

Chapter 2

Systems Biology of Death Receptor-Induced Apoptosis

Kolja Schleich and Inna N. Lavrik

Abstract Programmed cell death, termed apoptosis, plays a fundamental role in the development and homeostasis of multicellular organisms. Dysregulation of apoptosis can lead to numerous diseases, including autoimmune diseases, neurodegenerative diseases, and cancer. In mammalian cells apoptosis can be induced by intra- or extracellular stimuli. Extracellular stimuli comprise death ligands which lead to death receptor-induced apoptosis, referred to as extrinsic pathway. Intracellular signals, such as DNA damage, trigger the intrinsic pathway which results in the activation of Bcl-2 proteins and release of proapoptotic factors from the mitochondria into the cytosol. Apoptosis is executed by a family of cysteine proteases, the caspases, which eventually lead to the apoptotic phenotype, such as chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage, and formation of apoptotic bodies. The focus of this chapter is on understanding the signaling complexity of the extrinsic apoptotic pathway using systems biology. We summarize the main signaling paradigms and the major models of the extrinsic pathway. The development of these models has elucidated new insights into the regulation of apoptosis.

K. Schleich

Division of Immunogenetics, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany

Bioquant, 69120 Heidelberg, Germany

I.N. Lavrik (✉)

Department of Translational Inflammation Research, Institute of Experimental Internal Medicine, Otto von Guericke University, Leipziger Str. 44, 39120, Magdeburg, Germany

e-mail: inna.lavrik@med.ovgu.de

2.1 Death Receptor-Mediated Apoptosis

Death receptors (DR) belong to the tumor necrosis factor (TNF) family of proteins and are characterized by extracellular cysteine rich domains (CRD) and an intracellular ~80 amino acid long motif, the death domain (DD) (Ashkenazi 1998; Krammer et al. 2007). The best characterized DRs are CD95 (also named Fas/APO-1), TNFR1, TRAIL receptor 1 (TRAIL-R1), and TRAIL-R2 (Ashkenazi 1998; Krammer 2000; Krammer et al. 2007). Other DRs include DR3 and DR6, EDA-R, and NGF-R (Ashkenazi 1998; Krammer et al. 2007; Lavrik et al. 2005a). Death ligands (DL) are assumed to be homotrimeric (Yan and Shi 2005) and exist in a membrane-bound or a soluble form.

The CD95-induced apoptotic pathway is one of the best-studied signaling pathways. The natural ligand of CD95, CD95L, is expressed on a variety of cells, such as cytotoxic T cells, as a type II membrane protein (Krammer 2000; Rathmell et al. 1995). Cleavage of the membrane-bound to the soluble form reduces its apoptosis-inducing potential by more than 1,000-fold (Schneider 1998). Stimulation of CD95 with its ligand or with agonistic anti-CD95 antibodies, such as anti-APO-1 (Trauth et al. 1989), triggers the oligomerization of CD95 (Fig. 2.1). This leads to the recruitment of Fas-associated death domain (FADD) through DD interactions, as well as procaspase-8, procaspase-10, and cellular FLICE inhibitory proteins (c-FLIPs) via N-terminal death effector domains (DED), and formation of the death-inducing signaling complex (DISC). In the DISC procaspase-8/10 are activated by dimerization and internal cleavage (Lavrik et al. 2005b), which is regulated by c-FLIP proteins. Recently, it has been reported that ubiquitylation plays an important role in caspase-8 activation (Jin et al. 2009). Recent structural analyses challenge the concept of a trimeric ligand binding to a trimeric receptor. Scott et al. (2009) reported a tetrameric conformation of the CD95/FADD complex. Wang et al. (2010) suggest that the minimal signaling unit of CD95L is at least hexameric and found an asymmetric conformation of 5–7 CD95 bound to 5 FADD molecules.

Triggering of CD95 has also been reported to induce nonapoptotic pathways, such as NF- κ B, AKT, and ERK (Neumann et al. 2010; Peter et al. 2007). However, the detailed mechanisms of the induction of CD95-mediated nonapoptotic pathways are not elucidated yet.

Activated caspase-8 is released from the DISC and activates effector caspases-3 and -7, which cleave a variety of substrates, such as DFF45/ICAD, thus releasing the DNase DFF40/CAD. DFF40/CAD is responsible for the fragmentation of chromosomes (Strasser et al. 2009; Yan and Shi 2005). Additionally, caspase-8 cleaves the BH3-only Bcl-2 protein Bid. The C-terminal part, tBid, then translocates to the mitochondria resulting in mitochondrial outer membrane permeabilization (MOMP) and the release of proapoptotic factors into the cytosol, such as cytochrome c, Apaf-1, or endonuclease G (Li 1998; Yan and Shi 2005). Recently, it has been reported that caspase-8 cleaves Bid in a specific complex at the mitochondria, which involves cardiolipin (Gonzalvez et al. 2008; Schug et al. 2011). This results in the

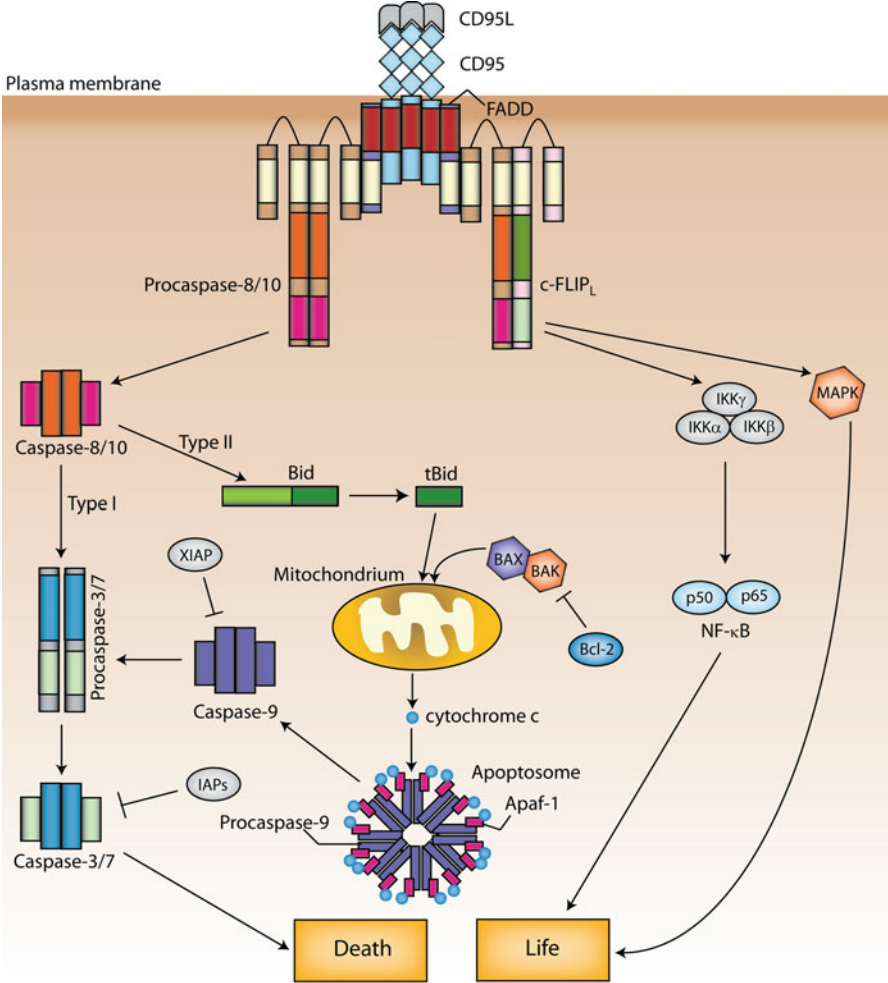


Fig. 2.1 Overview of CD95-induced pathways. Stimulation of CD95 leads to the formation of the death-inducing signaling complex (DISC), which includes at least CD95, FADD, procaspase-8, procaspase-10, and c-FLIP proteins. Caspase-8 is activated at the DISC which is regulated by c-FLIP proteins. In the cytosol caspase-8 cleaves and activates procaspase-3 or the Bcl-2 protein Bid. The truncated form of Bid (tBid) translocates to the mitochondria, which leads to mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c, as well as other proapoptotic proteins from the mitochondria into the cytosol. Cytochrome c is involved in apoptosome formation and procaspase-9 activation. Procaspase-9 in turn also activates procaspase-3 resulting in caspase-3 activity and eventually cell death. Additionally CD95 stimulation can lead to the activation of nonapoptotic pathways, such as NF- κ B or MAPK and cell survival

formation of another complex, the apoptosome, including cytochrome c, Apaf-1, ATP and procaspase-9, and activation of procaspase-9 in this complex (Boatright et al. 2003; Shi 2002). Caspase-9 also cleaves and activates caspase-3.

In CD95-induced apoptosis two different cell types are distinguished: Type I and Type II (Barnhart et al. 2003; Scaffidi et al. 1998). Type I cells [e.g., thymocytes (Strasser et al. 2009)] are characterized by high amounts of CD95 DISC, which results in very efficient procaspase-8 activation, leading to massive activation of caspase-3 and cell death. Type II cells [e.g., hepatocytes (Strasser et al. 2009)], on the other side, are characterized by lower amounts of CD95 DISC formation, that results in less active procaspase-8 and require signal amplification through tBid-mediated mitochondrial permeabilization.

2.2 Effectors and Regulators of Extrinsic Apoptosis

DR-induced apoptosis is regulated at several levels and involves numerous protein families, from the DISC to effector caspases. The interplay between the different levels of regulation provides a significant complexity, which can be understood better using systems biology. In the following sections, we will give a summary of the different protein families and their roles in extrinsic apoptosis.

2.2.1 *Caspases as Major Effector Molecules of Apoptosis Pathway*

Caspases are cysteine proteases and are the effector molecules of the apoptotic machinery (Fuentes-Prior and Salvesen 2004; Lavrik et al. 2005b). There are apoptotic, as well as inflammatory caspases. The apoptotic caspases are divided into initiator caspases, including caspase-2, -8, -9, and -10, and effector caspases, such as caspase-7 and -3 (Fuentes-Prior and Salvesen 2004). All caspases are present in the cell as inactive zymogens referred to as procaspases and are activated by internal cleavage (Fuentes-Prior and Salvesen 2004; Yan and Shi 2005). Initiator caspases act upstream of effector caspases and activate them through cleavage. Effector caspases then cleave a variety of cellular substrates, eventually resulting in cell death (Fuentes-Prior and Salvesen 2004). All caspases share a common structure. Caspase monomers consist of a large (~20 kDa) and small (~10 kDa) subunit (Fig. 2.2). Initiator caspases additionally have specific recruitment domains at their N-terminus. Procaspase-8 and -10 have two tandem DED through which they interact with FADD at the DISC, procaspase-9 possesses a caspase-recruitment domain (CARD) which is required for recruitment to the apoptosome via interactions with Apaf-1 (Fuentes-Prior and Salvesen 2004; Krammer et al. 2007; Lavrik et al. 2005b; Yan and Shi 2005).

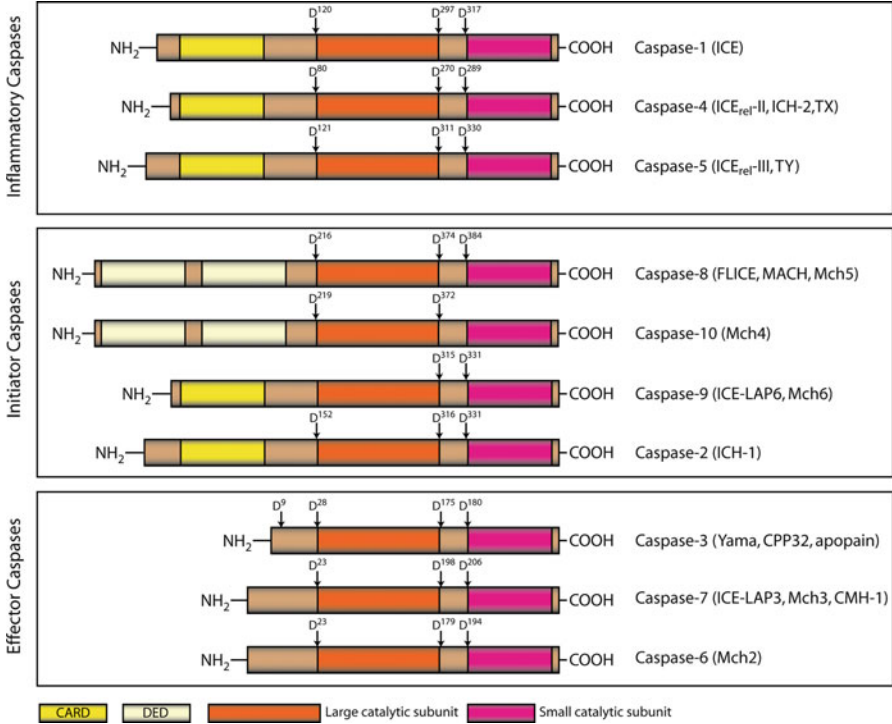


Fig. 2.2 Structural organization of caspases. Caspases are generally divided into inflammatory and apoptotic caspases. Apoptotic caspases are further divided into initiator and effector caspases. Caspases possess a large (p20) and small (p10) subunit. Initiator caspases additionally have DEDs (procaspase-8/10) or CARD domains (procaspase-9) at their N-terminus

Active caspases are heterotetramers consisting of two large and two small subunits (Fig. 2.3). Initiator caspases are present in the cytosol as monomers and are activated by dimerization or oligomerization at caspase-activating platforms and cleavage between the large and small subunits only stabilizes the dimer (Fuentes-Prior and Salvesen 2004; Hughes et al. 2009; Krammer et al. 2007; Lavrik et al. 2005b; Oberst et al. 2010). It was shown that both dimerization and interdomain cleavage are required for full activation of caspase-8 (Hughes et al. 2009; Keller et al. 2009; Oberst et al. 2010). Effector caspases on the contrary are present as inactive dimers and are readily activated by internal cleavage (Fuentes-Prior and Salvesen 2004; Oberst et al. 2010). This cleavage is performed by initiator caspases. The conceptual difference between the two classes is that there is no proteolytic enzyme upstream of initiator caspases. Consequently, initiator caspases exhibit low zymogenicity, which is defined as the ratio of activity between the cleaved and the uncleaved form. While initiator caspases have highest ratios of 10 (caspase-9) or 100 (caspase-8), the ratio for caspase-3 is more than 10,000 (Fuentes-Prior and Salvesen 2004).

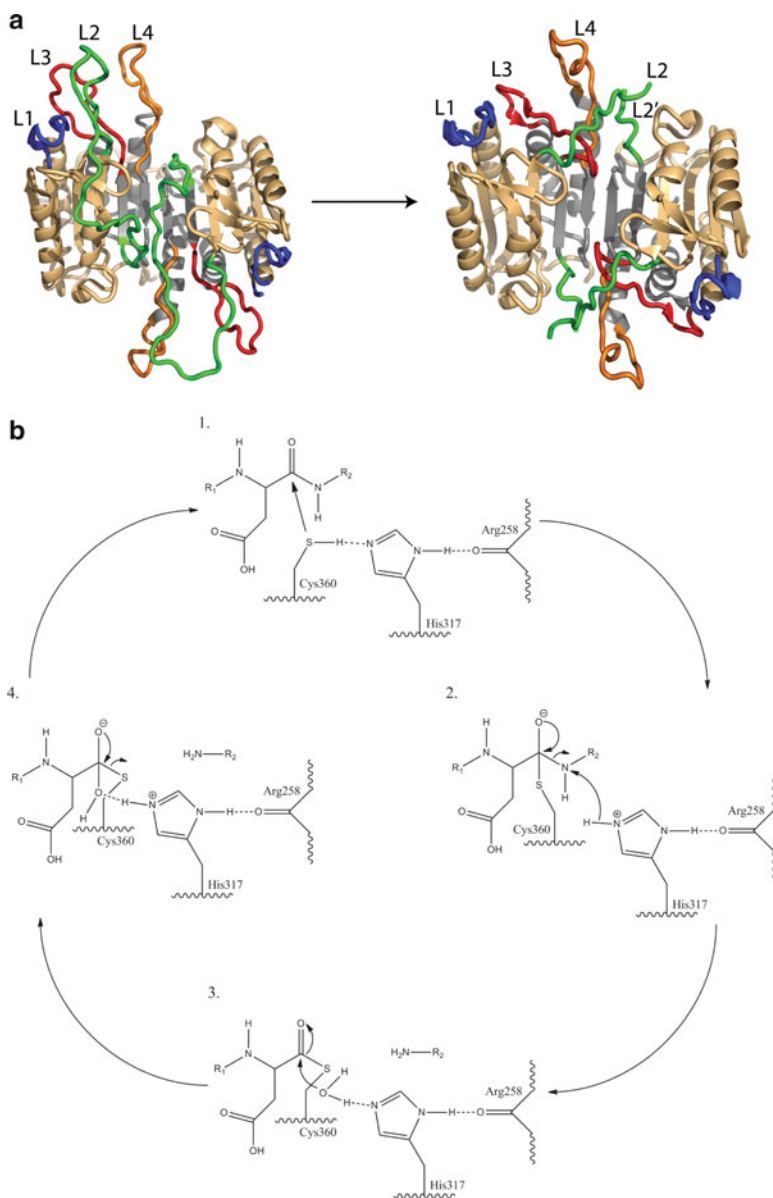


Fig. 2.3 Structure and suggested catalytic mechanism of caspases. (a) Crystal structure of procaspase-7 (left, PDB 1GQF) and active caspase-7 (right, PDB 1RHJ). The large and small subunits are colored *light orange* and *gray*, respectively. Cleavage of the intersubunit linker (L2) results in a conformational change in the active site and activation of caspase-7. The active site is formed by five loops, loops L1–L4 from one monomer and loop L2' from the adjacent monomer. The structure was generated using PyMOL (Schrodinger, 2010). (b) The catalytic mechanism of caspases is suggested to be similar to serine proteases (Fuentes-Prior and Salvesen 2004). The cysteine acts as nucleophile and forms a tetrahedral intermediate with the substrate (step 2). A nearby histidine is critical for the subsequent hydrolysis of the substrate (step 3) [adapted from Fesik (2000)]

The active site of caspases is highly conserved and is formed by five protruding loops: L1, L2, L3, and L4 from one monomer and loop L2' from the adjacent monomer (Fig. 2.3) (Fuentes-Prior and Salvesen 2004; Yan and Shi 2005). The catalytic cysteine residue is positioned in the loop L2 which determines, together with L1, L3, and L4 substrate specificity, recognizing specific tetrapeptide sequences. The L2' loop from the adjacent monomer has mainly a stabilizing function of the active site (Fuentes-Prior and Salvesen 2004; Yan and Shi 2005). Initiator and effector caspases have a fundamental difference in active site architecture, which explains their different modes of activation. The intersubunit linker (L2) of initiator caspases is longer than that of effector caspases, which allows the formation of the active site also in its uncleaved form and results in the low zymogenicity (Fuentes-Prior and Salvesen 2004). The catalytic mechanism of caspases is suggested to be similar to serine proteases (Fuentes-Prior and Salvesen 2004). The cysteine acts as nucleophile and forms a tetrahedral intermediate with the substrate (Fig. 2.3b, step 2). A nearby histidine is critical for the subsequent hydrolysis of the substrate (Fig. 2.3b, step 3) (Fesik 2000; Fuentes-Prior and Salvesen 2004).

2.2.1.1 Caspase-8/10 Activation at the DISC

Two isoforms of procaspase-8 procaspase-8a (p55) and -8b (p53) are recruited to the DISC (Scaffidi et al. 1997). After binding to the DISC, procaspase-8a/b (p55/p53) undergoes processing, thus generating active caspase-8 (Lavrik et al. 2005b; Medema et al. 1997; Muzio et al. 1996) (Fig. 2.4). This processing has been suggested to occur via dimerization of two procaspase-8 monomers followed by a conformational change, leading to autoactivation of procaspase-8 homodimers (Chang et al. 2002; Golks et al. 2006b; Hughes et al. 2009; Yu et al. 2009). Procaspase-8a/b (p55/p53) processing at the DISC also results in the generation of the N-terminal cleavage products p43/p41, the prodomains p26/p24, as well as the C-terminal cleavage products p30, p18, and p10 (Golks et al. 2006b; Hoffmann et al. 2009; Hughes et al. 2009; Medema et al. 1997). Active caspase-8 heterotetramers (p10/p18)₂ generated at the DISC initiate the execution of apoptosis (Krammer et al. 2007). Recently, it has been reported that the cleavage products p30 and p43/p41 also possess catalytic activity leading to apoptosis initiation (Hoffmann et al. 2009; Hughes et al. 2009). Hence, procaspase-8 processing at the DISC initiates apoptosis through the generation of several catalytically active cleavage products. Recently it has been reported that full activation of caspase-8 at the DISC requires Cullin3-mediated polyubiquitination in the C-terminal part of caspase-8 (Jin et al. 2009).

Three isoforms of caspase-10 namely procaspase-10a, procaspase-10c, and procaspase-10d were reported to be bound to the DISC (Sprick et al. 2002). Procaspase-10 is also activated at the DISC via generation of homodimers, leading to the formation of an active heterotetramer. However, whether caspase-10 can

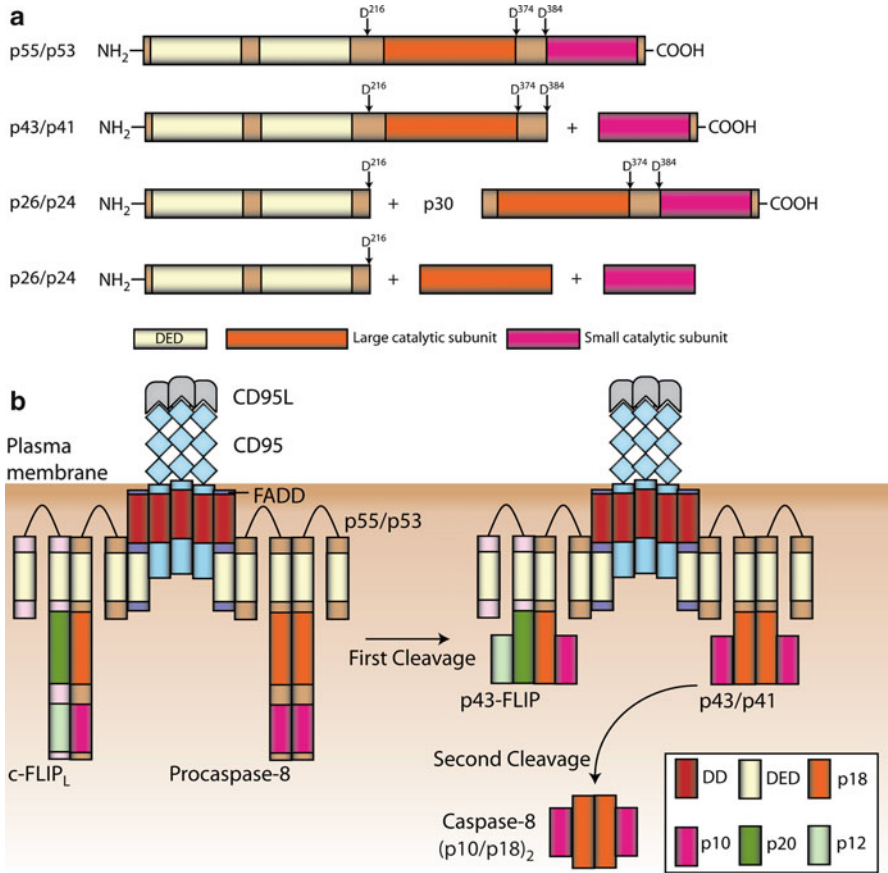


Fig. 2.4 Caspase-8 cleavage products and processing at the DISC. **(a)** Procaspase-8 can be cleaved between the large and small subunit or between the prodomain and the large catalytic subunit, resulting in numerous different cleavage products. **(b)** Procaspase-8 homodimers at the DISC can be processed to the active caspase-8 heterotetramers (p10/p18)₂ via the intermediate p43/p41. The procaspase-8/c-FLIP_L heterodimer can only be processed to the intermediate p43-FLIP

trigger cell death in the absence of caspase-8 in response to CD95 stimulation is controversial (Fischer et al. 2006; Mühlethaler-Mottet et al. 2011; Sprick et al. 2002).

2.2.2 *c-FLIP Proteins Regulate Caspase Activation at the DISC*

Three c-FLIP isoforms and two cleavage products have been characterized so far (Golks et al. 2005, 2006a; Scaffidi et al. 1999; Ueffing et al. 2008) (Fig. 2.5). Three isoforms include one long, c-FLIP_L, and two short variants, c-FLIP_S and c-FLIP_R. All three c-FLIP isoforms possess two tandem DED domains at their

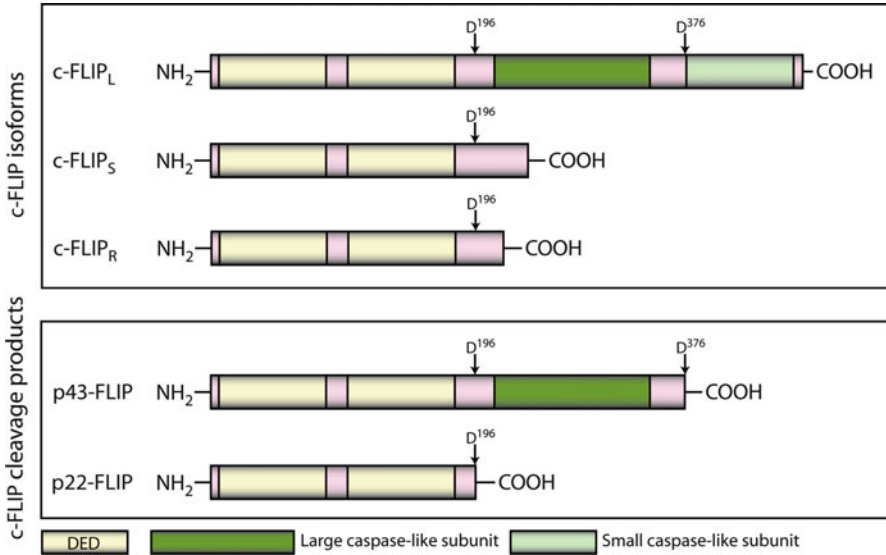


Fig. 2.5 c-FLIP isoforms and cleavage products. Three isoforms of c-FLIP proteins exist, one long (c-FLIP_L) and two short variants (c-FLIP_S and c-FLIP_R). All isoforms contain two tandem DEDs which are required for DISC recruitment. c-FLIP_L additionally has a large and small caspase-like subunit, which are catalytically inactive. C-FLIP_L can be cleaved by caspase-8 at different positions generating the N-terminal fragment p43-FLIP or N-terminal fragment p22-FLIP

N-terminus. c-FLIP_L additionally contains catalytically inactive caspase-like domains (p20 and p12). The two short isoforms, c-FLIP_S and c-FLIP_R, block DR-induced apoptosis by inhibition of procaspase-8 activation at the DISC (Golks et al. 2005; Krueger et al. 2001). This has been suggested to occur through the formation of catalytically inactive procaspase-8/c-FLIP_{R/S} heterodimers. c-FLIP_L can play both a pro- and an antiapoptotic role. It can act as an antiapoptotic molecule, functioning in a way analogous to c-FLIP_S when it is present at high concentrations at the DISC (Chang et al. 2002; Krueger et al. 2001). c-FLIP_L can act proapoptotic when expressed at lower concentrations, in combination with strong receptor stimulation or in the presence of high amounts of either of the short c-FLIP isoforms c-FLIP_S or c-FLIP_R. Under these conditions c-FLIP_L facilitates the activation of procaspase-8 at the DISC (Fricker et al. 2010). This occurs via the formation of catalytically active procaspase-8/c-FLIP_L heterodimers in which the procaspase-8 active loop is stabilized by c-FLIP_L (Micheau et al. 2002; Yu et al. 2009), thereby increasing the catalytic activity of procaspase-8.

2.2.3 IAP Family of Proteins

The Inhibitors of Apoptosis (IAP) proteins directly inhibit caspases. They all share a conserved sequence motif of 70–80 amino acids, the baculoviral IAP repeat (BIR)

domain, which is arranged around a coordinated zinc atom (Fuentes-Prior and Salvesen 2004; Shi 2002), of which each family member can possess up to three copies (Deveraux and Reed 1999). There are six human IAPs, which include XIAP, c-IAP1, c-IAP2, NAIP, Bruce, and survivin (Deveraux and Reed 1999). Numerous mammalian IAPs, as well as IAPs in flies and viruses, possess a C-terminal RING domain, however, the requirement of this domain for apoptosis suppression remains unclear (Deveraux and Reed 1999). There are reports that both domains are required for their antiapoptotic function in insects (Clem and Miller 1994; Harvey et al. 1997), however, c-IAP1, c-IAP2, and XIAP in humans could still inhibit apoptosis when lacking the RING domain (Deveraux and Reed 1999; Hay et al. 1995).

XIAP directly inhibits active caspase-3. After MOMP Smac is released from the mitochondria into the cytosol and relieves XIAP-mediated inhibition. This causes a delay between receptor-mediated initiator phase and final commitment to cell death in type II cells (Albeck et al. 2008; Fuentes-Prior and Salvesen 2004). XIAP also contains a RING domain with E3 ubiquitin ligase activity, which promotes caspase-3 degradation by the proteasome (Albeck et al. 2008; Fuentes-Prior and Salvesen 2004).

2.2.4 Bcl-2 Family of Proteins

The Bcl-2 family of proteins play a key role in the regulation of apoptosis at the mitochondrial level and are essential for extrinsic apoptosis in type II cells or intrinsic apoptosis (Adams 1998; Yan and Shi 2005). The Bcl-2 protein family comprises at least 15 members with pro- as well as antiapoptotic functions (Adams 1998). All family members share a conserved structural motif, the Bcl-2 homology domain (BH1-BH4) (Adams 1998; Yan and Shi 2005). Prosurvival family members include Bcl-2 and Bcl-X_L. The proapoptotic Bcl-2 family members can be further subdivided into multidomain proteins, represented by Bax and Bak, and BH3-only proteins, such as Bid (Adams 1998; Yan and Shi 2005). The balance of pro- and antiapoptotic Bcl-2 family members determines the apoptosis induction. Proapoptotic Bcl-2 proteins cause the release of proapoptotic factors from the mitochondria by inducing MOMP. This process is triggered either in the extrinsic pathway through cleavage of Bid by caspase-8 or in the intrinsic pathway which is mainly controlled by the tumor suppressor p53 (Yu and Zhang 2005). The Bcl-2 family is described in detail in the Chaps. 4 and 5.

2.3 CD95-Induced Nonapoptotic Pathways

Accumulating evidence suggests that stimulation of CD95 can also induce nonapoptotic pathways, such as tumor growth and invasion, as well as proliferation and programmed necrosis, termed necroptosis (Chen et al. 2010; Choi et al. 2010; Geserick et al. 2009; Lee et al. 2011; Steller et al. 2011; Strasser et al. 2009; Tang

et al. 2010). CD95-mediated nonapoptotic signaling occurs via induction of NF- κ B, Akt and mitogen-activated protein kinases (MAPK) pathways. These, however, are not well understood, but have been reported to require caspase-8 activity (Kober et al. 2011; Lee et al. 2011; Nakajima et al. 2008; Neumann et al. 2010; Shikama et al. 2003; van Raam and Salvesen 2011). C-FLIP proteins play a very important role in the regulation of caspase-8 activation as well as induction of nonapoptotic pathways. It could be shown that the cleavage product of c-FLIP_L p43-FLIP directly interacts with the IKK complex, leading to the activation of NF- κ B (Neumann et al. 2010). Other prominent players in nonapoptotic pathways are receptor-interacting protein 1 (RIP1) and RIP3 which are important for CD95-induced necroptosis, as well as activation of NF- κ B (Geserick et al. 2009; Kamarajan et al. 2010).

2.3.1 CD95-Mediated NF- κ B Activation

The eukaryotic transcription factor NF- κ B was originally discovered transcribing the immunoglobulin kappa light chain gene in B cells (Sen and Baltimore 1986). NF- κ B can be activated following a variety of stimuli, including bacterial lipopolysaccharide (LPS), T cell receptor (TCR) signaling, different cytokines, such as TNF α , interleukin 1 (IL-1) and IL-2, viral infections, UV and X-ray radiation, nitric oxide, and also CD95 (Ghosh et al. 1998; Hayden and Ghosh 2004; Legembre et al. 2004; Verma et al. 1995). The NF- κ B transcription family comprises multiple members, including RelA (p65), NF- κ B1 (p50, p105), NF- κ B2 (p52, p100), c-Rel, and RelB (Ghosh et al. 1998; Li and Verma 2002; Rasper et al. 1998; Verma et al. 1995). All NF- κ B proteins share a conserved N-terminal 300-amino acid motif, the Rel homology domain (RHD), which contains a dimerization, nuclear localization as well as DNA-binding domain (Ghosh and Hayden 2008). NF- κ B proteins form homo- or heterodimers with each other, except for RelB. The most prominent dimer which is commonly referred to as NF- κ B is the heterodimer of p65 with either p50 or p52 (Li and Verma 2002). Importantly, only c-Rel, RelA, and RelB possess a transactivation domain and thus act as transcriptional activators, while other NF- κ B proteins act as transcriptional repressors (Ghosh and Hayden 2008). In the absence of activating stimuli NF- κ B dimers are inhibited by I κ B (inhibitor of NF- κ B) proteins via ankyrin-repeat motifs and masking their nuclear localization signal (Hayden et al. 2006). I κ B proteins are phosphorylated by I κ B kinases (IKKs) and subsequently degraded by the proteasome (Li and Verma 2002). The IKK complex, consisting of IKK α , IKK β , and NEMO (IKK γ) regulates NF- κ B activation following various stimuli and lack of either IKK complex component blocks NF- κ B activation (Ghosh and Hayden 2008).

Besides its well studied proapoptotic function, CD95 also has nonapoptotic functions. It was shown that T cell proliferation under suboptimal CD3 stimulation could be enhanced by CD95 costimulation (Alderson et al. 1993; Paulsen and Janssen 2011; Paulsen et al. 2011). Additionally, CD95-knockout mice had

abnormally low lymphocyte levels (Hao et al. 2004). Further, cancer cells were found to produce CD95L and inhibition of CD95 reduced tumor size in several cancer mouse models (Chen et al. 2010). The mechanism of the nonapoptotic functions of CD95 is largely unknown. It could be shown from different independent groups that CD95 stimulation triggers NF- κ B activation, the exact mechanism, however, remains elusive (Kataoka et al. 2000; Kreuz et al. 2004; Neumann et al. 2010; O'Reilly et al. 2004). Numerous groups have reported that CD95-mediated activation of NF- κ B, ERK, and MAPK requires caspase-8 activity and is controlled by c-FLIP proteins (Fricker et al. 2010; Golks et al. 2006a; Hughes et al. 2009; Kataoka et al. 2000; Kober et al. 2011; Kreuz et al. 2004; Nakajima et al. 2008; Neumann et al. 2010; Shikama et al. 2003; van Raam and Salvesen 2011). Our group could show in a systems biology study that p43-FLIP mediates NF- κ B activation by direct interaction with the IKK complex (Neumann et al. 2010). However, the exact details of molecular mechanism of nonapoptotic signaling of CD95 will be addressed in further studies.

2.3.2 The Function of RIP in CD95-Mediated Nonapoptotic Pathways

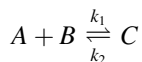
The receptor-interacting protein (RIP) family plays a major role in CD95-mediated necrosis, as well as in life and death decisions after DR stimulation (Geserick et al. 2009, 2010; Kamarajan et al. 2010; Kreuz et al. 2004; Moquin and Chan 2010; Shikama et al. 2003; Vandenabeele et al. 2010a). The most prominent member is RIP1, a serine/threonine kinase, which also possesses one C-terminal DD, a RIP homotypic interaction motif (RHIM) and a ubiquitylation site (Geserick et al. 2009; Moquin and Chan 2010; Vandenabeele et al. 2010a, b). The DD of RIP1 allows it to interact with DR or FADD. Another important player is RIP3, a homolog of RIP1, lacking the DD. RIP1 and RIP3 can interact via their RHIM domains (Moquin and Chan 2010; Vandenabeele et al. 2010b). RIP1 can form another cytosolic complex, termed ripoptosome, which consists of at least RIP1, Caspase-8/10, and FADD and is regulated by cIAPs and c-FLIP proteins (Feoktistova et al. 2011; Tenev et al. 2011). The essential role of RIP1 for CD95-induced necroptosis, however, is controversial and it may be that both RIP1 and RIP3 are required (Bertrand and Vandenabeele 2010; Wong et al. 2010). While the kinase domain of RIP1 is required for the execution of necroptosis, ubiquitylation by cIAP1/2 has been reported to be needed for its nonapoptotic activities (Moquin and Chan 2010; Vandenabeele et al. 2010a, b). The exact roles of RIP1 and RIP3 in nonapoptotic pathways induced by CD95 are still unresolved and will be addressed in models of extrinsic apoptosis in the chapter by Zhivotovsky (Calzone et al. 2010). Especially the mechanistic interactions leading to nonapoptotic pathways after death receptor stimulation are poorly understood.

2.4 Mathematical Formalisms Used in Modeling of the Extrinsic Pathway

There are numerous different modeling formalisms to describe cellular systems and some of them have been applied for modeling the extrinsic pathway. The simplest model is a graph, which only qualitatively describes a system and cannot be used for simulation. Further, apoptosis signaling has been described by Boolean models (Calzone et al. 2010; Mai and Liu 2009; Saez-Rodriguez et al. 2009; Schlatter et al. 2009). The advantage of Boolean models is that simulation and optimization can be done very fast and no kinetic parameters need to be known, because each variable can be either on or off, represented by 1 or 0, respectively. The disadvantage, however, is that Boolean models are not quantitative and cannot represent reaction kinetics. Other modeling approaches which were also applied to apoptosis modeling include Bayesian models and petri nets (Heiner et al. 2004; Yang 2005). The most common approach used in apoptosis modeling is based on ordinary differential equations (ODEs), which describe cellular reactions based on mass-action kinetics and can describe biochemical networks with adequate accuracy. A first order ODE has the form $\dot{x} = f(t, x(t))$. Coupled differential equations can be used to describe the changes in protein concentrations depending on the system state. ODEs cannot describe diffusion in a cell. Diffusion is mostly neglected when modeling a signaling pathway, based on the general assumption that molecule diffusion is much faster compared to signal transduction. In order to include diffusion, partial differential equations (PDE) can be applied.

Contrary to models based on differential equations, which are deterministic, stochastic models use probabilities that two molecules interact with each other. This can be also used in agent-based models (Bonabeau 2002; Brown et al. 2011; Macal and North 2005, 2009; Roche et al. 2011). Each molecule is represented by an agent with certain properties based on biological knowledge. Agent-based models take into account local heterogeneity of molecules, while other approaches only consider molecule populations.

ODEs are constructed based on mass-action kinetics. The following chemical reaction:



transforms into the following system of ODEs:

$$\begin{aligned}\frac{d[A]}{dt} &= \frac{d[B]}{dt} = -k_1 \cdot A \cdot B + k_2 \cdot C \\ \frac{d[C]}{dt} &= k_1 \cdot A \cdot B - k_2 \cdot C\end{aligned}$$

Usually ODEs cannot be solved analytically, but are solved numerically using computer programs, such as Matlab. The larger the model, the more kinetic

parameters the system has. Measuring a large amount of parameters is often not possible resulting in unknown parameters. Either the system needs to be simplified to reduce parameter complexity or parameters need to be estimated. When estimating parameters the system should describe the system as accurately as possible. Parameter estimation can be done by the method of least-squares to fit experimental data to the model. Oftentimes the model includes many kinetic parameters compared to few experimental data, which gives multiple solutions for the parameter estimation. Therefore, sensitivity analysis is applied to test the robustness of the model upon parameter changes.

2.4.1 Modeling Extrinsic Apoptosis

The first model of CD95-mediated apoptosis was published more than a decade ago by Fussenegger et al. (2000), which was not based on experimental data, but only mechanistically described the apoptotic pathway. Albeck et al. (2008) quantitatively described death receptor-induced signaling with a focus on MOMP. Others focused on the intrinsic pathway and mitochondrial permeabilization (Düssmann et al. 2010; Rehm et al. 2009). The following section gives a review of systems biology studies of the CD95 signaling pathway as prototypic extrinsic pathway, all using an ODE-based modeling approach.

2.4.1.1 Revealing a Threshold Mechanism in CD95-Induced Apoptosis

In Bentele et al. (2004) the CD95 pathway topology was reconstructed by literature and database research. Initially, a model was generated consisting of about 70 molecules, 80 reactions, and more than 120 unknown parameters. The relatively high number of parameters did not allow parameter estimation using experimental data. Thus, subunits of varying information qualities were added. Well-known interactions were modeled mechanistically, and two “black boxes” were introduced for mitochondria and the degradation process. Black boxes were defined by their input–output behavior. For simplification the DISC was modeled as one complex, without taking into account its stoichiometry. These steps led to a model of CD95-induced apoptosis with 41 molecules (including complexes, e.g., DISC), 32 reactions, and two black boxes, namely mitochondria and degradation. The mitochondria black box takes the concentration of Bcl-X_L/Bcl-2 and tBid as input and triggers the release of cytochrome c if the concentration of tBid reaches a certain threshold compared to the concentrations of the antiapoptotic proteins. For degradation, a decay function was introduced, depending on the apoptotic activity of the different molecules. This model still had over 50 unknown parameters and was further simplified by applying sensitivity analysis, which tests the system stability upon parameter changes. Due to the large number of unknown parameters, the sensitivities for many randomly chosen points, covering a

range of three orders of magnitude, were calculated. This analysis indicated that most sensitivities of the system were highly robust to changes in parameters. This analysis led to a simplified model of the DISC system in which the different c-FLIP isoforms were not distinguished and it was assumed that c-FLIP proteins block procaspase-8 activation at the DISC. Furthermore, the system dimensionality could be reduced from 58 to 18 unknown parameters. Based on this structured information model of CD95-induced cell death, experiments were designed to measure time-dependent concentrations of 15 different molecules after stimulation of CD95. The experiments were performed in the type I B lymphoblastoid cell line SKW6.4. The cells were stimulated with agonistic anti-APO-1 antibodies and the concentrations of the molecules of interest were measured by Western blot. In the first approach, kinetics of molecule concentrations were measured with an oversaturated ligand concentration of 5 $\mu\text{g/ml}$ anti-APO-1, corresponding to a ligand/receptor ratio of about 5:1. The mathematical model could describe well the experimental data under these conditions. However, the system was still underdetermined, meaning different model parameters could match the same experimental data. Therefore, the same kinetics were measured for lower ligand concentrations and the parameters were estimated based on these different stimulation conditions. The resulting model could fit different stimulation scenarios. Most importantly, the model predicted a critical ligand concentration, below which apoptosis is abrogated, which could be verified experimentally (Bentele et al. 2004; Lavrik et al. 2007). It could be shown experimentally that c-FLIP proteins play a critical role in maintaining the threshold behavior of CD95 signaling. Upon low ligand concentration only few receptors would be activated and c-FLIP proteins could block all binding sites for caspase-8, thus preventing induction of apoptosis. High ligand concentrations, however, could lead to the activation of many receptors and caspase-8 would outnumber c-FLIP proteins at the DISC, allowing its activation and initiation of apoptosis.

2.4.1.2 The Stoichiometry of the DISC Determines Life and Death at CD95

In Neumann et al. (2010) life/death decisions after CD95 stimulation were analyzed. In particular, we focused on the crosstalk between the apoptotic and NF- κ B signaling pathways. Initially a model was designed in which a trimerized ligand binds to trimerized CD95 further leading to the recruitment of three FADD molecules and DISC formation. To each of the FADD molecules either procaspase-8, c-FLIP_L, or c-FLIP_S could be recruited via DED interactions. The order of binding of the DED proteins to the DISC gave rise to different intermediates. Fully formed DISCs were divided into three groups. The first group contained at least two procaspase-8 molecules leading to activation of procaspase-8 and apoptosis. The second group contained at least one procaspase-8 and one c-FLIP_L molecule and would lead to the activation of NF- κ B via generation of the c-FLIP_L cleavage product p43-FLIP. The model assumed a direct interaction between p43-

FLIP and the IKK complex, leading to the phosphorylation and degradation of I κ B and translocation of p65 to the nucleus. This prediction could be also verified experimentally. The third group of DISCs comprised all other conformations leading to a blocked state which neither initiates apoptosis nor NF- κ B activation. The model was simulated using coupled ODEs and the large number of unknown parameters was reduced by assuming irreversible reactions. The remaining unknown parameters were estimated from experimental Western blot data by the method of least-squares. The model could well describe experimental data and postulated direct interaction between p43-FLIP and the IKK complex. Interestingly effector caspase-activity was not required to reproduce the dynamics of NF- κ B activation after CD95 stimulation. In addition, the model postulated that apoptotic and nonapoptotic pathways diverge at the DISC. This hypothesis was tested experimentally by inhibiting the apoptotic branch using the pan-caspase inhibitor zVAD-fmk, which blocks caspase activation downstream of the DISC, but not at the DISC (Golks et al. 2006b; Hughes et al. 2009). We stimulated zVAD-fmk pretreated CD95 overexpressing HeLa cells (HeLa-CD95) and studied NF- κ B activation as well as apoptotic signaling. We could see p65 translocation to the nucleus using mCherry-tagged p65 and fluorescence microscopy, but could not detect apoptosis, thus validating the model prediction. In order to study whether the complete model might hide key features due to its complexity we performed a step-wise model reduction. Parameter estimation was done after each reducing step and its performance compared to the original complete model. A simpler model usually gives more reliable parameter estimation, because very complex models are prone to overfitting. We came up with a simplified model, in which CD95 and FADD were assumed to be pretrimerized (RF). Upon ligand (L) binding, the L/RF complex could further recruit procaspase-8, c-FLIP_L, or c-FLIP_S. Two procaspase-8 molecules in the DISC would lead to the generation of active caspase-8 heterotetramers (p18/p10)₂ via p43/p41, while the presence of one molecule of procaspase-8 and c-FLIP_L initiates the NF- κ B pathway via the cleavage product p43-FLIP leading to nonlinear dynamics. Recruitment of c-FLIP_S to the DISC leads to inhibition. This model could still well describe experimental data and also fit with the previously found threshold behavior of CD95-signaling (Bentele et al. 2004). Interestingly, the threshold concentration did not depend on the number of CD95 on the cell surface, but determines the rates of apoptotic and nonapoptotic signaling, which was found by comparing HeLa wt and HeLa-CD95 (Neumann et al. 2010). Importantly, we revealed that the stoichiometry of DISC components and especially the ratios of c-FLIP isoforms to procaspase-8 and the concentration of their cleavage products p43/p41 and p43-FLIP play a crucial role in the life/death decisions in CD95 signaling.

2.4.1.3 Dual Function of c-FLIP_L in Procaspase-8 Processing and Cell Death

In Fricker et al. (2010) we also focused on the CD95 DISC and studied the role of c-FLIP proteins in the activation of procaspase-8 and cell death. We studied the

influence of c-FLIP on caspase-8 activation in HeLa-CD95 cells which stably overexpress c-FLIP_L (HeLa-CD95-F_L), c-FLIP_S (HeLa-CD95-F_S), or c-FLIP_R (HeLa-CD95-F_R). Procaspase-8 activation was studied after stimulation of these cell lines for different times with CD95L, followed by CD95 DISC Immunoprecipitation (IP) and Western blot analysis. Overexpression of either of the short isoforms of c-FLIP resulted in inhibition of procaspase-8 processing, even after several hours. Interestingly overexpression of c-FLIP_L strongly accelerated the first cleavage step to p43/p41, which agreed with previous studies (Golks et al. 2005; Krueger et al. 2001; Micheau et al. 2002). This effect was also observed for different concentrations of CD95L. Even though the first cleavage of procaspase-8 was enhanced, c-FLIP_L overexpressing HeLa-CD95 cells were less sensitive to apoptosis induction compared to normal HeLa-CD95. Stimulation with lower concentrations of CD95L resulted in complete inhibition of procaspase-8 processing as well as caspase-3 and PARP cleavage in c-FLIP_L overexpressing HeLa-CD95 cells, but not in normal HeLa-CD95 cells. Stimulation with a high dose of ligand led to accelerated caspase-8 processing and cleavage of caspase-3 and PARP. Essentially, we observed nonlinear effects of c-FLIP_L overexpression on procaspase-8 processing and cell death. To further study these effects, we built a mathematical model of caspase-8 processing at the DISC using coupled ODEs. The model involved CD95L which binds to CD95, further causing the recruitment of FADD to form the DISC. Contrary to the model of Neumann et al. (2010), we considered the complex of CD95L/CD95/FADD as monomer which can further recruit one DED protein. Procaspase-8 can form three different dimers at the DISC: procaspase-8 homodimers, procaspase-8/c-FLIP_L heterodimers, and procaspase-8/c-FLIP_{S/R} heterodimers. Procaspase-8 homodimers could be fully processed to p43/p41 and p18, procaspase-8/c-FLIP_L heterodimers could be only processed to p43/p41, but not p18, and procaspase-8/c-FLIP_{S/R} heterodimers could not be processed at all. This assumption was experimentally validated in HeLa-CD95-c-FLIP_{L/R} cells, overexpressing both the short and long isoforms of c-FLIP. Furthermore, procaspase-8 homodimers and procaspase-8/c-FLIP_L heterodimers were assumed to be catalytically active and cleave other molecules at the DISC. Fully processed caspase-8 heterotetramers would be released into the cytosol and replaced by new DED proteins, causing a turnover of caspase-8 at the DISC. Additionally, to reduce model complexity, procaspase-8a and procaspase-8b were considered as one entity, because they had been reported to have similar catalytic properties (Hughes et al. 2009). In addition, processing of procaspase-8 to p30 was neglected due to much lower amounts compared to p43/p41 (Hoffmann et al. 2009). Essentially, we showed that c-FLIP_L only acts proapoptotic under certain conditions and depends upon the strength of stimulation. At moderate concentrations at the DISC combined with strong stimulation of CD95 or high amounts of one of the short c-FLIP isoforms at the DISC c-FLIP_L plays a proapoptotic role, while high amounts of c-FLIP_L at the DISC results in inhibition of apoptotic signaling. Due to its high affinity to the DISC (Chang et al. 2002), c-FLIP_L could be preferentially recruited to the DISC and compete with procaspase-8 for binding sites. In addition c-FLIP_L could block procaspase-8 turnover at the DISC by blocking the final cleavage step to p18.

Finally, the role of c-FLIP_L in procaspase-8 processing is further modulated by the short c-FLIP isoforms. High levels of c-FLIP_{R/S} in the cell cause a sensitizing effect of c-FLIP_L. These findings demonstrate that c-FLIP proteins are critical regulators of life and death decisions in CD95 signaling. Further, they demonstrate the importance of the stoichiometry of the DISC in this decision process. Changes in DISC components could shift the signaling from apoptotic to nonapoptotic or vice versa. In addition the data show the complex interplay of the different components at the DISC, because the action of c-FLIP_L does not only depend on its own concentration but also on the concentration of the short variants at the DISC as well as the strength of stimulation.

2.5 Conclusions

CD95-induced apoptosis is one of the best-studied signaling pathways, making it especially interesting for modeling. Stimulation of CD95 can induce, both, apoptotic and nonapoptotic responses. The apoptotic response upon CD95 stimulation is very well defined. CD95 stimulation leads to the formation of the DISC and activation of the initiator procaspases-8 and -10 that, in turn, triggers the apoptotic cascade.

Apoptosis has been subject of intense modeling, using different modeling formalisms, including Boolean modeling (Calzone et al. 2010; Mai and Liu 2009; Saez-Rodriguez et al. 2009; Schlatter et al. 2009), Bayesian modeling, petri nets (Heiner et al. 2004; Yang 2005), and ODEs (Albeck et al. 2008; Bentele et al. 2004; Cui et al. 2008; Fricker et al. 2010; Fussenegger et al. 2000; Kober et al. 2011; Legewie et al. 2006; Neumann et al. 2010; Rehm et al. 2006; Spencer and Sorger 2011). We could successfully model extrinsic apoptosis using coupled ODEs based on biochemical data and derive reasonable biological conclusions. Using this systems biology approach, we could reveal the threshold mechanism of CD95-induced apoptosis (Bentele et al. 2004; Lavrik et al. 2007). Furthermore, we could show that the stoichiometry of the DISC is central to life and death decisions upon CD95-stimulation (Neumann et al. 2010). In the same study, we showed that p43-FLIP directly interacts with the IKK complex leading to NF- κ B activation after CD95 stimulation. In addition, we could gain detailed insights into the role of c-FLIP proteins in the activation of apoptosis or nonapoptotic pathways and showed that c-FLIP_L can exhibit pro- or antiapoptotic functions, depending on the strength of stimulation, as well as its concentration at the DISC (Fricker et al. 2010).

Our results depicted the important function of the DISC in the activation of apoptotic as well as nonapoptotic responses. Especially the DISC stoichiometry and the complex interplay between DISC components decide the signaling outcome. The stoichiometry of the DISC, however, has only been studied for CD95 and FADD (Scott et al. 2009; Wang et al. 2010), but no conclusive data on all major components, including caspase-8/10 as well as c-FLIP proteins are available.

Determining the stoichiometry of the CD95 DISC and comparing apoptotic vs. nonapoptotic cells could provide key insights into this decision process, which is still not understood conclusively. Analysis of the CD95 DISC stoichiometry using mathematical modeling should provide new insights into the regulation of CD95 signaling. Furthermore, it could be possible to alter the DISC stoichiometry pharmacologically, thus switching between the two signaling outcomes in CD95. This would add new therapies for diseases with dysfunctional apoptosis, such as neurodegenerative disorders or cancer.

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