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Review

The modular nature of apoptotic signaling proteins

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Abstract. Apoptosis, initiated by a variety of stimuli, is a physiological process that engages a well-ordered signaling cascade, eventually leading to the controlled death of the cell. The most extensively studied apoptotic stimulus is the binding of death receptors related to CD95 (Fas/Apo1) by their respective ligands. During the last years, a considerable number of proteins have been identified which act together in the receptor-proximal part of the signaling pathway. Based on localized regions of sequence similarity, it has been predicted that these proteins consist of several independently folding domains. In several cases these predictions have been confirmed by structural studies; in other cases they are at least supported by experimental data. This review focuses on the three most widespread domain families found in the apoptotic signaling proteins: the death domain, the death effector domain and the caspase recruitment domain. The recently discovered analogies between these domains, both in structure and in function, have shed some light on the overall architecture of the pathway leading from death receptor ligation to the activation of caspases and eventually to the apoptotic phenotype.

Key words. Modular; apoptosis; controlled death; structural studies.

Introduction

Apoptosis, a form of programmed cell death, is a mechanism used by multicellular organisms to dispose of unwanted cells in an orderly fashion [1]. A central feature of apoptosis, as compared with necrotic cell death, is the permanent containment of cellular material in membranous structures [2]. Subsequently, these apoptotic particles can be phagocytosed without any leaking of the potentially dangerous intracellular enzymes [3]. There are several reasons why a cell might activate the apoptotic pathway. Physiological apoptosis occurs during embryonic development in situations where some cells have fulfilled their function and are no longer needed or where cells have failed to make the proper contacts [4–6]. A very important and well-studied form of apoptosis is at work in the development of the immune system: the removal of autoreactive T-cells or of thymocytes with incompletely rearranged T-cell receptor genes is accomplished by triggering the apoptotic pathway in those cells [7]. Besides this developmentally programmed cell death, apoptosis is also used as a last line of defense against cellular damage. Cells which contain irreparable damage in their DNA either halt their cell cycle and activate repair mechanisms or, failing that, undergo apoptosis [8, 9]. By that drastic measure, the organism is protected from the proliferation of genetically altered cells, thus reducing the chance of tumor formation [10]. In addition, cells under excessive oxidative stress or otherwise severely damaged try to enter the apoptotic pathway instead of becoming necrotic [11, 12].

A third reason for apoptosis lies in the containment of infection with viruses, bacteria or fungi. This pathogen-induced cell death is of particular importance for plants, which lack the defense mechanism provided by an immune system [13, 14]. Even for mammals with their

highly developed immune system apoptosis might be an attractive way of removing cells infected with viruses or intracellular pathogens. Since these cells cannot be rescued anyway, it is advantageous for the host organism if its cells can detect their own infection and undergo apoptosis before the pathogen is disseminated any further. It is therefore not surprising that viruses have evolved elaborate strategies to evade host cell apoptosis, at least until the assembly of the viral progeny has been completed [15–17].

Apoptosis research has applications not only in basic research but also in clinical medicine [18]. To mention only one example, the importance of p53-induced apoptosis in radiation therapy of cancer is well recognized nowadays [19]. Initially, it had been thought that the radiation itself inflicts sufficient damage on cancer cells to kill them directly. A number of observations argue against this idea. Most important, cancer cells defective for p53 or some other genes are resistant to the radiation doses that would kill a normal cell, demonstrating that the cell has to assist in triggering its own death. In addition to radiotherapy, most if not all of the commonly used chemotherapeutic agents are thought to trigger apoptosis instead of killing cells directly [20–22].

Due to its importance and its far-reaching applications, apoptosis research is a very active field. There are a number of excellent reviews highlighting the various aspects of apoptosis, including death receptors [23], upstream signaling events [24–27], the role of caspases [28, 29], the role of the mitochondrion [30, 31] and the Bcl-2 family of apoptosis regulators [32–34]. This review focuses on the modular nature of apoptotic signaling proteins, putting special emphasis on the three death-adaptor domains found in the early signaling components of death receptor-induced apoptosis.

The death domain

The first glimpse of a modular structural element in apoptotic signal transduction was the discovery of a local similarity in the cytoplasmic region of the two death receptors Fas (CD95/Apo1) and 55 kDa tumor necrosis factor receptor (TNF-R55) (TNF-R1) [35, 36]. These two receptors belong to a large family of type I transmembrane proteins which is characterized by the occurrence of two to four copies of a cysteine-rich domain in the extracellular region [23]. In contrast, the cytoplasmic portions of these proteins are not generally related. Fas and TNF-R55 appeared to be the only exceptions, since these two proteins share an intracellular region of about 80 residues that are moderately well conserved. Since at that time these two receptors were the only members of the TNF-R family that signal cell death, and the conserved domain was found to be important for this signaling, it was termed death domain (DD). The role of the other then known members of the TNF-R family (TNF-R75, CD27, CD30, CD40, OX40, 4-1BB, NGF-R, LTβ-R) was seen in other pathways rather than death induction.

With the exception of NGF-R, the similarity of the extracellular ligand binding domain of the receptors is paralleled by the structural similarity of their ligands. NGF-R is a low-affinity receptor for neurotrophins, including nerve growth factor (NGF). All other known members of the TNF-R family bind to relatives of TNF, a type II transmembrane protein. X-ray diffraction studies were performed on cocrystals of the soluble extracellular portion of TNF-R55 in complex with TNF-β [37]. In the resulting three-dimensional (3D) structure, both ligand and receptor were trimers, suggesting that it is the induced trimerization of the receptor which leads to the generation of a signal.

Some diversity in this recognition process is evident: not all members of the TNF family occur as type II transmembrane proteins with their N-terminus located in the cytoplasm. Some of the ligands lack a transmembrane region and thus are secreted into the extracellular space. Other ligands, including TNF itself, possess a membrane-proximal cleavage site which is recognized by metalloproteases. These ligands are normally membrane-bound, but can be rapidly shed upon activation of the protease [38, 39]. As a further complication, not all of the TNF-R family members bind to homotrimeric ligands. Lymphotoxin-β receptor binds to a heterotrimer consisting of two molecules of lymphotoxin-β and one molecule of either lymphotoxin-α or TNF [40]. While only LTβ-R has been shown to bind to a heterotrimeric ligand, it cannot be excluded that other receptors share this binding mode, either obligatorily or facultative.

According to the paradigm of induced receptor trimerization, the cytoplasmic portions of the receptors must generate a signal when brought into contact. A caveat applies here, as there is still no reliable data on receptor stoichiometry in the uninduced form. For example, it is conceivable that a receptor normally occurs as a dimer, which upon binding of the ligand forms a trimer of dimers. Irrespective of the number of receptor subunits in the signaling complex, the mechanism by which the induced proximity of the receptor cytoplasmic tails leads to the generation of a signal was the first central issue in understanding apoptosis by death receptors.

Relatively soon it became clear that the death domain of Fas and TNF-R55 is the site of interaction with the downstream targets in apoptotic signaling. Experiments using alanine scanning or deletion mutagenesis demonstrated that an intact DD is required for the induction of apoptosis, whereas the membrane-proximal part of the receptor's intracellular domain is less important [35,

36]. The region of detectable sequence similarity between the intracellular regions of Fas and TNF-R55 corresponds well with the minimal region of TNR-R55 that still is able to confer cytotoxicity in these experiments. A point mutation Ile → Asn within the DD of Fas is the cause of the lymphoproliferative disease phenotype observed in *lpr* mice [41].

The further elucidation of the signaling pathway originating at Fas or TNF-R55 was made possible by the identification of factors binding to the DDs of the two receptors. Using the yeast two-hybrid system, several groups independently found that three proteins interact with the DD of one or both of the receptors. Fas-associated death domain protein (FADD)/Mort1 [42, 43] and receptor interacting protein (RIP) [44] were found to bind to the DD of Fas, whereas TNF-receptor associated death domain protein (TRADD) [45] was identified as an interactor with the TNF-R55 intracellular region. All three proteins share one distinctive feature: they all contain DDs, relatively closely related to the DDs of the receptors. In addition, the DD of the three newly identified proteins was in all cases responsible for binding to the receptors. It was therefore obvious that, at least in this case, the DD functions by mediating heterodimerization with other DDs. More recent studies using the RIP DD have demonstrated that it binds with high affinity to TRADD and that under physiological conditions RIP is localized in the TNF-R55 signaling complex rather than being associated with Fas. [46]. In addition to its strong interaction with Fas, FADD is also found associated with TNF-R55, an interaction that is mediated by TRADD [47, 48]. Overexpression of either protein induced apoptosis, with FADD being the most effective in this respect [42–45, 47]. Soon it became clear that these three proteins by themselves are not directly responsible for the phenotypic changes seen in apoptosis or for the activation of Caspase-3 (CPP-32), a protease with a crucial role in this process. The role of FADD, TRADD and RIP lies in the transduction of the signal originating from Fas or TNF-R55 to downstream targets. For that reason, the three proteins are frequently referred to as death adaptor protein.

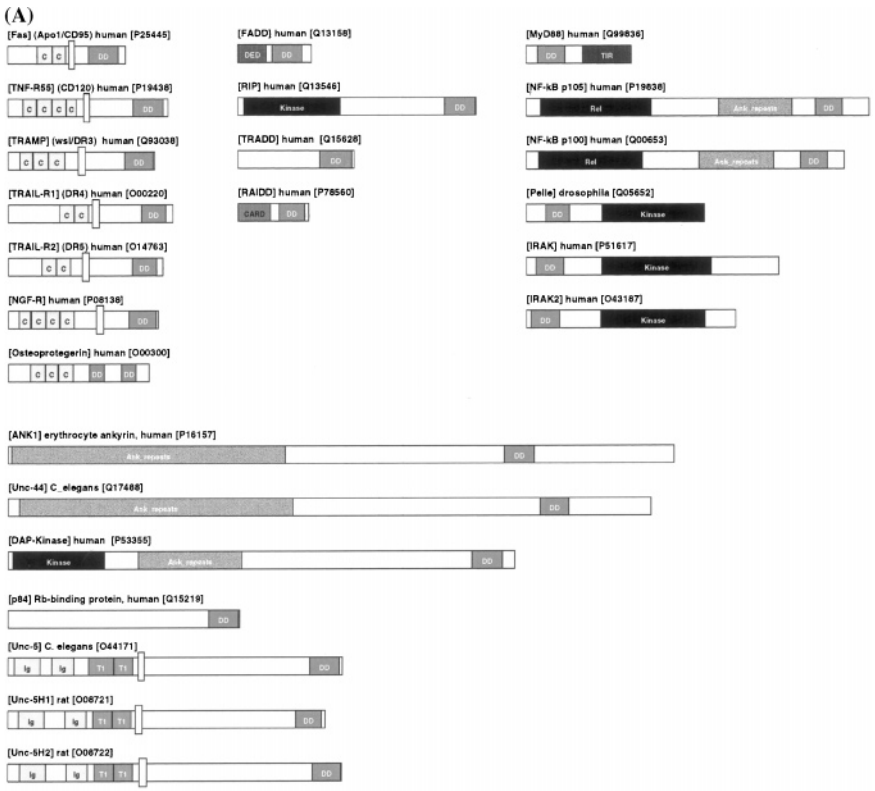
The DDs of Fas, TNF-R55, FADD, TRADD and RIP form a heterogeneous family, with pairwise sequence similarities ranging from 17% to 32% amino acid identity. The fact that DD/DD heterodimerizations are crucial for the first steps of death receptor signaling has prompted several groups to search for new DD proteins, with the rationale that those proteins might either constitute additional death receptors or new adaptor proteins. In two initial screens, using the BLAST [49] program or generalized profiles [50], respectively, several known proteins were found to contain regions related to the DD [51, 52]. Both studies identified putative DDs in NGF-R, a further member of the TNF-R family, in the death-associated protein kinase (DAP-kinase), in the myeloid differentiation protein MyD88, in mammalian ankyrin and its nematode homologue Unc-44, in the nematode neuronal guidance protein Unc-5 and in the *Drosophila* protein kinase Pelle, which plays a role in signaling from the receptor Toll to the nuclear factor κB (NF-κB)-like transcription factor Dorsal. In addition, one study also found a DD in NF-κB which did not reach the more stringent significance criteria of the second report. Later, more DDs were identified by both sequence analysis and experimental methods. RAIDD, a fourth death adaptor protein, was demonstrated to bind the DD of RIP in the TNF-R55 signaling complex [53].

Searches with DD profiles and sequences in expressed sequence tag (EST) databases led to the identification of a number of TNF-receptor members with DDs. Most of these receptors were shown to be death receptors, that is to induce cell death upon binding to their cognate ligand or upon overexpression. These proteins include Wsl/TRAMP/DR3 [54–56], TRAIL-R1/DR4 [57] and TRAIL-R2/DR5 [58–61]. Including Fas and TNF-R55, there are now five death receptors in the TNF receptor family. Osteoprotegerin [62], another member of this family, is an exceptional case in several respects. First, it is a secreted protein and lacks a transmembrane domain. In the C-terminal region, the protein contains two DDs which are the only examples of extracellular DDs—all other DDs are assumed to be localized in the cytoplasm. Since osteoprotegerin and the low-affinity NGF receptor do not signal apoptosis upon ligation, they are not grouped with the death receptors. A complete list of proteins with DDs is shown in figure 1.

DD structure

Several of the DDs discussed in the previous paragraph and shown in figure 1 are so divergent that a sequence similarity cannot be detected by conventional sequence comparison and database search methods. More sophisticated methods, like the generalized profile technique [50] or Hidden Markov Model (HMM) searches [63], are necessary to establish the relationship of the DD family and statistical significance. This fact, in combination with the less-than-impressive-looking family alignment, raises the question whether there is some independent evidence supporting this grouping. An even more important question is whether an evolutionary relationship between all DDs also implies some common function.

Support for a meaningful relationship of divergent DDs comes from an analysis of 3D structures. The solution structure of the Fas DD, obtained by nuclear magnetic resonance (NMR) spectroscopy, was the first to become



(B)

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Fas          230 SKYITTIAGVM....TLSQVKGFVRKNGVNEAKIDEIKNDNVQ......DTAEQKVQLLRNWHQLHG.KKEAYDTLIKDLKKANLCTLAEKIQTII
TNF-R55      356 PATIYAVVENV....PPLRWKEFVRRLGLSDHEIDRLELQNGR......CLREAQYSMLATWRRRTPRREATLELLGRVLRDMDLLGCLEDIEEAL
TRAMP        332 GPQLYDVMDAV....PARRWKEFVRTLGLREAEIEAVEVEIGR.......FRDQQYEMLKRWRQQQP...AGLGAVYAALERMGLDGCVEDLRSRL
TRAIL-R1     361 TETLMLFFDKFANIVPFDSWDQLMRQLDLTKNEIDVVRAGTA.......GPGDALYAMLMKWVNKTG.RNASIHTLLDALERMEERHAKEKIQDLL
TRAIL-R2     335 TETIRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAA.......GHRDTLYTMLIKWVNKTG.RDASVHTLLDALETLGERLAKQKIEDHL
NGF-R        344 REEVEKLNGS....ACDTWRHLAGELGYQPEHIDSFTHE...........ACPVRALLASWATQD...SATLDALLAALRRIQRADLVESLCSES
OPG     NT   278 EEAFFRFAVPT..KIIPNWLSVLVDSLPGTKVNAESVERIKRR.....HSSQEQTFQLLKLWKHQNK.AQDIVKKIIQDIDLCEN.SVQRHIGHAN
OPG     CT   203 ENSVQRHIGHA..NLTFEQLRSLMESLPGKKVGAEDIEKTIKA.....CKPSDQILKLLSLWRIKNG.DQDTLKGLMHALKHSKTYHFPKTVTQSL
FADD          97 LCAAFNVICDN....VGKDWRRLARQLKVSDTKIDSIEDRYPR......NLTERVRESIRIWKNTEK.ENATVAHLVGALRSCQMNLVADLVQEVQ
RIP          583 TDKHLDPIREN....LGKHWKNCARKLGFTQSQIDEIDHDYER.....DGLKEKVYQMLQKWVMREGIKGATVGKLAQALHQCSRIDLISSIIYVS
TRADD        231 SLKDQQTFARS....VGLKWRKVGRSLQRGCRAIRDPALDSLAYEYEREGLYEQAFQLLRRQVQAEG.RRATIQRLVPALEENELTSLAEDILGLT
RAIDD        116 SDRQINQIAQR....LGPEWEPVVLSLGLSQTDIYRCKANHPH......NVQSQVVEAFIRWRQRFG.KQATFQSLHNGLRAVEV.DPSLLIHMLE
ANK1        1402 AEMKMAVISEH....LGLSWAELARELQFSVEDINRIRVENPN......SLLEQSVALLNLWVIREG.QNANVENLYTALQSIDRGDIVNMIEGSG
ANK2        3536 IEERLAYIADH....LGFSWTELARELDETEEQIHQIRIENPN......SLQDQSQYLLKIWLERDG.KHATDTNLVECLTKINRMDIVHLVETNT
Unc-44      1499 EKDIPEFVHQNVLKGIGADWPRLGRALEVPHRDIQHIRQNYP.........GQECKNTLKIWIHLKK.EDANQDNLDQALRQIGRDEIVRSIAYGE
DAP-Kinase  1311 RRKLSRLIDPP..DPLGKDWCLLAMNLGPDLVAKYNTNNGAP....KDFLPSPLHALLREWTTYP...ESTVGTLVSKLREIGRRDAADLILKAS
K12C11.4    1316 RCELACLIDPP..HAMGRDWSILAVKLQLTDQVPDVDSTGQSL.........SRTDQLLNEWAIHHP.EQASVGNLCRILVEIGRCDARDAIYRTV
Unc-5        855 KDELARLIDMP..NESHSDWRGLAKKLHYDRYLQFFASFPD..........CSPTSLLIDLWEASSSGSARAVPDLIQTLRVVGRPDAVMVIERFL
Unc-5H1      816 RQKIIASIDPP..CSRGADWRTLAQKLHIDSHLSFFASK............PSPTAMILNLWEARHF.PNGNIGQLAAAVAGIGQPDAGLFTVSEA
Unc-5H2      863 RQKICNSLDAP..NSRGNDWRLLAQKLSVDRYLNYFATK............ASPTGVILDLWEARQQ.DDGDLNSLASALEEVGKSEMLVAYTTDG
p84          570 TGEQIEVFANK....LGEQWKILAPYLEVKDSEIRQIECDSE.......DMKMRAKQLLVAWQDQEG.VHATPENLGNALNKSGLSDLAESITNDN
MyD88         32 RLSFFLNVRTQ....VAADWTALAEEMDFEYLEIRQLETQ..........ADPTGRLLDAWQGRPG...ASVGRLLELLTKIGRDDVMPLEIGPSI
NF-kB105     817 KLQLYKLIEIP...DPDKNWATLAQKLGIGILNNAFRLS.............PAPSKTLVDNLEVSG....GTVRELVDALRQVGYTDAIEVIQAAS
NF-kB100     774 LQNLEQLLDGP...EAQGSWAELAERLGIRSLVDTYRQT............TSPSGSLIRSVELAG....GDIAGLIPALSDVGLEEGVRLIRGPE
Pelle         42 RAQLCAHLDAL.....DVWQQLATAVKLYPDQVEQISSQKQR.......GRSASNEPLNIWGGQY...NHTVQTLFALFKKIKLHNAVRLIKDYV
IRAK          27 MCRFYKVMDAL....EPADWCQFAALIVRDQTELRLCERSG...........QRTASVIWPWINRNA.RVADIVHITTHLQLLRARDIITAWHPPA
IRAK2         13 LDDICRNMDAL....SEWDWMEFASYVITDLTQIRKIKSMERV......QGVSITREILWWWGMRQ....AIVQQLVDLLCRIELYRAAQIILNWK
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Figure 1. The death domain. (*A*) Domain structure of representative proteins containing DDs, drawn to scale. Whenever available, the human sequence is depicted. Orthologs from closely related organisms are not shown. Homology domains are indicated by shaded boxes; the domain names are abbreviated as follows: DD for death domain, DED for death effector domain, CARD for caspase recruitment domain, C for TNF-receptor cysteine-rich region, Ank_repeats for regions containing multiple copies of the ankyrin repeat, Ig for immunoglobulin domain, T1 for thrombospondin type 1 domain, Rel for rel/NF-κB DNA binding domain, TIR for Toll/IL1-R domain. Membrane-spanning regions are drawn by vertical open bars. Accession numbers of the proteins are given in square brackets. Accession numbers starting with the letters O, P, Q are from SwissProt/TrEMBL. Otherwise, no such accession number is available, and the GenBank/EMBL accession number of the corresponding DNA is shown instead. (*B*) Multiple alignment of the DDs shown in (*A*). Positions with more than 50% identical or similar amino acids are shown on black or grey background, respectively. (*C*) Dendrogram constructed from the multiple alignment of (*B*) using the neighbor-joining algorithm [108].

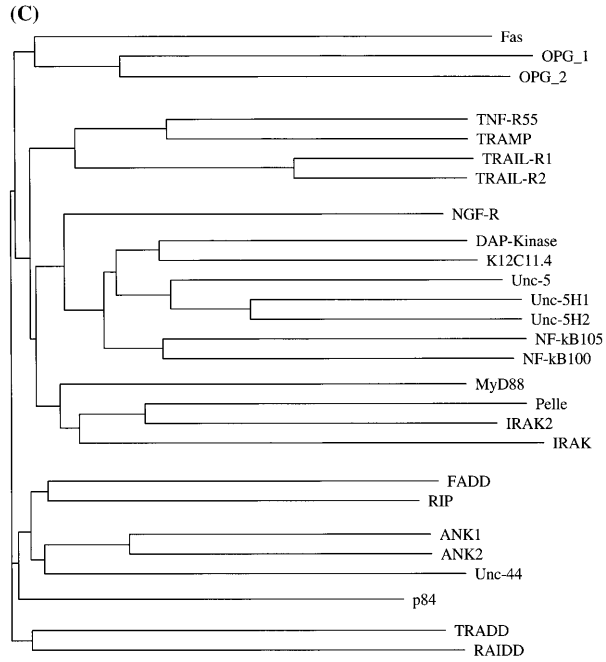


Figure 1. Continued.

available [64]. The Fas DD is relatively closely related in sequence to the DDs of the other death receptors and to that of TRADD, FADD and RAIDD. Its solution structure exhibits a novel fold, consisting of six antiparallel α-helices which form a compact bundle. The N- and C-termini of the domain are in close vicinity, a property that allows the DD to be inserted into a heterologous sequence with minimal structural disruption, a hallmark of truly modular domains. The well-ordered region in the NMR structure is in good agreement with the domain boundaries determined by sequence comparisons and truncation experiments. Analysis of the electrostatic properties of the surface revealed a high proportion of charged residues. The structural study also included several examples of site-directed mutagenesis of the Fas DD and allowed a molecular interpretation of resulting binding data as well as data obtained from other mutagenesis experiments. The substitution of several charged residues on the surface of helices $\alpha 2$ and $\alpha 3$ abolished self-association and FADD binding, making this region a likely interaction surface [64, 65]. No information about a likely interaction stoichiometry could be derived from the DD structure.

The 3D structure of the NGF-R DD was the second to be determined [66]. The DD of NGF-R is only very distantly related to the classical DDs. The fact that the fold of the NGF-R DD is very similar to the fold of the Fas DD can be considered as a proof of the distant sequence relationship detected by profile analysis. Helices $\alpha 2$ to $\alpha 6$ of the two DD structures are similar in orientation and can be superimposed with a good root mean square (RMS) value. However, helix $\alpha 1$ has a very different orientation in the two structures, reflecting that fact that there is only little sequence conservation in this region within the DD family. The NGF-R is not only untypical in its divergent DD, it also binds to a ligand not belonging to the TNF family. Not much is known about potential interaction partners of its cytoplasmic region.

A better understanding of the structural requirements of DD/DD interactions will probably require more 3D structures of classical DDs to be solved. Even more desirable would be the structure of a cocrystal of two DDs caught in the act of binding to each other.

DD interactions

Does the fact that the first characterized DDs interact with each other in the receptor-proximal steps of apoptotic signaling mean that all other DDs also interact with each other and play a role in apoptosis? According to current knowledge, the answer to the first question is probably yes. All DDs studied so far have been shown to interact with at least one other DD, either homotypically or heterotypically with a different DD protein. In the death receptors, the DD might be involved in the trimerization of the intracellular domain upon binding of a ligand to the extracellular domain. The DDs of Fas, TRAIL-R1 and TRAIL-R2 bind to the DD of FADD. The DD of TNF-R55 binds to TRADD, which in turn binds to the DDs of FADD and RIP, the latter of which binds the DD of RAIDD. In all of these cases, it is not clear whether the interaction is a dimerization or a trimerization, or whether the stoichiometry is variable. The solution structures of the death domains of Fas and NGF-R, together with the available mutagenesis data, also give no clear idea of how this interaction looks at the molecular level. No interaction data are available on the other DDs shown in figure 1. However, biological knowledge about the pathways in which these proteins work allows some educated guesses. Osteoprotegerin is a decoy receptor for TRANCE/RANK-L and is involved in the regulation of osteoclastogenesis. Its two DDs probably help to keep this soluble receptor in a trimeric form, since a soluble receptor cannot rely on ligand-induced trimerization. For the DD of NGF-R, no target has been identified either. Here, too, it is conceivable that the DD induces formation of a constitutive complex of two or three receptor monomers, a hypothesis supported by the no-

tion that in contrast to the death receptors, NGF-R generates a signal when it is no longer bound by a ligand. NF-κB is a proinflammatory transcription factor, which is activated by TNF and interleukin 1 (IL1) signaling [67]. Pelle and tube are two *Drosophila* proteins acting in the signaling pathway from the surface receptor Toll to the transcription factor Dorsal, an insect homologue of NF-κB [68]. Pelle has two mammalian homologues called interleukin 1 receptor associated kinase and IRAK2, signaling from IL1-R to NF-κB [67, 69, 70]. It appears that MyD88, a DD protein that interacts with IL1-R, together with IRAK and possibly a mammalian Tube homologue, forms an interaction chain connecting IL1-R with NF-κB [69, 71]. No interaction partners have been described for DAP-kinase, ankyrin/Unc-44 and Unc-5. Since these three proteins contain the only known DDs in the almost completed *Caenorhabditis elegans* genome, it is likely that two of them, if not all three, form a complex in the nematode. The axonal guidance proteins Unc-5 and Unc-44 have multiple homologues in mammals, suggesting that a similar pathway of cell contact regulation is conserved throughout evolution of multicellular animals.

The latter examples show that probably not all DDs are involved in death signaling. The common denominator in DD function is the interaction with other DDs. The extensive recruitment of DD-based interaction networks for the signaling of apoptosis seems to be an evolutionarily recent invention, parallel to the occurrence of death receptors.

The death effector domain

After the discovery of the three classical death adaptor proteins FADD, TRADD and RIP, and the appreciation of their role in signal transduction, the identification of downstream components became a central issue. Upon overexpression, each of the three proteins was able to induce apoptosis. However, an important difference became apparent when using truncated versions of the proteins, containing either the DD or the non-DD portion. In the case of TRADD and RIP, the two adaptors found associated with the TNF-R55 complex, an intact DD was necessary for triggering cell death. In contrast, overexpression of the FADD DD was ineffective, whereas overexpression of a protein containing only the non-DD C-terminal part consistently induced apoptosis [42, 43, 47]. To reflect this fact, the C-terminal domain of FADD was hence termed death effector domain (DED). The general importance of FADD and the DED in death receptor-induced apoptosis was underscored by the finding that the FADD DD binds to the TRADD DD, thus recruiting FADD to the TNF-R55 signaling complex. In addition, FADD was found associated with other death receptors like Wse (TRAMP/DR3) and TRAIL-R (DR4) [54, 72], although this result is not supported by all published reports. FADD uses its DD to associate with the receptors, either directly in the case of Fas or indirectly via the TRADD DD in the case of TNF-R55. The TRADD DD, in complex with TNF-R55, can also recruit RIP, which in turn can recruit RAIDD. The resulting alternative route to apoptosis induction will be discussed below in the context of the caspase recruitment domain (CARD). TRADD and RIP also have a role outside of cell death signaling. They are able to recruit members of the TRAF (TNF-receptor associated factor) family to the receptor complex, an interaction that subsequently leads to NF-κB activation [73]. The multitude of proteins in the TNF-R55 complex, with signaling pathways branching into several directions, certainly contributes to the pleiotropic nature of TNF effects.

The apoptosis-inducing activity of the DED initiated a search for interaction partners which might constitute downstream components in death receptor signaling pathways. In parallel, exhaustive sequence analysis was applied to identify further proteins with regions similar to the DED, with the rationale that those proteins might also be capable of inducing apoptosis. The experimental approach was faster to yield results: using a yeast two-hybrid screen, a DED-interacting protein with a very interesting domain structure was identified [74, 75]. This protein, initially termed Fas-linked ICE-like protease (FLICE) or Mach, and now renamed to caspase-8, contains two DEDs at the N-terminus. The C-terminus strongly resembles the caspases, a group of proteases related to interleukin-1β converting enzyme (ICE), with a well-established connection to the execution phase of apoptosis. Caspases are known to be synthesized as inactive precursors that need to be activated by proteolytic cleavage, usually performed by other caspases [28, 29]. The resulting activation cascade, or possibly even a feedback network, eventually activates caspase-3 (CPP32), an enzyme that is known to cleave a number of noncaspase apoptotic substrates [28, 29]. Initially, it was not clear which caspase was the most upstream one, and how the signal was transduced from the death receptors to the proteolytic cascade. The identification of a caspase that interacts with the death adaptor molecule FADD, and which is found in the Fas signaling complex, made it a prime candidate for this function. Recently, it was demonstrated that the induced proximity of two or more molecules of caspase-8 in

the signaling complex is sufficient for autoproteolytic activation of this enzyme [76]. The complex formed by Fas, FADD and caspase-8 is occasionally referred to as DISC (death-inducing signaling complex).

A second interesting aspect of the caspase-8 domain structure was the presence of two DEDs at the N-terminus, which is also the region that interacts with the DED of FADD. Like the DD, the DED thus appears to be a heterodimerization domain. The region of detectable sequence conservation between the DED of FADD and the two DEDs of caspase-8 spans approximately 80 residues, which is similar in size to the DD.

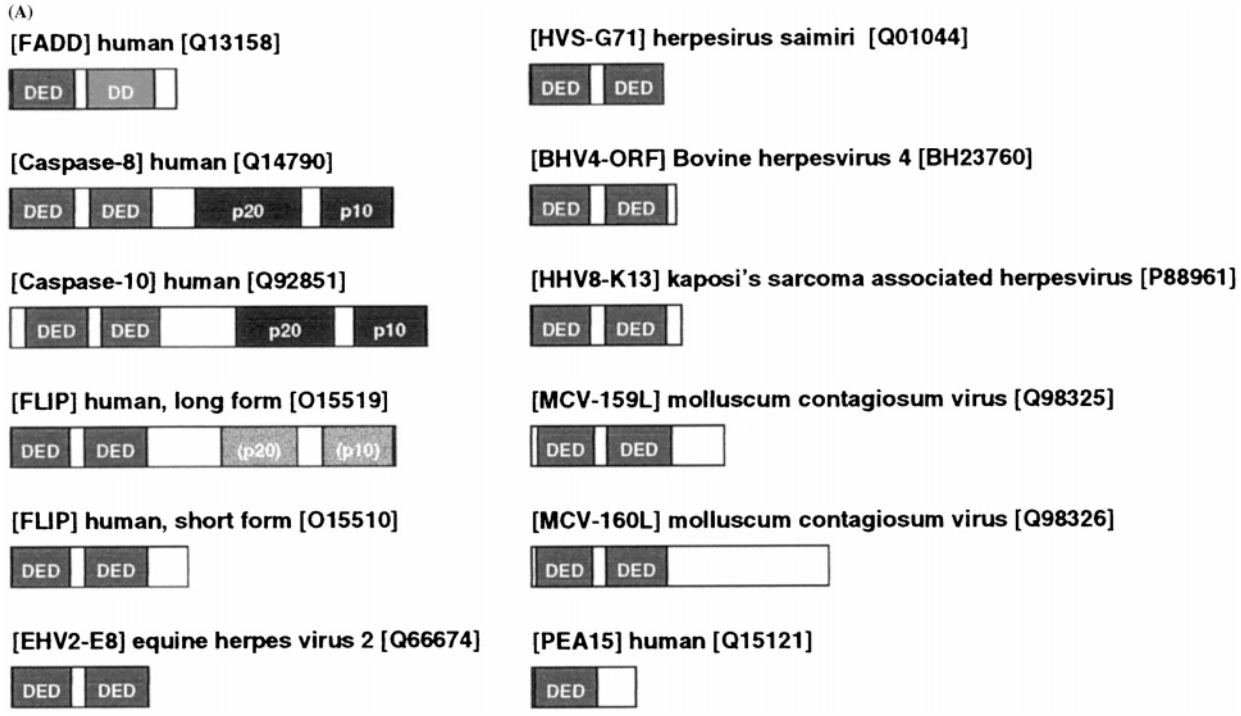
The in silico search for further DED-containing proteins was hampered by the fact that at the time FADD was the only known DED protein. Using profile techniques, it was possible to identify a single copy of the DED at the N-terminus of the astrocytic phosphoprotein PEA-15, and two DED copies in members of a protein family encoded by several γ-herpesviruses and the poxvirus MCV (molluscum contagiosum virus) [77]. The experimental discovery of caspase-8/FLICE and its interaction with FADD directed the attention of the field to these viral proteins, since their double DED arrangement strongly resembled the N-terminus of caspase-8. It is a well-known fact that viruses frequently encode proteins with a specific function in evading the host's immune system, or in the prevention of host cell apoptosis [15–17]. It was thus an obvious idea that the viral DED proteins might interfere with the apoptosis-inducing FADD/caspase-8 interaction. Indeed, three independent studies demonstrated that several of the viral DED proteins, namely E8 from equine herpes virus 2 (EHV2), open reading frame (ORF) 71 from herpesvirus saimiri, K13 from human herpesvirus 8 (HHV8, Kaposi sarcoma associated-herpesvirus) and MC159 from MCV, were able to bind the DEDs of FADD and/or caspase-8, and to disrupt the interaction of the two cellular proteins [77–79]. For that reason, the viral proteins were termed v-FLIPs (viral FLICE inhibitory proteins). Among the tested proteins, only MC160 from MCV was not able to function as a v-FLIP. The role of this ORF, which in the MCV genome is adjacent to functional v-FLIP MC159, as well as its interaction partner still remain elusive.

It is not uncommon for viral host interaction proteins to be derived from cellular proteins after adaption to the virus's needs. The v-FLIPs are relatively closely related to a host-encoded protein termed c-FLIP [80–84]. The cellular protein occurs in a number of different splice forms. The most prominent ones are a short form (c-FLIP$_S$) whose architecture is essentially the same as those of the v-FLIP family. In addition, a long form (c-FLIP$_L$) has a domain structure resembling that of FLICE. The two N-terminal DEDs are followed by a linker and the two domains (p10 and p20) necessary to constitute a caspase. However, in contrast to FLICE, the long form of c-FLIP is not an active caspase. The two catalytically important cysteine and histidine residues are replaced by noncatalytic residues, and no caspase activity could be observed. Since the inactive caspase domain is able to interact with the catalytic domain of caspase-8/FLICE, the long form of c-FLIP is an even more potent inhibitor of FADD-mediated FLICE activation. Since c-FLIP was identified independently by at least eight different groups, a number of other names are also in use, among them CASPER, CLARP, MRIT, I-FLICE, CASH and usurpin. The c-FLIP protein is clearly related to caspase-8/FLICE by evolution. The two proteins have the same domain structure and share considerable sequence homology. In addition, the genes for c-FLIP, caspase-8/FLICE and the FLICE-related caspase-10 lie adjacent to each other on chromosome 2q33–34 [84].

Apparently, there have been multiple events of viral FLIP acquisition: the v-FLIPs of the γ-herpesviruses are relatively closely related to each other and to c-FLIP. In contrast, as can be seen from figure 2C, the v-FLIP of herpesvirus saimiri is very different and its C-terminal DED is more closely related to the N-terminal DED of the same protein than to the C-terminal DED of other FLIPs. It is conceivable that this virus acquired a single DED from the host genome and duplicated it later. MCV as a poxvirus belongs to a very different viral class; it must be assumed that MCV acquired its v-FLIP independently from the herpesviruses. The domain structure and sequence conservation of DED-containing proteins is shown in figure 2.

DED structure

Based on similarities in size, function, predicted secondary structure and a marginal sequence homology, an evolutionary relationship between the DD and the DED has been proposed [85]. This prediction was confirmed recently, when the NMR-derived solution structure of the FADD DD became available [86]. Like the two known structures of DDs, the DED forms a bundle of six antiparallel helices. The C_α atoms of the FADD DED and the NGF-R DD structures can be superimposed with a root mean square (RMS) distance of 2.1 Å. The ordered part of the structure corresponds well with the domain boundaries determined from sequence analysis. The



(B)

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FADD             3 PSLVLLHSVSSSLSSSE.LTELKFLCLGRVGKR.KLERVQSGLDLFSMLLEQNDLEPGHTELLRSLLASLRRHDLLRRVDD
Caspase-8  NT    2 DFSRNLYDIGEQLDSED.LASLKFLSLDYIPQR.KQEPIKDALMLFQRLQEKRMLEESNLSFLKELLFRINRLDLLITYLN
Caspase-8  CT  100 AYRVMLYQISEEVSRSE.LRSFKFLLQEEISKC.KLDDDMNLLDIFIEMEKRVILGEGKLDILKRVCAQINK.SLLKIINE
Caspase-10 NT   19 SFREKLLIIDSNLGVQD.VENLKFLCIGLVENK.KLEKSSSASDVFEHLLAGDLLSEEDPFFLAELLYIIRQKKLLQHLNC
Caspase-10 CT  114 LFRNMLYELSEGIDSEN.LKDMIFLLKDSLLK.....TEMTSLSFVAFLEKQGKIDEDNLTCLEDLCKTVVP.KLLRNIEK
FLIP       NT    6 MSAEVIHQVEEALDTDE.KEMLLFLCRDVAIDV....VPPNVRDLLDILRERGKLSVGDL...AELLYRVRRFDLLKRILK
FLIP       CT   97 DYRVLMAEIGEDLDKSD.VSSLIFLMKDYMGRG.KISKEKSFLDLVVELEKLNLVAPDQLDLLEKCLKNIHRIDLKTKIQK
EHV-E8     NT    1 MSHYSMIDTYFSLDEDE.TETYLYLCRDLLKNK...GEFQCTRDAFKFLSDYACLSAANQ...MELLFRVGRLDLIRRIFG
EHV-E8     CT   92 PFRCLMALVNDFLSDKE.VEEMYFLCAPRLESHLEPGSKKSFLRLASLLEDLELLGGDKLTFLRHLLTTIGRADLVKNLQV
HVS-71     NT    2 DLKTTVLHITDSFTDEE.MYCLLFLINGCLFR......SCNAVNISDLLIETLSKSTQWDICLMQCLYVLRKIGLLLNLFQ
HVS-71     CT   93 THVLTLVNVNNNLTAKD.EKRLCFILDQFPRN.VAAPSVILCVFSNMLCEMHVLECLCQ..LKKCLKQIGRSDLAKTV..
BHV4-ORF   NT    2 VTRDVLLAIETHLNQNEKTFVMYFLLDPYIP.........KECEDFLPTLENLHSKRKIIYPILLISLMYILQRPDLLRSIFL
BHV4-ORF   CT   94 PYKQLIFSIGQNIDDED.LISIKFTSMNYIGKS..PSKIKNYLDWVRALEKVAMVGPDNLDLFETLFKQIHRMDIVKMIKN
HHV8-K13   NT    2 ATYEVLCEVARKLGTDD.REVVLFLNVFIEQ....PTLAQLIGALRALKEEGRLTFPLL...AECLFRAGRRDLLRDLLH
HHV8-K13   CT   93 PYQLTVLHVDGELCARD.IRSLIFLSKDTIGSR...STPQTFLHWVYCMENLDLLGPTDVDALMSMLRSLSRVDLQRQVQT
MCV-159L   NT    8 PSLPFLRHLLEELDSHE.DSLLLFLCHDAAFG......CTTVTQAICSLSQQRKLTLAAL...VEMLYVLQRMDLLKSRFG
MCV-159L   CT   95 RYRKLMVCVGEELDSSELRALRLFACNLNPSLSTALSESSRFVELVLALENVGLVSPSSVSVLADMLRTLRRLDLCQQLVE
MCV-160L   NT    8 IPFSFLRNLLAELDASE.HEVLRFLCRDVAFA......SKTAEDALRALQRRRLLTLSSM...AELLCALRRFDVLKVRFG
MCV-160L   CT   95 QYRLQVAAINNMVGSEDLRVMCLCAGKLLPP....SCTPRCLVDLVSALEDAGAISPQDVSVLVTLLHAVCRYDLSVALSA
PEA15            3 EYGTLFQDLTNNLTLED.LEQLKSACKEDIPSE.KSEEITTGSAWFSFLESHNKLDKDNLSILEHLFEISRRPDLLTMVVD
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(C)

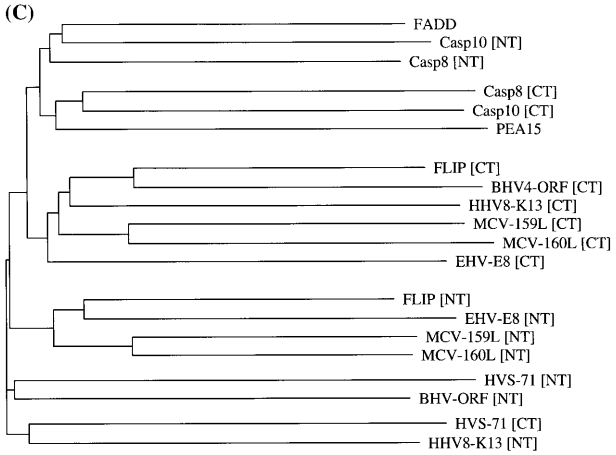

Figure 2. The death effector domain. (*A*) Domain structure of representative proteins containing DEDs, drawn to scale. Whenever available, the human sequence is depicted. Orthologs from closely related organisms are not shown. Homology domains are indicated by shaded boxes. Domain names are abbreviated as described in figure 1, with the following addtions: p20 or p10 (on grey background) for subunits of caspase catalytic domains, same on light grey background for catalytically inactive caspase domains. (B) Multiple alignment of the DEDs shown in (*A*). Positions with more than 50% identical or similar amino acids are shown on black or grey background, respectively. (*C*) Dendrogram constructed from the multiple alignment of (*B*) using the neighbor-joining algorithm [108].

surface of the DED structure contains an extended hydrophobic patch that might form the domain interaction surface. However, it is not clear whether a dimer or a trimer is the preferred composition of the complex. Another interesting problem lies in the structural basis for the specificity of DED interactions. While the DD and DED structures are generally similar, DD and DED interactions have only been found with members of the same domain class—no DD/DED interaction has been described as yet. No direct clues for the origins of this specificity can be obtained from the currently available structures. Again, a cocrystal of two interacting domains would be very useful to address this question.

The caspase recruitment domain

The discovery of RAIDD, a death adaptor protein with a novel domain structure, has revealed interesting parallels in the recruitment of caspases to the various death receptors [53]. On the one hand, FADD binds to the Fas receptor by means of its C-terminal death domain and recruits caspase-8 by its N-terminal DED. On the other hand, RAIDD engages its DD, which is also located at the C-terminus, to bind to the TNF-R55 signaling complex. The N-terminus of RAIDD, which has no detectable similarity to the DED of FADD, binds to the pro-domain of caspase-2 (ICH-1), thereby recruiting the caspase to the receptor complex [53]. As in the case of FADD, overexpression of RAIDD induces apoptosis. A dominant-negative suppression of the RAIDD/caspase-2 interaction does not abolish TNF-induced apoptosis; this is not too surprising since TNF-R55 can also signal cell death by the TRADD/FADD pathway. Interestingly, the N-terminal region of RAIDD has a remarkable sequence similarity to the N-terminus of caspase-2. The two sequences are 32% identical over 80 residues, which corresponds to the entire pro-sequence of caspase-2. As a third protein, the cell death caspase Ced-3 from the nematode *C. elegans* contains in its pro-domain a region with obvious similarity to the N-terminus of RAIDD and caspase-2. In fact, the pro-domain of Ced-3 is able to bind the N-terminus of RAIDD, although this interaction is hardly physiological, given that the proteins come from two very different organisms.

Evidently, this is the third example of a heterodimerization domain used in apoptosis signaling pathways. This idea is strongly supported by results from exhaustive sequence database searches. By using profile and HMM methods, the family of proteins containing similarity to this domain could be extended considerably [85]. Most important, all of the proteins containing this domain are thought to function in cell death signaling; in the meantime, their mutual interactions could be demonstrated in several instances. In all cases studied, the function of this domain is to recruit caspases to an upstream signaling complex. For that reason, the domain has been termed CARD for caspase recruitment domain. When using sensitive sequence comparison methods, it can be seen that the CARD is not restricted to caspase-2 and Ced-3 but is present also in other caspases including caspase-1 (ICE), caspase-4 (ICH-2), caspase-5 (ICH-3), caspase-9 (Mch6) and the recently described caspases 11, 12 and 13. In addition, caspase recruitment domains are found at the N-terminus of the *C. elegans* cell death protein Ced-4 and its mammalian homologue APAF1, in the central region of two cellular inhibitor of apoptosis (c-IAP) proteins, and at the N-terminus of a recently identified RIP-like kinase termed CARDIAK (for CARD containing ICE-associated kinase), RICK or RIP2 [87–89]. A viral CARD protein is also known: in analogy to the ORF E8 of equine herpesvirus 2, which encodes a v-FLIP, the protein E10 contains a CARD domain [85]. Including the Bcl-2 like protein E5, this virus possesses at least three different proteins with a role in the inhibition of host cell apoptosis [17]. An overview of the domain structure and the sequence conservation of CARD domain-containing proteins is given in figure 3.

For several of these CARD proteins, interaction partners have been described, and their role in the induction of apoptosis is widely appreciated. In the nematode *C. elegans*, the two proapoptotic genes Ced-4 and Ced-3 have been shown to bind to each other [90, 91]. Experiments with truncated mutants have detected a twofold interaction between the proteins: one of them is mediated by the two N-terminal CARD domains; the other is mediated by the C-terminal portions of the two molecules. APAF1, the mammalian homologue of Ced-4, interacts with caspase-9 (Mch6, APAF3) and thus induces apoptosis in response to the liberation of cytochrome c from mitochondria [92, 93]. CARDIAK (RICK/RIP2), a protein containing a C-terminal kinase domain related to RIP, binds to caspase-1 (ICE), mediated by their respective CARD domains [89]. Binding of this protein to the CARD domains of IAP1 and IAP2 has also been described [87–89]. The physiological role of the CARDIAK interactions still remains to be established.

For the other CARD proteins, binding partners have not yet been identified. However, potential binding partners are suggested by the pathways the proteins are known to work in. While the IAP proteins are homologues of caspase inhibitors first described in

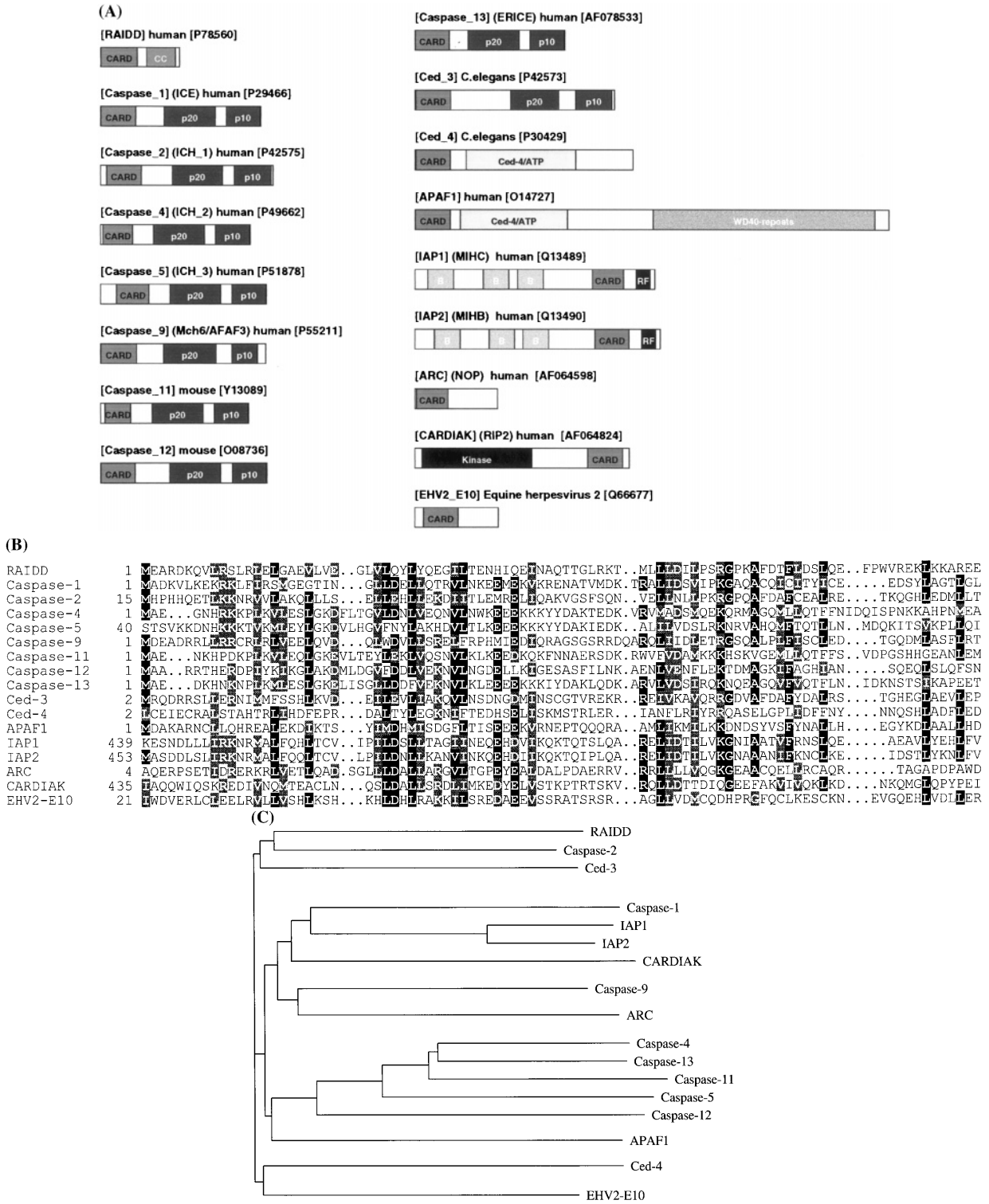

Figure 3. The caspase recruitment domain. (*A*) Domain structure of representative proteins containing caspase recruitment domains, drawn to scale. Whenever available, the human sequence is depicted. Orthologs from closely related organisms are not shown. Homology domains are indicated by shaded boxes. Domain names are abbreviated as described in figures 1 and 2, with the following addtions: Ced-4/ATP for Ced-4 type ATPase domain, B for a single BIR repeat domain, RF for a RING-type Zn-finger. (*B*) Multiple alignment of the caspase recruitment domains shown in (*A*). Positions with more than 50% identical or similar amino acids are shown on black or grey background, respectively. (*C*) Dendrogram constructed from the multiple alignment of (*B*) using the neighbor-joining algorithm [108].

baculoviruses [94, 95], it is not entirely clear whether the role of IAP1 and IAP2 is predominantly in apoptosis inhibition. Both proteins have been described to be components of the TNF-R75 and CD40 signaling complexes. These two receptors are known to engage caspases, although their main role is not in cell death signaling. It is conceivable that the cellular IAPs serve to recruit one or more of the CARD caspases to these nonapoptotic receptors. The γ-herpesvirus protein E10 is of particular interest since it underscores the parallels to the disruption of DED signaling by E8. Interaction partners of E10 are not yet known, but it is tempting to speculate that the interaction of RAIDD with caspase-2 or of APAF1 with caspase-9 are likely targets.

CARD structure

During the database searches for CARD proteins, cross-matches between the CARD-specific profiles and proteins containing DDs or DEDs were noted [85]. In addition, all three domains have approximately the same size; the prediction of the secondary structure elements by the PHD program [96] revealed a similar arrangement of five to six α-helices. Since all three domains have a propensity to form heterodimers or -trimers, it has been suggested that they are related by evolution and possess an identical fold [85]. As has been discussed above, the fold of the classical and nonclassical DDs is very similar to the fold of the DED. Recently, the 3D structure of the RAIDD CARD domain has been analyzed by NMR spectroscopy [97]. In agreement with the predictions, the architecture of the CARD domain is similar to both the DD and the DED fold. Again, the well-ordered part of the structure corresponds to the region where sequence similarity is observed. In the CARD structure, the orientation of helices α1 and α6 of the six-helix bundle is somewhat different from the arrangement found in the DDs. Nevertheless, the topology is identical, and the structural core formed by the four-helix bundle comprising helices α2–5 can be superimposed reasonably well. In order to identify the regions conferring specificity to the CARD/CARD interaction, the authors used their structure as a template for modeling the corresponding domains in Ced-3, Ced-4, APAF1 and caspase-9. Based on the analysis of the surface charge distribution in the NMR-derived structure and in the models, the authors suggest an interaction mode where helices 1, 3 and 4 of one domain bind to a surface formed by helices 2, 5 and 6 of the other domain. In this interaction mode, the specificity is determined mainly by charged surface patches. This is opposed to the model of the DED-mediated FADD/FLICE interaction, where hydrophobic surface regions have been implicated [86].

The modular nature of caspase activation

Members of the superfamily of six helix bundle interaction domains, comprising DD, DED and CARD, are used in multiple ways in the intermediate layer between death receptor ligation and caspase activation. The current knowledge about these death adaptor interaction pathways is summarized in figure 4. The final interaction in each of the death adaptor cascades is the association of an adaptor with the prodomain of a caspase. Caspases are also the driving force in the execution phase of apoptosis by cleaving a number of substrates, including cytoskeletal proteins, kinases and nucleases. It is most likely the combined effect of these cleavages that eventually leads to the expression of the apoptotic phenotype. However, there is ample evidence that the caspases activated by the death receptors are different from those cleaving the death substrates. All caspases are synthesized as inactive pro-forms and can be activated by the combination of proteolytic removal of an N-terminal pro-sequence and an internal cleavage separating the p20 and p10 catalytic subunits [28, 29]. This activation cleavage invariably requires aspartate-directed proteases, like the caspases themselves or granzyme B. It is therefore reasonable to assume a whole caspase activation cascade, having the receptor-activated caspases in the top layer and the execution caspases in the bottom layer. The intermediate layers are less well understood. Caspase research is hampered by the lack of specific inhibitors for single caspases and by the fact that these enzymes are quite promiscuous in cleaving each other in vitro. It is not clear which of these reactions is really relevant in vivo. At least in some cases, noncaspase proteins might be required for the intermediate layers of the caspase cascade. Recently, Bid, a protein that contains a BH3-motif implicated in binding to members of the Bcl-2 family, has been shown to be cleaved by caspase-8 [98, 99]. This cleavage leads to an efflux of cytochrome c from the mitochondria, which in turn is able to activate caspase-9 through the APAF1 CARD-mediated pathway.

The caspases can be grouped into subfamilies by different criteria. One possible criterion is cleavage specificity, which is correlated with the sequence and structural details of the catalytic domain. While all caspases cleave proteins after an aspartate residue, it is known that other sequence requirements have to be

fulfilled in order to make a peptide a good caspase substrate. A number of systematic studies have addressed this specificity, which typically resides in a four-residue region upstream of the cleavage site [100, 101]. Caspase-1 (ICE) for example prefers the sequence YVHD, which occurs in its physiological substrate IL1β. Caspase-3 (CPP32), a typical execution caspase, by contrast prefers the sequence DEVD, which is also recognized by several other caspases. A totally different classification criterion is the nature of the pro-domain, which is correlated with the mode of activation. Several caspases possess a short pro-domain of only a few residues. This group comprises caspase-3 (CPP32), caspase-6 (Mch2), and caspase-7 (Mch3, ICE-LAP3), all caspases with a bona fide role in the execution phase of apoptosis [100, 101]. The other caspases have much longer pro-domains; interestingly, all of them contain either two DED domains or a single CARD domain within their pro-sequence. It is likely that these enzymes are, by means of their pro-domain, recruited to various signaling complexes, in a stoichiometry that brings two or more caspase molecules into close proximity. In two cases, the DED-containing caspase-8 and the CARD-containing caspase-9, it has been shown that this induced proximity is sufficient for autoactivation of the caspase [76, 102]. The short pro-domain caspases, on the other hand, lack the recruitment domains and are most likely cleaved by a heterologous caspase.

The two classifications are truly independent: caspase-8 and caspase-3 have a similar cleavage specificity, although the former has a double DED pro-domain and the latter has a short pro-domain. The modularity of activation domain and catalytic domain is exemplified by Ced-3. The minimalistic apoptosis signaling pathway in *C. elegans* uses Ced-3 as the sole caspase. The catalytic domain of Ced-3 is closely related to the execution caspases, whereas the CARD-containing pro-domain is closely related to the receptor activated caspase-2. The nematode thus makes use of a single caspase that is activated by induced proximity but nevertheless is able to cleave the typical death substrates.

The limits of domain detection

A modular architecture is the hallmark of many signal transduction proteins involved in a wide variety of pathways. The detection of homology domains in such proteins has proved a valuable research tool, since specific functions can be attributed to many of them. The presence of such a domain in an otherwise uncharacterized protein of interest frequently allows a prediction of the protein's function and suggests further experiments. Some of the domain families are very divergent in sequence and might go undetected by conventional methods of sequence database searches. Even in the absence of readily detectable sequence relatedness, the structure and function of those domains can be conserved, at least in part. A number of freely available programs and servers accessible via

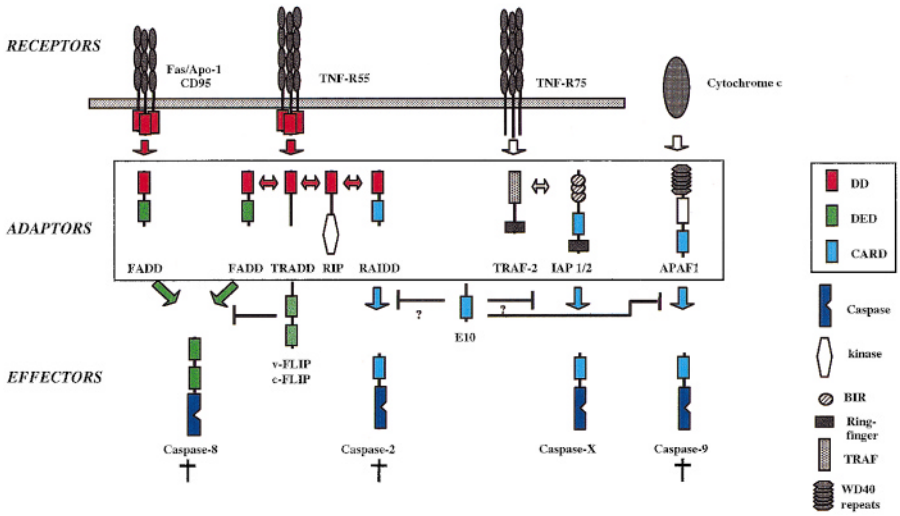

Figure 4. Overview of caspase activation pathways. The associations between death receptors and death adaptors, leading to caspase activation, are depicted. DDs, DEDs and CARDs are indicated by boxes in red, green or cyan, respectively. Interactions mediated by these domains are represented by arrows in the corresponding color. The symbols for the other domains are explained in the figure.

the Internet are devoted to the sensitive detection of these domains. Two useful WWW addresses are "http://www.isrec.isb-sib.ch/software/PFSCAN_form.html", which allows searches in the PROSITE profile database [103], and "http://www.sanger.ac.uk/Pfam", which searches Pfam [104]. Functions encoded by such homology domains include various catalytic activities, as well as interaction with other proteins, small molecules, lipid membranes, DNA or other subcellular structures. The domains described in the previous paragraphs are prominent examples here. The fact that they are all heterodimerization or -trimerization domains adds another dimension of usefulness to their detection. When looking for interaction partners of DD proteins, one might use a yeast two-hybrid screen or similar experimental techniques. In many cases, however, it is simpler to just scan the sequence databases for other DD and test the candidates for binding subsequently. Up to now, DDs have been found to interact exclusively with other DDs, but never with death effector- or caspase recruitment domains. DEDs and CARDs appear to be equally subfamily-specific in their interactions.

It might even be possible further narrow down this rule by speculating that closely related domains have a higher propensity to interact with each other. While there are several exceptions, a number of observations seems to support this stricter rule. First, many DDs, DEDs and CARDs appear to form homodimers. Second, when analyzing the dendrograms shown in figures 1C, 2C and 3C, there are several instances where interacting domains group together. In the DD dendrogram, FADD forms a cluster with RIP; so does TRADD with RAIDD and MyD88 with the Pelle/IRAK family. Even more conspicuous are the groupings in the CARD dendrogram, where RAIDD forms a cluster with caspase-2 and CARDIAK/Rip2 forms a cluster with caspase-1 and the IAPs. All of these groups have been described to also form interaction partners. One testable prediction derived from this hypothesis would be an interaction of DAP-kinase DD with Unc-5.

Because of the great deal of attention that the three domain families have received recently, their high degree of sequence divergence and the resulting difficulties in their identification, it might be not surprising that a number of DD, DED and CARD instances have been postulated that do not stand up to closer scrutiny. A classical case is the *Drosophila* protein reaper, a small death-inducing protein that has some apparent sequence similarity to the DD of TNF-R55. As has been discussed previously [51], the residues conserved between reaper and TNF-R55 are not the ones that are generally conserved in the DD family, suggesting that the similarity observed might just be a coincidence. While the alignment between reaper and TNF-R55 looks tempting, one should keep in mind that in today's large protein databases (with more than 300,000 nonredundant protein sequences), there are dozens if not hundreds of proteins that would give an even better-looking alignment with reaper. The visual inspection of alignments can be misleading, and the identification of new domains or domain instances should always be based on a rigorous statistical analysis, taking into account the size of the database. In the meantime, there is also experimental evidence arguing against a DD-type interaction in reaper-induced apoptosis [105]. Other proteins, for which death domains of dubious significance have been postulated, are MADD, Siva and Par-1. Similarly, there is not much statistical support for the existence of a DED in Ced-4, which has been suggested to reside in the N-terminus [106] or in the central region [107]. Since the former one colocalizes with the CARD, which is related to the DED, there might be some justification for this suggestion.

The power and relative ease of motif detection in sequence databases, in combination with the availability of large databases containing uncharacterized sequence material, has gradually changed the way apoptosis research is done today. The first components of the apoptotic signaling pathways (like e.g. Fas or TNF-R55) have been isolated by biological screens. Later, the focus shifted towards the detection of interaction partners (like e.g. FADD, TRADD, RIP), mainly by using the yeast two-hybrid system or other interaction traps. Most of the recently described signaling components were first identified in silico, by doing database searches with sequences or sequence motifs. A role of these proteins in apoptosis was only demonstrated a posteriori. FLIP, CARDIAK, ARC and the TRAIL receptors are just a few published examples. The characterization of several new proteins harboring DDs, DEDs and CARDs is well on its way and will certainly introduce new facets to this fascinating and most important signaling pathway.

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