Molecular Analysis of the TRAIL DISC and its signalling pathways

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Abstract

The TNF-receptor family plays a decisive role during the ontogenesis and homeostasis of multicellular organisms. This importance is apparent by the multitude of diseases which are caused by defects in either the ligands, receptors, or the associated signal transduction processes of the TNF family.

The receptors of the TNF family exert their effects mainly via two groups of intracellular signal transduction pathways. Stimulation of the receptors of the TNF family in many cases leads to changes in gene expression. One important pathway in this process is the activation of the NF-kB signalling cascade. The second, equally important pathway utilized by members of the TNF family is the triggering of the caspase cascade. This pathway is utilized mainly by a subgroup of the TNF-receptor family, the death receptors. The death receptors are characterized by the presence of an intracellular protein domain, the death domain (DD). This domain is necessary for direct coupling of the death receptors to activation of the caspase cascade. Caspase activation occurs in a protein complex termed DISC (death inducing signalling complex). Amongst the death receptors, the TRAIL system stands out due to its complex receptor system. This consists of two death-receptors, TRAIL-R1 and TRAIL-R2 as well as two receptors containing no or a truncated death domain, TRAIL-R3 and –R4, respectively. The cytokine TRAIL is of special interest due to its ability to kill tumor cells while normal cells are resistant to TRAIL. These properties make TRAIL a promising drug candidate for tumor therapy. Yet, preceding the clinical development, it is obligatory to better understand the molecular basis of the action and signalling of TRAIL. This knowledge will help to evaluate possible risks as well as fields of application.

At the beginning of this work, the composition of the TRAIL-DISC was controversially discussed. This was caused by the protein overexpression utilized in previous experiments.

In this work, a method was devised to isolate the *native* TRAIL-DISC for the first time. Using this method it was shown that the composition of the TRAIL-DISC identical to the CD95-DISC, regarding the already known components. It was shown that the initiator caspase, caspase-8 as well as the adaptor protein FADD are components of the TRAIL-DISC. Using cell lines deficient in either one of these two proteins it could further be shown that these two proteins are essential for normal apoptosis induction by

TRAIL. It could further be shown that no qualitative difference exists between the TRAIL-R1 and TRAIL-R2 DISC.

The involvement of Caspase-10 in TRAIL induced signal transduction has been discussed controversially as well. In this work, it could be shown that a majority of commercially available antibodies against this protein are unspecific. Using a specific antibody, which was identified in this work, it was demonstrated that three caspase-10 isoforms are expressed in cell lines. For the first time it could be demonstrated that caspase-10, like caspase-8, is recruited to the native TRAIL- and CD95-DISC. Further, it was shown that caspase-10 cannot substitute for caspase-8 and thus, possibly serves a function different from apoptosis induction.

The results of this work are the basis for further studies of the signal transduction in the TNF-Receptor family, where still some fundamental questions are unresolved. One of these is unquestionably the function and of caspase-10 and the pathways, it is involved in.

Zusammenfassung

Die TNF-Rezeptorfamilie spielt eine entscheidende Rolle bei der Ontogenese und Homöostase von vielzelligen Organismen. Deutlich wird diese wichtige Rolle durch eine Vielzahl von Erkrankungen, die durch Defekte in den Rezeptoren und Liganden der TNF-Familie oder durch Störung der durch diese Moleküle verwendeten Signalketten hervorgerufen werden.

Die Rezeptoren der TNF-Rezeptorfamilie vermitteln ihre Effekte hauptsächlich mittels zwei Gruppen intrazellulärer Signaltransduktionswege. Die Stimulation von Rezeptoren der TNF-Rezeptorfamilie führt in vielen Fällen zu einer Aktivierung von Signaltransduktionswegen, die eine Veränderung der Genexpression hervorrufen. Ein wichtiger Weg ist dabei sicherlich die Aktivierung der NF-kB Signalkaskade. Die zweite wichtige Signaltransduktionsmöglichkeit, die von den Mitgliedern der TNF-Rezeptorfamilie verwendet wird, ist das Auslösen von Apoptose durch Aktivierung der Caspase-Kaskade. Dieser Signalweg wird hauptsächlich durch eine Subgruppe der TNF-Rezeptorfamilie verwendet, den so genannten Todesrezeptoren. Diese Rezeptorgruppe ist gekennzeichnet durch das Vorhandensein eines bestimmten Proteinmotivs in ihrem intrazellulären Teil, der Todesdomäne (DD). Diese Domäne ist nötig für die direkte Kopplung der Todesrezeptoren an die Aktivierung der Caspasen. Die Aktivierung der Caspasen erfolgt in einem Proteinkomplex, dem DISC (death inducing signaling complex, Tod induzierender Signalkomplex). Der DISC assembliert nach Stimulation der Todesrezeptoren an der Todesdomäne. Unter Todesrezeptoren ist das TRAIL-System herausragend durch sein komplexes Rezeptorsystem. Dieses besteht aus zwei Todesrezeptoren, TRAIL-R1 und TRAIL-R2, sowie zwei Rezeptoren die keine oder eine unvollständige Todesdomäne besitzen, nämlich TRAIL-R3 und TRAIL-R4. Das TRAIL-System ist von besonderem Interesse, da TRAIL in der Lage ist in Tumorzellen Apoptose auszulösen, während normale Körperzellen resistent gegen TRAIL sind. Diese Eigenschaften machen TRAIL zu einem viel versprechenden Wirkstoffkandidaten für die Tumortherapie. Vor der Weiterentwicklung für einen möglichen klinischen Einsatz ist es jedoch nötig, die Grundlagen der Signaltransduktion des TRAIL-Systems zu erforschen, um sowohl mögliche Einsatzgebiete als auch potentielle Risiken abzuschätzen.

Die Zusammensetzung des TRAIL-DISCs wurde zu Beginn dieser Arbeit kontrovers

diskutiert. Dies war darauf zurückzuführen, dass vorherige Experimente mit Hilfe von Protein-Überexpressionsexperimenten durchgeführt wurden.

In dieser Arbeit konnte eine Methode entwickelt werden, um erstmals den *nativen* TRAIL-DISC zu isolieren. Mit Hilfe dieser Methode konnte gezeigt werden, dass der TRAIL-DISC in Bezug auf die bekannten intrazellulär rekrutierten Komponenten identisch zu dem CD95-DISC ist. So sind im TRAIL-DISC sowohl die Initiatorcaspase Caspase-8 als auch das Adapterprotein FADD enthalten. Durch Verwendung von Zelllinien, die defizient in jeweils einem dieser beiden Proteine sind, konnte weiter gezeigt werden, dass diese beiden Proteine essentiell für die normale Apoptoseinduktion durch diese beiden Rezeptoren sind. Des Weiteren konnte gezeigt werden, dass es auf dieser Ebene keinen qualitativen Unterschied in der Zusammensetzung des TRAIL-R1- und des TRAIL-R2- DISCs gibt.

Die Beteiligung von Caspase-10 an der TRAIL-vermittelten Signaltransduktion wurde ebenso kontrovers diskutiert. In dieser Arbeit konnte gezeigt werden, dass während ein Grossteil der kommerziellen Antikörper gegen Caspase-10 unspezifisch ist, mit Hilfe eines in dieser Arbeit identifizierten spezifischen Antikörpers die in Zellen exprimierten Caspase-10-Isoformen identifiziert werden können. Es konnte auch erstmalig gezeigt werden dass auch Caspase-10, wie Caspase-8 in dem nativen TRAIL- und CD95-DISC enthalten ist. Abschließend wurde gezeigt, dass Caspase-10 die Funktion von Caspase-8 nicht ersetzen kann, und somit möglicherweise primär nicht der Induktion von Apoptose, sondern vielmehr der Vermittlung von anderen Signalen dient.

Die Ergebnisse dieser Arbeit legen die Grundlage für weitere Untersuchungen der Signaltransduktion der TNF-Rezeptorfamilie, die noch einige offene Fragen bereithält. Eine dieser Fragen ist sicherlich die Funktion der Caspase-10 und Identität der Signalwege, an denen sie beteiligt ist.

Contents

I INTRODUCTION

For a multicellular organism to function properly, a well orchestrated interplay between the different cells making up the organism is needed. This communication is mediated by soluble and insoluble factors which, when brought together in different combinations, concentrations and varying temporal and spatial patterns results in a vast number of instructions a cell can receive. One prominent family of signaling molecules, the TNF-receptor and -ligand family has received much and early attention. This is attributed to its involvement in regulating processes as diverse as organogenesis and homeostasis, the genesis and function of the immune system and the physiological form of cell death, apoptosis. Being involved in these diverse processes, it is not surprising that disturbances in the normal function of the members of the TNF family are involved in diseases as diverse as cancer, stroke, sepsis, autoimmune disorders and immune deficiencies. Thus understanding the normal physiological role of and the signaling used by the TNF family members is central for understanding key physiological processes. In addition, it will also help us comprehending of and finding cures for diseases caused by defects in these signaling cascades.

1 The TNF superfamily

1.1 History of the identification of “Tumor Necrosis Factor”

Observation of TNF bioactivity first occurred in the late 1800s when surgeon Dr. W. B. Coley noticed that cancer patients who developed bacterial infections had tumors that became necrotic[2]. In hopes of curing cancer, Coley started injecting cancer patients with supernatants derived from certain bacterial cultures. These injections, which were called "Coley's toxins" showed some success in producing necrosis of tumors but had negative side effects.

A quarter of a century later it was shown that life bacteria were in fact not needed for

this anti-tumor effect to occur but it was not until the middle of the 20th century that a substance capable of inducing hemorrhagic tumor necrosis in tumor-bearing mice was isolated from cell-free extracts obtained from gram-negative bacteria. This substance turned out to be lipopolysaccharide (LPS), a major constituent of the cell wall of gram-negative bacteria. However, LPS (also called endotoxin) did not kill the cells but rather acted as the inducer of a factor that was found in the serum of LPS-treated animals. This factor was then named tumor necrosis factor (TNF)[3].

After the cloning of human TNF[4] and mouse TNF[5], it was realized that TNF shared considerable sequence homology to a factor that has been shown to induce lymphocyte killing, coined lympohotoxin[6]. These two factors were the first glimpse at what turned out to become a complex network of interacting ligand and receptor members of the TNF/TNF-R superfamily (TNF-SF, TNF-R-SF)[7].

1.2 The ligands of the TNF superfamily

TNF family ligands are mainly expressed as transmembrane proteins with the exception of LTα which is exclusively produced in a soluble form. An overview over the currently known family members is shown in **Table 1.** For several TNF-SF members soluble ligands have been described to exist under physiological conditions. These ligands can be generated either by proteolytic cleavage of the transmembrane precursor[8-10] or by alternative splicing of the ligand mRNA. It is still a matter of debate whether the soluble ligands possess different signaling properties or might even block signaling by membrane bound receptors[11, 12]. Information about the three dimensional structure of the ligand is currently available for TNF[13, 14], TRAIL(APO-2L)[15, 16], CD40L[17], LT[18], TALL-1 (BAFF, BLyS, zTNF4)[19], and mRANKL[20] revealing that these proteins adopt a typical "jellyroll" sandwich topology forming a trimeric ligand structure. Additional biochemical evidence like gel filtration experiments also support the trimeric nature of the TNF-SF ligands, at least for the soluble forms. However, variations of these structures have been described.

- Soluble TRAIL has been reported to exist in a stable dimeric form that exhibits only marginal activity[21]. This form is the result of interchain disulfide bridge formation which probably occurs under expression conditions favoring disulfide bond formation between the free cysteine observed in the TRAIL monomer instead of the

coordination of a zinc atom by all three TRAIL monomers in the complex. So far, TRAIL is the only known TNF family ligand which coordinates a metal ion, necessary for its complete activity[16, 21, 22].

- Dependent on pH, crystals of TNF contained dimers which are oriented head to tail[23].
- TALL-1, a ligand for non death-receptor members of the TNF-R family, has been reported to form a large virus like assembly consisting of multiple trimers at pH 7.5. At lower pH values, only single trimers were observed [19]

1.3 The receptors of the TNF-R superfamily

The TNF-R-SF consists of a large group of mostly membrane-associated receptors with 29 family members (**Table 1**). The extracellular domains of these receptors are characterized by the presence of up to 6 copies of a typical fold, the cysteine rich domain (CRD) in the extracellular part[24].

A subgroup of these receptors, the death receptors, can trigger cell death when engaged by their respective ligand. This ability has first been discovered for TNF-R1 and CD95 (APO-1/Fas). These two receptors share an intracellular motif, the death domain (DD), named after its absolute requirement for transmitting TNF mediated cytotoxicity[25]. Soon after the initial recognition of the death domain, it was established that also the ability of CD95 to transmit cell death relies on the presence of an intact DD, as naturally occurring mutations in this domain abolished CD95 mediated cytotoxicity[26, 27]. Later, it was shown that the lpr mutation in the CD95 death domain locally unfolds the alpha helix 3 and thus abolishes FADD binding explaining the observed phenotype. Additional TNF-R family members which contain a DD were subsequently identified. These are TRAIL-R1 (DR4), TRAIL-R2 (APO-2, DR5, KILLER, TRICK), TRAMP (APO-3, DR3, Wsl1, LARD, TR3, TNFRSF12), DR6, the p75 NGFR and EDAR[28]. However, not all of these DD-containing receptors when triggered are capable of efficiently signaling cell death. Of the above mentioned receptors, the ability to induce apoptosis is well established for CD95, TNF-R1, TRAIL-R1 and TRAIL-R2. The remaining DD containing members seem to be primarily involved in activating other signaling pathways like the NF-κB, JNK or MAPK pathways when triggered.

The crystal structures of two TNF-R family members in complex with their respective ligands are known. Both the structures for TNF-R/TNF[23] and that for TRAIL/TRAIL-R2[22, 29, 30] show a trimeric ligand structure binding three receptor monomers. The binding of the receptor to the ligand occurs at the ligand monomer-monomer interface. These molecular assemblies also suggest a possible mode of receptor activation by the ligands, a trimerization of receptor molecules by a trimeric ligand.

Ligand Symbol	Gen-bank ID	Aliases	Receptor Symbol	Genbank ID	Aliases
TNFSF1	X01393	LTA, TNFB, LT	**TNFRSF1A**	M75866	p55-R, CD120a, TNF-R-I p55, TNF-R, TNFR1, TNFAR, TNF-R55, p55TNFR, TNFR60
TNFSF2	X02910	TNF, TNFA, DIF	**TNFRSF1B**	M32315	CD120b, p75, TNF-R, TNF-R-II, TNFR80, TNFR2,TNF-R75, TNFBR, p75TNFR
TNFSF3	L11016	LTB, TNFC, p33	**TNFRSF3**	L04270	LTBR, TNFR2-RP, CD18, TNFR-RP, TNFCR, TNF-R-III
TNFSF4	D90224	OX-40L, gp34, TXGP1	**TNFRSF4**	X75962	OX40, ACT35, TXGP1L
TNFSF5	X67878	CD40LG, IMD3, HIGM1, CD40L, hCD40L, TRAP, CD154, gp39	**TNFRSF5**	X60592	p50, Bp50, CD40
TNFSF6	U11821	FasL, APO-1L, APT1LG1	**TNFRSF6**	M67454	CD95, FAS, APO-1, APT1
			TNFRSF6B	AF104419	DcR3, M68, TR6, HGNC:15888, NHL, DKFZP434C013, KIAA1088, bK3184A7.3, C20orf41
TNFSF7	L08096	CD70, CD27L, CD27LG	**TNFRSF7**	M63928	Tp55, S152, CD27
TNFSF8	L09753	CD30LG	**TNFRSF8**	M83554	Ki-1, D1S166E, CD30
TNFSF9	U03398	4-1BB-L	**TNFRSF9**	L12964	4-1BB, CD137, ILA
TNFSF10	U37518	TRAIL, Apo-2L, TL2	**TNFRSF10A**	U90875	TRAIL-R1, DR4, Apo2
			TNFRSF10B	AF012628	TRAIL-R2, DR5, KILLER, TRICK2A, TRICKB
			TNFRSF10C	AF012536	TRAIL-R3, DcR1, LIT, TRID
			TNFRSF10D	AF029761	TRAIL-R4, DcR2, TRUNDD
TNFSF11	AF013171	TRANCE, RANKL, OPGL, ODF	**TNFRSF11A**	AF018253	RANK
			TNFRSF11B	U94332	OPG, OCIF, TR1
TNFSF12	AF030099	TWEAK, DR3LG, APO3L	**TNFRSF12**	U72763	DR3, TRAMP, WSL-1, LARD, WSL-LR,DDR3, TR3, APO-3
			TNFRSF12L	?	DR3L
TNFSF13	NM_003808	APRIL	**TNFRSF13B**	AF023614	TACI

TNFSF13B	AF136293	BAFF, THANK, BLYS, TALL-1, TALL1, **TNFSF20**	**TNFRSF13C**	AF373846	BAFFR
TNFSF14	AF036581	LIGHT, LTg, HVEM-L	**TNFRSF14**	U70321	HVEM, ATAR, TR2, LIGHTR, HVEA
TNFSF15	AF039390	TL1, VEGI	**TNFRSF15**	-	-
TNFSF16	-	-	NGFR	M14764	**TNFRSF16**, p75NTR
TNFSF17	-	-	**TNFRSF17**	Z29574	BCMA, **TNFRSF13**
TNFSF18	AF125303	AITRL TL6 hGITRL	**TNFRSF18**	AF125304	AITR, GITR
TNFSF19	-	-	**TNFRSF19**	AB040434	TAJ-alpha, TROY, TAJ, TRADE
-	-	-	**TNFRSF19L**	AF319553	FLJ14993, RELT
TNFSF20-withdrawn	-	-	**TNFRSF20**	-	-
TNFSF21	-	-	**TNFRSF21**	AF068868	DR6
TNFSF22	-	-	**TNFRSF22**	?	SOBa, Tnfrh2, 2810028K06Rik
TNFSF23	-	-	**TNFRSF23**	?	mSOB, Tnfrh1

Table 1 Known TNF-R and TNF-L Family members, their official symbols and their database accession number. Additionally their alternative names are given. Adapted from: http://www.gene.ucl.ac.uk/nomenclature/genefamily/tnftop.html

1.4 The TNF System

TNF can bind two receptors, TNF-R1 (p55TNF-R) and TNF-R2 (p75 TNF-R) (reviewed by Wajant et al.[31]). Only TNF-R1 contains a death domain and is capable of potently inducing cell death when triggered, although the cryptic ability to induce cell death has also been described for TNF-R2[32-35]. The protein complex involved in TNF-R2-mediated apoptosis has been proposed to involve TRAF-2, RIP and FADD[35]. In addition to their principal ligand TNF, TNF-R1 and TNF-R2 can also be triggered by the principally secreted cytokine LTα which consists of homotrimeric LT ($LT\alpha_3$). Additional complexity arises from the fact that a second membrane bound ligand, LTβ can form a heteromeric complex with LTα, yielding $LT\alpha_1\beta_2$ which neither binds to TNF-R1 nor to TNF-R2 but instead to a third receptor named LTβ-R. A complex interplay between these three different receptors and ligands plays an important role in the development of secondary lymphoid tissues[36].

1.5 The CD95 (APO-1/Fas) system

In 1989 two groups reported on the identification of monoclonal antibodies which ac-

tively induced apoptosis in target cells[37, 38]. Upon cloning of the respective antigens it was found out that both antibodies reacted with the very same receptor on the cell surface, namely the 48 kDa transmembrane receptor which was initially called Fas[39] and APO-1[40] and is now called CD95. One year later, the ligand for CD95 was identified[41].

Having both, the receptor and ligand at hand, the physiological function of the CD95 system began to be unraveled. First, it was established, that autocrine T cell suicide upon T cell activation, also called activation-induced cell death (AICD), was due to the interaction of CD95 with its ligand[8, 9, 42, 43]. The central importance of the CD95/CD95-L interactions in AICD and further immunological processes is illustrated by the phenotype caused by a defect in this system.

In mice, several mutations in the CD95 signaling cascade are described. The observed effects in affected mice include accumulation of double negative ($CD4^-$ /$CD8^-$) thymocytes, leading to enlarged spleen and lymph nodes. On some genetic backgrounds the cell numbers observed are up to 200-fold higher than normal controls[44]. In addition, a massive autoimmune syndrome is seen with 50 percent of the mice dying at the age of 5 month due to glomerulonephritis.

In the *lpr* mouse, the CD95 receptor expression is affected by the insertion of a retrotransposon into the second exon of the gene, leading to aberrant splicing and a strongly reduced expression of the CD95 receptor[45-48]

In the lpr^{cg} mutation a point mutation in the intracellular domain of the CD95 receptor leads to impaired signal transduction. In gld (generalized lymphoproliferative disease) mice in contrast, a point mutation in the CD95-ligand prevents binding of the ligand to the CD95 receptor and thus, signal transduction. Similar defects can be found in humans, also leading to autoimmune diseases, called autoimmune lymphoproliferative syndrome, ALPS[26, 27]. ALPS patients suffer from lymphoadenopathy, autoimmune syndromes and an accumulation of non-malignant T cells[49].

Another important physiological role for the CD95 system was discovered when it was shown that immune privilege in eye and testis was due to CD95L expression in these tissues[50, 51]. In addition, a pathological role was attributed to CD95L when expressed on certain tumors which had been shown to be immune-privileged as well[52]. In the following years the CD95 system has been shown to be involved in a plethora of physiological and pathophysiological situations. However, the desired pro-apoptotic therapeutic activity,

namely the induction of apoptosis in tumor cells, longed for since the identification of the tumor-necrotizing properties of the bacterial extracts discovered by Coley in 1893, was again impeded: agonists of the CD95 receptor, both antibodies and the CD95L, were highly toxic upon systemic administration[53].

1.6 The TRAIL (APO-2L) system

In 1995 and 1996 Wiley et al. and Pitti et al. independently identified a novel member of the TNF-SF solely due to its homology to the CD95 ligand[54, 55]. Due to this homology it was not surprising to see that this new protein which was named TRAIL for "TNF-related apoptosis inducing ligand" and APO-2L for its close homology to APO-1L (CD95L/FasL), respectively, was capably of inducing apoptosis. Interestingly, however, TRAIL induced apoptosis in many different tumor cell lines but not in the majority of the normal cell types that were tested. This property of TRAIL prompted the testing of its antitumor potential *in vivo*. It was shown that TRAIL (APO-2L) was capable of inhibiting tumor growth *in vivo* and that it acted synergistically with standard chemotherapeutics and thereby achieved even more striking anti-tumor effects[56-61].

At the biochemical level it was of course interesting to identify the receptor for TRAIL which was responsible for the induction of cell death on the surface of the target cell. The outcome of the resulting cloning race was surprising. With many different receptors at its disposal TRAIL emerged as the most promiscuous of all cytokines known[62, 63]. TRAIL can bind two apoptosis-inducing receptors, TRAIL-R1 (DR4)[64] and TRAIL-R2 (Killer, DR5, TRICK2)[65-71], two additional cell-bound receptors incapable of transmitting an apoptotic signal, TRAIL-R3 (LIT, DcR1)[65, 67, 68, 72, 73] and TRAIL-R4 (TRUNDD, DcR2)[74-76], and, lastly, a soluble receptor called osteoprotegerin (OPG)[77]. OPG binds TRAIL with reduced affinity at physiological temperatures[78]. The prominent physiological function of OPG is most likely the regulation of bone formation in concert with its high affinity ligand, the osteoclast differentiation factor (ODF, OPGL, RANKL, TRANCE)[79]. However, OPG has also been proposed to be a survival factor for some cancer cells, possibly by inhibiting TRAIL-mediated apoptosis[80].

At first it seemed as if the existence of these functionally distinct receptors might provide an answer to the differential sensitivity to TRAIL observed between normal and trans-

formed cells. The initial findings indicated that TRAIL-R3 and TRAIL-R4 may act as so-called ”decoy receptors” by competing with TRAIL-R1 and TRAIL-R2 for binding of TRAIL[68, 76]. However, these results were obtained solely by relying on overexpression data. By using monoclonal antibodies specific for each one of the individual surface-bound TRAIL receptors it was recently shown for various cellular systems that TRAIL resistance is controlled intracellularly rather than at the level of TRAIL-R3 and/or TRAIL–R4, the putative ”decoy receptors”[81]. In addition, several surveys of cancer cell lines did not show any[82-85] or only a limited[86] correlation between TRAIL sensitivity and TRAIL-R3 and –R4 expression. Yet most of these studies relied on investigating mRNA levels rather than surface expression of the receptors. Even looking at the surface expression might be misleading, as it has been shown that TRAIL-R3 and –R4 can be localized inside the cell[87] and become redistributed after TRAIL stimulation. Whether this mechanism is of general importance in TRAIL resistance of primary cells remains to be determined.

In mice, so far only one TRAIL death receptor, has been identified[88]. In addition, two TRAIL decoy receptors, mTRAILDcR1 and mTRAIL-DcR2 have been found[89]. Interestingly, the overall sequence structures of these murine decoy receptors differ markedly from that of the human TRAIL-receptors –3 and –4, suggesting that the appearance of TRAIL-decoy receptors represents a more recent evolutionary event.

1.7 Other DD-containing receptors

Apart from the well characterized death receptors CD95, TNF-R1, TRAIL-R1 and TRAIL-R2 several other DD containing receptors have been identified in recent years. These receptors are TRAMP (DR3, Wsl1, Apo-3, LARD, TR3, TNFRSF12)[90-95], DR6[96] and EDAR[28]. However, they differ widely in their ability to trigger cell death and much less is known about the pathways these receptors utilize to transmit their downstream signals. From the data available it seems as if these receptors’ main function is not to induce apoptosis, but rather to transmit signals utilized during adaptation of the immune response or regulating developmental processes[7].

2 Programmed cell death: Apoptosis

During the life span of a multicellular organism, most cells in such an organism die at a certain point. The decision to die serves the common purpose of all cells in such organisms, which is self-propagation. Multicellular organisms have evolved a system where a single cell either by itself decides to die (intrinsic pathway) or where specialized other cells, so-called effector cells, take this decision for a certain cell if this cell is recognized as potentially harmful (extrinsic pathway). Examples for such potentially dangerous cells are plentiful and include e.g. virally or oncogenically transformed cells, which are recognized as such and are therefore killed by the effector cells of the immune system. For many years it was unknown how exactly these cells kill their targets and it was only after decades of research that we finally got a glimpse. We now know that beside other mechanisms, programmed cell death by apoptosis induced by certain members of the TNF family is crucial for these targeted cell deletion mechanisms.

2.1 Apoptosis versus necrosis

The first recognition of apoptosis or 'programmed cell death' dates back to the 19th century, when Carl Vogt described that single cells could undergo a predictable ('programmed') cell death[97]. In 1972, Kerr, Wyllie and Currie noted that the morphology of toxin-treated liver cells resembled that of dying embryonal cells durin ontogenesis, and coined the name 'Apoptosis' for this phenomenon[98]. It was soon recognized, that apoptosis played a role not only in ontogenesis but also during a variety of different processes, e.g. during the elimination of potentially dangerous cells like, oncogenically[99] or virally transformed cells[100], homeostasis of tissues and the immune system[101].

Apoptosis is a well-orchestrated chain of events that ultimately leads to the destruction and phagocytosis of the apoptotic cells. Key events during apoptosis include chromatin condensation with subsequent fragmentation of the DNA[102], presentation of phagocytosis signals like phosphatidylserine on the outer leaflet of the plasma membrane[103], degradation of a variety of cellular proteins and 'blebbing', a morphological change which can be observed light microscopically[104]. All these processes ultimately serve one goal: the safe de-

struction and elimination of the apoptotic cell.

Besides apoptosis there is another form of cell death termed necrosis. Necrosis is cell death without the proper exceution of a defined the apoptotic program. Such death occurs e.g. due to excessive mechanical or otherwise detrimental damage to the cell. During necrosis, the cytosol and cell organelles are released into the intracellular space, ultimately leading to an inflammatory reaction.

However, it is becoming more and more evident that apoptosis and necrosis constitute the two ends of a spectrum, that covers all forms of cell death including mixtures of both forms[105]. Although different names have been proposed the observed processes most likely, represent an incomplete already described apoptotic program. In that respect it seem noteworthy to mention that it has been that cells undergoing apoptosis can be forced to switch to necrosis by depletion of cellular ATP[106].

2.2 Caspases, the executioners of cell death

Amongst the key enzymes responsible for the execution of the apoptotic program are the caspases[107]. Caspases are proteases belonging to the group of cysteinyl-aspartases, hence the name. Caspases are evolutionarily conserved and members of the caspase family can be found in organisms as diverse as flies, worms, fish and man. The existence of enzymes having similar functions to caspases has also been described for plants[108].

All caspases are synthesized as zymogens (procaspases) sharing a common domain structure consisting of a large (p10) and a small (p20) catalytic subunit, and an N-terminal prodomain of varying length.

Caspases can be grouped based either on the architecture of their N-terminal prodomain or on their function. The caspases 1, 4, 5 and 11 are largely involved in mediating inflammatory signals by proteolytically maturing cytokines of the IL-1β family. Caspases 2, 9, 8 and 10 are apical caspases in the caspase cascade, while caspases 3, 6 and 7 are involved in executing the apoptotic program by degrading a variety of cellular substrates[104]. The mode of activation and the physiological substrates of caspase-14 are not yet determined, although it possibly plays a role during differentiation of epidermal cells[109-118].

Two fundamentally different groups of caspases can be discerned when grouped according to their prodomain. The first group is characterized by a long prodomain forming a

protein-protein interaction domain that is characterized by six alpha-helical loops. These prodomains allow for the recruitment of the procaspases into a protein complex. Recruitment into the complex usually results in activation of the respective caspase. Long prodomain caspases are caspase-1, -2, -4, -5, -9, -11 and –12 with an N-terminal CARD (caspase activating recruitment domain). The activation complexes for these caspases are either the apoptosome for caspase-9[119] or the recently proposed inflammasome for caspase-1,- 4-, -5[120] and possibly their murine homologs caspases –11 and 12 respectively. Caspase-2 has been proposed to play a role in the apoptotic response after DNA damage[121], and the existence of a caspase-2-specific activation complex has been hinted at recently[122]. Caspase-8 and –10 with their DED (death effector domain) are utilized during death receptor mediated apoptosis by their activation at the death inducing signaling complexes (DISCs)[123]. Caspase-3, -6, –7 and -14 in contrast contain only a short prodomain and their activation is usually achieved by proteolytic cleavage by the more upstream long prodomain caspases.

2.3 Two pathways to apoptosis

Two principle pathways for the initiation of cell death exist. Each of these two pathways is triggered by a specific set of signals under different conditions. Central to both pathways is the outcome, the activation of the executioner caspases and thus the initiation of apoptosis.

2.3.1 The intrinsic apoptotic pathway

The intrinsic, cell autonomous pathway is triggered by activation of caspases from inside the cell. Central to this intrinsic pathway is the formation of an intracellular caspase-9 activating complex, the apoptosome[119]. Central to the activation of this pathway are the mitochondria. By interaction of caspase-9 with a co-factor, Apaf-1 in combination with cytochrome c the apoptosome is formed. This molecular assembly of the apoptosome leads to activation of caspase-9. Examples of events triggering apoptosome formation include genotoxic stress, cytokine withdrawal and detachment of adherent cells (anoikis). Common to all these events is that they lead to efflux of cytochrome c, usually located in the lumen between the inner and outer mitochondrial membrane, from the mitochondria.

Key to regulating the integrity of the mitochondrial membrane are proteins of the Bcl-2

family. This protein family consists of pro- and anti-apoptotic members, the balance of their activities controlling cytochrome c release. Members of the antiapoptic Bcl-2 family like Bcl-2, Bcl-X_l, Bcl-w, Mcl-1, and Bfl-1/A1 are associated with the mitochondrial outer membrane, maintaining their integrity. The large proapoptotic members, Bax, Bak, Bok (Mtd) associate with the outer mitochondrial membrane during apoptosis, breaching its integrity. Apoptosis initiation not only requires these large Bcl-2-related proteins, but also smaller family members, the BH3 only proteins. These pro-apoptotic proteins act as sentinels over various cellular organelles and processes. Different apoptotic stimuli lead to the activation of these BH3 only proteins. The mechanisms by which these proteins become activated are different for each family member. DNA damage leads to p53-dependent transcriptional upregulation of Noxa and PUMA (Bbc3) providing a link between p53 upregulation and apoptosis. Growth factor withdrawal leads to a dephosphorylation of Bad, releasing it from its interaction partner 14-3-3. Two additional BH3 only proteins, Bim and Bmf, are usually sequestered at cytoskeletal components. Bmf is released after UV irradiation or induction of Anoikis. Bim has been shown to be released after induction of Ca^{++} flux or cytokine withdrawal. In addition, Bim has been shown to be upregulated by the JNK/FKHR pathway. The protein Bid provides a link between the extrinsic, receptor-mediated pathway, and the mitochondrial pathway. This protein can be cleaved by caspase-8, inducing its translocation to the mitochondria[124, 125].

The current view of this process is that BH3-only proteins act as a ligand for the Bcl-2 family members Bax and Bak, which enhance mitochondrial permeability upon binding of the BH3 only members. The molecular details of this process are not yet fully resolved. Current theories involve channel formation by the pro-apoptotic members, an attractive hypothesis in the view of the structural similarity between Bax/Bak/Bok and bacterial channel forming proteins. Other views are that these proteins interact with a channel already present on the mitochondria and induce the constant opening of this channel [126]. Regardless of their mechanism of action, what can be taken as experimentally confirmed is that the antiapoptotic Bcl-2 family members counter the effects of the BH3-only family members and Bax/Bak/Bok and thus serve to prevent the release of cytochrome c and other pro-apoptotic molecules from the mitochondria.

2.3.2 Formation of the death- inducing signaling complex (DISC)

In the extrinsic, receptor mediated pathway, cell death is initiated by stimulation of members of the death receptor family, leading to formation of a death inducing signaling complex (DISC) at the receptor. Central to this pathway are the initiator caspases 8 and 10.

Soon after discovery of CD95, it was observed that following stimulation several proteins termed CAPs (for cytotoxicity-dependent APO-1(Fas/CD95) associated proteins) are recruited to the CD95 receptor. This protein complex has been named 'DISC' for death inducing signaling complex[127]. The first clues for the identity of these proteins came in the same year with the identification of FADD/MORT1, a protein that bound to the intracellular domain of CD95[128, 129]. It was found that this protein when overexpressed induced cell death which could be blocked by caspase inhibitors. Still, it remained unclear how the recruitment of this catalytically inactive protein to the DISC could lead to activation of caspases. However, only one year later two groups provided the unexpected and exciting solution to this problem. Two complementary cloning approaches led to the identification of a protease of the caspase family (now known as caspase-8), which is involved in CD95 and TNF-R1 induced apoptosis[130, 131]. Further, it was found that binding of this caspase to the CD95-receptor complex leads to activation of its proteolytic activity [132]. This milestone discovery, the identification of a caspase as an integral component of the DISC, provided the missing link that coupled death receptor ligation to activation of the proapoptotic caspases.

2.4 DISC components and mode of DISC assembly

2.4.1 Interaction domains

The interactions between the DISC components are mediated by conserved protein motifs which interact in a homotypic mode. Two prominent domains involved in these interactions are the death domain (DD) and the death effector domain (DED) (**Figure 1**)[133]. Structural studies showed that both domains adopt a fold consisting of six antiparallel helices. This evolutionarily conserved motif is also present in a third protein domain which mediates protein-protein interactions, the CARD domain[134-141]. Protein-protein interactions between these protein folds occur in a homotypic manner between DDs, DEDs and

CARDs, respectively[133]. While the interactions between DDs are mainly mediated by electrostatic interactions[136, 137], DED domain interactions preferably utilize hydrophobic interactions[134], although, recently an additional surface patch, mediating ionic interactions in the FADD DED was defined[142].

2.4.2 FADD/MORT1

FADD/MORT1 has been originally identified in a yeast two hybrid screen with the CD95 death domain as a bait[128, 129]. It contains both a DD and a DED and acts as an adapter between DD-containing receptors and the DED-domain-containing caspases, namely caspase-8 and caspase-10. It is generally believed that FADD binds directly to the death domains of CD95, TRAIL-R1 and TRAIL-R2 by interaction of its death domain with the death domains of the receptors. This model is supported by in vitro experiments which show that FADD and the death domains of the receptors interact. The central role of FADD as an adaptor linking death receptors to caspase activation is further supported by experiments using cell lines or mouse embryonic fibroblasts (MEFs) deficient in FADD. In these systems, FADD has been shown to be essential for recruitment of caspase-8 and caspase-10 not only to the CD95 DISC, but also to the TRAIL-R1 and TRAIL-R2 DISCs as could be shown in this work (**Fig. 13**)[143-145]. In addition, cells lacking FADD are resistant to CD95-, TNF- and TRAIL-mediated apoptosis[146-149]. Blockage of CD95- and TNF-mediated killing could also be observed when a dominant negative version of FADD was overexpressed[150]. However, cell death after overexpression of TNF-R1 in MEFs derived from FADD deficient mice was only partially blocked, opening the possibility for a FADD-independent death pathway, at least for TNF-R1[146].

Amongst the death receptors, TNF-R1 differs from CD95 and TRAIL-R1 and -R2. Recruitment of FADD to TNF-R1 does not seem to be mediated by direct interaction of the DD of FADD with the DD of TNF-R1. Instead, it has been proposed that an additional molecule, the TNF-R1-associated DD-containing protein (TRADD) acts as a bridge between TNF-R1 and FADD[130, 151].

In addition to its function in pro-apoptotic pathways, another possible function for FADD was determined by the analysis of mice that either lack FADD in their T cell compartment or overexpress a dominant negative version of FADD under a T cell specific promoter[148, 152-154]. Interestingly, lymphocytes derived from these mice are not only resis-

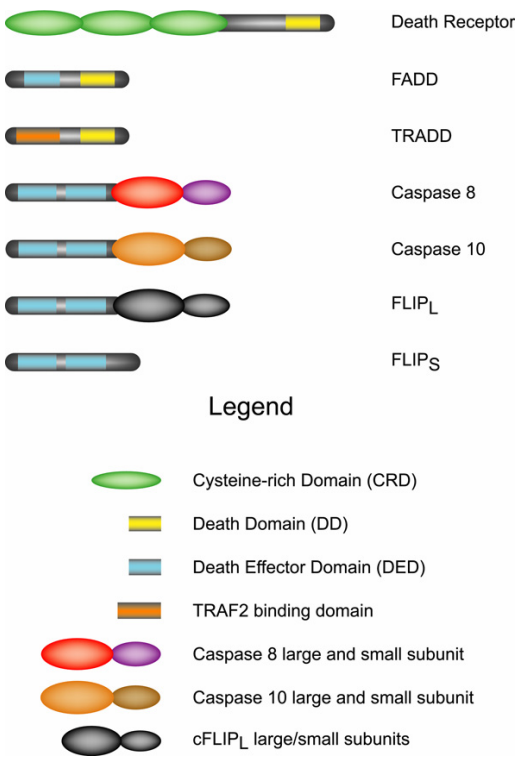

Fig. 1. Overview of the domain structure of DISC associated proteins.

tant to death-receptor-mediated apoptosis, but also show a defect in lymphocyte proliferation**.** In line with these results, FADD has been shown to be phosphorylated by a cell cycle regulated kinase also suggesting a connection between FADD and the regulation of cell proliferation[155]. This idea is further corroborated by a recent publication showing that indeed the reversible phosphorylation of FADD is necessary for proper cell-cyle progression in T cells[156].

2.4.3 The initiator caspases

2.4.3.1 Caspase-8

The discovery of caspase-8 (FLICE, Mach, Mch5) as a component of the CD95 DISC yielded the missing link between the activation of death receptors and cell death induced by activation of caspases[128, 129, 157]. Among the initiator caspases, only caspase-8 and its close homologue, caspase-10, contain two DEDs in their prodomain. These death effector domains have been shown to mediate the interaction between the adapter protein FADD and each one of these caspases.

Caspases are synthesized as inactive proenzymes. Maturation of caspases to the active enzyme occurs by proteolytic separation of the large and small catalytic subunit from the pro-enzyme. Once liberated, two large and two small subunits form the active tetrameric enzyme[158, 159]. Caspase-8 is expressed in at least three splice variants of which two, caspase-8a and caspase-8b, are catalytically active[160]. The third expressed variant, termed CAP3 represents a truncated form that lacks catalytic activity[160].

Experiments using cell lines or mouse cells deficient in caspase-8 suggest that caspase-8 is the major caspase necessary for death receptor-mediated apoptosis. In cell lines deficient in caspase-8, death receptor-mediated apoptosis is strongly suppressed (this work (**Fig.**

8,14)) and ref.[144, 145, 161]. However, residual cell death can be observed after longer stimulation and/or stronger stimuli also in the absence of caspase-8, suggesting the existence of an alternative pathway which does not rely on caspase-8 (this work **Fig.14** and refs.[143, 162-164]). Homozygous deletion of the caspase-8 locus in mice leads to embryonic lethality, and murine embryonic fibroblasts (MEF) derived from these mice are resistant to CD95-, TRAMP- and TNF-induced cell death providing evidence for a non-redundant role of caspase-8 in the embryonic development in mice[165]. Recent reports also link caspase-8 to activation of peripheral T cells by the phenotype described for two patients with a mutation in in this caspase[166]. In contrast to the effects seen in ALPS patients, these kindred had a defect in activation of naive lymphocytes leading to immune deficiency rather than an autoimmune syndrome. Similar defects have been described in mice with a lymphocyte specific deletion of caspase-8[167].

2.4.3.2 Caspase-10

Caspase-10 is, in addition to caspase-8, the only caspase containing two death effector domains in its prodomain[168-170]. Like caspase-8, caspase-10 has also been shown to be recruited to the CD95 and TRAIL-R DISCs, where it is activated with kinetics comparable to caspase-8 (this work **Fig.11** and[143, 162, 164]). Due to its high homology to caspase-8 and obviously identical activation pattern, it seemed logical that caspase-10 would also mediate TRAIL- and CD95-induced apoptosis, even in the absence of caspase-8. Under certain circumstances this can been observed, although apoptosis induction by caspase-10 alone is much less efficient than by caspase-8, making it unlikely that caspase-10 is a backup caspase for receptor-mediated apoptosis. Yet, it might serve to transmit signals other than cell death emanating from stimulated death receptors. In addition, it might play a more prominent role in apoptosis induced by other receptors of the TNF family. Interestingly, in a number of tumor cell lines analyzed caspase-10 is downregulated at the protein level by a posttranscriptional mechanism, pointing towards a possible role of this caspase in tumor suppression.

While both, a caspase-8 and a caspase-10 homologue have been identified in Xenopus laevis[171], to date no mouse caspase-10 gene has been identified and might be absent in this species, at least in the laboratory strains analyzed thus far.

2.4.4 cFLIP

The first FLICE-inhibitory protein (FLIP, also called Casper, CASH, MRIT, FLAME-1, I-FLICE, CLARP and Usurpin; for a recent review see Krueger et al.[172]) that was discovered was a viral protein (v-FLIP) capable of inhibiting CD95-, TRAIL- and TRAMP- mediated apoptosis[173-175]. Later two cellular homologues, $cFLIP_L$ and $cFLIP_S$ were identified[176-183]. Of the multiple splice variants that were originally reported only two forms, $cFLIP_L$ and $cFLIP_S$ have been shown to exist on the protein level[160]. The primary structure of cFLIP resembles that of caspase-8 and caspase-10. Both variants contain the tandem DED, enabling this protein to interact with FADD and thus to be recruited to the DISC complexes. The longer variant in addition contains domains which are similar to the large and small subunit of the initiator caspases. Yet, despite this high homology, $cFLIP_L$ does not contain any catalytic activity, because residues critical in forming the active site, which are conserved in all caspases are missing in this protein. Therefore, $cFLIP_L$ can interact with initiator caspases but $cFLIP_L$ itself is not a caspase. $cFLIP_L$ can also be cleaved by caspase-8 between the region homologous to the large and the small subunit. When present in high amounts, $cFLIP_L$ and $cFLIP_S$ have been shown to block death-receptor-mediated cell death by interfering with caspase activation at the DISC[184]. However, the unique role of cFLIP solely as an inhibitor of apoptosis is challenged by recent reports showing, that $cFLIP_L$, when present in lower amounts can in fact activate caspase-8[185, 186].

2.4.5 TRADD and TNF-R1 DISC assembly

TRADD, for TNF-R1-associated DD-containing protein[151], has been found in a yeast two hybrid screen to bind to the DD of TNF-R1. TRADD contains a C-terminal DD and an N-terminal domain capable of interacting with TRAF2. TRADD has been suggested to bind to the death domain of TNF-R1 via its death domain[151]. The presence of TRADD in the TNF-R1 signaling complex has also been confirmed to occur after stimulation with TNF-α under native conditions[151, 187, 188]. The proposed role of TRADD in this complex is to form a docking platform for recruitment of FADD, which subsequently leads to activation of caspase-8, analogous to the CD95 and TRAIL-R DISCs.

Amongst the death receptors, TNF-R1 differs in the mode of DISC assembly. In contrast to CD95 and TRAIL-R1/-R2, TNF-R1 does not bind FADD directly via its death domain. Rather it is proposed that FADD binding to TNF-R1 occurs via the adapter protein

TRADD. TRADD is suggested to bind to the TNF-R1 death domain via homotypic interactions with its death domain. Then, TNF-R1-bound TRADD is able to recruit FADD to the TNF-R1 DISC which leads to recruitment and subsequently activation of caspase-8. This proposed model however leaves several questions unanswered. Association of FADD with TNF-R1 has only been observed after simultaneous overexpression of FADD, TRADD and TNF-R1. Even in this experiment, the interaction of FADD with the TNF-R1/TRADD complex was weak[187]. In fact, recruitment of FADD to the native TNF-R1 DISC has never been reported so far.

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

Another peculiarity of the TNF-R1 DISC concerns the proposed interactions in this complex. TRADD has been suggested to bind RIP and FADD via a homotypic interaction between the DD of TRADD and the DD of RIP or TRADD. It is currently unknown how this dual binding is supposed to be achieved as both, the death domains of FADD and RIP would occupy the same binding patch TRADD is suggested to use for binding to the TNF-R1 DD. A recently proposed alternative model, which could explain this dichotomy proposes that the interactions between DDs (and DEDs) in the DISC lead to the formation of another interaction motif, providing more than one binding site per protein[189].

2.5 Mechanism of caspase activation at the DISC

2.5.1 Binding of the ligand and receptor multimerization

All receptors of the TNF-R family need to be crosslinked by multimeric ligands to become activated. In the case of the death receptors, the intracellular assembly of death domains creates a binding surface for the adapter molecule FADD which in turn recruits further signaling molecules like caspases-8 and -10 (see **Figure 2**).

The crystal structure of the extracellular domain of death receptors in complex with their respective soluble ligands yielded important first insights into the mechanism of DISC formation. Both complexes crystallized so far, TNF-R1 and TRAIL-R2 in complex with their ligands TNF-α and TRAIL, respectively, show a trimeric ligand binding to a receptor trimer[22, 23, 29, 30]. These data suggested that the minimum requirement for the active complex formed on the cell surface is a trimer as well. The model of receptor activation deduced from these data led to the following model: monomeric death receptors become trimerized by their ligands leading to intracellular crowding of their death domains. Alternatively, a conformational change in receptor structure upon ligand binding could also be involved. Recently it was proposed that in fact two adjacent trimeric ligands are necessary for activating the CD95 receptors[190]. Whether this is a general phenomenon or a peculiarity of the cell lines analyzed remains to be determined. Distinct crosslinking requirements have, however, been also reported for TRAIL-R1 and –R2. TRAIL-R1 has been reported to be activated by trimerized ligands whereas TRAIL-R2 needed higher order crosslinking[191].

Recent genetic and biochemical evidence however, revealed the existence of a for pre ligand assembly domain (PLAD) in several members of the death receptor family[192, 193]. It

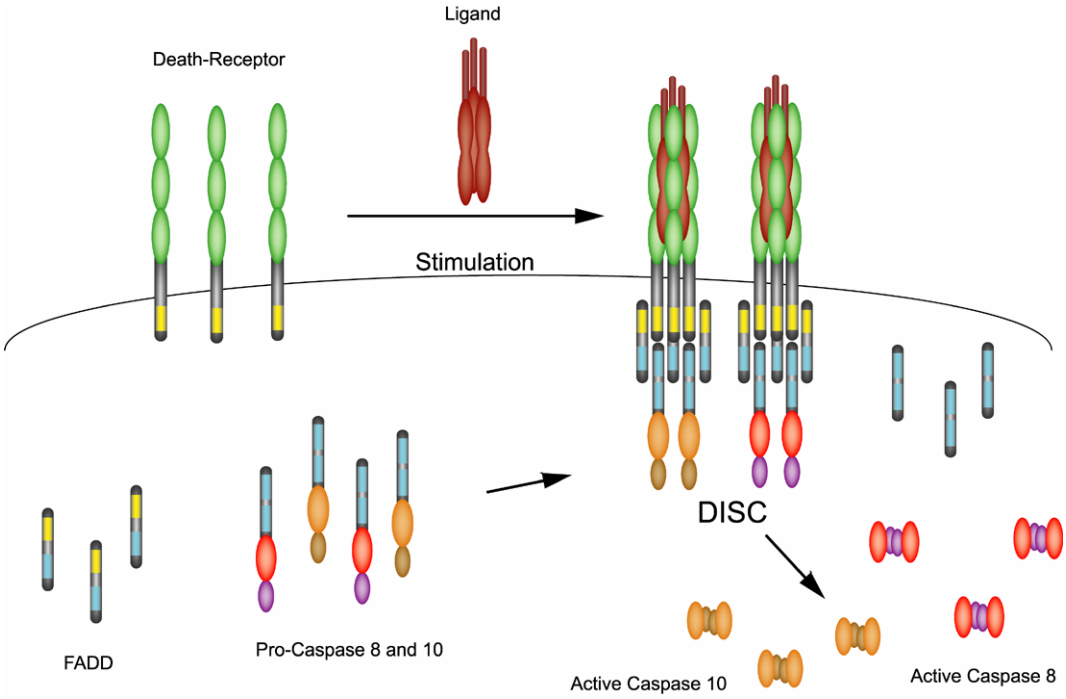

Fig. 2. Stimulation-dependent formation of the DISC results in caspase activation emanating from the activated DISC.

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

Interestingly, the PLAD is located opposite of the receptors' ligand binding site. Thus the formation of stable trimers as proposed would result in a receptor trimer with all its ligand binding sites turned outwards. Generation of a stable hexameric ligand-receptor-

complex would require a 180° turn in two of the receptor subunits to orient them towards the ligand. An alternative model which is consistent with the reported observations was also proposed[194]. Here, receptor dimers or trimers are formed through PLAD domain interactions. Addition of the ligand could then lead to higher order cluster formation where these pre-associated receptor dimers or trimers are crosslinked by the ligand trimers.

Receptors for which a PLAD domain has been reported to date are CD95, TNF-R1, CD40, TRAIL-R1 and TRAIL-R2. Given the general similarities in the members of the TNF- and TNF-R superfamilies it is quite likely that this phenomenon is not restricted to the above mentioned receptors but that it is of more general importance in this family of proteins.

2.5.2 Binding of FADD

The clustering of the receptors leads to the binding of the adapter molecule FADD at the death domain of CD95 and TRAIL-receptors –1 and -2. This binding is rapid and is detectable within seconds after receptor triggering[127, 132, 150]. Both the phosphorylated and the non-phosphorylated forms of FADD are equally well recruited to the CD95 and TRAIL DISC[155]. Importantly, binding of FADD to the death receptors is only observed after receptor stimulation. Immunoprecipitations of unstimulated death receptors do not contain co-precipitated FADD. Thus it is the ligand-induced crosslinking that creates the binding surface on the receptors' DD for the DD of FADD.

2.5.3 Recruitment of procaspases 8 and 10 to the DISC

The FADD molecules bound to the oligomerized receptors are thought to form a binding platform for recruitment of procaspase-8 and -10 to the DISC complexes. This recruitment is mediated via homotypic interactions between the FADD DED and the procaspase-8 and -10 DED, respectively. The recruitment of caspase-8 and caspase-10 occurs simultaneously with FADD binding. As in the case of FADD, the association of these two caspases with the death receptors can only be detected after receptor triggering. This is not surprising since FADD binding to the receptor is required for the binding of caspase-8 and –10. Complexes of caspase-8/10 and FADD without receptor stimulation have so far not been detected, highlighting the function of stimulated death receptors as a platform mediating interactions of the proteins necessary to initiate the caspase cascade.

2.5.4 The activation cycle

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

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

These observations led to the model of "induced proximity" which describes the mode of caspase activation by an increase in local concentration[195-197]. In the case of death receptor signaling, this increase is mediated by FADD-dependent recruitment of procaspase-8 and -10 to the oligomerized receptors. Probably the receptor-recruited caspases form a complex which resembles the active tetrameric complex formed in solution by activated caspases. A conformational change in the structure upon interaction with FADD in the DISC could also play a role in activation of the enzymatic activity. As has been shown recently, the key process in activating the initiator caspases is indeed the dimerization of the zymogens, with the cleavage step being dispensable for activating the initiator caspases[186, 198-200]. Thus, the DISC provides the scaffold for alignment of procaspase molecules in a manner that allows for their auto activation.

After recruitment of the procaspases to the DISC, proteolytic cleavage between the large and the small subunits occurs, with the intermediate products still being bound to the DISC complex. Subsequently a second cleavage event between the prodomain and the large subunit occurs, thereby liberating the large subunit from the prodomain. The processed subunits now form the active caspase enzyme, consisting of two large and two small subunits. These two subunits can still be coprecipitated with the active DISC[201]. At this stage, the remaining prodomain detaches from the DISC and allows for a new proenzyme to be recruited to and activated at this complex. How this exchange of the remaining prodomain for a new caspase-8/10 proenzyme takes place is elusive. Possibly, the affinity of

the procaspase for the DISC is higher than that of the prodomain, either due to a posttranslational modification or a conformational change.

2.5.5 Caspase inhibition by cFLIP

Both cFLIP splice variants are recruited to the CD95 and the TRAIL DISC complexes along with caspase-8 and caspase-10. It has recently been shown that, although both molecules can efficiently inhibit caspase activation at the DISC when overexpressed, they differ in their mode of inhibition[184]. When $cFLIP_L$ is present in the DISC, the initial cleavage between the large and the small subunits of the initiator caspases is observed. Concomitantly, $cFLIP_L$ is also cleaved leading to the appearance of a $cFLIP_L$ fragment which stays bound to the DISC.

The presence of high amounts of $cFLIP_S$ in the DISC in contrast seems to block at the initial caspase-8 cleavage step as no intermediate caspase products are observed and only full length caspase-8 can be found in the DISC. In addition to its observed function in suppression of death receptor-mediated caspase activation, other functions have been proposed for cFLIP. It was reported that cFLIP can activate NF-κB, JNK and ERK pathways in response to death receptor stimulation[202-204]. However, the relevance of these pathways in a true physiological setting remains to be determined, as all experiments to date used overexpression of cFLIP at levels that were several fold higher than naturally occurring levels.

2.6 Propagation of caspase activation

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

required for this enzyme to become active. This maturation step can be inhibited by the so called inhibitors of apoptosis proteins, the IAPs which interact with caspases in a manner that inhibits the final autocatalytic step of caspase activation.

2.7 Amplification loops and the role of proteins of the Bcl-2 family

Activation of the initiator caspases at the DISC does not necessarily lead to immediate and rapid cell death by activation of the downstream caspases (**Figure 3**). In some settings, a cell can tolerate remarkable amounts of DISC-activated caspase-8 without committing itself to apoptosis. The obvious explanation for this phenomenon would be that the activation and/or activity of the downstream caspases may be inhibited. This idea was supported by the recent identification of several proteins involved in the regulation of effector caspase activation. On the on side, there are the IAPs as just mentioned. These proteins were first discovered as baculoviral proteins, the vIAPs, which were able to inhibit apoptosis when overexpressed[205, 206]. Subsequently, the cellular counterparts, the cIAPs were identified with the human IAP family currently consisting of eight members (reviewed in[207]). These IAPs are able to inhibit apoptosis by blocking caspase-3, -7 and –9 by distinct mechanisms, with XIAP being the most potent[207]. Recent experiments suggested that XIAP inhibits caspase-3 and –7 maturation by blocking the second autocatalytic cleavage step[208-212]. Caspase-9 is inhibited by XIAP sequestering it in a monomeric state and thus inhibiting allosteric changes necessary for caspase-9 activation[213]. These findings explain why some cells are able to tolerate active initiator caspases without dying. In this setting, the initial processing of caspase-3 is readily observed, yet the fully matured enzyme can not be detected[214, 215].

This linear pathway of caspase activation, however, is not the whole story. It was observed that in some situations the death-receptor mediated pathway can be modulated in part by the expression of antiapoptotic molecules of the Bcl-2 family. This led to the classification of two cell types with regard to CD95-induced apoptosis[216]. Type I cells formed a strong DISC after stimulation and were independent of mitochondrial pathways in their commitment to cell death. Type II cells in contrast formed only little DISC and depended on the mitochondrial release of pro-apoptotic molecules (with cytochrome c being the only one known at that time). However, it is becoming clear that the mitochondria act as inte-

grators of a plethora of signals governing cellular integrity. This is achieved by influencing the balance between pro- and antiapoptotic Bcl-2 family members[217, 218].

At first, it remained unclear how mitochondria can be involved in death-receptor mediated pathways. A solution to this puzzling finding was provided by the identification of Bid, a protein which provided the link between the death receptors and the mitochondria[124, 125]. Bid can be cleaved by activated caspases to its truncated form tBid, which then in turn can act at the mitochondrial membrane to promote the release of proapoptotic molecules. The best defined molecule so far being known to be released from the mitochondria is cytochrome c. Upon its release from mitochondria, cytochrome c promotes the formation of

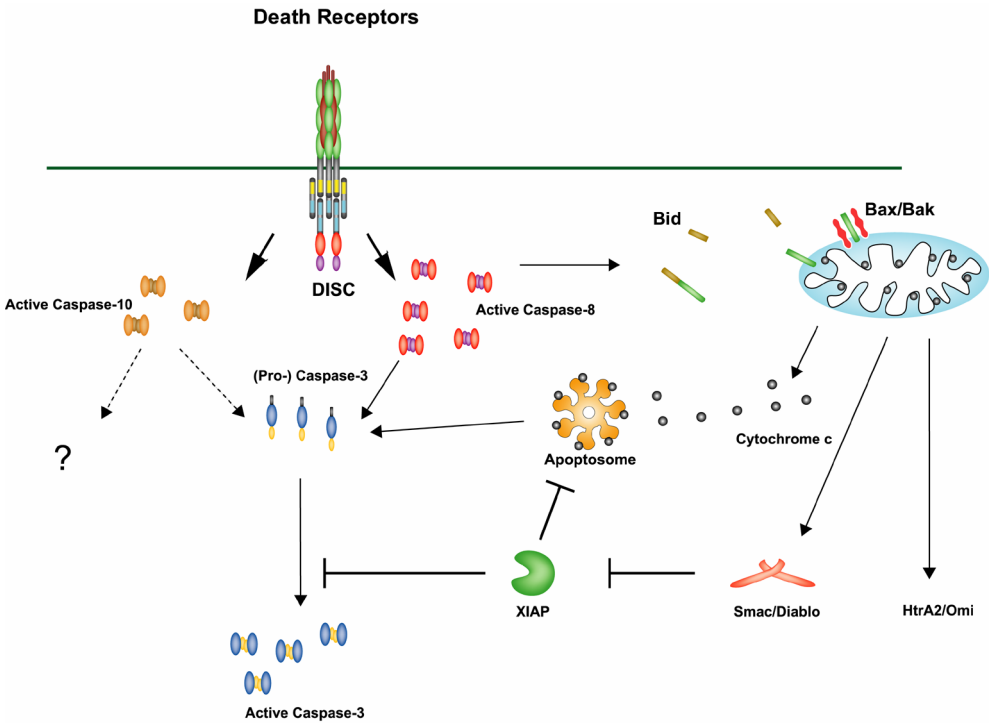

Fig. 3. Interplay between death-receptor-mediated caspase activation and mitochondrial pathways in apoptosis.

the apoptosome consisting of Apaf-1, caspase-9 and cytochrome c.

Once formed, the apoptosome mediates the activation of caspase-9 and subsequent cleavage of effector caspases within the apoptosome, thus providing additional activated caspase-3 molecules. Another important molecule released from mitochondria upon their

apoptotic activation is the recently discovered Smac (Second mitochondria derived activator of caspases)[219] in humans and its mouse homologue Diablo (direct IAP binding protein with low pI)[220] Interestingly, Smac/Diablo has been shown to inhibit XIAP by direct interaction, thereby interfering with its binding to caspase-3 and –7 . Thus, upon Smac/Diablo release from mitochondria, the inhibition of effector caspase maturation is relieved, allowing for full activation of caspase-3 and/or –7 and, thus execution of cell death. Recently, another molecule named HtrA2/Omi, also being capable of interfering with XIAPs inhibitory action was identified, adding additional complexity to the inhibitory machinery[207].

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

2.8 The integration of death-inhibiting and promoting signals

The outcome of death receptor triggering is regulated at several steps. The first step is binding of the ligand to its receptors, a step which might be regulated by the presence of soluble or membrane bound decoy receptors. Second, the amount of DISC complexes formed is naturally dependent on the amount of receptors that are expressed at a cells' surface. Third, the ability of death receptors to cluster and initiate signaling might be regulated as exemplified by the type I/type II dichotomy. Fourth, the activation of initiator caspases at the DISC can be blocked by the simultaneous presence of FLIP proteins or other elusive inhibitory proteins in the DISC, inhibiting the first activation step of the caspase cascade. Once activated, initiator caspases activate two principal downstream pathways. The first one aims at the initial cleavage step that is necessary for activation of executioner caspases. The maturation of these 'primed' executioners might be blocked by high levels of IAPs. However, high levels of primed caspase-3 might overcome cellular levels of IAPs and thus circumvent the need for removal of this inhibition. Thus if the ratio of caspase-activation at the DISC to XIAP or other inhibitory molecules is high enough, no amplification-loop might be needed. Alternatively expression of IAPs might be low or absent.

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

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

3 Aims of this work

The TNF superfamily plays an important role during the ontogenesis und maintenance of multicellular organisms. This important role is evident by a multitude of diseases which result from defects in TNF-SF members or a disturbance in their downstream signaling[7].

The TNF-SF signals relay its effect by two fundamentally different mechanisms. The first pathway, shared by virtually all members of the TNF-R SF, is the induction of target genes by activating members of the NF-κB, JNK and p38-MAPK pathways.

A second mechanism is the direct activation of the caspase cascade leading to apoptosis. This signaling pathway is utilized by a subgroup of the TNF-R superfamily, the death receptors. These receptors are characterized by a distinct intracellular protein motif, the death domain (DD), in the intracellular portion of these receptors. This domain is necessary for directly coupling the death receptors to activation of the caspase cascade.

Amongst the death receptors the TRAIL receptor system is of outstanding complexity[221]. The ligand TRAIL can bind two apoptosis inducing receptors, TRAIL-R1 and TRAIL-R2. Two additional receptors, TRAIL-R3 and TRAIL-R4 exist containing no or an incomplete death domain, respectively. Additionally, TRAIL can also bind the soluble receptor OPG. TRAIL has achieved much attention due to its ability to induce cell death in tumor cells in vitro and in vivo while normal cells are resistant to TRAIL. In addition, TRAIL can kill tumors regardless of their p53 status. Together, these findings make TRAIL a promising new agent for treating cancers[222].

Before TRAIL can be utilized in a clinical setting however, more information is needed about the signal transduction pathways utilized by TRAIL. These findings will help to judge possible fields of application as well as potential risks of using TRAIL clinically.

The death-inducing signaling complex (DISC) which forms after triggering the receptor has a central role in TRAIL signaling pathways as it is regarded as the proximal signaling event after receptor stimulation[123].

At the beginning of this work the molecular composition of the TRAIL DISC was controversially discussed. Most of the previous experiments conducted to elucidate the TRAIL

DISC were performed employing protein overexpression systems. These experiments tend to yield misleading results, as protein overexpression tends to promote unspecific protein interaction by misfolded and aberrantly localized proteins. Especially the nature of the apoptosis initiating caspase and the involvement of the adaptor protein FADD, which is essential for CD95 and TNF induced apoptosis, were unresolved. In addition, the differential signaling properties, if any, of the two TRAIL death receptors, TRAIL-R1 and TRAIL-R2 were unknown[145].

The aim of this work was therefore first, to develop a method to precipitate the native TRAIL DISC. By using this method, the molecular composition of the native TRAIL DISC should be elucidated. Of special interest were the questions, wether the adaptor protein FADD and the initiator caspase caspase-8 are also of central importance in TRAIL mediated apoptosis, as it has been reported for TNF-R1 and CD95. As two TRAIL death-receptors are frequently coexpressed, it should also be determined whether TRAIL-R1 and TRAIL-R2 utilize different signaling pathways. Finally, the expression pattern of caspase-10 had to be analyzed, as well as the contribution of this caspase in TRAIL- and CD95-induced apoptosis

II MATERIALS AND METHODS

1 Material

1.1 Chemicals

Except where stated differently, all chemicals were of at least analytical grade and purchased from by Sigma-Aldrich (Taufkirchen, Germany), Amersham Biosciences (Freiburg, Germany), Serva (Heidelberg, Germany) or Merck (Darmstadt)

1.2 Common Buffers

PBS (Phosphate buffered Saline)

137 mM	NaCl
8,1 mM	Na_2HPO_4
2,7 mM	KCl
1,5 mM	KH_2PO_4
pH = 7.4	

TAE (Tris Acetate EDTA)

40 mM	Tris Base
20 mM	Acetic acid
10 mM	EDTA

MOPS-SDS Running Buffer

50 mM	MOPS
50 mM	Tris-base
0.1% (w/v)	SDS
1 mM	EDTA
pH = 7.7	

MES-SDS Running Buffer

50 mM	MES
50 mM	Tris-base
0.1% (w/v)	SDS
1 mM	EDTA
pH = 7.3	

Lysis-Buffer (Eukaryotic cells)

20 mM	Tris-HCl pH 7.4 (at 4°C)
130 mM	NaCl
10 % (w/v)	Glycerol
1% (w/v)	Triton X-100
1 x	Complete Protease Inhibitors EDTA free (Roche)
2 mM	EDTA

Lysis buffer (Bacteria)

30 mM	Tris-HCl pH 8.0
50 mM	NaCl
1% (v/v)	Triton X-100
2 mM	KCl
2 mM	EDTA

1.3 Culture media

1.3.1 Media for culturing bacteria

LB

10 g/l	Tryptone
10 g/l	NaCl
5 g/l	Yeast ex-tract
pH adjusted to 7.4 with NaOH	

LB-Agar plates were prepared by addition of 15 g/l Bacto-Agar before heating.

1.3.2 Media for culturing eukaryotic cells

All culture media were from Gibco/Invitrogen. RPMI 1640 contained Glutamax I, a more stable Glutamine source, instead of Glutamine. All media were supplied with 15 mM HEPES pH 7.4 (Gibco/Invitrogen) and 10 % FCS (Gibco) before use.

Cells were cultured without the addition of antibiotics with the following exceptions:

For large scale culture in roller bottles, 10 µg/ml Gentamycin (Gibco/Invitrogen) were added to prevent microbial contamination.

For selection of stably transfected cells either Blasticidin S (Invitrogen), G418 (Gibco/Invitrogen), Puromycin (Sigma-Aldrich) or Hygromycin (Invitrogen) were used.

1.4 Biological Material

1.4.1 Bacterial strains

Strain designation	Purpose	Reference, Supplier
TOP10	Propagation of plasmids	Invitrogen

XL-10 gold	Propagation of plasmids	Stratagene
XL-1 blue	Propagation of plasmids	Stratagene
DH5α	Propagation of plasmids	Clontech
BL21 (DE3)	Protein expression	Invitrogen
BL21 (DE3) pLysS	Protein expression	Invitrogen
TOP 10 F'	Propagation of plasmids	Invitrogen

1.4.2 Eukaryotic cells

Cell line	Reference, Supplier	Medium
BJAB	EBV-negative Burkitt lymphoma cell line	RPMI 1640 10% FCS
Jurkat A3	Juo et al[147]	RPMI 1640 10% FSC
Jurkat FADD def	Juo et al [147]	RPMI 1640 10 % FSC
Jurkat caspase-8 def	Juo et al [147]	RPMI 1640 10% FCS
Jurkat FADD def TR1/TR2	Invitrogen	RPMI 1640 10% FCS
BL60	Burkitt`s lymphoma	RPMI 1640 10% FCS
K50	BL60 stably transfected with CD95[38]	RPMI 1640 10% FCS
HaCat	Boukamp et al.[223]	DMEM 10% FCS
CEM	human T cell leukemia	RPMI 1640 10% FCS
CHO K1	Chinese hamster ovary cell line	HAMs F12 10% FCS

1.5 Antibodies

Antibody	Antigen	Supplier, Reference
FADD (IgG1)	FADD	Transduction Laboratories, San Diego, CA
FADD (IgG1)	FADD	Pharmingen, San Diego, CA
Caspase-10 4C1	Prodomain of caspase-10	MBL International (Watertown, MA)
Caspase-10 #67041N	Unknown	Pharmingen, San Diego, CA
Caspase-10 #06-836	Unknown	Upstate Biotechnology
Caspase-10 Ab-2	Unknown	Oncogene Sciences
Caspase-10 MAB834	Unknown	R&D Systems
C15	human Caspase-8, p18 subunit	Scaffidi et al.[160].
NF-6	human cFLIP	Scaffidi et al.[224]
M2	FLAG epitope	Sigma Aldrich, Taufkirchen, Germany

HS101 (IgG1)	TRAIL-R1 extracellular domain	
HS102 (IgG1)	TRAIL-R2 extracellular domain	
HS103 (IgG1)	TRAIL-R3 extracellular domain	
HS104 (IgG1)	TRAIL-R4 extracellular domain	
anti APO IgG3	CD95 extracellular domain	Trauth et al.[38]
anti APO IgG1	CD95 extracellular domain	Dhein et al.[225]
anti IgG1, HRP	mouse IgG1	Southern Biotechnology, Birmingham, USA
anti IgG2a, HRP	mouse IgG2a	Southern Biotechnology, Birmingham, USA
anti IgG2b, HRP	mouse IgG2b	Southern Biotechnology, Birmingham, USA
anti κ, HRP	mouse κ light-chain	Southern Biotechnology, Birmingham, USA
anti rabbit, HRP	rabbit IgG	Southern Biotechnology, Birmingham, USA
M272	TRAIL-R1	Immunex Corp., Seattle, USA[226]
M271	TRAIL-R1	Immunex Corp., Seattle, USA[226]
M413	TRAIL-R2	Immunex Corp., Seattle, USA[226]
M270	TRAIL-R1	Immunex Corp., Seattle, USA[226]
M414	TRAIL-R2	Immunex Corp., Seattle, USA[226]
M415	TRAIL-R2	Immunex Corp., Seattle, USA[226]
	Caspase-3	Transduction Laboratories, San Diego, CA
	ERK	St. Cruz
	RIP	Transduction Laboratories (San Diego, CA)
	TRADD	Transduction Laboratories (San Diego, CA)

1.6 TNF-Superfamily ligands

Ligand	Reference, Supplier
LZ-CD95L	Walczak et al.[59]
LZ-TRAIL	Walczak et al.[59]
FLAG-TRAIL	Keogh et al.[227]
FLAG-CD95L	Alexis, Grünberg, Germany

1.7 Materials for molecular biology

1.7.1 Vectors

Vector name	Purpose	Reference, Supplier
pEFV5 His- TOPO	Vector for eukaryotic expression	Invitrogen
pEFV5 His- TOPO Caspase-10a	Eukaryotic expression of Caspase-10a	Sprick et al.[164]
pEFV5 His- TOPO Caspase-10d	Eukaryotic expression of Caspase-10d	Sprick et al.[164]
pEFV5 His- TOPO Caspase-10c	Eukaryotic expression of Caspase-10c	Sprick et al.[164]
pCR T7 NT TOPO	Vector for bacterial expression	Sprick et al.[164]
pCR T7 NT TOPO Casp 10 Prodomain	Bacterial expression of caspase-10 prodomain	Sprick et al.[164]
pET28a	Bacterial expression vector	Novagen
pET 28a Caspase-10d Δ prodomain	Expression of active caspase-10d	Sprick et al.[164]
pET 28a Caspase-10a Δ prodomain CEM	Expression of active caspase-10a	Sprick et al.[164]
pET 28a Caspase-8 Δ prodomain	Expression of active caspase-8	Sprick et al.[164]
pcDNA 3.1 Hygro	Vector for eukaryotic expression	Invitrogen
pcDNA 3.1 Hygro TRAIL-R1	Vector for stable expression of human TRAIL-R1	Grosse-Wilde (unpublished)

1.7.2 Oligonucleotides

Name	Sequence (5'-3')
CASP10UP	atgaaatctcaaggtcaacattggtattcc
CASP10LOW	ctatattgaaagtgcatccaggggcacagg
Casp10 N Up	aaatctcaaggtcaacattg
Casp10 N Low STOP	ggactcctgcggtaaggctt

1.7.3 Enzymes and kits

Enzyme/Kit	Supplier
Pfu Turbo DNA Polymerase	Stratagene
Superscript II first strand kit	Invitrogen
Taq DNA Polymerase	Sigma, Taufkirchen, Germany
Restriction enzymes	New England Biolabs Roche

2 Methods

2.1 Cell biological methods

2.1.1 Cell culture

Cells were cultured in the appropriate culture medium without antibiotics except when explicitly stated. Culture conditions were 37°C at 96% relative humidity and 5% CO2 except for cells cultured in DMEM, where 10% CO_2 was used.

2.1.2 Transfection and complementation of Jurkat A3 control, FADD^{def}, and FLICE^{def} cells

Jurkat A3 control, FADD^{def}, and FLICE^{def} cells were transfected essentially as described [161] with slight modifications. In brief, 6 x 10^6 cells were transfected with a total of 20 µg DNA by electroporation with an expression plasmid coding for a spectrin-GFP fusion protein [1] together with an expression plasmid coding for the respective missing signaling component, or together with empty vector in the controls, at a ratio of 3 to 1 (0.4 cm cuvette, 250 V, 950 µF, Biorad Gene-Pulser). The expression plasmids pRSV-HA-FADD, pRSV-Caspase-8 or empty control vector are described elsewhere[147, 161]. One hour after transfection, cells were purified over a Ficoll gradient to remove dead cells. After each transfection, half of the cells were either left untreated or stimulated with 1 µg/ml LZ-

TRAIL. After 10 h of incubation apoptosis was quantified by FSC/SSC analysis of the GFP-positive population on a FACSscan cytometer (Beckton Dickinson).

2.1.3 Stable transfection of caspase-10 and TRAIL-R1 in Jurkat caspase-8 deficient cells

First, a caspase-8-deficient cell line stably expressing TRAIL-R1 was generated by transfecting the parental caspase-8 deficient cell line with the expression vector pCDNA3 Hygro coding for TRAIL-R1. After initial selection in Hygromycin B (200µg/ml), single clones were obtained by two rounds of limiting dilution cloning. Clones were analyzed by surface staining for expression of TRAIL-R1 and one clone, denoted clone 1D2, was chosen.

Caspase-8-deficient cell lines stably overexpressing caspase-10d were generated by transfecting the caspase-8-deficient cell line with the pEFV5His-TOPO-Casp-10d vector using Xtreme-GeneQ2 (Roche, Mannheim). After initial selection in Blasticidin-S (10 µg/ml), single clones were obtained by limited dilution cloning. To exclude the introduction of mutations in caspase-10 during the generation of the stable clones, the cDNA encoded by the plasmid was re-isolated from the single clones, sequenced and found to be without mutations.

2.1.4 Generation of the cell line FADD^{def} TR1/TR2

The TRAIL-R1- and -R2-expressing FADD-deficient Jurkat cell line TR1/2 (clone 6D2-1D2) was derived from the original Jurkat FADD^{def} cell line by transfection with an expression plasmid coding for the complete TRAIL-R1 open reading frame. The transfected pool was subjected to two rounds of limiting dilution cloning, choosing the cell line with the highest TRAIL-R1 surface expression as evaluated by FACS staining with a TRAIL-R1-specific monoclonal antibody (HS102).

2.1.5 Transient transfection of CHO cells.

CHO cells were transiently transfected with the expression plasmids for caspase-10a, -10d or a control vector with Fugene transfection reagent according to the manufacturer's (Roche, Mannheim, Germany) instructions. 24h after transfection, cell lysates were collected and analyzed.

2.1.6 FACS analysis

Cells ($5x10^6$) were incubated with the indicated Abs or control mIgG1 at 10 µg/ml followed by biotinylated secondary goat anti-mouse antibodies (Southern Biotechnology Associates, CA) and Streptavidin-PE (Pharmingen, Hamburg, Germany) for 20 minutes on ice. Antibodies and conjugates were diluted in FACS buffer (PBS containing 2% FCS and 1 % Sodium Azide). After each incubation, the cells were washed two times with FACS buffer. Surface staining was analyzed on a FACS-Calibur (Becton Dickinson, Heidelberg, Germany).

2.1.7 Quantification of apoptotic cell death

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described[228]. Briefly, 2.5 x 10^5 cells were incubated in 24-well plates (Costar, Cambridge, MA) with or without apoptotic stimuli in 0.5 ml medium at 37°C. Cells were collected by centrifugation at 600 x g for 10 min at 4°C, washed twice with PBS and then resuspended in 100 µl lysis solution containing 0.1% (v/v) Triton-X-100, 0.1% (w/v) sodium citrate and 50 µg/ml propidium iodide (PI). Apoptosis was quantitatively determined by flow cytometry after incubation at 4°C in the dark for at least 24 h as cells containing nuclei with subdiploid DNA content. Alternatively, apoptosis was determined by a drop in the forward scatter to sideward scatter (FSC/SSC) profile of apoptotic in comparison to living cells. Percentage of specific cell death was calculated as follows:

100 x (% experimental cell death - % spontaneous cell death) / (100 - % spontaneous cell death).

2.2 Molecular biological methods

2.2.1 Cloning of Caspase-10 isoforms

Plasmids coding for the caspase-10 isoforms were obtained by amplification of the different caspase-10 open reading frames by PCR with Pfu polymerase. Primers used for amplification were: CASP10UP and CASP10LOW (See II1.7.2.). PCR fragments were ligated into pEFV5His-TOPO vector (Invitrogen, Karlsruhe, Germany) for eukaryotic expression. The stop codon contained in the caspase-10 cDNA was included to generate an

untagged version of caspase-10. Insert containing vectors were fully sequenced and verified to contain the desired products without mutations.

2.2.2 Site-directed mutagenesis

Site-directed mutagenesis was preformed to correct errors in the caspase-10 clones obtained after the initial PCR-based cloning step. Mutagenesis was performed using the QuickChange Kit (Stratagene), which employs a PCR-based method amplifying the whole initial plasmid which contains the sequence to be mutated. Essentially, the procedures were performed according to the manufacturer's protocol.

2.3 Protein biochemical Methods

2.3.1 2D Gel analysis

For 2D analysis cells were directly lysed for 1h in 2D sample buffer (7M Urea, 2M Thiourea, 4%CHAPS, 2mM Tris-Butylphosphine, supplemented with complete protease inhibitors (Roche, Mannheim, Germany). Subsequently, the samples were clarified by centrifugation (50000g, 30 minutes). For the first dimension, protein samples were diluted in 2D rehydration buffer (2D sample buffer supplemented with bromophenol blue, 1% ampholytes pH 5-8 (Bio-Rad, München, Germany, 10% (v/v) glycerol). The first dimension isoelectric focusing (IEF) was performed with ReadyStrip precast IPG strips pH 5-8 essentially according to the manufacturer's instructions (Bio-Rad, München, Germany). After electrofocussing, the strips were incubated two times for 15 minutes in equilibration buffer (50mM Tris pH 8.8, 6M Urea, 30% Glycerol, 2% (w/v) SDS, 10 mg/ml DTT). The second dimension was run on precast 4-12% NuPage Bis-Tris gels (Invitrogen, Karlsruhe, Germany) with MOPS running buffer and subsequently gels were blotted onto nitrocellulose membrane.

2.3.2 Preparation of cell lysates

Cells were harvested by centrifugation at 300 x g for 10 min at 4°C, washed twice with ice cold PBS and lysates were prepared by resuspending the resulting cell pellets in 100 µl lysis buffer per 1 x10^7 cells (30 mM Tris-HCl pH 7.5, 120 mM NaCl, 10 % Glycerol 1% Triton X-100) supplemented with Complete^{TM} protease inhibitors (Roche Diagnostics,

Mannheim, Germany) according to the manufacturer's instructions. After 30 min incubation on ice, the lysates were centrifuged once at 15,000 x g at 4°C to remove nuclei. For the lysis of primary T cells the protease inhibitor concentration was increased five times.

2.3.3 Biotinylation of LZ-TRAIL

Bio-LZ-TRAIL was prepared by incubation of purified LZ-TRAIL at 1 mg/ml with Sulfo-NHS-LC-Biotin at 1 mg/ml (Pierce) for 1 h on ice before the reaction was stopped by adding 1/10 volume of 1 M Tris-HCl at pH 7.5. Unincorporated biotin was removed from Bio-LZ-TRAIL preparations by buffer exchange into 150 mM NaCl, 30 mM HEPES pH 7.5 on PD-10 columns (Amersham Pharmacia Biotech). Protein preparations were checked for purity and incorporation of biotin by SDS-PAGE. The biological activity of Bio-LZ-TRAIL was determined by its apoptosis-inducing capacity and found to be comparable to non-biotinylated LZ-TRAIL.

2.3.4 DISC precipitation using biotinylated LZ-TRAIL

Initially, DISC analysis was performed ligand affinity precipitations by using biotinylated LZ-TRAIL (Bio-LZ-TRAIL) in combination with Streptavidin beads (Pierce). For ligand affinity precipitation 3 x 10^8 cells were used per sample. Cells were washed twice with 50 ml RPMI medium at 37°C and subsequently incubated for the indicated time periods at 37°C and a cell density of 1 x 10^8/ml in the presence of 1 µg/ml Bio-LZ-TRAIL or, for the unstimulated control, in the absence of Bio-LZ-TRAIL. In the case of differential TRAIL receptor DISC analysis we preincubated the cells with 10 µg/ml TRAIL-R1 and/or TRAIL-R2-blocking mAbs for 15 min before stimulation with Bio-LZ-TRAIL. DISC formation was stopped by addition at least 15 volumes of ice-cold PBS. Cells were then washed twice with 50 ml ice-cold PBS before cell lysates were prepared. 3 x 10^8 cells were lysed by addition of 4.5 ml lysis buffer. The resulting protein complexes were precipitated from the lysates by co-incubation with 20 µl Streptavidin Beads (Pierce) for 2-4 h on an end-over-end shaker at 4°C. For the precipitation of the non-stimulated receptors, Bio-LZ-TRAIL was added to the lysates prepared from non-stimulated cells at 1 µg/ml to control for protein association to non-stimulated receptor(s). Ligand affinity precipitates were washed 4 times with lysis buffer before the protein complexes were eluted from the beads by addition of 15 µl 2 x standard reducing sample buffer. Subsequently, proteins were separated on SDS-PAGE before presence or absence of antigens was determined in the

different precipitates by Western blot analysis.

2.3.5 DISC precipitation using FLAG-tagged ligands

In the experiments using either FLAG-tagged CD95-Ligand (FLAG-CD95L) or FLAG-tagged TRAIL (FLAG-TRAIL), the procedures for DISC precipitation were as follows: for ligand affinity precipitation 1-5 x 10^7 cells were used per sample. Cells were washed with 50 ml RPMI medium at 37°C and subsequently incubated for the indicated time periods at 37°C and a cell density of 5 x 10^7/ml in the presence of 1 µg/ml FLAG-TRAIL or FLAG CD95-L pre-complexed with 2 µg/ml anti-FLAG M2 for 15 minutes, or, for the unstimulated control, in the absence of the respective ligand. In the case of differential TRAIL receptor DISC analysis we preincubated the cells with 10 µg/ml TRAIL-R1 and/or TRAIL-R2-blocking mAbs for 15 min before stimulation with FLAG-TRAIL. DISC formation was stopped by addition of at least 15 volumes of ice-cold PBS. Cells were then washed twice with 50 ml ice cold PBS before cell lysates were prepared. Cells were lysed by addition of 1 ml lysis buffer. The resulting protein complexes were precipitated from the lysates by co-incubation with 20 µl Protein G Beads (Roche, Mannheim, Germany) for 2-4 hours on an end-over-end shaker at 4°C. For the precipitation of the non-stimulated receptors, FLAG-TRAIL/FLAG-CD95L plus M2 was added to the lysates prepared from non-stimulated cells at 200 ng to control for protein association with non-stimulated receptor(s). Ligand affinity precipitates were washed 5 times with lysis buffer before the protein complexes were eluted from the beads by addition of 15 µl 2 x standard reducing sample buffer. Subsequently, proteins were separated on SDS-PAGE before detection of DISC components by western blot analysis.

2.3.6 Western blot analysis

For Western blot analysis the resulting post nuclear supernatants or DISC precipitates were supplemented with 2-fold concentrated standard reducing sample buffer (2 x RSB). Subsequently, lysate containing 20 µg of protein as determined by the BCA method (Pierce, Rockford, IL) or proteins eluted from beads after ligand affinity immunoprecipitation were separated on 4-12% NuPage Bis-Tris gradient gels (Novex, San Diego, CA) in MOPS buffer according to the manufacturer's instructions. After protein transfer onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by electroblotting, membranes were blocked with 5% non-fat dry milk (NFDM) in PBS/Tween (PBS

containing 0,05% Tween-20) for at least 1 h, washed with PBS/Tween, and incubated in PBS/Tween containing 3% non-fat dry milk (NFDM) and primary antibodies as required. After 5 washes for 3 min each in PBS/Tween the blots were incubated with HRPO-conjugated isotype-specific secondary antibody diluted 1:20,000 in PBS. After washing 5 times for 3 min with PBS/Tween the blots were developed by enhanced chemoluminescence (ECL) using SuperSignal West Dura substrate following the manufacturer's protocol (Perbio Science, Bonn, Germany).

For stripping, blots were incubated in 50 mM glycine HCl 500 mM NaCl pH 2.3 for 20 min at room temperature. Subsequently, blots were washed two times for 10 min in PBS/Tween and blocked again.

2.3.7 Bacterial expression and purification of the caspase-10 prodomain

The caspase-10 prodomain was amplified by PCR from a vector containing full length caspase-10d (using the primers as described in II1.7.2) and subcloned into pCRT7NT-Topo (Invitrogen, Karlsruhe, Germany). His-tagged prodomain was expressed in *E.coli* BL21 (DE3) pLysS and purified on a Ni^{2+} column under denaturing conditions.

III RESULTS

1 FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2

At the beginning of this work, little was known about the composition of the native signaling complexes formed by the two death inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2. Especially the requirement for the adaptor protein FADD for apoptosis induction was controversially debated. While some experiments conducted in mouse embryonic fibroblasts (MEF) deficient for FADD argued for the ability, at least of TRAIL-R1, to induce cell death independent from FADD[146], while other experiments suggested that interactions of FADD with both TRAIL receptors are either necessary[70, 71, 229, 230] or not[64, 69, 231, 232] necessary depending on the overexpression system used. Additionally, the initiator caspase utilized by the TRAIL death receptors for TRAIL induced apoptosis was controversially debated[64, 69, 81, 233]. Besides caspase-8 and FADD, additional molecules like RIP and TRADD had been suggested to play a role in TRAIL-mediated apoptosis[70]. This controversy largely was caused by the nature of the experimental systems used. Mostly, protein overexpression systems where at least one of the molecules under investigation were transiently transfected and overexpressed were used. As protein overexpression may lead to aggregation, non-natural localization and unspecific interactions, the conflicting results could be anticipated. To analyze the physiological function of the TRAIL system it was, therefore, necessary to elucidate the composition of the TRAIL signaling complex. To represent the physiological state to the best possible extent, it was imperative to analyze cell systems which contain native levels of the TRAIL signaling components.

1.1 BL60, BJAB and CEM cells are susceptible to TRAIL-R1- and TRAIL-R2-induced apoptosis whereas Jurkat cells only express functional TRAIL-R2

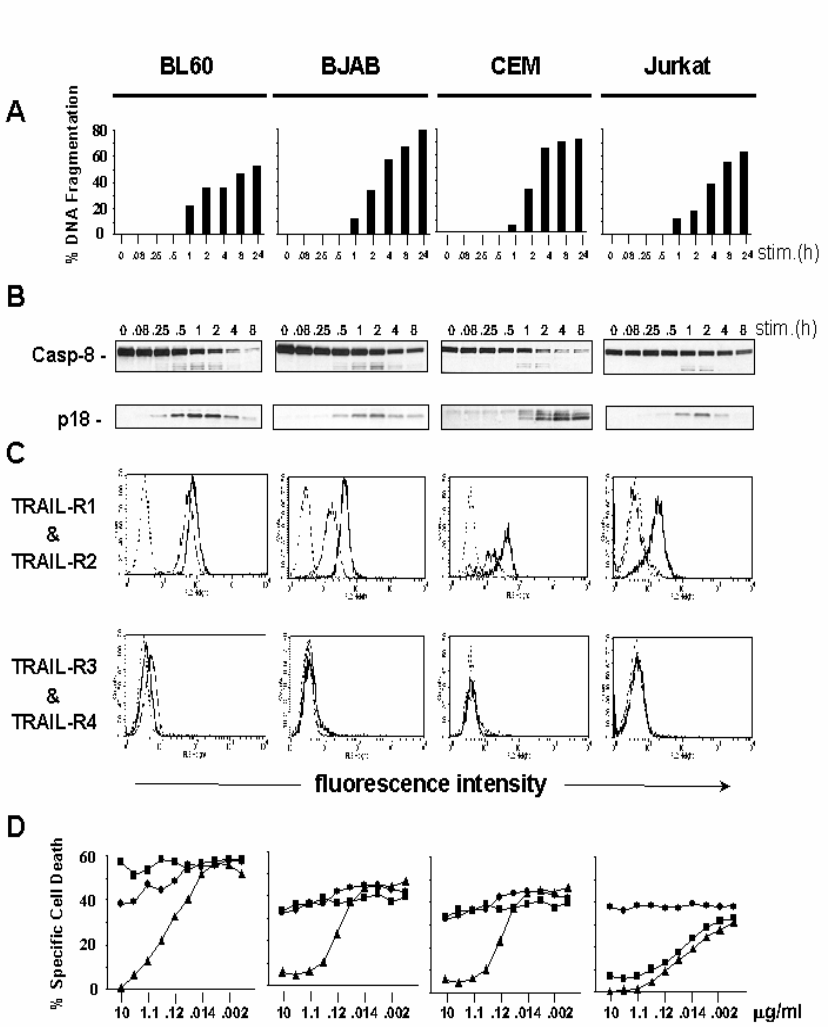

Fig. 4. Analysis of kinetics of TRAIL-induced apoptosis, caspase-8 cleavage, receptor expression profile, and functional expression of TRAIL-R1 and TRAIL–R2 in BL60, BJAB, CEM, and Jurkat cells.

(A,B) Cells were stimulated with 1 µg/ml LZ-TRAIL for the indicated time periods followed by analysis of percentage of cells with subdiploid DNA content (A) and Western blot analysis of caspase-8 (p55/53) and its p18 cleavage product (B). (C) FACS analysis of surface expression of TRAIL-R1 (C upper panels, solid lines), TRAIL-R2 (C; upper panels, solid bold lines), TRAIL-R3 (C; lower panels, solid lines), and TRAIL-R4 (C; lower panels, solid bold lines) as compared to an isotype-matched control mIgG1 mAb (C; dashed lines). (D) Identification of TRAIL-R1- and TRAIL-R2-inhibiting mAbs. Cells were treated with 100 ng/ml LZ-TRAIL after 30 min pre-incubation with or without blocking TRAIL-R1- and/or TRAIL-R2-specific mAbs at the indicated concentrations. The percentage of cell death was determined by FSC/SSC analysis and plotted against the concentration of mAbs specific for TRAIL-R1 (●), TRAIL-R2 (■), or both apoptosis-inducing TRAIL receptors (▲).

In order to dissect the apoptosis-inducing events immediately following receptor stimu-

lation by TRAIL in lymphoid cells the human B cell lines BL60 and BJAB, and the human T cell lines CEM and Jurkat were chosen as model systems. These cell lines were characterized by determination of TRAIL sensitivity, analysis of the time course of caspase-8 activation, and their TRAIL receptor expression profile. Next, the functionality of TRAIL-R1 and TRAIL-R2 signaling was analyzed separately. All four cell lines were susceptible to TRAIL-induced apoptosis (**Fig. 4A**). In these experiments a recombinant form of TRAIL, LZ-TRAIL, that forms stable trimers due to the addition of a trimerization motif was used[71]. In accordance with studies on non-lymphoid cell lines[81, 233] stimulation of BL60, BJAB, CEM, and Jurkat cells with LZ-TRAIL led to early cleavage and activation of caspase-8 (**Fig. 4B**) prior to cleavage of caspase-3 and PARP (data not shown). This supports an initiating role for caspase-8 during TRAIL-induced apoptosis also in lymphoid cells. While BL60, BJAB, and CEM cells expressed both apoptosis-inducing TRAIL receptors, only TRAIL-R2 was present on the surface of Jurkat cells (**Fig. 4C**, top panels). TRAIL-R3 and TRAIL-R4 were not detectable on the surface of these cell lines (**Fig. 4C**, bottom panels).

It was then analyzed whether TRAIL-R1 and/or TRAIL-R2 were functionally expressed. After preincubation with soluble blocking mAbs against TRAIL-R1, TRAIL-R2, or a combination thereof the different cells were treated with LZ-TRAIL (**Fig. 4D**). While the combination of TRAIL-R1- and TRAIL-R2-specific mAbs inhibited TRAIL-induced apoptosis in all four cell lines, pre-incubation with anti-TRAIL-R2 alone blocked TRAIL-induced apoptosis only in Jurkat cells. Preincubation with blocking anti-TRAIL-R1 alone did not inhibit TRAIL-induced apoptosis in any of the cell lines tested. Thus, BL60, BJAB, and CEM cells are susceptible to apoptosis mediated by ligand-induced crosslinking of either TRAIL-R1 or TRAIL-R2 whereas Jurkat cells can only be killed via TRAIL-induced crosslinking of TRAIL-R2. This data shows that these antibodies are suitable for differential analysis of the individual protein complexes formed upon TRAIL-mediated crosslinking of the two different apoptosis-inducing TRAIL receptors under native conditions.

1.2 TRAIL-dependent recruitment of caspase-8 and FADD/MORT1 to the TRAIL DISC in BL60 cells

The active caspase-8 subunit p18 could be detected as early as 15 min after TRAIL stimulation (**Fig. 4B**) suggesting that caspase-8 might be the caspase responsible for initia-

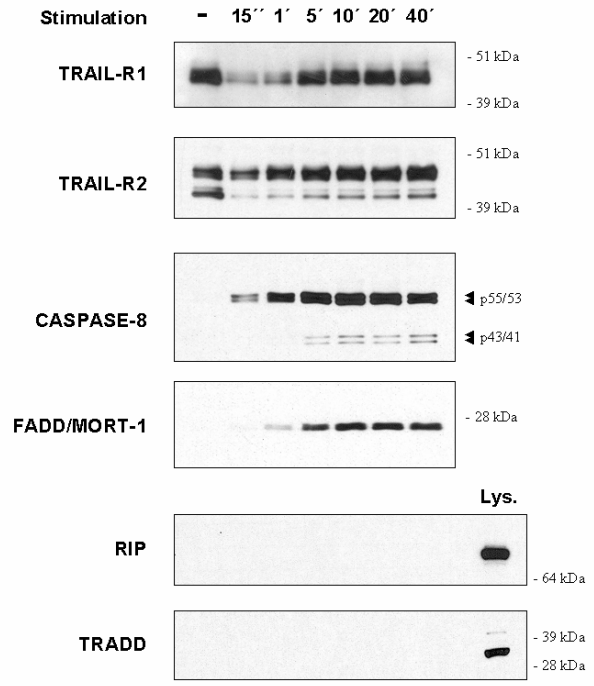

Fig. 5. Kinetics of DISC assembly in BL60 cells.
BL60 cells were either stimulated with 1 µg/ml biotinylated LZ-TRAIL (Bio-LZ-TRAIL) for the indicated time periods or left untreated (-) before cell lysis. 1 µg/ml Bio-LZ-TRAIL was added to the lysates in the untreated control. Bio-LZ-TRAIL-bound protein complexes were analyzed by SDS-PAGE and Western blot following affinity precipitation with Streptavidin beads. For RIP, and TRADD lysate controls (Lys.) prepared from BL60 cells are shown.

tion of TRAIL-induced apoptosis. The *native* protein complex that initiates TRAIL-induced apoptosis was analyzed for presence of the various signaling proteins that have been suggested to play a role at the onset of TRAIL-induced apoptosis (**Fig. 5**). BL60 cells were incubated either in the absence or presence of biotinylated LZ-TRAIL (Bio-LZ-TRAIL) for various time periods. Both TRAIL-R1 and TRAIL-R2 were precipitated with Bio-LZ-TRAIL already after 15 sec of stimulation. When analyzing the precipitated protein complex for the presence of caspase-8 and FADD/MORT1 it was found that both signaling proteins were recruited as early as 15 sec to 1 min after stimulation with Bio-LZ-TRAIL. In addition, cleavage fragments of caspase-8 (p43/41), indicative of activation of the proform of this enzyme (p55/53), were detectable within 1 to 5 min of stimulation.

In contrast, caspase-8 and FADD/MORT1 did not associate with non-stimulated TRAIL receptors which were precipitated with Bio-LZ-TRAIL from the lysates of mock-treated

cells (**Fig. 5**, left lane). Thus, recruitment of FADD/MORT1 and caspase-8 was dependent on ligand-induced receptor crosslinking. The amount of FADD/MORT1 and caspase-8 recruited to the TRAIL receptors increased in a time-dependent manner reaching maximum levels between 5 and 20 min of stimulation. From these results, it can be concluded that TRAIL stimulation leads to the formation of an apoptosis-initiating protein complex, the TRAIL DISC. FADD/MORT1, caspase-8, and the apoptosis-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, are integral components of this signaling complex. However, although prominently present in the lysates of BL60 cells, RIP, and TRADD could not be detected associated with the TRAIL DISC at any time point analyzed (**Fig. 5**, bottom panels).

1.3 FADD/MORT1 and caspase-8 form part of the TRAIL DISC in various lymphoid cell lines

In the next set of experiments it was studied whether FADD/MORT1 and caspase-8 recruitment to the TRAIL DISC can also be observed in other cell lines that express both apoptosis-inducing TRAIL receptors. Therefore, the formation of the TRAIL DISC in BL60 cells was compared with the BJAB and CEM TRAIL DISC. Cells were incubated

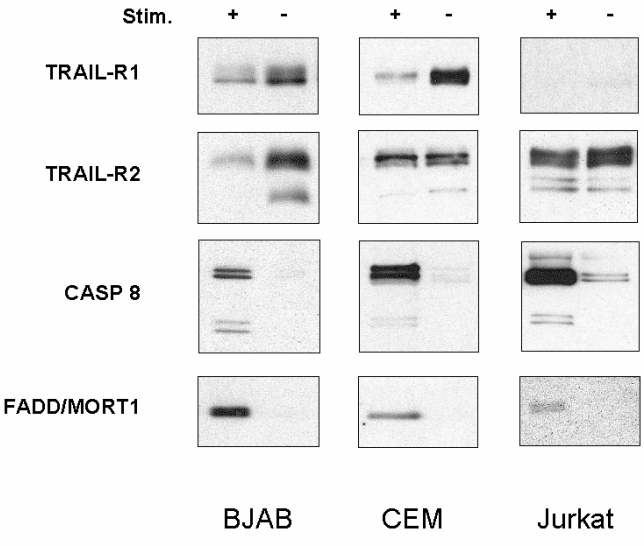

Fig.6. TRAIL DISC analysis in BJAB, CEM, and Jurkat cells.
BJAB, CEM, and Jurkat cells were left untreated or were treated for 20 min with 1 µg/ml Bio-LZ-TRAIL before cell lysis. 1 µg/ml Bio-LZ-TRAIL was added to lysates from unstimulated cells for precipitation of unstimulated TRAIL receptors. Bio-LZ-TRAIL-bound protein complexes were analyzed for presence of TRAIL-R1, TRAIL-R2, FADD/MORT1 and Caspase-8 by SDS-PAGE and Western blot analysis following affinity precipitation from the lysates with Streptavidin beads.

for 20 min in the presence or absence of Bio-LZ-TRAIL before analyzing the proteins

complexed with the biotinylated ligand. TRAIL-R1 and TRAIL-R2 associated with Bio-LZ-TRAIL in BJAB and CEM cells and both, FADD/MORT1 and caspase-8 were recruited to the TRAIL DISC (**Fig. 6**). This association was stimulation-dependent since Bio-LZ-TRAIL added after cell lysis precipitated the non-stimulated receptors but not cspase-8 and FADD/MORT1. Thus, these two signaling proteins, previously identified as integral components of the native CD95 DISC[127, 130, 131]also form part of the native TRAIL DISC in various cell lines.

Jurkat cells only express TRAIL-R2 on their surface (**Fig. 4C**). Thus, Jurkat cells served to test whether crosslinking of TRAIL-R2 in the absence of TRAIL-R1 may also lead to recruitment of FADD/MORT1 and caspase-8. Stimulation of Jurkat cells with Bio-LZ-TRAIL induced recruitment of TRAIL-R2, caspase-8, and FADD/MORT1 in the absence of TRAIL-R1 (**Fig. 6**). Under non-stimulatory conditions, only TRAIL-R2 was bound to TRAIL indicating that recruitment of FADD/MORT1 and caspase-8 to TRAIL-R2 is dependent on TRAIL-induced crosslinking of TRAIL-R2 on intact Jurkat cells. Thus, homomeric TRAIL-R2 complexes are sufficient for recruitment of FADD/MORT1 and caspase-8 in the absence of TRAIL-R1.

1.4 Homomeric TRAIL-R1 and TRAIL-R2 DISCs recruit FADD/MORT1 and caspase-8 in BL60 cells

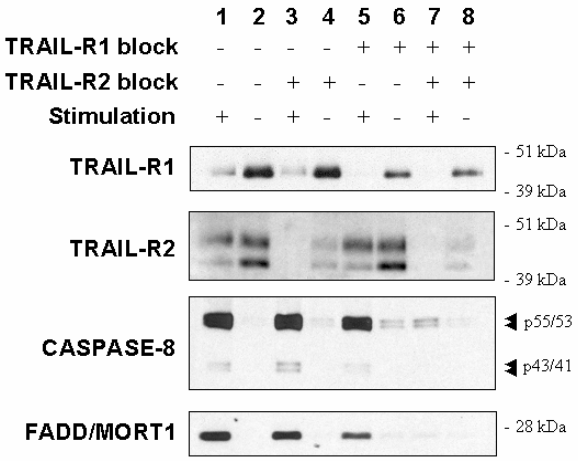

Fig.7. TRAIL stimluation induces recruitment of FADD/MORT1 and caspase-8 to homomeric TRAIL-R1 and TRAIL-R2 DISCs in BL60 cells.
After 30 min preincubation with 10 µg/ml blocking TRAIL-R1- and/or TRAIL-R2-specific mAbs cells were either stimulated with 1 µg/ml Bio-LZ-TRAIL (+) for 20 min or left untreated (-) before cell lysis. 1 µg/ml Bio-LZ-TRAIL was added to the unstimulated samples for precipitation of unstimulated TRAIL receptors from lysates. Bio-LZ-TRAIL-bound protein complexes were analyzed for presence of TRAIL-R1, TRAIL-R2, FADD and caspase-8 by SDS-PAGE and Western blot.

Since both apoptosis-inducing TRAIL receptors associated with TRAIL upon stimulation, FADD/MORT1 and caspase-8 might have been recruited to homomeric TRAIL-R1, homomeric TRAIL-R2, or heteromeric complexes containing TRAIL-R1 and TRAIL-R2. To differentiate between the proteins associated with homomeric TRAIL-R1 and TRAIL-R2 DISC, respectively, the two receptors were stimulated separately. This was achieved by preincubating BL60 cells in the presence or absence of blocking monoclonal antibodies (mAb) against TRAIL-R1 and TRAIL-R2, respectively, before stimulation with Bio-LZ-TRAIL (**Fig. 7**, odd numbered lanes).

In the unstimulated controls (**Fig. 7**, even numbered lanes), Bio-LZ-TRAIL was added after cell lysis and precipitated TRAIL-R1 and TRAIL-R2 irrespective of whether surface-expressed TRAIL receptors were blocked by mAb preincubation of intact cells or not. This indicates that, also in BL60 cells substantial amounts of TRAIL-R1 and, to a lesser extent TRAIL-R2, were present intracellular (described for melanoma cells by Zhang et al.[148]) and, thus, could not be blocked by mAb preincubation of intact cells. Western blot analysis

revealed that in the presence of TRAIL-R2 blocking mAb only TRAIL-R1 was precipitated upon stimulation with Bio-LZ-TRAIL (**Fig. 7**, lane 3, top two panels) and vice versa (**Fig. 7**, lane 5, top two panels). Thus, both homomeric TRAIL-R1 and TRAIL-R2 DISCs were formed upon stimulation with Bio-LZ-TRAIL. Stimulated homomeric TRAIL-R1 and TRAIL-R2 recruited pro-caspase-8 (p55/53), cleavage intermediates of caspase-8 (p43/41), and FADD/MORT1 (**Fig. 7**, lane 3 & 5). This recruitment was dependent upon ligand stimulation of TRAIL receptors on intact cells since caspase-8 and FADD/MORT1 were not bound to unstimulated TRAIL receptors (**Fig. 7**, lanes 4 & 6). Thus, FADD/MORT1 and caspase-8 are integral components of both, the native homomeric TRAIL-R1 DISC and the native homomeric TRAIL-R2 DISC in BL60 cells.

1.5 FADD/MORT1 and caspase-8 are essential for TRAIL-R2-induced apoptosis in Jurkat cells

The mere presence of a protein in the DISC does not imply functional importance at the initiation of apoptosis. Other proteins have been shown to associate with CD95 or TNF-R1[234, 235]. However, for the CD95 system, an essential function could so far only be attributed to FADD/MORT1 and caspase-8 as cells deficient for one of these two signaling proteins were resistant to CD95-mediated apoptosis. In addition to experiments with cells from mice deficient for FADD/MORT1[146, 148] or caspase-8[165] that showed the requirement of these two signaling proteins, respectively, it was shown that Jurkat cells deficient for either one of these two proteins did not undergo CD95-mediated apoptosis[147, 161]. These FADD/MORT1-deficient (FADD^{def}) and caspase-8-deficient (Casp-8^{def}) Jurkat cells expressed normal levels of caspase-3 but showed the expected deficiencies in FADD/MORT1 and caspase-8 expression, respectively (**Fig. 8A**). In addition, the TRAIL receptor surface expression pattern of the resistant clones was identical to that of Jurkat A3 control cells as TRAIL-R2 was expressed while TRAIL-R1, -R3, and –R4 were not present (**Fig. 8B**). In order to determine whether caspase-8 and FADD/MORT1 are necessary for TRAIL-R2-induced apoptosis in Jurkat cells FADD^{def}, casp-8^{def}, and Jurkat A3 control cells were treated with LZ-TRAIL. Both the FADD^{def} and casp-8^{def} Jurkat cells were resistant to TRAIL-induced apoptosis while Jurkat A3 cells underwent TRAIL-induced apop-

tosis in a dose-dependent manner (**Fig. 8C**).

FADD/MORT1 and caspase-8 deficiency do not necessarily constitute the only defects

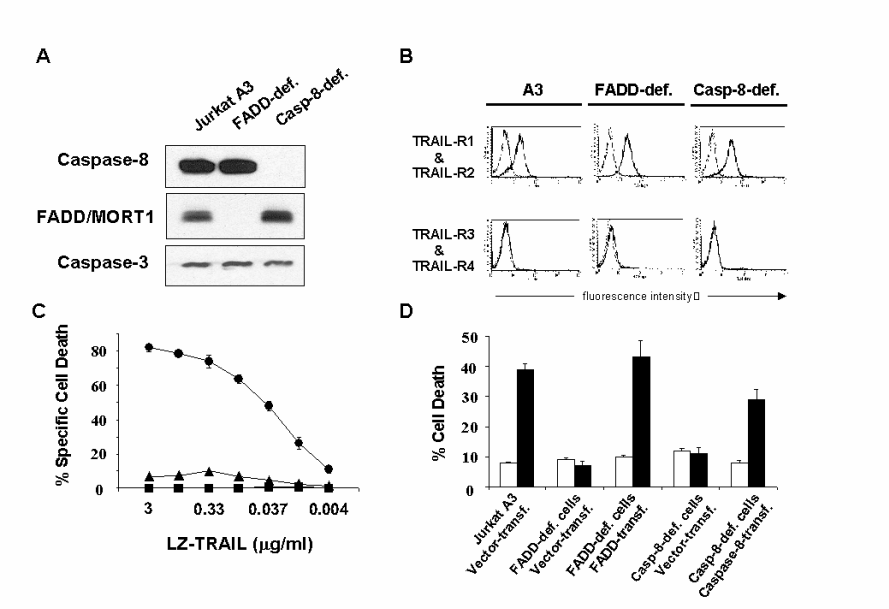

Fig.8. FADD and Caspase-8 Are Necessary for TRAIL-R2-Induced Apoptosis. (A) Western Blot analysis for expression levels of FADD/MORT1, caspase-8, and caspase-3 in Jurkat A3, FADD/MORT1 ^{def} and caspase-8 ^{def} Jurkat cells. Migration positions of the detected proteins are indicated. (B) FACS analysis of surface expression of TRAIL-R1 (A; solid line), TRAIL-R2 (A; solid bold line) TRAIL-R3 (B; solid line), and TRAIL-R4 (B; solid bold line) as compared to an isotype-matched control mIgG1 mAb (B; dashed line) on Jurkat A3, FADD^{def} and casp-8^{def} cells. (C) Jurkat A3 (●), FADD^{def} (■) and Casp-8 ^{def} (▲) cells were treated with the indicated concentrations of LZ-TRAIL or were left untreated. Cell death was determined 12 h after stimulation. All data points are the mean (+/- SD) of six independent experiments. (D) Jurkat A3, FADD^{def}, and casp-8^{def} cells were transfected with spectrin-GFP fusion protein [1] together with an expression plasmid coding for the respective missing signalling component or together with empty vector in the controls. Cells were incubated for 10 h in the absence (open bars) or presence (black bars) of 1 µg/ml LZ-TRAIL before cell death was determined by FSC/SSC analysis in the GFP-positive cell population.

of these mutated cell lines. Therefore, it was tested whether ectopic expression of FADD/MORT1 or caspase-8 resensitized the respective Jurkat clones for TRAIL-induced apoptosis. While Jurkat A3 cells transfected with vector control were killed by LZ-TRAIL, both Jurkat FADD^{def} and Jurkat casp-8^{def} remained TRAIL resistant when transfected with vector alone. However, reconstitution of the missing protein resensitized both cell lines for TRAIL-induced apoptosis (**Fig. 8D**). Since TRAIL resistance was overcome by re-expression of FADD/MORT1 in Jurkat-FADD^{def} and by re-expression of caspase-8 in the caspase-8^{def} Jurkat cells, TRAIL resistance was due to deficiency in the respective signal-

ing proteins. Therefore, it can be concluded that FADD/MORT1 and caspase-8 are essential for TRAIL-R2-induced apoptosis and cannot be substituted for by other endogenous proteins in Jurkat cells.

2 Caspase-10 is recruited to and activated at the native TRAIL and CD95 DISCs in a FADD-dependant manner but can not functionally substitute caspase-8.

Caspase-10 (Mch4, FLICE-2) is the second DED-containing caspase besides caspase-8. It was identified by homology cloning[168, 169]. The FLICE-2 and Mch4 isoforms represent different splice variants derived from the same gene. Later, two additional isoforms were identified[170]. Mch4 is now known as caspase-10a, FLICE-2 as caspase-10b, and the two recently identified variants as caspase-10c and caspase-10d. While the caspase-10a, -10b and –10d isoforms represent proteins that contain both, the large and small catalytic subunits, the caspase-10c variant represents a truncated form yielding a catalytically inactive molecule. While previous studies on the function of caspase-10 relied on overexpression of the different isoforms or catalytically inactive variants, no detailed study under native conditions with the endogenously expressed proteins has been conducted so far. Protein overexpression experiments are prone to artifacts, adding at least a cautionary note to the results obtained in these systems. Further, the expression and distribution of the different caspase-10 isoforms has been unclear, as only mRNA levels have been investigated.

In addition, caspase-10 has been implied to play a causative role in a rare immune disorder called ALPSII (for autoimmune lymphoproliferate syndrome). Wang et al identified two mutations in caspase-10 in patients suffering from ALPSII[236]. In this study, it was proposed that TRAIL resistance of mature DC and activated peripheral T cells from ALPS II patients was due to the mutated non-functional caspase-10 and causative for the disease. Later, one of the mutations identified in this study has been found to be a common polymorphism in the Danish population[237]. Although to date, besides the ALPS II patient identified by Wang et al., no other individual homozygous for this caspase-10 mutation has been described. This finding raised the question whether this mutation in caspase-10 alone is causative for the disease.

Therefore it was of interest to, (i) study the endogenous expression levels of caspase-10, (ii) determine whether it forms part of the TRAIL and the CD95 DISC under native conditions, and (iii) define whether caspase-8 and caspase-10 are functionally redundant or might fulfill different functions

2.1 Caspase-10 is expressed in three isoforms

The role of caspase-10 in CD95- and TRAIL-mediated apoptosis has been controversial. At least four splice variants of caspase-10 have been reported[168-170]. Three of the reported caspase-10 isoforms contain the large and the small subunits of caspase-10 and yield potentially active enzyme. The fourth variant, caspase-10c, codes for a truncated form that lacks the catalytic subunits and, thus, represents a catalytically inactive form of caspase-10 reminiscent of the CAP3 form of caspase-8[127, 131]. To investigate the potential role of caspase-10 during TRAIL- and CD95L-mediated apoptosis it was first necessary to identify a caspase-10-specific antibody. Amongst a panel of commercially available antibodies that was tested only one antibody specifically recognized bands at 55 and 59 kDa in Chinese hamster ovary (CHO) cells transfected with expression plasmids encoding recombinant caspase-10a and -10d, respectively (**Fig. 9A**). The epitope recognized by this antibody is localized in the death-effector-domain-containing prodomain as the antibody also detects the recombinantly expressed His-tagged prodomain (**Fig. 9A**). All splice variants of caspase-10 reported thus far contain this prodomain. Therefore, this antibody should recognize all known isoforms of caspase-10 while all other antibodies that were tested neither reacted with the recombinant prodomain nor with overexpressed caspase-10a or -10d in CHO cells (data not shown).

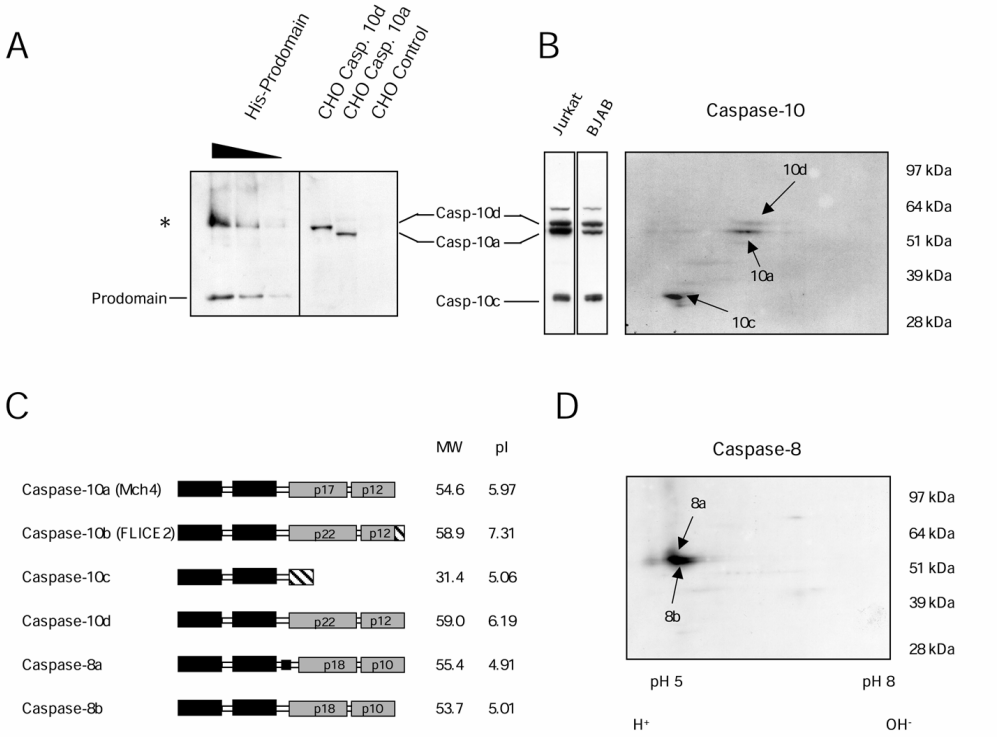

Fig.9. Three isoforms of caspase-10 are expressed in BJAB and Jurkat cell lines.
(A) Specificity of the caspase-10 antibody was determined by blotting decreasing amounts of bacterially expressed His-tagged caspase-10 prodomain or lysates from CHO-cells transfected with expression plasmids for human Caspase-10d, -10a or a control plasmid. The asterisk denotes a co-purified protein resulting from inefficient termination of translation. (B) Three endogenous isoforms of caspase-10 can be detected in lysates from BJAB and Jurkat cells. 10 µg proteins from the indicated cell lines were resolved on SDS page, blotted and detected with the caspase-10 antibody. Lysates from BJAB cells were resolved on 2D gels spanning pH 5- pH 8, blotted and subsequently detected with caspase-10 antibody. The pI and MW of the large caspase-10 isoforms correspond to caspase-10d and caspase-10a, the small protein to the pI and MW of caspase-10c. (C) Overview of the calculated MW and pI of the reported caspase-10 and caspase-8 isoforms. (D) As a control for the pI determination, the 2D-gel blot was reprobed with an antibody to caspase-8, showing the caspase-8 isoform at the expected positions.

Having identified a specific antibody for caspase-10 it was investigated which isoforms of caspase-10 are expressed in the human Burkitt's lymphoma cell line BJAB and the human T cell line Jurkat. Two large isoforms of caspase-10 (p55/p59) were detected in the lysates derived from both cell lines (**Fig. 9B**), reminiscent of the two large isoforms of caspase-8. At about 28 kDa an additional isoform of caspase-10 was detected. To determine to which of the published isoforms the individual bands correspond a two-dimensional (2D)

gel electrophoretic analysis was performed (**Fig. 9B**). The calculated molecular weights and isoelectric points (pI) for the different isoforms of caspase-10 are shown in **Fig. 9C**. As a control, the same blot was calibrated by detection of caspase-8 showing that the 2D-determined pI of the p55 and p53 caspase-8 isoforms exactly matched their predicted pI of 4.9 and 5.0, respectively (**Fig. 9D**). Determination of the pI of the two expressed large isoforms of caspase-10 revealed that they represent caspase-10d (p59) and caspase-10a (p55). This analysis further excluded that one of them could represent caspase-10b (calculated pI 7.3). As the pI of the 28 kDa band matched the pI of caspase-10c this protein spot most likely corresponded to this isoform of caspase-10.

2.2 Caspase-10 is cleaved early during CD95- and TRAIL-induced apoptosis

Caspase-8 has been shown to be the apoptosis-initiating caspase in the CD95 and the TRAIL DISCs and, thus far, caspase-8 has been thought to be the earliest caspase to become cleaved during death receptor-mediated apoptosis. Knowing that caspase-8 is cleaved during TRAIL- and CD95L-induced apoptosis it was next analyzed whether caspase-10 might also be processed during death receptor-mediated apoptosis and whether this cleavage occurs prior to, concomitantly with, or following caspase-8 cleavage (**Fig. 10**). During TRAIL- and CD95L-induced apoptosis the two large isoforms of caspase-10 are cleaved resulting in two intermediate cleavage products of caspase-10a and -10d, p43 and p47, respectively (**Fig. 10**). Further cleavage of these two intermediates results in the appearance of a 25 kDa band representing the prodomain of caspase-10. Also, the 28kD caspase-10c is cleaved during TRAIL- and CD95-induced apoptosis. Thus, caspase-10 is present in both BJAB and Jurkat cells in three different isoforms and all of these isoforms are cleaved during TRAIL- and CD95-mediated apoptosis. Depending on cell type and stimulus this cleavage was apparent between 15 and 30 minutes after onset of the incubation with TRAIL or CD95L concomitantly with the appearance of caspase-8 cleavage in the cytoplasm.

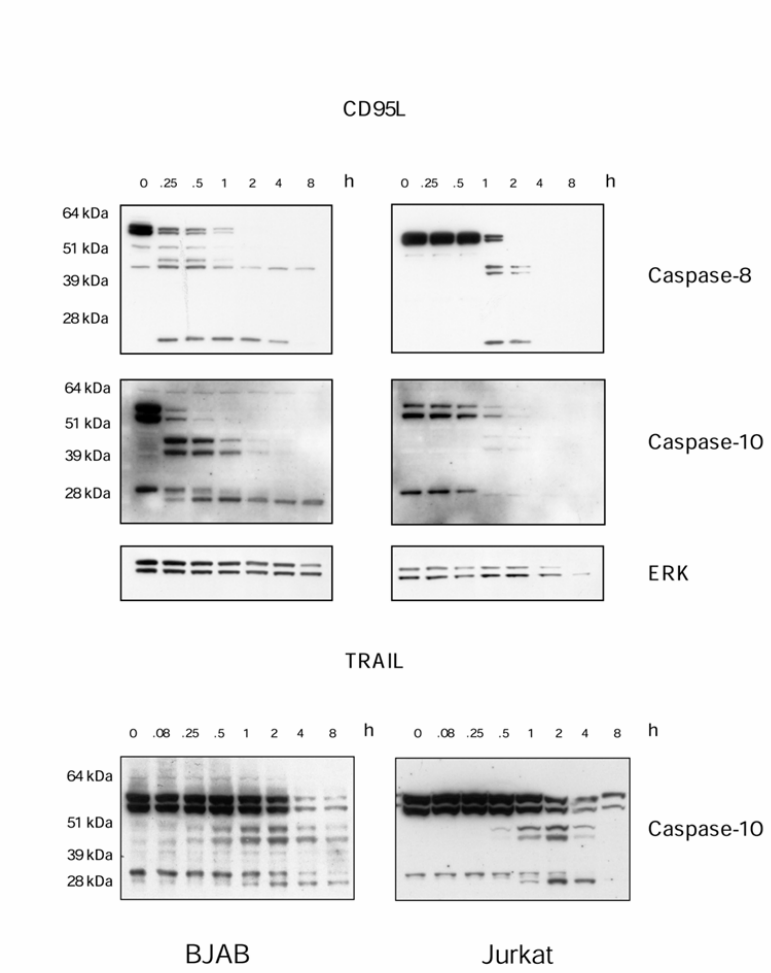

Fig. 10. Caspase-10 is processed in TRAIL- and CD95L-sensitive cell lines after stimulation with the respective ligands.

Cells were stimulated for the indicated time periods with TRAIL (1 µg/ml) or CD95L (500 ng/ml). Lysates were prepared, and analysed by western blot with antibodies to caspase-8 and caspase-10. As a control, the blot containing lysates from cells stimulated with CD95L was reprobed with an antibody to ERK. Cleavage of all caspase-10 isoforms can be observed as early as 15 to 30 minutes for stimulation with CD95L (upper panel) or TRAIL (lower panel), respectively. Cleavage of caspase-10 leads to the appearance of two intermediate products, p47 and p43 and a smaller band corresponding to the prodomain at 25 kDa. The cleavage pattern is similar to that observed for caspase-8 which is included as a control. The lower amount of ERK control observed after 8h of stimulation is the result of almost complete cell death observed at this time point.

2.3 Caspase-10 is recruited to the CD95 and the TRAIL DISC in BJAB and Jurkat cells

Like caspase-8, caspase-10 contains the tandem DEDs necessary for homotypic interaction with the adapter protein FADD, making recruitment of caspase-10 to death receptor-bound FADD-containing complexes possible. Caspase-8 is recruited to the CD95, the

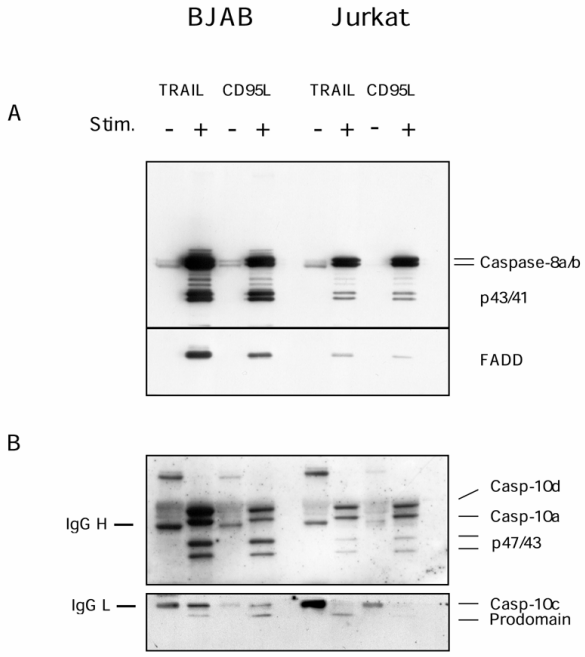

Fig. 11. Caspase-10 is recruited to the native TRAIL and CD95 DISCs.
Precipitation of the DISC complexes which form upon stimulation with TRAIL and CD95L was performed in BJAB and Jurkat cells. (**A**) To control for DISC formation, the western blots were probed for the known DISC components caspase-8 and FADD showing recruitment of these proteins to the TRAIL and the CD95 DISC in BJAB and Jurkat cells. (**B**) The DISC complexes were also analysed for the presence of caspase-10, showing that caspase-10 is recruited to the TRAIL DISC and the CD95 DISC in both cell lines tested. All three caspase-10 isoforms associate with both complexes. In addition, the intermediate products p47, p43 and the fragment corresponding to the prodomain, p25, are also associated with the CD95 and the TRAIL DISCs. The light chain of the M2 anti-FLAG antibody migrates at the same MW as the caspase-10 isoform. Correlation of heavy and light chain levels reveals caspase-10c association with crosslinked receptors.

TRAIL-R1 and the TRAIL-R2 signaling complexes after ligand-induced stimulation of the respective receptors in a FADD-dependent fashion. As results concerning protein-protein interactions that solely rely on transient protein expression are prone to artifacts the receptor complexes were analyzed under native conditions.

The death-inducing signaling complexes (DISCs) that form after stimulation with CD95L and TRAIL in both, Jurkat and BJAB cells were compared and it was tested whether caspase-10 is recruited to these complexes (**Fig. 11**). To control for efficiency of DISC formation the recruitment of caspase-8 and FADD, two already known components of the TRAIL and CD95 DISC was also analyzed (**Fig. 11A**). Whereas none of the proteins analyzed were bound to the non-stimulated receptors a stimulation-dependent recruitment of FADD and caspase-8 to CD95 and the TRAIL death receptors could be observed, as

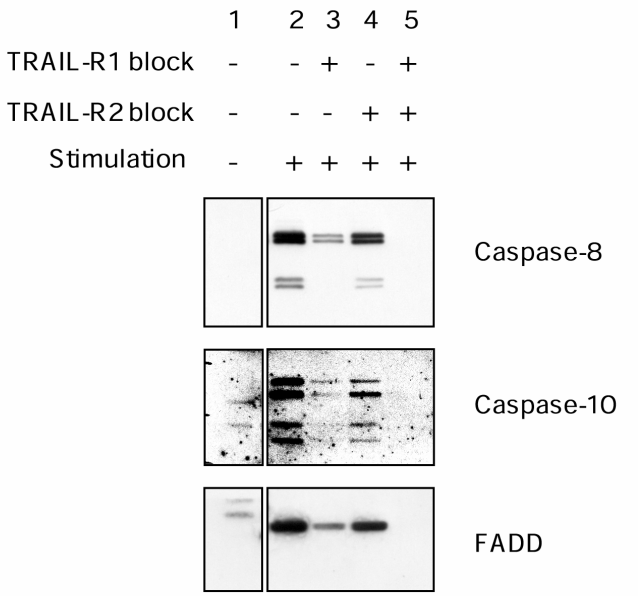

Fig. 12. Caspase-10 associates with homomeric TRAIL-R1 and TRAIL-R2 complexes.
Differential TRAIL DISC analysis was carried out by blocking either TRAIL-R1 or TRAIL-R2 with monoclonal antibodies before DISC analysis was performed. While precipitation of the unstimulated receptors shows no association of the proteins analysed (lane 1), under conditions where both receptors are stimulated (lane 2) caspase-10 associates with both, TRAIL-R2 complexes (lane 3) and TRAIL-R1 complexes (lane 4). Blockage of both receptors completely abolishes DISC formation (lane 5).

described before[144, 145, 188]. For caspase-10 the analysis revealed ligand-induced recruitment of all three expressed caspase-10 isoforms to the native TRAIL DISC and the native CD95 DISC in both cell types (**Fig. 11B**). Like for caspase-8, the products of the first activation step of caspase-10a and -10d (p43/p47) were also associated with the TRAIL and the CD95 DISC. These two forms represent the prodomain with the p17 subunit of caspase-10a and –10d, respectively after separation of the p12 subunit by proteolytic cleavage. In addition, the prodomain is also apparent in both DISCs (Fig. 3B, lower panel).

2.4 Caspase-10 is recruited to the TRAIL-R1 and the TRAIL-R2 DISC

So far no biochemical differences in recruitment of adaptor and effector proteins between TRAIL-R1 and TRAIL-R2 have been identified. To investigate whether caspase-10 is recruited selectively to one of the apoptosis-inducing TRAIL receptors, differential DISC analysis for TRAIL-R1 and TRAIL-R2 on BJAB cells was performed (**Fig. 12**). Either of the two receptors was blocked by incubation with a monoclonal antibody specific for TRAIL-R1 or TRAIL-R2 before stimulation of the other receptor by the ligand. Efficiency of inhibition of the receptors was demonstrated by complete inhibition of FADD and caspase-8 recruitment upon simultaneous TRAIL-R1 and TRAIL-R2 blockage. The analysis of the resulting complexes shows that both, caspase-10a and -10d were recruited to both receptors independently of the other receptor.

2.5 FADD is necessary for the recruitment of caspase-10 to the CD95, the TRAIL-R1 and the TRAIL-R2 DISC

FADD has been shown to be required for the recruitment and subsequent activation of caspase-8 to the TRAIL-R2 and the CD95 DISC. To test whether FADD is also required for recruitment of caspase-10 to the CD95, TRAIL-R1 and TRAIL-R2 DISC the respective protein complexes in cells that lack FADD but express all three receptors was investigated (**Fig. 13**). As the original FADD-deficient Jurkat cells only expressed TRAIL-R2[144, 145] first, a stable subclone of this cell line, expressing TRAIL-R1, was established. This was achieved by stably transfecting the original FADD-deficient Jurkat cells with an expression plasmid coding for TRAIL-R1. After two rounds of limiting dilution cloning one clone was selected. This clone which was named Jurkat FADD^{def}–TR1/2 expressed TRAIL-R1 and TRAIL-R2 on its surface (**Fig. 13A**). The protein complexes that form upon stimulation with CD95L and TRAIL are shown in **Fig. 13B**. While the CD95 and TRAIL DISC were efficiently formed in the parental Jurkat cells by incubation of these cells with CD95L and TRAIL, respectively, the absence of FADD resulted in failure of the crosslinked receptors to recruit caspase-8 and caspase-10. Thus, FADD is not only necessary for recruitment and activation of caspase-8 but also for recruitment and activation of caspase-10 at the TRAIL and CD95 DISC.

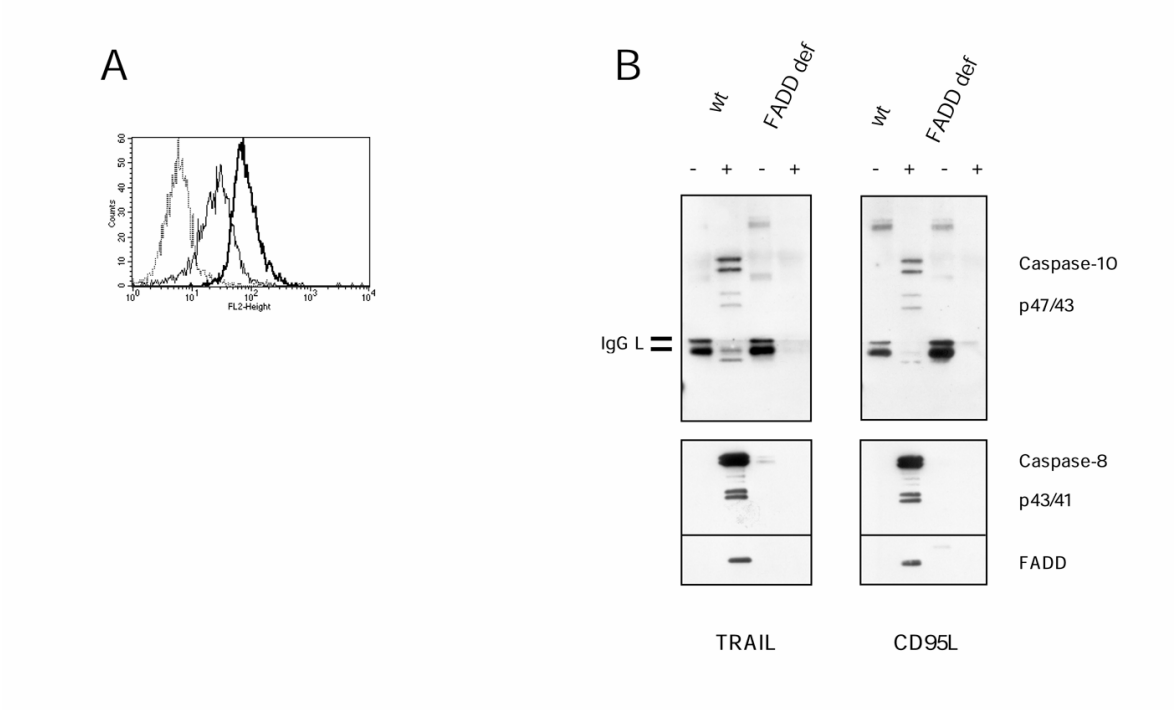

Fig. 13. FADD is the adapter protein for caspase-10 recruitment to TRAIL-R1, TRAIL-R2 and CD95. (**A**) FACS stain of the FADD deficient cell line FADD^{def} TR1/TR2. A surface stain for TRAIL-R1 (thin line) and TRAIL-R2 (thick line) shows expression of both TRAIL-R1 and TRAIL-R2. Dotted line, IgG control. (**B**) DISC analysis was performed with TRAIL and CD95L on wild type and FADD^{def} TR1/TR2 Jurkat cells. Western blot analysis of the precipitated protein complexes shows that caspase-8, caspase-10 and FADD are efficiently recruited to the TRAIL DISC and the CD95 DISC in the parental wild type (wt) cells. In the absence of the adapter protein FADD in the cell line Jurkat FADD^{def} TR1/TR2 (FADD def) caspase-10 as well as caspase-8 are neither recruited to the TRAIL-DISC nor to the CD95-DISC.

2.6 Caspase-10 is not necessary for death-receptor mediated apoptosis and caspase-8 activation at the DISC

As both, caspase-8 and caspase-10 are present in the DISC complexes it could be possible that both are needed for apoptosis to be initiated at the death receptors CD95 and the TRAIL receptors. It was noted that several cell lines, including the Burkitt's lymphoma cell line BL60 and the CD95-transfected BL60 subclone K50, do not express detectable levels of caspase-10. Although K50 cells are devoid of detectable levels of caspase-10

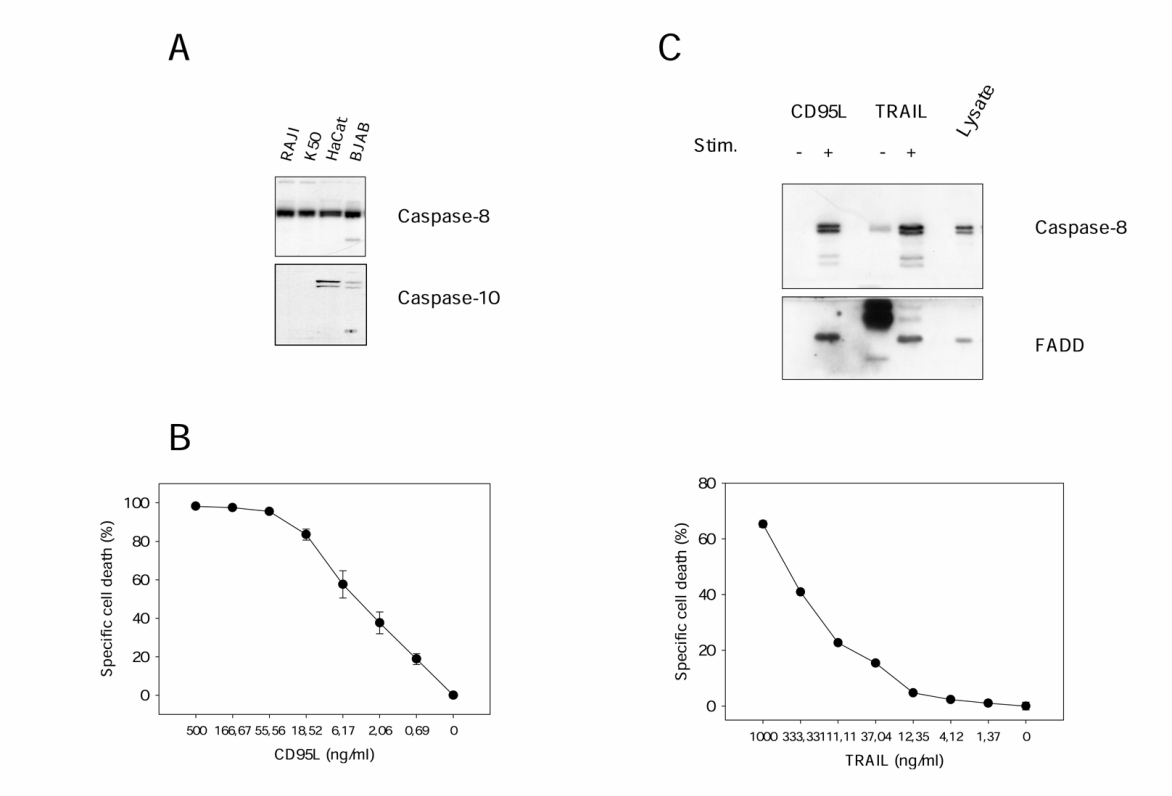

Fig. 14. Caspase 10 is not required for for TRAIL and CD95L mediated cell death and activation of caspase-8. (**A**) Western blot of lysates of different cell lines shows that some cell lines do not express detectable levels of caspase-10. (**B**) Cells from the cell line K50 were incubated in CD95L or TRAIL at the concentrations indicated. Cell death was measured after 16 h by PI exclusion and FSC/SSC analysis. Both ligands induce cell in the absence of detectable levels of caspase-10. (**C**) Analysis of the TRAIL and CD95 DISCs in K50 cells shows that caspase-8 is recruited and activated at both DISC complexes in the absence of caspase-10. The DISCs were precipitated and analysed as in the experiment presented in Figure 4B.

(**Fig. 14A**), stimulation with CD95L or TRAIL resulted in normal apoptosis induction (**Fig. 14B**). In addition, caspase-8 is recruited to both, the CD95 and the TRAIL DISCs in this cell line and activated as indicated by the presence of the cleavage products, p43/41 in the DISC (**Fig. 14C**). Thus, caspase-10 is dispensable for activation of caspase-8 at the DISC and concomitant apoptosis induction.

2.7 Caspase-10 can not functionally substitute caspase-8, although it is recruited to the DISC in the absence of caspase-8.

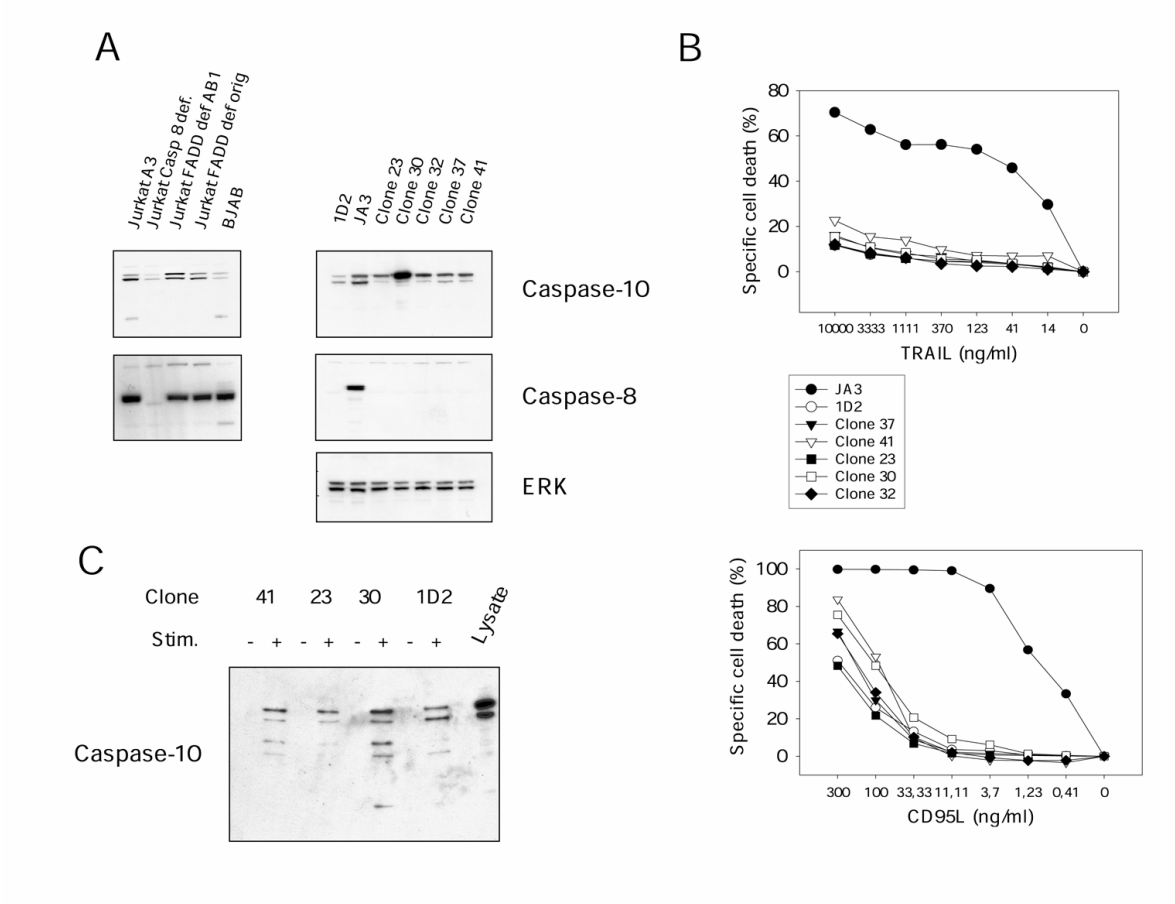

Fig. 15. Caspase 10 can not functionally substitute loss of caspase-8.
(**A**) Comparison of the expression levels of caspase-10 in different cell lines and clones stably transfected with caspase-10. Cell lysates from the cell lines indicated were resolved by SDS Page and blotted. The blots were probed with antibodies against caspase-10 and caspase-8. (**B**) High expression levels of caspase-10 do not sensitize caspase-8 deficient cell lines to TRAIL or CD95L induced apoptosis. Different cell lines were incubated with either TRAIL (**upper panel**) or CD95L (**lower panel**) at the concentrations indicated. Cell death was assessed after 24 h by measuring the drop in FSC/SSC and PI exclusion. (**C**) Caspase-10 is recruited to and activated at the CD95 DISC in the absence of caspase-8. Cells were stimulated with CD95L at 100 ng/ml for 15 minutes. After lysis, the DISC complexes were precipitated with an antibody against CD95 (anti APO-1), resolved on SDS and blotted. The blots were probed for Caspase-10, showing recruitment of caspase-10 in caspase-8 deficient cell line 1D2 and in the caspase-10 transfected caspase-8 deficient cell lines. Clone 41, 23 and 30. Caspase-10 is recruited to the CD95 DISC in all cell lines even in the absence of caspase-8. Also, in all cases caspase-10 is activated in these complexes as indicated by the appearance of the p47/p43 fragments. **L** denotes the lane containing control lysate from unstimulated clone 41.

In earlier experiments it has been shown that Jurkat cells deficient in caspase-8 are defective in apoptosis induction after stimulation with CD95L or TRAIL (**Fig. 8**). However, analysis of expression of caspase-10 in this cell line revealed that the levels of caspase-10 are substantially reduced compared to the parental cell line (**Fig. 15A**). To test whether caspase-10 can induce apoptosis in the absence of caspase-8, stable clones that expressed

caspase-10d in the absence of caspase-8 were established. To expand the analysis of the TRAIL DISC to TRAIL-R1 first a caspase-8 deficient cell line stably transfected with a TRAIL-R1 expression plasmid was generated, as the Jurkat caspase-8^{def} cell lines normally express only TRAIL-R2 (**Fig. 8**). A resulting clone was transfected with an expression plasmid for caspase-10. Several independent caspase-10 expressing clones were chosen for analysis. The amounts of caspase-10 expressed in these clones ranged from relatively low levels to amounts highly exceeding the amount of caspase-10 present in the parental cell line, the parental Jurkat caspase-8^{def} and also, the amounts present in primary T cells (not shown). Expression of the TRAIL receptors and CD95 was comparable in the different cell lines as determined by surface staining (not shown). Next, TRAIL sensitivity of these cell lines in comparison to the parental clones was determined. Surprisingly, apoptosis induction was not significantly higher in caspase-10 transfected versus non-transfected caspase-8 deficient cell lines (**Fig. 15B** upper panel).

Stimulation with CD95L leads to induction of cell death in the caspase-8-deficient cell lines, although much higher amounts of CD95L are needed comparing caspase-8-deficient cell lines to the caspase-8-expressing control cell line (**Fig. 15B** lower panel). Recently, cell death induction by CD95L in caspase-8-deficient Jurkat cells was described[163, 238]. However, as seen for stimulation with TRAIL, overexpression of caspase-10 does not lead to a sensitization to CD95L induced apoptosis when compared to the parental caspase-8-deficient cell line. The inability of caspase-10 to substitute for caspase-8 could be due to its inability to be activated at the DISC complexes in the absence of caspase-8. However, analysis of the DISC shows that caspase-10 is recruited to and activated at the CD95 DISC also in the absence of caspase-8 (**Fig. 15C**). These results show that although caspase-10 is recruited to and activated at the DISC, it can not functionally substitute for caspase-8 in apoptosis induction.

2.8 Caspase-10 is expressed in primary human T cells and is cleaved upon CD95-induced apoptosis of pre-activated human T cells

Mutations in caspase-10 have been shown to result in the human autoimmune lymphoproliferative syndrome type II (ALPS II) that is characterized by a defect in T cell and dendritic cell apoptosis[236]. To test whether caspase-10 might also play a role in apoptosis induction in primary cells, pre-activated human T cells were stimulated with CD95L. Then, it was analyzed whether caspase-10 is cleaved during the resulting CD95-L induced apoptosis. This analysis showed that caspase-10a, -10d and –10c were present in lysates obtained from these cells and that all caspase-10 isoforms were cleaved following CD95 stimulation (**Fig. 16.**). The observed cleavage pattern was essentially identical to the pattern observed in BJAB and Jurkat cells (**Fig. 10**). The proteolytic activation of caspase-10 during apoptosis of activated T cells is indicative of a role for this caspase in the transmission of signals emanating from death receptors not only in transformed cell lines but also in primary cells of the immune system.

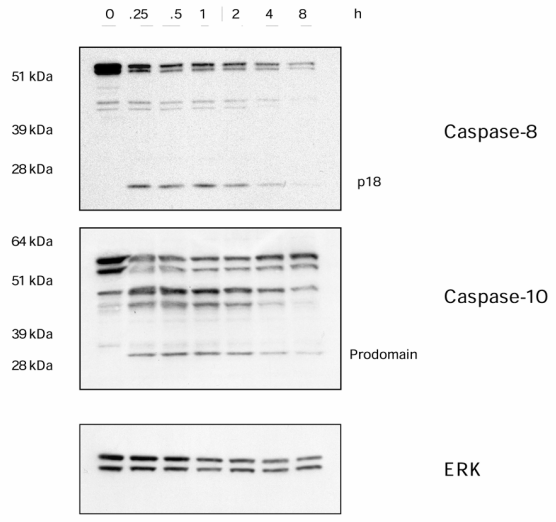

Fig. 16. Caspase-10 is expressed and rapidly processed in primary T cells after stimulation with CD95L.
Activated primary T cells were stimulated with CD95L (500 ng/ml) for the time periods indicated, lysed and subjected to western blot analysis. Processing of caspase-10 and caspase-8 can be detected simultaneously at 15 min. after stimulation as indicated by the appearance of the p18 subunit of caspase-8 (upper panel) or the processed prodomain of caspase-10 (lower panel), respectively. The cleavage fragments of caspase-10 p47, p43 and the prodomain, p25, correspond to the pattern detected in BJAB and Jurkat cell lines (Fig. 2).

IV DISCUSSION

The superfamilies of TNF ligands and receptors play an important role during processes as diverse as organogenesis and homeostasis, the genesis and function of the immune system and the physiological form of cell death, apoptosis[7]. Being involved in these processes, it is not surprising that disturbances in the normal functions of the TNF family are involved in various diseases. Due to their potency in regulating cellular processes, many of the TNF superfamily members are attractive therapeutic targets and, in fact, some of them are already used clinically[239]. The TNF superfamily member TRAIL has received much attention due to its apparent ability to induce cell death in tumor cells while sparing normal cells, rendering it an attractive novel drug candidate for the fight against malignancies[59, 60].

Especially in the light of more recent reports that certain recombinant forms of TRAIL may exert an apoptosis-inducing effect on some normal human cells, most importantly hepatocytes[240, 241], it is imperative to better understand the signaling mechanisms employed by this ligand before the clinical development of TRAIL. This knowledge will not only help to evaluate possible areas of application. It will also help to predict which potential side effects and contraindications of use can be expected. In addition, by better understanding the signaling mechanisms triggered by death receptors it is also possible to discover further cellular targets which might be used for therapeutic or prognostic purposes.

In TRAIL signaling, the first known intracellular step is the formation of the protein complex termed the TRAIL DISC. At the onset of this work, the composition of this protein complex was controversially discussed, primarily owing to the protein overexpression methods employed. In fact, nothing was known about the composition of the *native* TRAIL DISC.

1 FADD and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptors 1 and 2

Overexpression of proteins can lead to unspecific protein-protein interactions due to

misfolding and aberrant protein localization. Thus, protein interactions which do not occur under physiological conditions might be observed in overexpression experiments. To study the native TRAIL DISC, first a method needed to be devised to precipitate this complex. Ligand affinity precipitation utilizing a biotinylated version of the ligand, Bio-LZ-TRAIL (for "biotinylated leucine-zipper TRAIL") was utilized. This form of recombinant TRAIL allowed for stimulation of the TRAIL receptors and subsequent precipitation of the resulting protein complex by means of the affinity tag, biotin. The addition of a leucine zipper trimerization motif leads to a stabilization of the trimeric structure[59].

As a first step, the involvement of the adaptor protein FADD and the initiator caspase caspase-8 in TRAIL induced apoptosis was analyzed.

1.1 FADD is the central adaptor for caspase-8 and caspase-10 recruitment to the TRAIL and CD95 DISC and is essential for apoptosis mediated by these receptors

It has been controversial whether the adaptor protein FADD and caspase-8 play a key role in TRAIL-induced apoptosis[242]. In some overexpression systems dominant-negative FADD (FADD-DN) inhibited TRAIL-induced apoptosis[67, 70, 71, 230]. In other studies FADD-DN overexpression did not prevent TRAIL-induced apoptosis[64, 65, 68, 69]. Co-immunoprecipitations of overexpressed cytoplasmic domains of TRAIL-R1 or TRAIL-R2 with FADD supported a role for FADD in TRAIL-induced apoptosis[67, 70]. Yet, opposite results were obtained in another study as FADD was not co-immunoprecipitated with overexpressed TRAIL-R2 but only with CD95[69]. Murine embryonic fibroblasts (MEF) from FADD-deficient mice underwent apoptosis upon overexpression of human TRAIL-R1 [146]. Thus, the role of FADD in TRAIL-induced apoptosis was still unclear.

In this work, it could be shown that under non-overexpression conditions, endogenous FADD is recruited to both, TRAIL-R1 and TRAIL-R2 homomeric complexes (**Fig. 5, 6, 7**) as well as to heteromeric complexes containing both receptors seconds after receptor stimulation. In addition, Jurkat cells lacking FADD are completely resistant to the actions of TRAIL (**Fig. 8**). Reconstitution of these cells with FADD rescues the apoptosis sensitivity of these cells (**Fig. 8**). As normal Jurkat FADD^{def} cells express only TRAIL-R2, a clone stably expressing TRAIL-R1 was generated (**Fig. 13**). These cells were also found to be resistant to TRAIL-induced apoptosis contrasting earlier results by Yeh et al.[146], which

implied that overexpression of TRAIL-R1 induces apoptosis in MEF derived from FADD-deficient mice. However additional, more carefully conducted studies in MEF from FADD-deficient mice by another group also demonstrate an absolute requirement for FADD during TRAIL-R1- and TRAIL-R2- induced apoptosis[149]. The FADD-independent apoptosis induction observed by Yeh et al.[146] might represent an overexpression artifact or it could be speculated that alternative pathways can be triggered by TRAIL-R1 under certain circumstances. The nature of these pathways is unclear, but could possibly involve activation of pathways leading to transcriptional events.

Taken together, the results obtained establish FADD as the central adaptor protein in death-receptor-mediated apoptosis. In this work FADD has been shown to be crucial for recruitment of caspase-8 and caspase-10 to the TRAIL and CD95 DISC and subsequent apoptosis induction.

1.2 The role of caspase-8 and caspase-10 in TRAIL- and CD95-induced apoptosis

The identity of the caspase(s) involved in the initiation of TRAIL-mediated apoptosis was also controversial. Under native conditions, early cleavage of caspase-8 has been observed following TRAIL stimulation[81, 233]. Yet, overexpression studies with dominant-negative forms of caspase-8 and -10 either suggested caspase-10 alone[64] or a combination of caspase-8 and -10[69] as initiator caspase(s) during TRAIL-induced apoptosis. In addition, TRAIL resistance of mature DC and activated peripheral T cells from ALPS II patients was suggested to be due to mutated non-functional caspase-10 and to be causative for the disease[236]. Mice deficient in caspase-8 die in utero probably due to defective heart development[165]. Embryonic fibroblasts derived from these mice, as well as lymphocytes from mice with a lymphocyte-specific caspase-8 deletion[167], have been shown to be resistant to CD95-, TNF- and TRAMP-induced apoptosis.

The expression pattern and the physiological role of the second DED-containing caspase, namely caspase-10 were controversially discussed. Four different isoforms of caspase-10 (a-d) had been described on the mRNA level[168-170]. Yet, no previous study so far unambiguously determined which if these mRNAs are actually translated. A similar uncer-

tainty existed for caspase-8, with at least eight isoforms described on the mRNA level. Careful studies using a specific antibody revealed that only three of these isoforms are expressed at a detectable level[160]. Previous studies on the function of caspase-10 relied on overexpression of the different isoforms or catalytically inactive variants[168-170]. Thus far, the analysis of function and expression of caspase-10 was hampered by the unavailability of specific antibodies. In this work, an antibody specific for the prodomain of caspase-10, which is capable of recognizing all theoretically existing isoforms of caspase-10 was, identified (**Fig. 9A**). Using the newly identified antibody it is shown that like for caspase-8, two large isoforms of caspase-10 are expressed (**Fig. 9B**). These isoforms were identified by 2D-gel-electrophoresis to represent caspase-10a (Mch4)[168] and caspase-10d[170] (**Fig. 9C**). Conversely, the previously described caspase-10b (FLICE-2)[169] could not be detected in any of the cell lines tested although it should be readily detectable with the antibody used. In addition, a smaller isoform, caspase-10c, is detected (**Fig. 9B**). This isoform yields a catalytically inactive form that is reminiscent of the CAP3 isoform of caspase-8[127]. The function and significance of the small isoforms of the two caspases for regulation of DISC function remains to be determined. However, the conserved pattern of presence of these caspase-8 and –10 short isoforms is indicative of a functional role of these proteins. In addition, a recent report shows that these DED-only variants of caspase-8 and caspase-10 can inhibit TRAIL- and CD95-mediated apoptosis when overexpressed[243]. This inhibitory function was predictable given their close resemblance to the inhibitory molecule vFLIP and $cFLIP_s$[173, 224] (reviewed in[172]). Taken together, caspase-8 and caspase-10 are not only structurally homologous, but are also similar in their isoform pattern with two large forms and a truncated form being expressed.

With the detected expression of two DED-containing initiator caspases, it was of interest to identify the role of these two caspases in TRAIL- and CD95-induced apoptosis, as they both share the protein motif, which potentially allows for their recruitment to the death receptors via FADD. It was of special interest to elucidate whether both initiator caspases are necessary for TRAIL- and CD95-induced apoptosis, or alternatively, play a redundant role with both proteins being equally capable of initiating death-receptor-mediated apoptosis. A third possibility is that both caspases fulfil different, non-redundant roles in death receptor signalling.

Like caspase-8 (**Fig. 4B**), caspase-10 is processed early after stimulation of sensitive

cells with either CD95L or TRAIL (**Fig. 10**).

Both, endogenous caspase-8 and caspase-10 are recruited to the native TRAIL and CD95 DISCs within seconds following stimulation (**Fig. 5**) and (**Fig. 11 A, B**). Both caspases are rapidly processed at the DISC, as apparent from the occurrence of smaller caspase-8 and –10 fragments in the DISC. All three different isoforms of endogenous caspase-10 are recruited to the native TRAIL and CD95 DISC (**Fig. 11B**), comparable to what has been previously described for caspase-8[132, 160]. The sequence of activation at the DISC is also comparable to that of caspase-8. In the first cleavage step, the small subunit is cleaved. Then the large subunit is cleaved from the remaining DISC-bound p43 and p47 fragments respectively.

1.3 Differential signaling properties of TRAIL-R1 and TRAIL-R2

So far, it was unclear whether TRAIL-induced apoptosis may be differentially regulated by the triggering of the two different apoptosis-inducing TRAIL receptors. Here it is shown that TRAIL-induced crosslinking of either TRAIL-R1 or TRAIL-R2 leads to the recruitment of both FADD/MORT1 and caspase-8 to the individual receptor-specific DISCs (**Fig. 7**). Additionally, caspase-10 which was identified as a new DISC component during this work is also recruited to both, TRAIL-R1 and TRAIL-2 independent of each other (**Fig. 12**). Apart from the homomeric receptor complexes also heteromeric complexes consisting of TRAIL-R1 and TRAIL-R2 may exist. It cannot be excluded that these complexes may signal apoptosis via an additional pathway different from the FADD/MORT1 and caspase-8/caspase-10 involving pathway. Of special interest are the signaling pathways which trigger NF-κB activation after TRAIL stimulation[214]. Apart from the possible involvement of RIP[244, 245] little is known about the pathways and proteins involved in TRAIL-induced activation of NF-κB.

1.4 Role of caspase-8 and caspase-10 in apoptosis induction

The co-occurrence of caspase-10 and caspase-8 in the CD95 and the TRAIL DISC raises the question whether caspase-10 may act in the same pathway as caspase-8 either in

a redundant or an enhancing fashion in death-receptor-mediated apoptosis, or whether caspase-10 may act as a transmitter of signals other than apoptosis induction that emanate from TRAIL death receptors and CD95.

Loss of caspase-8 expression almost completely abrogates sensitivity to TRAIL, with cell death induction being only barely detectable (**Fig. 15**). This insensitivity to TRAIL can be rescued by re-expressing caspase-8 (**Fig. 8**) clearly confirming the necessity for caspase-8 with regards to death induction by TRAIL.

During the course of this work two additional reports appeared showing that caspase-10 is recruited to the DISC[143, 162]. In one of these studies cell death was observed in caspase-8-deficient cells after prolonged stimulation with TRAIL or CD95L and was proposed to be mediated by caspase-10[143].

This is in line with the results shown in **Fig. 15** that both death ligands can induce cell death in the caspase-8-deficient Jurkat cells. However, titration of TRAIL and CD95L show that extremely high concentrations of these ligands are needed for death induction when compared to the caspase-8-expressing Jurkat cells. It seemed most likely that this residual cell death is mediated by caspase-10, which is also recruited to both DISCs in these cell lines in the absence of caspase-8 as shown in **Fig. 15C**.

The reduced sensitivity of the caspase-8-deficient cells to TRAIL and CD95L has been attributed to be due to lower expression levels of caspase-10 in these cells[143, 162]. In both studies, transient transfection of caspase-8-deficient cells with caspase-10 sensitized these cells for CD95L- and TRAIL-induced apoptosis, arguing for redundant roles of caspase-10 and caspase-8 in death receptor-mediated apoptosis.

In this work, several independent caspase-10-overexpressing clones, which had been established from the caspase-8-deficient cell line, were analyzed. Surprisingly, stable expression of caspase-10 in these clones did not increase the apoptosis susceptibility when these cells were stimulated with TRAIL or CD95L. The levels of caspase-10 expression we observed exceeded the level found in wild type Jurkat cells (**Fig. 15A**) and in several primary cell types (data not shown) analysed by several fold. These data strongly argue against a role of caspase-10 as a second redundant initiator caspase in the TRAIL and CD95 system. Possibly, the transient expression system utilized in the previous reports leads to caspase-10 levels, which are much higher than the ones observed in the clones generated during this study. Such exceedingly high levels may surpass the physiological levels that can be

tolerated by the cells, explaining the observed increase in sensitivity to TRAIL and CD95L. In addition, caspase-10 is not necessary for normal apoptosis to proceed, as cell lines deficient in caspase-10 show no apparent defect in apoptosis (**Fig. 14**).

Several brain tumors show a loss of caspase-8 expression[82, 246-252], rendering these tumors insensitive to death-receptor induced apoptosis. In some of these tumor cell lines caspase-10 expression was also analyzed with mixed results. Some authors noted unchanged expression of caspase-10 while others found a decrease in caspase-10 expression as well. Caspase-10 expression was analyzed either by RT-PCR or western blot. However, in the light of the poor quality of most commercially available antibodies, and the posttranscriptional regulation of caspase-10, these results certainly need confirmation as only one study employed an antibody which was confirmed to be specific for caspase-10[252].

Taken together, while some cell death is observed in the absence of caspase-8, caspase-10 is unable to fully substitute for a loss of caspase-8, even at high expression levels (**Fig. 15**) suggesting that it serves a function different from that of caspase-8. A physiological role of caspase-10 is suggested by the fact that it is processed after stimulation of death receptors not only on tumor cell lines (**Fig. 10**) but also on primary activated T cells (**Fig. 16**).

What is the physiological function of caspase-10 and what are the processes it is involved? Interestingly, caspase-10 has been found to be downregulated in about 50 percent of all tumor cell lines analyzed in one study[143]. Additionally, inactivating mutations in caspase-10 but not in caspase-8 have been found in gastric cancers[253]. This high ratio of caspase-10 inactivation suggests a role of caspase-10 as a tumor suppressor. The exact pathways involving a possible caspase-10 function in tumor suppression *in vivo* are still elusive. Caspase-10 may diversify the apoptotic signal that emanates from the CD95 and TRAIL death receptors by targeting a different set of proteins for proteolysis than caspase-8. Alternatively, pathways not necessarily involving apoptosis induction may be relevant. As caspase-10 cannot fully substitute for a loss of caspase-8 in apoptosis induction, it might serve different functions after triggering other members of the TNF superfamily. Not all caspases are primarily involved in apoptosis induction; e.g. caspase-1, -4 and –5 function by maturing cytokines of the interleukin family and the drosophila caspase-8 ortholog DREDD is involved in the activation of innate immunity[254-256].

Interestingly, caspase-10 has been reported to be selectively upregulated by several

stimuli that trigger innate immune responses[257], by heat shock[258] and by TGF-β[259]. Also, arsenic trioxide[260] induced apoptosis in the treatment of promyelocytic leukemia and glucocorticoid induced cell death in pre-B leukemic cells[261] have been implicated to involve caspase-10.

Future studies will aim at identifying the cellular substrates and interaction partner of caspase-10 in a physiological context. The absence of caspase-10 in the mouse strains analyzed thus far by Celera Genomics and the NCBI sequencing consortium precludes the generation of caspase-10 knockout animals in this species. The apparent lack of caspase-10 in the mouse genome raises also the question as to why this might be, especially in the light of the fact that a caspase-10 ortholog has been described in *xenopus laevis*[171]. Either the inbred mouse strains used for sequencing are not under selective pressure for keeping the caspase-10 gene (e.g. due to the relatively pathogen free conditions they are kept under), or the evolution of a caspase-10 gene is a peculiarity of only some clades of the vertebrate lineage.

No matter what the exact role of caspase-10 will be, focussing on the molecular interactions of this protein which, following this work can now be regarded as a bona fide DISC constituent, will help in elucidation of the signals which emanate from cell surface receptors of the TNF superfamily.

V APPENDIX

1 Abbreviations

2D-Gel	two-dimensional gel-electrophoresis
$\Delta\Psi_m$	mitochondrial transmembrane potential
λ	Wavelength
µg	Microgram
µL	Microlitre
µM	micromolar
Fig.	Figure
ActD	Actinomycine D
AICD	***A**ctivation **i**nduced **c**ell **d**eath*
AIF	**A**poptosis **i**nducing **F**actor
ALPS	***a**uto-immune **l**ymphoproliferative **s**yndrome*
Apaf-1	*Apoptosis-**p**rotein-**a**ssociated-**f**actor-1*
APC	***A**ntigen **p**resenting **c**ell*
AA	Amino acid
ATP	**A**denosine **T**ri**p**hosphat
Bcl-2	***B** **c**ell **l**ymphoma gene **2***
Bcl-xL	**B** **c**ell **l**ymphoma gene **x** (**l**ong form)
BCR	**B-c**ell **r**eceptor (B-cell-receptor)
BH	**B**cl-2 **H**omologydomain
Bid	***B**H-3 **I**nteracting **D**omain*
Bp	Base pair
BSA	***B**ovine **s**erum **a**lbumin*
C.	*Caenorhabditis*
CAD	***C**aspase-**a**ctivated **D**NAse*

CARD	***C**aspase **a**ctivation and **r**ecruitment **d**omain*
°C	Degrees Centigrade
CD	***c**luster of **d**ifferentiation*
CHAPS	((3-chloramidopropyl)-dimethylammonio)-propanesulfate
CHX	Cycloheximide
CRD	***c**ysteine **r**ich **d**omain*
cIAP	***c**ellular **i**nhibitor of **a**poptosis **p**rotein*
D.	*Drosophila*
DD	***d**eath **d**omain*
DcR	***d**e**c**oy **r**eceptor*
DED	***d**eath **e**ffector **d**omain*
DISC	***d**eath-**i**nducing **s**ignalling **c**omplex*
DMSO	Dimethylsulfoxid
DNA	***d**eoxyribon**u**cleic **a**cid*
DNTP	Desoxyribonukleosidtriphosphate
DR	***d**eath **r**eceptor*
ds	double-strand
E.	*Escherichia*
EAE	experimental autoimmune-Encephalomyelitis
EGF	**e**pidermal **g**rowth **f**actor
ECL	***e**nhanced **c**hemo**l**uminescence*
EDAR	**e**cto**d**yspl**a**sin **r**eceptor
EDARADD	**e**cto**d**yspl**a**sin **r**eceptor **a**ssociated **d**eath **d**omain protein
et al.	*et alii*
EST	***e**xpressed **s**equence **t**ag*
Fc	***c**rystallizing **f**ragment*
FACScan	***F**luorescence **A**ctivated **C**ell **S**can*
FADD	***F**as-**a**ssociated **d**eath **d**omain prot*ein
FADD-DN	dominant negative FADD
FCS	***f**oetal **c**alf **s**erum*
FLIP	**FL**ICE **i**nhibiting **p**rotein
fmk	Fluoromethylketone

FSC	***f**orward **sc**atter*
g	Gravitational constant
GFP	**g**reen **f**luorescent **p**rotein
GPI	**G**lykosyl**p**hosphatidyl**i**nositol
h	*hora* (hour)
HRP	***h**orseradish **p**eroxidase*
hu	human
IAP	***i**nhibitor of **a**poptosis **p**rotein*
IFN	Interferon
Ig	**I**mmuno**g**lobulin
IL	**I**nter**l**eukin
IP	**I**mmuno**p**recipitation
JNK	c-**J**un **N**-terminal **K**inase
kB	Kilobase
kDa	Kilodalton
L	Ligand
LIT	**L**ymphocyte-**I**nhibitor of **T**RAIL
LTα	Lymphotoxin-alpha
LZT	Leucine-Zipper-TRAIL
M	Molar (mol/L)
mA	Milliampere
ME	Mercaptoethanol
mg	Milligram
MHC	*major histocompatibility complex*
MES	Morpholino Ethane Sulfonic Acid
min	Minute
mL	Millilitre
mM	Millimolar
MOPS	Morpholino Propane Sulfonic Acid
mRNA	***m**essenger **r**ibo**n**ucleic **a**cid*
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromid
m	murine

MW	molecular weight
NF-κB	**N**uclear **F**actor **κB**
ng	Nanogram
NK-Zellen	**N**atural killer cells
nM	Nanomolar
NP-40	Nonidet P-40
nt	Nukleotide
OPG	**O**steo**p**rote**g**erin
ORF	***O**pen **r**eading **f**rame*
PAGE	**P**oly**a**crylamid-**G**el **e**lektrophoresis
PARP	**p**oly-(**A**DP-**r**ibose)-**P**olymerase
PBS	***p**hosphate **b**uffered **s**aline*
PCR	Polymerase chain reaction
PE	β-Phycoerythrin
Pfu	***p**laque **f**orming **u**nít*
pH	*potentia hydrogenii*
p*I*	isoelectric point
PI	**P**ropidium**i**odid
PLAD	***p**re-**l**igand-binding **a**ssembly **d**omain*
PNGase	**P**eptid-**N**-**G**lycosid**ase**
PTPC	***p**ermeability **t**ransition **p**ore **c**omplex*
R	Receptor
RIP	***R**eceptor **i**nteracting **p**rotein*
RNA	**r**ibonucleid **a**cid
rpm	*rounds per minute*
RT	Room temperature
SA	Streptavidin
SDS	**S**odium**d**odecyl**s**ulfat
SDS-PAGE	Sodium-dodecylsulfat Polyacrylamide-Gelelektrophoresis
Serpin	Serin-Protease-Inhibitor
SF	Superfamily
SODD	***s**ilencer **o**f **d**eath **d**omain*

SP	Signalpeptide
SSC	***s**ide **s**catter*
Tab.	Table
TCR	***T** **c**ell **r**eceptor*
Tg	transgenic
TE	Tris-EDTA
TM	**T**rans**m**embrandomain
tk	**T**hymidin**k**inase
TNF	***T**umor **n**ecrosis **f**actor*
TRADD	***T**NF-**R**eceptor **a**ssociated **d**eath **d**omain*
TRAF	***T**NF-**R**eceptor **a**ssociated **f**actor*
TRAIL	***T**NF-**r**elated **A**poptosis-**i**nducing **l**igand*
TRANCE	***T**NF-**r**elated **a**ctivation-i**n**duced **c**ytokin**e***
TRID	***T**RAIL **r**eceptor without an **i**ntracellular **d**omain*
TRUNDD	*TRAIL receptor with a **trun**cated **d**eath **d**omain*
Tris	Tris(hydroxymethyl)-methylamine
TWEAK	***t**umor necrosis factor family member that **weak**ly induces apoptosis*
UTR	***un**t**r**anslated **r**egion*
UV	ultraviolet
WT	Wildtype
XEDAR	**X**-linked **e**cto**d**yspl**a**sin-A2 **r**eceptor
X-Gal	5-Brom-4-Chlor-3-indoyl-β-D-Galactopyranosid
zVAD-fmk	Benzyloxycarbonyl-Val-Ala-Asp-(O-Methyl)-Fluoromethylketone

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Original publications

Sprick, M.R., Weigand, M.A., Rieser, E., Rauch, C.T., Juo, P., Blenis, J., Krammer, P.H. and Walczak, H. (2000) FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity*, 12, 599-609.

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