Review

Peptides in Apoptosis Research

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Abstract: Apoptosis is a complex process that plays a central role in physiological and pathological cell death. This fast evolving research area has experienced incredible development in the past few years. Progress in the knowledge of the structure of many of the main molecular actors of the apoptotic signal transduction pathways has driven the design of synthetic peptides that in some cases can function as simplified versions of their parent proteins. These molecules are contributing to a better understanding of the activity and regulation of apoptotic proteins and also are setting the basis for the discovery of effective drugs to combat important diseases related to apoptosis. Most applications of peptides in apoptosis research are so far related to caspases, caspase regulatory proteins, such as IAPs and Smac, and proteins of the Bcl-2 family. Additionally, important perspectives are open to other systems, such as the macromolecular assemblies that are responsible for the activation of initiator caspases. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Apoptosis is the key biological process for the physiological death of metazoan cells. It is conserved through evolution and programmed at the genetic level and is necessary for cell recycling and normal tissue homeostasis [1–3]. Apoptosis is of special importance in processes like animal development, tissue regeneration, the functioning of the immune system and in the control and behaviour of pathological states. A complex network of pathways (Plate 1) links the fate of the cell to specific death or survival signals, which may be present in the cell surroundings or in the cytoplasmic interior [4–6]. Whenever

physiologically required, or as a consequence of cell cycle malfunctioning, toxicity, or at the periphery of acute lesions, a complete biochemical machinery will be activated and the cell will be prompted to die. But a failure in the regulation of apoptosis can be the origin of tissue degeneration or uncontrolled cell proliferation, giving rise to diseases such as degenerative disorders or cancer [7–9]. This pivotal role of apoptosis has attracted enormous interest, making it a preferred focus for drug discovery. Both the activation and the inhibition of apoptosis may be of interest for therapeutic use [10,11].

In this paper we review recent developments in the design and characterisation of peptides that have been used as tools for the study of apoptosis, and also as powerful regulators of this process for the control of related diseases. We start with a summary of the state-of-the-art of the field, which refers mainly to mammals. Similar signalling pathways and close homologues of apoptotic proteins are also known for other organisms, such

as *Drosophila melanogaster* and *Caenorhabditis elegans*, and are described in various recent excellent reviews [5,6,12,13]. After this overview, we continue with an update of the contributions of peptide research to the specific topic of apoptosis.

APOPTOTIC PATHWAYS

The complete process of apoptosis involves a large number of different proteins mainly located in the cell membrane, the cytoplasm, the mitochondrion and the nucleus [5]. According to their function, these proteins can be classified as receptors, adaptors, regulators and executioners. This last group is represented by caspases [14–16], a family of cysteine aspartyl proteases which can be, in principle, of two types: effector caspases (e.g. caspases-3 and -7), responsible for the disassembly of cellular components, and initiator caspases (e.g. caspases-8, -9 and -10), responsible for activation of the effector caspases. Caspases are highly conserved through evolution and share similarities in amino acid sequence, structure and substrate specificity. At least 14 mammalian caspases are known (Table 1) [16]. Some of them are not involved in apoptosis, but participate in the activation of cytokines and in the inflammatory response, like caspase-1 (usually known as interleukin-1-β-converting enzyme or ICE). Apoptotic caspases cleave selectively a critical set of cellular proteins in a coordinated manner leading to cell death. More than 60 caspase substrates have been identified to date [16]. Caspase processing is highly specific and means activation or inactivation, depending on the cases, of their protein substrates. The majority of these substrates, like procaspases, proteins of the Bcl-2 family, cell cycle regulators, cytoskeleton proteins, DNAases, transcription factors, signalling molecules, etc. are involved, directly or indirectly, in the regulation or execution of apoptosis, although for many of them the functional consequences of the cleavage are not yet known.

Because of the fatal consequences of apoptosis malfunctioning, the activation of caspases is highly controlled, mainly at two levels. The first involves regulation of their processing and activation. The second implies direct inhibition of active caspases [17]. Caspases are synthesised as inactive zymogens or proenzymes known as procaspases, but are rapidly activated in large amounts by proteolysis in response to apoptotic signals. Procaspases contain two domains: a pro-domain of variable length

and a conserved protease domain made of a small (p10) and a large (p20) subunit, joined by a short flexible linker that contains the cleavage site for caspase activation [18]. Initiator and inflammatory-related caspases have long pro-domains that are important for activation. In contrast, effector caspases have short pro-domains that seem to be dispensable [18]. According to the *induced proximity* model [19,20], the mechanism of activation of initiator caspases involves procaspase oligomerisation, aided by adaptor proteins, and auto-processing after association. On the other hand, effector caspases are transactivated by initiator caspases in a two step process. First, a partially active intermediate is generated by cleavage at a linker region, and in a second step the intermediate cleaves itself at the level of the short pro-domain to generate the active enzyme. Effector caspases can also auto-activate and then contribute to the development of amplifying cascades.

The structure of the mature active enzymes is known for caspase-1 [21,22], caspase-3 [23,24], caspase-7 [25,26] and caspase-8 [27–29]. All of them are tetramers consisting of a homodimer of two p10/p20 heterodimers. Each heterodimer forms a single globular structure with a six-stranded β-sheet flanked on either side by five α-helices. The enzyme has two independent active sites formed by amino acids of both p10 and p20.

Proteolytic maturation of dormant caspases is generally launched through either of two pathways, as shown schematically in Plate 1. Each of these routes is represented by a different initiator caspase and both converge at the level of activation of effector caspase-3. The mitochondria-mediated or intrinsic pathway [30] responds to most apoptotic signals and is triggered by the liberation of proapoptotic proteins from the mitochondrial inter-membrane space in a process regulated by proteins of the Bcl-2 family [31–35]. The central event of this pathway is the formation of an initiator caspase holoenzyme made of various copies of caspase-9, the adaptor protein called Apaf-1 (apoptotic protease activating factor 1), mitochondrial cytochrome-*c* (Cyt-*c*) and ATP [20,36,37]. In this macro-molecular complex, known as the apoptosome, caspase-9 is believed to be auto-proteolytically activated by a mechanism of *induced proximity*. In turn, the active caspase-9 holoenzyme can activate downstream effector caspases like caspase-3. This system is tightly regulated by a number of proteins [17]. Thus, the IAPs (inhibitor of apoptosis proteins) are multi-functional inhibitors that bind strongly to active
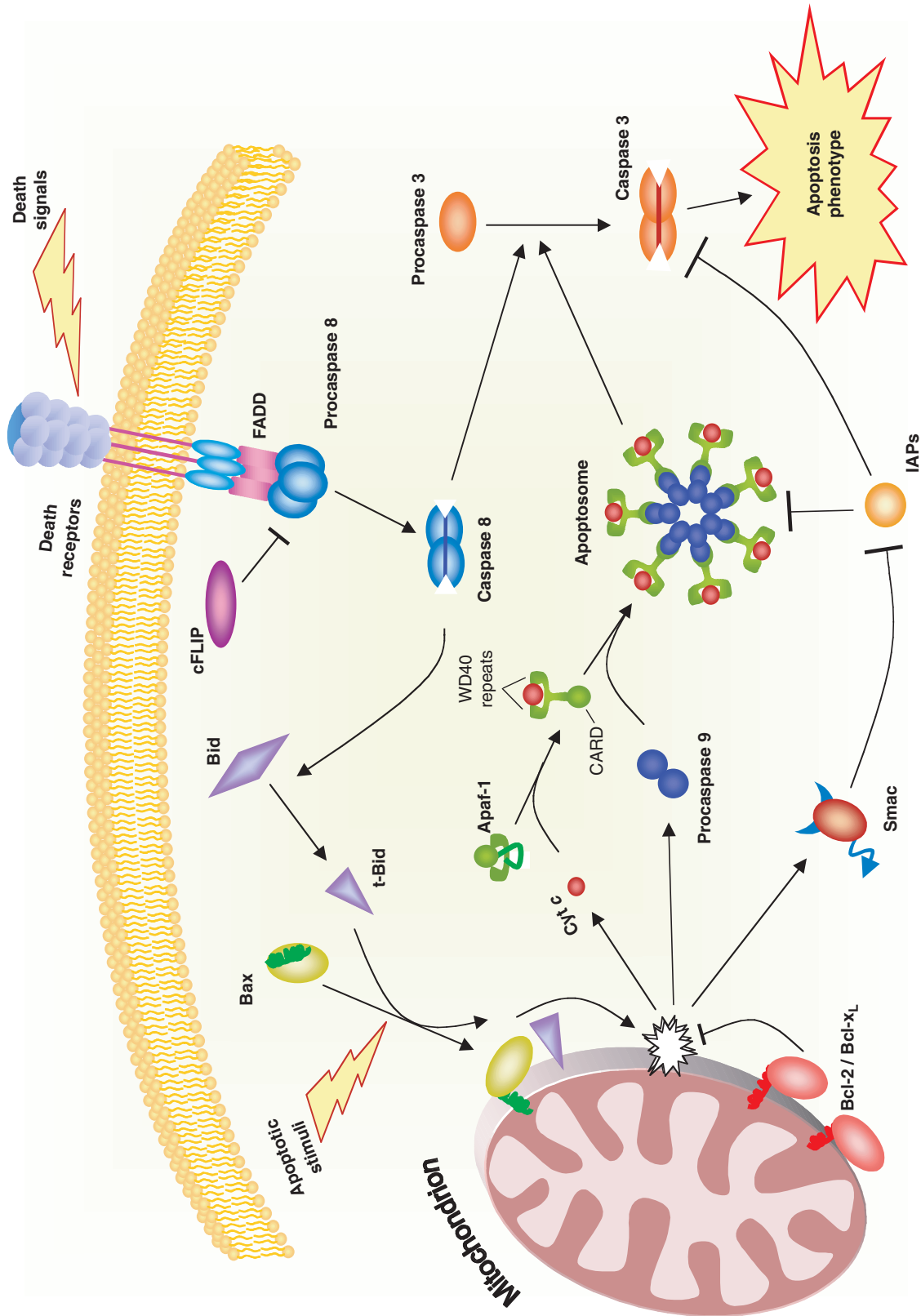
Plate 1   Biochemical routes to apoptosis. The main protagonists of the signal transduction pathways of mammalian apoptosis are depicted schematically.
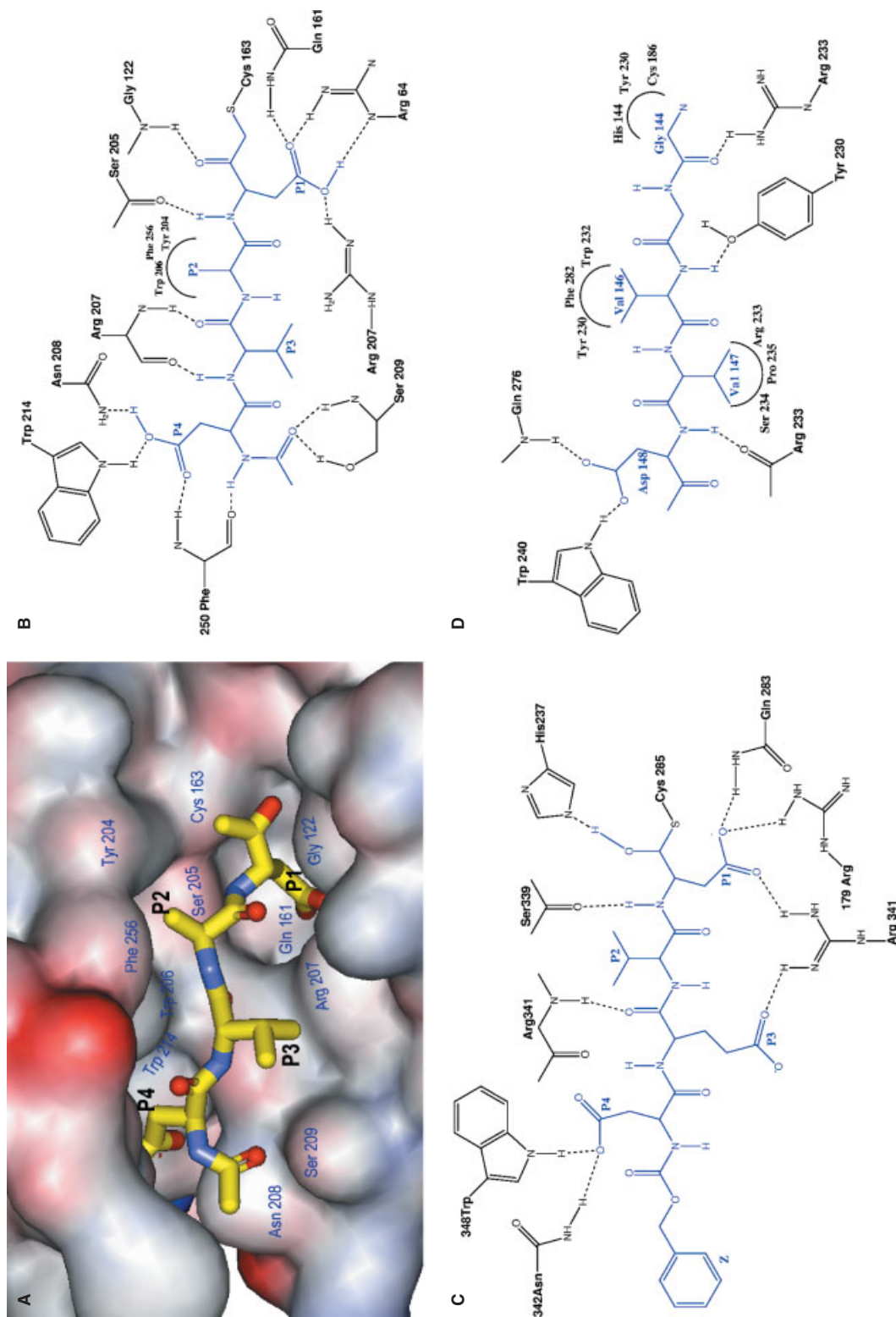
Plate 2 Schematic representation of different structures of caspase/inhibitor complexes. (**A**) Caspase-3/Ac-DVAD-*fmk* complex (structural data from reference [24] and PDB ID 1CP3). A soft accessible surface of the protein is shown, coloured according to the electrostatic potential. The inhibitor peptide is shown in a stick representation (carbon atoms in yellow colour, oxygen atoms in red and nitrogen atoms in blue). The P1–P4 peptide subsites are labelled black. Important residues from the protein are labelled with blue characters. A scheme of the same structure, indicating the most important interactions, is drawn in (**B**), with residues of the caspase in black and the tetrapeptide inhibitor in blue. For comparison, a similar scheme corresponding to the structure of the caspase-8/*z*-DEVD-*cho* complex [29] is shown in (**C**) with the structure of the complex of the BIR2-linker region of a natural caspase inhibitor (XIAP) with caspase-7 shown in (**D**). Schemes (**B**), (**C**) and (**D**) are based on similar drawings from references [24], [29] and [71], respectively.

caspases-3 and -9 and block their action [38–41]. Conversely, other proteins like Smac (second mitochondrial activator of caspases) [42], also known as DIABLO (direct IAP binding protein with low pI) [43] have a proapoptotic effect since they can block the binding of IAPs to caspase-3 and caspase-9. A higher-level control mechanism consists of keeping proapoptotic factors like procaspase-9, Cyt-*c*, Smac and AIF (apoptosis inducing factor) compartmentalised inside the mitochondrial inter-membrane space. Export of these proteins to the cytoplasm is regulated by proteins of the Bcl-2 family, made of two antagonistic groups with pro- and antiapoptotic effects [35,44].

Alternatively, activation of caspase-3 may also occur via an extrinsic pathway initiated by specific death signals (Plate 1) [45], which can be recognised by integral membrane proteins called death receptors (DR). Binding of extracellular death signals to the DR induces the formation of trimeric DR complexes in the cytoplasmic membrane. The DR clusters recruit multiple procaspase-8 molecules in the cytoplasm via an adaptor protein called FADD (Fas-associated death domain protein) and facilitate the auto-proteolytic activation of initiator caspase-8 [46]. Then, caspase-8 activates caspase-3 and the apoptotic pathway enters a point of no return. Caspase-8 activation can be blocked through the binding of c-FLIP (a degenerate caspase

homologue) [47] to FADD. Additionally, in certain cases, caspase-8 can indirectly induce the intrinsic pathway by cleavage of Bid, a proapoptotic member of the Bcl-2 family [48,49].

Although many aspects of these apoptotic pathways are still not clearly defined, an increasing amount of detailed structural and functional data has accumulated throughout the past decade. Caspase substrates and inhibitors, functional motifs and domains of different apoptotic executioners and regulators, and protein–protein complexes have inspired the design of peptides which can be used as highly specific tools to investigate physiological cell death and also in many cases as promising therapeutic agents.

CASPASE RELATED PEPTIDES

Peptidic Substrates

Caspases have cysteine in their active site and digest polypeptide substrates after a P1-aspartate residue in a three to five residue motif (see Table 1). Variations within this general cleavage site determine distinct specificities among caspases, as can be recognised from the sequences of their natural substrates [50]. This specificity has been investigated in detail through the study of the

Table 1 Specificity for Binding of Substrates and Peptide Inhibitors to Mammalian Caspases. Inhibition constants are taken from reference [58]. For Comparison, The Inhibition Constants Corresponding to the Natural Protein Inhibitor CrmA from Baculovirus are also shown

Caspase	Function	Recognition motif	Type	$t_{1/2}$ (s) z-VAD-*fmk*	K_i (nM)				
					Ac-DEVD-*cho*	Ac-YVAD-*cho*	Ac-WEHD-*cho*	Ac-IETD-*cho*	CrmA
1	Cytokine	WEHD	(I)	2.5	18	0.7	0.05	<6	0.01
4	activators	(W/L) EHD	(I)	130	132	362	97	400	1.1
5		(W/L) EHD	(I)	5.3	205	163	43	223	<0.1
11				—	—	—	—	—	—
12				—	—	—	—	—	—
13				—	—	—	—	—	—
14				—	—	—	—	—	—
2	Apoptosis	DEHD	(II)	2400	1710	$>10^4$	$>10^4$	9400	$>10^4$
8	initiators	LETD	(III)	2.5	0.9	352	21.1	1.05	<0.3
9		LEHD	(III)	3.9	60	970	508	108	<2.3
10				—	12	408	330	27	17
3	Apoptosis	DEVD	(II)	43	0.2	$>10^4$	1960	195	1600
6	effectors	VEHD	(III)	98	31	$>10^4$	3090	5.6	1300
7		DEVD	(II)	39	1.6	$>10^4$	$>10^4$	3280	$>10^4$

kinetic properties of synthetic chromogenic, *p*-nitroaniline-peptide derivatives that can function as caspase substrates [51]. Most significant differences in specificities for different caspases depend on the residue at the P4 position of the substrate, Asp being preferred for caspases-2, -3 and -7, hydrophobic residues for caspases-1 and -4 and the *β*-branched residue Val for caspase-6. The preference at P3 for several caspases is Glu, while a wide range of amino acids is tolerated at P2. Furthermore, only caspase-2 requires a P5 residue, preferably hydrophobic, in the peptide substrates [51].

A wider scan of sequence specificity for caspase cleavage was performed using a combinatorial approach with fluorogenic peptide substrates [52]. A positional scanning substrate combinatorial library with the general peptide structure *Ac*-XXXD-*amc* (*amc*, 7-amino-4-methylcoumarin) was synthesised considering the absolute requirement of caspases for cleavage after an Asp residue and the ability of tetra-peptides bound to *amc* to function as fluorogenic substrates. The library of peptide substrates consisted on three sub-libraries, each of them with one position defined and the other two positions with approximately equimolar mixtures of the natural amino acids. Specificities for the S2, S3 and S4 sub-sites of the binding pocket were determined for ten different caspases and granzyme B (a serine protease which also displays specificity for Asp at P1) by measuring the rate of *amc* production for each positionally defined amino acid. The results were used to divide caspases into three major groups (Table 1): group I is formed by caspases-1, -4 and -5 which prefer the sequence WEHD; group II is formed by caspases-2, -3 and -7, and they cleave mainly at the sequence DEXD; and group III prefer the sequence (L/V)EXD and is formed by caspases-6, -8 and -9. The specificities agree with significant structural differences found in the active sites of caspases-1, -3, -7 and -8 and in enzyme/inhibitor complexes [25] (see below).

Caspase Activator Peptides

RGD-peptides, normally used as inhibitors of integrin–ligand interactions can induce integrin-independent apoptosis in different cell lines [53]. The RGD-motif is present in many integrin ligands, but also in caspases-2, -3, -7 and -8. Moreover, procaspases-1 and -3 have potential RGD-binding sites (DDA and DDM motifs, respectively) near their sites for processing and activation. Using a breast carcinoma cell line with a functional deletion in

the caspase-3 gene, it was confirmed that apoptosis induction by RGD-peptides is due to caspase-3 activation [53]. RGD-peptides are also able to promote self-activation of purified procaspase-3, suggesting that binding of these peptides to the DDM site in the pro-enzyme induces a conformational change that allows auto-processing. The two Asp residues in the DDM-motif, together with an additional Asp, located just before this tripeptide, have been reported to constitute a *safety catch* that, through multiple ionic interactions, keeps the caspase-3 pro-enzyme in a dormant state [54]. However, this latter regulatory mechanism has been questioned [18].

The ability of peptides containing the RGD-motif to inhibit tumour metastasis [55] and induce endothelial-cell apoptosis in models of angiogenesis [56] is thought to be due to their capacity to inhibit integrin–ligand interactions. The newly found procaspase-3 target should now be considered as a direct way to induce apoptosis in RGD-peptides treated cells.

First Generation of Caspase Inhibitors: Pseudosubstrate Peptides

In general, a good caspase inhibitor can be any P1-Asp pseudosubstrate tetrapeptide with the appropriate P4-residue. However, as can be seen in the structure of caspase-inhibitor complexes (Plate 2), the presence of an electrophile to attack the thiol side-chain of the Cys residue in the caspase active-site renders the inhibitor more effective. The *C*-terminal aldehyde (*cho*) or ketone derivatives of P1-Asp tetrapeptides are potent reversible competitive inhibitors (Table 1), while halomethyl ketone derivatives react irreversibly with the Cys residue at the active site [57]. These features were the basis for the design of a family of widely used peptide derivatives of fluoromethylketone (*fmk*) or chloromethylketone (*cmk*), which are common tools for the analysis of caspase activity in experimental models of apoptosis. They display a wide range of selectivity and potency, which in general corresponds to the substrate-sequence specificity of the different caspases.

A good understanding of the principal determinants of caspase specificity has come with the structures of caspase-1 [21,22], caspase-3 [23,24], caspase-7 [25] and caspase-8 [27–29] complexed with different peptide inhibitors. In all these cases the peptides appeared bound to a surface cleft of the enzymes in an extended conformation (Plate 2A), with H-bond patterns between the polar main chain

atoms equivalent to the network of an antiparallel β-sheet. Other important stabilising interactions involve the amino acid side chains of both the peptide and the enzyme. Thus, in the structure of the caspase-3/Ac-DVAD-*fmk* complex [24] the P1-Asp forms salt bridges with Arg[64] and Arg[207] and a H-bond with Glu[161] in a deep pocket of the protein at subsite S1. The residue at P2 is stabilised through hydrophobic interactions with the aromatic residues Tyr[204], Trp[206] and Phe[256] at subsite S2, and the P4-Asp participates in H-bonds with the side chains of Asn[208] and Trp[214] and the main chain of Phe[250] in a narrow pocket at subsite S4 (Plate 2A,B). The size of this pocket avoids the entrance of large aromatic residues determining the selectivity at the level of P4. In fact, this latter feature is the main difference between the binding pockets of caspase-3/-7 [23–25] and caspase-1 [21,22], where S4 is made of smaller residues and allows Trp to be present at P4.

Caspase-8 structures are available in complex with z-EVD-*cmk* [28], Ac-IETD-*cho* [27] and z-DEVD-*cho* [29]. These inhibitors are also bound in an antiparallel beta-sheet conformation (Plate 2C). The environments provided by S1 and S2 are conserved with respect to caspases-1 and -3, and the P1-Asp interacts again with positively charged residues at the S1 pocket while interactions at the S2 pocket are hydrophobic. The complex with z-DEVD-*cho* [29] is interesting because this molecule is a specific inhibitor of group II caspases, in which caspase-8 is not included (Table 1). It had been accepted that differences in substrate specificity between caspases-3/-7 and -8 arise mainly from subsite S4. However, the structure of the caspase-8/z-DEVD-*cho* complex demonstrates more tolerance for residue P4 than originally assumed, due to flexibility of the loop that forms S4. Thus, P4-Asp forms H-bonds with Asn[342] and Trp[348] of caspase-8 in a way similar to caspase-3 (Plate 2B,C). These observations agree with kinetic data [58] and suggest that the general classification based on the screening of a combinatorial library [52] should probably be revised.

The structures of caspase/peptide-inhibitor complexes provide also a mechanism of inhibition. The ketone-peptide derivatives form a thioether bond with the side chain of the active-site Cys, and the carbonyl of the methyl ketone group interacts with an amide proton in the *oxyanion hole* [22,24] (Gly[122] in Plate 2B). However, aldehyde inhibitors form a tetrahedral thiohemiacetal structure when bound with the thiol group of the Cys in the active site.

In this latter case, the hydroxyl group of the thio-hemiacetal is hydrogen bonded to the imidazole side chain of the active site His [22,23]. Both complexes seem to represent different states of the tetrahedral intermediate of the proteolytic reaction [24].

An important concern when using the above described inhibitors is that, at the concentrations normally used in studies to demonstrate the involvement of caspases in cellular events, these peptides may not be sufficiently selective [50,59]. For example, the widely used z-VAD-*fmk* can strongly inhibit non-apoptotic ICE (caspase-1), apart from caspases-3, -5, -7, -8 and -9, and Ac-DEVD-*cho* is a good inhibitor of caspase-3, but also of caspases-1, -7, -8 and -10 (Table 1). On the other hand, using biotinylated peptide inhibitors to affinity-label active caspases in crude extracts it has been shown that z-VAD-*fmk*, z-DEVD-*fmk* and YVAD-*cmk* inhibit efficiently proteases from the family of cathepsins apart from caspases [59]. Despite these limitations, peptide inhibitors have been extensively utilised in preclinical trials to prove the potential therapeutic use of inhibition of caspases. Thus, they have been remarkably successful in decreasing apoptosis and improving survival in different models of ischaemia-reperfusion injury, traumatic injury, neurodegeneration and infectious diseases [60]. This first generation of peptide inhibitors is evolving towards new designs of nonpeptide molecules that are intended to improve cell-membrane permeability as well as their stability inside the cells [61].

Basis for the Next Generation of Caspase Regulators: Physiological Inhibitors and Activators of Caspases

In addition to the regulatory mechanisms that control caspase activation, cells have also developed mechanisms for direct inhibition of active caspases. One of them is post-translational modification, like phosphorylation of caspase-9 [62] or S-nitrosylation at the active site of caspase-3 [63]. But undoubtedly the major regulators of caspases are inhibitory proteins [60]. An important source of these proteins are viruses, which have developed potent inhibitors to block the apoptotic response in infected host cells. The best known examples of viral caspase inhibitors are the cytokine response modifier A (CrmA, included in Table 1 for comparison) [64], p35 [65] and the inhibitor of apoptotic proteins or IAPs [38]. The latter were first identified in baculoviruses, although various members of this family

have been found in *Drosophila* and at least seven in mammals [66,67]. IAPs from animal cells function as a safety brake to maintain the concentration of active effector caspases-3 and -7 below the threshold required to initiate cell disassembly. Moreover, they can also act upstream of these caspases precluding the initiation of the mitochondrial apoptotic pathway by direct inhibition of apical caspase-9 and so prevent the consequences of accidental or spontaneous pro-enzyme activation [41]. The control mechanism is completed with Smac/DIABLO (see Plate 1), a proapoptotic protein released from the mitochondrion which can bind to the IAPs and abort their inhibitory function [42,43].

The IAPs contain one to three conserved zinc-finger like domains, called BIR (baculoviral IAP repeat), and in some cases a *C*-terminal RING motif [68,41]. Five different IAPs have been discovered in human cells. The best known is the X-chromosome linked IAP (XIAP) [39], which has three BIR domains. Inhibition of caspases-3 and -7 by XIAP depends on domain BIR2 plus a linker sequence between this domain and BIR1 (L-BIR2) [69]. The mechanism of inhibition has been investigated by site directed mutagenesis [69] and structural studies [26,70,71]. The *N*-terminal linker region of L-BIR2 can compete with tetrapeptide inhibitors for binding at the active site and is responsible for caspase-3/-7 inhibition. In fact, the crystal structures of the caspase-3/L-BIR2 [70] and caspase-7/L-BIR2 [71,26] complexes show that the linker is tightly bound at the caspase active site (Plate 3B). But surprisingly, it is placed in an orientation opposite to that corresponding to pseudo-substrate tetrapeptides (Plate 2D). The bound linker has an α-β hook structure and makes extensive hydrophobic and H-bond interactions with residues at the caspase active site (Plate 3). In contrast, the BIR2 domain itself does not seem to contribute to the stability of the complex [26,71,70]. However, a peptide corresponding to the *N*-terminal linker sequence alone is unable by itself to inhibit caspase-3 [69]. It appears that BIR2 is required to promote a productive conformation (or to avoid an unproductive conformation) of the linker peptide. Moreover, this secondary role of BIR2 is not specific, since the linker fused to either the *N* or the *C* terminus of BIR1 [69], and even to a completely unrelated protein such as the glutathione-S transferase, is able to inhibit caspases-3 and -7 [71]. On the other hand, the main, specific function of BIR2 can be binding to Smac to eliminate the inhibitory effect of XIAP on caspases-3 and -7 [72]. Such a binding interaction

is possible through a binding pocket which is similar to the docking site found in BIR3 for the specific interaction of the proapoptotic protein Smac [70] (see below).

The mechanism of inhibition of caspase-9 by XIAP is different and involves only the BIR3 domain (the adjacent linker region is dispensable in this case) [73]. The activating processing of caspase-9 exposes the *N* terminus of the small subunit. The first four residues of this *N*-terminal sequence share similarity with the *N* terminus of Smac and other homologous proteins from *Drosophila* (Plate 4A), and this tetrapeptide is demonstrated to be necessary for caspase-9 binding to XIAP and inhibition [74]. Thus, the proapoptotic activity of Smac is exerted by the competitive displacing and releasing of caspase-9 from its binding to XIAP. Supporting this model, peptides with the sequence of the *N*-terminal IAP binding motif are able to mimic Smac and suppress inhibition of caspase-9 [72,75] (Table 2). The structures of the complex of domain BIR3 with Smac [76] and with an *N*-terminal 9-mer peptide from Smac [75] show the first four residues (Ala-Val-Pro-Ile) bound to a surface groove of BIR3 (Plate 4B). The peptide ligand adopts an extended conformation with a kink at the Pro residue, and the structure is stabilised by ionic interactions between the *N* terminus of the Smac peptide and Glu[314] from BIR3 and various H-bond and hydrophobic interactions involving backbone and side chain groups of both the peptide and BIR3. The contribution of the *N*-terminal residue of the peptide (Ala[1']) to the total binding energy appears to be determinant as demonstrated by site directed mutagenesis [75] (Table 2).

Although a structure of the complex of BIR3 with caspase-9 is not available, the fact that two residues of XIAP that are important for inhibition of caspase-9, as shown by site-directed mutagenesis [73], mediate critical contacts with the Smac tetrapeptide, proves that caspase-9 and Smac use the same binding pocket. This model for physiological caspase-9 inhibition and activation has important implications [77]. First, XIAP selectively interacts and inhibits processed caspase-9, but not procaspase-9, which can also be active [78]. Second, it shows that a conserved XIAP-interaction motif in caspase-9 and Smac mediates opposing effects [74]. And third, the small subunit of caspase-9 can be further cleaved by downstream caspases at the *N*-terminus, immediately after the XIAP binding motif, releasing a 15-residue peptide that would be available to bind XIAPs and block their inhibitory
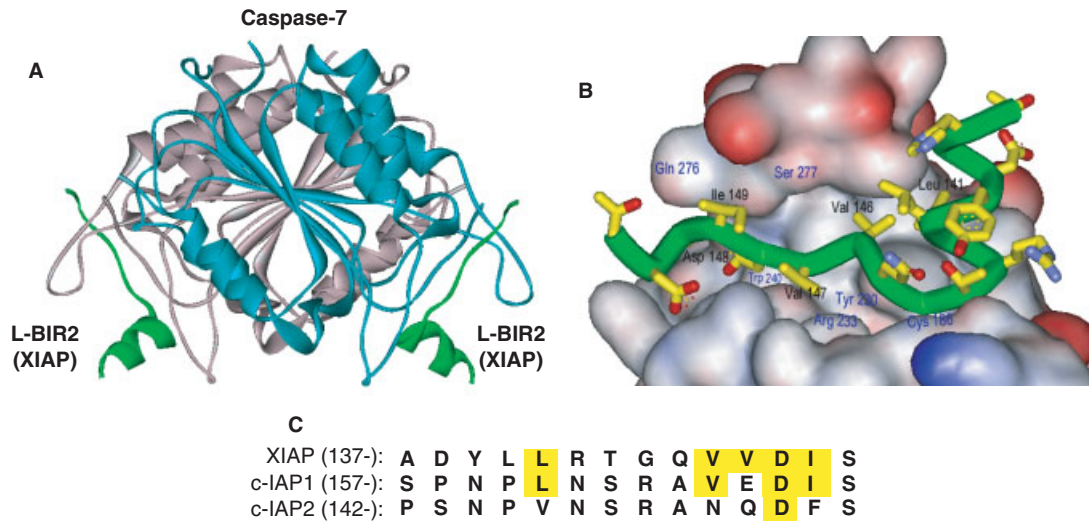
Plate 3 (**A**) Structure of the complex formed between the caspase-7 tetramer and the linker region of the L-BIR2 domain of XIAP (structure coordinates from PDB ID 1I51, reference [71]). (**B**) View of the linker (main chain in green, carbon atoms in yellow, oxygen atoms in red, nitrogen atoms in blue) bound to the active-site cleft of the enzyme. The catalytic residue Cys[186] and residues from the linker (in black characters) and the enzyme (in blue characters) that participate in relevant interactions are indicated. Compare this structure with the structures in Figure 2. (**C**) Comparison of the sequences of the linker region from three mammalian IAPs. Most relevant residues, according to the structural [26,70,71] and mutagenesis [69] studies, are highlighted in yellow.
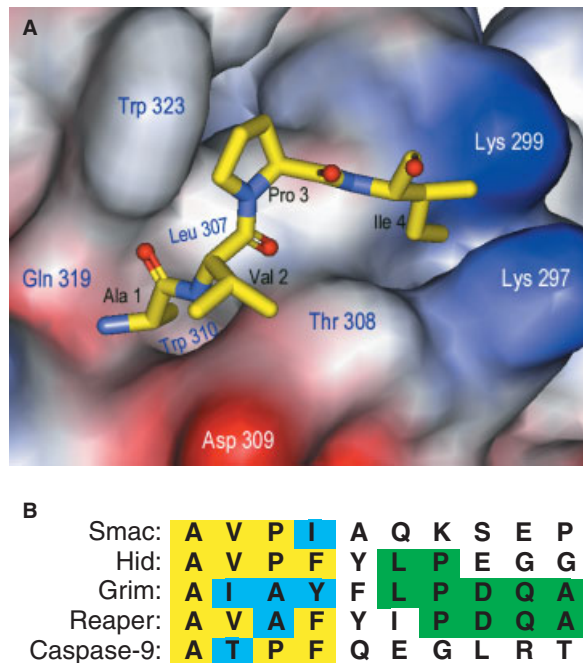

Plate 4 (**A**) Interaction of an *N*-terminal peptide from Smac (stick representation, same colour coding as in Figure 2) with the BIR3 domain of XIAP (electrostatic potential surface). The structure was generated using coordinates from PDB ID 1G3F [75]. Only the first four residues of the peptide, out of nine, are shown here. These residues, labelled with black characters, make extensive contact at the binding cleft of the protein. Amino acids from the XIAP-BIR3 domain involved in the interaction are labelled with blue and white characters. (**B**) Comparison of the sequence of the *N* terminus of mature human Smac [42] with sequences of homologous proteins from *Drosophila* (Hid, Grim and Reaper) [146,147,148] and the *N* terminus of human caspase-9 [149].
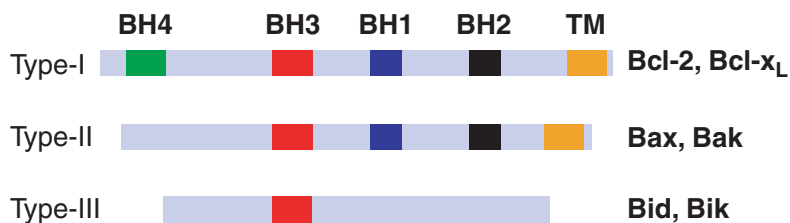
Plate 5   The three types of proteins of the Bcl-2 family and most representative members. The Bcl-2 homology domains are indicated.
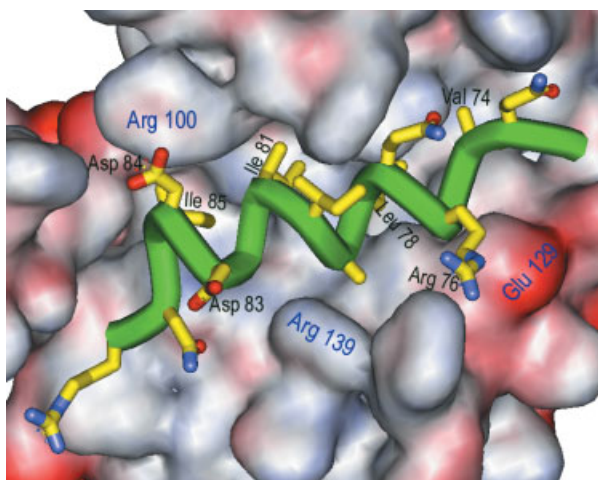

Plate 6   Structure of a peptide corresponding to the BH3 domain from Bak as viewed in the complex formed with Bcl-x_L (PDB ID 1BXL [97]). Residues of the peptide important for the stability of the complex are labelled in black. The peptide seats in a groove of the protein made of hydrophobic residues. Some charged residues at the edges of this groove (labelled blue) may participate in ionic interactions with charged partners from the BH3 peptide.

Table 4   Sequences and Binding Properties of BH3(Bak), aPP, A Library Designed by Protein Grafting Considering a Minimum Conflict Alignment and Peptides Selected from their Ability to Bind Bcl-2 [131]

Peptide	Sequence[a]	Bcl-2 K_d (nM)[b]	Bcl-x_L K_d (nM)[§]
Bak (72–87)	. . . . .GQVGRQLAIIGDDINR . .	4933 ± 450	350 ± 40
aPP	GPSQPTYPGDDAPVEDLIRFYNDLQQYLNVVTRHRY		
PPBH3 library	GPSQPTYPGDDAPVEDLIRFVXRLLXYIXDXINR . .		
PPBH3-1	. . . . .FVGRLLAYFGDTINR . .	52 ± 5	7 ± 2
PPBH3-2	. . . . .FVGRLLRYFGDEINR . .	80 ± 12	—
PPBH3-3	. . . . .FVGRLLAYFGDDINR . .	120 ± 20	—

[a] Residues in red contribute to binding of BH3(Bak) to Bcl-x_L. Residues in blue contribute to folding of aPP. Residues in green represent randomised positions. F^{28}, in bold red, is a selected unexpected mutation which the authors believe is functionally significant [131].
[b] Measured by fluorescence polarisation. Data correspond to peptide derivatives carrying a *C*-terminal cysteine and labelled with acetamidofluorescein.

Table 2 Sequence and Dissociation Constants of Natural and
Variant Smac Peptides for the BIR3 and BIR2 Domains of XIAP

Peptide/protein[a]	K_D (µM)[b]	
	BIR3	BIR2
Mature Smac (1–183)	0.42 ± 0.02	2.3 ± 0.3
1—AVPIAQKSEPHSLSSEALMR—20	0.69 ± 0.05	6.9 ± 0.7
AVPIAQKSE..........	0.43 ± 0.060	6.0 ± 0.9
GVPIAQKSE..........	>1000	>1000
CH₃ CO–AVPIAQKSE..........	>1000	>1000
A**A**PIAQKSE..........	12 ± 2	56 ± 5
AV**A**IAQKSE..........	20 ± 4	4.0 ± 0.9
AVP**A**AQKSE..........	34 ± 7	18 ± 2
AVPI**G**QKSE..........	1.2 ± 0.4	10 ± 2
AVPIA**A**KSE..........	0.43 ± 0.08	3.5 ± 0.2
AVPIAQ**A**SE..........	0.43 ± 0.08	7.1 ± 0.6
AVPIA–NH₂..........	0.64 ± 0.07	5.5 ± 0.5
AVP**FYLPEG**..........	0.13 ± 0.03	4.9 ± 0.8
MAVP**FYLPEGGADDVAS**....	>1000	>1000

[a] Mutated residues are shown with bold characters.
[b] Data from reference [75]. Measured by fluorescence polarisation in
a competition assay using a peptide based on Smac labelled with
6-carboxyfluorescein succinimidylester (FAM) at the *C* terminus, with
the sequence AVPIAQKSEK(FAM).

action. Thus, this model postulates the possibility
of a positive feedback through the release *in situ* of
proapoptotic peptides.

The Apoptosome

As mentioned above, the apoptosome is the caspase-
9 holoenzyme and constitutes the apical caspase of
the mitochondrial apoptotic pathway. This macro-
molecular complex is formed by Apaf-1, procaspase-
9, Cyt-*c* and ATP (or dATP) [36,20], and according
to a low resolution structure there would be seven
units of each of these components in the active
apoptosome [37]. Apaf-1 is central for the correct
assembly and activity of the apoptosome and the
importance of this protein is illustrated by the fact
that it is frequently inactivated in a number of can-
cers [79,9].

The assembly of the apoptosome is mediated
by three domains of Apaf: an *N*-terminal caspase
recruitment domain (CARD) [80], a central CED-4
homology motif that binds dATP/ATP, and 12–13
WD40 repeats at the *C*-terminal half that are
involved in binding to Cyt-*c* [20]. A pre-apoptosome
complex can be formed with Apaf-1 and Cyt-*c*
in the presence of dATP/ATP, and this can then

bind procaspase-9 in a subsequent step [37]. It
has been proposed that the WD40-repeats domain
may trap the CARD domain and keep Apaf-1
in an inactivate conformation [37]. According to
this model, the function of Cyt-*c* is to release
this *safety brake* by displacing the CARD domain
from its intra-molecular binding. Thus, the initial
Cyt-*c*:Apaf-1 binding is a critical step in the
formation of an active apoptosome, and therefore
could represent a potential therapeutic target.
Horse heart Cyt-*c* can initiate caspase activation
in an *in vitro* reconstitution system containing
this latter protein, Apaf-1, dATP, procaspase-9
and procaspase-3 [20,81,82]. However, the highly
homologous yeast Cyt-*c* can not substitute horse
Cyt-*c* to initiate activation [83]. The basis of the
interaction between Cyt-*c* and Apaf-1 have been
defined through an extensive mutational study of
potentially relevant amino acid residues of Cyt-
c [84]. A number of residues were found to be
important for the formation of an active apoptosome.
These residues are widely distributed on the surface
of Cyt-*c*, including residues of the lysine-rich face
(Lys 7, 25 and 72), normally used by Cyt-*c* to
interact with its electron-transfer partners, but
also residues in an opposite face, centred around

residues 62–65. These results suggest that Apaf-1 recognition involves a large area of the Cyt-*c* surface, in agreement with the model proposed by Acehan *et al.* [37].

We have initiated in our laboratory the search for synthetic peptides and non-peptide molecules that could interfere with the Cyt-*c*:Apaf-1 interaction and modulate the formation of the apoptosome. We are currently using an assay based on a totally *in vitro* reconstituted apoptosome system. From the screening of peptoid-based combinatorial libraries [85] we have obtained promising results that might be the basis of the development of molecules that inhibit the apoptosome-mediated activation of caspase-9 (unpublished results).

PEPTIDES BASED ON PROTEINS OF THE BCL-2 FAMILY

Proteins of the Bcl-2 family regulate the release of apoptotic factors from mitochondria. They can be of three types, depending on the presence of different homology domains BH1-BH4 [35,44] (Plate 5). Antiapoptotic members such as Bcl-2 and Bcl-x_L (type-I Bcl-2 proteins) present all four homology domains BH1–BH4. Proapoptotic members, however, can be of two types: type-II Bcl-2 proteins, like Bax and Bak, which have domains BH1, BH2 and BH3, and type-III Bcl-2 proteins, like Bid and Bad, which have only domain BH3. These latter proteins must be modified in order to expose a functional BH3 domain, which in the case of Bid is achieved by truncation performed by caspase-8 [86–88].

Structures of representative members of each type are known and they all are remarkably similar [87–93]. Additionally, type-I and type-II Bcl-2 proteins have a hydrophobic *C*-terminal sequence that controls their subcellular localisation. In type-I Bcl-2 proteins this *C*-terminal segment functions as a permanent anchor to the cytoplasmic side of outer mitochondrial and nuclear membranes as well as the membrane of the endoplasmic reticulum [94–96]. However, in type-II Bcl-2 proteins, the *C*-terminal segment is partially buried in a hydrophobic cleft on the surface of the protein [91]. This latter group of proapoptotic proteins are normally soluble in the cytoplasm, but in the presence of apoptotic signals the *C*-terminal segment binds and inserts into the mitochondrial membrane. The hydrophobic cleft, made by regions of the BH1, BH2 and BH3 domains, is a common feature of type-I and type-II Bcl-2 proteins [91,97,98]. This region functions as a docking site for binding of the BH3 domain of other Bcl-2 proteins, this interaction being responsible for the formation of heterodimers between different members of the family.

It has been proposed that the antiapoptotic function of type-I Bcl-2 proteins consists of binding proapoptotic type-II and type-III Bcl2-2 proteins through their BH3 domain to block their action [31,32,99,100]. On the other hand, a mechanism for triggering proapoptotic activity could be the induction of insertion in the outer mitochondrial membrane of type-II Bcl-2 proteins by type-III Bcl-2 proteins. According to this model, the formation of heterodimers through interactions involving the BH3 domain of an active type-III partner (like truncated Bid, t-Bid) and the hydrophobic cleft of a type-II (like Bax) would displace the *C*-terminal segment of the type-II Bcl-2 protein and promote its insertion and oligomerisation in the membrane [34,101–103]. In the outer mitochondrial membrane Bax and Bid facilitate export of apoptotic factors, but is not clear whether they form a pore [104–108] or regulate other existing mitochondrial pores [109–115].

The different domains found in the sequence of Bcl-2 proteins have inspired the design and synthesis of a number of active peptides and non-peptidic molecules [116] which are used to investigate the function and mechanism of action of these systems, but also as potential therapeutic agents. It should be mentioned that a number of cancer cells overexpress antiapoptotic, type-I Bcl-2 proteins that prevent the action of proapoptotic type-II and type-III members of the family and often make cancer cells resistant to conventional therapies [9,117]. In the following sections we give an account of the most important peptides derived from Bcl-2 proteins and summarise their properties.

N-terminal Peptides

The *N*-terminal BH4 domain, characteristic of type-I Bcl-2 proteins, has been shown to be essential for inhibition of apoptosis [118,119]. The role of this helical domain [89,90,120] in controlling mitochondrial changes dependent on antiapoptotic Bcl-2 proteins, has been investigated by Shimizu and coworkers using synthetic peptides derived from the sequence of the BH4 domain of Bcl-2 and Bcl-x_L [121]. In their study, the authors focus on the capacity of these peptides to regulate the voltage dependent anion channel (VDAC), which has been related to the apoptotic release of Cyt-*c* and to the

regulation of this release by agonists and antagonists of the Bcl-2 family [122]. By using HeLa cells transfected with DNA corresponding to Bcl-x_L and a BH4 deletion mutant lacking amino acids 3–23 (ΔBH4) it was demonstrated that Bcl-x_L, but not ΔBH4 prevents Cyt-*c* release from the mitochondria after apoptotic stimuli. A similar effect was observed using isolated mitochondria and recombinant Bcl-x_L and ΔBH4 proteins [121]. Coimmunoprecipitation analysis reveals that both Bcl-x_L and ΔBH4 bind to VDAC to a similar extent. However, only binding of Bcl-x_L, but not ΔBH4, inhibits channel activity of VDAC-containing liposomes (VDAC-cLip). Thus, it appears that the BH4 domain, by itself, can inhibit the activity of VDAC, although it is not the major contributor to the binding of Bcl-x_L to the channel [121]. The use of BH4 derived peptides supports such a hypothesis. BH4-peptides from human Bcl-x_L (4-SNRELVVDFLSYKLSQKGYS-23) and from human Bcl-2 (7-TGYDNREIVMKYIHYKLSQRGYEW-30) inhibit sucrose uptake in VDAC-cLip while having no significant effect on plain liposomes [121]. These peptides can also prevent apoptotic mitochondrial changes, like Ca^{2+} induced $\Delta\psi$ loss and Cyt-*c* release. Additionally, a change of conserved residues at the BH4 domain (residues Leu^8, Val^9, Phe^{12}, Leu^{13} and Leu^{17}), which have been shown to be crucial for the antiapoptotic activity [120], also decreases inhibition of the activity of VDAC and the outcome of apoptotic mitochondrial changes caused by BH4-peptides [121]. These five amino acids line up on one face of the BH4 α-helix and are important for the stabilisation of its structure, which might be required for the interaction with VDAC. The inhibitory effects of BH4-peptides are somewhat smaller than the effects of the corresponding complete recombinant Bcl-x_L proteins. The differences may be due to contributions of the additional binding regions of Bcl-x_L to the inhibitory action of the BH4 domain, by either increasing affinity or facilitating proper folding [121]. The ability of the BH4-peptide to prevent apoptosis was also tested. In this case, to facilitate transport of the peptide into cells, a chimeric version was used which contained a synthetic peptide from the protein transduction domain of HIV TAT [123] with an eosin-labelled cysteine residue at the *N*-terminus (eosin-C-RKKRRQRRR) fused to the BH4 peptide of human Bcl-x_L (TAT-BH4). The TAT-BH4 peptide enters cells efficiently, where it localises mainly at the mitochondria and prevents apoptosis [121]. BH4 peptides alone can also prevent fluid percussion trauma-induced hippocampus cell death [124].

The Suicide Domain

Domain BH3, which is common to all three types of Bcl-2 proteins, has been extensively studied. A region from the proapoptotic protein Bax containing BH3 was termed the *suicide domain* [125] because insertion of this particular sequence in place of the corresponding sequence of the antiapoptotic Bcl-2 converted the latter from an inhibitor into an activator of cell death. In a number of studies it was reported that the BH3 domain mediates interaction between different Bcl-2 proteins [125–129]. This interaction has been characterised in detail through the structural analysis of complexes formed between a soluble active variant of Bcl-x_L (i.e. a mutant where the *C*-terminal membrane anchoring domain was deleted) and peptides corresponding to the BH3 domains of Bak [97] and Bad [98]. The structure of the truncated form of Bcl-x_L when complexed to the BH3 peptides is very similar to the x-ray and NMR structures of uncomplexed Bcl-x_L [89,90]. In the complexes, the BH3 peptides adopt an α-helical structure and bind to a hydrophobic cleft formed by regions from the BH1, BH2 and BH3 domains of Bcl-x_L (Plate 6). The *N*-terminal residues of the peptide interact with residues of the BH1 region of Bcl-x_L, while the *C*-terminal end is mainly in contact with residues of the BH2 and BH3 regions. The Bcl-x_L/BH3(Bak) complex is stabilised by hydrophobic interactions between residues of the BH3(Bak) peptide and residues in the hydrophobic cleft of Bcl-x_L (Tyr^{101}, Leu^{108}, Val^{126}, Phe^{146}) [97]. The contribution of these interactions to the stability of the complex was evaluated by measuring the binding affinities of wild type and mutant BH3(Bak) peptides [92,93,97,98,]. In agreement with the structural data, mutation to Ala of Val^{74}, Leu^{78}, Ile^{81} and Ile^{85} from the peptide results in an important reduction of the binding ability to Bcl-x_L (Table 3). Specially important is the contribution of Leu^{78}, which is conserved within the Bcl-2 family of proteins. There are also ionic interactions between residues of opposite charge from the peptide (Arg^{76}, Asp^{83}, Asp^{84}) and from the protein (Glu^{129}, Arg^{139}, Arg^{100}). Mutation of peptide residues Arg^{76}, and specially Asp^{83}, destabilises the complex. Asp^{83} interacts with Arg^{139} from Bcl-x_L, and both are highly conserved in the family of Bcl-2 proteins. The contribution of Arg^{76} to the peptide/protein interaction is smaller, and mutation of Asp^{84} hardly affects the stability of the complex, despite the charge–charge interaction between this residue and Arg^{100} from Bcl-x_L [97].

The selectivity of the interactions between different members of the Bcl-2 family depends on the characteristics of the binding of the BH3 domain of one protein to the hydrophobic cleft of its partner. As shown in Table 3, there are important differences in the binding of a particular BH3 peptide to different proteins of the Bcl-2 family. For example, the BH3(Bcl-x_L) peptide shows a very low affinity for Bcl-x_L, which indicates that this protein cannot homo-dimerise through interactions depending on the BH3 domain. On the other hand, Bcl-x_L,

Bcl-2 and a Bcl-2 homologue from Kaposi sarcoma virus (KSHV Bcl-2) show a very different selectivity for the BH3(Bak), BH3(Bad) and BH3(Bax) peptides [92,93,97,98]. Since there is a correlation between binding of the BH3 peptides and binding of the corresponding full-length proteins, the selectivity observed may be important for the mechanism of control of apoptosis. The different binding properties are due to both differences in the BH3 peptide sequences and differences in the characteristics of the binding groove of Bcl-x_L, Bcl-2 and KSHV

Table 3 Dissociation Constants of Different BH3 Peptides to Bcl-x_L, Bcl-2 and KSHV Bcl-2. The Sequences of the Peptides Correspond to Sequences of Different Bcl-2 Proteins, as Indicated

Peptide	Sequence[a]	K_d (nM)		
		Bcl-x_L	Bcl-2	KSHV Bcl-2
Bak (wt)	72 ....GQVGRQLAIIGDDINRRYDSEFQ 94	200[b]	—	—
	77 ....QLAIIGDDINR....... 87	No binding[b]	—	—
	72 ....GQVGRQLAIIGDDINR....... 87	340[b]	12 710[d]	<50[e]
Bak (mutant)	....GQ**A**GRQLAIIGDDINR.......	15 000[b]	—	>10 000[e]
	....GQVGA**Q**LAIIGDDINR.......	3300[b]	—	—
	....GQVGRQ**A**AIIGDDINR.......	270 000[b]	—	>10 000[e]
	....GQVGRQLAA**I**GDDINR.......	1000[b]	—	—
	....GQVGRQLAI**A**GDDINR.......	17 000[b]	—	—
	....GQVGRQLAII**A**DDINR.......	500[b]	—	—
	....GQVGRQLAIIG**A**DINR.......	41 000[b]	—	149[e]
	....GQVGRQLAIIGD**A**INR.......	140[b]	—	—
	....GQVGRQLAIIGDD**A**NR.......	93 000[b]	—	>10 000[e]
Bad (wt)	140 NLWAAQRYGRELRRMSDEFVDSFKK... 164	0.6[c]	15[d]	3900[e]
	145 ....QRYGRELRRMSDEFVD....... 160	50 000[c]	—	—
Bad (mutant)	**AAA**AAQRYGRELRRMSDEFVDSFKK...	0.5[c]	—	—
	NLWAAQRYGRELRRMSDEFVD**A**FKK...	0.3[c]	—	—
	NLWAAQRYGRELRRMSDEFVDS**A**KK...	2.1[c]	—	—
	NLWAAQRYGRELRRMSDEFVDSF**A**K...	1.2[c]	—	—
	NLWAAQRYGRELRRMSDEFVDSFK**A**...	0.2[c]	—	—
	....QRYGRELRRMSDEFV**R**.......	3300[c]	—	—
	....Q**Q**YA**R**ELR**IM**A**DEFV**R**.......	439[c]	—	—
	....**DD**YA**R**ELR**MM**A**DEFV**R**.......	203[c]	—	—
Bcl-2 (wt)	91 ....PVVHLALRQAGDDFSR....... 106	6400[b]	—	—
Bax (wt)	57 ....KKLSECLKRIGDELDS....... 72	13 000[e]	5200[e]	980[e]
Bik (wt)	55 ....DALALRLACIGDEMDV....... 70	15 000[b]	—	—
Bcl-x_L (wt)	84 ....AAVKQALREAGDEFEL....... 99	325 000[b]	—	—

[a] Mutated residues are shown with bold characters.

[b] Data from reference [97]. Measured from changes of the fluorescence emission of Trp residues of Bcl-x_L as a function of peptide concentration.

[c] Data from reference [98] Measured by a fluorescence polarisation-based competition assay using a fluorescein-labelled Bad peptide as a probe.

[d] Data from reference [92] Measured by a fluorescence polarisation-based competition assay using a 6-carboxyfluorescein-succinimidyl ester-labelled Bad peptide as a probe.

[e] Data from reference [93] Measured by a fluorescence polarisation-based competition assay using a fluorescein-labelled Bak peptide as a probe.

Bcl-2 [92,93,97]. Although the binding grooves are hydrophobic in all three cases, there are changes at the amino acid level that confer significantly different properties to the contact surfaces of these grooves. Thus, the higher affinity of Bcl-x_L for the BH3(Bad) and BH3(Bak) peptides, compared with that of Bcl-2 has been attributed to differences at the electrostatic surface of the binding groove of these two proteins [92]. Similarly, mutations of important residues in the BH3(Bak) peptide affect binding to Bcl-x_L to a different extent than binding to KSHV Bcl-2 (Table 3), due to differences in the docking groove of these two proteins [93].

Binding of BH3 peptides derived from Bad to Bcl-x_L has been found to depend dramatically on the length of the peptide (Table 3). The 25-residue long BH3(Bad) forms additional interactions with Bcl-x_L compared with the 16-mer BH3(Bak) peptide. However, site-directed mutagenesis demonstrates that the additional contacts are not responsible for the increased affinity of the 25-mer [98]. A careful analysis led to the conclusion that the low affinity of the BH3(Bad) 16-mer could be due to its reduced helix propensity, which may originate from charge repulsion between Asp[160], Asp[156] and Glu[157] in the bound α-helical state [98]. In agreement with this idea, mutation of Asp[160] to Arg showed an increased affinity of the 16-mer for Bcl-x_L (Table 3). The BH3(Bad) 16-mer peptide was further evolved to increase its intrinsic helix propensity by molecular design with the help of the program AGADIR [130], while maintaining those residues described as important for the native contacts with the protein (Tyr[147], Leu[151], Asp[156], Phe[158] and Val[159]) [98]. As a result, two peptides were designed whose binding constants were improved by more than two orders of magnitude when compared with the wild type BH3(Bad) 16-mer (see Table 3), suggesting that, in fact, the ability to adopt an α-helix is crucial for the binding of BH3 peptides to Bcl-x_L. This study demonstrates that the accessibility of the bound conformation is an important factor that, together with the intermolecular contacts, determines the stability of a protein/peptide complex. Moreover, a recent report describes the design of a miniature protein which binds to Bcl-2 and to Bcl-x_L in the nanomolar concentration range [131]. The authors use the strategy of *protein grafting and evolution* that consists of the introduction of the residues required for functional recognition in a small, but well-folded, non-functional scaffold, which is similar to the avian pancreatic polypeptide (aPP) [132]. After

definition of a minimum conflict alignment between the sequences of the BH3(Bak) peptide and aPP, and fixing the important residues for BH3(Bak) to Bcl-x_L binding and aPP structural stability, the remaining residues of the overlapping region were varied across all 20 amino acids and the potential sequences were displayed in a M13 phage library (Table 4). A selection based on their ability to bind to Bcl-2 yielded high affinity, well-folded miniature proteins that perform orders of magnitude better than the parent BH3(Bak) peptide (Table 4). Moreover, the designed miniproteins discriminate between Bcl-x_L and Bcl-2, showing a preference similar to wild type BH3(Bak) [131].

Synthetic peptides corresponding to the BH3 domain have been also studied *in vivo*. In order to access the interior of the cells the peptides are delivered fused to the Antennapedia homoprotein internalisation domain [133]. For example, treatment of HeLa cells with Antennapedia-BH3(Bak) fusion peptides results in a rapid induction of apoptosis [134]. In an alternative approach, a fatty acid was used as a carrier to drive the functional peptides inside the cells [135]. BH3(Bad) peptides attached to decanoic acid, which can still bind to Bcl-2 efficiently, are able to enter myeloid leukemia HL-60 cells and induce changes characteristic of apoptosis. The effect is probably due to binding to and blocking Bcl-2, which is overexpressed in these cells, and is accompanied by caspase-3 activation. Additionally, these peptide derivatives reduce tumour growth in mice challenged with HL-60 cells while having no apparent toxicity in normal mice [135].

Although it is accepted that induction of apoptosis by peptides derived from BH3 domains is related to their ability to bind to Bcl-2 proteins, the mechanism of their action remains controversial and there are conflicting reports on whether these peptides can by themselves release Cyt-*c* [110,134,136–138]. It is also controversial whether the BH3 induced apoptosis depends on mitochondrial permeability transition [109–111,115]. In a recent study using a BH3(Bax) peptide, it is reported that the release of Cyt-*c* induced by this molecule is a non-selective, cell specific process which depends on the presence of endogenous Bax and is regulated by Bcl-2, but is independent of mitochondrial permeability transition [138]. The authors propose that the BH3 peptide acts as a BH3-only (type-III) protein, like t-Bid [34,101], by mediating Bax oligomerisation and insertion in the outer mitochondrial membrane (see below).

C-terminal Peptides

Another important segment of the sequence of type-I and type-II Bcl-2 proteins is the hydrophobic *C*-terminal domain. This region regulates constitutive anchoring of type-I Bcl-2 proteins, mainly to the mitochondrial outer membrane [96]. Although the mechanism for organelle recognition and membrane insertion is not known, there is evidence that targeting is a specific saturable process that depends on the presence of a relatively short hydrophobic sequence at the *C*-terminus of the protein flanked by positively charged residues [139,140]. In the case of type-II proteins like Bax, the *C*-terminal domain docks constitutively at the hydrophobic groove of the surface of the protein [91]. Targeting of Bax to the mitochondrial membrane occurs after apoptosis induction, probably as a consequence of a conformational change [141] aided by displacement of the *C*-terminus from the hydrophobic groove by a type-III, BH3-only Bcl-2 protein (see above) [34,91].

The structures of peptides corresponding to the *C*-terminal domain of Bcl-2 [142], Bax [143] and Bak [144] have been investigated in model lipid membranes by infrared spectroscopy. The Bcl-2 and Bak *C*-terminal peptides present an aggregate β-sheet structure in D_2O but are mainly α-helical in multilamelar phosphatidylcholine membranes, as deduced from the amide I' bands with a maximum in the region of $1620–1635 \text{ cm}^{-1}$ in water and $1648–1658 \text{ cm}^{-1}$ in the presence of phospholipids. Peptide insertion in the membrane is further supported by a broadening of the lipid phase transition, as observed by differential scanning calorimetry, and an increased polarisation of the hydrophobic fluorescence probe 1,6-diphenyl-1,3,5-hexatriene. Moreover, the *C*-terminal peptides from Bcl-2 and Bak destabilise unilamelar phospholipid vesicles and induce the release of encapsulated fluorescein. The *C*-terminal peptide corresponding to Bax also inserts into phospholipid membranes and induces changes of the physical properties of the bilayer. However, in this case the peptide forms aggregates even in the presence of lipids [143]. A possible connection between the formation of these aggregates and the oligomerisation of Bax in the membrane [145] deserves further investigations.

CONCLUSIONS AND PERSPECTIVES

Progress in the molecular basis of apoptosis defines a new framework for the final depiction of this complex process. The use of synthetic peptides together with the availability of structural information from key protein–protein complexes has significantly contributed to the understanding of cellular events at the molecular level. These developments have also opened important questions and perspectives that will be the driving force for future research. The biochemical basis of apoptotic inhibition by IAPs and activation by Smac/DIABLO is considered a source for new modulators of apoptosis. Understanding the structural basis of these processes has permitted the design and synthesis of small peptides that are able to mimic the functions of their parent proteins. From this knowledge, further efforts will be directed towards the development of a new generation of caspase inhibitors and activators which may constitute lead compounds for the discovery of potent regulators of apoptosis.

The Bcl-2 regulators are inspiring a large number of drug discovery programmes although important aspects of their function are still unknown. For example, the relationship between these proteins and mitochondrial channels like VDAC and PTP and the role of the latter systems in apoptosis is controversial. Also, it is not clear what are the molecular and mechanistic implications of the homo- and hetero-dimerisations of members of the Bcl-2 family and this may be an obstacle for the development and successful application of new active molecules based on the BH3 domain. Major advances are still waiting for answers to important questions: how are the various internal apoptotic stimuli transmitted to the mitochondria and how do Bcl-2 family members control the release of proapoptotic proteins from the inter-membrane space of mitochondria into the cytosol? Bcl-2 family proteins are known to control the permeability of mitochondria, but are they the primary components of the pores or do they regulate the status of other existing pores? To answer some of these questions an investigation of the insertion of Bcl-2 proteins in the membrane and their structure in lipidic environments should be undertaken.

The control of the assembly of the large macromolecular complexes that perform activation of apical caspases will also be the focus of continuing attention. The apoptosome is one such system, and the first simplified peptidic molecules, able to promote cytochrome-*c* independent activation of caspases, may soon appear. Additionally, the structural characterisation of homophilic-interaction motifs like CARD opens good possibilities for the design

of peptides to target the recognition of procaspase-9 by Apaf-1.

Finally, although apoptosis is a complicated pathway, as the biochemical and molecular complexities are elucidated, new therapeutic strategies will arise and synthetic peptides will be key molecules to validate such strategies. Probably, the full control of the apoptotic pathway will relay in a multi-drug therapy due to the different regulatory points that control the process in the cell. An apoptosis-activation based therapy would represent a new class of weapons against cancer, which may be based on inhibitors of type-I Bcl-2 proteins combined with Cyt-*c*-independent activators of the apoptosome and inhibitors of IAPs. In contrast, an apoptosis-inhibition based therapy, to be directed against stroke and neurodegenerative diseases, would consist of molecules that efficiently inhibit the formation of the apoptosome or direct inhibitors of caspases.

Acknowledgements

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