1. Introduction

1.1 Programmed Cell Death

Life requires death. Multicellular organisms eliminate redundant, damaged or infected cells by a stereotypic program of cell suicide termed apoptosis. Apoptosis is an essential physiological process involved in a variety of biological events. Examples of physiological apoptosis include the hormonally regulated involution of the tadpole tail during development, negative selection of lymphocytes to delete autoreactive or non-reactive cells, widespread cell death of neuronal cells during the self-assembly of the central nervous system, and the formation of digits by involution of interdigital cells in the primitive limb paddle [1-3].

Severe disturbance of homeostasis of any particular cell population or lineage can cause major pathologies in multicellular organisms. Not surprisingly, therefore, substantial evidence indicates that alterations in control of cell death/survival contribute to the pathogenesis of many human diseases. Diseases linked with suppression of apoptosis include cancer, autoimmune disorders (e.g. systemic lupus erythematosus) and viral infections (e.g. herpes viruses, poxviruses, adenoviruses); diseases in which increased apoptosis is an element include AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury (e.g. stroke, myocardial infarction), toxin-induced liver disease (e.g. alcohol) and some autoimmune disorders [4-5].
Apoptosis was first described by Kerr et al. in 1972, and is defined by the morphological appearance of the dying cell, which includes blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells), and cell shrinkage [6]. Biochemical features of apoptosis include internucleosomal cleavage of DNA, leading to an oligonucleosomal "ladder" ; phosphatidylserine (PS) externalization; and proteolytic cleavage of a number of intracellular substrates. As a result of the plasma membrane changes, apoptotic cells are rapidly phagocytosed prior to the release of intracellular contents and without induction of an inflammatory response. [7-8]

Genetic analysis have identified four genes that act sequentially to control the onset of apoptosis in ***C. elegans*** : Among these four components, **CED-3** (**ce**ll death **d**efective) is a member of a class of cysteine proteases that C-terminally cleave after aspartate residues (**caspases**). Activation of **CED-3** is likely to be mediated by an adaptor protein **CED-4** . The other gene, **CED-9**, acts to antagonize the killing activity of **CED-3** and **-4**[9-10]. The negative regulation of **CED-3** can be removed by **EGL-1** [11]. During ***C. elegans***

development, the interplay among **CED-3, CED-4, CED-9** and **EGL-1** results in the death of 131 cells at precise times and in precise locations [12].

In mammalian cells, although 14 other members are now known, **Caspase-9** have been identified as the homologue of *C.elegans* **CED-3** protein. The **CED-9** protein was found to be homologue to mammalian **Bcl-2** family proteins (antiapoptotic members, especially **Bcl-2** and **Bcl-X$_L$**). In addition **EGL-1** has been characterized as a homologous to **Bid** and **Bad**, proapoptotic members of Bcl-2 family proteins. A mammalian **CED-4** homologue **Apaf-1** (apoptotic protease activating factor), have been also identified (Fig. 1.1.), [13-15]. All these studies revealed that the core of this cell suicide program is, evolutionarily conserved from worm to human.

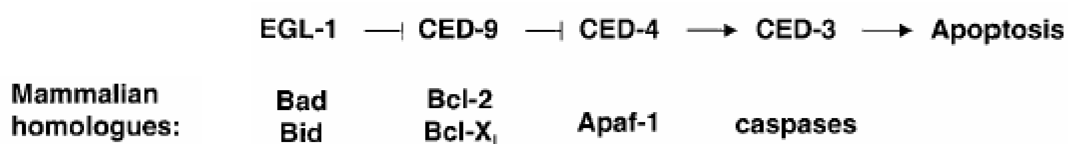

Fig. 1.1. *C.elegans* apoptotic proteins and mammalian homologues

During the last decade, tremendous progress has been made in understanding apoptosis. At the molecular level apoptosis is found to be a tightly regulated process and mainly driven by the caspase cascade.The term caspases stands for cysteinyl aspartate specific protease as showed in (Fig1.2.), [13-14].

1.2 Caspases

Caspases play an essential role during apoptotic cell death. These enzymes define a new class of cysteine proteases which is a multi-gene family with more than a dozen distinct mammalian family members. [15]

To date, 14 caspases have been identified in mammals. Of these, eleven have been cloned in human, with caspases-11, -12 and -14 only conclusively identified in the mouse thus far (Fig. 1.3.), [15-17] Multiple caspases are also present in other organisms; four have been identified in Drosophila melanogaster, at least two active caspases have been found in Caenorhabditis elegans, two have been found in Xenopus laevis, and one in the insect Spodoptera frugiperda.[18, 19]

Caspase cysteinyl aspartate-specific proteinase
cysteinyl aspartate-specific proteinase

Fig 1.2. Origin of Caspase nomenclature (from [20]).

Caspase family and functional subfamilies

A phylogenetic analysis indicates that the gene family is composed of two major sub-families: the mammalian counterparts of CED-3 ; those that are thought to be centrally involved in cell death (caspases -2, -3, -6, -7, -8, -9, and -10) and those most closely-related to caspase-1(ICE) whose primary role seems to be cytokine processing (caspases -1, -4, -5 and -11), (Fig. 1.3.), [20-21]. At present insufficient data available to enable caspases -13 and -14 to be categorized. However these caspases demonstrate higher degree of sequence similarity to caspase-1. Although in terms of its primary structure, caspase -12 also appears to be related to the cytokine processing subfamily, recently it has been found that caspase-12 is involved in endoplasmic reticulum (ER) stress induced apoptosis. [112-114]

Alternatively, these proteases can be subdivided on the basis of their substrate specificities which has been defined by a positional scanning combinatorial substrate library [22, 23]. To this approach, the proteases fall into only three specificity subgroups. Group I caspases (caspase-1, -4, and -5) cleave at (W/L)EHD tetrapeptide motifs and are probably involved in cytokine activation. Apoptotic caspases can be further subdivide into two groups as effectors and initiators [29]. Group II, (effector) caspases include -3, and -7 (and also CED-3), and cleave at DEXD tetrapeptide motif. Caspase-6 is also included in the effector caspases which cleaves at VEXD tetrapeptide motif. These are believed to be the main executioner of apoptotic process. [30]. Group III (activator) caspases include caspase-2, -8, -9 and -10 and cleave at (I/V/L)EXD tetrapeptide sequences (Fig.1.3.). There are two exceptions to this general ordering based on the substrate specificity, which remain unresolved, are caspase-6 and caspase-2 which can also play an initiator or effector role, respectively. Generally initiator caspases act upstream of effectors to activate them [22-23].

Caspases recognize a tetrameric primary (P4-P1) sequence in their substrates with a distinctive requirement for an aspartic acid residue in the substrate P1 position. Group I caspases (1, 4, 5, 13) are tolerant of liberal substitutions in P4 but prefer bulky hydrophobic amino acids such as Tyr or Trp. This preference is found to be consistent with their role in cytokine processing. The group II caspases (2, 3, 7) are substantially more stringent, requiring an Asp in P4. This specificity and stringency is found to be nearly indistinguishable from that

of C. elegans CED-3. The preferred cleavage motif (DEXD) for group II caspases appears in many proteins that are cleaved during cell death, consistent with group II caspases being the major effectors of cell death. Group III caspases (6, 8, 9, 10), on the other hand, prefer branched chain aliphatic amino acids in P4; residues that are found at the maturation site of most group II and group III caspases. This specificity is also found to be consistent with the group III enzymes being upstream activators of the group II effector caspases [20,22-25].

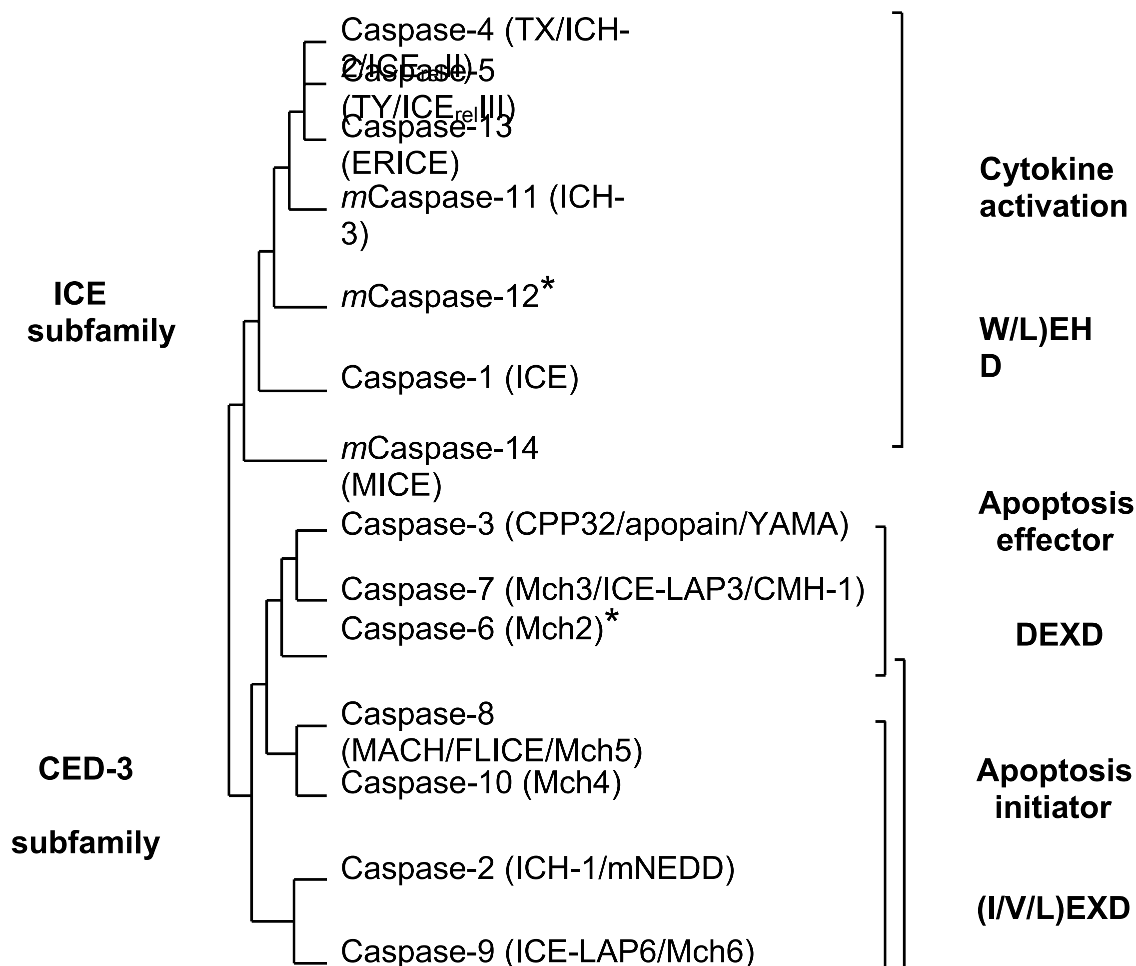

Fig. 1.3. Phylogenetic scheme of the caspase subfamilies[20]. * Substrate specificity of caspase-12 is not determined. Caspase-6 cleaves at VEXD tetrapeptide motif.

Structural features of caspases

Caspases share similarities in amino acid sequence, structure, and substrate specificity. They are all expressed as proenzymes (30-50 kD) that contain three domains : an NH_2 terminal domain, a large subunit (20 kD) containing the active site cysteine within a conserved **QAC*X*G** motif, and a C-terminal small subunit (10 kD) (Fig. 1.4.), [14,15,20].

An aspartate cleavage site is present between the prodomain and the large subunit, and an interdomain linker containing one or two aspartate cleavage site is present between the large and small subunits. Activation accompanies proteolysis of the interdomain linker and usually results in subsequent removal of the prodomain. The active enzymes function as tetramers,

consisting of two large/small subunit heterodimers (17-20), (Fig. 1.4.). The heterodimers each contain an active site composed of residues from both the small and large subunits.

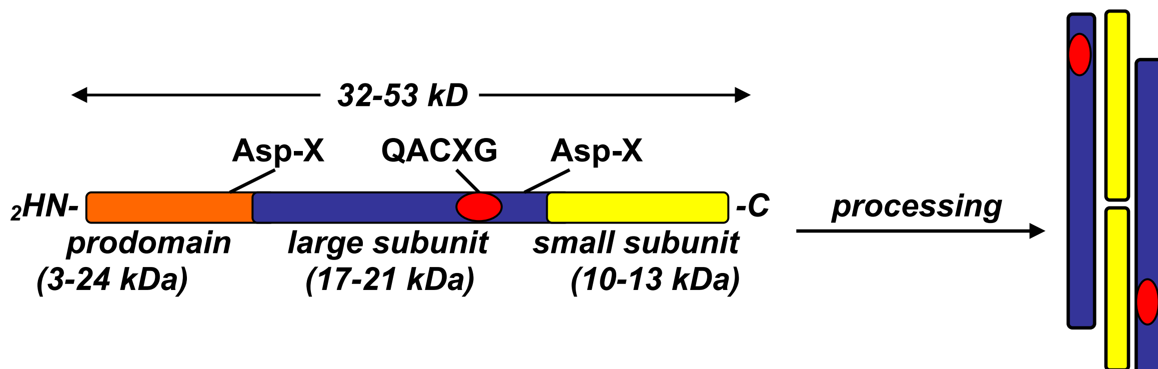

Figure 1.4. Caspase proenzyme organization and proteolytic activation

The presence of Asp at the maturation cleavage sites allows caspases to auto-activate or to be activated by other caspases as part of an amplification cascade.

Table 1.1. Caspase prodomains vary in length and sequence [15].

Zymogen	Prodomain and motif	Active subunits
kDa		*kDa*
Apoptotic initiators		
Caspase-2 (51)	Long, CARD	20/12
Caspase-8 (55)	Long, DED	18/11
Caspase-9 (45)	Long, CARD	17/10
Caspase-10 (55)	Long, DED	17/12
Apoptotic executioners		
Caspase-3 (32)	Short	17/12
Caspase-6 (34)	Short	18/11
Caspase-7 (35)	Short	20/12
Cytokine processors		
Caspase-1 (45)	Long, CARD	20/10
Caspase-4 (43)	Long, CARD	20/10
Caspase-5 (48)	Long	20/10
mCaspase-11 (42)	Long	20/10
mCaspase-12 (50)	Long, CARD	37/10
Caspase-13 (43)	Long	20/10
mCaspase-14 (30)	Short	20/10
Invertebrate caspases		
CED-3 (56)	Long, CARD	17/14

Prodomain structures vary considerably between different caspase family members ranging from small peptides with unknown (if any) function (e.g. caspases-3, -6, -7) to large domains that contain sequence motifs such as CARD (caspase activation and recruitment domain) and

DED (death effector domain) which are involved in recruitment-activation (e.g. caspases-2, -8, -9, -10). (Table 1.1.), [14-15].

Quaternary structure and mechanism of procaspase activation

The first crystal structures of inhibitor bound active caspase-1 [29-30], and later the homologous structures of either inhibitor or substrate bound active caspases -3, -7, -8 [28-33] and recently the crystal structures of procaspase-7, and active capsase-7 without any bound cofactors [34-36] have been revealed that both active and zymogen caspases are composed of two catalytic subunits (a heterotetramer consisting of two large and two small subunits) each containing six-stranded β-sheet and five α-helices. Four surface loops (L1-L4) extending from these core structural elements, are forming a potential catalytic site (Fig. 1.6.A), [28-30]. The procaspase-7 zymogen comprises two closely associated heterodimers (with a large and a small subunit). Based on previous structural studies two models have been proposed to explain the mechanism of procaspase activation [28]. In the first model (the process and association model) each heterodimer (p20p10) was proposed to be derived from the same single polypeptide. Following cleavage from interdomain linkage, an extensive reordering of the proenzyme takes place thus the p20 C-terminus and the p10 N-terminus pairs in a cis position (distant from each other) to form an active site. In contrast, the second model (the association and process model) proposed that two molecules of procaspase associate and interdigitate, naturally forming a caspase-like intermediate and then the interdomain linker and prodomain are removed to form a mature proenzyme. Indeed crystal structure studies of procaspase and active caspase-7 confirmed the latter model. The N-terminus of the small subunit (L2' loop) in one heterodimer traverses along the interface between to heterodimers and comes close to the C-terminus of the large subunit (L2 loop) in the same heterodimer [34-36]. This observation strongly suggested that each heterodimer is derived from a single contiguous polypeptide. The same study on caspase-7 has been also revealed the differences between the structure of procaspase and active caspase-7 and thus, allowed us to understand the procaspase inactivity and the conformational changes leading to procaspase activation [34]. Compared to active caspase-7 without any bound cofactors, the structure of the zymogen shows significant differences (Fig1.6.A-C). The conformation of active site loops of procaspase-7, particularly that of L2' and L2 loops, is ineffective for substrate/inhibitor binding. The underlying restriction is found to be the covalent linkage in the interdomain region that prevents the formation of a productive conformation.

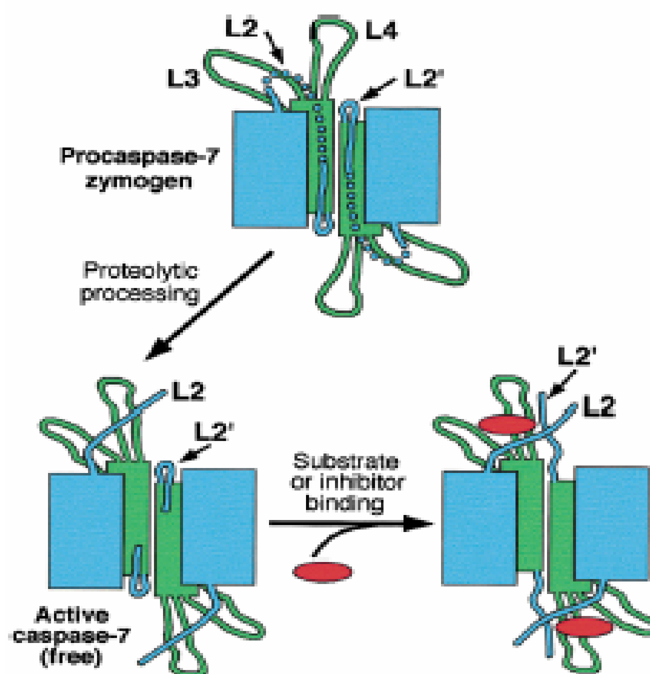

Fig1.5. Shematic diagram of caspase activation and substrate/inhibitor binding [34].

Cleavage after Asp^{403} leads to some rearrangements in loops L2, L3, L4, which adopt conformations that are similar to those in the inhibitor/substrate bound caspase-7 and produces a free N-terminus in the small subunit (numbering of the residues given according to Fig. 1.7) (Fig1.5.). These changes make the active caspase-7 ready for substrate/inhibitor binding. The critical L2' loop, however keeps the closed conformation (presumably in a lower energy state). Inhibitor substrate binding removes the energy barrier and induces the flipping of the L2' loop. [34-37], (Fig. 1.5.). Sequence alignments have been revealed that important residues such as Asp^{398} on L2 and Pro^{424} on L2' (for loop formation, or maintenance of the loop in a productive conformation, or stabilization of the loop), are generally conserved among the other caspases [28-34]. These observation indicating that the activation mechanism observed for caspase-7 likely true for the other caspases, such as caspases-3 and -6.

Another study has revealed the structural differences of caspase-7 and caspase-9. Because the procaspase-9 exhibits basal level of activity in the absence of any proteolytic cleavage, there should be differences. According to a recent study, compared to caspase-7, caspase-9 has been found to contain an expanded L2 loop which may allow enough conformational flexibility such that caspase-9 does not need an interdomain cleavage. Association of caspase-9 lead to an activation to so that it can exhibit basal level of activity in the absence of any proteolytic cleavage [38-39].

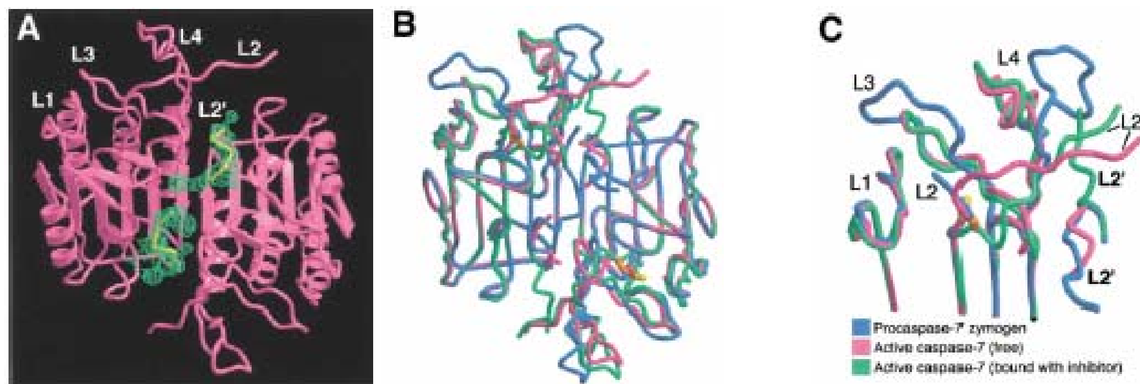

Fig.1.6. Structural comparison of the unprocessed procaspase-7, and isolated active caspase-7 without any bound cofactors. **A)** Overall structure of active caspase-7, the four loops that define the catalytic groove are indicated **B)** Superposition of the active caspase-7 without bound cofactors (pink) the procaspase-7 (blue) and the active caspase-7 bound with an inhibitor XIAP fragment (green) **C)** Close up comparison of the four loops that define the catalytic groove. [34].

Catalytic mechanism of caspases

Caspase catalytic activity shows a typical cysteine protease mechanism involving a catalytic Cys^{390}-His^{337} dyad plus an `oxyanion hole' involving Gly^{338} and Cys^{390} (all of which are conserved in all caspases, numbering of the residues based on the alignmets in Fig.1.7.), (Fig.1.8.). Analysis of the active sites of caspases showed that following substrate binding Cys^{390} (in p20) is positioned to the imidazole of a His^{337} (in p20), which attracts the proton from the cysteine and enhances its nucleophilic property (Fig.1.8.), [28-30]. This nucleophilic attack is stabilized by the oxyanion hole. In many protease the side-chain of a third residue plays a significant role in the promotion of catalysis. In caspases this third component is not a side-chain, but rather is proposed to be the backbone carbonyl group of residue 276 which in caspase 1 is a proline and caspase 3 is a threonine, (Fig.1.7.) [20, 28, 40, 41].

Following substrate binding, the carboxylate side chain of the P_1 Asp (in substrate terapeptide motif P_4-P_1) is buried in a deep pocket in the caspase, termed as S_1 site. In the S_1 site, the side chains of Arg^{277} (p20) and Arg^{459} (both conserved in all caspases, indicated with stars in Fig.1.7.) participate in a direct charge-charge interaction with the Asp residue of the substrate, thus, contributing the selective recognition of Asp in the P1 position (Fig.1.8.). The tight physical dimensions of S_1 has no tolerance for anything other than Asp in this position. The side chains of P_2 and P_3 are mainly exposed to solvent which is consistent with the less stringent requirement at these positions. One of the major differences among caspases is the S_4 subsite. For example in caspase-1 S_4 subsite is a large shallow hydrophobic depression, prefers Tyr and Trp at this position and the corresponding site in caspase-3 is a narrow pocket which prefers Asp side chains in this position.

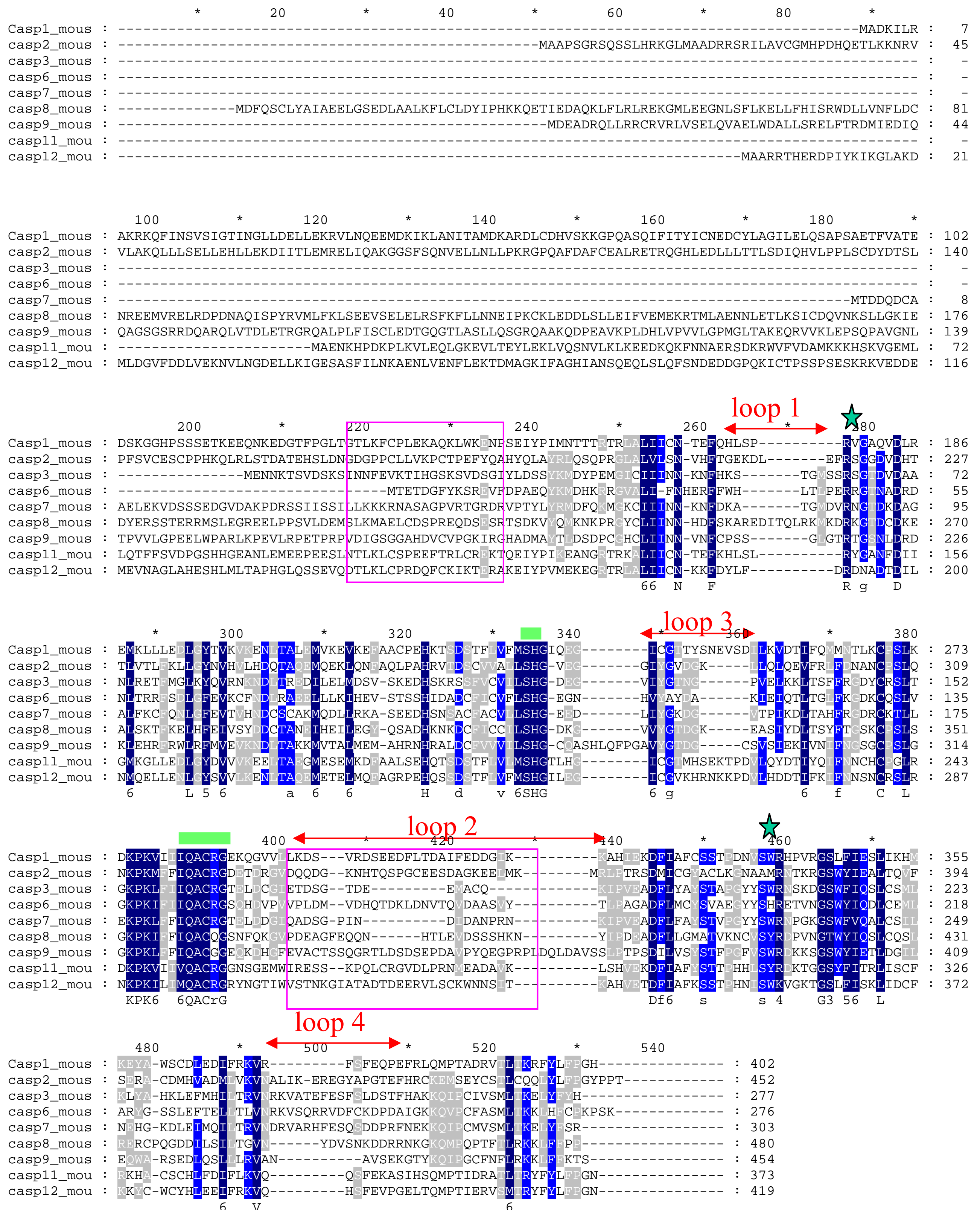

Fig.1.7. Sequence alignment of important structural elements and conserved residues for substrate binding and catalysis for mouse caspases. Four loops that define the catalytic groove highlighted with red arrows (loop numbers are given according to the [34]. The conserved motif containing the active site cysteine and the oxyanion hole highlighted in green. The Arg residues which contribute to the selective recognition of Asp in the P_1 position are indicated by stars. The residues surrounded with pink squares containing the prodomain and interdomain cleavge sites.

Varying preferences in P_4 enable the caspases assignment to one of the three generic subgroups (I, II, III) as described under caspase family and functional subfamilies. Of the critical residues discussed above, Cys^{390} and His^{337} are contributed by the large subunit. Residues lining the S_1 pocket are contributed by both subunits. The S_2, S_3, and S_4 subsites are contributed by the small subunit. Thus, both subunits contribute to the active site [20, 28, 40-41]

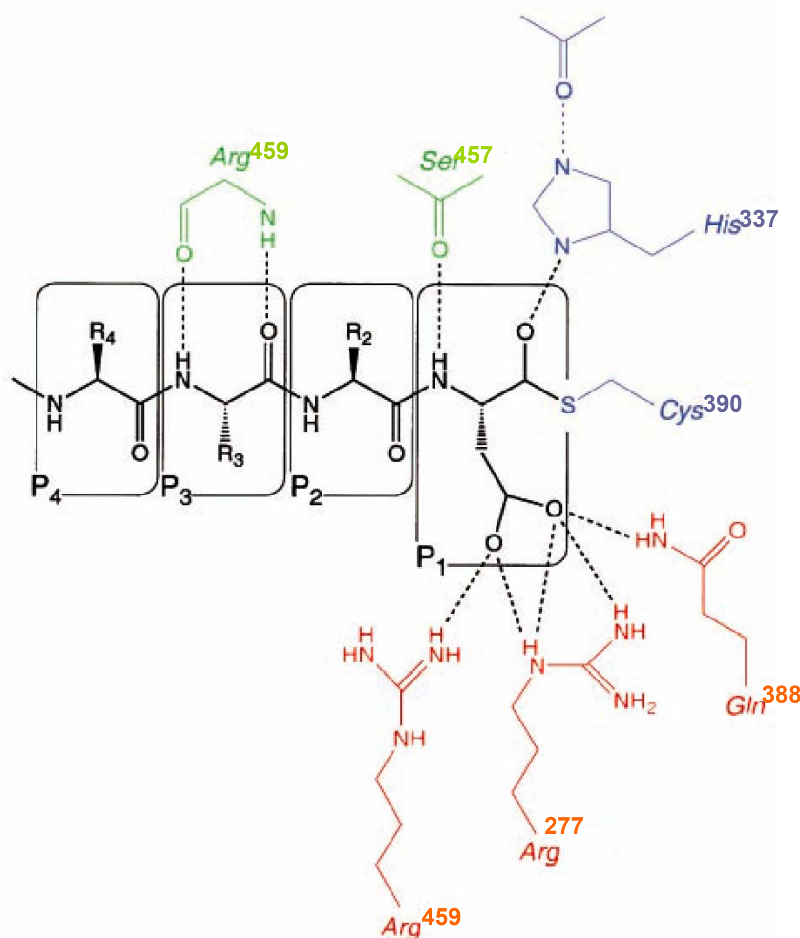

Fig. 1.8. Major polar interactions within the inhibitor bound caspase active site. P_1 Asp (red), the catalytic dyad (blue), and mainchain interactions (green). The P_4 network of interactions vary between the enzymes and accounting in part for major differences in their respective substrates[20]. The numbering of the residues based on the sequence alignment in Fig.1.7.

Substrate specificities of Caspases

The sufficiency of P_4-P_1 tetrapeptide motif for caspase recognition and high affinity binding has formed the basis for fluorogenic and chromogenic synthetic substrate design of caspases. Previously, in a survey of peptide substrates spaning known or suspected cleavage sites within caspases and/or their substrates, several groups have been identified different fluorogenic and chromogenic substrates [22-23]. Table 1.2. and 1.3. lists the chromogenic and

fluorogenic substrates and their corresponding K_m values for the recombinant human caspases, respectively [22-23, 42].

Table 1.2. K_m values for the cleavage of the chromogenic substrates of recombinant human caspases

	K_m (μM)					
Substrate	Casp-1	Casp-4	Casp-2	Casp-3	Casp-7	Casp-6
Ac-YVAD-pNA	23	874	NC	29000	NC	NC
Ac-YVED-pNA	7.3	31	ND	370	490	1200
Ac-DEVD-pNA	18	32	NC	11	12	180
Ac-VEID-pNA	46	205	NC	250	570	30
Ac-LEVD-pNA	8.5	44	ND	ND	ND	160
Ac-VDVAD-pNA	ND	ND	53	67	200	ND

ND: Not determined, NC: No catalysis

Table 1.3. K_m values for cleavage of the fluorescent substrates of recombinant human caspases

	Enzyme	**Optimal Sequence**	**Substrate**	**K_m [μM]**
GroupI	Casp-1	WEHD	WEHD.amc	4
	Casp-4	WEHD	WEHD.amc	31
	Casp-5	WEHD	WEHD.amc	15
GroupII	Casp-3	DEVD	DEVD.amc	5
	Casp-7	DEVD	DEVD.amc	15
	Casp-2	DEHD	DEHD.amc	n.d.
GroupIII	Casp-6	VEHD	VEHD.amc	170
	Casp-8	LETD	DEVD.amc	4
	Casp-9	LEHD	LEHD.amc	408
	Casp-10	LEXD	VEHD.amc	42

Table 1.4. Inhibiton of caspases by tetrapeptide aldehyde inhibitors [43]

	Enzyme	**Inhibitors**		
		K_i [nM]		
		WEHD-cho	**DEVD.cho**	**IETD.cho**
GroupI	Casp-1	0.056	18	<6
	Casp-4	97	132	400
	Casp-5	43	205	223
GroupII	Casp-3	>10000	0.23	9400
	Casp-7	1960	1.6	195
	Casp-2	>10000	1710	3280
GroupIII	Casp-6	3090	31	5.6
	Casp-8	21.1	0.92	1.05
	Casp-9	508	60	108
	Casp-10	330	12	27

An intimate understanding of individual substrate specificities of the caspases permitted the design of the potent peptide based inhibitors, as well [43]. Table 1.4. summarizes the specificity of caspase inhibitors.

Caspase substrates during apoptosis

Caspases participate in apoptosis in a manner of a well-planned and executed operation. They cut off contacts with surrounding cells [44-45] reorganize the cytoskeleton [46-49], shut down DNA replication and repair [50], interrupt splicing [51], destroy DNA[52-54], disrupt the nuclear structure [55-56], induce the cell to display signals that mark it for phagocytosis, and disintegrate the cell into apoptotic bodies [57]. Table 1.5. lists polypeptides cleaved in apoptotic cells in a caspase-dependent fashion, as well as polypeptides cleaved by caspases under cell free conditions. The responsible caspase (where known), and the sequence of the cleavage sites (where known) was indicated.

Table.1.5. Polypeptides cleaved during apoptosis [15].

Polypeptides	Cleavage site	Responsible caspase	Proposed effect of cleavage
Abundant cytoplasmic proteins			
Gelsolin	DQTD/G	3	Calcium-intensive actin cleavage
Gas-2	SRVD/G	?	Cytoskeleton rearrangement
Fodrin (alpha II spectrin)	DETD/S DSLD/S	3	Plasma membraneblebbing
Beta II spectrin	DEVDIS	3	Unknown
β-Catenin	?	3	↓α-Catenin binding and cell cell-contact
Cytokeratin 18	VEVD/A	3, 6, 7	?
Abundant nuclear proteins			
Lamin A	VEID/N	6	Nuclear lamina disassembly
Lamin B_1	VEVD/S	6, ?3	Nuclear lamina disassembly
NuMA	?	3, 6	Nuclear shape changes
HnRNP proteins	?	3	↓RNA processing
(C1 and C2) 70-kDa protein of U1 snRNP	DGPD/G	3	↓RNA processing
mdm2	DVPD/C	3, 6, 7	unknown, still binds p53
Proteins involved in DNA metabolism and repair			
PARP	DEVD/G	3, 7, 9	↓Synthesis of poly(ADP-ribose)
DNA-PKcs	DEVD/N	3	↓ Activity in some studies
Replication factor C large sb.	DEVD/G	3	N-terminal fragment inhibits DNA replication
Topoisomerase I	DDVD/Y	3	Unknown
Protein kinases			
PKCδ	DMQD/D	3	Constitutively active kinase
PKCτ	DEVD/K	3	Constitutively active kinase
PKCε	SATD	3	?
PKC-related kinase 2	DITD/C	3	?Constitutively active kinase
PKN	LGTD/S	3	Constitutively active kinase
Calcium/calmodulin dependent protein kinase IV	PAPD/A	3	Unknown

Table 1.5. continued

Polypeptides	Cleavage site	Responsible caspase	Proposed effect of cleavage
p21-activated kinase 2	SHVD/G	3, 8	Constitutively active kinase
PITSLRE kinase α2-1	YVPD/S	3	Constitutively active kinase
Mst1 kinase	DEMD/S	?	Constitutively active kinase
Mst2 kinase	DELD/S	?	Constitutively active kinase
Focal adhesion kinase	DQTD/S VSWD/S	3, 7, 6	Loss of paxilin binding, ? Translocation nucleus,↓ activity
MEKK-1	DTVD/G	3	↓Binding to 14-3-3, Δ subcellular distribution, constitutively activation
Wee1kinase	?	3, 7, 8,	Probably inactivation, leading to Cdk activation
Other proteins involved in signal transduction and gene expression			
Pro-interleukin-1β	FEAD/G YVHD/A	1	Key mediator of inflammation
Prointerleukin-1-6	SSTD/S	3	T lymphocyte chemotaxis
Prointerleukin-18	LESD/N	1	induces synthesis of IFN γ
Ras-GTPase activating protein	DTVD/G	3	Inactivation of survival signalling from extracellular receptors.
D4-GDP dissociation inhibitor	DELD/S	3	No demonstrated effect
Other proteins involved in signal transduction and gene expression			
Protein phosphatase 2a subunit-α	DEQD/S	3	↓Activity toward non-cdk substrates
Cytosolic phospholipase A_2	DELD/A	3	Activation
Stat 1	MELD/G	3	↓Transcription after IFN-α, γ
NF_KB_P50, p65	?	3	↓NF_KB dependent transcription
I_KB	DRHD/S	3	?Generates constitutive inhibitor of NF_KB
Steroid response element binding proteins	DEPD/S SEPD/S	3, 7	Nonphysiological cleavage
DCC	LSVD/R	3	Product facilitates ligand withdrawal induced apoptosis
Calpastatin	ALDD/S LSSD/F ALAD/S	1, 3, 7	Decreased inhibition of calpain
Proteins involved in regulation of cellcycle and proliferation			
$p21^{WAF1}$	DHVD/L	3, 7	Loss of N-terminal cdk inhibitory domain from nucleus
$p27^{kip1}$	DPSD/S	3, 7	↓p27 in cyclin E-cdk complexes
Rb (retinoblastoma) protein	DEAD/G	3	Unopposed E2F-1 action
CDC27	?	3	↓Ubiquitin ligase, stabilization of cyclins Aand B
Proteins involved in human genetic diseases			
Huntington	DSVD/L	3, 7	Possible non physiologocal cleavage
Dentatorubral pallidalysian atrophy protein	DSLD/G	3	No known effect
Presenilin-1	ARQD/S	?	Unknown
Presenilin-2	DSYD/S	3	Generatesantiapoptotic fragments
Apoptotic regulatory proteins			
Bcl-2	DAGD/V	3	Generatesproapoptotic fragments
$Bcl-X_L$	HLAD/S	1, 3	Generatesproapoptotic fragments
$FLIP_L$	LEVD/G	3, 8, 10	Unknown
Bid	LQTD/G	8	Generatesproapoptotic fragments
Bax	FIQD/R	?	Unknown
ICAD	DEPD/S	3	Liberates active CAD endonuclease

Natural inhibitors of caspases as regulators

Identification of caspase inhibitors has come out of work on viruses, which attenuate apoptosis to circumvent the normal host response, to infection. Four distinct classes of viral inhibitors have been described: these viral inhibitors may directly inhibit caspases as exemplified by **p35, CrmA**, family of **IAP** (inhibitors of apoptosis) proteins or inhibit caspase adaptor interactions as exemplified by **v-FLIP** (viral-FLICE Inhibitory Protein) [58,71].

CrmA is a serpin that directly targets the active site of mature caspases[58-59]. It has an active-site loop that is easily accessible to caspases. After being cleaved by a caspase, however, CrmA stays bound to the caspase and blocks the active site [60]. CrmA is limited to the group I and group III caspases (except for caspase-6) and granzyme B. The dissociation constants range from 0.01 nM for caspase-1 and 0.34 nM for caspase-8 to over 1 mM for the group II caspases [60-61]. Similar to CrmA, the baculovirus protein p35 also targets mature caspases and serves as a suicide substrate [58,62, 64]. The inhibition requires a substrate-like sequence containing Asp–Gln–Met–Asp-^{87}–Gly which fits well with the caspase active site. **p35** is a broad-spectrum caspase-specific inhibitor; it inhibits human caspase-1, -3, -6, -7, -8, and -10 with *Ki'* s of from less than 0.1 to less than 9 nM. However, it does not inhibit granzyme B [62-64].

In contrast to CrmA and p35, **IAPs** are not active-site-specific inhibitors, and their inhibition of apoptosis does not require cleavage by caspases [58,64]. The baculovirus IAPs, Op-IAP and Cp-IAP, were identified by their ability to functionally replace p35 [58,64]. While no cellular homologues of CrmA and p35 have been identified so far, the cellular homologues of IAPs constitute a major family of caspase regulators. These proteins are characterized by the presence of a homologous domain named the baculoviral IAP repeat (**BIR**) domain and IAPs containing from one to three BIR domains have been described. By this criterion, IAP family proteins have been identified in a variety of organisms, from viruses to mammals. IAP family members in mammals includes **cIAP1, cIAP2, XIAP, NAIP, Livin, KIAP, Bruce/Apollon,** and **Survivin** [64-65]. With the exception of last two, which are thought to have cell cycle functions, these proteins bind to and inhibit both initiator caspases such as caspase-9 and effector caspases such as caspase-3 and –7 [36-37, 64-65]. Among the above IAPs, **XIAP** is the most potent inhibitor of caspases and apoptosis. XIAP can physically interact with and block caspase activity. This mechanism has since been demonstrated to operate for XIAP, cIAP1 and cIAP2, and is specific for caspase-3, -7 and –9, making IAPs the only known cellular inhibitors of caspases which target the enzyme directly

rather than indirectly affecting their activation [36-39, 66]. The XIAP BIR2/caspase complex consist of two heterodimers of an active caspase complex with two BIR2 domains. The catalytic site of each active caspase heterodimer is occupied by one of BIR2 molecule. The BIR2 and BIR3 domain of XIAP inhibit caspase-3 and caspase-9 by very different mechanims. For example, the BIR3 domain binds to the NH_2-terminus of the small subunit of caspase-9, by recognizing a peptide motif (**ATPF)**, which becomes exposed after cleaved at Asp^{315}. The availability of this peptide allows free XIAP to bind cleaved caspase-9, thereby obstructing substrate entry into the active site of the enzyme [66].

Induction of cell death requires efficient elimination of the caspase inhibition enforced by IAPs. Therefore cellular mechanisms which overcome caspase inhibition by IAPs, are also existing. **Smac** or its murine homolog **Diablo** is an apoptosis-promoting mitochondrial factor that antagonizes the function of IAPs by interacting with their BIR domains [67-68]. Specifically the binding is mediated by a four aminoacid motif at the N-terminus of Smac/Diablo via hydrogen bonds and hydrophobic interactions with a surface groove on BIR3 of XIAP. Smac/Diablo can also interact with BIR2 of XIAP but not BIR1. To overcome the inhibition of caspase, binding to the BIR domain adjacent to the linker is destabilizing the IAP-caspase- interaction, thus releasing the active enzyme. However Smac/Diablo competes with caspase-9 for binding to the same pocket of the BIR domain of XIAP via the common binding motif **(AVPI)** [67-68]. Recently an other protein, a serine protease, **HtrA2** or the murine homolog **Omi** has been identified as a mitochondrial direct IAP-binding protein which is released from the mitochondria upon induction of apoptosis [70]. Like Smac/Diablo, the mature **HtrA2**/**Omi** protein contains a conserved IAP binding motif **(AVPS)** at its N-terminus and binds to IAPs in a similiar manner to Smac/Diablo and disrupt the interaction of caspase-9 with XIAP [70].Thus, although IAPs use different mechanisms to inhibit initiator and effector caspases, the mechanism of antagonizing IAPs is conserved and requires interaction with the conserved BIR domain of IAPs. (In *Drosophila*, the cell death inducing proteins **Reaper, Hid** and **Grim** use the conserved IAP binding motif at their N-terminus to interact with and inhibit the endogenous fly IAP [64-65]).

v-FLIPs represent another group of viral apoptotic inhibitors. v-FLIPs contain two DEDs that are similar to those in the N-terminal region of procaspase-8. They inhibit apoptosis mediated by death receptors through competition with procaspases for recruitment to the death receptor complex [71-72]. Cellular homologues of v-FLIP have been identified, and they come as both a long form and a short form, termed c-FLIPL and c-FLIPS, respectively. c-FLIPS is similar to v-FLIP and contains only the DEDs. Overexpression of

this form inhibits apoptosis mediated by Fas and related death receptors. In contrast, c-FLIPL is strikingly similar to procaspase-8 and -10, comprising two NH_2-terminal DEDs and a COOH-terminal caspase-like domain [73].

Caspase inhibition as a therapeutic approach

Defects in the physiological pathways for apoptosis make important contributions to multiple diseases. Infact, it is estimated that either too little or too much cell death is involved in over half of the diseases for which adequate therapies do not currently exist [4-5, 14]. The control of caspases as a key and central component of the biochemical pathway that mediates apoptotic cell death is an attractive first step in modulating this process. Caspase activation for the treatment of disorders where insufficient apoptosis occurs (e.g. cancer) represents a substantial challenge. For example, Trojan Horse genetherapy approaches has been described for HIV infection, in which a TAT-caspase-3 construct containing a HIV protease recognition motif, selectively induces apoptosis in HIV infected cells only [74].
On the other hand, caspase inhibition for the treatment of disorders where excessive apoptosis occurs (e.g. neurodegeneration) appears to be more useful to therapeutic interventions with classical small molecule inhibitors [75]. Direct evidence in support of caspase inhibitors as therapetic agents in neurodegenerative diseases has been come, *in vivo* with the finding that mice expressing exon1-Huntington with an expanded polyglutamine tract had 20% longer life span when treated with the broad spectrum caspase inhibitor z-VAD.fmk or when crossed to animals expressing a dominant-negative Caspase-1, when compared to control untreated animals [76-78]. In addition, peptidyl caspase inhibitors provide substantial protection in stroke models of focal transient and permanent ischemia as well as myocardial infarction and have been used to demonstrate *in vivo* efficacy in mouse models of hepatic injury, sepsis, amylotrophic lateral sclerosis (ALS) and several other diseases [75]. Thus, *in vivo* inhibition of caspases may be useful as a therapeutic approach for the above mentioned diseases.

1.3 The Bcl-2 Protein Family

Bcl-2 was first discovered as a proto-oncogene in follicular B-cell lymphoma [79-80]. Subsequently, it was identified as a mammalian homologue to the apoptosis repressor **Ced-9** in *C. elegans* [81-82]. Since then, at least 19 Bcl-2 family members have been identified in mammalian cells. These members possess at least one of four conserved motifs known as Bcl-2 homology domains (**BH1-BH4**). The Bcl-2 family members can be subdivided into three categories according to their function and structure (Fig. 1.7.) [83-84].

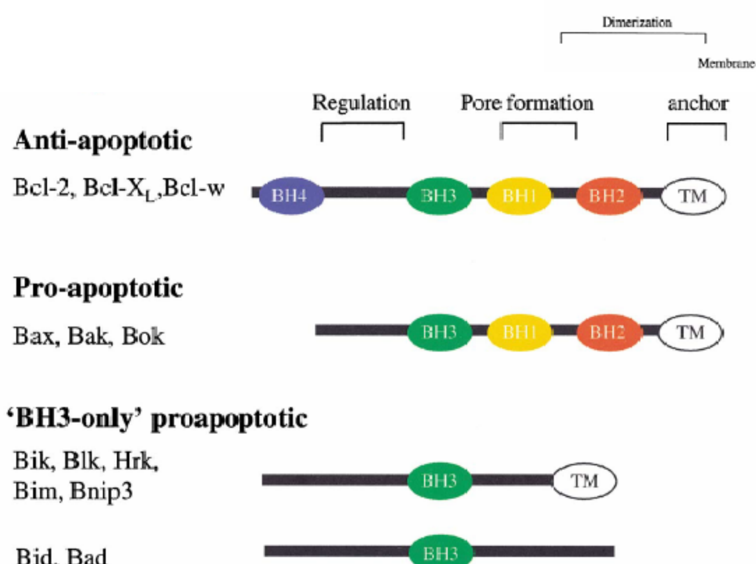

Fig 1.9. The members of the Bcl-2 family. Three subfamilies are indicated: the anti-apoptotic Bcl-2 members promote cell survival, whereas pro-apoptotic and BH3-only members facilitate apoptosis. BH1-BH4 are conserved sequence motifs. Several functional domains of Bcl-2 are shown. A membrane-anchoringdomain (TM) is not carried by all members of the family [83].

(1) Antiapoptotic members, such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1 (Bfl-1), NR-13, and Boo (Diva), all of which exert anti-celldeath activity and contain at least BH1 and BH2 (those most similar to Bcl-2 contain all four BH domains). Viral members of this group include E1B-19K, BHRF1, KS-Bcl-2, ORF16, and LMW5-HL. **(2) Pro-apoptotic members**, such as Bax, Bak, and Bok (Mtd), which share sequence homology in BH1, BH2, and BH3, but not in BH4 domain. **(3) 'BH3-only' pro-apoptotic members**, which include Bid, Bad, Bim, Bik, Blk, Hrk (DP5), Bnip3, BimL, and Noxa, and possess only the central short BH3 domain, (Fig.1.9.), [83-84].

Bcl-2 homology domains have been shown to be involved in protein-protein interactions. The BH1 and BH2domains are required for Bcl-2 and Bcl-X_L to interact with Bax and to supress apoptosis. The BH3 domain of proapototic proteins such as Bax, Bak, or Bad is sufficient but not required for their binding to Bcl-2 or Bcl-X_L and to promote apoptosis [83-84]. The fourth domain, BH4, is found in the N-terminal region of antiapoptotic proteins only (except the proapotoic Bcl-X_S that olso contains a BH4 domain). Mutants of Bcl-2 lacking the BH4 domain not only loose their antiapoptotic activity but behave like killer proteins [84-85].

One of the unique features of Bcl-2 family proteins is found to be heterodimerization between anti-apoptotic and pro-apoptotic proteins, which is considered to inhibit the biological activity of their partners [83-85]. In addition to the regulation of apoptosis by heterodimerization of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, some protein members have been suggested to regulate apoptosis independently of

each other, based on the observations with transgenic and knockout mice [85]. This notion is found to be consistent with the findings that some Bcl-2 family members such as Bcl-2, Bcl-xL, and Bax, can form ion channels in synthetic lipid membranes. Although one of the BH3-only proteins, Bid, shares very limited sequence homology with Bcl-2 and Bax which have channel-forming ability, it has been shown that the solution structure of Bid is strikingly similar to Bcl-xL and Bid indeed creates an ion channel on synthetic lipid membranes [86].

Subcellular localization studies have shown that Bcl-2 and Bcl-X_L reside on the mitochondrial outer membrane, while the proapoptotic family members may be either cytosolic or present on the mitochondrial membrane. They are also found elsewhere in ER and nuclear envelope [83].

Mitochondria and the functions of Bcl-2 family

There is accumulating evidence that mitochondria play an essential role in many forms of apoptosis by releasing apoptogenic factors, such as cytochrome c from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis [87-88]. In living cells, anti-apoptotic members of the Bcl-2 family of proteins predominantly prevent mitochondrial changes. During apoptosis, the proapoptotic Bcl-2 family members are activated leading to the exposure of the pro-apoptotic BH3 domain, may occur via several mechanisms, including dephosphorylation (e.g., Bad) and proteolytic cleavage by caspases (e.g., Bid) and translocate to the mitochondria. Bax translocation to the mitochondria involves homo-oligomerization. The translocation of Bax, Bid, or Bad to the mitochondria can then induce a dramatic event, release of the proteins contained within the intermembrane space, including one key protein, cytochrome c. Cytochrome c is encoded by a nuclear gene, but when it is imported into the mitochondria, it is coupled with a heme group to become holocytochrome c, and it is only this form that functions to induce caspase activation [84,88-89]. In contrast to proapoptotic members the anti-apoptotic proteins Bcl-2 and Bcl-XL work to prevent cytochrome c release from mitochondria, and thereby preserve cell survival [85-87].

1.4 Caspase Cascades in Apoptosis

Until recently there were two well characterized pathways for the cleavage and thus activation of the executioner caspases : (a) A mitochondrial pathway in which cytochrome c, Apaf-1, and caspase-9 interact as a caspase activating complex (apoptosome), (b) A receptor mediated recruitment of caspase-8 in to the death inducing signalling complex (DISC). Recently a third

pathway has been identified (c) An Endoplasmic Reticulum (ER) specific pathway, in which caspase-12 is activated in response to ER-stress, (Fig 1.10.), [93].

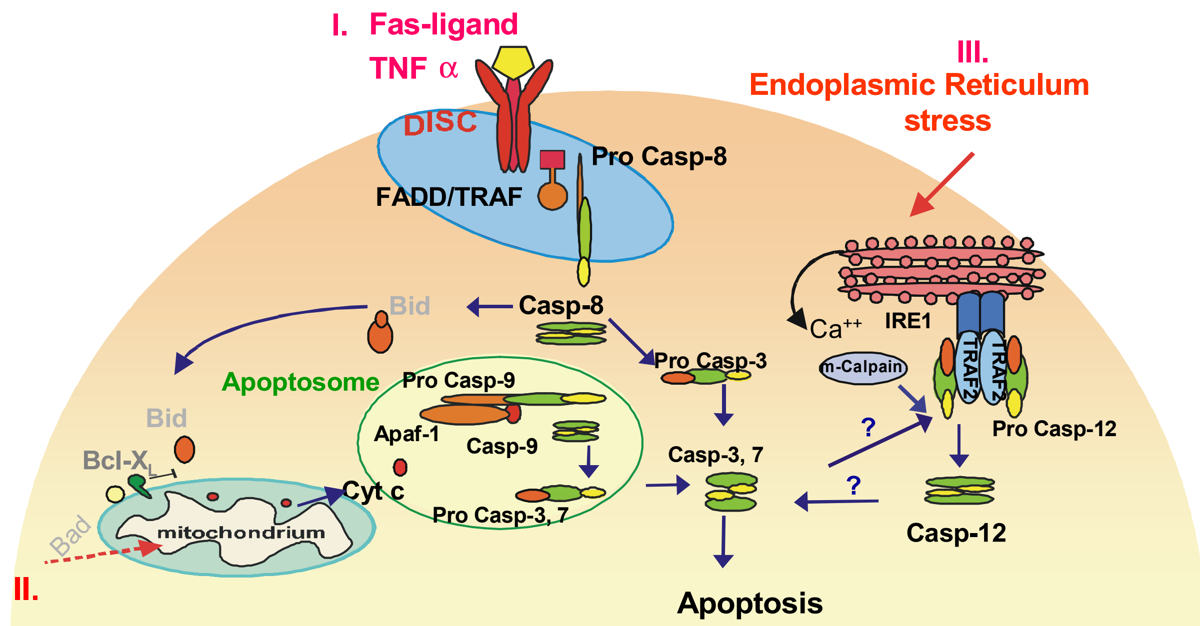

Fig 1.10. Three distinct signalling pathways in apoptosis. The question marks denote the possible but unconfirmed pathways.

Caspase cascades in receptor mediated apoptosis

Within the plasma membrane of many cells when triggered by their corresponding death ligands, death receptors initiate rapid activation of caspases and the induction of apoptosis. Death receptors are members of the TNF (tumor necrosis factor) receptor superfamily [94-95]. They possess both cysteine-rich extracellular domains and an intracellular cytoplasmic sequence known as the death domain (DD). The bestknown death receptors are **CD95/Fas/Apo1**, **TNFR1**, **TNFR2**, **DR3/Wsl-1/Tramp**, **DR4/TRAIL-R1** (TNF-**R**elated Apoptosis-**I**nducing **L**igand **R**eceptor-1), **DR5/ TRAIL-R2/TRICK2/Killer**, and **DR6** [96-97]. Triggering of death receptors with their cognate ligands or agonistic antibodies results in receptor trimerization and recruitment of adapter proteins. For example, **CD95L** (CD95 ligand) interacts with and induces trimerization of CD95 receptors, resulting in clustering of the receptor's cytosolic **DD** (Death Domain) and recruitment of the adapter molecule, **FADD** (Fas-associated death domain; also termed Mort1). FADD contains a C-terminal DD, which enables it to bind trimerized CD95 receptors through DD–DD interactions, as well as an N-terminal DED (death effector domain), which can associate with similar DEDs located in the prodomain of caspase-8. This complex of proteins is referred to as the **DISC** (**D**eath-**I**nducing **S**ignaling **C**omplex), [98-101]. It is proposed that as more procaspase-8 molecules are

recruited to this complex, they begin to cluster and undergo transcatalysis to generate active caspase-8 (Fig.1.10.)

Similarly, trimerized TNFR1 can recruit the adapter protein TRADD (**T**NF**R**-**A**ssociated **D**eath **D**omain), which consequently recruits FADD and procaspase-8. TRADD, however, also binds the serine-threonine kinase RIP (receptor-interacting protein) and in turn, the adapter molecule RAIDD/CRADD through a series of homophilic DD–DD interactions. RAIDD is interesting in that its N-terminus shares sequence similarity with the prodomains of caspase-2, caspase-9, and CED-3, and it recruits and activates procaspase-2 [96-97]. However, the physiological importance of caspase-2 in this context is unclear. Caspase-8 is the major apical caspase in TNFR1- and CD95-activated caspase cascades [96-98]. However, caspase-10, like caspase-8, also contains two FADD-like DEDs in its N-terminal prodomain and is apparently recruited following ligation of TNFR1 and DR5. Thus, caspase-10 may also serve as an apical caspase in some receptor-mediated caspase cascades [98].

Triggering of cell death through DR3, DR4, and DR5 receptors, is less well characterized. In contrast to other TNF family receptors, stimulation of DR4 and DR5 receptors using artificially trimerized TRAIL results in selective killing of tumor cells both *in vitro* and *in vivo* with little or no side-effects [98, 101]. Unfortunately, the signaling pathways downstream of DR4 and DR5 are largely unknown.

Death receptors and mitochondria

Some investigators have found that Bcl-2 can prevent Fas-mediated apoptosis in certain cell types, while in others, it cannot. However, in some cases it has been found that Bcl-2 family members never interfere with Fas-mediated apoptosis [91]. If Bcl-2 can sometimes interfere with death mediated through Fas, is this through a new function of the anti-apoptotic protein or its role in the mitochondrial pathway ? Evidence indicated to the role of the mitochondria. One of the BH3-only proteins, Bid, is cleaved by caspase-8 (activated by ligation of the death receptors) and translocates to the mitochondria, where it triggers cytochrome c release most likely through interaction with Bax or Bak. Bcl-2 can prevent this Bid mediated effect . In cells, expression of Bid can sensitize for TNF-induced apoptosis [85]. In vitro, the ability of caspase-8 to activate caspase-3 is greatly facilitated by the presence of Bid and mitochondria These observations suggest that when caspase-8 is limiting, the action of Bid on mitochondria can determine whether a cell will undergo apoptosis (via cytochrome c release and the activation of caspase-9) [101-102].

Caspase cascades in stress induced apoptosis

In stress-induced apoptosis, a number of cellular signals induce perturbations in the mitochondria resulting in the release of proapoptotic molecules, including AIF (apoptosis inducing factor) and cytochrome *c*, from the intermembrane space into the cytoplasm [103]. Cytochrome *c* release appears to be a common occurrence in apoptosis, and the mechanisms controlling its release are still under intensive investigation. They may include opening of a mitochondrial permeability transition pore, the presence of a specific channel for cytochrome *c* in the outer mitochondrial membrane or mitochondrial swelling and rupture of the outer membrane without a loss in membrane potential [102]. Cytochrome *c* interacts with Apaf-1 (Apoptotic protease activating factor-1) which can bind, dATP/ATP and procaspase-9 to form a complex known as the "apoptosome", (Fig. 1.10.), [104-108]. Apaf-1, was the first identified mammalian homologue of the *C. elegans* gene product CED-4, is a 130-kDa protein with a region homologous to CED-4 domain. However, Apaf-1 appeared more evolutionarily advanced because, in addition to CED-4 domain, it also contained an N-terminal caspase recruitment domain (CARD) and a C-terminal domain containing multiple WD-40 repeats (WDR) [104]. In a mechanism that is not clearly understood, cytochrome *c* appears to interact with WDR in Apaf-1 whereas dATP/ ATP binds the nucleotide binding domain [106]. Cytochrome c and dATP/ATP, acting in concert to induce a conformational change in Apaf-1 that allows it to undergo self oligomerization via its CED-4 domain into a large (~700 kDa) caspase activating complexe [106-110]. In this 700 kDa apoptosome complex, procaspase-9 is simultaneously recruited to the newly exposed N-terminal of Apaf-1, where it undergoes auto-catalytic activation. Procaspase-9 binds to Apaf-1, in a ratio of 1:1. Once activated, caspase-9 can recruit and activate the effector caspases, -3 and -7 within the apoptosome (Fig. 1.10.), [106-109]. Caspase-3 then processes caspase-6 and -2 and in addition feed back to process additional caspase-9 [109-110]. Crystallographic studies indicate that Apaf-1 binds caspase-9 through CARD-CARD interactions, and the multiple WDRs may provide a "docking" region for effector caspases [111-114].

Recently another large protein complex (~1.4MDa) which contains Apaf-1 has been also identified. For reasons that are not entirely clear, the ~700 kD complex is present in a different conformation and exhibits significantly greater activity than the ~1.4MDa complex. The caspase inhibitor XIAP also associates with the apoptosome, where it inhibits the activity of processed caspase-9. More importantly it is proposed that perhaps XIAP is situated to inhibit effector caspases-3 and –7 following their activation and might prevent their release from the apoptosome [115].

ER stress induced pathway of apoptosis

The ER plays a key role in folding, modifying and sorting newly synthesized proteins, maintaining intracellular Ca^{2+} homeostasis, and synthesizing lipids and sterols. When these processes are disturbed, at least three major ER stress-induced signaling pathways can be activated: (1) the unfolded protein response (UPR), which leads to induction of ER-localized proteins, including chaperones such as GRP78 (BiP), GRP94 and calreticulin, protein disulfide isomerase (PDI) and the transcription factor, CHOP (GADD153); (2) the ER-overload response (EOR) pathway, which leads to activation of NF-κB and, consequently, production of interferons and cytokines; and (3) phosphorylation of eukaryotic translation initiation factor (eIF-2α), which inhibits initiation of translation and, thus, blocks protein synthesis. Each of these pathways enables the cell to deal with accumulated or incorrectly folded proteins in the ER. However, when the ER stress is too great, the cell might die via apoptosis through mechanisms that are currently not very clear [116-117]. ER stress can also lead to the release of Ca^{2+} from the ER. High cytosolic Ca^{2+} can induce a loss in the mitochondrial inner transmembrane potential ($\Delta\psi m$), which results in the release of cytochrome *c* and formation of the apoptosome, as previously described. Ca^{2+} might also, promote the direct associatiotion of mitochondria with smooth subdomains of the ER, which could facilitate the movement of proapoptotic signals or proteins from stressed ER to mitochondria. ER stress or other apoptotic stimuli might also activate caspases at the ER surface and induce apoptosis [116]. Murine caspase-12, a member of the ICE subfamily of caspases with high homology to caspase-1 (39% identity), is ubiquitously expressed in mouse tissues and resides predominantly on the outer ER membrane. Caspase- 12 is activated by chemicals that disrupts the Ca^{2+} homeostasis and induce ER stress [e.g. thapsigargin, calcimycin (A23187), brefeldin A or tunicamycin] but not by agents that engage the mitochondrial pathway or by death receptors[118]. Although Caspase-12 has been identified as playing a role in ER stress-induced apoptosis in renal tubular epithelium and in amyloid-β neurotoxicity, which implicates the human orthologue of caspase-12 as a therapeutic target for Alzheimer's disease [118], most recently another group has found that human caspase-12 has acquired deleterious mutations and functional caspase-12 is lost in human and that it can not play a role in Alzheimer's disease [119]. The precise mechanism(s) responsible for activation of caspase-12 is currently unclear, although m-calpain has been shown as implicated in [120]. Because caspase-12 contains a long prodomain, it is presumed to be an

'initiator caspase' and to associate with an adapter protein located in the ER membrane. In this regard, TNF-receptor-associated factor 2 (TRAF2), an adapter protein that associates with TNF receptor complexes and induces activation of c-Jun N-terminal kinases (JNKs), is similarly recruited to the ER membrane following ER stress, where it interacts with the cytoplasmic domain of the ER sensor IRE1α (Fig.1.10), [116,121]. In any event, IRE1α–TRAF2 complexes are also capable of activating JNKs, and JNK activity appears to be fundamentally important for ER-stress-induced apoptosis [116].

Recent work also suggests that TRAF2 plays a direct role in the activation of caspase-12 through an induced proximity model, similar to that proposed for FADD and APAF-1-mediated activation of caspases-8 and -9, respectively [121]. Once activated, caspase-12 probably activates effector caspases to induce apoptosis. Active caspase-7 also appears to localized to the ER during apoptosis, which raises the possibility that it might be a preferred substrate of caspase-12, though it has been shown that caspase-7 cleaves caspase-12 in a recent study [122-123]. Putting these data together, further investigations have to be done to elucidate the mechanisms that are involved in caspase-12 activation, or the role of caspase-12 in apoptosis and its cellular targets.

1.5 Cell Death of AKR-2B Cells

Cell death of AKR-2B cells was demonstrated in earlier publications [124-129]. Density arrested AKR-2B cells die in response to serum starvation [124]. After serum removal, death starts after a delay of 90 min and 50% of AKR-2B cells die within 6h. showing known morphological changes of apotosis *e.g.*, chromatin condensation, membrane blebbing and membrane disrupture. Remarkably there is no DNA fragmentation and no loss of mitochodrial potential [126]. Multiple signalling pathways which interfere with the activation of caspase-3 like activity have been identified. Protective efects of caspase inhibitors have been described [127]. An ICE-like protease inhibitor YVAD-cmk and two other caspase inhibitors YVAD-amc and DEVD-amc protected cells from death. In addition, a pronounced DEVDase activity was detected reaching a maximum after 3h of serum removal [127, 129]. These results strongly indicated the existence and involvement of caspases in AKR-2B cell death. Using different approaches, the expression of mRNA of all known caspases, which are believed to be involved in apoptosis, has been detected. According to these results, with the exception of Caspase-14 all caspases are present in AKR-2B cells at RNA level. Considerable importance of the caspases during cell death of AKR cells has been revealed by using other specific caspase inhibitors and by determination of the specific activity of the caspases. Beside

DEVDase activity, constutive VEIDase and IETDase activities, were also detected. The present mixture of caspase activity is dominated by DEVDase which has been confirmed by ionexchange chromotography and represented as a single enzyme after 2D-SDS-PAGE and affinity labeling [129].
Recently, it has been also shown that ATP and adenosine prevent via defferent pathways the activation of caspases in apoptotic AKR-2B cells [128]. Finally the involvement of the receptor mediated pathway in caspase activation have been studied by means of overexpression of CrmA; a cowpox virus derived Caspase-8 and thus receptor mediated pathway inhibitor. Since overexpression of CrmA does not prevent serum starvation induced apoptosis involvement of receptor mediated pathway in AKR-2B cell death excluded [129].

1.6 The Aim of The Study

Apoptosis is an essential physiological process for multicellular organisms for the selective elimination of cells, which is involved in a variety of biological events. Molecular biological studies of apoptosis have made fairly rapid progress in unveiling the machinery of apoptosis. The framework of the apoptotic signal transduction pathway appears to be as follows: various proapoptotic signals initially activate separate signalling pathways which eventually converge into a common mechanism driven by a unique family of cysteine proteases, called caspases. Activation of caspases requires formation of large protein complexes such as DISC involved in receptor mediated extrinsic pathway, or APOPTOSOME in mitochondria mediated intrinsic pathway.
Density arrested AKR-2B cells die rapidly in response to serum starvation or treatment by Anisomycin. Cell death is associated with typical hallmarks of apoptosis including membrane blebbing and chromatin condensation but lacks energy dissipation in mitochondria and intranucleosomal fragmentation. During apoptosis a considerable DEVDase activity has been detected which seemed to be represented by a single enzyme[129]. This enzyme had typical effector caspase characteristics, like caspase-3, but exhibited an unusual high K_M values of ~100 µM and its large subunit exhibited a molecular weight of 19 kDa, instead of expected 17 kDa.
Thus the first aim of the study was firstly to generate a recombinant mcaspase-3 to define the precise molecular weight of active caspase-3, and clarify the discrepancies to be able to assign the DEVDase activity to caspase-3. Secondly the major effector caspase in AKR-2B cells which is activated during apotosis should be identified to further examine its localization and to find out explanations for the lack of the internucleosomal fragmentation.

As activation of caspases represents a points of no return in the apoptotic process, it was important to understand the mechanisms of underlying the activation and regulation of caspase cascades. Therefore the characterization of the process of caspase activation during AKR-2B cells should be examined. In this regard examination of the the involvement of the mitochondria mediated intrinsic pathway and classical APOPTOSOME complex formation or the ER stress mediated third pathway should be examined since the receptor mediated extrinsic pathway has been already excluded in caspase activation in AKR-2B cells [129].