFadden and Richard W. Moyer

Introduction

There have been many excellent reviews on caspase structure and function[1-6] and key features will only briefly be discussed here to set the framework for our discussion of virus encoded caspase inhibitors. Caspases (Cysteine-dependent Aspartate Specific Proteases)[7] are members of the C14 protease family based on the Barrett and Rawlings classification.[8] There are currently 13 known mammalian caspases. Caspases-1, -4, -5, -11 and -12 are considered to be associated with regulation of inflammation and function to cleave and activate pro-inflammatory cytokines. Caspases-2, -3, -6, -7, -8, -9 and -10 are considered to be pro-apoptotic caspases which function to cleave and activate pro-apoptotic substrates. Pro-apoptotic caspases can be further divided into initiator caspases (-2, -8, -9 and -10) that function to cleave and activate effector caspases (-3, -6 and -7). All caspases are characterized catalytically by a nearly absolute specificity for proteins and peptide substrates containing aspartic acid in the P1 or P1-P1' cleavage position[9] and the use of a cysteine side chain nucleophile to assist hydrolysis of the peptide bond.[10] Therefore, caspases are thiol proteases. The specificity for aspartic acid is quite rare and among proteases is shared only by granzyme B, a serine protease[11,12] and itself an activator of caspases.[13,14] Granzyme B, a component of cytotoic T lymphocyte involved in apoptosis[14] will not be further discussed here.

A concise, informative grouping of the caspase family has been articulated by Chang and Yang.[1] All caspases are synthesized as inactive zymogens which are activated through proteolytic cleavage. The initial proenzyme consists of a protease domain preceded by an N-terminal prodomain of varying length (2-25 kD), which is cleaved and removed during zymogen activation. Internal cleavage of the protease domain into subunits of approximately 20 and 10 kD is also required to produce active enzyme. Some caspases have a short linker between the small and large subunits which is likewise eliminated during activation. All domains are linked by Asp-X bonds, a specificity reminiscent of the caspases themselves. Indeed, this specificity is consistent with a hierarchical activation of the so-called executioner caspases by the initiator caspases seen during apoptosis.

Although cleaved during activation, some of the longer N-terminal prodomains, which can range from six to over 200 amino acids, are functional domains in the sense they are required for activation and in some cases recruitment of the caspases. Generally, inflammatory caspases and caspases involved in initiating apoptosis have longer domains (>100 amino acids), whereas effector caspases, those involved in the execution phase of apoptosis are shorter, generally 30 amino acids or less. Two types of functional elements are found in the prodomains; (1) the caspase Recruitment Domain (CARD) and (2) the Death Effector Domain (DED). These domains are structurally similar in the sense that both are six helix bundles[15-18] but DED domains are primarily hydrophobic whereas CARD domains are hydrophilic. DED domains are found only within caspases-8 and –10, apical caspases associated with death receptor triggered cell death where they function in the recruitment of the caspase to the receptor together

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with adapter molecules. The function of the CARD domain is less specific as they are found in caspases which are involved in cytokine regulation (caspases-1, -4, -5) as well as caspases which lead to cell death (caspase-2 and -9). The function, if any, of the short N-terminal peptides, found in other caspases is not known. Since the viral caspase inhibitors, CrmA/SPI-2, p35 and the IAPs, interact with functional caspases, we will not discuss further either the prodomains or the regions which separate the small and large subunits of the mature caspases as all are removed during the activation process.

The three dimensional features of all active caspases are surprisingly similar and have been succinctly summarized[2] and are only recapitulated here. Caspase-1 provided the first crystallographic insight into these common features.[19,20] Later information from caspase-3,[21,22] -8[23,24] and -7[25] allowed further definition and refinement of a number of these common features. The basic structural unit is a heterodimer containing one large, 20 kD (p20) and one small, 10 kD (p10) subunit and allows definition of the "caspase fold". Active enzymes contain two heterodimers p10/p20, which then topologically defines a rather unique overall quaternary structure (p10/p20) and topology. A single heterodimer consists of five parallel (a-e) and one antiparallel (f) β-sheets which form a twisted β-sheet structure with two α helices (H2 and H3) on one side and three α helices (H1, H4 and H5) on the other, roughly in parallel to the β strands. Each heterodimer contains an active site, meaning that each enzyme is comprised of two heterdimers contains two active sites. The active site is located at the C-terminal end of the parallel β strands within each p10/p20 heterdimer. Although the fold and overall structural features are common for all caspases, there are structural differences around the active site.

The substrate is recognized by a cleft formed by the loop regions of the p10 and p20 subunits. The cleft recognizes a tetrapeptide located N-terminal to the canonical cleavage site Asp-X.[2] The four amino acids to the left of the cleavage site define the specificity of caspases with P_1 (aspartic) being nearly inviolate. After P_1, the P_4 residue is the most important[4,26] with the most stringent specificity being for the group II caspases where a P_4 of aspartic acid is found in most protein substrates.[4]

CrmA:/SPI-2 -Family: A Poxvirus Encoded Inhibitory Serpins and Caspase Inhibitors

The CrmA (cytokine response modifier protein A) protein of cowpox virus (CPV) is a member of the serine proteinase inhibitor (serpin) superfamily, and is classified as a member of Clade N.[27] In other orthopoxviruses, such as vaccinia, ectromelia and rabbitpox viruses, this gene is known as the SPI-2 (Serine Proteinase Inhibitor–2). For this review, *crmA* will be used throughout to refer to all orthopoxvirus *crmA/spi-2* genes.

Serpin Mechanistic Considerations Relevant to CrmA

The CrmA protein acts as an inhibitory serpin and achieves inhibition of proteinase activity through acting as a suicide substrate. All inhibitory serpins possess a metastable, energy-rich conformation that is required for their inhibitory activity.[28,29] The classic serpin conformation is highly conserved among family members and comprises a conserved secondary structure consisting of β-sheets A, B and C plus at least seven α-helices (most serpins have 9), designated A to I. One of these helices, helix D, in some serpins, is thought to be associated with modulation by other proteins. In the cases of both antithrombin and heparin cofactor 2, the D-helix contains heparin-binding elements which serve to activate these serpins for inhibition of their respective proteinases.[30-32] The nuclear translocation signal in protease inhibitor-10 resides in the interhelical loop between the C and D helix,[33] as does the region of plasminogen activator inhibitor 2 associated with inhibiting apoptosis. Hence, this region of the protein is associated with co-factor regulation or modulation. The Reactive Center Loop (RCL), the region of the serpin which directly interacts with the proteinase, consists of approximately 17 amino acid residues and is flexible and exposed, tethered in between β-sheets A and C. The accepted nomenclature for serpins[34] defines an approximate RCL length of 17 amino acids where the P_1

and P_1' residues are those cleaved during scissile bond formation reaction with the proteinase. The P_1 residue in particular is very important in defining susceptibility and specificity towards a particular proteinase.

The mechanism of inhibition of caspases by CrmA is through the typical irreversible suicide substrate mechanism (Fig. 1). Inhibitory serpins first form a noncovalent Michaelis-like complex through interactions with amino acid residues which flank the scissile bond (P_1-P_1'). In the case of caspases, attack by the caspase active site cysteine (rather than serine, typical of most proteases) leads to a covalent thiol ester linkage between the active site cysteine of the proteinase and the P_1 residue. Cleavage of the P_1-P_1' serpin peptide bond is typical of the initial stages of ester or thiolester bond hydrolysis. The P_1 residue of CrmA is aspartic acid, a rather unusual P_1 residue for serpins. It is believed at this stage, that the RCL begins to insert into the β-sheet A together with the attached proteinase. The loop insertion leads to a profound 70 Å translocation of the proteinase with a concomitant distortion of the active site. Proteinase inactivation results from compression and restraint of the proteinase against the body of the serpin which in turn is dependent on the length of the RCL. The energetics of the process derive from the fact that the cleaved loop-inserted conformation is less energy rich than the native structure of the serpin. The end result of the conformational rearrangement is the kinetic trapping of the acyl intermediate as the deacylation step leading to hydrolysis is slowed by 6-8 orders of magnitude. In practical terms, the half-life of such trapped intermediates may be hours to weeks.

Precisely when the proteinase-serpin complex is committed to the stable intermediate is not known, but this commitment is not absolute. A non-inhibitory pathway in which the proteinase substrate is hydrolyzed releasing the cleaved serpin and active proteinase always accompanies the inhibitory pathway of a serpin. If the RCL movement is impeded in some way, successful deacylation may occur before the intermediate is irreversibly trapped. The ratio of inhibitory serpin complex (loop insertion) to the cleaved serpin (hydrolysis) is a measure of the relative competition between the two pathways. This ratio is defined as a stoichiometry of inhibition or the number of moles of serpin needed to inhibit one mole of proteinase as a kinetically trapped complex.

Normally, serpin-proteinase complexes are quite stable and can readily be detected as "band shifts" on SDS-denaturing gels. However, inhibition of caspases operates through a thiolester bond, where the intrinsic instability of thiol esters[35] makes simple detection more difficult. These complexes, unlike simple esters, do not survive the conditions typically employed to perform SDS-polyacrylamide gel electrophoresis. Such thiolester complexes can, however, be detected on non-denaturing gels.[36,37]

Recently, the cleaved form of CrmA has been crystallized.[38,39] CrmA has been defined as a minimal serpin because it lacks the entire D-helix, half of the A-helix and a small portion of the E-helix, all of which are highly conserved throughout the serpin superfamily. Despite these deficiencies, the overall serpin architecture and fold[40] have been maintained. Since the D-helix missing in CrmA is responsible for interactions with other molecules, it has been proposed that CrmA is not regulated by external cofactors[38] (Fig. 2). However, one of the most profound differences between CrmA and other serpins is the addition of a novel antiparallel β-strand of β-sheet A (residues 53-57),[39] designated S1'a, which could functionally substitute in place of the missing D-helix. This new strand, which extends from amino acids 53-57, is characterized by the typical antiparallel hydrogen bonding with S1'a. This feature and region of CrmA is unique, even compared to the other highly related members of the orthopoxvirus CrmA/SPI-2 family. Generally, other orthopoxvirus members of this family are >90% identical to CrmA, with the exception of this region which is quite divergent.[41] Finally, CrmA contains a highly charged antiparallel strand for b-sheet A of length and sequence unique to CrmA.[38,39]

The Biology of CrmA

The crmA gene was originally discovered as being required for development of red, hemorrhagic pocks on the chorioallantoic membrane of cowpox virus infected embryonated chicken

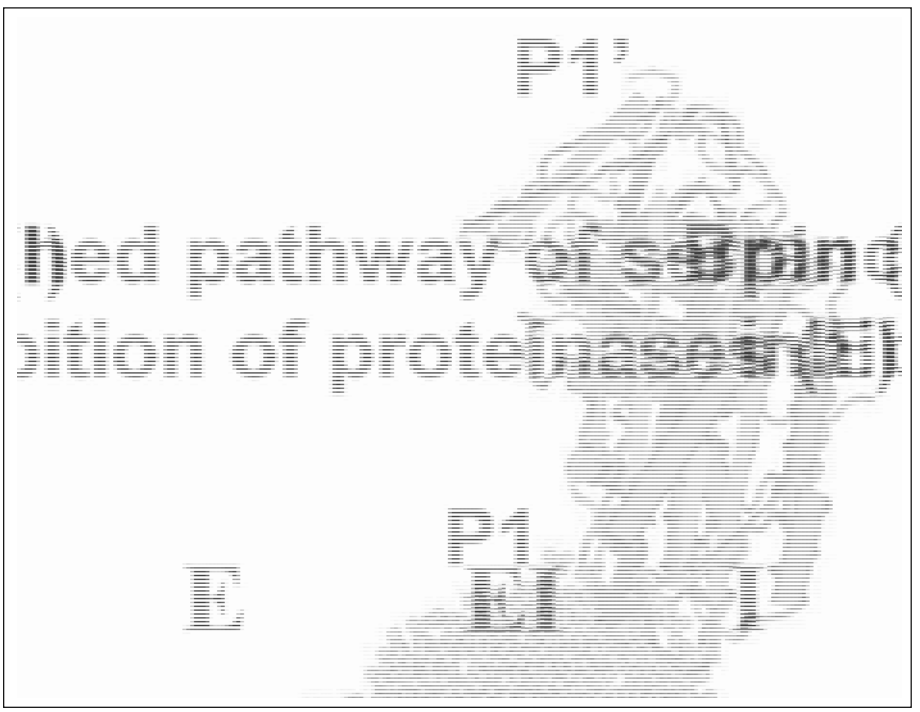

Figure 1. General inhibition mechanism of serpins. All serpins act as 1:1 pseudosubstrate inhibitors (I) against proteinase enzymes (E) that first form enzyme/inhibitor (EI) complexes which partition into either the inhibitory complex (EI*) pathway or the cleaved substrate (E +I) pathway. When formed, the EI* complex is very long lived and thus serpins act as suicide inhibitors.

eggs. The relatedness of the CrmA protein to serpins was also immediately recognized.[42] Deletion of the CrmA gene of cowpox virus led to production of white, rather than red, pocks which were characterized by development of an intense inflammatory cell influx into the developing lesion[42-44] (Fig. 3). Subsequently, CrmA was shown to be a potent inhibitor of IL-1β activation via caspase-1.[45] SPI-2/CrmA is also likely to be a physiological inhibitor of caspases-8 and possibly –10.[36,46,47] It is the activity against caspase-8 and perhaps -10, which is responsible for the well-documented anti-apoptotic activity of CrmA in a variety of ectopic settings such as following withdrawal of serum,[48] withdrawal of nerve growth factor,[49] death receptor ligation[50] and detachment from the extracellular matrix.[51] In a similar fashion and in a more natural setting, deletion of CrmA from cowpox virus leads to induction of apoptosis in infected swine kidney cells.[52] CrmA also inhibits the serine proteinases granzyme B and the protease E of Streptomyces griesus.[53,54] Hence, this viral serpin is classified as a cross class inhibitor being active against both thio- and serine-proteinases. Recently cleaved derivatives of CrmA have also been crystallized.[38,39] Deletion of CrmA from cowpox, vaccinia or rabbitpox viruses leads to differing levels of attenuation (ranging from modest to none) in infected mice[55,56] (Turner, P.C. and Moyer, R. W., unpublished results). The virus-specific attenuation will be further discussed below.

CrmA Specificity Towards Caspases

While it is clear that the serpin P_1 residue is important in determining specificity, it is likewise equally clear that other interactions are also extremely important. CrmA is an excellent

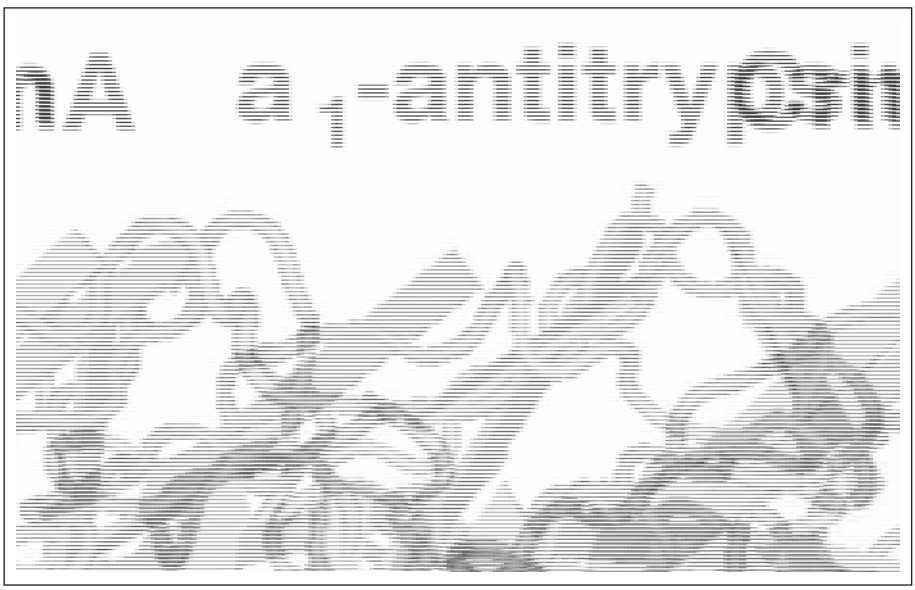

Figure 2. Structural comparison of the cleaved forms of cellular and viral serpins. The cleaved forms of a-antitrypsin and CrmA are compared, illustrating the absence of helix D (hD) in CrmA which is replaced by the S_1'a helix. For details, see refs. 38 and 39.

inhibitor of caspases-1 and -8 but a weak inhibitor of caspase-3.[36] Examination of the RCL of CrmA that shows the sequence Leu-Val-Ala (P_4-P_2) precedes the P_1 aspartic acid residue. This sequence is not ideal for caspase-3. However, when the P_4-P_2 residues were changed to Asp-Glu-Val, which is the preferred substrate for caspase-3, the inhibition rate against caspases-1 and -8 dropped dramatically with only a moderate increase in the inhibition of caspase-3.[38] On the other hand, when the RCL amino acids P_4-P_1 (Leu-Val-Ala-Asp), were changed to those of the baculovirus anti-apoptosis protein p35 (Asp-Gln-Met-Asp), lymphoid cells were protected against radiation and dexamethasone induced apoptotic death mediated by direct activation of caspase-3. Again, however, the P_4-P_1 change Asp-Glu-Val-Asp offered no added protection.[57] Recently, a change of the P_1 Asp residue to Glu was shown to be significant during attempts made to direct specificity of CrmA inhibition towards more distant relatives of caspases. Specifically, the P_1 mutant Asp-> Arg was designed towards the arginine-specific clostripain and gingipains; the Asp-> Lys mutant was targeted towards the lysine specific gingpain-K; and the Asp-> Gln mutant was constructed in an attempt to inhibit the asparagine-specific legumain. Of these constructs, only the Asp-> Lys mutant was effective against gingpain-K.[35] While these results highlight the significance of the RCL in determining activity against caspases, there is not a strict correlation between RCL optimization and specificity towards individual caspases. This lack of correlation supports the notion that interactions separate from those that govern substrate specificity permit inhibition of some but not all caspases.

A possible model for the specificity of CrmA based on X-ray crystal structure has been proposed[38] (and Guy Salvesen, personal communication). Basically, CrmA can readily dock to both caspases-1 and -8; however docking to caspases-3 and -7 is prevented by one particular surface loop that is specifically found in these executioner caspases. This 10 amino acid insertion present in caspase-3, but absent in caspase-1, and defines the important S4 sub-site specificity of caspase-3. This loop of caspase-3 overlaps with Phenylalanine 227 at the end of the S1β loop in CrmA causing unfavorable interactions and thereby explaining the inability of

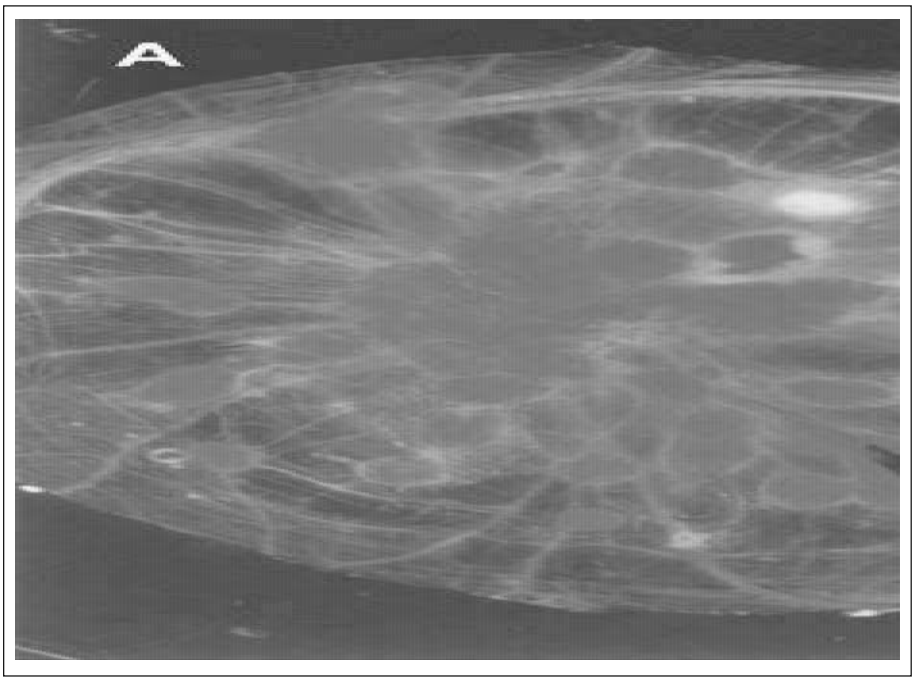

Figure 3. CrmA-minus mutants of cowpoxvirus form white pocks on chicken chorioallantoic membranes The initial report describing CrmA mutants of cowpox noted that the red pocks caused by wild-type virus cowpox infection on chicken chorioallantoic membranes turn white due to increased infiltration of inflammatory cells. When infected CAMS are stained with nitrotetrazolium blue, white inflammatory plaques (A) turn dark blue (B). For further details, see ref. 42.

CrmA to inhibit caspase-3. Implicit in this model is the importance of secondary interactions distal to the RCL, which can negate or modify interactions predicted solely by sequence considerations.

The Role of CrmA in Infections and Cytokine Regulation

Following the discovery that CrmA was a potent inhibitor of caspase-1, a member of the expanding caspase family, it was anticipated that CrmA would act as a powerful regulator of IL-1β or apoptosis during natural infections. IL-1β regulates functions associated with inflammation and immune processes. The importance of IL-1β in poxvirus infections was further emphasized when it was found that orthopoxviruses also encode an additional secreted protein (ORF B15R) which serves to bind extracellular IL–1β.[58-60] Deletion of the B15R ORF leads to infections of mice characterized by elevated fever and increased morbidity which was not effected by deletion of CrmA.[61,62] No such effects were noted upon CrmA deletion. It is now believed that the role of the B15R ORF is to control fever during infections and that CrmA may function to control more localized inflammation as reflected in the remarkable mediation of inflammation on chicken chorioallantoic membranes by CrmA.

In addition to cleavage of pro-IL-1β, caspase-1 also cleaves pro-IL-18, a cytokine structurally related to IL-1β. Both precursors are cleaved by caspase-1 to yield active cytokine.[63,64] One role of IL-18 is to control the levels of interferon gamma (IFN-γ). It is an interesting parallel to note that IFN-γ action, like IL-1β, is controlled extracellularly in poxvirus infections by a secreted IFN-γ binding protein[65-70] as well as by the additional intracellular viral proteins E3L[71-77]

and K3L.[75,78-82] Recently, a secreted protein from ectromelia virus (p13), distinct from CrmA, has been characterized that specifically binds to and inactivates IL-18 and inhibits subsequent NK cell responses.[83,84] Collectively, these results suggest again that, like IL-1β, the effects of CrmA on IL-18/IFN-γ mediated pathways are again limited to localized, rather than systemic effects.

Despite the numerous examples of CrmA regulating apoptosis when expressed ectopically, within the context of a natural infection, data indicating CrmA control of apoptosis during virus infection is very limited. It has been clearly demonstrated that induction of apoptosis in cowpox virus (CPV) infected swine kidney cells occurs readily in the absence of CrmA[52] and that CrmA functions to control caspase induction in these cells.[85,86] It was also suggested that CrmA has an effect on Fas/APO-1 and granule mediate killing by cytotoxic T-lymphocytes.[87] However, more recently, it appears that inhibition of CTL mediated lysis of target cells is limited to alloreactive but not MHC-restricted CTLs.[88]

Differences of CrmA Genes Between Cowpox (CPV), Rabbitpox Virus (RPV) and Vaccinia

As more complete poxvirus genomic sequences become available it is clear that many, but not all poxviruses encode serpins (Table.1). The most notable exceptions are molluscum contagiosum and the parapoxviruses. Viruses within the orthopox and leporipox genera each encode three, whereas the number encoded by other genera is variable ranging from one in the capripox- and suipoxvirus genera to five within the avipoxviruses. Based on predicted P_1 residues, the most widely conserved serpins are those with P_1=Asp, typical of CrmA.

Within the orthopoxvirus genus, the CrmA genes found within individual orthopoxviruses, i.e., cowpoxvirus, vaccinia and rabbitpoxvirus, are highly conserved. A number of experiments suggest these proteins may not be functionally equivalent. While CrmA of CPV can control inflammation, the equivalent SPI-2 (B13R) gene of VV does not.[42,89] Unlike wild-type CPV infections, when swine kidney cells are infected with cowpox virus deleted for CrmA, apoptosis results. However, wild type rabbitpox virus (RPV), which contains a fully functional CrmA gene, cannot block apoptosis in infected swine cells. Additionally, the CrmA of CPV could control all apoptosis induced by either RPV or RPV deleted for CrmA suggesting that the two proteins are not functionally equivalent.[85]

In various animal models, non-equivalence has again been suggested. Examination of recombinant ectromelia and vaccinia viruses containing IL-4 show a markedly increased pathogenicity of ectromelia IL-4 recombinants compared the vaccinia IL-4 recombinants. This difference is proposed to result from the failure of the CrmA/SPI–2 of vaccinia to control Fas mediated caspases in infected target cells[90] even though the respective crmA genes are very similar except for the S_1'A region.[41] Finally, using an intranasal model of infection, deletion of CrmA from CPV led to a modest attenuation[91] whereas deletion of the comparable gene from VV gave no attenuation.[56]

One is able to tentatively speculate that these differences might reside within the molecule. While highly conserved, there are two divergent regions within the orthopoxvirus genus CrmA molecules. The first is within the RCL (amino acids P_5 and P_6) and the second is from amino acids 50-60. Amino acids 50-60 comprise the novel S_1'a strand discussed earlier which are unique to these serpins.[39] The sequence heterogeneity in these regions most likely accounts for the differences in function noted among the various CrmA molecules.

CrmA-Like Serpins within other Poxvirus Genera

If one compares the sequences of CrmA-like serpins between different genera, there is a great deal of sequence divergence. The best studied examples are those of SERP2 of myxoma virus and CrmA/SPI-2 of rabbitpox virus. SERP2, unlike CrmA, is a potent virulence factor in the pathogenesis of myxoma virus in rabbits.[92,93] While both serpins contain a P_1 residue of Asp, the sequence of the two proteins is quite divergent, although both are clearly serpins.

Table 1. Viral members of the CrmA/SPI-2 family of caspase inhibitors

	AT	CPV CrmA	VV SPI-2	RPV SPI-2	ECV	Variola	Camelpox	MYX SERP2	LSDV	YLDV	SPV	FPV010	FPV251
							% Identity						
AT		**28.7**	**27.7**	**27.4**	**28.2**	**28.0**	**27.9**	**27.7**	**30.2**	**31.5**	**25.0**	**30.1**	**32.4**
CPV crmA	39.5		**93.3**	**93.0**	**93.0**	**90.3**	**92.1**	**34.7**	**39.3**	**40.3**	**36.9**	**29.4**	**27.4**
VV SPI-2	38.4	94.4		**98.0**	**94.5**	**92.4**	**93.9**	**35.4**	**37.0**	**40.1**	**36.0**	**28.6**	**26.0**
RPV SPI-2	38.4	93.8	98.3		**94.2**	**93.9**	**95.3**	**35.1**	**37.7**	**40.4**	**36.3**	**28.6**	**26.0**
ECV	38.6	94.1	95.1	94.5		**93.0**	**95.3**	**34.5**	**38.1**	**39.9**	**35.0**	**27.5**	**26.0**
Variola	38.4	92.1	93.6	94.8	93.6		**96.2**	**34.9**	**38.0**	**38.3**	**35.4**	**28.3**	**28.1**
Camelpox	38.3	93.5	94.8	95.9	95.6	96.5		**34.9**	**38.7**	**38.6**	**36.3**	**27.5**	**26.7**
MYX SERP2	38.3	45.0	46.3	46.0	44.8	46.2	45.6		**37.7**	**45.8**	**34.8**	**30.2**	**35.8**
LSDV	40.4	49.5	47.6	48.2	48.9	48.8	49.2	48.2		**45.2**	**44.3**	**31.0**	**33.8**
YLDV	42.4	52.1	51.7	51.7	52.7	51.7	51.7	56.4	57.3		**39.9**	**29.5**	**31.0**
SPV SPI-7	37.2	48.7	47.1	47.5	48.4	47.8	48.7	46.8	55.1	50.6		**28.2**	**32.1**
FPV010	42.1	41.7	40.1	40.4	39.0	39.5	39.0	41.8	43.6	43.2	40.6		**91.8**
FPV251	42.8	37.0	34.9	35.6	35.6	37.7	36.3	47.3	46.2	43.7	42.9	92.5	
							% Similarity						

Percentage of similarity and identity of the poxvirus P1=Asp serpins are indicated. The serpins are divided into two clusters. The first cluster contains orthopoxvirus serpins from cowpox virus (CPV), vaccinia virus (VV), rabbitpox virus (RPV), ectromelia virus (ECV), variola and camelpox viruses. The second cluster lists serpins from other poxvirus genera; myxoma virus (MYX), lumpy skin disease virus (LSDV), Yaba-like disease virus (YLDV), swinepox virus (SPV), and fowlpox viruses (FPV). Anti-trypsin (AT) is also included for comparison.

SERP2, like CrmA, also inhibits caspases-1 and -8, although SERP2 is less effective at inhibiting either caspase than is CrmA.[37] Infection of RK-13 cells by myxoma virus deleted from SERP2 leads to apoptosis, a system analogous to infection of swine kidney cells by CPV deleted from CrmA. Infections of RK-13 cells by myxoma virus recombinants replacing SERP2 with CrmA do not undergo apoptosis. However, the reciprocal experiment, where swine kidney cells are infected with a CPV construct in which SERP2 replaces CrmA, leads to apoptosis. Analysis of the CPV SERP2 recombinant on chicken chorioallantoic membranes likewise shows inflammatory pocks, indicating that SERP2 cannot control inflammation under these conditions (R. Nathaniel and R. W. Moyer, unpublished results).

The sole serpin encoded by swinepoxvirus has a predicted P_1-Asp and an RCL most similar to that of CrmA, although the sequence is otherwise quite diverged from that of CrmA. Numerous attempts have failed to demonstrate any inhibitory activity of this serpin towards caspases (P. Musy, P. Turner and R. W. Moyer, unpublished results). Clearly, these related serpins are not functionally equivalent and, while species specifity might account for this, another possibility is that there may well exist as yet unidentified target proteinases or functions of the poxvirus serpins.

Viral Inhibitor of Apoptosis Proteins (vIAPs)

The inhibitor of apoptosis (iap) genes were first discovered in baculoviruses (Cydia pomoella granulovirus, CpGViap) based on an assay designed to rescue Autographa californica nuclear polyhedrosis virus (AcMNPV) deleted for p35 (the classical annihilator mutant).[94,95] At least 10 distinct baculoviruses are known to contain one or more iap genes, which can be subdivided into three clades. In addition to baculoviruses, IAPs have been found in entomopoxviruses[96,97] and African swine fever virus[98] which do not readily fit into the three clades defined by the baculoviruses, suggesting significant sequence if not functional divergence (Fig. 4). The family of IAP proteins has been expanded by their discovery in organisms as diverse as yeast and mammals (for reviews see refs. 99-104).

The IAP family of proteins is characterized by a novel domain of approximately 70 amino acids termed the "Baculovirus IAP Repeat" or BIR, the terminology stemming from the original discoveries of Miller and colleagues.[94,95] While up to three BIR domains can occur in cellular IAPs, only one or two are found in viral IAPs. The baculovirus IAP, with demonstrated anti-apoptotic activity, such as those from CpGV and OpMNPV, each have two BIR domains[94,95] as does both the IAP-1 and IAP-2 from Epiphyas postvittana MNPV (EppoMNPV-1 and -2).[105] On the other hand, the IAP from African swine fever virus which contains only one BIR, is also active.[106] Other viruses that encode IAP proteins with only one BIR include those from the entomopoxviruses[96,97] and the Chiloiridescent virusIAP, a member of the iridovirus family.[107] The defining BIR core structural motif ($Cx_2Cx_6Wx_3Dx_5Hx_6C$) reveals an unusual spacing of histidine and cystine residues which produces a novel zinc-binding fold.[108-110] These structural studies suggest that the 70 residue core region forms four short α-helices linked by a number of loops. Within the BIR core are interactions between the hydrophobic residues and a zinc atom coordinated by three cysteines and the histidine. The surface of the BIR contains a large number of hydrophobic regions and conserved charged amino acids which may participate in interactions of the IAPs with other proteins. While a BIR domain is required for inhibition of apoptosis, not all BIR domains have a anti-apoptotic function (see below).

Some of the vIAPs also contain a RING (Really Interesting Gene) finger domain. The RING domain[111] has been associated with cellular ubiquitination reactions[112] and functionally the RING domains can be involved in control of apoptosis under some conditions but are not required in others, indicating an environment specific function (for review see ref. 100). Additional levels of complexity in cellular proteins is provided in certain of the human cellular IAPs which can additionally contain a CARD,[113] a ubiquitin-conjugating (UBC) domain such as found in BRUCE, an unusually large (528 kD) BIR containing protein[114] or a NACHT domain. NACHT refers to a novel NTPase domain found in neuronal IAP (NIAP).[115] The

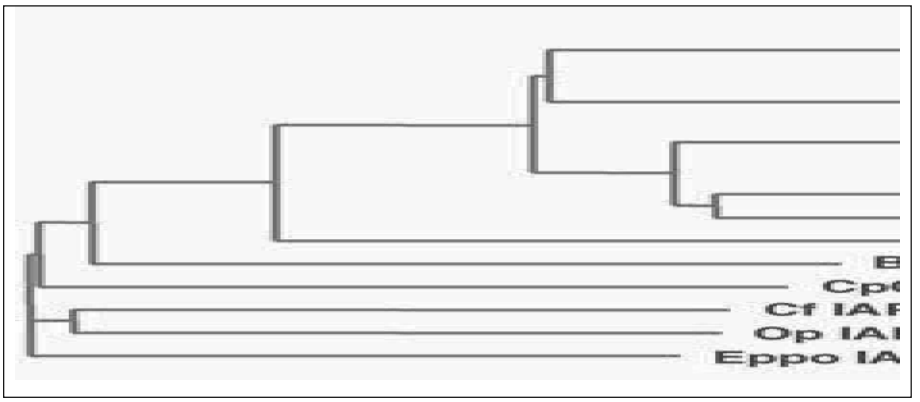

Figure 4. Phylogenetic analysis and grouping of baculovirus, entomopoxvirus and African swine fever virus IAPs. The baculoviruses Ac, Autographa californica MNPV; Cf, Choristoneura fumiferana MNPV; CpGV, Cydia pomonella granulovirus; Eppo, Epiphyas postvittana MNPV; Op, Orgyia pseudotsugata MNPV; Busu, Buzura suppressaria SNPV. Non-baculoviruses include MSV, Melanoplus sanguinipes EPV; AmEPV, Amsacta moorei EPV; ASFV, African swine fever virus. The tree was generated using AlignX, a component of Vector NTI v5.5 (Informax Inc). It is difficult to definitively place any of the non-baculovirus IAPs within any of the three accepted baculovirus clades indicating significant divergence.

focus of this review is on the viral IAP proteins and we only discuss cellular IAPs within context of understanding the function and mechanism of action of vIAPs as caspase inhibitors.

The classical definition of an IAP protein is that it contains a BIR element and functions to inhibit apoptosis. Mammalian IAPs typically contain two or more of these elements. Studies with a variety of IAPs suggest that they inhibit caspases-3, -7 and -9 but not caspases-1, -6, -8, -10.[116-118] The minimum number of BIR domains required for caspase inhibition is one.[119] The human XIAP protein, which contains three BIR domains, illustrates several interesting features relevant to caspase inhibition and BIR function. Inhibition of caspases-3 and -7 and concordant anti-apoptotic activity was localized to the second BIR of XIAP,[119] whereas the third BIR and RING domain is required for inhibition of caspase-9.[109,120] These results suggest that BIR domains within the same or different proteins are not functionally equivalent and may have additional functions other than to interact with caspases.

Mechanism of IAP-Mediated Caspase Inhibition

The vIAPs from African Swine fever,[106] two of the four IAPs from Epiphyas postvittana[105] and those from baculoviruses CpGV and OpMNPV[94,95] can each act to inhibit apoptosis under defined conditions by inhibiting caspases. IAPs bind to susceptible caspases at either a 1:1 or 2:1 ratio, indicative perhaps of the presence of two active sites per active caspase molecule.[118,121] While the IAPs, like p35 and CrmA-like serpins, all competitively inhibit caspase, only the IAPs inhibit through a mechanism which does not require peptide bond hydrolysis.[117,118] It has been proposed[100] that IAPs could still function as competitive inhibitors of caspases in much the same way as the Kunitz, Kazal and Eglin families of serine proteinase inhibitors, which contain structural loops that conform and adapt to the catalytic pocket of their respective proteinases.[122] A similar mechanism governs cystatin inhibition of papain-family members.[123] In all cases, a loop region of the inhibitor binds to the catalytic groove of the proteinase without scissile bond formation. Recently, a crystallographic analysis of the second BIR of the XIAP protein in complex with caspases-3 or -7 showed, perhaps surprisingly, that the BIR domain has almost no direct role in inhibition as all important inhibitory interactions are made by the flexible region which precedes the BIR domain.[124-126]

Instead of direct and intimate contact, a model for inhibition has been articulated whereby the main function of the BIR domain may be to align the inhibitor and stabilize inhibitory actions mediated by what are defined as the "hook", "line" and "sinker" regions located within the upstream N-terminal inter-domain linker region.[126] The BIR and relevant adjacent regions can be linearly depicted as "N-terminal interdomain region-hook-line-sinker-BIR-C-terminus". This model then proposes that the substrate pocket on the caspase consists of subpockets designated S_4, S_3, S_2, S_1 and S_1'. The "hook" region interacts with S_1 and S_1' exosites. From the hook, two peptide bonds (the "line") stretch across the binding cleft (exosites S_2 and S_3) and connect to the "sinker" and the BIR domain. The sinker and part of the BIR domain interact with only S_4 with most of the interaction coming from the sinker region. Most of the inhibitory interactions stem from the most distal hook region which prevents catalysis through steric blockage of substrate access. While most of the structural insight has come from the human XIAP, it seems likely that the overall mechanism for caspase inhibition is likely to be conserved for the viral IAPs.

P35

Baculoviruses encode members of two distinct classes of apoptosis inhibitors, namely, viral IAP and p35.[99,127,128] Of these, the p35 family members are notable for their broad inhibitory spectrum that includes a wide variety of caspases identified from nematodes, insects and mammals.[129-133] To date, no cellular homologs of baculovirus p35 have been identified, but the crystal structure of the native p35 and its inhibitory complex have both been determined[134,135] and it is still possible that related protein folds will be discovered as ongoing genomic and proteomic analysis of mammalian genomes continues. The inhibitory spectrum of p35 includes nematode CED-3, Drosophila Sf-caspase-1, the mammalian caspases-1, -3, -6, -7, -8, -10 and gingipain–K.[35,129-133] The p35 protein acts as a suicide inhibitor of caspases, with a 1:1 stoichiometry reminiscent of serpins, but the inhibitory complex is characterized by a distinctive protected thioester linkage between the caspase and p35.[129,130,134-136] In fact, structural analysis of the inhibitory complex between p35 and caspase-8 reveals a unique active site configuration that protects the intermediary thio-ester link from solvent hydrolysis.[135] P35 is cleaved during the inhibitory complex formation, and this cleavage is necessary for stability of the inhibitory complex but other protein-protein contacts also contribute to the caspase inhibition.[135,136] The deduced structure of the uncomplexed p35 reveals an exposed reactive site loop that includes the caspase cleavage site at the apex aspartate residue.[134] Following cleavage of the p35 scissile bond, the interaction between the reactive centre loop and the B-sheets of the p35 body stabilize the assembled inhibitory complex.[137] The p35/caspase complex is long-lived such that both components are effectively sequestered and conformational changes resulting from the cleavage of p35 render the complexed p35 energetically more stable than the uncleaved form.[138]

P35, a Broad Spectrum Caspase Inhibitor in Vitro and in Vivo

For example, ectopic expression of p35 inhibits apoptosis in developing Drosophila embryos,[139,140] C. elegans[141] and mice.[142,143] Interestingly, p35 does not inhibit the caspase DRONC, which is one of the two known initiator caspases in Drosophila, as opposed to the downstream executioner Sf-caspase-1 that is inhibitable by p35.[144-146] When expressed constituitively in transformed insect (Sf9) cells, p35 induces increased resistance to apoptosis caused by actinomycin D or nutrient withdrawal, and also increases the secretion levels of ectopically-expressed glycoproteins.[147] P35 itself does not inhibit the apical caspase that activates Sf-caspase-1, although vIAP appears to be capable of inhibiting this activity.[146,148]

The in vitro inhibition spectrum of p35 is quite impressive, particularly for downstream effector caspases that mediate the execution phase of apoptosis.[149,150] Table 2 summarizes the inhibitory constants of p35 for various mammalian caspases and provides a useful comparison with the inhibitory spectrum exhibited by CrmA.

Table 2. Inhibition of mammalian caspases by viral caspase inhibitors

Enzyme	Inhibitory Constants (Ki) CrmA	P35
Caspase 1	0.004-.0.1	9
Caspase 2	>10,000	Inh.
Caspase 3	1600	0.1
Caspase 4	1	-
Caspase 5	<.01	-
Caspase 6	110	0.4
Caspase 7	>10,000	2
Caspase 8	<0.3-0.95	0.5
Caspase 9	<2	-
Caspase 10	17	7

The numbers represent Ki measured as nM values (see also Ekert et al, 1999; ref. 133)

Conclusions

The three viral inhibitors considered here are the best studied examples of virus-encoded caspase modulators, but other examples have also been reported in the literature. For example, the Adenovirus 14.7-kD protein and the UL35 protein of human cytomegalovirus (denoted vICA) have both been reported to bind and inhibit caspase–8.[151,152] However, the analysis of these proteins is still at the early stages. Nevertheless, it is likely that many more inhibitors of caspases will continue to be discovered in other virus systems. Given the extensive range of diverse mechanisms by which viruses are already known to modulate the apoptosis cascade (reviewed in refs.153-159), the field of caspase manipulation by viruses seems destined to grow considerably in the future.

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CHAPTER 7

Apoptosis Dependent and Independent Functions of Caspases

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Introduction

The study of cell death in the nematode *Caenorhabditis elegans* has led to the identification of several proteins which are responsible for orchestrating cell death. For each of these proteins, termed Ced for cell death defective, numerous mammalian homologues have been described (for review see ref. 1). The mammalian homologues of Ced-3, caspases, are a family of proteases involved in apoptosis execution. Caspases require an aspartic acid residue in the P_1 position of their substrates and have an active site cysteine that mediates such cleavage. To date, 14 mammalian caspases have been identified. Caspases are divided into three groups: Group I caspases (caspases -1, -4, -5, -14, and murine -11, and -12) cleave the substrate sequence (W/L)EHD. This sequence is absent in most apoptosis substrates and these caspases are responsible for the generation of inflammatory signals and immune regulation rather than in the signaling of cell death. Group II (caspases -2, -3, -7) which prefer a DEXD motif, and group III caspases (caspases -6, -8, -9, -10) which prefer a (L/V)E(T/H)D sequence are involved in the execution of apoptosis.[2] Caspases -8, -9, and -10 (initiator caspases) initiate the propagation of the apoptotic signals whereas caspases -3, -6, and -7 (effector caspases) execute the apoptotic program by cleaving numerous cellular proteins.

In addition to the well characterized role of caspases in apoptosis, growing evidence suggests their participation in other cellular processes such as development, cell cycle, cell proliferation, cell migration, and receptor internalization. This Chapter summarizes the recent advances in the understanding of apoptosis dependent and independent functions of caspases.

Regulation of Apoptosis by a Combination of Initiator and Effector Caspases

The requirement for caspases in the execution of apoptosis has been well documented. Activation of caspases can occur by two distinct pathways: an extrinsic death receptor-mediated pathway and an intrinsic mitochondrially-mediated pathway. In either of these pathways initiator caspases are activated by oligomerization following an apoptotic signal. Initiator caspases cleave and activate effector caspases which then cleave diverse cellular proteins resulting in apoptosis. The best characterized extrinsic pathway is that initiated by activation of the death receptor CD95 (Fig. 1) (for review see ref. 3). The cytoplasmic domain of the CD95 receptor has no intrinsic activity but contains a death domain which can mediate protein interactions.

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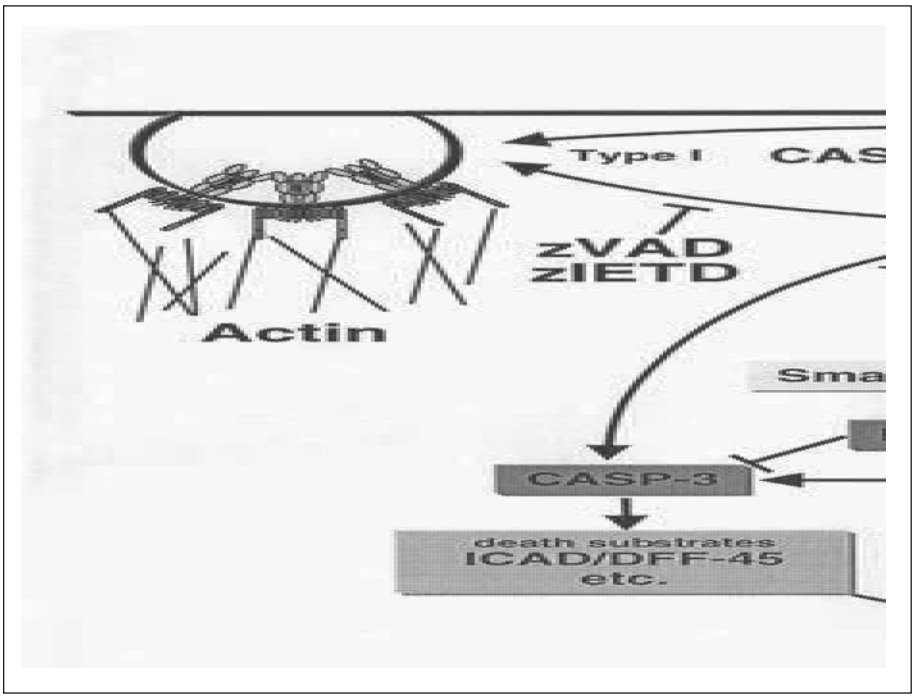

Figure 1. The role of initiator and effector caspases in CD95 mediated apoptosis. The amount of caspase-8 generated at the CD95 death-inducing signaling complex (DISC) determines whether a cell undergoes apoptosis through a mitochondrial dependent (Type II) or mitochondrial independent (Type I) pathway. In addition to activation of downstream effector caspases the initial activation of caspase-8 at the DISC also regulates clustering and internalization of CD95 in Type I cells.[78] CD95 is shown as a trimer with bound CD95 ligand.

Activation of CD95 by its ligand, CD95L, results in recruitment of the adaptor protein FADD (also called Mort1) through the death domain. Following recruitment of FADD, caspase-8 is recruited to the receptor and oligomerization drives its activation through autoproteolytic cleavage.[4,5,6,7] caspase-8 then activates downstream effector caspases such as caspase-3, resulting in apoptosis. The intrinsic apoptotic pathway involves activation of the apoptotic function of mitochondria, including the release of cytochrome c, and activation of caspase-9. Caspase-9 reacts with the cytosolic apoptotic protease activating factor-1 (Apaf-1) and, in the presence of dATP and cytochrome c, oligomerizes to form the apoptotically active complex termed the apoptosome. The activation of caspase-9 in the apoptosome then allows it to cleave caspase-3.[8]

We have described the existence of two distinct pathways of CD95-mediated apoptosis signaling that diverge downstream of caspase-8 activation.[9] In this model of CD95 mediated apoptosis the relative amounts of initiator and effector caspases determine whether a cell uses a mitochondrially dependent (Type II cells) or independent (Type I cells) pathway (Fig. 1). Type I cells have developed a mechanism to bypass mitochondrial functions by activating large amounts of caspase-8 at the DISC that, in turn, activate caspase-3 directly, with no reliance on mitochondrial amplification. This signaling pathway is unaffected by Bcl-2 or Bcl-X_L. In Type II cells, the DISC forms very inefficiently and as a consequence only very small amounts of caspase-8 are generated at the DISC, sufficient to affect mitochondria, but not enough to

activate caspase-3 directly. In Type II cells, small amounts of active caspase-8 cleave the proapoptotic Bcl-2 family member Bid. This generates a 15-kD fragment that translocates to mitochondria, and this, in turn induces cytochrome c release via an undetermined mechanism.[10,11] This leads to the initiation of a mitochondria-dependent apoptotic signaling pathway. Bid induced mitochondrial activation can be blocked by Bcl-2 or Bcl-x_L, a characteristic of Type II cells.

Role of Caspases in Development, Cell Proliferation and Cell Cycle

Studies in caspase deficient mice have elucidated many of the functions of these molecules. They have also illustrated the requirement for caspases in multiple aspects of development, as several of the caspase deletions led to grossly malformed embryos. Deletion of caspases –3, -8, and -9 had a profound impact on the development of the deficient mouse embryos and on the response of tissues to apoptotic stimuli. Deficiency of caspases-8, -3 and -9 resulted in embryonic or perinatal lethality.[12,13,14,15] Deletion of the gene encoding caspase-8 led to profound defects in embryo formation, causing severe abdominal hemorrhage, and defective cardiac muscle development. Hemorrhage in abdominal areas and hyperemia in major blood vessels as well as in lung and retina were observed.[14] caspase-9 deficient mice showed central nervous system defects reminiscent of caspase-3 deficient animals, but with even more profound defects in brain development.[12,13] The similarity of these defects suggests a general pathway of apoptosis, though the greater severity in caspase-9 deficient animals suggests that it may activate other caspases that could potentially replace caspase-3 in the cascade. Caspase $7^{-/-}$ mice died during embryogenesis, whereas caspase-$2^{-/-}$, -$6^{-/-}$, and -$12^{-/-}$ mice developed generally normally.[16,17,18] These data suggest that certain caspases are crucial for the homeostasis of certain tissues, whereas other caspases are redundant and can compensate for one another.

Although gene inactivation studies revealed new insights into caspase function and have greatly enhanced our knowledge on the role of individual caspases, a number of areas should still be addressed. Despite the essential role of caspases, particularly caspase-8 in death receptor induced apoptosis, none of the phenotypes of caspase knock-out mice resembled that of mice carrying mutations in either the CD95 receptor or CD95 ligand. These animals accumulate abnormal $CD4^-CD8^-$ T cells, show autoantibody formation, lymphadenopathy and splenomegaly.[19] It is possible that the phenotypes may not solely be caused by defective apoptosis signaling through CD95, but may additionally be due to other signaling properties of the receptor. It is important to note that the disorders of certain caspase deficient mice are predominantly of one specific tissue such as the heart in caspase-$8^{-/-}$ mice or the brain in caspase-$9^{-/-}$ or caspase-$3^{-/-}$ mice. Studies in tissue specific knock-out mice will explore the role of specific caspases in the development of other organs.

Caspase-8 is not the only initiator caspase that is recruited to death receptors. Another recently identified caspase of the Ced-3 family, with a 28 % identity with caspase-8 was identified and termed caspase-10. Caspase-10 exists as several alternatively spliced forms, caspase-10 a-d.[20] One study demonstrated that caspase-10b was inhibited by a caspase inhibitor with the sequence DEVD specificity, but not with YVAD, suggesting that the sequence specificity of this caspase is similar to apoptosis activating caspases rather than caspases involved in cytokine maturation.[20] The significant homology of caspase-10 with caspase-8 suggested that this caspase may be involved in death receptor signaling; caspase-10 contains two tandem DED, further suggesting that the caspase may be recruited to the DISC of the activated receptor. Caspase-10 has been shown to associate with the adaptor FADD in vitro and in transfected cells, and to induce apoptosis when overexpressed.[20,21] After much of controversy it was recently established that caspase-10 is recruited to the DISC along with caspase-8 upon stimulation of CD95 (ref. 22 and unpublished data). A recent study has suggested that caspase-10 mutations may be

involved in Autoimmune Lymphoproliferative Syndrome (ALPS) Type II, as certain patients who harbor mutations in this caspase but in no other apoptotic molecules, developed this disorder.[23] Aside from these few studies, little is known about the role of caspase-10 in apoptosis, a fact compounded by the lack of a caspase-10 orthologue in mice.

Evidence for the role of the adaptor protein FADD and caspases in cell proliferation was provided by studies in knockout mice. FADD/MORT1 is essential for CD95-induced apoptosis as FADD deficient thymocytes and Jurkat T cells do not undergo apoptosis upon CD95 stimulation.[24,25] FADD is also involved in apoptosis-induction by other death receptors. Overexpression of a dominant negative mutant of FADD (FADD-DN) inhibits TNF-R1, TRAIL receptor and DR3-induced apoptosis.[26-29] In addition to the defect in apoptosis, thymocytes and peripheral T cells from FADD$^{-/-}$ mice or from mice expressing FADD-DN under the control of the proximal lck promoter showed a defect in activation-induced proliferation despite normal production of IL-2.[30-32] Similarly, FADD$^{-/-}$ T cells in chimeric mice on the RAG-1$^{-/-}$ background also showed impaired proliferation following activation.[33] Expression of FADD-DN inhibits proliferation of T cells and fibroblasts by inhibiting cell cycle entry at the G0 to S transition.[34] Recently Zhang et al showed that T cells from FADD$^{-/-}$ mice show dysregulated expression of various cell cycle proteins such as increased levels the cdk inhibitor p21, and constitutive activation of the cyclin dependent kinases cdk2 and cdk6.[34] The above findings suggest that FADD is a key regulator of T-cell development and cell proliferation in addition to its role in apoptosis. We recently demonstrated that FADD is specifically phosphorylated at serine 194 during the G2/M transition of the cell cycle, and is not phosphorylated in cells arrested in G0/G1.[35] This phosphorylation does not appear to be important for apoptosis signaling and thus may be important for an apoptosis independent activity such as cell proliferation or cell cycle control. Whether the effect of FADD in cell proliferation requires caspases-8 or CD95 is not currently established. However it is worth mentioning that under certain circumstances CD95 can induce proliferation rather than apoptosis[36,37] suggesting that CD95 play a direct role in regulating cell proliferation.

The role of caspase-8 in cell proliferation has been more difficult to delineate since caspase-8 deficient mice were embryonic lethal[14] and chimeric mice carrying T cells deficient for caspase-8 have not been generated. However, various reports have highlighted the role of caspases including caspase-8 in cellular proliferation. The first report showed that in PHA activated Jurkat cells a caspase-3-like protease was activated. In addition, it was shown that caspase inhibitors blocked IL-2 production following anti-CD3 plus anti-CD28 stimulation of Jurkat cells.[38] Two other reports have shown that the earliest upstream initiator caspase, most likely caspase-8 is activated after CD3 ligation.[39,40] Both reports demonstrated that T cell receptor (TCR) stimulation of human peripheral T cells results in caspase activation in the absence of apoptosis. However, these studies differed with regard to the extent of caspase activation and which substrates were cleaved. Whereas Kennedy et al[39] observed only caspase-8 and no caspase-3 activation with 24 hrs of stimulation, Alam et al[40] found activation of caspase -3, -6 and -7 and proteolysis of the substrates poly (ADP-ribose) polymerase-1 (PARP-1) and lamin B in cells 16 hrs to 4 d after stimulation. In addition Kennedy et al demonstrated that the CD95 receptor provides a costimulatory signal during T cell activation since T cell proliferation could be significantly blocked by a Fas-Fc construct. This observation confirmed previous reports that suggested that stimulation of CD95 enhances proliferation of peripheral T cells induced by TCR stimulation.[36] Although these findings proposed a new function of caspases in the regulation of cell proliferation, various other data must be reconciled. For example, transgenic mice that overexpress CrmA, a potent viral inhibitor of caspase-1 and caspase-8 do not have a block in T cell proliferation.[41] Additionally, the CD95 mutations observed in *lpr* mice or ALPS patients do not cause a defect in T cell proliferation.[19,42] It is possible that other members of

the death receptor family such as TNF-R, the TRAIL receptors and DR3 that also signal through FADD and caspase-8 can replace the function of CD95 as a costimulatory molecule.

It has as well been proposed that caspases may play a role in the regulation of cell cycle by serving as additional checkpoints that ensure that only healthy cells complete the cell cycle.[43] A recent report demonstrated the presence of active caspase-3 in mitotic and postmitotic cells of the rat forebrain.[44] Active caspase-3 was localized predominantly to the nuclei of cells undergoing cell division in the proliferative regions of the forebrain. Caspases have been shown to cleave various cell cycle regulatory proteins during apoptosis such as the retinoblastoma protein (Rb),[45] Wee1,[46] Cdc2[47] and Cdc27.[46] It is conceivable that activation of caspases during cell cycle results in the cleavage and inactivation of proteins that act as negative regulators of the cell cycle machinery. For example, $p21^{CIP1}$ and $p27^{KIP1}$ are cleaved by caspase-3 like proteases during apoptosis in human endothelial cells.[48] $p21^{CIP1}$ and $p27^{KIP1}$ bind to and block the activation of cyclin /Cdk2 complexes, preventing progression through G1 to S phase. Cleavage of $p21^{CIP1}$ and $p27^{KIP1}$ by caspases results in decreased association with cyclin/cdk2 complexes leading to a dramatic increase in Cdk2 activity. Additionally, cleavage of the Wee1 kinase by caspases results in its inactivation and an increase in Cdc2 activity that could allow progression through the G2/M checkpoint.[46] caspase dependent cleavage of $p27^{KIP1}$ in nonapoptotic proliferating lymphoid cells has also been reported.[49,50] The caspase activity detected in proliferating BJAB cells induces the cleavage of the tetrapeptide substrate Ac-IETD-Amc and is inhibited by Ac-IETD-CHO but not by zVAD-fmk in vitro.[49] However, Jurkat cells deficient for caspase-8 also show a proliferation dependent caspase activity that is abrogated by Ac-IETD-CHO.[49] Further studies are necessary to determine the non-apoptotic caspase that is involved in regulation of $p27^{KIP1}$ and possible other cell cycle regulatory proteins.

If caspases are activated during the cell cycle, the mechanism of how cleavage of substrates is restricted to cell cycle regulators while not resulting in disintegration of the cell is the most puzzling. The answer to this dilemma may lie in the subcellular compartmentalization of caspases or in the accessibility of substrates. The localization of some caspases has been studied and they have been found to exhibit a diverse localization pattern encompassing the plasma membrane, the mitochondria, the ER and the nucleus in addition to the cytoplasm. For example, we recently showed that caspase-6 is preferentially activated in the nucleus after apoptosis induction by the apoptosis inducing molecule DEDD.[51] We have also recently observed that procaspase-8 is localized to the outer mitochondrial membrane in MCF7 cells, and that active caspase-8 is sequestered and inactivated there by the action of Bcl-X_L and the protein BAR (bifunctional apoptosis regulator) (ref. 52 and unpublished data). Selective processing of substrates by caspases has been described following TCR stimulation.[40] Cleavage of PARP-1, lamin B, and Wee1 was observed in this study, whereas DNA fragmentation factor (DFF45) and replication factor C (RFC140) were not cleaved.[40] Cleavage of the latter substrates would be fatal for cells which are not undergoing apoptosis, since it would lead to inhibition of DNA replication and to DNA fragmentation. Another possibility for regulating the substrates that caspases cleave is that these substrates may be modified thereby making the caspase cleavage site inaccessible. For example, phosphorylation of serine residues adjacent to the caspase-3 cleavage site of presenilin-2 has been shown to protect the caspase cleavage site.[53] Therefore, locally selective activation of caspases, for example in the nucleus, and selective processing of substrates could reconcile apparent contradiction between cell cycle regulation and the apoptotic functions of caspases.

Inactivation of Survival Pathways by Caspases

Caspases have been shown to act as negative regulators of survival pathways. For example, caspases negatively regulate the activation of the prosurvival pathway orchestrated by NF-κB

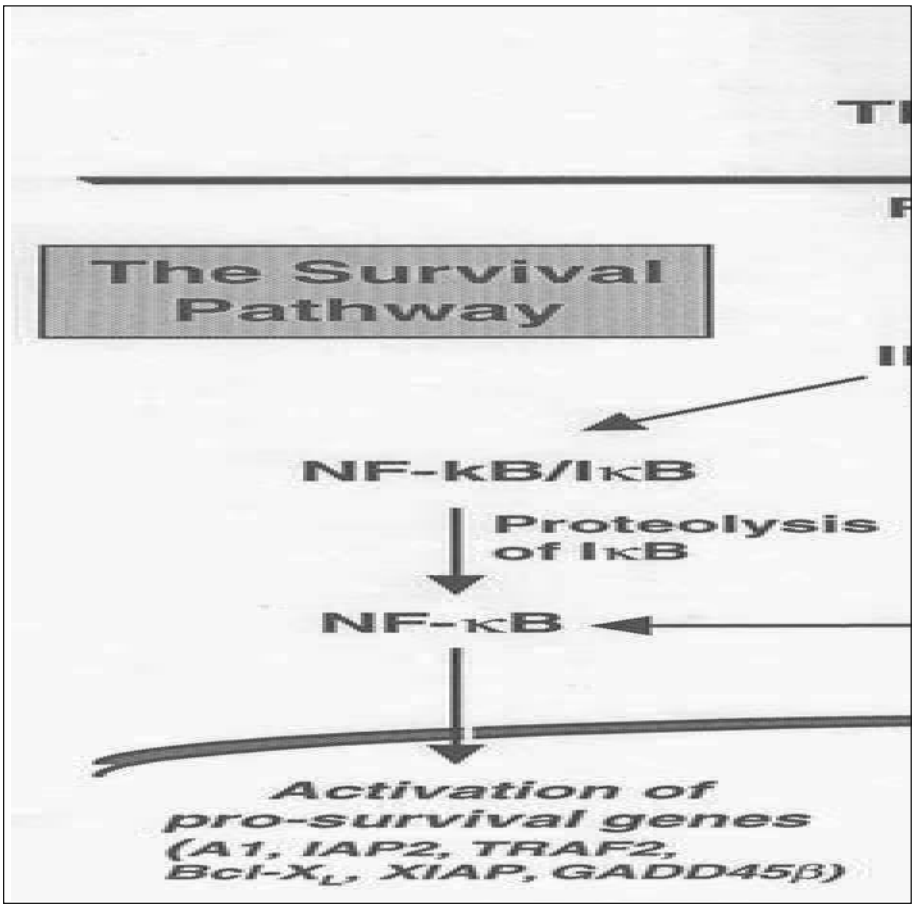

Figure 2. Inactivation of the NF-κB orchestrated prosurvival pathway by caspase activation. TNF-R1 initiates a prosurvival pathway through TRAF2/RIP and activation of the IKK complex resulting in transcriptional upregulation of a number of prosurvival genes. The most recent genes identified are XIAP and GADD45β.[83,84] Under certain circumstances triggering TNF-R1 activates a proapoptotic pathway by activation of caspase-8 through TRADD and FADD. When a proapoptotic signal prevails many components of the antiapoptotic pathway are inactivated by caspase cleavage. See text for details. TNF-R1 is shown as a trimer with bound TNF.

(Fig. 2). Activation of most death receptors has been shown lead to activation of two opposing pathways: induction of apoptosis through recruitment of caspase-8 or cell survival by the activation of the transcription factor NF-κB. Modulation of the response in favor of NF-κB protects cells from apoptosis whereas failure to activate NF-κB results increased cell death.[54-56] A number of the crucial proteins for the activation of NF-κB through TNF stimulation have been shown to be substrates for caspases (Fig. 2). The serine threonine kinase RIP and the TNF receptor associated factor 1 (TRAF1),[57-61] are cleaved by caspase-8 during apoptosis. In both cases cleavage by caspases results in the generation of a C-terminal fragment that acts as dominant negative molecule preventing NF-κB activation, and subsequently leading to the amplification of the death signal. Recently it has been shown that the β subunit of the IκB kinase

complex (IKKβ) can be inactivated by caspase-3 cleavage.[62] Furthermore, both the main subunits of the transcription factor NF-κB itself, p50 and p65, are substrates for caspase-3.[63,64] Cleavage of p65/RelA or p50 result in their inactivation and loss of their transcriptional activity. Other molecules that may regulate cell proliferation pathways such as Vav1,[65] PKC,[66] STAT1[67] and MEKK-1[68] have also been shown to be possible caspase substrates. It is conceivable that caspase mediated inactivation of survival or proliferation pathways in nonapoptotic cells may be important in decreasing the activation state of cells in the termination of an immune response or under conditions of limited growth factors, for example.

Caspases in Cell Spreading and Migration

Caspases have been implicated in the control of cell movement. In one study treatment of NIH3T3 cells with a caspase inhibitor prevented their spreading and migration on a collagen coated surface.[69] These studies were performed with a broad spectrum caspase inhibitor, and a caspase-3 selective inhibitor was not effective at blocking migration. The specific caspase that may regulate this process therefore has not been identified.[69] A recent study showed that Langerhans cells from caspase-1 deficient mice were defective for migration upon antigenic stimulation. The study also demonstrated that the caspase-1 inhibitor Ac-YVAD-cmk was able to prevent migration of LC in vivo, as observed by application of a contact sensitizer.[70]

Additionally, data from our lab has demonstrated that the cytolinker plectin is an early caspase-8 substrate.[71] Plectin is a component of hemidesmosomes and thus may be involved in cell spreading. Plectin has been shown to be essential for actin rearrangement in CD95 stimulation as well as in activation of rho and Cdc42[72]. Thus caspases may potentially regulate these processes in cells which are or are not actively undergoing apoptosis. Multiple other cytoskeletal components have been identified as caspase substrates such as actin,[73] Gas2,[74] gelsolin,[75] vimentin,[76] cytokeratins[77] and caspases could potentially regulate the function of some of these proteins and thus cytoskeletal mediated migration or spreading.

Novel Function of Caspases: Role in Receptor Internalization

We have recently demonstrated that triggering CD95 results in receptor clustering followed by its internalization shortly after engagement of the receptor by either anti-CD95 mAb or CD95L.[78] Clustering of CD95 and its internalization can be prevented by pretreating cells with zVAD-fmk or zIETD-fmk. In addition in BJAB cells expressing a dominant negative form of FADD which therefore do not recruit caspase-8 to the receptor, CD95 clustering following stimulation was impaired. Our data favor caspase-8 as the caspase regulating this process because MCF7-Fas cells that do not express caspase-3 and mainly activate caspase-8 during the first 4 hrs after CD95 triggering,[71] very efficiently cluster and internalize CD95 (unpublished data). Furthermore, the caspase-8 selective inhibitor zIETD-fmk inhibits receptor internalization much more efficiently than the caspase-3/7 selective inhibitor zDEVD-fmk. To our knowledge this is the first evidence of the role of caspase-8 in receptor clustering and internalization.

Another member of the death receptor family, TNF-R1, also has been shown to internalize upon binding to its ligand TNF.[79] Internalization and signaling in TNF-R1 were inhibited by the transglutaminase inhibitor monodansylcadaverine (MDC). The involvement of caspases in TNF-R1 internalization has not been tested. Additionally, the caspase-8 substrate that is involved in CD95 clustering and internalization is currently unknown. In MCF7-Fas cells protected from apoptosis by stable expression of Bcl-X_L, the DISC inhibitor c-FLIP is the only known caspase substrate that is cleaved (unpublished data). However, clustering of CD95 and its internalization efficiently occurs and can be prevented by pretreating cells with zIETD-fmk, suggesting that very small quantities of active caspase-8 are sufficient to initiate this process.

The finding that nonapoptosing cells also internalize CD95 after stimulation demonstrates that internalization of CD95 is not an unspecific event due to changes in the membrane of apoptosing cells. The data rather support the notion that CD95 clustering and internalization are active processes regulated by activation of caspase-8.

Clustering of CD95 is dependent on its ligand and on caspases, however, this does not exclude the possibility that surface molecules in general could be specifically internalized in a caspase dependent fashion without being directly stimulated. Activation of caspases could trigger clustering and internalization of receptors including CD95. Such a mechanism could explain how agents that induce apoptosis by activating caspases could cause clustering of CD95 in a ligand independent fashion as has been shown for antitumor ether lipids, UV radiation or the herpes simplex thymidine kinase/ganciclovir gene therapy system.[80-82] Many mediators of receptor internalization are either protein kinases or GTP binding proteins. This novel mechanism involving caspase-8 may however not be restricted to CD95. For example, caspase-8 has been shown to be activated following T cell receptor activation in cells that do not apoptose, and its inhibition blocks T cell proliferation. Activation of caspases may be required to modulate activity of other receptors such as the T cell receptor by assisting in its down regulation. Future studies should address whether activation of caspases regulates surface expression of other surface molecules.

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CHAPTER 8

The Role of Caspases in Modulation of Cytokines and other Molecules in Apoptosis and Inflammation

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Introduction

Caspases are a large family of evolutionary conserved proteases. The first caspase, has been identified as the enzyme necessary for functional maturation of IL-1β.[1,2] This molecule, initially named interleukin-1β-converting-enzyme (ICE), is the founding member of the caspases:[3] the cysteine-containing (in a QACxG-motif) aspartate specific proteases. Soon it has been recognized that ICE is related to the proapoptotic molecule *ced-3* of the nematode *Caenorhabditis elegans*.[4] This finding suggested that caspases may be involved in regulation of apoptosis and initiated extensive research in this field. So far 14 caspases have been identified in animals and men. In addition, caspase-like proteases, the para- and metacaspases, have been identified in protozoa, fungi and plants, or *Dictyostelium* and metazoans, respectively.[5] It is well accepted now that caspases are essential for the regulation of apoptosis, but they also contribute to regulation of inflammation or cell cycle progression.[6-8] The caspases do so by catalysis of various substrates,[9,10] including caspases themselves, other enzymes, structure proteins, signal proteins, or cytokines (Table 1).

Although caspases act mainly within the cell, their production and activity is triggered and modulated by external signals, including cytokines, and the cytokine and caspase networks are closely "inter-connected" in various ways and at different levels. Thus, some caspases are essential for cytokine maturation (i.e. activation) and secretion, whereas others cleave cytokines, thereby inactivating them. In the following we briefly summarize informations regarding the interaction of cytokines and caspases in inflammation and apoptosis. We focus on the importance of caspases for production, activation or degradation of cytokines, summarize the influence that cytokines have on caspase-mediated cell apoptosis and present informations about possible interactions of cytokines and caspases at the level of transcription. We do not address the question of TNF-induced caspase activation because this subject is covered by other contributions in this issue.

The Role of Caspases in Modulation of Activity of Cytokines

Depending on their molecular biology the caspases are divided into three groups: the caspase-1, caspase-2 and caspase-3 families. The caspase-1 family includes caspase-1, -4, -5, -11, -12, -14. These caspases are believed to be involved primarily in the regulation of cytokine processing or in activation of caspases involved in cytokine processing. Thus, a caspase network[11], in analogy to that described for apoptosis, may be present also in regulation of cytokine-dependent inflammatory processes. The caspase-2 family consists of the apoptosis initiator caspases-2, -8, -9, -10, and the caspase-3 family consists of the apoptosis executioner

Caspases—Their Role in Cell Death and Cell Survival, edited by Marek Los & Henning Walczak.

Table 1. Caspases and their substrates[a]

*Ca*spase	References	Synonyms	Substrates[b]
1	2	ICE	IL-1β[1,2]; IL-18[37,38,41]; casp3; casp7[11]; actin[181]; ICE[78]; calpastatin[182]; PARP[183]; EGFR[54]; Nedd-4[184]; PITSLRE[185]
2	186,187	Ich-1_L; NEDD-2	PARP-1[183]; golgin[188]
3[c]	189-191	YAMA, prICE, PARP-1, CPP32, apopain	ICAD/DFF[192-194]; casp9; casp6[195];Apaf-1[196]; IL-2[49]; IL-16[47]; IL-18[41]; PARP[189-191]; actinN ENRfu [197]; vimentin[198]; gelsolin[199]; acinus[200]; Bcl-2[201]; T-cell receptor[53]; hILP/XIAP[103]; casp9[90]; SREBP[202]; Vav-1[204]; mdm2[205]; APP[206]; EGFR[54]; Nedd-4[184]; STAT-1[207]; NF-κB[208]; I-κB[169]; TRAF-1[55]; spectrin[209]; PKC[210]; MEKK[211]; pp125[FAK 212]; FAK[213]; PITSLRE[185]; PKC-theta [214]; p21WAF1/CIP1[215]; ROCK-1u [216]
4	44,217	Ich-2, TX, ICE rel II	casp1[44]; PARP-1[183]; casp3[218]; IL-1β[219]
5	220,221	ICE rel III, TY	Max[222]; IL-1β (partial cleavage)[219] casp1[45]
6[c]	223,224	Mch-2	casp3[225]; PARP-1[224]; Nedd-4[184]; vimentin[198]; lamin[223]; TRAF-1[55]; keratin[226]; β-catenin/ plakoglobin[227]; SATB-1[228]; AP-2alpha[171]; FAK[213]
7	229	Mch-3; ICE-LAP-3; CMH-1	casp12[230]; PARP[231]; T-cell receptor[53]; vimentin[198]; Nedd-4[184]; EMAP-II[51]; EGFR[54]; Max[222]
8	232,233	FLICE; Mch-5; MACH	GATA-1[234]; casp13Rfu [235]; casp14[236]; all procaspases[11]; TRAF-1[55,165]; BID[237,238]; RIP[166,167]
9	239,240	Mch-6; ICE-LAP-6	casp3[112,241]; casp9fu [241]; casp7[241]
10	88	Mch-4; FLICE-2	casp14[236]; TRAF-1[55]
11[d]	242,243	TX; mIch-3	ICE[75]; casp3[86]; IL-1β (partial cleavage); NRfu [219]
12	242	mIch-4	ER-specific apoptosis[244];
13	235	ERICE	casp3[219]; IL-1β[219]
14	12,236,245		associates with caspase-1, -2, -4, -8, -10

[a] General remark: due to space limitations this table of substrates cannot make the claim to be exhaustive, however, examples are provided for the various groups of substrates targeted by caspases.

[b] Although numerous molecules are listed as substrates of particular caspases some molecules, for example fodrin[246,247], are also cleaved by other enzymes.

[c] Possibly posttranslational modifications result in multiple species of casp-3 and -6 in apoptotic cells[248]

[d] Murine casp-11 is presumably homologous to human caspase-4[242] or caspase-5[45]

caspases-3, -6, -7. From the phylogenetic point of view caspase-2 appears to form an own group, whereas the other members of the caspase-2 family (caspase-8, -9, -10) are more related to the caspase-3 subfamily.[12] An important characteristic of the caspases is the size of their prodomain. Some caspases may contain motifs of the death domain superfamilies, such as

CARD or DED,[13] as summarized in a recent review.[14] The caspases use a nucleophile hydrolysis mechanism.[15] They cleave their substrates behind aspartate and contain a cysteine in a QACxG-sequence (x = G, Q, or R) in their active centre. The four aminoterminal amino acids in P_4 to P_1 are important for the substrate specificity, and the tertiary structure of the substrate also appears to influence cleavage.[15,16]

Caspase-1 (ICE) was identified by its capacity to activate the IL-1β precursor (proIL-1β)[1,2,17], although the majority of the described caspases target apoptotic substrates rather than cytokines. However, there are examples of other caspases, including caspase-1, -3, -4, -5, -7 and -11, which either process cytokines or contribute to cytokine activation through activation of the respective cytokine-processing caspase.

IL-1β is produced as a precursor molecule[18-20] and has to be cleaved behind aspartate at position 116 to become active.[1,2] The mature IL-1β potently activates an enormous variety of biological activities.[21] The protein necessary to exert full biological activity contains amino acids 120-266 in human IL-1β[22] and amino acids 131-270 in murine IL-1β.[23] The necessity of a properly maturated IL-1β for biological activity is explained by the observation that the IL-1 receptor type I[24-26] does not interact with the IL-1β proform and, therefore, does not induce a signal.[27] From the protein pattern in Western blot it has been concluded that caspase-1 first cleaves the IL-1β proform at position 27-28 before it hydrolyzes at position 116-117.[28] Experiments in ICE $^{-/-}$ mice showed that in FasL-stimulated PMN other caspases may also be involved in cleavage of preIL-1β.[29] These mice expectedly did not produce IL-1β following LPS stimulation, whereas a synthetic FasL stimulated IL-1β production. However, the caspase responsible for IL-1β maturation in ICE$^{-/-}$ mice has not been identified, thus, it appears possible that another enzyme, activated by caspases, is involved in this process.

Besides caspases there are other enzymes which also can cleave the IL-1β precursor resulting in biologically active IL-1β. These enzymes include elastase, cathepsins, metalloproteinases, trypsin, or chymotrypsin.[30-32] The mast cell chymase also cleaves preIL-1β.[33] Furthermore, cleavage of preIL-1β by a cysteine protease from *Streptococcus pyogenes*, resulted in a biologically active product, with one additional amino acid terminal of the normal cleavage site.[34] Thus, the limited proteolysis through these enzymes results in aminotermini not far from the caspase-1-derived aminoterminus.[18,35] These informations point out that during inflammation IL-1β may be activated not only by caspase-1, and that IL-1β is relatively stable to degradation. Indeed, it has been shown, that interferon-γ and tumor necrosis factor-α, but not IL-1, were inactivated by alkaline protease or elastase.[36]

Another target of caspase-1 is IL-18.[37-39] Although this molecule, unlike proIL-1β, is constitutively expressed in whole blood cells or freshly isolated mononuclear cells[40], it also needs to be activated by caspase-1. There is some evidence, however, that caspase-3 may also be involved in cleavage of preIL-18[41], although the cleavage products may be inactive. Further evidence about the contribution of caspases other than caspase-1 for the activation of IL-18 is derived from experiments showing that pan caspase-, but not ICE-specific-inhibitors, blocked IL-18-stimulated production of TH1-cell cytokine production (IFN) in BALB/c mice.[42] The caspase-4 and -5, in addition to caspase-1, are regarded as cytokine activators or inflammatory caspases.[8,16,43] Although both do not cleave IL-1β as potent as caspase-1, they are thought to be involved in activation of caspase-1.[44,45]

High expression of caspase-3 has been detected in the absence of apoptosis, suggesting that caspase-3 may participate in processes other than apoptosis.[46] Thus, caspase-3 may be involved in inflammatory processes by cleaving cytokines. IL-16 is a proinflammatory, CD8-cell-derived lymphocyte-attractant, produced as a proform (80 kD) and active as a tetramer of the mature (14-17 kD) form. This molecule is activated by caspase-3, but not by caspase-1, caspase-2, or granzyme-B.[47,48] It has also been reported that caspase-3-like activity is important for the release of the T-cell mitogen IL-2,[49] possibly by caspase-mediated calcineurin cleavage.[50] caspase-7 cleaves the proform of the molecule EMAP-II (endothelial monocyte-activating polypeptide II). This interesting proinflammatory cytokine and chemoattractant is derived from the aminoacyl-tRNA synthase complex.[51,52]

Besides cytokines, cytokine receptors or other receptors (even T-cell receptors[53]) may be targets for caspases. The growth / survival promoting signalling by EGF receptors is abrogated by cleavage of the receptor through caspase-1, caspase-3, or caspase-7.[54] Also, the cleavage of the receptor adaptor molecule TRAF-1 has been reported. This mechanism enhances apoptosis by blocking NF-κB.[55] Cleavage of glutamate receptors has been investigated in inhibition experiments, and is thought to prevent necrosis and favours apoptosis.[56]

Caspase-Dependent Modulation of Cytokine Production

The above paragraphs indicated that caspases are involved in enzymatic activation or degradation of cytokines. However, the caspase network may also interfere with the production of cytokines at steps prior to processing of a certain cytokine. Evidence for this suggestion has been derived from experiments applying caspase inhibitors. For example, in the model of heterotopic cardiac transplantation combined with ischemia-reperfusion, the inhibition of caspase-3 by DEVD-CHO prevented the otherwise characteristic increase of TNF production.[57] In IL-1-activated fibroblasts the inhibition of caspase-3 also prevented IL-16 production.[58] On the other hand, besides their capacity to induce caspase activity, apoptotic stimuli may also activate cytokine production. Thus, in FasL-mediated smooth muscle cell-(SMC)-apoptosis IL-1α production was increased and was possibly involved in the enhanced levels of chemokines observed in the vessel wall.[59] The IL-1 in SMC is probably expressed at the surface of the cells and can induce the cytokine production of adjacent cells.[60] IL-1 is a very potent activator of cardiovascular cell cytokine production[61, 62] and these mediators are thought to be involved in cardiovascular diseases.[63-66] The involvement of IL-1α, rather than IL-1β, may be explained by a possible interference during production of both IL-1 isoforms, as indicated by results obtained in $ICE^{-/-}$ mice, which show that not only IL-1β, but also IL-1α production is impaired in these mice.[67] The above results are in line with results obtained in transgenic mice overexpressing FasL in the heart. These mice developed normally, but had increased expression of TNF, IL-1β, IL-6 or TGF-β.[68] Furthermore, in human MNC and cell lines increased IL-4 production was observed in response to various apoptosis inducing agents.[69] Apoptosis and cytokine production in parallel are observed during phagocytosis of microorganisms. Phagocytosis of viable serum-opsonized bacteria resulted in caspase-dependent monocyte apoptosis.[70,71] The same cells produced biologically active IL-1β and TNF-α in amounts correlating with the number of engulfed microorganisms (Pryjma, unpublished data). Moreover, employing a single-cell RT-PCR technique, both IL-1 and IL-12 were detected in cells loaded with bacteria (Guzik and Pryjma, unpublished data). However, it remaines to be determined whether or not caspases are involved in every case when cytokine production parallels apoptosis. It has been shown that caspase inhibitors effectively blocked both DNA fragmentation and IL-4 production in toxin-treated MNC[69], but although effectively preventing apoptosis, did not influence IL-10 production by FasL-treated monocytes[72] or ultraviolet light-treated lymphocytes.[73]

Regulation of Cytokine Processing by Caspases

The activity of the caspases has to be tightly regulated, no matter if the caspases are involved in apoptosis or in cytokine processing. The mechanisms involved in caspase regulation include activation of the enzyme zymogens, inhibition of the caspases or postranslational modifications. The regulation of caspase-1 activity and production of IL-1β is not completely understood. However, it has been shown that LPS co-induces caspase-1 and IL-1β,[74] and caspase-11 or caspase-5 are thought to be required for caspase-1 activation.[45,75] On the other hand, other enzymes, such as tripeptidyl peptidase II or cathepsin-B, may also activate caspase-1.[76,77] It has been suggested that caspases may autoactivate.[78,79] Thus, it has been proposed that caspase-1 or caspase-8 are autoactivated by oligomerization.[80] Other possible molecular mechanisms of autoactivation are now evolving. Molecules have been identified, which can interact with caspase-1, thereby possibly regulating its function.[81,82] The RIP-like kinase RIP-2 (CARDIAK; RICK) can bind to the CARD-motif[83] of caspase-1 and activate it.[84] This activation can be

inhibited by COP[85] or ICEBERG,[82] through interaction with both the CARD of caspase-1 and that of RIP-2, thereby blocking autoactivation of caspase-1.

Interestingly, evidence has been provided that, besides caspase-1, caspase-11may also activate caspase-3.[86] Other caspase-3 activators are granzyme-B[87] or caspase-10.[88] Recently a component of the DISC complex has been identified to be an activator of caspase-8.[89] Furthermore, caspase-9 is activated in a feedback amplification pathway by caspase-3.[90] caspase-9, as well as other caspases can also be activated by autoproteolysis.[90-92]

On the other hand, inhibitors of capases,[43] including potassium-ions,[93] serpins,[94,95] soluble receptors,[96] other cell-associated molecules[97] or caspase inactivators[98] may contribute to regulation of caspase-1 function. Caspases involved in apoptosis are targets for a variety of apoptosis inhibitors,[43] including the caspase-2 and -8 inhibitor ARC (apoptosis inhibitor with CARD domain),[99] or other inhibitors of apoptosis (IAPs;[100]). Thus, it has been shown, that a baculovirus IAP can potently reduce ICE- or caspase-2-induced apoptosis.[101] Some of these inhibitors, i.e. CrmA, p35 or hILP remain bound to the caspase after cleavage (suicide inactivators).[94,102,103]

Inactivation of caspases may also be obtained by other mechanisms, i.e. by enzymatic inactivation. Thus, it has been shown that calpain can inactivate caspases-7, -8 and -9.[98] Furthermore, caspases may be targets for phosphorylation.[104] As an example, phosphorylation of caspase-9 through the kinases Akt and p21-Ras inactivated caspase-9.[105] In addition, in recent years the influence of the vasodilator NO on caspase activity has been discovered. It has been shown that NO can block apoptosis by S-nitrosylation of the active center cystein of the caspases.[106] This process is reversible, since in FasL-induced apoptosis S-nitrosylated caspase-3 could be denytrosylated,[107] resulting in activation of caspase-3. And finally, S-nitrosylation also inhibited caspase-1 activity, followed by reduced release of IL-1 and IFN-γ from RAW264.7 macrophages.[108]

Caspase-1, IL-1 and Apoptosis

Although caspase-1 was the first caspase described and initiated a new era in apoptosis research, caspase-1 is believed to be no major player in apoptosis. However, in one of the early publications caspase-1 overexpression in fibroblasts was investigated and the experiments showed induction of apoptosis.[109] Surprisingly, mice devoid of caspase-1 developed normally.[67] The cells of these mice did not export IL-1α or IL-1β after LPS stimulation. The production of TNF and IL-6 was also diminished and the mice were resistant to endotoxic shock.[110] Interestingly, thymocytes of these animals were resistant to Fas-induced, but not to radiation- or dexamethasone-induced apoptosis, suggesting a role of caspase-1 in Fas-mediated apoptosis.[67] In contrast to ICE$^{-/-}$ mice, caspase-3, -9 or -8 knockout mice showed impaired neuronal or heart developement.[111-113] These data indicate, that caspase-1 has no major role in developemental programmed cell death, but it may be important in other types of physiological cell death, such as Fas-mediated apoptosis. As already indicated above caspase-1, but not caspase-3 or -11, appears to be involved in apoptosis of macrophages infected with bacteria.[114,115] Under these conditions caspase-1 binds a *Shigella* invasion plasmid ((Ipa)B) or the invasin SipB of *Salmonella* and becomes activated.[114,115] In addition, macrophages isolated from caspase-1$^{-/-}$ mice were not susceptible to *Shigella*-induced apoptosis.[114] On the other hand, caspase-1 is also involved in TGF-β-induced apoptosis of T-cells,[116] and thymocytes of mice harbouring a disrupted ICE gene are resistant to apoptosis induced by Fas antibody.[67]

In addition to a possible role of caspase-1 in certain types of apoptosis the major cleavage product of caspase-1, the mature IL-1β, may also have a role in the interaction of caspases and cytokines during regulation of apoptosis. Under these conditions IL-1 may act depending on the cell type and differentiation,[117] i.e. depending on the IL-1 receptor status. Thus, in spontaneous PMN apoptosis ICE or IL-1, as well as LPS stimulation, which results in IL-1 production, delay the onset of apoptosis.[118] In addition, following antisense-mediated SOD-1 downregulation apoptosis was detected in PC12 cells, and was accompanyied by IL-1β production. The addition of IL-1-Ra or IL-1β antibody prevented cell death.[119] On the other hand, blocking of ICE or IL-1 accelerated the spontaneous apoptosis of PMN, although ICE

expression is augmented during Fas-mediated PMN apoptosis.[120] By contrast, in monocytes, the spontaneous apoptosis, which occurs under serum free conditions, is independent of caspase-1.[121] The caspase-1 activator IL-11 may be involved in these processes. Thus, Hisahara et al[122] reported that caspase-11 is essential for apoptosis of oligodendrocytes during mice EAE and Kang et al[86] provided evidence that caspase-11 can activate caspase-3 in a similar way as caspase-8 or caspase-9.

Cytokines both Augment and Block Caspase-Dependent Apoptosis

The above data indicate that cytokines on one hand are targets for caspases, but on the other hand they can be involved—besides being involved in regulation of inflammatory processes—in regulation of apoptosis. In the following paragraph we summarize some of the data about the role of cytokines other than IL-1β in regulation of apoptosis.

Despite of the well known ability of TNF to induce various biological responses, including apoptosis (reviewed in ref. 123) recently another cytokine, namely IL-10, was also shown to induce apoptosis. As shown by Schmidt et al[124] IL-10 induced (or greatly augmented) Fas-FasL- or caspase-8-dependent apoptosis in human peripheral blood monocytes (MNC). Similarly, stromal-derived factor 1α induced $CD4^+$ T-cell apoptosis by up-regulation of CD95 and CD95L.[125] In contrast, cells deprived of proper stimulation by survival factors, including stimulation with cytokines, die by apoptosis. Interleukin-3, nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), or platelet-derived growth factor all protect cells from undergoing apoptotic cell death. Their cognate receptors belong to the family of protein tyrosine kinase receptors which are implicated in the activation of phosphatidylinositol-3 kinase (PI3-K). Rescue of PC12 pheochromocytoma cells with NGF or IGF-1 depends on the activation of PI3-K.[126] Also, downstream from PI3-K, Akt (also known as protein kinase B (PKB)) was found to be critical for the prevention of apoptotic cell death.[127, 128] Other cytokines, known to prevent or delay apoptosis, use different mechanisms. VEGF induced by hypoxia, prevented apoptosis of serum-deprived cells by activation of the MAPK/ERK pathway[129] and a similar mechanism seems to operate in some cell lines upon triggering of TNF-R1.[130] Human growth hormone was shown to protect monocytes and promonocytic U937 cells from Fas-mediated cell death by enhancing the expression of the antiapoptotic oncoprotein Bcl-2, as well as the level of Bcl-2α mRNA,[131] while TGF-β prevented apoptosis of microglia by induction of FLICE-inhibitory protein (FLIP).[132] Also, chemokines influence caspase acivity. IL-8 protected PMN from apoptosis by a mechanism independent of Fas and TNF-R, resulting in reduced caspase-3 activity.[133] During inflammation or allergic responses also GM-CSF, G-CSF or IL-5 delayed the apoptosis of neutrophils or eosinophils, respectively (reviewed in refs.134,135).

Cytokines may differentially affect pro- and anti-apoptotic pathways even in the same cell type. For example IFN-α/β sensitizes murine fibroblasts to FADD-dependent apoptosis induced by dsRNA or influenza virus infection but render them resistant to caspase-9 dependent cytolysis induced by vesicular stomatitis virus.[136] IL-6, despite its growth promoting effect, enhanced IFN-α-induced apoptosis of myeloma cells.[137] In erythroid progenitor cells IFN-γ provides a survival signal despite the parallel up-regulation of Fas.[138] Whereas, in microglia, Fas and FasL up-regulation observed after IFN-γ treatment is followed by apoptosis.[139] Activated T-cells in the presence of IL-2, become sensitive to Fas-induced death due to down-regulation of FLIP. Furthermore, IL-2 acting via IL-2Rβ through STAT-5 increased FasL expression.[140,141] Thus, although IL-2 promotes T-cell growth, at the same time it sensitizes the same cell type for apoptosis.

Increased activity of caspases-1, -3, -6, and -9 was reported in mice undergoing cancer cachexia,[142] a condition in which cytokines are most likely involved. Another in vivo example of close connection of cytokine production and apoptosis is sepsis. Under this clinical condition apoptosis was detected in several organs including the myocardium. Of interest is a recent observation that application of the caspase inhibitors z-VAD-fmk or z-DEVD.cmk, even 2 hours after injection of endotoxin, reduced caspase-3 activation and myocardial dysfunction.[143,144]

The differential effect of cytokines on cells may depend on the type of receptors involved. A well known example is the TNF-R1 / TNF-R2 receptor system: TNF-R1 induces apoptosis, while TNF-R2 stimulates proliferation.[123] However, this is not a dogma, since TNF-R2 may also assist in triggering TNF-R1 apoptotic signaling.[145] Furthermore, other findings clearly showed that in T-cells TNF-R2 is capable of conferring both cell death and growth.[146]

Studies employing murine caspase$^{-/-}$ mice suggest that these enzymes are involved not only in apoptosis and in cytokine maturation, but also in cell growth and differentiation. Apoptosis and cell division, although driven by different effector mechanisms, are closely linked at several levels.[147] Thus, receptors for some cytokines (mostly those containing β_c- or γ_c-chains) arrest apoptosis by promotion of cell division and/or differentiation. Since various cell types have different requirements for proliferation and differentiation signals, the same cytokine (e.g., IL-7) may or may not be critical for survival of cells which belong to the same lineage (e.g., lymphocytes).[148-150] Furthermore, developing tymocytes lacking FADD expression became arrested at the immature CD4 / CD8 stage (before TCR expression) which could mean that the death receptor signaling complex may also be necessary for cell growth and differentiation. Inhibitors of caspase activity were recently shown to block CD3-induced proliferation and interleukin-2 production by human T-cells. In keeping, rapid cleavage of caspase-8, without detectable processing of caspase-3, was observed after CD3 stimulation.[151] These data point to the possible role of death receptors during promotion of T-cell growth, together with IL-2.

Caspases in the Regulation of Transcription

Cells express their genes responding to environemental changes, in particular in response to specific extracellular signaling molecules. In turn, the cell changes the microenvironment by synthesis and release of cytokines, soluble receptors, enzymes, etc. The way a cell will respond to a stimulus depends very much on the context, namely the repertoire and sequence of incoming signals. The multiple extracellular signals converge within the cell and may have differential impact on gene expression. Since gene expression is initiated at the level of transcription it is widely accepted that cellular fates are derivative of gene transcription. Cell activation by cytokines, as well as expression of cytokines, bases upon a molecular machinery including transcription as the major regulatory pathway. Therefore the following paragraphs will show that communication between caspases and cytokines occurs also at transcriptional level.

Many caspase substrates have been described including: cytokines, repair proteins, proteins of Bcl-2 family, RNA binding proteins (La-1, U1-70kD), structural proteins (lamins, keratin, spectrin), molecules of signaling pathways (kinases, PLC-γ), proteins controlling the cell cycle (Rb), transcription factors and their regulatory proteins (I-κB, SP1, SREBP), integrin signaling (HEF-1) and many others (reviewed in[152-154]). Evidently, some of the caspase substrates do not seem to be directly involved in the apoptotic machinery, which means that caspases may function in other cellular processes. The possibility that two apparently conflicting cellular proceses (i.e. activation of caspases and transcription) are triggered by the same receptor raises the question about the influence of caspases on transcriptional regulation of cellular functions.

The TNF receptors can induce the caspase cascade, which results in cell death. On the other hand, by means of TRAF-2 and cIAP, they can initiate kinases and the corresponding transcription factors AP-1 or NF-κB, which may result in survival.[123] NF-κB has been reported to be an anti-apoptotic factor that plays a major role in cell survival, protecting cells against apoptosis induced by various agents.[49,155-159] It has been demonstrated that IFN-α pretreatment suppressed TNF-induced apoptosis[160] which correlates with the inhibition of both, the phosphorylation and degradation of I-κBα. Interestingly, macrophages require constitutive NF-κB activation to maintain viability by A1 expression and mitochondrial homeostasis. In keeping, suppression of NF-κB activation induced a time-dependent loss of mitochondrial transmembrane potential and DNA fragmentation.[161]

Apoptotic cascades and transcription-activating pathways appear to work separately, although some 'cross-talk' between the pathways has been reported. Inhibition of NF-κB activating

pathways up-regulates the caspase cascade. The suppressive influence of NF-κB on the caspase cascade is still controversial. It has been assumed that the activity of NF-κB regulates the expression of anti-apoptotic proteins such as TRAF-1, TRAF-2, cIAP-1, cIAP-2, the Bcl-2 family, A1, Bcl-X_L, and IEX-1L. Inhibition of these proteins may result in up-regulation of apoptosis, while overexpression may prevent apoptosis. The latest data show that over-expression of these proteins does not completely block apoptosis, which leads to the conclusion that NF-κB regulates also the expression of other suppressive proteins. NDED (NF-κB-inducible death effector domain-containing protein) probably plays a major role in the anti-apoptotic activity of NF-κB. Overexpression of NDED suppressed the TNF-mediated apoptosis in NF-κB-deficient cells. NDED inhibited TNF-mediated but not etoposide-mediated apoptosis, which is presumably due to selective downregulation of caspase-8.[162]

In the case of two signaling pathways induced by the same ligand (for example by TNF-α or Apo3L), activation of one pathway may switch-off the activity of the alternative pathway. Indeed, inhibitors of apoptotic pathways promote the survival pathway. c-FLIP (an inhibitor of caspase-8) activates the NF-κB and Erk signaling pathways, and since NF-κB enhances the expression of c-FLIP, it prolonges the inhibition by positive feedback.[163,164] The opposite is also true, since caspases down-regulate the survival pathway. Caspases cleave adaptor proteins, such as RIP, TRAF, or cIAP. Recently, it has been shown that TRAF-1 is a substrate for caspase-8 in apoptosis induced by TNF-α or FasL. Probably, in receptor-mediated apoptosis, caspase-8 switches off the survival pathway by proteolysis of competing molecules involved in signal transduction. In UV-induced apoptosis, where no involvement of caspase-8 is observed, there is also no proteolysis of TRAF-1. It should be noted that, while TRAF-1 is cleaved by caspase-8, it is rather TRAF-2 which is important for the survival pathway. It has been shown that the C-terminal TRAF-1 fragment has the activity of a dominant negative form of TRAF-2, blocking the induction of NF-κB by TNF-α[55,165] Among the substrates of caspase-8 there is also another molecule of the survival pathway, the serine/threonine kinase RIP which inhibits the TRADD-FADD interaction.[166,167] RIP is processed by caspase-8 into a dominant negative fragment. It has been reported that one of the cleavage products, RIPc, enhances the interaction between TRADD and FADD and increases the sensitivity to TNF-α. Since TRADD and-FADD interaction is indispensable during death receptor-induced apoptosis, the caspase-8 resistant RIP mutants protect cells against TNF-α-induced apoptosis.[167]

Recent findings reveal caspase-dependent cleavage of the hematopoietic specific adaptor protein Gads. Gads is a SH2 and SH3 domain-containing, hematopoietic-specific adaptor protein. It acts by linking SLP-76, bound by the carboxy-terminal Gads SH3 domain, to tyrosine phosphorylated LAT, which contains binding sites for the Gads SH2 domain. Caspase cleavage takes place within a 120 amino acid unique region between the SH2 and SH3 domain. It makes the interaction between molecules SLP-76 and LAT impossible and alters signaling of the T-cell receptor.[168] Thus, cleavage of signaling molecules may lead to branching of one signaling pathway or interference between two distinct pathways.

Apoptotic proteases can cleave and inactivate further survival signaling molecules, such as Akt/PKB, phospholipase C (PLC-γ1), and Bcl-2. Recently, it has been found that caspase-3 participates in the proteolytic cleavage of epidermal growth factor receptor (EGFR), which plays a crucial role in anti-apoptotic signaling.[54] This cleavage abrogates the activation of EGFR-dependent downstream survival signaling molecules. Caspases can directly down-regulate the activity of NF-κB in two dictinct ways. Caspase-3 is involved in amino-terminal truncation of I-κBα. The cleavage product ΔN tightly binds NF-κB, suppresses its activation and sensitizes cells to death induced by TNF-α. ΔN is also resistant to degradation depending on factors activating NF-κB.[169] Surprisingly, NF-κB is neutralized during apoptosis induced by other factors, such as gamma radiation, which do not deliver direct survival signals to the cell. Recently, it has been reported that caspases can also cleave the p65 subunit of NF-κB.[170] Several reports have linked another transcription factor—activating protein 2α (AP-2α) to apoptosis. AP-2α is cleaved by caspases prior to the DNA-fragmentation phase during apoptosis. Caspases

cleave AP-2α behind Asp_1 of the sequence Asp-Arg-His-Asp_1 (DRHD). The cleavage is followed by degradation of the transcription factor. It seems that AP-2α is an important survival factor since caspase-resistant mutation of AP-2α confers resistance to TNF-α-induced apoptosis.[171]

Besides signal transduction molecules caspases can directly cleave transcription factors, which would lead to silencing of those genes which are unspecific to certain cells. It has been demonstrated recently that caspase activity is an important negative regulator during erythroid differentiation through caspase-mediated degradation of the transcription factor GATA-1.[172] During transcription-dependent apoptosis caspases may function as positive regulators. They can activate transcription factors, which control expression of apoptosis-related genes. This process can take place by limited proteolysis or protein-protein interaction. It has been reported that cells can survive despite the presence of activated caspases in their cytoplasm, suggesting that caspases function apart from apoptosis.[151,168,173,174] This is not surprising since caspases contribute to the maturation of various cytokines. IL-1, like other proinflammatory cytokines provides a survival signal to many cell types. In some other cells NF-κB activated by IL-1β can have proapoptotic activity as described above.

Although cell proliferation and cell death are opposing and mutually contradictory, some evidence suggests that these two events are linked (as mentioned earlier). Caspases and cell cycle regulators share one purpose, the maintenance of genomic stability. The cell cycle regulators constitute the first checkpoints, which exist to interrupt cell cycle progression when damage of the genome is detected. If the damage is not repaired caspase-dependent cell death takes place. We may infer that caspases impose the final decision on the cell cycle renewal or on apoptosis. They can decide in favour of cell cycle progression through proteolysis of cell cycle repressors, or they can execute apoptosis through induction of the apoptotic cascade.

It has been reported that caspases take part in differentiation of progenitor cells during erythropoiesis, proliferation of T-lymphocytes or lens fiber development.[154] Several caspase knockouts reveal caspase influence on growth and development. For instance, caspase-$3^{-/-}$ mice are born at a lower frequency and are smaller than their littermates. Caspase-$8^{-/-}$ and $FADD^{-/-}$ mice exhibit impaired heart muscle development with thin trabeculae and ventricular musculature. In the immune system, hematopoietic precursor cells from these knockout mice reveal a strongly impaired colony-forming activity and a defect in maintaining sufficient numbers of T-cell progenitors entering thymic development.[172] It has been reported that caspase-3 is active in mitotic and postmitotic cells of the rat forebrain. A wave of active caspase-3 positive cells are dividing in the proliferative zones and subsequently migrating to the bulb, as they differentiate into neurons.[175]

Many authors have considered caspases as indispensable regulators of the replication machinery (reviewed in refs. 147, 174). Some substrates of caspases, such as topoisomerase-1 and nuclear replication factor MCM-3 function as 'mitosis entry blocker'. Caspases also cleave negative cell cycle regulators like Wee1, an inhibitor of the cell cycle regulatory kinases CDK2 and Cdc2, Cdc27, and a component of the anaphase-promoting complex. Moreover, the cycline inhibitors p21*Waf1* and p27*Kip1* are targeted by caspases. Caspases are activated during unscheduled cell cycle progression. The activity of caspases during proliferation can explain why they are generally not deleted or silenced in most tumors. An example is the regulation of the abundance of cdkI p27 (KIP-1) by caspases. The repression of caspases resulted in the accumulation of full-length inhibitor molecules, as well as a decrease in cell proliferation.[173] Recent studies have revealed that a variety of malignant tumors express Fas and/or its ligand FasL. However, tumor cells expressing Fas are not always susceptible to Fas-mediated cell death, and the biological significance of simultaneous expression of Fas and FasL in the same tumor is not known. In gliomas Fas-mediated caspase activation promotes cell cycle progression by a mechanism closely linked to the MEK-ERK pathway.[176] The data strongly suggest that FasL in gliomas may play a role of an autocrine growth factor.

Recently explored connections between DNA lesions, cell cycle and the apoptotic machinery may permit us to pose apparent questions: do caspases promote DNA-repair and cell cycle progression? Is transcription also involved? Many experimental data connect transcription with cell cycle and apoptosis. A paramount example is that of heat shock protein 90 (HSP90). Stress-induced activation of transcription begins with the dissociation of heat shock factor 1 (HSF-1) from inactive complexes with HSP90.[177] HSF-1 monomers form active trimers, which bind to promoters of several *hsp*. Released HSP90 prevents oligomerisation of Apaf-1 and subsequent activation of procaspase-9.[178] The same pool of HSP90 may simultaneously stabilize the cyclin-dependent kinase, Cdc2.[179] Several hours later transcriptional activation caused by HSF-1 results in additional amounts of HSP90, accumulating within the cell. This example clearly demonstrates how activation of transcription by stressogenic agents may result in cytoprotection and cell cycle promotion. In the above example particular attention should be paid to the fact that many non-lethal stressogenic factors (including heat shock) have the ability to activate caspases other than caspase-9.

What is the role of the transiently activated caspases in cells which recover from stress and do not enter apoptosis? Transcription-coupled repair enables UV-damaged cells to progress through the S-phase and prevents the induction of apoptosis.[180] But how is transcription (and transcription-coupled repair) initiated in UV-damaged cells? Should we postulate that caspases play a role of upstream activators for cytoprotective/repair mechanisms? The nearest future will certainly give specific answers to the above questions, but we already witness changes of the paradigm in research concerning caspases. In the past decade caspases were pictured as 'death squad' and their role within a cell was limited to autocatalytic destruction. Now this one-sided and oversimplified view is substituted by a different apprehension. Caspases seem much more like proteinases involved in many processes besides apoptosis, including regulation of cell cycle and inflammatory processes.

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CHAPTER 9

Caspases, Bcl-2 Family Proteins and Other Components of the Death Machinery: Their Role in the Regulation of the Immune Response

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Abstract

The prime directive of the immune system is to defend the host. The threats can be external in the form of microbial pathogens or internal in the form of rebellious autoreactive or malignant clones. The central dogma is that infected or aberrant cells must be destroyed quickly and innocuously to avoid significant cellular conflict and pathology. Apoptosis is the molecular program used by the immune system to implement the prime directive. The program is used to activate a set of caspases, which destroy cells that have been targeted to die. The apoptotic program can be activated internally when the molecular operating system detects cellular perturbations or externally via death ligands. It is critical for the development, maintenance and effectiveness of the immune system. As with any program it is susceptible to corruption and internal errors, which can result in host pathology.

Introduction

Programmed cell death is fundamental to the development, function and maintenance of the immune system. The apoptotic program is critical for the day-to-day running of both the innate and acquired arms of the immune system.[1] It is important in host defence against pathogens and perhaps cancer. However, when things go wrong and the program crashes or is hijacked and corrupted by pathogens the normal physiological immune response is replaced by pathological processes. For example, excessive cell death has been observed in some infections and inappropriate lymphocyte survival can lead to autoimmune disease or malignancies of the hemopoietic system.[2-4]

The apoptotic program utilizes as part of its hardware a set of aspartate-specific cysteine proteases called caspases, which systematically dismantle the cell.[5] These enzymes that cleave and consequently destroy critical cellular substrates are normally maintained in an inactive zymogen state so that the default switch setting is off.[6] It is only when one of the elaborate cellular subroutines and sensors is activated that the master downstream program is triggered and the switch is thrown to activate effectors of apoptosis.

In this chapter we will explore the role of apoptosis in both the normal physiological functioning of the immune system and also in pathological disease states. We will dissect the elaborate subroutines that control the apoptotic program. We will meet such players as FADD (also

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called MORT1) that function as adapters to coordinate the flow of extracellular data to the central processing unit, which initiates the effector program subroutine.

Overview of the Immune System

The Purpose of an Apoptotic Program

The primary function of the immune system is host defence and surveillance. It must recognize and eradicate pathogens and aberrant cells but must leave normal cells unharmed. Consequently the immune system must incorporate a program that facilitates the killing of pathogens and also a fail-safe suicide program for removal of self-reactive clones that would otherwise attack normal tissues.[7,8] Self-tolerance and removal of autoreactive lymphocytes is essential for normal immune function.

The immune system has two arms, an innate defence system and an acquired response element. The innate immune response consists of the ready and immediate host defences including physical, chemical and microbiological barriers.[9] The cellular elements of the innate system are phagocytic cells including neutrophils, monocytes and macrophages,[10] cells that release inflammatory mediators including basophils, mast cells and eosinophils[11] and executioner cells called natural killer cells.[12] The phagocytic cells have an arsenal of weapons including cytokines such as interferon and free radicals. The other molecular components of the innate response include complement and acute phase proteins. These mechanisms are highly conserved through evolution and function as the first and only line of defence in many species that have no adaptive immune system.[13]

The acquired or adaptive immune response requires priming with antigen and the effectors are T and B lymphocytes. The two arms are highly integrated.[14] Early defence is coordinated through the innate arm. It is non-specific but rapid, whereas the adaptive response takes some time to coordinate but is specific to the invading pathogen.[15] Moreover, the adaptive arm develops memory and on rechallenge with the same pathogen it arms quickly and is able to deploy rapidly and decisively.[16] Apoptotic cell death plays a role in both arms of the immune system. It is involved in destruction of infected cells, and in down-regulating the adaptive immune response.[1]

The Innate Immune Response and Apoptosis

The apoptotic program and caspases are inherent to all cells. Cellular perturbations caused by virus infection can trigger a trip wire that activates the cell suicide program. In the majority of cases this aids in the elimination of virus at the expense of the host cell and constitutes an early and ready defence in the innate immune system.[1,17] The importance of this apoptotic program in innate immunity is highlighted by the number of anti-apoptotic mechanisms that viruses have developed to either avoid triggering the program or inactivate the operating system.[1] Activation of the apoptotic program in an aberrant form is sometimes deleterious to the host. Viruses may commandeer the program and use it to their advantage in destroying host tissue thereby facilitating viral dissemination.

The Adaptive Immune Response and Apoptosis

When the adaptive immune response is primed with antigen, one of the major mechanisms by which T lymphocytes kill infected cells (those that have not already committed suicide) is by activating the apoptotic program and associated caspases in these cells. This is achieved by two mechanisms, either by signalling through death receptors (like Fas/APO-1/CD95 and TNF-R1) or through the action of perforin and granzymes.[18] When the pathogens have been cleared and the adaptive response changes to standby mode, many of the expanded T lymphocyte effector cells are deleted by apoptosis probably to prevent inappropriate tissue destruction or lymphadenopathy, which can be a forerunner of malignancy.[19]

Overview of the Apoptotic Program, the Operating System and Caspases

Activation of the cellular suicide program results in a characteristic type of cell death called apoptosis.[20] This program is intrinsic to all cells that make up vertebrate and invertebrate multicellular animals including nematodes, insects and mammals.[21,22] The program has been conserved during the evolution of the animal kingdom and the final executioners are invariably caspases. Caspases can be divided into two groups according to their structure and function: "initiator caspases" and "effector caspases".[5] The former have characteristic protein – protein interaction domains that facilitate binding to adaptor proteins. Caspase-8 and caspase-10 (the latter is present in humans but not mice) have two death effector domains (DED) through which they interact with the adaptor protein FADD that has a single DED motif.[23,24] caspase-1, -2, -4, -5, -9, -11 and -12 have a different interaction domain called caspase recruitment domain (CARD).[5] caspase-9 through its CARD domain and by way of a homotypic interaction is able to bind the adaptor Apaf-1 which also has a CARD motif.[25-27] Adaptor molecules function to aggregate the initiator caspase zymogens and the induced proximity allows their low-level enzymatic activity to effect autocatalytic processing and throw the death switch on.[28] The auto-activated initiator caspases are then able to process and activate effector caspases including caspases -3, -6 and -7.[5] This sets in train a series of cascading and amplifying activation subroutines that proceed inexorably to cell collapse as critical cellular proteins are proteolytically destroyed. Caspase after caspase becomes activated and in turn inactive enzymes like CAD (caspase activated DNase) are liberated from their shackled state to become active and destructive proteins that, in the case of CAD, chew up the instruction code library of life, DNA.[6] Various sensors in the cell that detect intracellular perturbations or external death signals initiate the apoptotic program.[29,30] Mammals have two distinct pathways that converge and feed into the central apoptotic processing unit with its effector caspases.[31] The two pathways utilize different adaptor molecules and different initiator caspases.

The Death Receptor Pathway to Cell Death

Cell death can be induced through certain cell surface receptors that belong to the tumor necrosis factor receptor family (TNF-R).[32] These "death receptors" share, in their cytoplasmic portion, a homologous sequence called the death domain.[32-36] Interactions between such death receptors and their ligands are important in several physiological processes. The down-regulation of specific adaptive immune responses is in part achieved by inducing apoptosis in activated T cells via death receptor ligation.[19] Moreover expression of death ligands provides T cells and other cells of the immune system with a means to externally activate the apoptotic program in infected or aberrant cells that must be detected during immune surveillance.[32] When death ligands bind to their cognate death receptors they cause clustering of the receptors and their death domains. This clustering recruits, via a homotypic interaction, adaptor proteins that also have a death domain such as FADD and TRADD (Fig. 1).[35-37] The death ligand, FasL, binds and oligomorizes its receptor Fas (CD95/APO-1) and clustering of Fas then facilitates the recruitment of the adaptor protein FADD. In the case of TNF-R1 and, perhaps certain other death receptors, the intermediary adaptor TRADD is required before FADD can be recruited to activated receptors.[32] When FADD binds to Fas or other death receptors it is able to recruit procaspase-8 (and in humans also procaspase-10) via the DED-DED interaction described above.[23,24] Procaspase-8 (and procaspase-10) have low inherent activity but when they are recruited and clustered by FADD a critical level is achieved and the recruited zymogens are able to activate each other.[38,39] The activated caspase-8/10 molecules then set off the apoptotic chain reaction. Caspase-9 and its adaptor Apaf-1 are not required in the cell death program initiated through the clustering of cell surface death receptors.[38-44] These elements form part of a different operating system that ultimately converges with the death receptor induced apoptotic pathway at the point of effector caspase activation.

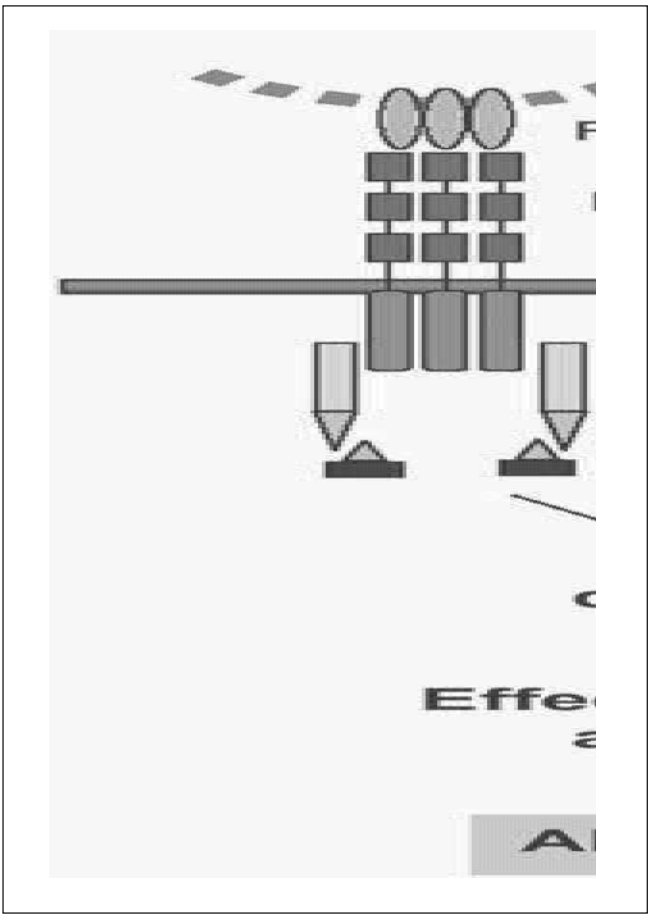

Figure 1. Apoptosis can be induced via death receptor signalling. FasL, TNF and TRAIL bind to their cognate receptors and recruit adaptor proteins FADD and TRADD. Caspase-8 is aggregated and activated to complete the death inducing signalling complex (DISC). Downstream effector caspases are activated and these cleave vital cellular substrates and cause cell death.

The cell surface death receptors have functions other than activating the apoptotic program. Ligation of TNF-R1 and some related receptors can activate transcription factors NF-κB and AP-1 via recruitment of various adaptor proteins and kinases like TRADD, RIP, NIK and TRAFs. Active NF-κB is in turn able to upregulate the expression of genes involved in inflammatory responses.[32,45] TNF upon binding to TNF-R1 can switch on the death program and instruct the cell to commit suicide or, if the death program is disabled for one or other reason, it can instruct the cell to survive and produce inflammatory cytokines.[46-48] The latter instruction set is issued more commonly probably because NF-κB is able to switch on genes that disable or deactivate the apoptotic program. The dual nature of death receptor signalling is an integral component of the "fuzzy logic" that the system uses to decide whether a cell should die or survive. The cell may receive many internal and external signals that contribute to a cellular milieu that is either conducive or obstructive in activating the apoptotic program.

FLIP molecules constitute one of the negative regulators of death receptor induced apoptosis. They resemble caspase-8 but do not possess its enzymatic activity. FLIP can bind to activated

FADD and thereby prevent the processing of caspase-8 and inhibit or interfere with the death receptor program.[49] Surprisingly in addition to its role in apoptosis, FADD may be critical in signalling pathways that promote cell proliferation and growth.[50-52] Like TRADD, which is able to flick the switch between activation of the cell death program and activation of the pro-survival and inflammatory pathway, FADD may have two faces and a dual role.

The Intrinsic Pathway to Cell Death

The operating system underpinning the intrinsic pathway involves regulatory elements known as the Bcl-2 family members. These members can be loosely divided into two groups, those that negatively regulate and those that positively regulate the intrinsic cell death program. Although caspase-8 and the adaptor FADD are essential elements of the apoptotic program induced by death receptor ligation, they are not involved in the apoptotic program activated by growth factor deprivation, glucocorticoids or DNA damage.[31,51,53-56] The initiator caspase-9 and the adaptor protein Apaf-1 are involved in the intrinsic apoptotic signalling program. Their role however, is not indispensable because at least in certain cell types overexpression of Bcl-2 or loss of certain pro-apoptotic Bcl-2 family members causes more severe apoptotic defects than loss of Apaf-1 or caspase 9.

The mammalian Bcl-2 protein family comprises at least 24 members encoded by 20 genes.[57] The various members function as sensors of cellular stress and they receive input from various sources including the endoplasmic reticulum, the cytoskeleton, the nucleus and the mitochondria (Fig. 2). The archetypal family member called Bcl-2 and its pro-survival homologues process most of the information collected by these sensors. Bcl-2 was the first member to be discovered when the encoding gene was found to be translocated in human follicular lymphoma.[58] Bcl-2 protects cells from growth factor deprivation or against exposure to cytotoxic drugs, taxol, cisplatin, glucocorticoids or ionising radiation.[59-62] However, it is not able to effectively abrogate the death program when it is activated by death receptors, at least in lymphocytes and myeloid cells.[31,63-67]

Seven of the Bcl-2 family members, Bcl-2, Bcl-X_L,[68] Bcl-w,[69] Boo,[70] A1,[71] Mcl-1[72] and Bcl-B[73] can inhibit apoptosis.[57] All the anti-apoptotic members share 3 or 4 homology domains called BH (Bcl-2 Homology) regions and they localize to the outer mitochondrial membrane and the cytoplasmic faces of the endoplasmic reticulum and nuclear envelope.[74-76] Seventeen other Bcl-2 family members promote apoptosis. These members include Bax,[77] Bcl-x_s (a splice variant of the *bcl-x* gene),[68] Bak,[78-81] Bok/Mtd,[82,83] Bad,[84] Bik,[85] Bid/Nbk,[86] Hrk/DP5,[87,88] Blk,[89] Bim/Bod,[90] Noxa,[91] Puma/Bbc3,[92-94] Bcl-G_L (long), Bcl-G_s (short)[95] and Bmf.[96] These pro-apoptotic members can be divided into two subgroups depending on the number of Bcl-2 homology domains they possess. Bax, Bok, Bak, Bcl-G_L and Bcl-X_s have multiple BH domains whereas Bik, Blk, Hrk, Bim, Bad Bid, Bcl-G_s, Puma, Noxa and Bmf only possess the short (9 to 16 residue) BH3 region and hence are sometimes called BH3-only proteins.[97]

The pro-apoptotic and anti-apoptotic Bcl-2 family members can physically interact and in some cases antagonize each other.[77,98] This is particularly important in the case of the BH3-only pro-apoptotic members because their ability to induce apoptosis is dependent on their ability to bind and antagonize the pro-survival members.[78,90,96,99] Many of the BH3-only proteins are sequestered from the pro-survival members in healthy cells so that antagonistic interactions are minimized. However, when these BH3-only stress sensors are activated they are liberated and they can block the pro-survival members and thereby activate the apoptotic program. For example, Bim and Bmf are sequestered to the microtubular dynein motor complex[100] and the myosin V actin motor complex respectively.[96] Certain apoptotic stimuli can cause release of Bim or Bmf and allow them to translocate to and antagonize the pro-survival Bcl-2 proteins, thereby initiating the apoptotic caspase cascade.

It is not clear how the multidomain pro-survival Bcl-2 family members are able to maintain the apoptotic program in a repressed state but somehow they must interfere with the activation

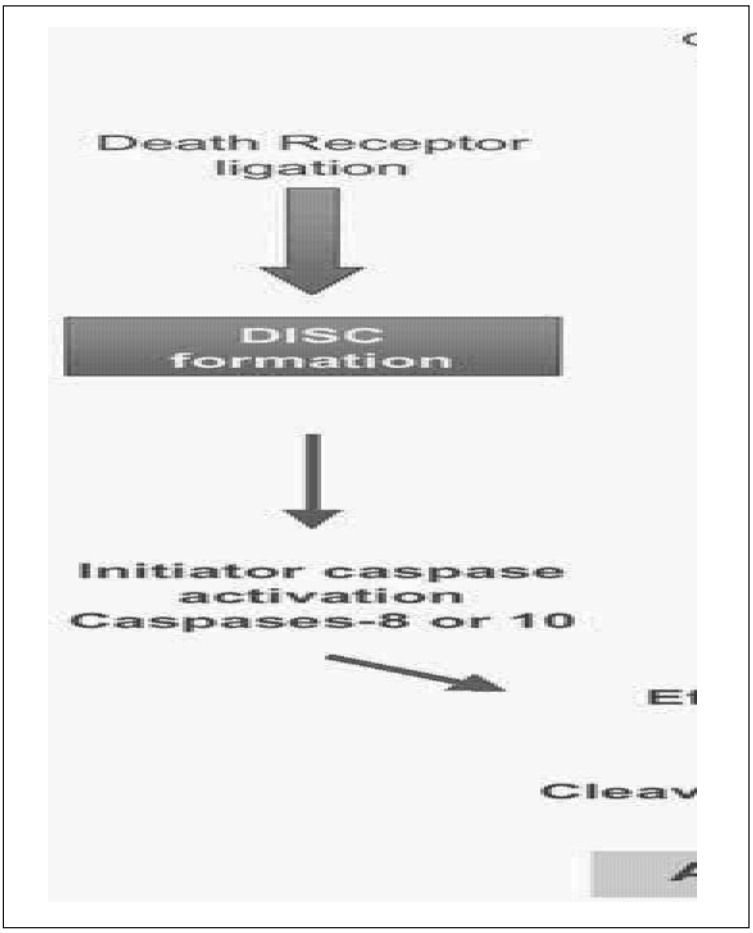

Figure. 2. The pathways to death. Apoptosis can be induced by death receptor signalling and DISC formation or via the activation of the intrinsic apoptotic program. The Bcl-2 family members form the backbone of the intrinsic apoptotic pathway. Growth factor deprivation, glucocorticoids and DNA damage activate pro-apoptotic Bcl-2 family members (via p53 in the cases of DNA damage, via glucocorticoid receptors in the case of glucocorticoids and via other signalling proteins in the case of cytokine withdrawal). The pro-apoptotic members antagonize pro-survival Bcl-2 homologues that normally block the activation of adaptor proteins and initiator caspases. Whichever path is taken the final program involves activation of effector caspases that dismantle vital cellular substrates and cause cell death.

of initiator caspases. In the case of caspase-9, the adaptor protein Apaf-1 is required to cluster the pro-enzyme and facilitate its autocatalytic activation. Apaf-1 mediated clustering of caspase-9 into an apoptosome requires the presence of cytochrome c, which is normally located inside the mitochondria.[26] Bcl-2 could either directly or indirectly regulate the activity of Apaf-1 or other adapters or it could prevent the release of cytochrome c from the mitochondria.[101-105] We believe that the Bcl-2 family members inhibit the activity of Ced-4/Apaf-1 related molecules and that the mitochondrial release of cytochrome c may function as an amplifier or positive feedback loop in the apoptotic cascade.[106] The pro-apoptotic molecules Bax and Bak are required to effect death induced by the BH3-only proteins and, Bcl-2 and its homologues appear to maintain Bax/Bak related proteins in an inactive state.[107,108] The BH3-only proteins

may directly or indirectly, by inactivating Bcl-2, promote the activation of Bax and Bak and thereby initiate the caspase cascade.

The ratio between pro-survival and pro-apoptotic Bcl-2 family members determines which direction the switch is flicked, cell death or cell survival. The levels and activity of pro- and anti-apoptotic Bcl-2 family members can be regulated by cytokines. Certain cytokines, such as IL-7, can upregulate the production of pro-survival Bcl-2 family members.[109-112] The amount or activity of pro-apoptotic Bcl-2 family members can also be regulated by extra-cellular ligands.[96,100,113,114]

DNA damage or cell cycle aberrations can induce cell death via a program subroutine that involves the tumor suppressor p53.[62,115-117] The mechanism by which p53 is able to activate the apoptotic program is unclear but it may involve increasing the expression of the pro-apoptotic (BH3-only) Bcl-2 family members Noxa or Puma.[91,94] The p53 apoptotic program ultimately utilizes the standard intrinsic operating system with caspase-9 and Apaf-1 to facilitate effector caspase activation[40-43,118] and it can be antagonized by pro-survival Bcl-2 family members.[62]

The Caspase Hardware

Regardless of the initiating event or the path taken, the final subroutine of the apoptotic program is common to all and involves the activation of effector caspases that function as the executioners of the cell death. Apart from their involvement in the apoptotic program, caspases can also be involved in other cellular processes. Caspases -1 and -11 have pro-inflammatory roles, as they are required for the processing of IL-1β and IL-18.[119-123] Indirectly they also control the production of IL-1α, Il-6, tumor necrosis factor-α (TNF) and interferon-γ (IFN-γ) in response to lipopolysaccharide (LPS) stimulus. Indeed mice that are deficient in caspase-1 do not succumb to a septic shock syndrome normally induced by LPS injection.[122-124] caspase-1 and caspase-11 deficient mice have no obvious abnormalities in developmental cell death.[123,124] Therefore it is believed that these caspases play no role in programmed cell death. Alternatively, they could have an important but redundant role that would only manifest in mice lacking two or more initiator caspases.

Mice deficient in caspase-8 die during embryogenesis due to defective myocardial development and they have reduced numbers of hemopoietic precursor cells.[56] This may indicate that caspase-8 has a role in cell growth and proliferation in addition to its role in death receptor induced apoptosis. Alternatively the phenotype may be due to defective apoptosis in other cells required for normal myogenesis. Mice lacking the initiator caspase-9 have brain overgrowth due to reduced apoptosis in neuronal tissue.[40,41] caspase-3 deficient mice show a similar but slightly less profound defect in the central nervous system.[125] The effects of caspase-9 and caspase-3 deficiencies in lymphoid and myeloid cells are not very severe indicating that other initiator and effector caspases may be more important for apoptosis in the hemopoietic system. Mice lacking caspase-2 have only minor abnormalities[126] and mice lacking caspase-12 develop normally, but their cells are relatively resistant to ER stress induced apoptosis.[127]

Granzyme B, a serine protease, can induce apoptosis by processing caspases at the P1 position.[128] Granzyme B released by T cells realizes its homicidal potential by activating caspases in target cells and thus activating the final elements of the apoptotic program.[129-131]

Deciphering the Operating Language of the Apoptotic Program in the Immune System

Most of our understanding of the apoptotic operating language comes from transgenic or gene knockout studies in mice.

Dissecting the Death Receptor Pathway

A deficiency of caspase-8 is embryonic lethal in mice possibly due to a cardiac defect. Embryonic fibroblasts obtained from caspase-8-deficient mice are completely resistant to death receptor induced apoptosis.[56] caspase-8 therefore, is an essential element of the death receptor

pathway despite reports that caspase-2 might be able to substitute for it in receptor-associated apoptosis.[126] Studies using transgenic mice that express CrmA, an inhibitor of caspase-8, in lymphocytes show that these lymphocytes are resistant to death receptor induced apoptosis.[54] Mice that have defects in FasL or Fas show a similar resistance to death receptor induced apoptosis, but in addition these mice develop T-cell hyperplasia and high levels of autoantibodies.[33] Mice deficient in FADD die during embryogenesis with a phenotype similar to that of caspase-8 deficient mice.[50,53] Interestingly, mature T cells that express a dominant interfering mutant of FADD (FADD-DN) or lack FADD, show a reduced proliferative potential in response to mitogens or antigens.[50,52] In contrast, lymphocytes from transgenic animals expressing CrmA proliferate normally in response to mitogenic stimulation, as do lymphocytes from mice with defective FasL or Fas.[51,54] Although the phenotype of the caspase-8 deficient mouse lends support to the theory that caspase-8 may be involved in the control of cell proliferation, the CrmA transgenic studies indicate that caspase-8 does not have a critical role in cell proliferation.

Thymocyte development and selection is dysregulated when the function of FADD is blocked. At an early stage of development $CD3^-4^-8^-$ pro-T cells differentiate to become $CD4^+8^+$ thymocytes after assembly of a functional T cell receptor β chain. Those cells that are unable to assemble a functional TCR β chain are culled at the pre-TCR checkpoint, but this is not the case in thymocytes expressing FADD-DN or in FADD-deficient pro-T cells from chimeric mice.[132,133] Interestingly, this phenomenon is not seen in mice lacking Fas, which indicates that other (death) receptors must be involved in this culling process. The normal proliferation of thymocytes as they progress from the $CD3^-4^-8^-$ pro-T to the $CD3^+4^+8^+$ thymocyte stage is severely impaired by FADD-DN expression.[133] Thus FADD plays a critical role in cell death and cell proliferation at the pre-TCR checkpoint.

It has been suggested that there is an element of cross talk between death receptor-induced apoptotic signalling and the intrinsic apoptotic program. Evidence suggests that activated caspase-8 can cleave Bid (a pro-apoptotic BH3-only Bcl-2 family member) to a truncated form, which is then able to activate the intrinsic pathway and thus amplify the apoptotic program.[105,134,135] Bid-deficient mice show some resistance to Fas-induced hepatocyte apoptosis but their lymphocytes are normal and remain sensitive to Fas-induced killing.[136] Thus, Bid may play a role in amplifying the death receptor signal through the intrinsic Bcl-2 apoptotic pathway in some but not all cells. Indeed, since Bid can also be cleaved by caspases other than caspase-8,[105,134,136] it may play a more general role as an amplifier in apoptosis signalling.

Dissecting the Intrinsic Apoptotic Signalling Pathway

Mice deficient in Bcl-2 have a defect in keeping mature lymphocytes and myeloid cells alive.[137] Differentiated T and B cells are highly vulnerable to accidental activation of the intrinsic apoptotic pathway. Bcl-2 deficient mice are born runted and within a few months die from renal failure secondary to polycystic kidney disease and they have excessive melanocyte death and neuronal cell death postnatally.[137,138]

Bcl-X has two isoforms, Bcl-X_L and Bcl-X_s, which inhibit or promote apoptosis respectively.[68] Mice that lack both isoforms die during embryogenesis due to overwhelming apoptosis of postmitotic differentiating neurons and fetal liver hemopoietic cells.[139] In an attempt to understand the relevance of Bcl-X in the immune system chimeric mice have been generated. In these mice mature Bcl-X deficient T and B cells function normally but immature $CD4^+8^+$ thymocytes are abnormally prone to apoptotic cell death.[139,140] This is in contrast to the Bcl-2 deficient animals described above in which lymphopoiesis proceeds normally but mature lymphocyte survival is severely impaired. This correlates precisely with the levels of each of these molecules in the respective cell types. A1-deficient mice have accelerated apoptosis in their neutrophil populations[141] and embryos lacking Mcl-1 die prior to implantation.[142] Transgenic overexpression of Bcl-X_L or Bcl-2 makes thymocytes resistant to a variety of apoptotic stimuli including gamma irradiation, glucocorticoids, and anti-CD3 treatment.[60-62,144] Overexpression

of Bcl-X_L within B lymphocytes causes marked accumulation of peripheral B cells in lymphoid organs and enhanced survival of developing and mature B cells in transgenic mice.[145]

Transgenic overexpression of Mcl-1 in hemopoietic cells enhances their viability when cells are cultured in vitro, particularly in cells of the myeloid lineage, but homeostasis is maintained with normal cell numbers in the whole animal.[143]

Mice lacking the BH3-only pro-apoptotic Bcl-2 family member Bim show aberrations in thymic development and their mature T and B cells do not die upon cytokine withdrawal. Consequently these mice develop lymphadenopathy, splenomegaly and elevated levels of immunoglobulin with plasmacytosis.[146] Over 50 % of Bim-deficient mice die during embryogenesis of unknown causes and many of those born die by one year due to SLE-like autoimmune complications with immune complex deposition in renal glomeruli and a vasculitis.[146]

The immune system, in an attempt to produce a large and varied repertoire of T cells that can recognize the spectrum of foreign antigens, produces as a byproduct T cells that recognize and could potentially be harmful to self. Autoreactive T cells must be deleted in the thymus or in peripheral lymphoid organs to protect the host from rebellious and self-destructive clones.[147] Apoptosis is responsible for the culling of autoreactive T-cells in the thymus.[148-150] This process is termed thymic negative selection. Cells that somehow escape this process may still be deleted in the periphery.[151] Given the thymic and peripheral T cell aberrations seen in Bim deficient mice, Bim could be one of the pro-apoptotic molecules involved in negative selection.[146, 239]

Bax deficient animals have very mild hemopoietic abnormalities and they also show a degree of neuronal hyperplasia.[152,153] Bak deficient mice appear normal.[154] Crosses between Bax and Bak-deficient animals generate $Bax^{-/-}Bak^{-/-}$ mice with lymphocytes and fibroblasts (and perhaps other cell types) that are refractory to many death stimuli that activate BH3-only-proteins.[107,108,154] These mice showed persistence of interdigital webbing and develop progressive lymphadenopathy.[154]

When the Program Crashes or is Corrupted

Cancer

Apoptosis is involved in the removal of aberrant cells that might otherwise give rise to tumors.[4] Mutations leading to reduced activity of pro-apoptotic Bcl-2 members, or mutations causing overexpression of pro-survival genes promote tumorigenesis. Bax is mutated in some leukeamias[155] and translocation of the *bcl-2* gene with its subsequent overexpression has been demonstrated in most follicular lymphomas and in some cases of chronic lymphocytic leukaemia and diffuse large cell lymphoma.[57] Bcl-2 transgenic mice develop lymphoid hyperplasia that can in turn develop into lymphomas.[156-160]

Many B lymphoid tumors have a translocation of the myc gene, which enhances cell proliferation and thereby contributes to oncogenesis. Deregulated Myc expression promotes cell cycling but also lowers the threshold for activation of the apoptotic program.[161-163] Mice that overexpress Myc and have the apoptotic program deactivated by overexpression of Bcl-2 develop lymphomas and mammary carcinomas at a rate that exceeds that seen in mice overexpressing either of the two oncogenes alone.[157,164,165]

Resistance to Fas-induced apoptosis has also been implicated in tumorigensis.[166-169] Human B non-Hodgkin's lymphoma cells are resistant to killing caused by agonist anti-Fas-antibodies.[170,171] Aberrations in Fas signalling could make malignant cells partially resistant to cytotoxic T cell mediated killing, but they remain sensitive to the action of perforin and granzymes.[172,173] It is not clear whether this or other consequences of deranged Fas signalling are responsible for transformation. Rag-1 deficient mice, in which the function of FADD has been blocked by transgenic expression of FADD-DN, develop thymic lymphomas over time.[133] FLIPs may be involved in deactivation of the death receptor program but evidence implicating their involvement in lymphoma disease is only circumstantial to date.[174,175]

Implications for Cancer Treatment

Chemotherapy used in the treatment of lymphoid malignancy can induce apoptosis of the rogue cells.[176] If the apoptotic program is impaired, cells could potentially become resistance to chemotherapy. It has been suggested that inactivation of p53 and/or overexpression of Bcl-2 (or any of its pro-survival homologues) is associated with chemotherapy and radiotherapy resistance.[62,177] Indeed, the tumor suppressor gene p53 is mutated in many types of cancer.[178] It is still controversial as to how important deactivation of the apoptotic program is in determining chemotherapy and or radiotherapy resistance in tumors that are not of hemopoietic origin.[179]

Autoimmunity

Cells of the immune system have an enormous potential to expand in response to stimulation. This is critical for their ability to deal with and eliminate invading organisms. The corollary of this, however, is that lymphocytes can develop into a dangerous population of cells if their growth and activity is not kept in check. We have speculated, on the basis of knockout studies, that the pro-apoptotic BH3-only protein Bim could play a role in negative selection in mice. Bcl-2 overexpression can interfere with the process of thymic negative selection,[60,61,180] but death receptor signaling via FADD and caspase-8 does not contribute to this process.[51,54]

Autoreactive T cells that escape thymic negative selection can still be deleted in the periphery to avoid the development of autoimmune disease. There are two potential ways in which such escapees can be dealt with in the periphery. One mechanism involves activation of death receptors and the other is triggered by limiting availability of essential growth factors.[151] T cells upregulate expression of death receptors in response to antigen stimulation and this primes their extrinsic apoptotic program.[19,181-183] Autoreactive clones that repeatedly make contact with self-antigen in the periphery will be particularly susceptible to death ligand induced apoptosis. Indeed *lpr* and *gld* mutant mice that have impaired death ligand mediated apoptosis, because of mutations in the Fas or Fas ligand gene, develop lymphoproliferative disorders and autoimmune disease.[184] If wild type Fas is restored in T cells of *lpr* mice they do not develop lymphoproliferative disease but they still succumb to autoimmune disease.[185] Therefore, Fas induced death or signaling in T cells alone is not sufficient to prevent autoimmune disease.

In humans defective Fas-signaling is the likely cause of a particular syndrome comprising lymphadenopathy and an SLE-like autoimmune disease called ALPS.[186,187] In type 1 ALPS there is a defect in Fas or FasL, but in a proportion of patients (ALPS type II) these defects are not present yet the patients still display an ALPS phenotype.[188] Capase-10 mutations have been defined in these cases,[189] but others suggest that these may just represent coincidental non-pathological polymorphisms of the gene locus given that a large proportion of healthy people carry these changes.[190]

Deletion of activated T and B cells can also be achieved by starving them of cytokines (in particular IL-2 in the case of T cells and IL-6 in the case of plasma cells). Withdrawal of these factors induces apoptosis via activation of the intrinsic program and this can be blocked by Bcl-2.[60,61,109,111,191] Bim-deficient lymphocytes are resistant to apoptosis induced by cytokine deprivation.[146] Moreover, B cells from transgenic mice that overexpress Bcl-2 are resistant to intrinsic death stimuli and the mice show sustained humoral immune responses with a plasmacytosis and consequently high level of serum immunoglobulins.[191] Bcl-2 overexpression or loss of Bim, on certain genetic backgrounds, leads to a fatal SLE-like autoimmune disease with high levels of autoantibodies to nuclear antigens.[146,191]

TNF-R Family Members Making Life and Death Decisions

We have met some of the TNF-R family members already in the form of death receptors like Fas, TNF-R1 and TRAIL receptors (DR4 and DR5). There are, however, other TNF-R family members, such as TNF-R2, CD30 and CD40, which do not signal death because they do not contain a death domain.[192] These receptors can still potentially trigger apoptosis indirectly by inducing the expression of membrane-bound TNF, which then causes cell death through

paracrine or autocrine TNF-R1 activation.[193] Recently, the BAFF receptors TACI, BCMA, and BAFF-R have been discovered. These receptors are distant relatives of the TNF-R family.[194-201] Signaling through these receptors inhibits the intrinsic apoptotic program in B cells, which is normally activated by B cell receptor ligation. Signaling through the BAFF receptors and also CD40 leads to increased levels of Bcl-2 in B cells, possibly by activating the NF-κB pathway, and this inhibits the intrinsic apoptotic program.[202-204] Increased levels of BAFF are found in certain strains of mice that develop SLE and transgenic mice that overexpress BAFF develop B cell lymphadenopathy, plasmacytosis and an SLE-like autoimmune disorder.[194,203,205,206] Activated B cells like T cells tend to commit suicide unless they are protected by the presence of growth factors. In the case of B cells these factors are likely to be CD40L and BAFF.

Infectious Diseases

The apoptotic machinery is integral to the function of the innate immune system.[207] Viruses encode a vast artillery of proteins that deactivate or corrupt the apoptotic program at practically every point including Fas death receptors, Bcl-2 members and caspases.[1] These proteins deactivate the apoptotic program to permit viral latency or corrupt and activate the program to facilitate viral dissemination.[208]

The first tripwire encountered by viruses attempting to infect cells is at the point of cell attachment. HIV has a viral coat protein called gp120 that attaches to the lymphocyte surface receptor CD4 and the chemokine receptors CCR5 or CXCR4.[209] Soluble or membrane associated gp120, on the surface of infected cells, can induce apoptosis in uninfected cells and this may contribute to viral pathogenicity.[210]

The Toll receptor family is a set of cell surface receptors used by the innate immune system to sense and signal the presence of microbes.[211] All bacteria express bacterial lipoproteins and these are potent activators of the Toll signaling system via Toll receptors (TLR).[212,213] Engagement of TLRs initiates a cascade of cellular signals that results in the activation of NF-κB, which regulates the expression of genes involved in inflammation and cytokine production. In addition, like the TNF-R family, TLRs via the adaptor protein MyD88 can recruit FADD, activate caspase-8 and thereby initiate apoptosis.[214] In essence then, the innate immune response through the Toll receptors can mobilize the immune forces and also instruct certain cells to die.

Cytotoxic T lymphocytes constitute a major arm of the adaptive immune system. One of their primary roles is to defend the host in the event of viral infection. CTLs kill virally infected cells by releasing perforin and granzymes and by expressing FasL, TNF and perhaps other ligands that activate the death receptor program in target cells.[215-217] Viruses have evolved mechanisms to inhibit this form of killing by downregulating cellular expression of MHC class I proteins so that the infected cells are no longer recognized by the immune system. This is highly effective, for as long as infected cells remain incognito they will not be detected by T cells and hence they will not be targets for death ligand or perforin/granzyme induced apoptosis. Certain viruses have evolved strategies to downregulate the expression of death receptors to directly prevent this form of killing.[218-220] Other viruses code for proteins that inhibit death receptor induced apoptosis by interfering with FADD/MORT1 mediated activation of caspase-8. Cytomegalovirus encodes a protein called vICA that binds to and inactivates caspase-8.[221] Many γ-herpes viruses encode a protein homologue of FLIP, called vFLIP, and adenovirus encodes the inhibitor RID/E3-14.7K.[222-225]

Given that pro-apoptotic Bcl-2 family members function as sensors of cellular perturbations, they may induce apoptosis if viral infection is detected. Therefore viruses have developed a whole array of Bcl-2 related death suppressers to prevent apoptosis.[17,226,227] Epstein-Barr virus (EBV), Human Herpes Virus 8 (HHV8) and adenovirus produce BHRF-1, KSbcl-2 and E1B-19K respectively.[228-231] HIV encodes the protein "tat" that increases the transcription of cellular Bcl-2.[232]

Depending on circumstances, pathogens may attempt to inhibit apoptosis to allow replication or alternatively they may promote apoptosis to facilitate dissemination and transmission and in doing so cause disease. Many of the pathogenic effects of viruses are due to excessive apoptosis. This is particularly the case with neurotropic viruses that induce apoptosis to produce disease[233] and with HIV, which enhances lymphocyte apoptosis induced by death receptors.[234,235] The bacteria *Neisseria meningitides* and *gonorrhoreae* produce porins that either stabilize or destabilize the mitochondrial membrane potential respectively. This can prevent or accelerate cytochrome c release and is thought to downregulate or enhance the apoptotic amplification loop respectively.[236,237] Cytomegalovirus may target the mitochondria by a different mechanism using the viral protein vMIA that may inhibit apoptosis.[238]

Viruses usually attempt to commandeer the host cell replication and cell cycle machinery. Cell cycle perturbations activate p53 and thereby activate the intrinsic apoptotic program. Many viruses therefore, encode proteins that antagonize the action of p53. These include adenovirus E1B-55K, human papillomavirus E6 and Simian virus 40 T-antigen proteins.[1]

Concluding Remarks

The apoptotic program with its two operating systems, one activated by death receptors and the other regulated by Bcl-2 family members, does not function in isolation. The program is necessarily subject to regulation from external and internal influences which dictate when, how, why, where and which cells die. Apoptosis and caspases are integral to the immune system. This system must develop, be instructed, cycle between massive expansion and catastrophic death, rebellious clones must be defeated, infectious agents must be eliminated and the prime imperative is to protect the host at any expense. The program itself is vulnerable to crashes, fatal errors and corruption that can result in disease. A better understanding of the operating system, the software and hardware will assist us in attempts to repair or manipulate the apoptotic program to prevent or treat diseases.

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Chapter 10

Learning from Deficiency: Gene Targeting of Caspases

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Introduction

For all multicellular organisms, cell number control is essential for proper organ formation during development, and for cellular homeostatsis in adults.[1] Such a critical task requires a delicate balancing act of cell proliferation and, as realized more recently, cell death. While much attention in the past has been given to the molecular mechanisms that control how cells divide and propagate, recent efforts unveiled an equally elaborate cellular machinery that governs the process of cell suicide, also known as apoptosis. At the very center of this tightly-regulated process is a group of intracellular cysteine proteases called caspases for cysteinyl aspartate-specific proteinases.[2] Upon receiving apoptotic stimuli, these otherwise latent proteases become activated and carry out cell destruction through proteolytically cleaving specific intracellular targets.

To date, approximately a dozen mammalian caspases have been identified through both genetic and biochemical means.[3] The presence of multiple caspases therefore raised the critical question of how individual caspases contribute to apoptosis in vivo. To definitely address this issue, we and others have generated several lines of caspase deficient mice using gene targeting strategy.[4] Analyses of these caspase deficient mice have contributed significantly to our current understanding of how caspases function in vivo. For example, studies using caspase-$3^{-/-}$ cells unequivocally established caspase-3 as the key caspase that mediates the execution of apoptosis in mammals.[5] Similarly, generation of caspase-9 deficient mice confirmed the existence of 'caspase cascade' regarding caspase activation during apoptosis, as suggested by elegant in vitro studies.[6,7] In this chapter, our aim is to provide an overview of what we have learned from caspase knockout mice, with a focus on some of the more surprising findings revealed.

Overview of Caspase Deficient Mice

Gene targeting in the past decade has become the standard approach to examine gene function in physiological settings. Among the ten known murine caspases, all but caspase-14 have been knocked out (see Table 1). Given the confusing nature of caspase nomenclature due to historical reasons, it is worth pointing out that caspase-11 and –12 have only been identified in mouse and are most likely the murine homologues of human caspase-4 and –5, respectively. In addition, caspase-10 probably only exists in human and recent reports have argued that caspase-13 is actually a bovine caspase gene, rather than a new human caspase, as originally reported.[8]

Based on sequence homology, prodomain function and substrate specificity, caspases are generally categorized into three groups: initiator caspases (caspase-8, –9 and -2), effector caspases (caspase-3, -6 and –7) and caspases that are primarily involved in mediating inflammatory

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Table 1. Murine caspases and caspase deficient mice

Caspase	Dev. Defects	Apoptotic Defects	Other Defects	References
Caspase-1	none	none	IL-1α, -1β & -18 processing, endotoxin resistant	12,13
Caspase-2	none	oocytes (decreased PCD) facial motor neurons (accelerated PCD)	N/D	42
Caspase-3	Neuronal (perinatally lethal*)	morphology/DNA fragmentation (MEFs, hepatocytes, thymocytes) Neutrophil turnover, lymphocyte AICD*		5,11
Caspase-6	none	N/D	B-cell maturation?	unpublished
Caspase-7	embryonic lethal	N/D	N/D	unpublished
Caspase-8	embryonic lethal	death-receptor pathway (MEFs)	N/D	10
Caspase-9	Neuronal, embryonic lethal**	mitochondrial pathway, (thymocytes)	N/D	6,7
Caspase-11	normal	none	L-1α, -1β & -18 processing, endotoxin (LPS) resistant	14
Caspase-12	normal	ER-pathway	N/D	15
Caspase-14	not yet reported			

* The perinatal lethality of caspase-3$^{-/-}$ mice and defective AICD observed in caspase-3$^{-/-}$ lymphocytes depend on genetic background.[6]

** A very small percentage of caspase-9 knockout mice (<2%) develop normally.
N/D=not determined

responses (caspase-1, -11 and –12).[9] According to the proposed 'cascade model' of caspase activation, multiple caspases are activated in the following step-wise manner during apoptosis.[2] In response to a given apoptotic stimulus, one upstream initiator caspase is first activated through adaptor-mediated autoprocessing. The activated initiator caspase in turn proteolytically activate several downstream effector caspases whose activation leads to the final destruction of apoptotic cells by cleaving various cellular targets. One can therefore predict that deficiency in individual caspases will likely result in defect in the initiation or execution of apoptosis, or in inflammatory responses.

The generation of mice deficient in these caspases has by and large confirmed such a prediction with a few exceptions. For example, deficiency in the initiator caspases-8 or –9 results in nearly complete block of downstream caspase activation and apoptosis induced through their respective pathways, demonstrating that initiator caspases are absolutely required for the progression of caspase-mediated cell death.[6,10] However, only caspase-3,[11] but not caspases-6 or –7, appears

to be required for apoptosis execution (unpublished data), raising the possibility of functional redundancy among downstream effort phase caspases or that caspase-6 and -7 are involved in other cellular functions. With respect to caspases that are thought to mediate inflammation, caspase-1$^{-/-}$ and –11$^{-/-}$ mice both indeed exhibit defective inflammatory response due to their inability to process and secret pro-inflammatory cytokine 'interleukin-1β and -1α.[12-14] caspase-12 deficiency, on the other hand, had no apparent effect on inflammation, but instead resulted in defect in apoptosis initiated through the endoplasmic reticulum (ER) pathway.[15]

Caspase Deficiency and Mammalian Development

Developmentally regulated apoptosis plays a prominent role during mammalian embryogenesis.[1] Complex tissue/organ formation often involves generation of excessive cells and their subsequence removal by apoptosis to ensure fine-tuning of the process. Thanks to the generation of various caspase deficient mice, it is now clear that several caspases are essential for mammalian development.

The best-studied example is the involvement of caspase-9 and -3 pathway in neuronal development. Mice deficient in either caspase-3 or -9 exhibit similar developmental blockage characterized by perinatal lethality.[5-7] Histological analysis of the surviving newborns revealed various neural phenotypes that likely resulted from supernumerary neurons during CNS development. For example, contrary to the smooth surface of the cerebrum mantel in normal rodents, the caspase-3$^{-/-}$ mouse brain exhibited multiple indentations reminiscent of gyria structures found in higher vertebrates, indicative of excessive brain cell mass. Also, BrdU-negative ectopic cell populations were frequently observed in various areas around the hippocampus region, some of which were even capable of adopting proper cellular deployment such as the formation of a 'double cortex'. Further examination of developing embryos deficient in either caspase-3 or –9 indeed confirmed our hypothesis that deletion in either caspase-3 or -9 resulted in severe developmental block due to aberrant neuronal apoptosis. As early as E12, a pronounced increase in cellularity was already evident in the proliferative zone along the ventricular area of the mutant embryos. Toluidine blue staining revealed that while pyknotic clusters could routinely be seen at the interventricular junctions in the wild-type E12 embryos, apoptotic cells were absent in the same areas of the caspase-3$^{-/-}$ or –9$^{-/-}$ embryos. Thus, the increased cellularity in the CNS associated with caspase-3 or –9 deficiency resulted directly from lack of apoptosis during early neuronal development. The presence of these superfluous cells were so profound that blockage of certain brain structures such as the aqueduct was frequently observed at later developmental stage.

In addition to caspase-3 and –9, knockout studies have also identified a few other caspases that are required for proper mammalian development, although the underlying mechanisms of their involvement are poorly understood. For example, deletion of caspase-7 resulted in very early developmental arrest during embryogenesis, yet the precise nature and cause of such arrest remains unknown (unpublished data). Also, mice deficient in caspase-8, the initiator caspase of the death receptor apoptotic pathway, are embryonically lethal with impaired cardiac development and abnormal erythrogenesis.[10] It is not clearly, however, whether these defects are primary or secondary effect resulting from caspase-8 deficiency and whether they reflect defective apoptosis during development. In fact, null mutation of FLIP,[16] the 'decoy' caspase-8 like molecule that antagonizes caspase-8 function in the death receptor apoptotic pathway, resulted in nearly identical developmental phenotypes,[17] therefore arguing for a critical role of the FADD/caspase-8/FLIP pathway during mammalian development that is not related to apoptosis.

The generation of these caspase knockout mice has not only provided critical insights into the role of caspases in mammalian development, but also revealed a few unexpected findings. First, genetic background strongly influences phenotypic penetrance of caspase-3 deficient mice.[18] As originally reported, caspase-3$^{-/-}$ mice under the mixed 129 and C57BL/6 background exhibited perinatal lethality and varied severity in their neuronal phenotype among individual animals. When backcrossed onto the C57BL/6 background, however, caspase-3

knockout mice can survive through adulthood with no obvious CNS defect. On the other hand, caspase-3$^{-/-}$ mice bred onto the pure 129 background are completely embryonic lethal, similar to that seen in the caspase-9 deficient animal (Kevin Roth, personal communication). Since the greatly improved survivability of caspase-3 KO under C57BL/6 was not observed with caspase-9$^{-/-}$ mice, it is likely that a genetic modifier capable of modulating the caspase-9/-3 activation pathway in developing neurons functions downstream of caspase-9, but either upstream or at the same level of caspase-3.

The second surprise is the apparent tissue-specificity of the developmental phenotypes observed in these caspase deficient mice, given the overlapping expression pattern, at least in adult animals, of these caspases in most, if not all tissues. Although one might argue for possible spatial and temporal regulation of expression among individual caspases during embryogenesis, an alternate explanation, perhaps more intriguing, is the possibility that distinct caspase pathways are being activated in different tissues/organs in response to certain yet to be discovered developmental cues.

Caspases in Apoptosis Execution

Apoptotic cells are characterized by a number of distinct morphological and biochemical changes such as cell shrinkage, cytoplasmic bleb formation, nuclear condensation and DNA fragmentation.[19] Previous studies using the broad-spectrum caspase inhibitor zVAD-fmk have concluded that most, if not all, of these cellular alterations can be attributed to caspase activity.[20] Such studies, however, have largely failed to distinguish the individual contribution of each effector caspase. Only with the generation of individual caspase knockout strains we have begun to address the precise involvement of each caspase during apoptosis execution.

So far, caspase-3 has clearly emerged as the single most important caspase during the execution phase of apoptosis. Using cells derived from caspase-3 deficient mice, we and others have shown that dying caspase-3$^{-/-}$ cells undergo an aberrant form of apoptosis, exhibiting drastically delayed cellular changes such as cytoplasmic bleb formation, nuclear and DNA fragmentation.[11,21] We further demonstrated that caspase-dependent cleavages of several intracellular proteins such as fodrin-α, DFF45/ICAD, lamin B and gelsolin, whose degradations have been linked to the various morphological and biochemical features associated with apoptosis, were impaired in caspase-3$^{-/-}$ thymocytes undergoing apoptosis.[11] These results strongly suggest that caspase-3 is the major effector caspase responsible for many, but not all, of the proteolytic events leading to cellular destruction. Importantly, similar results were also obtained in the human breast carcinoma line MCF-7,[22] which is 'naturally' deficient in caspase-3 expression, not only validating the results obtained with knockout cells, but also indicating that the essential role of caspase-3 in apoptosis execution is evolutionarily conserved.

In addition to caspase-3, caspase-6 and –7 are also thought to be important effector caspases given their sequence similarity to caspase-3.[23] In vitro experiments has previously suggested that caspase-6 was critical for the proteolysis of nuclear structural proteins such as lamin A and NUMA.[24] Thus, it came as quite a surprise when caspase-6 deficient mice exhibited no defect in apoptosis and dying caspase-6$^{-/-}$ cells underwent normal nuclear breakdown (unpublished data). Furthermore, cleavages of a number of caspase substrates in apoptotic caspase-6$^{-/-}$ thymocytes, including lamin B, also appeared normal, indicating that caspase-6 is dispensable for apoptosis execution. As for caspase-7, while the early embryonic lethality of caspase-7 KO mice has significantly hindered the scope of their characterization, the generation of caspase-7$^{-/-}$. embryonic stem (ES) cells using G418 selection has allowed us to examine its involvement during apoptosis execution. To our surprise, deletion of caspase-7 in ES cells did not result in any obvious defect in cell death or substrate cleavage induced by various stimuli such as UV irradiation and etopside, suggesting that caspase-7 is not required for apoptosis execution, at least in ES cells.

Taken together, gene targeting of caspase-3, -6 and –7 has provided critical insight into the role (and also lack of role) of these caspases in apoptosis execution. It confirmed previous

speculation that caspase-3 is the most important contributor of caspase-mediated proteolysis of cellular targets during apoptosis. In the same time, however, it also raised a number of questions as to what other caspases are involved in the effector phase and what role, if any, do caspase-6 and -7 have. Since it is clear that caspase-3 is not required for the cleavage of all substrates such as PARP-1, previously thought a caspase-3 target,[25,26] which caspase(s), then, is responsible for their proteolysis? Although one can argue for compensation by caspase-6 or -7 in the absence of caspase-3, we do not favor this possibility since the cleavage of many substrates in caspase-$3^{-/-}$ cells are defective. It is an unlikely scenario that selective compensation by other caspases on the cleavage of certain substrates, but not others, would occur.

The apparent lack of function for caspase-7 during apoptosis execution seen in caspase-$7^{-/-}$ ES cells is perhaps most surprising. All in vitro studies have concluded that the enzymatic activity of caspase-7 was indistinguishable from that of caspase-3.[27] Furthermore, unlike caspase-6 whose activation requires caspase-3 activity, caspase-7 is usually activated concurrently with caspase-3 in wild-type cells and caspase-7 activation appears normal in apoptotic caspase-$3^{-/-}$ cells (unpublished data). Based on these results, one would predict that caspase-7 should be able to compensate for caspase-3 in substrate cleavage in apoptotic caspase-3 null cells. It is therefore perplexing why caspase-7 failed compensate for caspase-3 deficiency in substrate cleavage. Our current hypothesis is that perhaps caspase-3 and –7 are differentially compartmentalized within the cell and the defective substrate cleavage observed in caspase-$3^{-/-}$. cells despite caspase-7 activation is due to inaccessibility of these substrates to caspase-7. The validity of this hypothesis should be testable experimentally.

The Plasticity of Caspase Activation

Much effort in the past few years has gone into understanding how caspases are activated during apoptosis. Results from both in vitro and in vivo studies support a 'branched cascade model' of caspase activation.[28,29] Briefly, apoptotic signaling first triggers adaptor-mediated direct activation of its corresponding initiator caspase such as caspase-8 or –9, likely through an autoprocessing mechanism. Activated initiator caspase in turn proteolytically activate two downstream effector caspases including caspase-3 and –7. Interestingly, the activation of the other presumed effector caspase, caspase-6, requires caspase-3 activity. To date, two major caspase acitivating pathways have been characterized in detail, namely the extrinsic pathway of caspase activation induced by death receptors and the intrinsic pathway of caspase activation involving mitochondria participation.

More recent evidence, however, suggested that the cellular pathways of caspase activation are more flexible than originally thought. One good example is the caspase activation triggered by death receptor signalling.[30] While death receptors universally induce FADD-dependent recruitment and activation of the initiator caspase-8 upon ligand engagement, the subsequent events could diverge depending on the cellular context. In addition to directly cleaving and activating caspase-3, caspase-8 can also trigger the intrinsic pathway through proteolytically activating a pro-apoptotic member of the Bcl-2 family, Bid, whose activation and subsequent translocation into the mitochondria result in cytochrome c release and caspase-9 activation.[31,32] The relevant contribution of the mitochondrial pathway to death receptor signaling is apparently cell type specific, but the underlying molecular basis remains poorly understood.[33]

To investigate potential plasticity of caspase activation in vivo, we took advantage of a well-established in vivo model of Fas-induced hepatocyte apoptosis.[34] Previous studies have demonstrated that injection of the agonistic anti-Fas antibody Jo2 induced massive hepatocyte apoptosis and led to rapid animal death. It has also been shown that Jo-2 induced liver damage and lethality is dependent on Bid-mediated mitochondrial pathway of caspase activation.[35] Indeed, Jo2 injection into wild type mice resulted in Bid translocation-induced cytochrome c release in hepatocytes and subsequent activation of both caspase-9 and –3. The Jo2-induced caspase activation pattern in caspase knockout mice, however, altered dramatically and demonstrated a great deal of flexibility.[36] Deficiency in caspase-3, for example, resulted in compensatory

activation of caspase-7 and –6 following Bid-induced caspase-9 activation. If the compensation of caspase-3 by caspase-7 and –6 is perhaps somewhat expected, the caspase activation pattern elicited in Jo2-treated caspase-9 knockout mice offered a total surprise. In the absence of caspase-9, Bid-translocation induced mitochondrial events triggered significant activation of caspase-2 and –6, revealing an alternative caspase-activating-pathway that was previously unknown. Overall, these results strongly suggest that caspase activation in vivo is not a rigid process, but rather consists of multiple pathways capable of compensating one another.

In addition to the compensatory mechanisms revealed by caspase-3 and –9 knockout mice, the early lethality in caspase-7 KO also suggests a novel pathway of caspase activation. Since no other caspase knockout exhibits developmental block at a similar stage, it is unlikely that one of the known initiator caspases is involved in the developmental step that requires caspase-7. Thus, caspase-7 may function as both an initiator and an effector caspase in response to the developmental cue and undergo direct activation.

Caspase Beyond Death

Caspase function has clearly expanded during evolution. While *ced-3*, the likely only functioning caspase in *C. elegans*, has no other apparent function other than to mediate programmed cell death during nematode development,[37] both in vitro and in vivo studies suggested that not all mammalian caspases function in apoptosis. In fact, caspase-1 was first identified as the interleukin-1β converting enzyme (ICE) and knockout studies have confirmed its main function as a critical mediator of inflammatory responses.[12,13] An almost identical role for caspase-11 has also been established based on the characterization of caspase-11 knockout mice, although the precise functional relationship between caspase-1 and –11 in IL-1 production has yet to be worked out.[14]

Additional involvement of caspases in biological processes other than apoptosis and inflammation has been further implicated from studies on caspase knockout mice.[4] As mentioned previously, null mutation of caspase-8 results in embryonic lethality characterized by defective cardiac development and several lines of evidence suggest that the developmental phenotype seen in caspase-$8^{-/-}$ embryos was probably not due to defective apoptosis. First, apoptosis is not known to play a prominent role during cardiac development. More importantly, FLIP deficiency, which resulted in enhanced apoptosis through death receptors, exhibited essentially the same developmental defects,[17] strongly suggesting the myocardiac abnormality as a result of caspase-8 is not apoptosis-related. Indeed, a number of in vitro studies have argued that caspase-8 mediated FLIP proteolysis could activate the NF-κB pathway and was required for T cell proliferation.[38,39]

Another clue for alternative caspase function comes from analysis of caspase-6 deficient mice. While no apparent apoptosis defect has been identified in these mutant mice, caspase-$6^{-/-}$ B cells appear somewhat abnormal phenotypically with no or very low expression of surface CD23 (unpublished data). Consistent with the role of caspase-6 in CD23 expression in B cells, CD40L-induced CD23 expression in WEHI 231 immature B cell line could be inhibited by the broad-spectrum caspase inhibitor zVAD-fmk, suggesting a potential role for caspase activity in B cell maturation. The functional significance of defective CD23 expression in caspase-$6^{-/-}$ B cells is currently under investigation.

Future Perspectives

The cloning of multiple mammalian caspases has presented us with the critical question of what in vivo role individual caspases plays and how these caspases functionally relate to each other in biological processes. Thanks to the generation of various caspase knockout mice, significant strides have been made toward understanding the contribution of many caspases during mammalian development, apoptosis and inflammatory responses. Despite the progress, we

are still faced with a number of important questions revealed by these knockout mice regarding the function of certain caspases and how caspase activities are regulated in vivo.

First, the early embryonic lethality associated with deficiency in caspase-8, -9 or –7 has greatly limited the characterization of these caspase knockout mice. As a result, much remains unknown about the function of caspase-8, -9 and –7. For example, is caspase-8 required for T-cell development and function, as one might expect from in vitro studies suggesting that caspase-8 activity is critical for T cell proliferation?[38] Similarly, despite our knowledge of caspase-9's involvement in neuronal apoptosis during development, very little is known about whether caspase-9 activity is absolutely required for apoptosis induced by various stimuli in other cell types. Obviously, the answer to such questions calls for the generation of conditional knockout mice, which would also allow assessment of the involvement of caspase-8 and -9 in various mouse models of human diseases. As the technology for creating both tissue-specific and temporally regulated conditional knockout mice continues to improve, we should expect a great deal of critical insights into the function of these caspases in physiological and pathological settings.

One intriguing finding from caspase knockout mice is the strong influence strain background exerts on the developmental phenotypes observed in caspase-3 deficient mice. As discussed before, while caspase-3 deficiency in the C57BL/6 background leads to dramatically improved animal survival compared to the original reported 129 and C57BL/6 mixed background, all pure 129 background caspase-$3^{-/-}$ mice are embryonically lethal. Interestingly, the requirement of caspase-3 for mediating the various morphological and biochemical changes associated with apoptosis did not alter with different backgrounds, suggesting that the genetic modifier is likely influencing the regulation of neuronal apoptosis during development, rather than exerting a direct effect on the caspase activation pathway. In past few years, phenotypic variation of gene knockout mice due to genetic background has been increasingly noticed and several attributing genetic modifiers have been isolated in a few cases through extensive backcrossing and phenotypic analysis.[40,41] The continuously improved sequence coverage of the entire mouse genome should greatly facilitate the isolation of genetic modifier that determines the viability of caspase-3 deficient mice.

Finally, despite our lack of understanding of its precise molecular mechanism, the compensatory pathways of caspase activation observed in caspase-3 and –9 knockout mice will likely have important implications.[36] As caspases have become attractive targets for therapeutic interventions for various diseases in which excessive apoptosis has been attributed to pathogenesis, selective inhibition of one or two upstream caspases that are involved in a particular disease has been suggested to be the ideal strategy. Our discovery, however, clearly indicates that cells have the capacity to activate caspases through compensatory pathways and efficient inhibition of caspase activity would require blocking such alternative pathways as well. On the other hand, mechanistic insights into how compensatory activation of caspase-2 and -6 can be achieved in the absence of caspase-3 and -9 may also provide the new means to 'jumpstart the death engines' in apoptosis-resistant tumor cells, whose regular apoptotic machinery, including caspases activating pathway, is almost certainly perturbed.

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CHAPTER 11

Caspase Activation in Cancer Therapy

Simone Fulda, Klaus-Michael Debatin

Abstract

Different anticancer therapies including cytotoxic drugs, γ-irradiation, suicide gene therapy or immunotherapy, appear to induce tumor cell death by activating key elements of apoptosis, the cell's intrinsic death program. Activation of the cascade of proteolytic enzymes known as caspases is a critical component of the execution phase of cell death in most forms of apoptosis. Two main caspase cascades, one triggered by death receptor stimulation and the other one initiated at the mitochondria, have been identified in response to various inducers of cellular stress such as DNA damage. Activation of caspases and apoptosis is tightly regulated at several levels, e.g., by Bcl-2 family members, by inhibitor of apoptosis proteins (IAPs) and upstream inhibitors such as FLIP. Failure to activate apoptotic pathways in response to drug treatment may lead to resistance of tumors cells to anticancer therapies. Therefore, factors affecting caspase activation might be important determinants of drug sensitivity. In addition to caspase-dependent apoptosis, caspase-independent forms of cell death may also play a role for treatment response. Insights into the mechanisms regulating caspase activation as well as other forms of cell death pathways provide a molecular basis for novel strategies targeting resistance of tumor cells.

Introduction

Killing of tumor cells by diverse cytotoxic approaches such as anticancer drugs, γ-irradiation, suicide genes or immunotherapy, has been shown to be mediated through induction of apoptosis in target cells.[1-7] Apoptosis or programmed cell death occurs upon the activation of a distinct, intrinsic cell death programs under certain physiological and pathological situations.[8] The underlying mechanism for initiation of an apoptosis response upon cytotoxic therapy may be different for various stimuli and is only partially understood. However, damage to DNA or to other critical molecules and/or sub-cellular structures appears to be a common early hit by some inducers which is then propagated by the cellular stress response.[9] Multiple stress-inducible molecules, e.g., JNK, MAPK/ERK, NF-κB or ceramide may have a profound impact on apoptosis pathways.[10-12] On the other hand cytotoxic T cells or NK cells may release compounds such as granzyme B which directly activates downstream apoptosis effector mechanisms inside the cell.[8] Apoptosis is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation and membrane blebbing.[8] Proteolytic enzymes such as caspases play an important role as effector molecules in apoptosis including cytotoxic therapy-induced cell death.[13-19] Because of the potential detrimental effects on cell survival in case of inappropriate caspase activity, activation of caspases has to be tightly controlled. The anti-apoptotic mechanisms regulating activation of caspases have also been postulated to be involved in drug resistance of tumor cells.[20-22] However, the concept that anticancer therapies primarily act by triggering apoptosis has also been challenged, since a consistent link between the ability of tumor cells to undergo apoptosis in vitro and their susceptibility to anticancer therapy in vivo has not always been observed.[23] Therefore, nonapoptotic

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modes of cell death, e.g., necrosis or some forms of cell death that cannot be easily classified, may mediate the cell death response to cytotoxic therapy.[24,25] Also, non-caspase dependent apoptosis has been found to be induced by anticancer drugs in some cells.[26-28] Thus, a better understanding of these diverse modes of tumor cell death following cytotoxic therapies will provide a molecular basis for new strategies targeting caspase-dependent and independent death pathways in apoptosis-resistant forms of cancer.

Caspases as Central Death Effector Molecules

Most signaling pathways activated by anticancer drugs ultimately result in activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (Fig. 1).[8,13-18] 12 human caspases with different substrate specificity have so far been identified that cleave next to aspartate residues.[13-18] Caspases are involved in apoptosis signaling and also in cytokine processing.[13-18] Caspases are synthesized as inactive zymogens and they are activated by proteolytic cleavage.[13-18] Upon activation, each caspase forms a tetramer of the two large and the two small subunits.[13-18] The hierarchy and partial substrate redundance allows to a form proteolytic, signalling cascade with positive feed-back properties.[13-18]

Caspases involved in apoptosis signaling are currently categorized into initiator and effector caspases, respectively.[13-18] Initiator caspases transduce various signals into protease activity and are directly linked to death inducing signaling complexes (DISCs): caspase-8 or caspase-10 via their death effector domain (DED) interact with adaptor proteins (FADD) recruited and bound to activated death receptors while caspase-9 is recruited to the apoptosome via its CARD domain.[13] Effector caspases cleave various cytoplasmatic or nuclear substrates prompting the occurrence of morphologic features of apoptosis.[13-18] For example, polynucleosomal DNA fragmentation is initiated by cleavage of ICAD (inhibitor of caspase-activated DNase), the inhibitor of the endonuclease CAD (caspase-activated DNase) that cleaves DNA into the characteristic oligomeric fragments.[8] DNA condensation is caused by AIF, a mitochondrial protein that translocates to the nucleus upon death triggering, and by Acinus, which stands for "apoptotic chromation condensation inducer in the nucleus".[29,30] AIF may also mediate caspase-independent cleavage of DNA into larger fragments.[29,30] Likewise, loss of overal cell shape is due to proteolysis of cytoskeletal proteins including fodrin, gelsolin, actin, plectrin, cytokeratin, while nuclear shrinking and budding occurs after degradation of lamin.[8]

Pathways of Caspase Activation

Activation of caspases can principially be triggered by two different mechanisms: according to the induced proximity model initiator caspases such as caspase-8 or –9 are activated in a multimeric complex, e.g., caspase-8 in the death inducing signaling complex (DISC) and caspase-9 at the apoptosome.[8,13,31-33] Alternatively, caspases are activated by catalytic processing of the zymogens at specific cleavage sites.[13] caspase activation can be initiated through different entry sites, e.g., at the plasma membrane by death receptor mediated signaling (receptor pathway) or at the mitochondria (mitochondrial pathway).[8,13] Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL receptors results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8.[31-33] Upon recruitment caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases.[31-33] The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), Smac/DIABLO, Omi/HtrA2, endonuclease G, caspase-2 or caspase-9 from the mitochondrial intermembrane space.[34-39] The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex.[40,41] Smac/DIABLO and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to IAPs, while AIF and endonuclease G cause DNA condensation.[30,38,39,42,43]

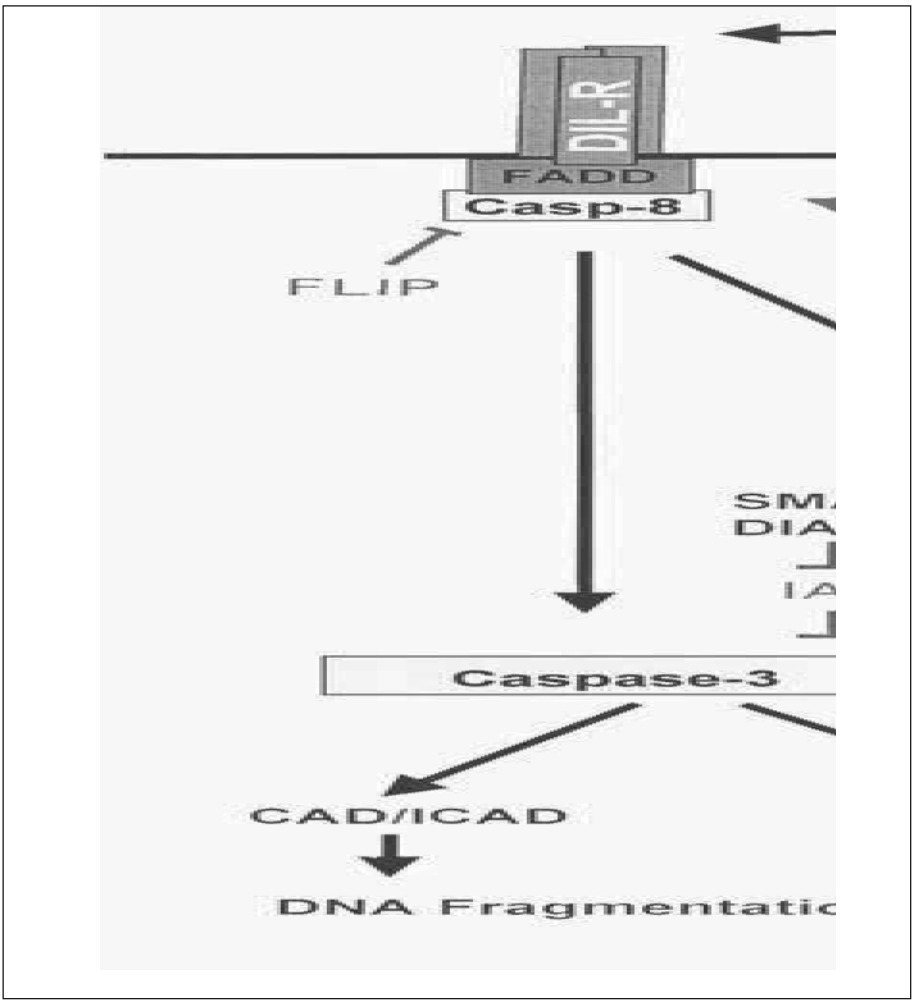

Figure 1. Activation of apoptosis pathways by anticancer therapy. Anticancer therapy-induced apoptotic pathways can be initiated through different entry sites, e.g., at the plasma membrane by death receptor mediated signaling (receptor pathway) or at the mitochondria (mitochondrial pathway). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily (DIL-R) such as CD95 (APO-1/Fas) or TRAIL receptors by death-inducing ligands (DIL) results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), or Smac/DIABLO from the mitochondrial intermembrane space. The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac/DIABLO promotes caspase activation through neutralizing the inhibitory effects to IAPs, while AIF causes DNA condensation. The receptor and the mitochondrial pathway can be interconnected at different levels, e.g., by Bid, a BH3 domain containing protein of the Bcl-2 family which assumes cytochrome-c-releasing activity upon cleavage by caspase-8. Activation of caspases is negatively regulated at the receptor level by FLIP which blocks caspase-8 activation, at the mitochondria by Bcl-2 family proteins and by inhibitor of apoptosis proteins (IAPs). See text for more details.

The receptor and the mitochondrial pathway can be interconnected at different levels.[44] Following death receptor stimulation activation of caspase-8 may result in cleavage of Bid, a BH3 domain containing protein of the Bcl-2 family which assumes cytochrome-c-releasing activity upon cleavage thereby initiating a mitochondrial amplification loop.[43-44] In addition, mitochondria-triggered caspase-6 cleavage may feed back to the receptor pathway by cleaving caspase-8.[16]

Signaling Pathways in Cancer Therapy

The relative contribution of the receptor and the mitochondrial pathway to drug-induced apoptosis has been a subject of controversial discussion.[1,2,4] A number of studies suggested that cancer therapy-triggered apoptosis involves the CD95 system by upregulating the expression of CD95L which then binds to its receptor and stimulates the receptor pathway in an autocrine or paracrine manner.[45-64] In support of this model, upregulation of CD95 mRNA and protein was found in a variety of tumor cell lines derived from T cell leukemia cells, neuroblastoma, malignant brain tumors, hepatoma, colon, breast or small lung cell carcinoma upon treatment with cytotoxic drugs such as doxorubicin, etoposide, cisplatin, 5-FU or bleomycin.[45-59] The increase in CD95L transcription and mRNA levels was found to be related to drug-induced activation of the transcription factors AP-1 and NF-κB.[55,60,61] Also, CD95 expression increased upon drug treatment, in particular in cells harboring wild-type p53, as the CD95 promotor contains p53 binding sites.[51,57,58] In addition, soluble antagonistic CD95 receptors, antagonistic CD95L antibodies or DN-FADD reduced drug-induced apoptosis under certain circumstances.[45,49,57,59] Moreover, it was recently demonstrated in vivo that 5-fluorouracil induced apoptosis in mouse thymocytes via activation of the CD95 system, since apoptosis was blocked by neutralizing CD95L antibodies or in *lpr* mice lacking a functional CD95 receptor.[62] Also, CD95L-independent activation of the receptor pathway through CD95 receptor oligomerization has been reported, e.g., by UV irradiation, cytotoxic drugs or suicide gene therapy using the herpes simplex thymidine kinase (HSV/TK) system.[65-67]

Other reports however challenged the concept that death receptor signaling is involved in drug-mediated cell death.[68-74] Antagonistic antibodies against CD95L or CD95 did not protect from cancer chemotherapeutica-induced death in several cell line models.[72-74] Although splenocytes from *lpr* mice exhibit decreased sensitivity to γ-irradiation, thymocytes of these mice did not show increased proliferation upon γ-irradiation or cytotoxic drugs.[68] Moreover, overexpression of FLIP, DN-FADD or the serpin CrmA that inhibits caspase-8 did not confer protection.[69,72-74] In addition, targeted disruption of genes involved in death receptor signaling suggested a dispensable role of the CD95 system in drug-induced apoptosis, at least in nontransformed cells. FADD$^{-/-}$ and caspase-8$^{-/-}$ fibroblasts are resistant to death receptor stimulation, but equally sensitive to cytotoxic drugs.[75,76] In contrast, caspase-9$^{-/-}$ embryonic stem cells and Apaf-1$^{-/-}$ thymocytes remain sensitive to death receptor triggering, however are resistant to cytotoxic drugs.[77,78] The discrepancies in data may be explained by the relative contribution of the death receptor versus the mitochondrial pathway depending on the cytotoxic drug, dose and kinetics or on differences between certain cell types. For CD95 signaling, 2 different cell types have been identified:[79] type I cells undergo CD95-triggered apoptosis independent of mitochondria, since caspase-8 is already efficiently activated at the DISC upstream of mitochondria. In contrast,type II cells depend on the mitochondrial pathway, since only little caspase-8 is recruited and activated at the DISC.[79] A similar cell type dependent signaling has also been identified in response to drug treatment.[54] Although the CD95 system is involved in anticancer drug-induced apoptosis under certain circumstances, the majority of cytotoxic drugs initiate cell death by triggering the cytochrome c/Apaf-1/caspase-9 dependent pathway through the mitochondria. Collectively, these data point to a crucial role of the mitochondrial pathway in drug-induced apoptosis, while the CD95 system may amplify and accelerate drug-induced apoptosis under certain conditions. Importantly, this amplification of the chemoresponse may be clinically meaningful, since it may critically affect the time required for execution of the death program.[80]

Regulation of Caspase Activation

Given the important role of caspases as effector molecules in various forms of cell death including drug-induced apoptosis, the ability of anticancer agents to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies. As a consequence, inhibition of caspase activation may be an important factor in chemoresistance. Given the central role of caspases for cell death execution one might expect a high frequency of caspase mutations in tumors. Interestingly however, screening for mutations in initiator or executioner caspases in a variety of human tumors has not reveiled a high frequency of genomic aberrations in caspase genes.[81,82] Instead, caspase expression and function appears to be epigenetically downregulated in tumors by mechanisms described below suggesting that restoration of a functional caspase system may be important to overcome resistance in tumors.[82,83]

Caspase Expression

First, expression levels of individual caspases may have an impact on their overall activity, since activation of caspases may simply be impaired by deficient expression levels of caspases.[84-86] For example, MCF-7 breast carcinoma cells completely lack caspase-3 expression due to a frameshift mutation within exon 3 of the caspase-3 gene.[85] These cells can be sensitized by transfection of pro caspase-3 towards treatment with cytotoxic drugs.[86] Next, caspase expression may be impaired by epigenetic alterations such as promotor hypermethylation. To this end, caspase-8 expression was found to be frequently inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumor and small lung cell carcinoma both in vitro and also in vivo in primary tumor samples.[82,83] Importantly, restoration of caspase-8 expression by gene transfer or by demethylation treatment sensitized resistant tumor cells for death-receptor- or drug-induced apoptosis.[82,83] Alternative splicing has been identified as another level of transcriptional regulation of caspase expression. The genes encoding procaspase-2 or procaspase-9 can generate short isoforms that prevent apoptosis in a dominant-negative fashion.[87,88] Conversely, enhanced transcription of caspase genes in response to cytotoxic treatment may increase expression levels. Thus, treatment with IFN-γ resulted in enhanced expression of caspase proteins mediated by direct activation of STAT-1, a downstream transcription factor involved in IFN-γ signaling.[89] Moreover, transcriptional upregulation of caspase-3 or –8 was reported upon drug treatment independent of STAT1.[90,91] In addition, subcellular compartmentalization of caspases may regulate their activation. Interestingly, in addition to their cytoplasmic localization, several caspases including caspase-2, -3 and –9 are found inside mitochondria.[92,93] Death signals targeting mitochondria trigger the translocation of caspases from the mitochondrial intermembrane space into the cytosol and also promote their nuclear translocation.[92-94]

Bcl-2 Proteins

Bcl-2 family proteins play a pivotal role in the regulation of the mitochondrial death pathway.[100-104] The family comprises anti-apoptotic members, e.g., Bcl-2, Bcl-X_L, Mcl-1, pro-apoptotic molecules such as Bax, Bak, Bad, as well as BH3 domain only molecules which link the death receptor pathway to the mitochondrial pathway (Bid, Bim, PUMA, Noxa).[100,101] Upon apoptosis induction proapoptotic Bcl-2 proteins with multidomains such as Bax or Bak translocate from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form a pore-like structure thereby promoting cytochrome c release.[102] The translocation to mitochondria can be triggered by so called "BH3-only" Bcl-2 family proteins.[100] BH3-only proteins include Bid, which is activated by caspase-8-mediated cleavage, Bim, a microtubule-associated protein, or Noxa and PUMA, two p53-induced proteins.[100,101] Bcl-2 or Bcl-X_L exert their anti-apoptotic function, at least in part, by sequestering BH3-only proteins in stable mitochondrial complexes thereby preventing activation and translocation of Bax

or Bak to mitochondria.[102] In addition, Bcl-2 and Bcl-X_L block apoptosis by preventing cytochrome c release through a direct effect on mitochondrial channels such as the voltage-dependent anion channel (VDAC) or the permeability transition pore complex (PTPC).[35,36] Several clinical correlative studies indicate that high expression of antiapoptotic Bcl-2 proteins confers a clinically relevant chemoresistant phenotype in various malignancies, including AML, ALL, CLL, multiple myeloma, prostate carcinoma, malignant brain tumors and neuroblastoma.[22,105-107] Likewise, reduced Bax level have been associated with poor responses to chemotherapy and shorter overall survival in breast or colorectal carcinoma.[108] Conversely, enhanced Bax expression correlated in several cell types with sensitivity to chemotherapy in vivo.[109]

Inhibitors of Apoptosis Proteins (IAPs)

The family of endogenous caspase inhibitors "inhibitor of apoptosis proteins" (IAPs) are highly conserved throughout evolution and comprise the human analogues XIAP, cIAP1, cIAP2, survivin, livin and ML-IAP.[110-112] Their common structural features consist of 1-3 baculovirus inhibitor repeat (BIR) domains, that mediate binding to caspases, and a RING domain, that acts as ubiquitin ligase thereby promoting ubiquitination and proteasomal degradation of the bound caspases.[110] IAPs have been reported to directly inhibit active caspase-3 and –7 and to block caspase-9 activation.[110] In addition to regulation of apoptosis, IAP members such as survivin have been found to be involved in the regulation of mitosis.[112113] The activity of IAPs are controlled at various levels, e.g., by the transcription factor NF-κB that has been reported to stimulate expression of cIAP1, cIAP and XIAP.[110] IAPs are negatively regulated by caspase-mediated cleavage.[110] In addition, Smac/DIABLO and Omi, two proteins released from mitochondria upon apoptosis induction, neutralize IAPs through their binding, thereby displacing them from caspases.[111] Likewise, XAF1 has been found to displace IAPs from bound caspases in the nucleus.[111] Inhibition of apoptosis by IAPs in response to cytotoxic therapy has been suggested by several experimental studies.[114-119] XIAP, cIAP1 or cIAP2 suppressed apoptosis in vitro following treatment with cisplatin, cytarabine, TRAIL, staurosporine or after γ-irradiation.[118,119] Also, increased IAPs expression correlated with poor treatment response in myeloid leukemia cells and elevated survivin expression predicted adverse prognosis in several tumors, e.g., neuroblastoma, AML, colon, lung and esophagus carcinoma.[114-117]

Death Receptors

Activation of caspases may also be controlled by upstream regulation at the level of death receptors. First, death receptor expression may vary between different cell types and can be downregulated in tumor cells, thus contributing to the escape from negative growth control.[31-33] Signaling by death receptors can be negatively regulated by proteins that associate with their cytoplasmatic domains, e.g., SODD, or by proteins such as FLIP that prevent the interaction between the adaptor molecule FADD and procaspase-8.[95-97] High FLIP expression which has been found in many tumor cells has been correlated with resistance to CD95- and TRAIL-induced apoptosis.[96] In addition, FLIP expression was associated with tumor escape from T-cell immunosurveillance and enhanced tumor progression in experimental studies in vivo, pointing to a role of FLIP as a tumor-progression factor.[97] The impact of FLIP on apoptosis sensitivity towards cytotoxic drugs may vary between cell types, since overexpression of FLIP did not confer protection against cytotoxic drugs in T cell leukemia cells, while FLIP antisense oligonucleotides sensitized osteosarcoma cells for cisplatin.[69,98] Elevated FLIP expression has been found in clinical samples from Burkitt lymphoma, pancreatic carcinoma, melanoma or neuroblastoma and in tumor cells that developed resistance upon chemotherapy suggesting that FLIP may play a role in chemoresistance of tumors.[97-99]

Caspase-Independent Cell Death

Although a large body of data point to an essential role of caspase-mediated tumor cell death upon cytotoxic therapy, this concept has also been challenged.[1-7,23] Thus, a clear, consistent

link between the cells' ability to undergo apoptosis and their susceptibility to anticancer therapy could not be observed.[23] In addition, the p53 status did not always correlate with the ability of a tumor cell to respond to treatment.[120] Cells harboring wild-type p53 may fail to respond and those lacking functional p53 may even respond better.[120] Moreover, nonapoptotic modes of cell death, e.g., necrosis or some forms of cell death that cannot be clearly classified, have also been taken into consideration as response to cytotoxic therapy.[23-28] Also, delayed regression of tumors upon e.g., irradiation has been taken as evidence against a predominant apoptotic mode of cell death, since apoptosis appears to be induced fairly rapidly in vitro and in vivo upon appropriate stimulation.[23] Although signaling pathways and molecules involved in these alternative forms of cell death have not yet exactly been defined, non-caspase proteases such as calpains or cathepsins, Bax or Bax-like molecules and AIF or endonuclease G may be involved.[8,23-28] The relative contribution of these different modes of cell death for chemoresponses in vitro and in vivo remains to be defined.

Role of Caspases for Treatment Response in vivo

What is the clinical impact of caspase expression and/or activity on individual patient's response to anticancer therapy in vivo? Unfortunately, this question is far from being answered yet. Aside from Bcl-2 family proteins, most molecules involved in the regulation of apoptosis including caspases have not extensively been studied in clinical specimens.[22,43] Although the potential relation between expression levels of procaspases in clinical samples and patients' response to chemotherapy has been addressed in several studies, the conclusive answer is still missing.[121-125] Some investigators postulated a correlation between procaspase-3 expression and clinical response, e.g., in leukemia, Hodgkin's disease or NSCLC.[121-124] However, this conclusion was not always based on a direct correlation between procaspase-3 expression levels and individual treatment responses and further studies did not confirm these findings. In bone marrow samples with predominance of leukemic blasts, a wide variation of caspase-2, -3, -7, -8, and-9 was found among different specimens, however, their level did not correlate with prognostic factors or response to induction chemotherapy.[125] Loss of spontaneous caspase-3 cleavage was reported in ALL samples at relapse compared to those at initial diagnosis.[107] As discussed before, while mutations in caspase genes have only infrequently been found in tumors, inactivation of caspase expression by epigenetic alterations such as promotor hypermethylation appears to be a primary mechanism of disabling of the caspase cascades in tumors.[81-83] How inactivation of caspase expression by DNA hypermethylation will correlate with clinical outcome remains a subject of future studies. The prospective study of clinical samples is further complexed by the necessity of multiparameter analysis. The expression level and activity of caspases is affected in vivo by positive and negative apoptosis regulators, such as Bcl-2 family proteins or IAPs.[100,110] Thus, an assessment of the impact of caspase expression and/or function on chemoresponse in vivo will require multiparamter analysis, e.g., by expression profiling.

Conclusion

Numerous studies over the last years have indicated that anticancer therapies primarily act by activating the apoptosis response pathway in tumor cells.[1-7] However, several points still remain to be addressed: First, most of the apoptosis signaling components have not been studied in clinical samples.[2,4,6,7,22] Second, many experimental studies indicate that alterations in components of the apoptotic machinery have an impact on sensitivity of tumor cells towards cytotoxic therapy, this premise remains to be tested in clinical settings.[1-5,22] Moreover, the biology that determines the individual responses of different tumors to cytotoxic therapies warrants further investigations to provide the basis for more specific therapeutic interventions. Finally, the concept that apoptosis represents the major mechanism by which tumor cells are eliminated by cytotoxic therapies may not universally apply and caspase-independent modes of cell death have also also to be considered.[23-28]

Nonetheless, studies on the regulation of apoptosis signaling pathways triggered by anticancer therapies have provided substantial insights into the molecular mechanisms regulating the response of tumor cells towards current therapies. Future studies on the role of apoptosis signaling molecules in individual tumors both in vitro and in vivo in tumor cells of patients under chemotherapy, e.g., by DNA microarrays or proteomic studies, may provide the basis for "tailored" tumor therapy and may identify new targets for therapeutic interventions.

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CHAPTER 12

In Situ Activation of Caspases Revealed by Affinity Labeling Their Enzymatic Sites

Jerzy Grabarek and Zbigniew Darzynkiewicz

Introduction

Activation of caspases is the key event of apoptosis as its initiates irreversible steps of the cell demise.[1-9] Several methods, therefore, have been developed to monitor this event. Most frequently the caspases involvement is probed indirectly, by testing whether their specific inhibitors, when administered together with the inducer of apoptosis, can prevent particular apoptotic episodes. Also indirectly, caspases activation can be revealed by the presence of the specific cleavage products that can be identified electrophoretically by a characteristic change in molecular weight upon the cleavage, confirmed immunochemically on Western blots. Antibodies also are available that detect the specific cleavage products such as a 89 kD fragment of poly(ADP-ribose) polymerase-1 (PARP-1).[10] The latter approach was adapted to cytometry, and has been utilized to correlate caspases activation with the cell cycle position[9] or collapse of the mitochondrial transmembrane electrochemical potential.[11]

The approaches to directly probe activation of caspases also have been developed. Analysis of caspases molecular weight is one of them. Namely, because the activation involves cleavage of the zymogen procaspases (Fig. 1) the cleavage products by virtue of their lower molecular weight compared with the zymogen can be separated electrophoretically and identified on Western blots. As in the case of the cleavage products of caspases, antibodies recently become available that recognize the epitope that is characteristic of the activated form of these proteases. Their activation, thus, can also be detected directly in situ, immunocytochemically.[12]

Still another approach utilizes peptide substrates that upon the caspase-induced cleavage generate colored or fluorescing products.[13-16] Their use was primarily restricted to cell extracts and therefore provided no information on individual cells, heterogeneity of cell populations or correlation with other cell attributes, on a cell by cell basis. Recently, however, the use of these substrates was adapted to individual cells whose fluorescence was measured by flow cytometry.[17]

We have recently described the use of fluorochrome-labeled inhibitors of caspases (FLICA) to monitor activation of these enzymes in live cells.[18-20] The use of enzyme active center specific inhibitors as affinity-labeling probes was introduced by us before to detect in situ activation of esterases,[21] mast cell serine proteases[22] and folate reductase.[23] Instead of radioisotope-labeling that in these earlier studies was detected by autoradiography, we are now tagging the inhibitors with fluorochromes, that are detected by fluorescence microscopy and can be measured by flow- or laser scanning- cytometry. The FLICA ligands are carboxyfluorescein (Fam)- or sulforhodamine B (Sr)-labeled peptide fluoromethyl ketones (fmk) that with 1:1 stoichiometry covalently bind to active centers of caspases (Fig. 1). These labeled inhibitors, similar as the unlabeled ones (e.g., zVAD-fmk) are permeant, and at least during short-term incubation, appear to be relatively nontoxic to the cell. Actually, the unlabeled analogs have

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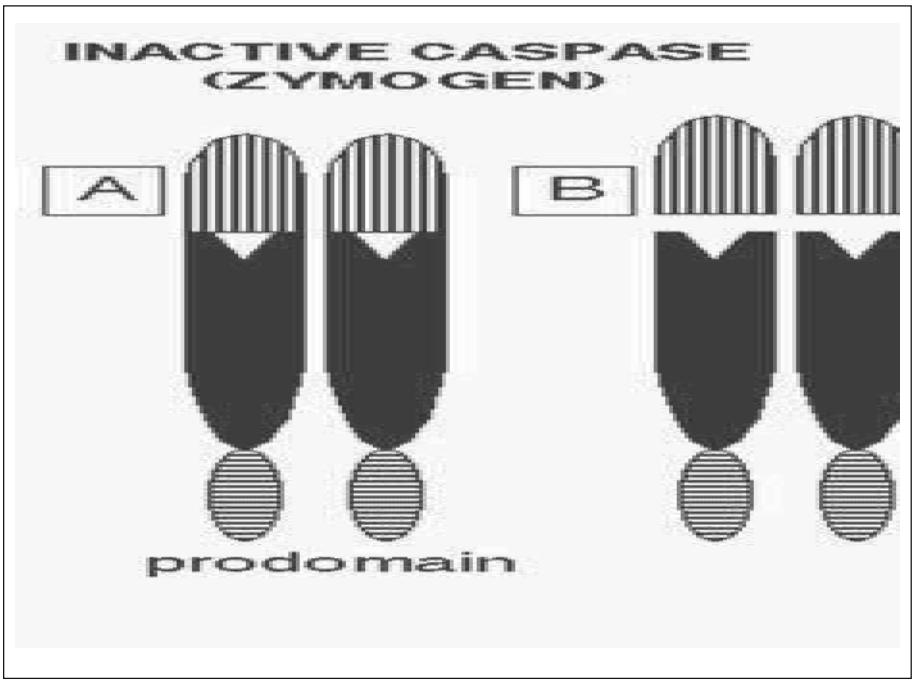

Figure 1. Schematic illustration of caspase activation and FLICA binding. Caspases are present in nonapoptotic cells as zymogens containing N-terminal prodomain attached to a large (~20 kD) catalytic subunit, which in turn is attached to a small (~10 kD) subunit (A). After induction of apoptosis the procaspases are first cleaved at Asp-X bonds between the large and small subunits (B). The second cleavage takes place also at Asp site and leads to separation of the prodomain (C). The subunits from two procaspase molecules then assemble into a hetero-tetramer to form the active enzyme that has two active centers at opposite ends (D). The active enzymatic centers are accessible to the substrates and also can bind FLICA (E). The covalent binding of FLICA is mediated by the halogen (fluoro- or chloro-) methyl ketone (fmk) moiety which interacts with the cysteine of the active center forming a thiomethyl ketone thereby irreversibly inactivating the enzyme.[25,26,30] The specificity of FLICA binding is provided by the sequence of four amino acids in the peptide moiety (e.g., VEID). It should be noted, however, that the three-aminoacid moiety VAD shows no specificity and is a pan-caspase inhibitor. The fluorescent tag (carboxyfluorescein, Fam) is located on the other end of FLICA molecule.

been reported to promote cell survival, protecting them from apoptosis.[24-27] Exposure of live cells to FLICA results in uptake of these reagents followed by their binding to activated caspases within the cells that undergo apoptosis. Unbound FLICA are removed from the nonapoptotic cells that lack activated caspases by rinsing the cells with wash-buffer. Cells labeled with FLICA can be examined by fluorescence microscopy, or subjected to quantitative analysis by flow- or laser scanning- cytometry (LSC).[28,29] The present review describes principles of this FLICA approach and discusses some applications of the methodology.

Affinity Labeling of Enzyme Centers with FLICA

Procaspases contain N-terminal prodomain followed by a large (~20 kD) then a small (~10 kD) catalytic subunit (Fig. 1A). The size of prodomain varies between caspases. A large prodomain size have initiator caspases while a small size have effector caspases. Two related motifs are present in prodomains: the death effector domain (DED) and caspase recruitment

domain (CARD).[4-6] The sequential steps of activation, formation of the enzymatically active hetero-tetramer and binding of FLICA are described in the legend to Figure 1.

Several FLICA are commercially available (e.g., from Immunochemistry Technologies, Mn USA; or Serologicals Corp. Norstar, Ga, USA), including fluorescein- or sulforhodamine B labeled-VAD-fmk, which contains the valyl-alanyl-aspartic acid residue sequence. This three amino acid target sequence allows this inhibitor to irreversibly bind to activated caspases-1, -3 -4, -5, -7, -8 and –9 making it multi-caspases marker. Other inhibitors such as these that contain VDVAD, DEVD, VEID, YVAD, LETD, LEHD, and AEVD peptide residues preferentially bind to activated caspases-2, -3, -6, -1, -8, -9, and -10, respectively.

It is difficult to assess, however, how indeed specific is in situ binding of individual FLICA designed to be markers for the respective caspases. As mentioned, Fam-VAD-fmk lacks specificity and binds to all caspases, perhaps with an exception of caspase-2 to which it has a low binding affinity.[25,26,30] The inhibitor with DEVD sequence designed to be caspase-3 specific is expected also to interact with several other caspases. Namely, the inhibitory constant (K_i) of Ac-DEVD-CHO is 0.2-2.2 nM for caspase-3, 0.9 nM for caspase-8 and 1.6 nM for caspase-7.[25] We observed that MCF-7 cells that are known to be caspase-3 null, were quite strongly labeled with Fam-DEVD-fmk.[19] This suggests that perhaps caspases-7 and -8, if not also other caspases, were labeled with Fam-DEVD-fmk in these cells. Other inhibitors also have strong affinity to more than a single caspase.[26,30] Moreover, since little is known about effective concentration of the used FLICA within the cell and also about their binding constants to the respective caspases in situ, one has to be careful in drawing the conclusions about their specificity based on binding to live cells. We observed, however, that when the cells were pre-treated with a high concentration of the unlabeled inhibitor zVAD-fmk the subsequent binding of Fam-VAD-fmk was reduced by over 90 %.[19] Likewise, in the presence of an excess of the caspase-3 substrate (Ac-DEVD-pNA) binding of Fam-DEVD-fmk was also diminished by over 90 %.[19] The FLICA binding sites within the cell, thus, can be competitively protected either by the unlabeled inhibitor or substrate. The specificity (or lack of it) of the FLICA with respect to the target caspases, thus, is comparable to that of their respective unlabeled analogs or substrates.

Subcellular Localization of FLICA-Binding Sites

Induction of apoptosis followed by cell exposure to FLICA makes them fluorochrome-labeled (Fig. 2). It is apparent under fluorescence microscopy that preferentially labeled are the cells that have altered morphology, characteristic of apoptosis (Fig. 3). It is difficult, however, to assess in live cells, particularly in the cells that become spherical and detaching, as most apoptotic cells are, the intracellular localization of the fluorochrome.

Because binding of FLICA to active centers of caspases is covalent, it is possible, after the binding occurred, to fix the cells (preferentially with formaldehyde), permeabilize them and counter-stain their DNA with a fluorochrome of another color than FLICA. Following such a treatment cell morphology and nuclear chromatin can be assessed with better clarity while the FLICA (Fam-VAD-fmk) fluorescence remains. It is quite apparent that most FLICA-labeled cells are detaching from slide, are distinctly smaller and have condensed chromatin (Fig. 3A, the asterisk labeled cells). Their green FLICA fluorescence has both the cytoplasmic localization and overlaps with red nuclear fluorescence resulting that nuclei fluorescence in yellow. However, because of the spherical geometry of apoptotic cells, without a help of confocal microscopy, it is difficult to discern whether the yellow fluorescence seen over the nucleus is due to the presence of FLICA in the nucleus, co-localizing with DNA, or to the layer of the cytoplasm underneath and above of the nucleus.

Interestingly, when the cells are first fixed and subsequently subjected to labeling with FLICA, both, apoptotic as well the non-apoptotic cells become labeled (Fig. 3C). The cell fixation makes the zymogen caspases in the nonapoptotic cells reactive with FLICA possibly by altering their conformation in such a way that the active centers become accessible to the inhibitors. The labeling has a very characteristic pattern: the most strongly and distinctly labeled are mito-

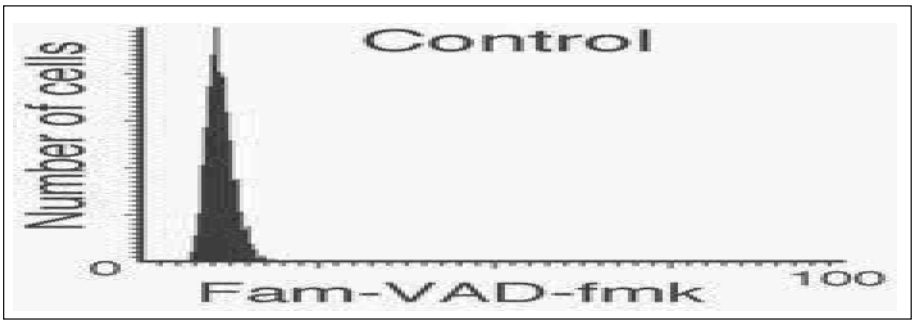

Figure 2. Detection of caspases activation by Fam-VAD-fmk binding. Exponentially growing HL-60 cells, untreated (control) or treated with camptothecin (CPT) or with tumor necrosis factor alpha (TNF) were incubated with for 1 h with 10 μM Fam-VAD-fmk and rinsed, as described.[67] Their green fluorescence (maximal pixel) of Fam-VAD-fmk bound to activated caspases was measured by laser scanning cytometry (LSC).[28,29] The frequency histograms show that large percentage of cells in the CPT or TNF-treated cultures become labeled with the probe.

chondria and nucleoli. Nucleoplasm shows a diffuse and rather weak fluorescence while fluorescence of the cytoplasm outside of mitochondria is even less intense. This pattern is consistent with the reported localization of caspases in mitochondria.[9,31,32] Although nuclear localization of several caspases also has been observed[33-35] their presence in nucleoli has not been yet demonstrated. However, the indirect evidence suggests that caspases may be localized, at least following their activation, in nucleoli. Thus, at early stage of apoptosis the accumulation of the 89 kD product of caspase-3 mediated cleavage of PARP-1 was most preeminent in nucleoli and perinucleolar areas.[10] Nucleolar segregation is also an early apoptotic event, and is associated with activation of caspases.[36-39] Nucleolar segregation was shown to initiate separation of RNA from DNA that culminated in packaging these nucleic acids into separate apoptotic bodies.[40] There is also evidence that the "upstream binding factor" (UBF), the protein regulating transcription of rDNA in the nucleolus, is the very early target of caspases during apoptosis.[41]

Detection of Caspase Activation Combined with Other Probes of Apoptosis

Simultaneous Analysis of Caspase Activation and Plasma Membrane Integrity

Multiparameter cytometry utilizing a combination of fluorochrome probes differing in emission or excitation wavelength allows one to correlate, within the same cells, the activation of caspases detected by FLICA with other apoptotic events. The evidence of a correlation, or lack of it, can reveal whether activation of caspases is, or is not, a prerequisite for the other event to take place. Such an analysis, if carried out sequentially at different time points after induction of apoptosis, also can reveal the time-gap between the caspase activation and the observed event.

Figure 4 illustrates an example of the analysis of caspases activation combined with the quest of integrity of plasma membrane. The green fluorescing pan-caspase inhibitor Fam-VAD-fmk was combined with the red fluorescing cationic fluorochrome propidium iodide (PI). The latter is excluded by live- and early- apoptotic but not by necrotic- and late-apoptotic cells.[42]

Based on the differences in binding Fam-VAD-fmk (FLICA) and PI one can distinguish four cell subpopulations (compartments) on the bi-variate scattergrams representing cellular green versus red fluorescence. They are: (A) the cells that are both FLICA- and PI- negative

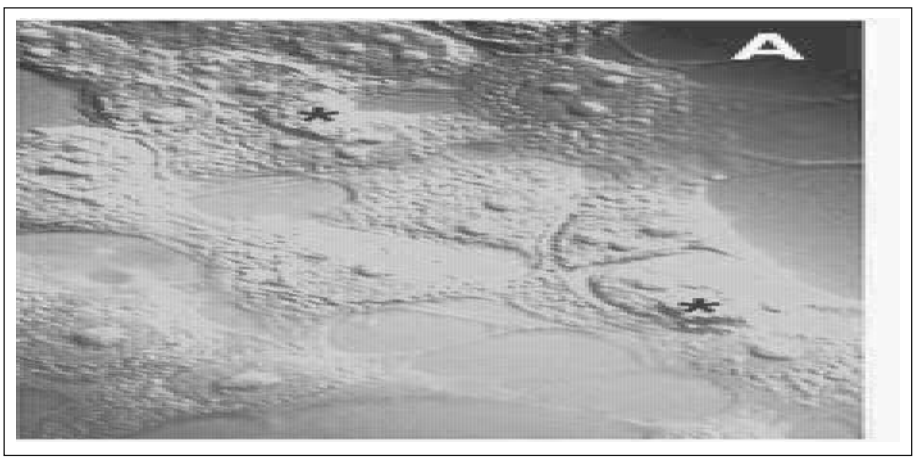

Figure 3. Photomicrographs of MCF-7 cells labeled with Fam-VAD-fmk. A,B) Live MCF-7 cells growing on the microscope slide cells were treated for 24 h with CPT, then exposed to Fam-VAD-fmk for 1 h, rinsed in PBS and fixed in 1 % formaldehyde followed by 70 % ethanol. Their DNA was then counterstained with 7-aminoactinomycin D. The same field of view was examined under interference (Nomarski) contrast (A) or fluorescence microscope (B).[19] (C) Untreated MCF-7 cells were fixed in 1 % formaldehyde, then in 70 % ethanol prior to labeling with Fam-VAD-fmk.[19]

(FLICA-/PI-); (B) the FLICA positive and PI negative cells (FLICA+/PI-); (C) the cells that are both FLICA and PI positive (FLICA+/PI+); and (D) the FLICA negative and PI positive cells (FLICA-/PI+).

These compartments represent the sequential changes that occur during apoptosis, involving activation of caspases followed by the loss of the plasma membrane integrity.[43] Thus, the compartment A represents live, nonapoptotic cells with inactive caspases and intact plasma membrane. Activation of caspases with no changes in plasma membrane integrity characterizes early apoptotic cells (compartment B). The cells that are more advanced in apoptosis have their caspases still active, able to bind FLICA. Their capability to exclude PI, however, is lost (compartment C). Finally, the very late apoptotic cells are characterized by loss of the abilities to bind FLICA and to exclude PI.[43]

The loss of plasma membrane integrity that manifests in cell inability to exclude cationic dyes such as PI or trypan blue characterizes the so-called "necrotic stage" of apoptosis.[44-46] The observed differential cell reactivity with FLICA allows one to subdivide the "necrotic stage" onto two sub-stages, the earlier one representing cells that still bind FLICA, and the late one, when the ability to bind these inhibitors is lost.[43] It is possible that at this late stage the caspases become either inhibited or degraded to the point that their active centers do not react with FLICA anymore. Such cells, thus, are indistinguishable from the genuine necrotic cells, that die by mode of necrosis ("oncosis" or "accidental cell death").[44,46]

Simultaneous Analysis of Caspase Activation and Annexin V Binding

Early during apoptosis the asymmetry of plasma membrane phospholipids is broken and phosphatidylserine becomes exposed on the outer leaflet of the membrane.[47,48] This event is considered to be one of the hallmarks of apoptosis. Because the anticoagulant protein annexin V binds with high affinity to phosphatidylserine, the fluorochrome-tagged annexin V is frequently used as a marker of apoptosis.[48] While in most instances the annexin V binding is caspase activation-dependent, there are situations when it appears to be independent.[49,50] It is desirable, therefore, to have an assay that simultaneously detects caspases activation and externalization of phosphatidylserine on plasma membrane of the same cells. Such an assay

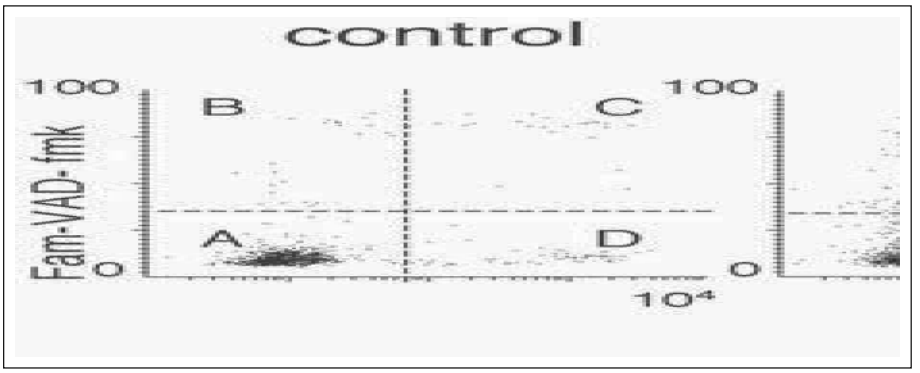

Figure 4. Scatterplots representing green fluorescence (Fam-VAD-fmk binding) versus intensity of PI fluorescence of HL-60 cells, untreated or treated with camptothecin (CPT). Exponentially growing HL-60 cells, untreated (control) or treated with 0.15 μM CPT for 2, 4 or 5 h were incubated for 1 h with Fam-VAD-fmk, rinsed and exposed for 5 min to 1 μg/ml of propidium iodide (PI) as described.[43] Their green (maximal pixel) and red (integral) fluorescence was measured by LSC.[28,29] Four distinct cell subpopulations can be identified based on the difference in staining with Fam-VAD-fmk and PI, as described in the text.

may also reveal the sequence- and time relationship- between caspases activation and loss of the asymmetry of the plasma membrane phospholipids, as reflected by the externalization of phospatidylserine.

The bi-variate scatterplots (Fig. 5) illustrate changes in cell ability to bind annexin V and Sr-VAD-fmk following induction of apoptosis by CPT. Similar as in the case of cell labeling with Fam-VAD-fmk versus PI (Fig. 4), based on the difference in Sr-VAD-fmk versus annexin V binding one also can identify on these scatterplots four cell populations. In quadrant A are the non-apoptotic Sr-VAD-fmk- and annexin V- negative cells. In quadrant B are the cells that have activated caspases but do not bind annexin V. The cells that have both, activated caspases and capability to bind annexin V are represented in quadrant C. Interestingly, the cells that show increased annexin V binding but no evidence of caspases activation are also apparent (quadrant D). Their presence may suggest that externalization of phosphatidylserine is caspase activation-independent. However, we observed that the presence of active caspases cannot be detected at the late stage of apoptosis (Fig. 4). It is possible, therefore, that the cells that bind annexin V and are Sr-VAD-fmk negative (Fig. 5, quadrant D) are the same, very late apoptotic cells, that do not bind Fam-VAD-fmk and cannot exclude PI (Fig. 4, quadrant D).

The Cell Cycle Phase-Specific Activation of Caspases

FLICA binds covalently to the active centers of the respective caspases forming a thiomethyl ketone II bond. The bond is stable and withstands cell fixation.[19] It is possible, therefore, to incubate live unfixed cells with FLICA in order to label the cells that activated their caspases, then rinse and fix them. The cells can be then probed with other fluorochromes, that require prior cell fixation and permeabilization. Cellular DNA, for example, can be stained with PI or 7-aminoactinomycin D (7-AAD) to correlate activation of caspases with the cell cycle position (Fig. 6). Likewise, DNA fragmentation (the presence of DNA strand breaks) can be detected by in situ labeling the 3'OH break termini with fluorochrome-tagged deoxynucleotides utilizing exogenous terminal deoxynucleotidyl transferase,[51] to find out whether caspases activation correlates with activation of the apoptotic DNase.

Figure 6 presents the measurement of fluorescence of HL-60 cells that, to induce apoptosis, were treated with tumor necrosis factor alpha (TNF) then labeled with Fam-VAD-fmk, fixed

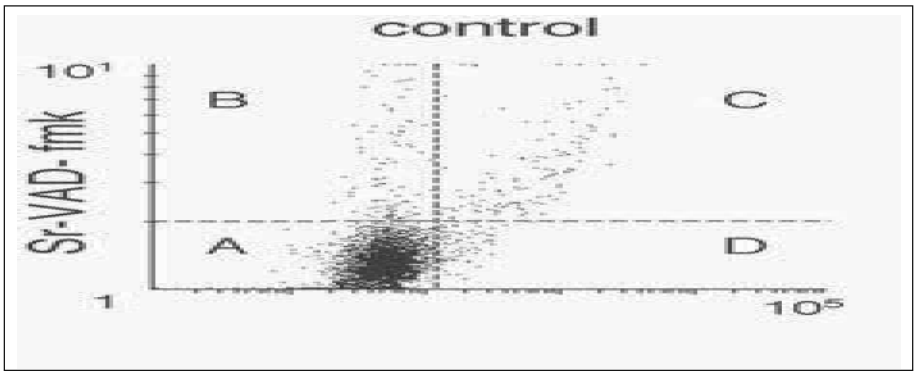

Figure 5. Scatterplots representing changes in binding of Sr-VAD-fmk and annexin V during induction of apoptosis. Exponentially growing HL cells, untreated (control) or treated with CPT for 3 or 5 h were exposed for 1 h to sulforhodamine B (Sr) labeled -VAD-fmk and to FITC-tagged annexin V. Their red and green fluorescence (both integral) was measured by LSC. The presence of Sr-VAD-fmk labeled/annexin V unlabeled cells indicates that activation of caspases precedes externalization of phosphatidylserine on plasma membrane.

and subsequently stained with PI. Their green and red fluorescence was measured by flow cytometry. The bi-variate distribution (scatterplot) representing cell labeling with these fluorochromes allows one to identify the population of cells with activated caspases (increased Fam-VAD-fmk fluorescence) and through the gating analysis, to reveal the cell cycle distribution (based on intensity of PI fluorescence) separately, in subpopulations of cells with activated- and non- activated caspases. (Fig. 6). The DNA content frequency histograms of these subpopulations, as shown in this figure, can be deconvoluted to reveal whether activation of caspases is cell cycle-phase specific. As it is evident, in the case induction of apoptosis by TNF, caspases are activated in all phases of the cell cycle (Fig. 6).

Stathmo-Apoptosis: The Use of FLICA to Arrest the Process of Apoptosis

The extent (incidence) of apoptosis in a cell population is commonly estimated based on the frequency of apoptotic cells (apoptotic index, AI). AI, thus, is a "snapshot" estimate of the percentage or fraction of apoptotic cells in that population at a particular time-point. However, because apoptosis is a transient, kinetic event, AI is an inaccurate measure of the incidence of apoptosis (Fig. 7). Namely, the entire apoptotic process, from its onset to the final cell disintegration, when the cell is no longer detectable, is often short and may be of variable duration.[46,52,53] For example, upon induction, apoptosis of cells of hematopoietic tumor lines (e.g., Jurkat, HL-60, U-937) progresses rapidly and the cells disintegrate within 3 -5 h. The duration of apoptosis in vivo appears to be even shorter. This is reflected by the fact that in tissues, under conditions of homeostasis, AI often approximates the mitotic index.[46,52,53] Because mitosis lasts ~1 h, apoptosis must be of comparable length of time. On the other hand apoptosis of epithelial or fibroblast cell lineage occurs with a 24 h delay upon induction, and the apoptotic process is much longer.[54] In addition, some inducers of apoptosis or factors in cellular environment may alter the duration of the apoptotic process e.g., by modulating formation and/or shedding of apoptotic bodies, rate of proteolysis or DNA degradation. Serine protease inhibitors, for example, significantly prolong apoptosis by preventing internucleosomal DNA degradation[51] and nuclear fragmentation.[55] The length of time-window during which the apoptotic cell can be identified also varies, as it depends on the marker (assay) that is being used, and expression of different markers is variable in time. In all these situations, therefore, when either

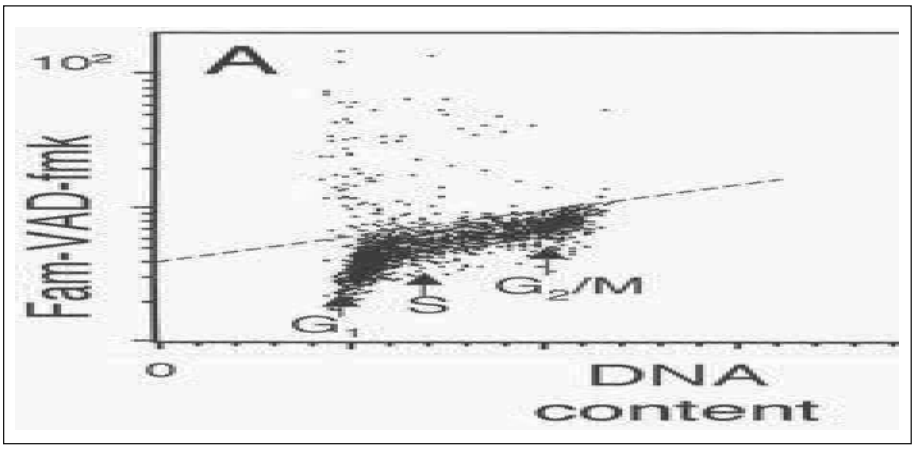

Figure 6. Analysis of the cell cycle distribution in populations of cells with activated and non-activated caspases. Exponentially growing HL-60 cells, untreated (A) and treated with TNF and cycloheximide for 5 h (B), were then exposed to Fam-VAD-fmk for 1 h, fixed in formaldehyde, permeabilized, incubated with RNase A and stained with PI, as described.[19] Cellular green and red fluorescence was measured by flow cytometry. The increased intensity of Fam-VAD-fmk fluorescence identifies cells with activated caspases. The insets show the DNA content frequency histograms of the gated cell populations of the cells with activated (top) and non-activated (bottom) caspases. In concordance with the published data that TNF induces apoptosis regardless of the cell cycle phase,[68] activation of caspases also occurs in cells in all phases of the cycle.

duration of apoptosis or of the time-window of its detection, varies, AI cannot accurately represent incidence of apoptosis (Fig. 7).

To obtain a more accurate assessment of the incidence of apoptosis, we have developed a stathmo-apoptosis assay[56] in which Fam-VAD-fmk is used to arrest cells in apoptosis thereby preventing their disintegration and loss from analysis (Fig. 8). Because the arrested (apoptotic) cells become fluorochrome labeled, they could easily be identified by fluorescence microscopy, as well as flow- or laser scanning- cytometry.[56] Fam-VAD-fmk, thus, has dual function in this assay, namely arresting the process of apoptosis and also serving as marker apoptotic cells. This approach is analogous to stathmo-kinesis, the assay that is used to estimate cell birth rate from a slope of the plot representing mitotic cell accumulation during their arrest by a mitotic poison.[57]

Because the process of apoptosis is halted at the stage of caspase activation the caspase-mediated events either do not occur in the arrested cell, or occur but at a much slower rate. Indeed, we observed that at 20 μM and higher concentration of Fam-VAD-fmk, the HL-60 or MCF-7 cells did not disintegrate for up to 48 h.[56] The arrested cells have still relatively high level of interference contrast when examined by microscopy, and exhibit higher intensity light scatter signal than the apoptotic cells growing in the absence of Fam-VAD-fmk, when analyzed by flow cytometry.[56] These arrested cells, however, are unable to exclude PI or trypan blue and cannot be revived when rinsed free of the inhibitor and grown in fresh medium.[56] It should be noted that the rate of cell entry to apoptosis is not affected by Fam-VAD-fmk.[56]

Arresting cells in apoptosis by Fam-VAD-fmk enables one to plot cumulative apoptotic index (CAI) as a function of time after administration of the inducer of apoptosis (Fig. 8). The plot reveals the rate of cell entry (kinetics) into apoptosis during the treatment. As it is evident in Figure 8, two distinct rates characterize the cells treated with camptothecin (CPT). During the initial 6 h approximately 50 % cells from the culture undergoes apoptosis at a rate of about

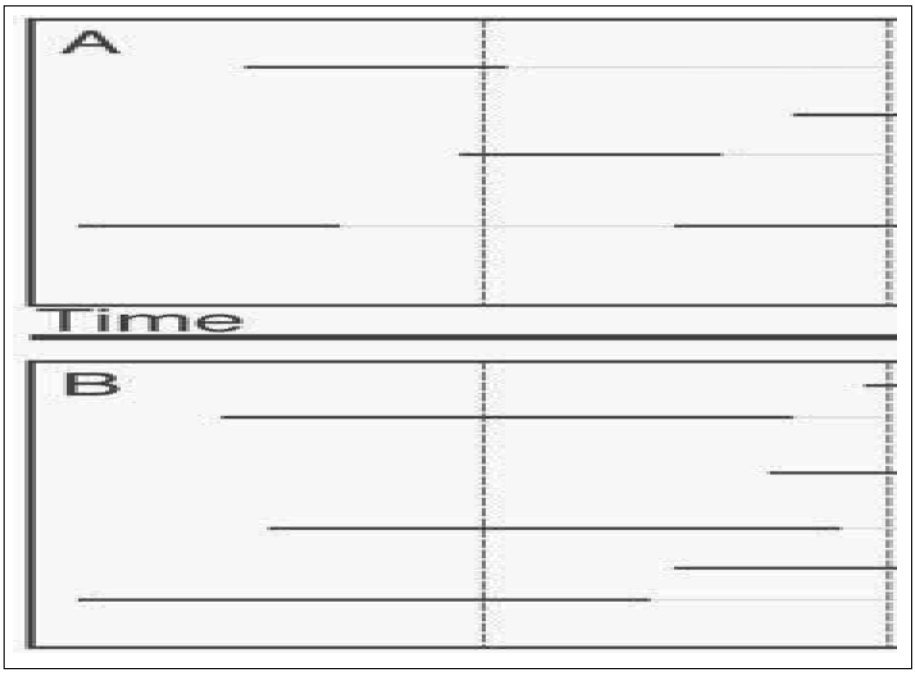

Figure 7. Dependence of the apoptotic index (AI) on duration of apoptosis. Duration of apoptosis of individual cells detected by a particular assay is represented by length of the lines along the time coordinate. Prolongation of apoptosis, as in panel B, results that the "snapshot" estimate of AI, at a given time point (dashed vertical lines), as is commonly done, results in a greater AI in B than A, despite that incidence of apoptosis in A and B is the same. The pan-caspase inhibitor Fam-VAD-fmk can be used to arrest the apoptotic process (stathmo-apoptosis) preventing cell loss, and by labeling cells entering apoptosis, to estimate cumulative AI over long period of time.[56]

8 % of cells per hour. The remaining cells are entering apoptosis for up to 48 h at a rate of ~1 % of cells per hour. Control cell cultures exposed to Fam-VAD-fmk in the absence of CPT show a minor increase in the percentage of labeled cells after a 48 h culture period (~9 %), consistent with the rate of spontaneous apoptosis in untreated cell cultures approximating of 0.2 % cells per hour.

To reveal whether the observed differences in rate of cell death may be related to the cell cycle phase the cell cycle distribution of the cells undergoing apoptosis (Fam-VAD-fmk labeled) at a faster rate i.e. early during the treatment (0-5 h), can be compared with that of the nonapoptotic cells in the same culture, as shown in Figure 6. When such a comparison was done we observed that in the CPT-treated cultures predominantly the S phase cells were undergoing apoptosis at the faster (~8 % cells per hour) rate.[56] The observation that the cells progressing through S phase are particularly sensitive to CPT is consistent with the wealth of the published data,[58-60] and with the mechanism of cell death that involves a collision of the DNA replication forks with the CPT-generated DNA lesions ("cleavable complexes") transforming these lesions into double stranded breaks that trigger apoptosis.[61] The slow rate of entrance to apoptosis observed between 8 and 48 h (~1 % cells per hour) represents predominantly G_1 cells that most likely were entering S phase during treatment with CPT. The stochastic nature of both slopes of the stathmo-apoptotic plot is consistent with the kinetics of cell progression through the cycle.[57,62]

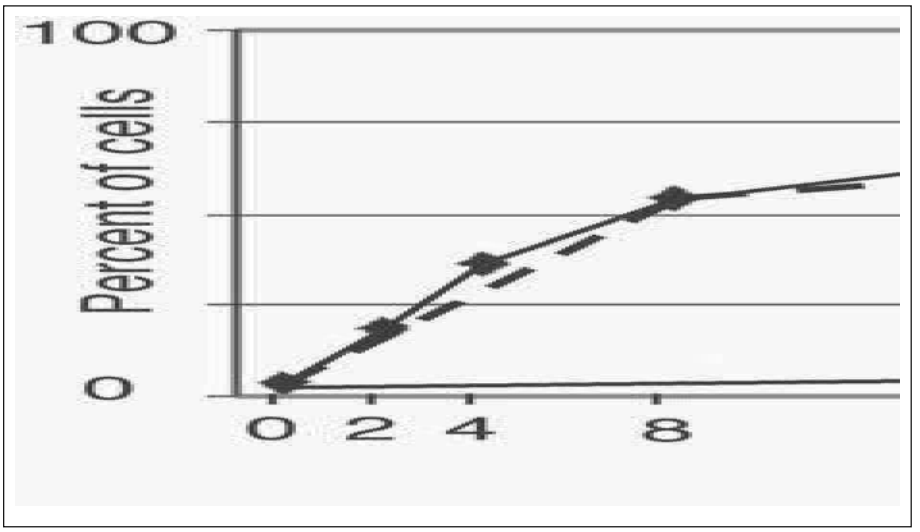

Figure 8. Kinetics of cell entry to apoptosis estimated by the stathmo-apoptosis assay.[56] Exponentially growing HL-60 cells were treated at time zero either with 20 μM of Fam-VAD-fmk only (control) or with 0.15 μM of CPT and 20 μM of Fam-VAD-fmk (CPT). The cells were collected at different time points and the percentage of cells labeled with Fam-VAD-fmk estimated as described.[56] This percentage is plotted as a function of time after administration of Fam-VAD-fmk (solid lines). The dashed line represents two distinct kinetic slopes of the early (0-8 h) and rapid (8% of cells per hour) versus the late (8-48 h) and slow (~1 % of cells per hour) rate of cell entry to apoptosis.

The ability by FLICA to arrest in apoptosis and trough an estimate of CAI to measure frequency of cells that at are committed to die may be of special value as a prognostic marker in analysis of sensitivity of tumor cells (e.g., leukemias, lymphomas, myelomas) to the treatment. Namely, during chemotherapy tumor cells are dying predominantly by mode of apoptosis.[63] Because they undergo apoptosis asynchronously, the single "snapshot" estimate of AI as it is conventionally done,[63] cannot reveal an incidence of apoptosis with adequate accuracy. In contrast, the ex vivo studies of the CAI of the patient's blood or bone marrow blasts taken after drug administration (at the time when the drug blood or bone marrow level already starts to fall) are expected to reveal the fraction of cells committed in vivo to die in response to the treatment, and thus be of predictive value.

It should be noted, however, that the stathmo-apoptosis approach based on the use of FLICA is applicable in analysis of the caspase-mediated apoptosis only. Other means of prevention of apoptotic cell disintegration and other means of their detection have to be used to apply this principle in studies of apoptosis that is not caspase-mediated.[49,50,64] Since serine proteases, in addition to caspases, appear to be also essential for completion of apoptosis[51,65.66] it is likely that inhibitors of serine proteases also may be used to ensure stathmo-apoptosis.

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CHAPTER 13

Other Methods of Caspase Activity Monitoring

Hubert Hug, Christof Burek and Marek Los

Theoretical Background

Caspases (**C**ysteine-**Asp**art-**ases**) are important effector molecules involved in apoptosis, though some of them can also participate in other physiological processes such as activation of pro-inflammatory cytokines and/or possibly regulation of cell activation and proliferation.[1,2] For detailed information regarding the protease family please refer to the first chapter of this book. Since the discovery of the prototype death protease *Ced-3*, in *Caenorhabditis elegans*[3] more than thirteen mammalian and invertebrate caspases have been described.[4] They can be divided into different sub-classes, based on structural similarities of either prodomain or their catalytic sub-units. Some of these structural similarities correlate with substrate specificity as indicated below (see Table 1). All mammalian caspases described so far are specific for aspartic acid in the P_1 position of their substrates. Caspases exist as latent zymogens that contain an N-terminal precursor domain followed by the region that forms the two subunits of the catalytic domain after proteolytic processing. The core of the catalytic center of these enzymes is formed by the conserved amino acid sequence: QACXG (for caspase-8, -10 X = Q; for caspase-9 X = G, for most of other known caspases X = R). The cysteine at the core of this peptide directly participates in catalysis and defines these proteases as cysteine proteases. The pro-forms of caspases are activated by proteolytic cleavage at specific aspartic residues (Fig. 1). Usually, an initial cleavage event occurs which separates the carboxy-terminal "short" subunit of the protease from the rest of the molecule, allowing assembly of an active protease that auto-catalytically cleaves off its N-terminal prodomain to generate the mature active enzyme. The proteolytic mechanism of caspase activation allows detailed detection of activation of single caspases by Western blotting. It is important to note that some pro-caspases can be cleaved by other proteases without the formation of an active subunit. Alternative cleavage products created this way remain inactive and they may mislead inexperienced investigators. Once activated, many caspases can propagate proteolytic activation of other family members by processing their pro-forms through cleavage at specified aspartic acid residues. The kinetics and mass balances of caspase activation and inhibition have been modeled and the model could be useful in the developing of new strategies to detect caspase activity.[5] A family of cellular inhibitors of caspases (IAPs) has been identified[6-8] (see chapter 5 for more details). In addition, some viruses are known to produce caspase inhibitory proteins such as CrmA of pox-viruses and p35 of the insect baculoviruses (more information in chapter 6). Several synthetic peptidyl inhibitors of caspases have been designed by taking advantage of the known specificity of caspases for certain substrates (see Table 1). Usually, in such an inhibitor the P_1 aspartate is modified by chloro-, fluoro-methyl-keton (cmk-, fmk-), or an aldehyde group. The aldehyde-based compounds are reversible inhibitors, whereas the cmk- and fmk-based reagents

Caspases—Their Role in Cell Death and Cell Survival, edited by Marek Los & Henning Walczak.

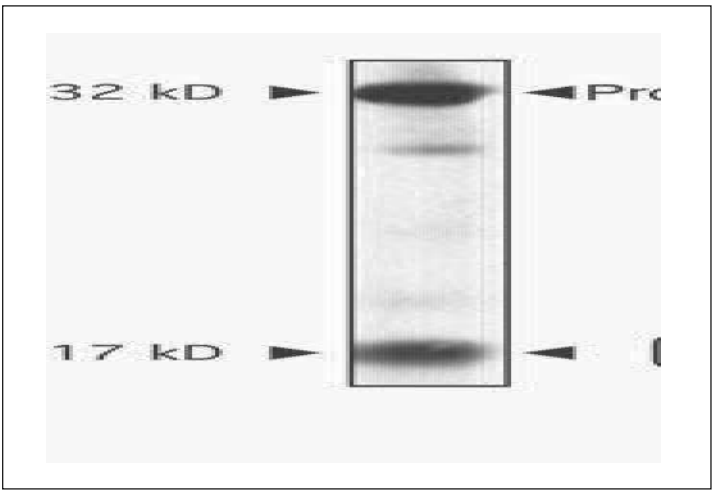

Figure 1. Schematic representation of caspase activation. Caspases exist as inactive pro-enzymes. Proteolytic (auto)activation leads to formation of an active hetero-tetramer containing two smaller and two larger sub-units, one of each derived from a common pro-enzyme polypeptide. Here, caspase-3 is shown as an example. The active p17 and p12 subunits (named after their molecular weight) are collectively called p20 (large subunit) and p10 (small subunit) respectively. The Western blot detection of caspase-3 activation was done as follows: Primary human fibroblasts were stimulated to die with 2.5 μM Staurosporin for 8 h. Cells were then harvested (see the Methodology section), proteins were resolved on 13.5 % SDS gel, blotted, and the specific, caspase-3 signal was detected using anti–caspase-3 monoclonal antibody (Transduction Laboratory, Heidelberg, Germany).

form covalent interactions with the active-site-cysteine of caspases and thus are irreversible inhibitors. Tetra-peptides are good inhibitors in vitro, but they usually poorly penetrate through cellular membranes. Therefore, they are more useful for caspase blockage in cellular extracts. For inhibition of caspases in intact cells tri-peptide compounds, especially benzyoxyl-carbony-Val-Ala-Asp-CH_2F (zVAD-fmk), were proven to be effective and useful as a control or reference substance for caspase research. The cellular and/or viral caspase inhibitors mentioned above often strongly influence the detected activation and/or activity of caspases. In this chapter we describe several methods to detect caspases as well as caspase activity in cells and cell extracts.

Detection of Caspase Activation by Western Blot

Western blotting is the method of choice to detect the expression level and the degree of proteolytic activation of pro-caspases (Fig. 1). Either the increase of a catalytic subunit and/or the decrease of the pro-enzyme are monitored. Many companies offer poly- and monoclonal isoenzyme-specific antibodies that recognize either the pro-enzyme form, a catalytic subunit, or both. Special attention needs the appearance and detection of intermediate processed caspase forms, which may remain inactive. In addition, the caspase activity can be monitored by the detection of cleavage products of various caspase substrates. By far, the most popular indicator of caspase activity (specific for caspase-3, -7 and -9) is the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1), but in principle cleavage products of any protein listed in Table 1 can be used to monitor the activity of certain caspase family members. Anti caspase antibodies can be obtained among others from the following companies: Alexis, Biocat, Calbiochem, Pharmingen, Promega, Roche, Transduction Laboratory, Upstate Biotechnology. Anti-PARP-1 antibodies,

Table 1. Cleavage sites of some known caspase substrates (examples)

Enzyme	Cleavage Site P_4 P_1	P_0	Substrate
Caspase-1	YVA**D**	G	optimised substrate
	FED**D**	G	Pro-caspase-1)
	IET**D**	S	Pro-caspase-3)
	YVH**D**	A	Pro-I Interleukin -1β
	FEA**D**	G	Pro-I Interleukin -1β
	LES**D**	N	Pro-Interleukin-18
	YVP**D**	S	p110 PITSLRE kinase α2-1
Caspase-3	DEV**D**	G	PARP-1
	DEV**D**	N	DNA- PKcs
	DQT**D**	G	Gelsolin
	DEP**D**	S	ICAD
	DEV**D**	G	Replication Factor C large subunit
	DET**D**	S	α-spectrin (foldrin)
	DSL**D**	S	α-spectrin (foldrin)
	DEV**D**	S	β-spectrin
	DSI**D**	S	β-spectrin
	DEP**D**	S	SREBP-1
	DGP**D**	G	70kD U1 snRNP
	DDV**D**	Y	Topoisomerase I
Caspase-6	VEI**D**	S	Lamin A
	VEI**D**	N	Lamin B
Caspase-8	LQT**D**	G	Bid
	LEV**D**	G	$FLIP_L$
CED-3	DQM**D**	G	Baculovirus p.35
	DNR**D**	G	CED-3
	LEA**D**	S	ICE-rel-III
	DMQ**D**	N	PKC-δ
	DEA**D**	G	Retinoblastoma Protein (Rb)

The P_1 aspartate is indicated in bold. It is advisable to consult ref. 17 for additional information concerning substrate sequence specificity for different caspases. There is an exception in *Drosophila*, where the P_1 residue can be either aspartate or glutamate.[18]

which specifically recognize the active p85 fragment, are available from Calbiochem and Promega. To quickly identify suppliers of necessary antibodies, or other reagents, authors recommend to use www.google.com, or another internet search engine. Usually specific information about Western blot conditions are included, and the standard protocol given below should be modified accordingly. Hints allowing quick identification of caspases involved in a pathway under investigation can be found in pathway databases, like e. g. TRANSPATH (transfac.gbf.de/TRANSFAC/), Path DB (www.ncgr.org/pathdb/), CSNDB (geo.nihs.go.jp/csndb) or BIND (www.bind.ca/cgi-bin/bind/dataman), (apoptotic pathways are not yet available).

Materials and Equipment

1. Lysis buffer: 20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.1 mM EGTA, 350 mM NaCl, 1 % Igepal CA630 (Sigma), 0.5 mM DTT, 20 % glycerol, 20 µl/ml protease inhibitor cocktail (Sigma, Cat. No. P8340)
2. BCA-Pierce-Protein Assay kit (Pierce, Cat. No. 23225)
3. Nitrocellulose membranes (e. g. Amersham-Pharmacia Biotech) or polyvinylidenedifluoride (PVDF) membranes (e. g. Millipore)
4. 10—20 % polyacrylamide gradient gels (e. g. Biocat)
5. SDS-PAGE electrophoresis apparatus and power supply (e. g. Biorad, Höfer, Biocat)
6. 5 x Tris-glycine running buffer: 25 mM Tris base, 250 mM glycine, 0.1 % SDS, pH 8.3
7. Semi dry blotter (Biorad)
8. Blotting buffer: 39 mM glycine, 48 mM Tris base, 0.037 % SDS, 20 % methanol
9. 2 x SDS loading dye: 100 mM Tris-HCl (pH 6.8), 4 % SDS, 0.2 % bromophenolblue, 20 % glycerol, 200 mM β-ME
10. Horseradish peroxidase (HRP)-conjugated anti-Ig antibody
11. 10 x TBS: 250 Tris-HCl (pH 7.4), 1.5 M NaCl
12. Blocking buffer: 1 x TBS, 5 % (w/v) skim milk (Merck)
13. 1 x TBST: 1 x TBS, 0.05 % Tween 20
14. Enhanced chemiluminescence Western detection system (Amersham-Pharmacia Biotech, RPN 2209)
15. Autoradiography films (Amersham-Pharmacia Biotech) and casette

Methodology

There are many more or less similar Western blotting methods described that may work for the detection of caspses (for a general description see refs. 9 and 10). We give an example of the protocol that we use. Special attention should be given to the suppliers recommendations.

1. 10^7 cells (10^6 cells/ml) are washed two times with 1 x PBS and then resuspended in 40 – 100 µl lysis buffer.
2. The protein concentration is determined by the BCA-Pierce-Protein Assay kit according to the protocol of the manufacturer.
3. Mix protein samples (50 µg per lane) with 2 x SDS loading dye.
4. Heat the samples at 95°C for 2 min to denature by SDS.
5. For Western analysis 50 µg of protein are separated on 10—20 % polyacrylamide gradient gels.
6. Proteins are then blotted onto nitrocellulose or PVDF membranes according to standard protocols (100 V for 1 h or 30 V overnight).
7. Membranes are then incubated it in 10 to 20 ml blocking buffer (membrane must be covered with solution). Gently agitate for 1 h at RT (or at 4°C overnight) using a rocker platform.
8. Wash the membrane two times in 20 ml TBST for 5 min with gentle agitation.
9. Incubate the membrane in 10 to 20 ml blocking buffer containing the first antibody. The monoclonal or polyclonal anti-caspase antibodies are diluted according to the manufacturers instructions. Incubate with gentle agitation for 1 h to overnight.
10. Wash the membrane two times in 20 ml TBST for 5 min with gentle agitation.
11. Incubate the membrane in 10 to 20 ml blocking buffer containing the secondary antibody. Secondary antibodies are usually anti-mouse or anti-rabbit IgG1 coupled to horseraddish peroxidase at a dilution of 1:5000. Incubate with gentle agitation for 1 h.
12. Wash the membrane two times in 20 ml TBST and one time in 20 ml TBS for 5 min, respectively, with gentle agitation.
13. Antibody reactive bands are detected by using the enhanced chemiluminescence Western detection system (Amersham-Pharmacia Biotech). Mix the detection solution 1 with solution 2 at a ratio of 1:1 (v/v). Incubate the membrane for 1 min at RT.
14. To reuse membranes, they can be stripped for 5 min in 0.2 M NaOH at room temperature.

Fluorescent Measurement of Caspase Activity in Intact Cells

The methodology described in this subheading supplements the fluorometric methods of caspase activity detection described in chapter 12. The approach is based on fluorescence increase of $(Aspartyl)_2$-Rhodamine 110 (D_2Rhodamine), (supplied by Alexis, Calbiochem, Biocat) upon removal of aspartyl groups from the substrate (see Note 1 for more information).[11,12] Liberation of each NH_4- group (previously occupied by aspartate) increases the fluorescence intensity of Rhodamine 110 one hundred fold (Fig. 2). Theoretically the signal from cells carrying active caspases should shift four decades on the logarithmic scale. In reality, not all substrate that entered the cell is processed and, in addition, the signal is partially absorbed by cytoplasmic contents. Therefore, the increase of signal intensity is usually lower than expected (1—1.5 on a log scale). The hypo-osmotic condition used in the protocol facilitates substrate loading into cells and β-mercapto ethanol or dithiothreitol (DTT) protects the cysteine residue in the active center of the caspases from oxidation. Please note that Rhodamine 110 has a wide fluorescence spectrum, and the signal collected in the FL-2 channel is only about 20 % weaker than in the FL-1 channel (which is usually used to detect the dye). Therefore, in three-channel flow cytometers only two-color fluorescence should be performed with the second signal being collected in the FL-3 channel (e.g cy5 surface staining or PI, 7-AAD).

Materials and Equipment

1. D_2Rhodamine, (50 mM); stock solution is stable for at least one month at -20°C or for one year at -70°C. A stock solution is prepared by diluting 63 mg of powdered D_2Rhodamine in 1 ml DMSO before 1 ml of absolute ethanol is added; the stock solution is then 50 mM. The dye dissolves very well in DMSO and the addition of ethanol keeps the stock liquid even at low temperatures, saving time upon stock usage. Since the dye is not very stable in H_2O, be careful not to contaminate the stock sol. with H_2O. Higher concentrated stock solutions i.e. 100—200 mM are also easily achievable. Protect from light. D_2Rhodamine is usually used at the final concentration of 50—60 μM, for approx. 5 x 10^5 cells in a volume of 1 ml.
2. β–mercapto ethanol (100 mM); Dissolve 347 μl of absolute β-ME in 50 ml of PBS. The solution is stable at 4°C for at least three months, protect from light.
3. PBS; Dissolve in 900 ml H_2O:
   8 g of NaCl
   0.2 g of KCl
   1.44 g, Na_2HPO_4
   0.24 g of KH_2PO_4
   Adjust pH to 7.3, and the volume to 1 L, store at 4°C (you will need it cold).
4. dH_2O; deionised H_2O, store at RT.
5. FACS® Brand Lysing Solution; (Becton Dickinson, San Jose, CA) Cat. No. 92-0002.
6. Flow cytometer (e.g., FACScan Becton Dickinson) equipped with blue-light laser (488 nm) and compatible plastic-ware, (e.g., FALCON #2058).
7. Centrifuge compatible with plastic-ware for flow cytometer.

Methodology

Detection of Caspase Activity in Isolated PBMC and Other Cells as Well as Cell Lines

1. Dilute an aliquot of D_2Rhodamine to 1 mM with H_2O.
2. Prepare 10^5—10^6 cells in 420 μl of culture medium, (see Notes 3, 4 and 6—10) for additional information).
3. Add β-mercapto-ethanol to a final concentration of 10 mM (50 μl of 100 mM stock sol.).
4. Add D2Rhodamine 110 to a final concentration of 60 μM (30 μl of 1 mM stock sol.).

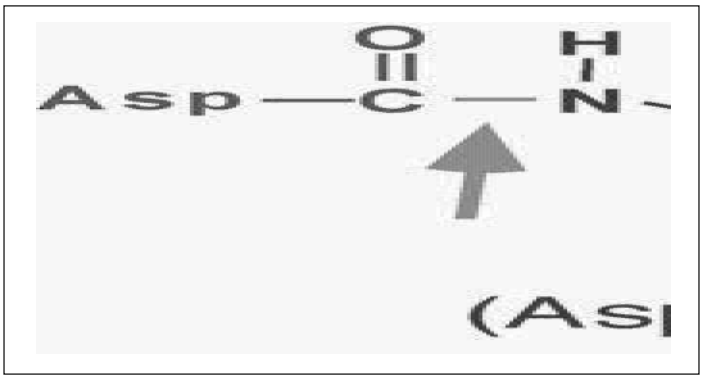

Figure 2. Structure and mechanism of action of D_2Rhodamine. Both aspartyl moieties are indicated in blue, cleavage sites are indicated by arrows. The removal of each aspartyl group causes a ~100 times fluorescence increase in vitro.

5. Add 750 µL of dH_2O (osmotic loading), (alternatively 1 -2 ml of FACS® Brand Lysing Solution can be used).
6. Incubate 8—10 min at 37°C.
7. Stop the reaction by pipetting 3 ml cold PBS (≤4°C). Keep cells on ice and proceed with additional staining steps e.g., for detection of specific cell subsets if desired.
8. Measure samples by flow cytometry (e.g., use FL-1 channel when FACScan is employed).

IMPORTANT: The samples have to be measured within three hours.

Detection of Caspase Activity in PBMC from Whole Blood

1. Prepare a 1 mM solution of D_2Rhodamine in H_2O, from the stock sol.
2. Take 200 µl of whole blood (heparinized), (see Notes 3—6 for additional information).
3. Add β-mercapto-ethanol to final concentration 10 mM (20 µl of 100 mM stock sol.).
4. Add D_2Rhodamine to a final concentration of 150 µM (30 µl of 1 mM stock sol.).
5. Add appropriate mAb if desired, and then 2 ml FACS® Brand Lysing Solution, (the staining works also with 1 ml of the lysing solution but a significant amount of erythrocytes will not be lysed).
6. Incubate 8—10 min at 37°C.
7. Stop the reaction by pipetting 2 ml cold PBS (≤4°C), (smaller volumes of cold PBS [e.g., 0.5 or 1 ml] can be added if this simplifies the following staining procedures). From now cells have to be stored on ice.
8. Perform the additional individual staining procedures (if desired).
9. At the end of the staining resuspend blood cells in about 100—200 µl of cold PBS and measure samples by flow cytometry (FL-1 channel, when FACScan is employed).

IMPORTANT: The samples have to be measured within three hours.

Detection of Caspase Activity with D_2Rhodamine in Adherent Cell Lines

Approximately 90 % confluent adherent cells in six well plates are washed once with 1 ml 1 x PBS and detached with 30 µl Trypsin-EDTA (Biochrome). (see notes 8 and 9.)

Cells are resuspended in 1 ml RPMI supplemented with 10 % FCS and centrifuged.

The cell pellet is then resuspended in 1 ml caspase-dye-solution (1 x PBS, 10 mM 2-mercapto-ethanol, 25 µM D_2Rhodamine). (Note that no osmotic shock is performed.)

Incubate cells for 8 to 10 min at 37°C.

After adding 5 ml 1 x PBS, cells are centrifuged. The supernatant is removed but 200 µl are left, in which the pellet is resuspended for flow cytometric analysis.

The cellular Rhodamine 110 green fluorescence (515—545 nm) is measured with excitation by a 488 nm argon laser on a FACScan flow cytometer in channel 1 (Becton Dickinson).[12] The fluorescence intensities of Rhodamine 110 measured in channel 1 and channel 2 are similar. A minimum of 10 000 events per sample are acquired, stored in listmode files and subsequently analysed with Cellquest˝ software (Becton Dickinson).

Alternatively, fluorescence can also be detected by confocal laser scanning microscopy.[13]

Detection of Caspase Activity in Cell Extracts

A number of caspase substrates have successfully been used for the detection of caspase activity in cellular extracts. Contrary to the detection of caspase activity in intact cells, fluorescent substrates used for measurement of caspase activity in cellular extracts do not need to be cell-permeable. This allows application of bigger, peptide based, caspase-sub-family-specific substrates like YVAD-AMC [N-acetyl-Tyr-Val-Ala-Asp-aminomethyl-coumarin] (caspase-1), VDVAD-AMC (caspase-2), DEVD-AMC (caspase-3), VEID-AMC (caspase-6), IETD-AMC (caspase-8), etc, as well as substrates based on other dyes. Unlike data from Western blot based detection of caspases, the data obtained by utilizing fluorometric- or colorymetric assays should be interpreted cautiously, since most if not all of these substrates are not absolutely specific for single caspases, they preferentially detect active members of a given caspase sub-family at best.

Materials and Equipment

1. CASPASE FLUORESCENT SUBSTRATES:
   DEVD-AMC (N-acetyl-Asâ˘Glu-Val-Asp-aminomethyl-coumarin) and other AMC-based substrates were obtained from Bachem, Heidelberg, Germany, a 66 mM stock solution was prepared in DMSO.
   D_2Rhodamine, a 50 mM stock solution was prepared in DMSO, prior to measurement 1 mM aliquots of D_2Rhodamine were prepared by diluting the stock solution with H_2O.
2. LYSIS BUFFER:
   20 mM HEPES pH 7.3
   84 mM KCl
   10 mM $MgCl_2$
   0.2 mM EDTA
   0.2 mM EGTA,
   0.5 % NP-40
   5 mM DTT
   (it can be supplemented with protease inhibitors: 5 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin)
   The buffer without DTT can be stored at RT, DTT should be added directly before use.
3. CASPASE BUFFER:
   50 mM HEPES pH 7.3
   100 mM NaCl
   0.1 % CHAPS (3-[cyclohexylamino]-1-propanesulfonic acid),
   10 mM DTT
   10 % Sacharose
   Prepare freshly before use; alternatively the solution without DTT can be stored in –20°C.
4. Bench-top centrifuge.
5. Spectrofluorometer, (see Note 2).
6. Water bath.

Methodology

1. Prepare ~10^6 cells per sample. Perform desired experimental procedures, (see Notes 1, 3, 10 and 11 for additional information).
2. At the end of an experiment, pellet cells in eppendorf tubes (ETs) by centrifugation at 1000 rpm, 4°C, for 10 min, in a bench-top centrifuge.
3. Discard supernatant, dry the pellets briefly by inverting the ETs on a paper towel (if necessary remove the rest of the liquid with a pipette or a piece of Whatman paper).
4. Lyse cells in 100 µl of lysis buffer for 10 min on ice, vortex samples vigorously about every two minutes.
5. Centrifuge extracts at 12 000 g for 20 min at 4°C and transfer supernatant into fresh ETs. Measure protein concentration (e.g., by BioRad protein assay according to the manufacturer's instruction), and adjust samples to same concentration with the lysis buffer, transfer 30 µl of the extract into a fresh ET and dilute it 1 : 10 with caspase assay buffer.
6. Add the dye (D_2Rhodamine to a final concentration of 10 µM–3 µl of 1 mM solution; or the DEVD-AMC to final concentration of 50 µM) and incubate for 10-20 min at 30°C.
7. In the meantime switch on the spectrofluorometer (the lamp needs usually 5–15 min to warm-up).
8. Terminate the reaction by placing samples on ice.
9. Transfer probes into 500 µl quartz cuvettes (glass cuvettes can be used if the excitation wavelength is 488 nm or longer) and measure in a spectrofluorometer (for D_2Rhodamine excitation: 488 nm; emission: 550 nm, for DEVD-AMC excitation: 360 nm, emission: 475 nm), if 1 ml cuvettes need to be used, samples can be diluted with 300 µl cold H_2O or PBS (600 µl of total volume is usually sufficient to fill 1 ml cuvette).

The blank cuvette should be filled with 30 µl lysis buffer, 270 µl caspase buffer, and 3 µl of 1 mM D_2Rhodamine, or 50 µM of DEVD-AMC respectively (do not forget to dilute the blank in case the experimental samples are diluted to a total volume of 600 µl).

Notes

1. D_2Rhodamine cannot distinguish between different caspases, if necessary DABCYL-xxxxxxx-EDANS -based substrates can be used where the caspase-specific sequence is placed between DABCYL and EDANS (i.e. DABCYL-DEVDAPK-EDANS for caspase-3 or DABCYL-YVADAPK-EDANS for caspase-1. For the design of new substrates the authors recommend previous publications.[14-17] 7-amino-4-trifluoromethyl-coumarin (AFC), or 7-Amino-4-methyl-coumarin (AMC) -based substrates have also been used successfully for detection of caspase activity in cell extracts,[8] and the appropriate kit can be obtained i.e. from Clontech, (Palo Alto CA).
2. The authors used spectrofluorometer Shimadzu RF-510 (Shimadzu Japan), equipped with an UV lamp.
3. It is advisable to stain each sample in duplicate and calculate the mean of two probes for the same sample.
4. Since the FSC / SSC signal will change slightly due to osmotic loading, for identification of specific blood cell sub-populations it is advisable to pre-stain a given cell population with antibody against a specific surface marker.
5. We have usually used 200 µl of blood per sample, but staining is also possible with 50 µl of blood. One can then either reduce the amount of used reagents or dilute the whole sample with PBS (room temp.) and follow the above protocol.
6. Inconsistency in the results can often be caused by carry-over of the dye on the external wall of the pipette tip.

7. To discriminate between effector and target cells: (i) FSC / SSC criteria can be used (which is quite difficult and not very precise); (ii) if cells differ significantly in their DNA content, Propidium Iodide or 7-amino-actinomycin-D (7-AAD) staining can be used; (iii) pre-staining with antibody against a specific surface marker for one of two cell populations can be used if it is sufficiently stable (please note that dead cells may stain unspecifically therefore target cells should be stained).
8. When staining adherent cells, it is better to pre-stain them for caspase activity in e.g., twelve-well-plate or a Petri dish, then scrape them off and then transfer to FACS-compatible tubes.
9. Staining of adherent cells is often less successful since detaching may cause spontaneous activation of caspases due to accompanying damage.
10. For best results start the staining procedure about 1 hours before typical apoptotic morphology is observed.
11. Some cell lines express large quantities of caspases, that can partially get activated during lysis, causing therefore high background. If the background signal is very high as compared to that one of the blank cuvette, it is recommended to, (i) shorten the incubation step at 30°C to 10 min or less, (ii) repeat the entire experiment by using ~2 x 10^5 cells, (iii) harvest cells earlier.

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CHAPTER 14

Caspases as Targets for Drug Development

Manuela Michalke, Anna Stepczynska, Malgorzata Burek, Truc Nguyen Bui, Karin Loser, Krzysztof Krzemieniecki and Marek Los

Abstract

Controlled cell proliferation, differentiation, activation and cell removal are the key events during the development and existence of multicellular organisms. Proliferating mammalian cells undertake a repeated sequence of DNA synthesis, mitosis, and cell division, a series of complicated processes that when going astray, may become deleterious not only to the particular cell, but also to the whole organism. Regulation and proper control of the cell cycle and of the programmed cell death (PCD, apoptosis) is therefore essential for mammalian development and tissue homeostasis. The molecular networks that regulate these processes are critical targets for drug development, gene therapy, and metabolic engineering. In this chapter we will focus on apoptotic pathways converging on caspase family proteases, summarizing "under development" pharmacological attempts towards genes, proteins, and intermolecular interactions presently known to control apoptosis. We also propose new potential molecular targets that may prove to be effective in controlling cell death in vivo.

Introduction

Programmed cell death is involved in almost every existence aspect of higher multicellular organisms. Multicellular animals developed controlled way of selective removal and replacement of their building blocks, a process tightly surveyed by the neighbor cells and intrinsic -mechanisms.[1,2] Caspases, the key effector molecules in apoptosis, together with a battery of triggers and regulators of their activity are among the most promising targets for pharmacological modulation of cell death. The search for caspase inhibitors was undertaken way before the discovery of these proteases as a key-effectors in apoptosis. The target of interest has been the interleukin-1β-converting enzyme (ICE, now caspase-1). Caspase-1, -4 and -5 are crucial regulators of secretion of inflammatory cytokines like IL-1ß, IL-16, IL-18 and indirectly IFN γ.[3,4] Therefore search programs focusing on single-caspase, or caspase-subfamily-specific inhibitors are pursued by a number of pharmaceutical companies (Table 1). In addition to caspases, modulators of their activity are also increasingly gaining the interest as potential targets for drug development. Among them the pro- and antiapoptotic Bcl-2 family members, especially the Bcl–2 death inhibitor itself, are amid the most frequent targets. In recent years a family of caspase inhibitors called IAPs that bind and inactivate already active caspases attracted attention of the pharmaceutical industry. The interest in IAPs increased with the discovery of IAP inhibitors, Smac/DIABLO and HtrA2, that allow an additional level of apoptosis modulation. Depending on the part of IAP which would become occupied by a designed inhibitor, the net outcome could be either caspase activation and apoptosis if the interaction with caspase is disrupted, or downregulation of caspase activity and apoptosis inhibition, if the interaction with Smac/DIABLO becomes disrupted.[5] Yet, another mechanism for apoptosis control can

Caspases—Their Role in Cell Death and Cell Survival, edited by Marek Los & Henning Walczak.

Table 1. Novel anticancer approaches, based on recent development of apoptosis research

Target	Approach	Brand or Code Name	Stage of Development	Company
Caspases	caspase inhibitor	IDN-5370	•protective towards apoptosis induction in cortical- and synaptic neurons •reduces infarct size in a rodent cardiac ischemia/reperfusion model, by more than 50%	Idun Pharmaceutical Inc.
Caspases	caspase inhibitor N-[(1,3-dimethylindole -2-carbonyl)] valinyl]- 3-amino-4-oxo-5- fluoropentanoic acid	IDN-1965	•ED50 by i.p. administration is 0.14 mg/kg, by i.v. administration is 0.04 mg/kg and by oral administration is 1.2 mg/kg •protects from anti-CD95-induced death and liver damage in murine system, (Hoglen et al, 2001, Pharmacol Exp Ther, 297:811-8 •increased survival in a Gaq-40 transgenic mouse model of heart failure (left ventricular hypertrophy, left ventricular dysfunction) •all treated animals showed improved fractional shortening and reduced left ventricular end-diastolic diameter compared with control, placebo-treated animals	Idun Pharmaceutical, Inc./ Mayo Foundation
Caspases	caspase inhibitor, preferences towards caspase-8		•animal study demonstrate protection of hepatocytes from TNF-, or galactosamine-induced apoptosis in a murine model (Jaeschke et al, 2000, Toxicol Appl Pharmacol, 169:77-83)	Maxim Pharmaceutical, Inc.
Caspases	caspase inhibitor	VX-799	•a potent small molecule caspase inhibitor •VX-799 was very effective in several animal models of bacterial sepsis •clinical trials in preparation	Vertex Pharmaceutical, Inc.
Caspases	caspase inhibitor	M-920 (L-826, 920)	•strongly (~80%) reduces mortality in a murine and rat sepsis model by preventing from sepsis-related apoptosis of B- and T- cells, (Hotchkiss et al, 2000, Nat Immunol, 496-501)	Merck Frosst Canada & Co.

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Table 1. Cont.

Target	Approach	Brand or Code Name	Stage of Development	Company
Caspase-3	highly selective caspase-3 inhibitor	M-791 (L-826, 791)	•strongly (~80%) reduces mortality in a murine and rat sepsis model by preventing from sepsis-related apoptosis of B- and T-cells, Hotchkiss et al, 2000, Nat Immunol, 496-501	Merck Frosst Canada & Co.
Caspase-3	selective activation of caspase-3		•caspase-3 zymogen is maintained in an inactive conformation by a regulatory triple-Asp-motif, so called "safety-catch" localized within a flexible loop near the large-subunit/small-subunit junction (Roy et al 2001, Proc Natl Acad Sci USA, 98:6132-6137) •the inhibitory mechanism depends on electrostatic interaction •screen for "small molecules" capable of disrupting the interaction is in progress	Merck Frosst Canada & Co.
Caspases	Peptide-based irreversible inhibitor		•in a rat model, a broad spectrum caspase inhibitor, zVADfmk (dose: 3 mg/kg, i.v.) when co-injected with endotoxin, completely prevented endotoxin-induced myocardial dysfunction evaluated at 4h and 14h following endotoxin challenge	INSERM, France, (non-profit, gov.-sponsored org.
Caspase-1, -4	Selective inhibitor originated from specific substrate peptide	pralnacasan VX-740, HMR-3480	•in a Type II collagen-induced rat rheumatoid arthritis model, pralnacasan is effective at 50 mg/kg, for over 60 days; well tolerated in animal models, (Randle et al, 2001, Expert Opin Investig Drugs, 10: 1207-1209) •encouraging results in phase I clinical studies, currently in phase II trials for rheumatoid arthritis treatment	Vertex Pharmaceutical Inc./Aventis Pharma AG

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Table 1. Cont.

Target	Approach	Brand or Code Name	Stage of Development	Company
Caspase-3	recombinant caspase-3 linked to an antibody		• recombinant caspase-3 linked to the antibody Herceptin (Genentech, Inc.) tested in animal tumor model	Immunex
Caspases	caspase activator	MX-2060	• "small molecule" caspase activator, a potential anticancer agent • tested in human cancer xenograft animal models	Maxim Pharmaceutical, Inc.
Bcl-2	antisense 18-mer-oligonucleotide (Phosphorothioate)	G-3139, Genasense	• very promising results in combination with a standard chemotherapy, (Chi et al, 2001, Clin Cancer Res, 7:3920-3927) • phase I/II studies of Genasense have demonstrated an excellent safety profile with toxicity observed in 20% of patients, fatigue in 10% and rash in 5%, the symptoms reverse upon withdrawal of treatment • in phase III trials for malignant melanoma, (Banerjee et al, 2001, Curr Opin Investig Drugs, 2:574-580)	Genta Inc.
Retinoid receptor-driven transcription, synergy with TRAIL	retinoic acid derivative: 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid	CD-437 AHPN	• mitochondria and caspase-3 dependent apoptosis • increases expression of Bad and down-regulates Bcl-2 expression • synergy effect between recombinant TRAIL and CD-437 observed in a number of cancer cell lines and in human tumor xenografts	Anderson Cancer Center, USA/CIRD Galderma
Survivin	antisense oligodeoxynucleotides		• following transfection of antisense oligonucleotides to mouse survivin mRNA, a time- and dose-dependent increase in polyploidy of approx. 2- to 3-fold and induction of apoptosis were observed in most of the tumor cell lines (Chen et al, 2000, Neoplasia, 2:235-241)	Isis Pharmaceuticals Abbott Laboratories
Smac/Diablo	exclusive rights patented		• exclusive rights to develop Smac-based therapy have been patented, • Smac inhibitor screening program have been started	Idun Pharmaceuticals

be applied. A number of cells express so called death receptors on the surface. They are able to activate caspases and induce apoptosis, when bound by appropriate ligand. A subfamily of caspases, termed apical/initiator caspases become activated upon enrollment to death inducing signaling complex (DISC), a multiprotein conglomerate recruited to death receptor within seconds, or minutes after its triggering.[6] Once activated, the initiator caspases trigger downstream/effector caspases and other components of the apoptotic machinery.[7,8] Modulation of interaction among DISC components, or triggering death receptors by naturally-occurring, or artificial ligand provides another mean of control of apoptotic process for clinical applications. Below we discuss in more details the progress, as well as positive and negative aspects of mentioned targets for drug development.

Modulation of Caspase Activity, Implications for Apoptosis and Cytokine Maturation

In lower eucaryotes, such as the worm *C. elegans*, caspases (*ced-3*) seems to be involved only in apoptosis. In higher eucaryotes, including mammals caspases form a large family comprising at least 12 members. Based on their differential substrate specificity, structural differences of their zymogens, preferred cellular localization, as well as known role in cellular processes, they can be divided into subfamilies with distinct role in cell (patho)physiology. Caspases are the key effector molecules in apoptosis. Their ability to proteolytically cleave selected cellular proteins, assures the progress and irreversibility of apoptosis.[8,9] The semi-hierarchical and partially-redundant organization of caspases (Fig. 1) guarantee strong amplification and rapid progression of the apoptotic process even if some family members are missing.[7-10] Some caspase family members including caspase-1, -4 and –5, are primarily involved in proteolytic activation of various important cytokines. Maturation-by-proteolysis of some key activatory cytokines like IL-1β, IL-16 and IL-18 allows their immediate secretion without the time-consuming process of de-novo synthesis. This way cells spare time, immediately mobilizing adequate immune response. Moreover, during viral attack, proteolytic signaling allows to mount a proper reaction under circumstances when shutting-off the cellular transcription and translation machinery is a powerful defense mechanism by itself. In addition to their well-established role in cell death and cytokine maturation, likely involvement in other crucial cellular processes, including activation, differentiation, and even in cell-cycle progression emerges (for a review see refs: 4 and 11). Although these areas of caspase action still largely await exact definition, they may be responsible for an unexpected results of caspase-based pharmacological approaches. Efforts are on the way to negatively- or positively modulate caspase activity for clinical purposes (Table 1). Discovery of drugs that selectively inhibit inflammatory-caspases (caspase-1, -4 and –5) may help to control some auto-immunoagressive diseases, like rheumatoid arthritis, as well as acute life threatening conditions such as sepsis. Inhibition of apoptotic caspases may be an approach of choice to slow-down, or even stop the progress of degenerative diseases like e.g., Alzheimer's disease or spinal lateral sclerosis. In contrast, selective activation of caspases, or at least lowering their activation-threshold, may be a powerful approach to combat cancer and eradicate some chronic viral infections. As indicated in the Table 1, caspases are by far the most popular targets for the development of drugs that should modulate the apoptotic process. A very interesting and potentially promising approach is followed by Merck (Merck Frosst, Canada). The discovery that caspase-3, the key effector caspase in apoptosis, is inhibited by an intramollecular electrostatic interaction, so called „safety-catch", a stretch of three aspartic acid molecules,[12] raises hope for rush development of small pharmacologically-active molecules capable of interfering with the electrostatic interaction, thus lowering the threshold of activation, or even activating the caspase. Comparable approach towards activation of caspases by "small molecules" is followed by Maxim Pharmaceuticals Inc (Table 1). Most of other attempts that are designed to directly activate caspases are in rather preliminary experimental stage. Significant advancement has been made by companies searching for specific caspase inhibitors. "Prove of principle" experimental data in animal models indicate that caspase inhibitors may

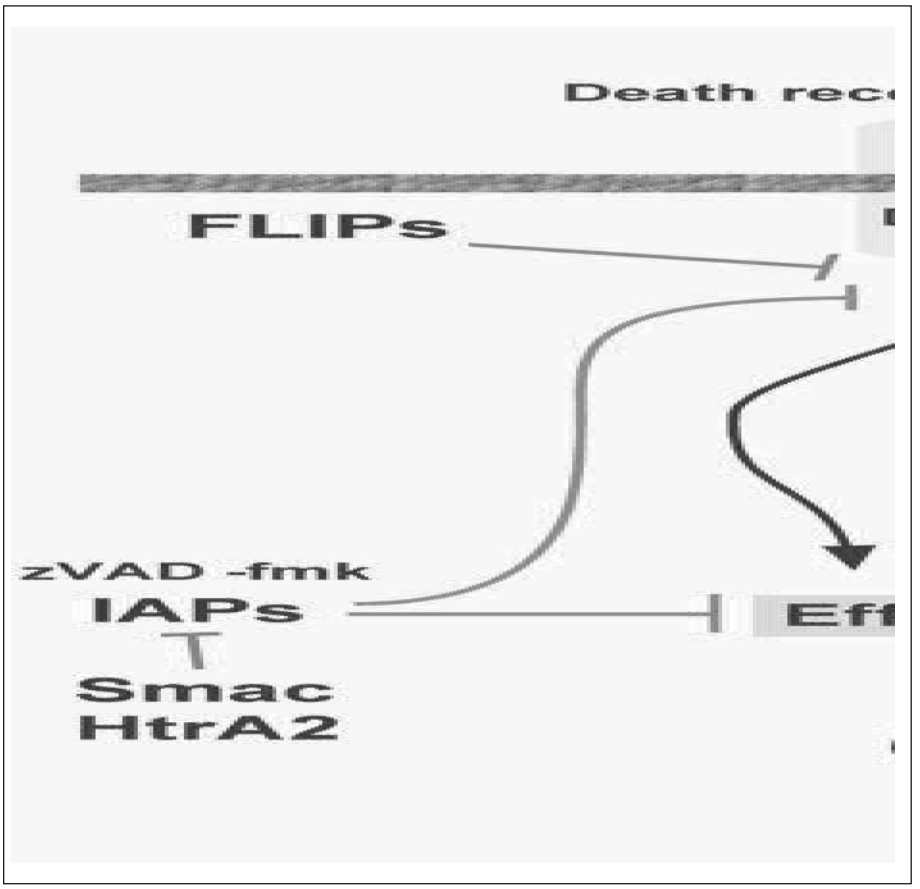

Figure 1. The principal cell death signaling pathways. Two best-characterized apoptotic pathways are indicated. Ligation of a death receptor leads to the recruitment of the adapter protein FADD and procaspase-8, which becomes cleaved and activated at the receptor complex, initiating caspase cascade. The mitochondria/apoptosome pathway (right) is triggered by a number of apoptotic stimuli. An early, not well understood step is the mitochondrial release of apoptosis inducing molecules (incl. cytochrome c, AIF, HtrA2 and Smac/DIABLO) into the cytosol. Initially, cytochrome c, together with dATP, associates with Apaf1. This event unmasks the CARD motif in Apaf1 and allows binding and activation of procaspase-9. Once activated, caspase-9 propagates the apoptotic signal. Positive feedback loops involving Bid, mitochondria, apoptosome, caspase-9, effector caspases and caspase –8 are able to amplify the death signal. The apoptosome pathway is further potentiated by AIF, through augmentation of mitochondrial release of cytochrome c and procaspase-9. Negative modulators of apoptosis such as FLIPs and IAPs may negatively influence the transmission of the apoptotic signal. Smac/DIABLO and HtrA2 may abolish apoptosis-inhibitory action of IAPs.

have therapeutic potential in the treatment of heart disease (Table 1), or stroke-related ischemia/reperfusion injury of the brain, liver and other organs.[13-16] The protective effect of caspase inhibitors in these circumstances can be at least in part related to the limitation of the inflammatory response by caspase inhibition.[17,18] Attempts to design inhibitors controlling the subfamily of inflammatory caspases have been made long before the role of caspases in apoptosis became obvious. Conducted later targeted disruption experiments in murine system fully confirmed the role of murine caspase-1 and –11 (the later is the murine homologue of

human caspase-4 and -5) as crucial for propagation of acute inflammatory response that relies on IL-1β and other cytokines.[19,20] caspase-1$^{(-/-)}$ mice had a major defect in the production of mature IL-1β and impaired IL-1α synthesis (Fig. 2). Secretion of TNF and IL-6 in response to LPS stimulation was also diminished in these targeted animals. In addition, macrophages from caspase-1$^{(-/-)}$ mice were defective in LPS-induced IFN-γ production,[21] and they were highly resistant to the lethal effects of endotoxin.[22] Almost identical phenotype was observed by caspase-11$^{(-/-)}$ mice.[23] The proinflammatory role of caspase-1 was strengthened by the finding that pharmacological blockade or genetic deletion of caspase-1 decreased necrosis, edema formation, and serum levels of amylase and lipase (both enzymes are indicators of pancreatic damage), during experimentally induced pancreatitis,[23] which was associated with dramatic survival benefits. Caspase inhibitors were also very protective in murine and rat experimental sepsis model (cecal ligation and puncture).[24] Application of either broad spectrum caspase inhibitor M-920, or the caspase-3 specific M-791 (both molecules synthesized by Merck) were equally protective. Both inhibitors protected 80—90 % of animals whereas only 10—20 % of control (solvent- or inactive molecule-treated) animals survived the experiment. The protective effect was likely due to the prevention of sepsis-related apoptosis of T- and B-cells that undergo massive apoptosis during sepsis.[25,26] Based on the striking phenotype similarity between caspase-1$^{(-/-)}$ and caspase-11$^{(-/-)}$, it has been proposed that caspase-1 is activated by a direct physical interaction with murine caspase-11.[27] However, as caspase-11 does not directly cleave either procaspase-1 or pro-IL-1β in vitro, likely yet to be discovered adapter/chaperon molecules may be required to assist this process in the cell. This hypothetical multiprotein complex, may appear to be an another promising target for selective modulation of the activity of inflammatory caspases without interference with the apoptotic cascade.

Modulation of the Mitochondrial Death Pathway: Pro- and Antiapoptotic Bcl-2 Family Members and Their Perspectives in the Clinic

Bcl-2 family proteins are important regulators of apoptosis.[28-31] The family comprises both antiapoptotic- (e.g., Bcl-2, Bcl-X_L) and proapoptotic proteins (e.g., Bax, Bid) with opposing biological functions; either inhibiting or promoting cell death (Fig. 1). Both subfamilies stay in equilibrium to each other in healthy cells. Antiapoptotic Bcl-2 family members inhibit apoptosis by blocking cytochrome c release from mitochondria,[32,33] thus preventing activation of the apoptosome pathway. In contrast, Bax, and truncated form of Bid induce cytochrome c release and caspase activation in vitro[34] and in vivo.[35,36] Overexpression of Bcl–2 could provide a survival advantage for cancer cells and it have been associated with increased frequency of lymphoma development in a murine model.[37] Loss of the proapoptotic protein Bax function seems to be important in the pathogenesis of colorectal cancers.[38]

Chemotherapy, radiation and most of other death stimuli induce cell death by triggering cytochrome c release from mitochondria and activation of caspases through the apoptosome pathway (Fig. 1). Bcl-2 prevents cytochrome c release, thereby it blocks cell death, it is a suitable target for the development of the anticancer therapy. Cells that upregulate expression of the *bcl-2* gene are significantly more resistant to a variety of noxious stimuli. Bcl-2 is frequently overexpressed in various malignancies, most commonly in a group of B-cell non-Hodgkin's lymphomas bearing t(14;18) chromosomal translocation. Thus, the antisense (AS) oligonucleotide, or phosphorothioate inhibition of Bcl-2 expression would shift the equilibrium in the cell towards proapoptotic family members. The AS approach shows high specificity of action for the selected target mRNA that is much higher than conventional small-molecule drugs.

The development of therapy targeting Bcl-2 expression is in the most advanced stage among all apoptosis-based newly-developed approaches. Genta Inc. has developed a series of AS sequences directed against different parts of the *bcl-2* gene that inhibit Bcl-2 expression to different degree. Genasense, (Table 1) the most promising AS phosphorothioate is a very specific towards Bcl-2 mRNA. Preclinical studies have shown that in human xenografts in a SCID

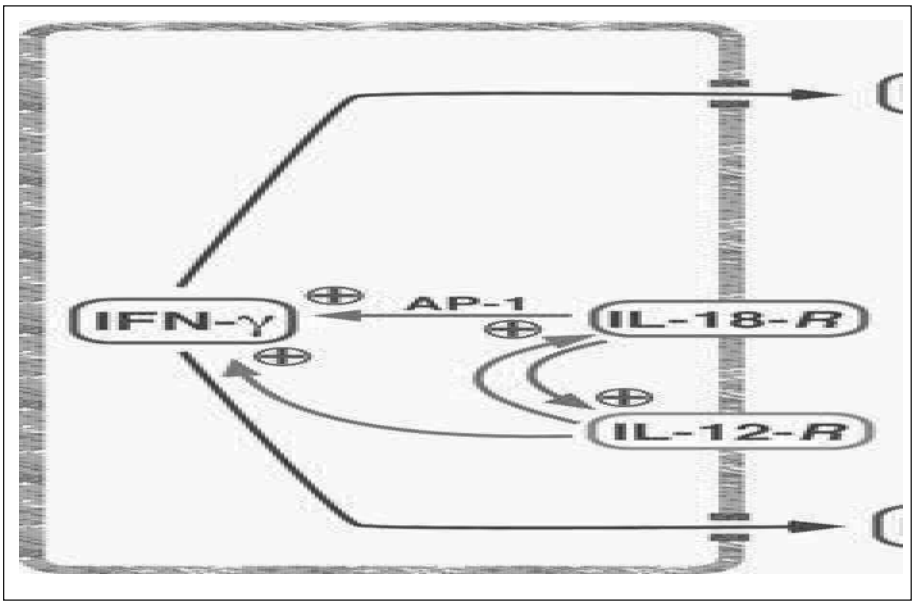

Figure 2. The role of caspase-1 and caspase-4, -5/11 in cytokine maturation, secretion and stimulatory events in the immune system. "+" next to arrowheads indicate stimulation on the transcriptional level; usually some components of the signalling pathway involved in the stimulation of transcription are also denoted (adapted from ref. 4). Please note LPS has been shown to increase the expression of caspase-4, data on LPS-regulated expression of caspase-5 and –11 are not available yet. "Scissors-like" signs indicate proteolysis. The " * " next to the arrow marking activation of caspase-4, -5/11 implies that the precise mechanism of this event is not known in details. The cross-talk between IL-12 and IFN-γ is not covered in detail in this chapter. More information regarding their regulation can be found in: Ma X et al, J Exp Med 1996; 183:147-157, Barbulescu K et al, J Immunol 1998; 160:3642-3647; Chang JT et al, Eur J Immunol 2000; 30:1113-1119.

mouse lymphoma model, Genasense compares favorably with Cytoxan, a drug used for lymphoma treatment. Combination of Genasense with Cytoxan markedly potentiates the efficacy of treatment. Similar results were obtained in another studies where Genasense was combined with Taxetir (currently the most effective drug for breast cancer treatment), to treat nude mice with xenografts of human breast cancer. Both Taxetir and Genasense were equally effective in extending the mean lifetime of mice, but the combination of both drugs led to full reversal of the tumor in all treated mice. The treated animals were cured and remained tumor free for at least 180 days, while controls died at around day 10. In a model of human melanoma xenografted into nude mice, administration of dacarbazine (DTIC) markedly decreased the size of tumors. Genasense in combination with DTIC was found to abolish tumors. Phase I and II clinical trials has demonstrated a biological response towards Genasense treatment. Most promising data were obtained on lymphoma therapy, where a sustained and complete reversal of the disease was demonstrated. A patient with an advanced stage of lymphoma exhibited complete remission after 18 months of treatment. Ex vivo examination of the patient material confirmed the elimination of the Bcl-2 protein 5 days after the begin of Genasense administration. Genasense also effectively decreased Bcl-2 protein expression in melanoma tumors. In combination with chemotherapy it was capable to induce partial remission of late-stage melanoma. In a study involving 25 patients with advanced-stage melanoma, with life expectancy <6 months, all patients responded to the combined therapy; treatment with Genasense increased life expectancy to ~17 months. In patients with acute myeloid leukemia treatment with Genasense

was also found to be promising. Treatment with Genasense for 5 to 7 days showed virtual elimination of Bcl-2, after which conventional chemotherapy was applied. This procedure resulted in complete remission in treated patients. The application of Genasense to treat bladder cancer resulted in a reduction in the size of the tumor after a single treatment of Genasense in a patient whose cancer was particularly resistant to chemotherapy. Phase I/II studies of Genasense have demonstrated an excellent safety profile with toxicity restricted to fever than 20 % of patients, fatigue in 10 % and rash in 5 %, (for a review see refs: 39 and 40). These are minor toxicities and are easily manageable and reverse quickly with the termination of treatment.

A significant limitation of Genasense and in general AS-based theropeutics is their inability to cross the blood-brain barrier. Thus brain methastases are inaccessible for this sort of therapy. In addition, the clinical studies on the predictive value of Bcl-2 family proteins in haematological malignancies or solid tumors (for a review see ref: 41) when taking into consideration the influence of Bcl-2 family members on cell proliferation. It has been observed that phosphorylated at the G2/M transition Bcl-2 delays the re-entry of resting NIH 3T3 cells into the cell cycle.[42] Moreover, Bcl-2 transgenic mice have impaired T cell proliferation, whereas transgenic overexpression of Bax accelerates cell cycle progression and apoptosis.[42,43] Cells overexpressing Bcl-2 also contain decreased levels of phosphorylated retinoblastoma protein, a key regulator of cell cycle progression.[44] Moreover, downregulation of Bcl-2 by AS approaches enhances proliferation of acute myeloid leukemia cells.[45] Finally, mutations that suppress the antiapoptotic activity of Bcl-2 also abolish the inhibitory effect on cell cycle transition. Thus the antiapoptotic activities of Bcl-2 may be linked and cell cycle suppressive activities of Bcl-2.[11,42,46] The careful evaluation of a large scale phase III clinical trials on Genasense and related AS-based approaches certainly will bring more light into the role of Bcl-2 family members in cell physiology and the efficacy of the respective AS therapy.

How to Control Already-Activated Caspases?—IAPs, Smac/DIABLO and Omi/HtrA2

The presence of activated caspases in a cell is not equivalent to the activation of the apoptotic process. Secretion of cytokines, or regulatory and effector functions of caspases during erythropoesis infers the presence of active caspases in cells under physiological conditions (for a review see refs: 4 and 11). It is the quantity and cellular localization of caspases that determines whether the cell will die.

IAPs (inhibitor of apoptosis proteins) are a family of proteins that contain BIR (baculoviral repeat) domains and in some cases, a zinc RING-finger domain.[47] The family members, X-linked IAP (XIAP), Livin/ML-IAP, cIAP-1 and cIAP-2, are believed to inhibit apoptosis through direct inhibition of caspases, although some of these proteins are also involved in additional signaling pathways.[48,49] XIAP, the most potent of these caspase inhibitors, selectively inhibits one of the active forms of caspase-9 (p35/p12 heterotetramer) through an interaction involving its BIR3 domain and the small subunit (p12) of caspase-9. In contrast, the BIR2 domain of XIAP, along with a few critical adjacent residues, is required to inhibit active caspases-3 and -7.[5,50,51] Consequently, XIAP is thought to interfere with death receptor-induced apoptosis by inhibiting effector caspases and mitochondrial-induced apoptosis by inhibiting both initiator and effector caspases.

The antiapoptotic activity of IAPs is subjected to regulation by a structural homologue of the Drosophila proteins, Reaper, Hid and Grim, has been identified, termed Smac/DIABLO.[52,53] This protein is normally localized to mitochondria, but like cytochrome c, is released into the cytosol during the early stages of apoptosis, where it promotes caspase activity by inhibiting IAPs, particularly XIAP. Smac does not resemble any protein with known function and represents a novel apoptosis regulator in mammalian cells. Although discovered quite recently Smac is among the most promising targets for tumor therapy. When overexpressed it sensitizes cells towards death stimuli,[54] potentially offering low side effects combined with reversal of

resistance towards cancer chemotherapeutica. Although the search for Smac inhibitors is in an early phase (Table 1), due to the relatively low molecular mass of 24 kD and relatively small interaction surface with IAPs the screen for a "small molecule" inhibitors has a great success chances.[55]

In contrast to Smac, the development of IAPs-based anticancer strategy may be more difficult. IAPs are the broadest caspase inhibitors in the cell, but due to their heterogeneity, and multiple caspase-inhibitory domains (eight human IAPs, containing in total 16 inhibitory domains, termed BIRs), it will be nearly impossible to target all of them with a single "small molecule" inhibitor. On the other hand, differential, tissue-specific expression pattern of IAPs may offer selective, tissue-specific modulation of caspase activity. For instance IAP-inhibiting molecules that do not target NAIP would spare central nervous system from adverse effects of cancer therapy. On the other hand, careful engineering of antisense molecules that target BIRs may reward in a "global" inhibitors of IAP expression.

Interestingly, another mitochondrial protein known as Omi/HtrA2 has just been identified which can bind XIAP.[56-59] HtrA2 is a serine protease whose mitochondrial targeting signal is proteolytically removed upon import into the mitochondrion to reveal an N-terminus conserved IAP (AVPS) binding site. During apoptosis HtrA2 is released from the mitochondrion and inhibits the function of XIAP in analogous manner to Smac. Binding of Smac/DIABLO, HtrA2 and perhaps other as yet unidentified proteins can antagonise the binding of XIAP to caspase-9 and thereby modulate the caspase cleavage activity of the apoptosome. The magnitude of the apoptotic stimulus as well as cellular levels of Smac, HtrA2, XIAP and other as yet unidentified proteins may contribute to the sensitivity of a particular cell type to apoptosis. Thus, analogically to Smac, HtrA2 appears to be a potential target for pharmacological modulation of apoptosis.

Much attention is currently being paid to survivin (Table 1), another IAP-family member that has been found to inhibit cell death by binding to caspases and the proapoptotic Smac.[52,60] Survivin is specifically induced in the G2/M phase and it appears to function both as a cell cycle regulator and apoptosis suppressor.[61] At the beginning of mitosis, survivin associates with microtubules of the mitotic spindle apparatus. Interestingly, caspase-3 and the CDK (cyclin-dependent kinase) inhibitor $p21^{Waf1}$ also colocalizes with survivin at the centrosomes. Interference with survivin function induces caspase-3 activity, apoptosis and produces a defect characterized by hyperploidy, multinucleation and supernumerary centrosomes.[61] However, the role of survivin as an apoptosis inhibitor has recently been challenged by some authors,[62] claiming that its role is restricted to mitosis. Indeed, survivin-like proteins that play a role exclusively in caryokinesis, have been identified in yeasts and *C. elegans*. Several of these genes show similar intron-exon structure, particularly around the BIR-encoding sequences.[63,64] Mouse embryos lacking survivin closely resemble the *C. elegans* BIR-$1^{(-/-)}$ phenotype. BIR-1 is the homologue of survivin, in *C. elegans*. In both species, the chromatin replicates, but cytokinesis is abnormal because cleavage furrows that begin to form are not completed. The phenotypes in both the mouse and *C. elegans* resemble those of embryos lacking INCENP (INner CENtromere Protein) homologues.[65] Furthermore, the localization of survivin homologues and INCENP homologues in the worm and in vertebrates is similar. These proteins localize to the centromeres until the metaphase-anaphase transition but then remain in the equatorial zone as the chromosomes separate, eventually localizing to the mid-body at telophase, after which they are degraded.[63,65] The function of the survivin and the INCENP-like proteins is conserved from yeast to vertebrates. These proteins are required to coordinate chromosome segregation with cytokinesis.[65]

Nevertheless, regardless if the survivin in addition to control of caryokinesis-related events, functions also as a caspase inhibitor, its downregulation may prove to be a new powerful anticancer tool. Initial experimens targeting survivin by specific ribozymes or with the antisense nucleotides, induced apoptosis in various cell lines or abolished cisplatin resistance.[66,67] Based on these promising results Isis Pharmaceuticals and Abbott Laboratories (Table 1) have launched the development of AS-based therapies targeting survivin.[67]

Receptor-Mediated PCD—Prospects and Limitations

Due to the experience of severe systemic toxicity of TNF and hepatotoxicity of CD95L/FasL in mice, these molecules are unlikely to be used in the clinic for cancer treatment. Therefore we focus on the third member of the TNF-related apoptosis inducing ligand family, TRAIL. This death ligand, known also as the Apo-2L, was cloned and preliminary characterized in mid-90s.[68] Unlike the other death receptor/ligand systems, the TRAIL appears to be more complex. Since the discovery of TRAIL, five receptors have been found to bind to TRAIL, and several intracellular checkpoints have been identified that regulate TRAIL sensitivity.[69,70] Numerous promising reports describe that TRAIL potently induces apoptosis in tumor or virally infected cells, but has little or no detectable cytotoxic effects on normal and non-transformed cells. Moreover, no overall toxicity was observed during different in vivo studies in mice and monkeys.[71,72] Preclinical safety studies in primates (cynomolgus monkeys) did not show adverse reactions even when substantial doses of recombinant TRAIL (10 mg/kg/day) were used.[72] The extreme liver toxicity (massive haemorrhagic liver necrosis) that has barred the in vivo testing of CD95L and TNF is not observed upon TRAIL treatment. Therefore, TRAIL is believed to be safe for use as anti-cancer agent without causing damage to nontransformed tissues.[73] From a therapeutic point of view, even more exciting is the finding that TRAIL induces apoptosis in a highly synergistic manner when combined with anticancer drugs or irradiation. The potentiation of cytotoxicity is especially observed in those tumor cells that are refractory to the treatment with either agent alone. The mechanisms which account for this potentiating effect may include the transcriptional induction of DR4 and DR5 TRAIL-receptors, the reduced expression of antiapoptotic molecules such as Bcl-2, Bcl-X_L or c-FLIP, and the upregulation of proteins with proapoptotic effects such as caspase-8 and FADD. A variety of malignancies, including common ones, like acute leukemia, breast cancer, colon cancer, lung cancer, melanoma and other malignant proliferative disorders refractory to standard treatment, regained sensitivity when co-treated with TRAIL.[74-79] What really determines the susceptibility of tumor cells and the resistance of nontransformed cells to TRAIL, is still largely unknown.

Another novel mechanism of action underlies the synergic effect of all-trans-retinoid-acid and its derivatives (Table 1). It has been shown that 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (code name: CD-437) not only synergizes with TRAIL, but furthermore it induces the expression of TRAIL, thus killing target cells in a TRAIL-autocrine or paracrine fashion.[80] The synergic effects of TRAIL and retinoids have been shown by a number of cancers including acute leukemia, lung- and prostate cancer.[78,80-82] Concerns have been raised after some primary cells, in particular hepatocytes, were found to be sensitive to TRAIL, however this is now considered to be dependent on the way the recombinant protein was engineered and also on the manner the primary cells were prepared. Although the data on TRAIL-clinical trials are not available yet, the bench- and animal experiments place this molecule among the most promising cancer-therapy approaches to be tested in the decade.

Perspectives

The development of "smart" cancer therapeutics, that selectively target only malignant cells will be one of the mayor challenges in cancer research during the 21st century. The exploitation of oncogene-directed treatment approaches targeting the mechanisms of uncontrolled proliferation will require prior better understanding of the numerous mechanisms underlying malignant diseases. Combinations of agents targeting different functions of a given oncoprotein complex, or different physiological processes, such as differentiation and apoptosis (CD-437, TRAIL, chemotherapeutica), are clearly bound to be more effective than single-agent protocols. Execution of clinical trials using multi-agent protocols without the necessity of testing them individually should help to bring effective therapies into the clinical setting at a faster rate.

The future of cancer therapy lays in treatment individualization and target selection. Doctors need to pick these patients who would benefit from a particular therapeutic approach.

DNA-chip based genetic diagnostic will allow proper identification of molecular targets that differentiate even among histologically-judged the same cancer. Malignancies with a common histologic origin and characteristics, need to receive an individualized therapy depending on the genetic trait of the particular group of cancer cells. Drugs that target molecular or genetic dearangements in tumors should be the primary goal for exploiting the knowledge of cancer biology that will continue to be elaborated in the coming decades. Prominent among the drug classes that will address this goal will be these targeting apoptotic pathways as well as other signal transduction cascades, which offer the potential for great activity with low toxicity. The final common pathway of tumorigenesis contains shared dearangements of cell cycle control, apoptosis, invasion and metastasis that will be relevant to all tumor types. More discrete, probably secondary changes are responsible for the individual clinical manifestation and they underlay the individual characteristic of a particular cancer case. Thus simultaneous targeting of both common- and individual characteristics of a given tumor will be the foundation of a successful clinical treatment.

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CHAPTER 15

Caspase-Independent Cell Death Mechanisms

Donat Kögel and Jochen H. M. Prehn

Introduction

Almost 30 years ago, Kerr and co-workers proposed the existence of an intrinsic cell death program and introduced the term apoptosis for the execution of this program.[1] Apoptosis is an active form of cell death enabling individual cells to commit suicide. In contrast, necrosis is a passive form of cell death induced by accidental damage of tissue and does not encompass activation of any specific cellular program during the death process. Initial classification of cell death into the apoptosis/necrosis dichotomy was mainly based on morphological criteria: hallmarks of apoptosis include membrane blebbing, cell shrinkage and chromatin condensation/fragmentation whereas necrosis typically is associated with early loss of plasma membrane integrity and swelling of the cell body. In recent years, significant progress has been made identifying key components of the apoptotic cell death machinery and deciphering the signaling pathways in which they are embedded. It is generally accepted that members of the caspase family of proteases are central executioners of apoptotic cell death (see other chapters of this book). For many years, apoptosis was thought to be a synonym for programmed cell death (PCD)*. However, an increasing number of studies substantiate the existence of caspase-independent forms of PCD. The initial model describing only one, stereotypical form of active cell death today is viewed as an oversimplification, because it is now generally accepted that multiple forms of PCD exist and that some forms of PCD do not require activation of caspases (Fig. 1). One single execution system, i.e., the caspases, could easily be overcome by viruses and transformed cells. Hence, alternative cell death pathways, acting as backup pathways, might have evolved during evolution.[2] This chapter will focus on the current cognoscenti of caspase-independent forms of PCD.

Multiple Forms of PCD

Although there is some controversy regarding the nomenclature of the different forms of PCD, in the majority of the current literature the term apoptosis in most cases is exclusively used for caspase-dependent cell death.[3] A broad classification of different PCD forms has recently been proposed by Leist and Jäättelä.[4] According to this classification which is based on both morphological and biochemical criteria, three different forms of PCD exist (Fig. 1) in addition to passive necrosis: 1) classical, caspase-dependent apoptosis associated with membrane blebbing, potent chromatin condensation/fragmentation, phosphatidylserine exposure, disruption of the cell into apoptotic bodies, activation of executioner caspases and internucleosomal DNA cleavage, 2) apoptosis-like PCD characterized by less compact chromatin condensation,

* The term programmed cell death was initially reserved for developmental cell death. It will be used interchangeably with active cell death in this chapter.

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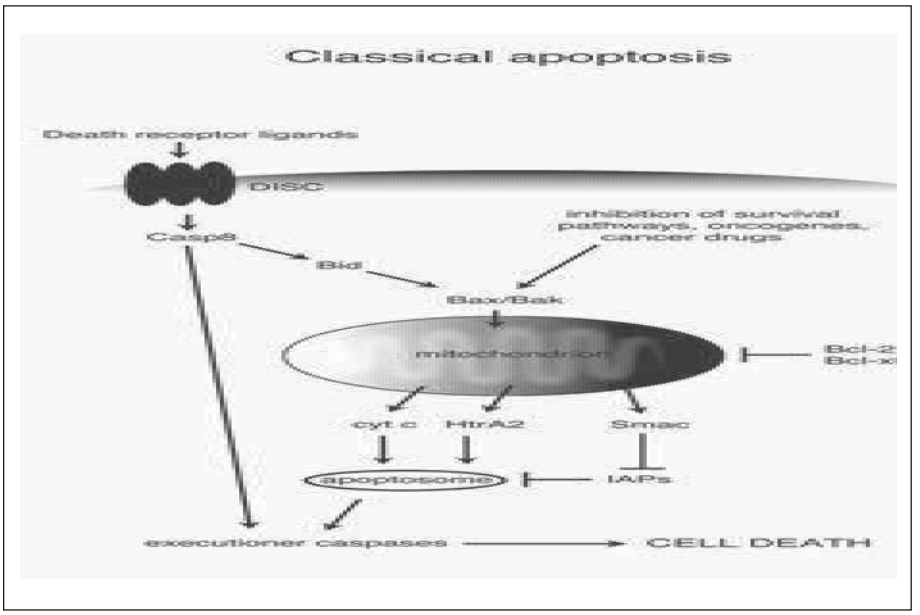

Figure 1. Proposed model for major cell death pathways implicated in apoptosis, apoptosis-like PCD and necrosis-like PCD. Classical, caspase-dependent apoptosis is activated by two major pathways: in the death receptor (extrinsic) pathway, executioner caspases are activated via the death receptor signaling complex (DISC) whereas the mitochondrial apoptosis pathway is triggered by activation of Bax and/or Bak. This is achieved by activation of BH3-only proteins like Bid, Bad and Bim. Both pathways are interconnected via caspase 8 (Casp8)-mediated cleavage of Bid. Signaling downstream of mitochondria comprises cytochrome c (cyt c)-mediated activation of the apoptosome, thus triggering effector caspase activation and execution of cell death. In addition, release of Smac and HtrA2 leads to sequestration of inhibitors of apoptosis (IAPs). Typically, apoptosis-like PCD occurs when the mitochondrial apoptosis pathway is activated via Bax and/or Bak in the absence of downstream caspase activation. In this case, caspase-independent mitochondrial death effectors apoptosis inducing factor (AIF), endonuclease G (endo G) and HtrA2 might trigger cell death by alternative mechanisms. Additionally, other proteases such as calpains and cathepsins might be involved in apoptosis-like PCD. Death receptor-activated necrosis-like PCD requires the enzymatic activity of the receptor interacting protein (RIP). Necrosis-like PCD is associated with mitochondrial permeability transition which leads to mitochondrial depolarization and increased mitochondrial ROS generation. However, cytochrome c is not released from the mitochondria and the classical mitochondrial apoptosis pathway is not activated. Necrosis-like PCD is inhibitable by antioxidants.

phosphatidylserine exposure, but absence of executioner caspase activation, and 3) necrosis-like PCD which occurs in the absence of both chromatin condensation and caspase activation.

In addition to this tripartite classification, there are more specialized forms of PCD not fitting into any of the three above models. These additional forms which are restricted to distinct cell types include paraptosis and dark cell death.[5,6] Yet another type of PCD is represented by autophagy, a process first described in yeast. In autophagy, which is characterized by prominent cytoplasmic vacuolization, cells are destroyed by degradation of cellular components via an autophagosomic-lysosomalpathway.[7] In this chapter, the biochemical features and genetic requirements for different forms of PCD will be discussed.

Caspase-Independent PCD Controlled by Bcl-2 Family Proteins

Activation of executioner caspases can occur after ligation of death receptors or via the release of proapoptotic factors from mitochondria. The latter pathway is controlled by Bcl-2

family proteins. In this pathway, activation of the initator caspase-9 occurs via binding of adaptor protein apoptotic protease activating factor-1 (Apaf-1) to the caspase recruitment domain (CARD). The association of caspase-9 and Apaf-1 and subsequent apoptosome formation is triggered by the proapoptotic factor cytochrome c. This factor resides in the mitochondrial intermembrane space where it participates in electron transport during respiration. During apoptosis, cytochrome c is released from the intermembrane space because of a significant increase in mitochondrial outer membrane permeability. This process is triggered and controlled by pro- and antiapoptotic Bcl-2 family proteins.[8] However, mitochondria as central integrators of PCD signaling pathways are able to release multiple factors that may trigger a caspase-independent cell death. Moreover, cell death may be caused by altered mitochondrial energetics directly due to the loss of cytochrome c.

Studies in Yeast

Caspase-independent cell death controlled by Bcl-2 family proteins and mitochondria has been initially studied in the fission yeast Schizosaccharomyces pombe, since the yeast genome does not contain any caspase genes. Nonetheless, overexpression of proapoptotic Bcl-2 family members Bax and Bak has been shown to cause cell death in *S. pombe*.[9] Bax- and Bak-induced cell death in yeast is associated with prominent cytosolic vacuolization and nuclear chromatin condensation. Furthermore, it can be inhibited by the anti-apoptotic counteraction of Bcl-X_L. This observation might indicate that the emergence of caspase-independent cell death pathways has occurred much earlier than classical caspase-dependent apoptosis during evolution. As a matter of fact, PCD has even been observed in bacteria.[10]

Studies in Genetically Altered Background

In concordance to the yeast model, overexpressed Bax and Bak are capable of inducing mitochondrial dysfunction-triggered death in cells lacking apoptosome-mediated caspase activation due to deficiency of caspase-3, caspase-9 or Apaf-1.[11] This mitochondrial step requires the activation of Bax and/or Bak, since Bax/Bak double knockouts are completely deficient to trigger the intrinsic apoptosis pathway.[11] Since it is well established that the major executioner caspase is caspase-3,[12] cells which lack expression of caspase-3 represent an often used experimental system to study the dependence of morphological and biochemical alterations on this particular caspase during cell death.[13,14] However, execution of cell death does not necessarily occur in caspase-independent fashion in cells devoid of caspase-3, since other executioner caspases such as caspase-6 and -7 might in part substitute for caspase-3 activity.[15,16]

Studies Using Caspase Inhibitors

Inhibition of the enzymatic activity is another widely used experimental approach to study the caspase-dependance of cell death. The employed caspase inhibitors are of either biological- (viral or cellular caspase inhibitors) or chemical-origin (synthetic peptide inhibitors). A myriad of experiments has been performed with various peptide inhibitors, either directed against individual caspases, or the zVAD-fmk broad-spectrum inhibitor. zVAD-fmk, the compound used in most studies, binds irreversibly to the catalytic site of caspases, forming a covalent inhibitor/enzyme complex. Generally, execution of cell death can be decelerated, but not prevented by abrogation of caspase activity.[17] It has been proposed that no single experimental system exists in which zVAD-fmk can save cells from dying.[18] This has been verified for multiple apoptotic stimuli[18] and both major cell death pathways, the death receptor pathway (see below) and the mitochondrial pathway.[17,19-21] These observations imply that in an individual cell receiving any given apoptotic death signal both caspase-dependent and caspase-independent cell death pathways are activated in parallel. In cells with inhibited caspase activity caspase-independent cell death mechanisms suffice to eventually cause cell death, albeit in a slower, less efficient manner.[17] Although active caspases are not a prerequisite for execution of cell death, the time frame and caspase-dependance of individual events during PCD might be

stimulus- and cell type-specific. For example, conflicting data exist on the dependance of membrane blebbing on caspase activity. Two recent reports describe caspase-dependent activation of serine/threonine kinase ROCK as critical step in initiation of membrane blebbing.[22,23] On the other hand, application of caspase inhibitors revealed that some of the early, cytoplasmic changes in apoptotic cell death do not depend on activation of caspases. In a seminal study by McCarthy and co-workers, cell shrinkage and membrane blebbing was not inhibited by abrogation of caspase activity with pan-caspases inhibitors after induction of cell death by a variety of stimuli including overexpression of oncogenes and induction of DNA damage.[17] In an alternative cell death pathway, members of the death-associated protein (DAP) kinase family might trigger membrane blebbing in caspase-independent fashion.[24] The catalytic domain of these kinases shares a high sequence homology to the myosin light chain kinase (MLCK). Of note, phosphorylation of myosin light chain (MLC) has been implicated in caspase-independent membrane blebbing.[25,26]

Although caspase-inhibitors can dramatically alter the death response of cells, the observed effects of these inhibitors must be discussed critically. Especially, it remains to be established if so called "pan-caspase inhibitors", such as the zVAD-fmk, really completely inhibit all proapoptotic caspases in the cell. The biggest drawbacks of the chemical caspase inhibitors are their limited stability and the gross differences in binding affinity to the individual caspase family members. In addition, other cysteine proteases such as calpains and cathepsins may also be inhibited by these compounds, thus aggravating interpretation of experimental data.

Mitochondrial Dysfunction Due to Loss of Cytochrome c

While many studies have focused on the role of cytochrome c release as the trigger of apoptosome activation,[27,28] loss of cytochrome c may also directly affect mitochondrial free radical and ATP production. Confocal time-lapse imaging experiments using cytochrome-c-GFP-expressing cells suggested that the release of cytochrome c during apoptosis is rapid and complete.[29,30] Cytochrome c normally transports electrons between mitochondrial complexes III and IV. A disruption of the mitochondrial electron flow caused by a significant loss of cytochrome c will maintain complex I and the ubiquinone at complex II in their reduced state. This condition may favor 1-electron reduction of molecular oxygen, presumably due to autooxidation processes.[31,32] This is also a potential mechanism for the known effect of complex III and IV inhibitors to increase the mitochondrial production of superoxide. Inhibition of mitochondrial electron flow and increased mitochondrial superoxide production secondary to cytochrome c release have been observed during Fas- and staurosporine-mediated apoptosis of Jurkat and HL60 cells.[33,34] Interestingly, cytochrome c release and superoxide production also occur at similar time points in the death cascade during trophic factor withdrawal- or staurosporine-induced apoptosis in neurons.[35-39] Moreover, in cell lines deficient in mitochondrial respiration (ρ^- cells), cytochrome c release and activation of apoptosis are preserved, while an increased superoxide production can not be detected.[39,40] Therefore, cytochrome c release may occur upstream of mitochondria-derived ROS production. The protective effects of antioxidants, SOD-mimetics, and superoxide dismutase overexpression in several apoptosis models suggest that the production of superoxide due to the loss of cytochrome c may play an important role in the execution of cell death, in particular in non-transformed cells.[35,37,39,41,42] In contrast, inhibition of executioner caspases reduces the biochemical and morphological signs of apoptosis, but does not necessarily inhibit cell death.[37,43,44] Therefore, mitochondrial superoxide production may significantly contribute to cell death during apoptosis, particularly in cell types that are sensitive to oxidant stress.

Mitochondria that have released their cytochrome c are likewise less capable of producing ATP. Mitochondria are able to maintain a mitochondrial membrane potential after the release of cytochrome c.[29,37,45] Evidence has been provided that this is caused by a reversal of the F_0F_1-ATPase operating in the reverse mode, hence even consuming ATP.[46,47] Re-addition of cytochrome c to isolated mitochondria that underwent an outer membrane permeability

increase likewise restores membrane potential and ATP production.[47,48] Evidence has also been provided that mitochondria are able to maintain their membrane potential in intact cells by diffusion of cytosolic released cytochrome c back to the mitochondrial inner membrane.[29,48] However, mitochondria will eventually depolarize after the release of cytochrome c, a process that is caspase-dependent in some systems.[45] Mitochondrial depolarization will lead to ATP depletion, followed by a disturbance of ion homeostasis, cellular Ca^{2+} overloading, and finally cellular necrosis. In cultured rat sympathetic neurons deprived of NGF in the presence of caspase inhibitors, cells can be rescued from cell death until the time point of mitochondrial depolarization.[49] Recent studies have shown that the opening of the permeability transition pore is involved in this final depolarization.[50]

Role of Other Proapoptotic Factors Released from Mitochondria in a Bcl-2-Dependent Manner

There are alternative signaling pathways leading to PCD-associated apoptotic events, such as degradation of chromosomal DNA. One of them is mediated by the apoptosis-inducing factor (AIF), a mitochondrial protein, that is released into the cytosol during execution of PCD (Table 1).[51] AIF, most likely the best studied gene product involved in caspase-independent cell death to date belongs to the gene family of oxidoreductases. However, the enzymatic activity of AIF is not required for its cell death-inducing properties. Apparently, upon activation of the intrinsic cell death pathway, both caspase-dependent (apoptosome/caspase-3/CAD/DFF-40) and caspase-independent (AIF) execution pathways can be triggered simultaneously, both leading to distinct nuclear events during PCD. In contrast to CAD/DFF–40, AIF induces large-scale DNA fragmentation, thus leading to chromatin condensation. Peripheral condensation of chromatin is an early nuclear event in classical apoptosis.[52,53] The DNA-degrading activity of AIF is caspase-independent as partial chromatinolysis and cell death caused by nuclear AIF is not inhibited by the presence of zVAD-fmk. In contrast, Bcl-2 overexpression inhibits AIF translocation from the mitochondria to the cytosol, thus abrogating AIF-triggered PCD.[52] Knockout studies revealed that AIF might control early morphogenesis during embryonal development.[54] The AIF gene of *Dictyostelium discoideum*, which has been recently identified,[55] is capable of inducing cell death. This suggests that AIF-based death pathway might be evolutionary older than the caspase-dependent death cascade.

Not only large-scale fragmentation, but internucleosomal DNA cleavage might also occur in caspase-independent fashion under certain circumstances. In CAD/DFF-40 knockout cells, DNA laddering, indicative of internucleosomal DNA cleavage, can be observed after induction of cell death, although to a lesser extent than in wild-type cells. Recently, a novel apoptotic DNase, the endonuclease G, capable of internucleosomal DNA processing was characterized. Just like AIF, endonuclease G is released from the mitochondria and translocates to the nucleus during PCD.[56]

Caspase-Independent Cell Death in Response to Death Receptor Signaling

Activation of the death receptor by binding of tumor necrosis factor-α (TNF) and Fas ligand (FasL) to their respective receptors can induce both classical apoptosis and necrosis-like PCD upon certain experimental conditions.[57,58] Furthermore, knockout studies revealed that necrosis-like PCD triggered by the extrinsic cell death pathway depends on both Fas-associated death domain (FADD)-mediated activation of the protein kinase receptor interacting protein (RIP). Interestingly, this type of cell death was shown to require the enzymatic activty of RIP which is dispensable for RIP-mediated activation of nuclear factor κB (NF-κB).[57] Although the molecular mechanisms of death receptor-mediated necrosis are poorly characterized, mitochondrial dysfunction[59,60] and non-caspase-proteases[61] seem to be critically involved in this process. In the presence of zVAD-fmk, death-receptor-mediated necrosis requires a mitochondrial step, although neither Bid cleavage, nor cytochrome c release are observed.[59,60] Instead,

Table 1.Genes implicated in caspase-independent cell death pathways

	Reference
Non-caspase Proteases	
calpains	69
serine proteases (HtrA2)	64, 68
cathepsins B	61
cathepsin D	70
granzyme A	65
granzyme B	67
Bcl-2 family members	
BNIP3	63
Mitochondrial death effectors	
apoptosis-inducing factor (AIF)	51
endonuclease G	56
HtrA2	
Protein kinases	
receptor-interacting protein (RIP)	57
apoptosis signal-regulating kinase (ASK1)	4
Jun N-terminal kinase (JNK)	4
Transcriptional regulators	
c-myc	81
Bin1	85
PML	88
Others	
Daxx	4
Ras	80
E4orf4	4
Src	4

this type of necrosis-like PCD is associated with increased production of ROS by the mitochondria.[57-59] ROS are released from the mitochondria during TNF-induced PCD, and antioxidants inhibit this form of cell death.[59,62]

Necrosis Controlled by Bcl-2 Family Members

BNIP3, a member of the Bcl-2 family and direct interaction partner of Bcl-2, induces necrosis-like cell death through mitochondrial permeability transition. Cell death triggered by BNIP3 is associated with translocation of BNIP3 to the outer mitochondrial membrane, loss of mitochondrial membrane potential and increased production of reactive oxygen species (ROS). However, BNIP3-mediated cell death is independent of Apaf-1, caspase activation and cytochrome c release.[63] It has been suggested that Bcl-2 exerts its anti-necrotic effect by complex formation with BNIP3.[60]

Involvement of Non-Caspase Proteases in PCD

In addition to caspases, other proteases such as serine proteases, cathepsins and calpains might be involved in PCD as well.[18] The general serine protease inhibitor AEBSF has been shown to inhibit oncogene-driven PCD in rat fibroblasts.[64] Granzymes A and B have been implicated in caspase-independent cell death pathways triggered by granule-mediated cytotoxicity of T lymphocytes. Granzyme B triggers the intrinsic cell death pathway via truncation of Bid in a caspase-independent cleavage event upstream of mitochondria.[65] However, granzyme B-induced cell death is significantly delayed by abrogation of caspase activity.[66] In contrast, granzyme A triggers caspase-independent cell death by activating the endonuclease granzyme A-activated DNase (GAAD), leading to single strand DNA nicking and chromatin condensation.[67] Another recently identified serine protease, HtrA2, which is released from the mitochondria during PCD, activates both caspase-dependent and caspase-independent cell death pathways. Caspase-independent cell death triggered by HtrA2 depends on its enzymatic activity.[68] Two members of the cathepsin family, cathepsin B and D, lysosomal proteins, have been suggested to translocate to the cytoplasm during PCD.[69] Under certain conditions cathepsin B can become the dominant execution protease in death receptor-induced PCD.[61] The PCD-related role of an another cathepsin family member, cathepsin D, has also been recently described.[70] Similiar to granzyme B, cathepsins have been implicated in cleavage and activation of Bid.[71]

Elevated cellular Ca^{2+} concentration during apoptosis, e.g., following mitochondrial dysfunction, may lead to the activation of PCD-related, Ca^{2+}-dependent enzymes, such as calpains or death associated protein (DAP) kinase. Like caspases, calpains are a family of cytosolic cysteine proteases, but require Ca^{2+} for their activity. Activation of calpains can be amplified by caspase cleavage of the endogenous calpain inhibitor calpastatin.[72] Calpains have been suggested to be involved in the regulation of caspase activity during apoptosis. The cleavage of upstream caspases-9 and -8, as well as executioner caspases-3 and -7 by calpains have been described. Calpain-cleaved procaspases-3 and -9 could still be activated by granzyme B.[73] In a study by Ruiz-Vela and co-workers,[74] calpain I-mediated proteolysis of procaspase-7 led to its activation. ER stress-induced apoptosis mediated via murine caspase-12 has also been shown to require calpain activation.[75] On the other hand, several reports support the role of calpains as negative regulators of caspase activity.[76,77] Calpain-generated fragments of caspases-7, -8 and -9 were inactive and/or unable to activate downstream executioner caspases, and calpain potently inhibited the ability of cytochrome c to activate executioner caspases. A recent study also demonstrated calpain-dependent cleavage of the cytochrome c-binding protein Apaf-1.[78] It is therefore conceivable that the upstream or concomitant activation of calpains exerts a negative feed-back signal on caspase activation.

A significant number of studies revealed that specific calpain inhibitors can inhibit PCD in many cases.[69] Interestingly, calpains promote apoptosis-like events during platelet activation and excitotoxic neuron death,[73,76] including chromatin condensation, phosphatidylserine exposure, caspase substrate cleavage and cell shrinkage, thus mimicking aspects of caspase-mediated apoptosis. Hence, calpains are among candidates for the execution of apoptosis-like PCD.

Oncogenic Transformation: Escape from PCD

One of the fundamental functions of PCD is to protect higher organisms from cancer. A number of oncogenes, including c-Myc, E2F and Ras have been shown to induce PCD upon overexpression in non-transformed cells, although the mechanism of oncogene-driven PCD remains elusive.[79] Importantly, both caspase-dependent and caspase-independent cell death mechanisms must be evaded by tumor cells during malignant transformation. A number of oncogene-driven caspase-independent forms of PCD have been described. Oncogenic Ras induces a caspase-independent and Bcl-2-insensitive form of PCD in human cancer cells.[80] In contrast, c–Myc triggers both caspase-dependent and caspase-independent cell death pathways.[81] Inhibition of PCD by tumor cells is achieved by either enhancement of anti-apoptotic

signaling pathways or inactivation of tumor suppressor genes. Survivin has been shown to protect tumor cells from both classical apoptosis and caspase-independent PCD.[82] A constitutively active mutant of Akt/protein kinase B(PKB) has been recently implicated in suppression of caspase-independent PCD.[83] In this study, ceramide-triggered cell death occurred in the presence of both zVAD-fmk and overexpressed Bcl-X_L in glioma cells. However, this type of PCD could be counteracted by the dominant-active Akt/PKB mutant.[83] Loss of function of tumor suppressors PML and Bin-1 might be implicated in abrogation of caspase-independent cell death.[84,85] The tumor suppressor PML is involved in cell death induced by a wide variety of stimuli known to activate classical caspase-dependent apoptosis.[86] In addition, a p53-coactivator function of PML has been recently established.[87] However, PML-triggered cell death does not require activation of caspases as zVAD-fmk may even enhance cell death induced by PML.[88] caspase-independent signaling by c-Myc seems to require Bin-1.[85] Similar to mutant Ras, PCD induced by Bin-1-overexpression cannot be rescued by zVAD-fmk or Bcl-2. However, Bin-1-triggered DNA degradation is abrogated by inhibition of serine proteases.[85] Execution of oncogene-driven caspase-independent cell death likely involves other non-caspase proteases, such as cathepsins and calpains.

Outlook

The discovery of alternative, caspase-independent cell death pathways increases our understanding of the evolution of PCD mechanisms, but also demands the search for new strategies for the treatment of disorders associated with a deregulation of PCD, such as cancer, ischemic and degenerative diseases. Apart from agents that inhibit the activity of caspases potential targets for future drug development are the Bcl-2 family proteins. New anticancer drugs that facilitate mitochondrial outer membrane permeability may help to modulate death pathways within the cell. Several novel cancer drugs activating caspase-independent death programs in tumor cells has already been described.[4] On the other hand, inhibitors of caspases, calpains, and cathepsins, but also antioxidants may prove beneficial for the treatment of ischemic and degenerative disorders involving a PCD component. Further experiments and clinical trials will reveal the effectiveness of these innovative therapies.

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