ROLE FOR TYROSINE KINASE LCK IN REGULATION OF APOPTOTIC PATHWAYS

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TO MY PARENTS

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CONTENTS

ABBREVIATIONS

aa	amino acid(s)
Amp	ampicillin
AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocase
Apaf-1	apoptotic protease-activating factor-1
ATP	adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X-protein
Bcl-2	B cell leukemia-2
BH	Bcl-2 homology domain
BIR	baculovirus IAP repeat
BSA	bovine serum albumin
CAD	caspase-activated DNase
CARD	caspase recruitment domain
Caspase	cysteinyl aspartate-specific protease
CD95L	CD95 ligand
CED	cell death-defective
C-terminal	carboxy terminal
DED	death effector domain
DD	death domain
DISC	death-inducing signalling complex
DMSO	dimethylsulfoxide
DN	double-negative

DNA	deoxyribonucleic acid
DR	death receptor
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemoluminescence
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FADD	Fas associated protein with death domain
FCS	fetal calf serum
GAS	gamma interferon-activated site
GSH	glutathione
GST	glutathione-S-transferase
HEPES	N-(2-hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
HIV	human immunodeficiency virus
IAP	inhibitor of apoptosis protein
ICE	interleukin-1ß converting enzyme
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IPTG	Isopropylthiogalactoside
IRF	interferon-regulated factor
ISRE	interferon-stimulated response element

JNK	Jun-N-terminal kinase
kD	kilodalton
MAPK	mitogen-activated kinase
MHC	major histocompatibiltiy complex
MPT	mitochondrial permeability transition
mRNA	messenger RNA
MW	molecular weight
NFAT	nuclear factor of activated T-cells
NF-□B	nuclear factor-kappaB
NGF	nerve growth factor
NP-40	Nonidet P-40
N-terminal	amino terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose)polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol myristylacetate
PMSF	phenylmethylsulfonylfluoride
PNK	polynucleotide kinase
PS	phosphatidylserine
RNA	ribonucleic acid

rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SH	Src homology
SMAC	second mitochondria-derived activator of caspase
STAT	signal transducer and activator of transcription
STE	sodium chloride / Tris / EDTA
Taq	*Thermus aquaticus*
TBE	Tris / boric acid / EDTA
TCR	T-cell receptor
TE	Tris / EDTA
TNF□	tumour necrosis factor □
TRAIL	TNF-related apoptosis inducing ligand
TNF-R1	TNF receptor-1
UV	ultraviolet light
VDAC	voltage-dependent anion channel
XIAP	X-chromosome linked IAP

1. INTRODUCTION

Cells are continuously exposed to a variety of environmental stresses and they are always required to integrate external stress signals in order to decide about “to live or to die”. A wide range of signalling pathways that are controlled by kinases make these cell fate decisions. Developmental programs and environmental stress trigger distinct and evolutionarily conserved kinases that relay signals mediating proliferation, survival and death (Caffrey et al., 1999; Widmann et al., 1999). One such kinase is Lck, which belongs to the Src family of kinases and is predominantly present in T-cells. It plays a pivotal role in T-cell development and signalling. Engagement of the T-cell antigen receptor (Alarcon et al., 2003) triggers signal transduction pathways that regulate the activation and differentiation of T-lymphocytes. Many of the individual components of this pathway are physically separated in resting cells, and upon receptor engagement, they assemble into functional complexes. Lck plays a very crucial function in this process. Lck has been extensively studied for its role in signal transduction in the context of T-cell activation, but it has been far less studied in the context of apoptosis. In this work, a potential role for Lck in apoptosis has been explored. Thus, the first introductory part of this thesis deals with apoptosis and introduces the major pathways involved in apoptosis and the underlying molecular mechanisms. Since Lck is a major mediator of T-cell signalling, the latter part of the introduction surveys T-cell receptor signalling and the kinases implicated in this process.

1.1 Programmed cell death: necrosis and apoptosis

For a long time, necrotic cell death was considered as a non-physiological alternative to programmed cell death, also termed apoptosis. The major difference between necrosis and apoptosis is that the occurrence of necrosis in response to acute lesions results in the leakage of cell contents and inflammation (Alison and Sarraf, 1995). In addition, necrosis has distinct morphological features and is accompanied by a rapid permeabilization of the plasma membrane (Figure-1). However, recent data show that, in contrast to necrosis caused by very extreme conditions, there are many examples, where this type of cell death might be a

normal physiological and programmed event (Proskuryakov et al., 2003). Various stimuli (e.g. cytokines, ischemia, heat, irradiation and pathogens) can cause apoptosis and necrosis in the same cell population. Furthermore, pathways such as death receptor and kinase cascades participate in both processes. By modulating these pathways it is possible to switch between necrosis and apoptosis (Proskuryakov et al., 2003). Therefore, it is proposed that necrosis, along with apoptosis, is an execution form of programmed cell death. More in particular, mitochondrial permeability transition (MPT) is involved in both apoptosis and necrosis. It has been demonstrated that inhibition of MPT by cyclosporin A protects cells against a variety of harmful conditions such as oxidative stress, ischemia or the exposure to TNF, CD95 (Fas) ligand and toxic chemicals (Lemasters et al., 2002). Apart from MPT, the execution of either necrosis or apoptosis depends on intracellular ATP levels. The maintenance of ATP is required for apoptosis, however, when intracellular ATP is depleted, cells mostly undergo necrotic cell death.

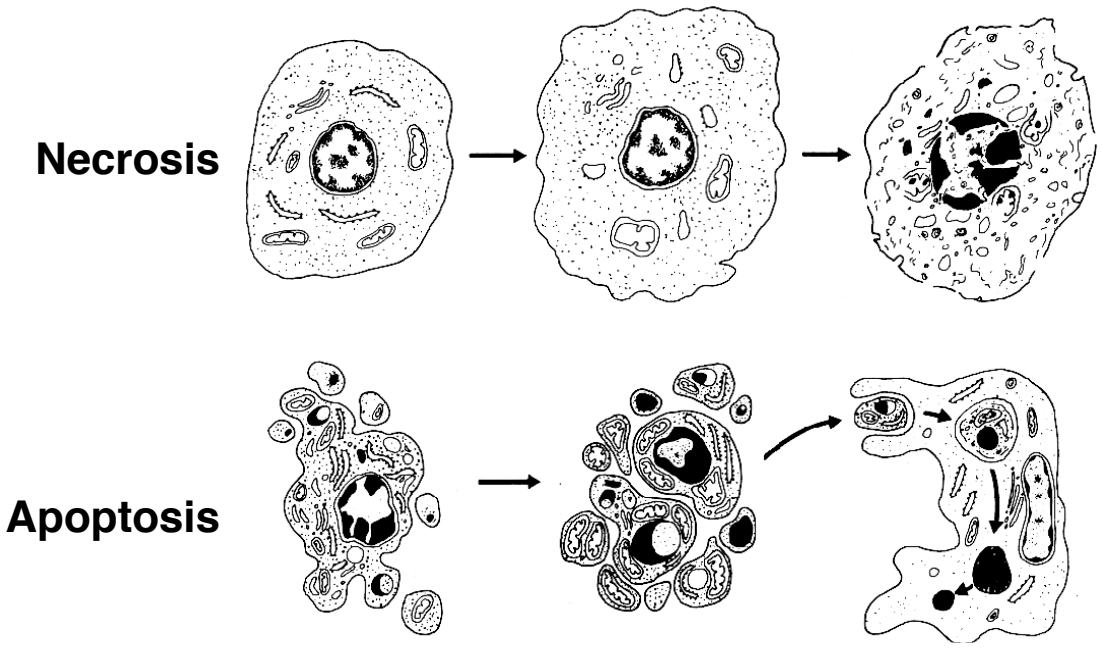

Figure 1: Comparison of apoptosis and necrosis. In apoptosis, cells show the typical morphological alterations including membrane blebbing, cell shrinkage as well as nuclear and cytoplasmic condensation. In contrast, necrosis leads to swelling of cells and mitochondria, the rupture of the plasma membrane, and the leakage of intracellular contents. Adapted from Kerr, 1972.

1.2 Morphological and biochemical changes during apoptosis

Apoptosis is a morphologically and biochemically distinct form of eukaryotic cell death that occurs under a variety of physiological and pathological conditions. The phenomenon of physiological cell death has been discovered independently several times over the past 150 years. The impact of apoptosis was widely recognised, not only after the discovery of a genetically controlled cell death in multi-cellular organisms such as *Caenorhabditis elegans* (Ellis and Horvitz, 1986), but also after the demonstration that abnormalities in cell death regulation can cause various diseases, such as cancer (Strasser et al, 1990; McDonnel and Korsmeyer, 1991), autoimmunity (Strasser et al., 1991; Watanabe-Fukunaga et al., 1992) and degenerative disorders (Barr and Tomei, 1994).

Kerr and colleagues introduced the term apoptosis in 1972 (Kerr et al., 1972). It is a mechanism that allows cells to commit suicide upon stimulation by an appropriate trigger. Kerr and colleagues published a detailed description of morphological changes associated with this form of cell death, which was initially also called programmed cell death (Kerr et al., 1972). This programme is activated for various reasons, e.g. when the cells are no longer needed within the body or when they become a threat to the health of the organism. Apoptosis is a normal process that occurs during embryonic development as well as in the maintenance of tissue homeostasis (Vaux et al., 1988). Cell death is important for the formation of structures such as fingers and toes, as the cells of the interdigital space are removed by apoptosis. Apoptosis also deletes structures that are no longer needed, for example during remodelling of the tadpole to form an adult by deleting its tail (Shi and Ishizuya-Oka.,1996). Apart from being involved in developmental processes, aberrant apoptosis can contribute to many diseases.

Definitive evidence for existence of a designed death programme came from the genetic studies of cell death in *C. elegans* (Ellis and Horvitz, 1986). During the development of the worm, from 1090 cells exactly 131 cells are destined to die by apoptosis. A set of genes, termed CED for cell death defective, regulates this cell death (Figure-2). One of these is CED-3, which has a protease activity and is absolutely required for apoptosis. CED-3 is activated by CED-4. In healthy cells, CED-4 is maintained in a functionally inactive state by association with CED-9.

The genes responsible for apoptosis in *C. elegans* have homologous genes with the same function in mammals. CED-9 is a structural and functional homologue of Bcl-2, which is a major anti-apoptotic protein in vertebrates. CED-4 is highly related to an apoptosis regulator in mammals, Apaf-1, which stands for apoptotic protease activating factor-1 (Zou et al., 1997). CED-3 is homologous to a family of mammalian proteases. The first identified member of this family was interleukin-1□-converting enzyme (ICE), which is now called caspase-1 (Alnemri, 1997). The function of the mammalian genes will be discussed in detail later.

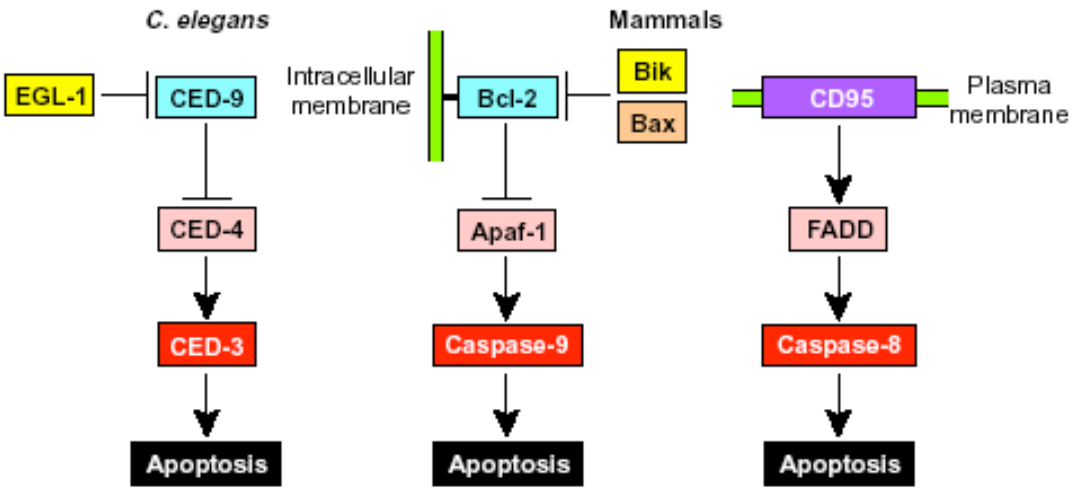

Figure 2: **Pathways to cell death in C. elegans and mammals.** The CED-9/Bcl-2 family integrates positive and negative signals and arbitrates whether apoptosis should occur. Activation of CED-4/Apaf-1 commits to apoptosis, and CED-3/caspases mediate the death process. In mammalian cells, the Bcl-2 family rules on signals from diverse cytotoxic stimuli. In contrast, signals induced by engagement of the "death receptor" CD95 proceed primarily through the adaptor FADD, which directly activates caspase-8 and largely bypasses members of the Bcl-2 family (Adams et al., 1998).

The aforementioned key regulators of apoptosis largely control the typical morphological alterations associated with apoptosis. Electron microscopic analysis has identified the classical morphological changes that occur during apoptosis, including chromatin condensation, cytoplasmic shrinkage and plasma membrane blebbing (Kerr et al., 1972; Wyllie et al., 1980). These studies have also noted that, during the early stages of apoptosis, no visible change occurs in mitochondria, endoplasmic reticulum, or Golgi apparatus. However, others

reported swelling of the outer mitochondrial membrane, which might be involved in the release of several proapoptotic factors from the mitochondrial intermembrane space (Vander Heiden et al., 1997). These include cytochrome c (Kluck et al., 1997) (Yang et al., 1997), Smac/Diablo, Omi/HtrA2, endonuclease G and apoptosis-inducing factor (AIF), a flavoprotein with oxidoreductase activity (Susin et al, 1999; Wang, 2001).

The biochemical changes induced during apoptosis include internucleosomal DNA cleavage (Wyllie, 1980) and externalisation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (Fadok and Henson, 1998). Techniques based on detecting some of these changes, such as staining of cell surface-exposed PS with annexin V (Koopman et al.,, 1994) and detection of cells with apparent subdiploid DNA content by staining with DNA-intercalating dyes (Nicoletti et al., 1991), are now standard tools for assessing apoptosis. These morphological and molecular changes are elicited by a broad range of physiological or experimentally applied death stimuli and are observed in cells from diverse tissue types and species. This is consistent with the idea that independent signalling pathways converge on common death effector machinery that is evolutionarily conserved (Hengartner and horvitz, 1994; Vaux and Strasser, 1996).

1.3 Caspases: the major component of the apoptotic machinery

The molecular circuitry of apoptosis began to emerge at an illuminating moment, when developmental genetics and mammalian biochemistry converged to reveal that cell death is launched by the activation of a class of cysteine proteases that are now called caspases (cysteinyl aspartate-specific proteases). Caspases comprise a family of intracellular proteases related to interleukin-1□-converting enzyme (ICE) and represent the effector arm of the cell death pathway (Shi, 2002). To date, 14 distinct mammalian caspases have been identified. Except for caspase-11, -13 (bovine), and -14 (mouse), all other caspases are present in human cells (Los et al., 1999; Strasser et al., 2000). Caspase-1 and -11 were shown to be mainly involved in the processing of proinflammatory cytokines (Kuida

et al., 1995; Li et al., 1995), whereas caspase-2, -3, -6, -7, -8, -9, -10 and -12 are involved in the initiation and execution of apoptosis (Figure-3).

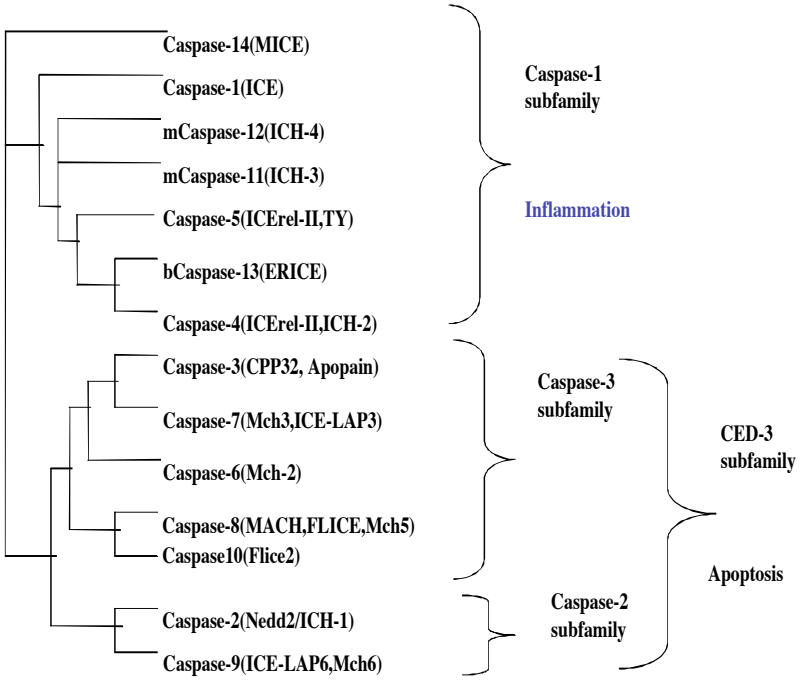

Figure 3. Cysteinyl aspartate-specific proteases (Caspases). The phylogenetic relationship of mammalian caspases appears to correlate with their function in either inflammation or cell death. The name in brackets refers to the old original names of the caspases. Abbreviations: MICE, mini ICE; ICH, ICE/CED homologue; TY, transcript Y; TX, transcript X; ICErel, ICE-related protein; CPP32, cysteine protease protein of molecular mass 32kDa; ICE-LAP, ICE-like apoptotic protease; CPP32/Mch homologue; FLICE, FADD-like ICE; mCaspase, mouse caspase; Mch, mammalian CED homologue; NEDD, neuronally expressed developmentally down-regulated protein; ERICE, evolutionary related ICE. Adapted from Shi et al. 2002.

Caspases are synthesized as inactive zymogens (procaspases) and must undergo proteolytic processing to their active forms at critical aspartate residues (Shi, 2002). An N-terminal prodomain is followed by sequences encoding a large and a small subunit. All cleavages that are involved in the activation of procaspases occur at the carboxyl side of aspartate residues (Earnshaw et al., 1999). The only known eukaryotic proteases with this specificity are the caspases themselves and the cytotoxic T lymphocyte serine protease, granzyme B (Andrade et al., 1998; Greenberg, 1996). Caspase prodomains range in length from 23 amino acids (aa)

for caspase-6 and -7 to 219 aa for caspase-10. Caspases with large prodomains are thought to be involved in the initiation of the apoptotic response (initiator caspases), and those harbouring a short prodomain are apparently activated by the initiator caspases. These are called effector caspases or executioner caspases, as they are involved in the cleavage of various protein substrates in the execution phase of apoptosis.

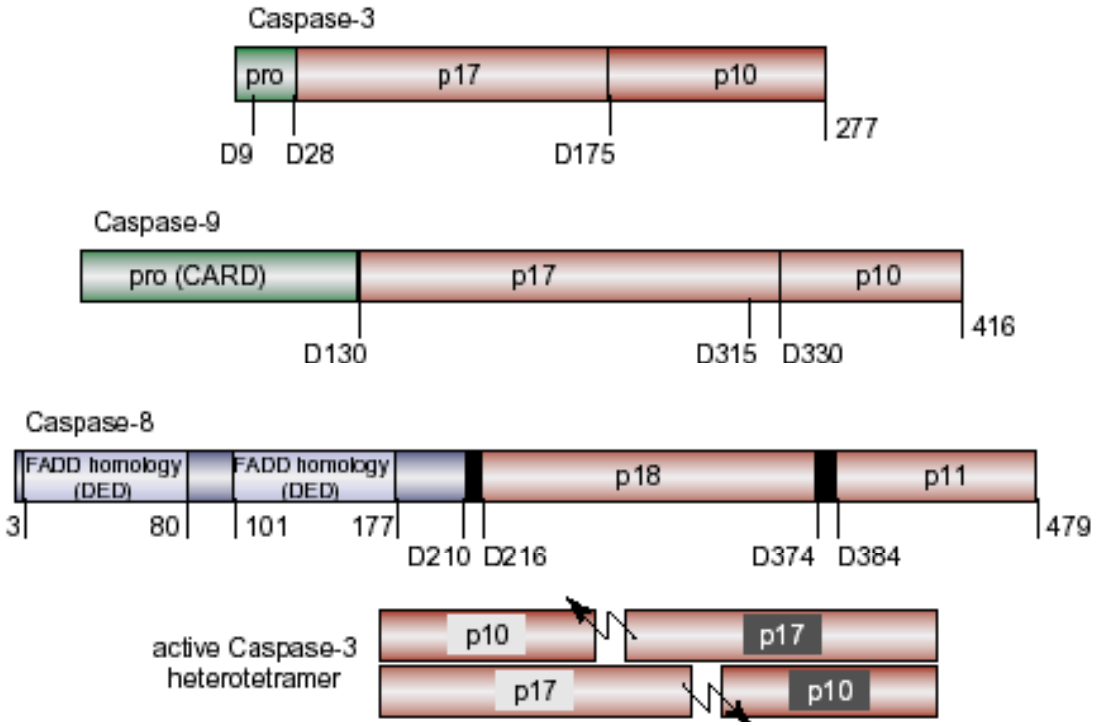

Figure 4: Caspase structure. Caspases are composed of an N-terminal prodomain, followed by a large and small subunit. Effector capases such as caspase-3 have a short prodomain. In contrast, initiator caspases, such as caspase-8 and -9 harbour a long prodomain that contains protein interaction motifs such as the CARD and DED. Following autoproteolytic cleavage caspases mature to the active protease, which consists of a heterotetrameric complex with two large and two small subunits. Each heterotetramer contains two catalytic sites as indicated by a flash.

On the basis of sequence alignments and functional studies two related motifs in the N-terminal region of the initiator caspases have been discovered: the death effector domain (DED) (Chinnaiyan et al., 1995) and a caspase recruitment domain (CARD) (Hofmann et al., 1997). The DED in caspase-8 and -10 is

necessary for the interaction of caspases with the adapter protein FADD (Fas associated protein with death domain) (Boldin et al., 1995; Chinnaiyan et al., 1995) and TRADD (tumour necrosis factor associated protein with death domain) in receptor mediated apoptosis (Hsu et al., 1995). The CARD domain is found in caspase-1, -2, -4 and -9. CARD was also shown to be involved in promoting interactions of these caspases (Figure-4).

Recent studies revealed that caspases have a distinct intracellular distribution, which sometimes changes after activation. For instance, it was reported that caspase-2 is distributed in the cytosol, microsomes and mitochondria (Zhivotovsky et al., 1999). Procaspase-3 and -9 are located in the cytosol and mitochondria, whereas procaspase-7 and -8 were detected exclusively in the cytosol. Upon induction of apoptosis the caspase distribution pattern can change. Caspase-2 was found only in the nuclei and caspase-3 was detected in cytosolic, mitochondrial and nuclear fractions. Caspase-7 was further found in the microsomal fraction, whereas caspase-8 was found in cytosol and to a smaller extent in mitochondrial, microsomal and nuclear fractions (Zhivotovsky et al., 1999).

1.4 Ways to die: extrinsic and intrinsic pathways

Two canonical pathways of caspase activation have been described, the extrinsic and the intrinsic pathway, which are either regulated by specialized cell surface receptors or by mitochondria, respectively (Green, 2000). In both pathways, high-molecular protein complexes are formed that first lead to the activation of initiator caspases. Upon activation of these tightly regulated initiator caspases, effector caspases are proteolytically activated, resulting in the cleavage of selected protein substrates and subsequent cell death.

1.4.1 The extrinsic pathway: death receptors

The interaction of cell surface receptors with their cognate ligands provides a fine-tuned mechanism to regulate life and death. Several members of the TNF receptor superfamily are capable of transmitting cytotoxic signals into the cytoplasm,

although many of these receptors display a wide range of functions unrelated to apoptosis.

Members of the TNF receptor superfamily form a group of more than twenty proteins with a wide range of different functions, such as regulation of cell survival, differentiation, immune regulation and control of gene expression. Their common structural features are cysteine-rich domains, which occur in two to six repeats in their extracellular region. Among the TNF receptor-related molecules, a novel subgroup has been defined which are termed the death receptors, as one of their most prominent functions is to induce programmed cell death (Schulze-Osthoff et al.,1998). Death receptors are structurally characterized by a similar intracellular region of about 80 amino acids, designated the death domain (DD), because it is required for the transmission of the cytotoxic signal. Homology searches in expressed sequence tag databases led to the identification of a number of DD-containing receptors. Currently, six different death receptors (DRs) are known, including CD95 (Fas, APO-1), TNF receptor-1, TNF-receptor-related-apoptosis-mediated-protein (TRAMP), two receptors which bind TNF-related apoptosis-inducing ligand (TRAIL), called TRAIL-R1 and TRAIL-R2 or DR4/5 (Figure-5) and death receptor-6 (DR6).

A major progress in the understanding of death receptor signalling was the definition of protein motifs that propagate the cytotoxic signals elicited upon receptor ligation. One proximal motif is the so-called death domain (DD), which is crucial for triggering cell death. Two other important motifs include the DED and CARD that are also found in caspases. The DD of death receptors is required for formation of a receptor complex by recruiting adapter proteins that also contain a DD motif. As revealed by NMR spectroscopy, the DD consists of a series of alpha-helices with many exposed charged amino acids that mediate self-association of the DD following ligand binding and receptor oligomerization (Huang et al., 1996).

Important DD proteins involved in death receptor signalling include the adaptor proteins FADD (MORT-1), RIP and TRADD. FADD has a high affinity to CD95, whereas it weakly binds to the DD in TNF-R (Boldin et al., 1995; Chinnaiyan et al., 1995). TNF-R1, in contrast, binds preferentially to TRADD, which by itself can then recruit FADD to the vicinity of TNF-R1. TRADD, in addition, recruits the serine

protein kinase RIP to TNF-R1 (Hsu et al., 1996). Although RIP was initially suggested to play an important role in apoptotic signalling, it is more crucial for TNF-mediated NF-□B activation. In a mutant cell line deficient in RIP, CD95 was not affected, whereas TNF-R1-mediated NF-□B activation was blocked (Ting et al., 1996). Overexpression of most DD proteins causes cell death indicating that these molecules are involved in apoptosis . In the case of FADD, transient expression of the N-terminal region was sufficient to cause apoptosis (Chinnaiyan et al., 1995). This part of FADD was therefore termed the death effector domain (DED). In contrast, overexpression of the C-terminal DD-containing part lacking the DED protected cells from CD95-mediated apoptosis and functioned as a dominant-negative mutant.

While most information regarding death pathways has been obtained from two-hybrid assays, for CD95 the signalling complexes have been also identified *in vivo*. Treatment of cells with agonistic anti-CD95 antibodies and subsequent co-immunoprecipitation led to the identification of receptor-associated proteins on two-dimensional gels. Together with the receptor these proteins form the death-inducing signalling complex (DISC) (Kischkel et al., 1995). Two spots were identified as differentially phosphorylated forms of FADD. Sequencing of the other proteins resulted in the identification of a molecule, which contained two DEDs at its N-terminus that associate with the DED of FADD (Muzio et al., 1996). At its C-terminus it had the typical catalytic domain of an ICE (interleukin-1ß converting enzyme)-like protease and was therefore termed FLICE (FADD-like ICE). It belongs to the cysteine proteases of the caspase family and is now referred to as caspase-8 (Alnemri et al., 1996). It is currently thought that the increased local concentration of procaspase-8 in the DISC likely leads to cross-cleavage. The resulting active caspase-8 then directly activates effector caspases such as caspase-3, -6 and -7. Identification of caspase-8 as part of the DISC therefore connected two different levels in apoptosis pathways, the receptor with the executioner caspases.

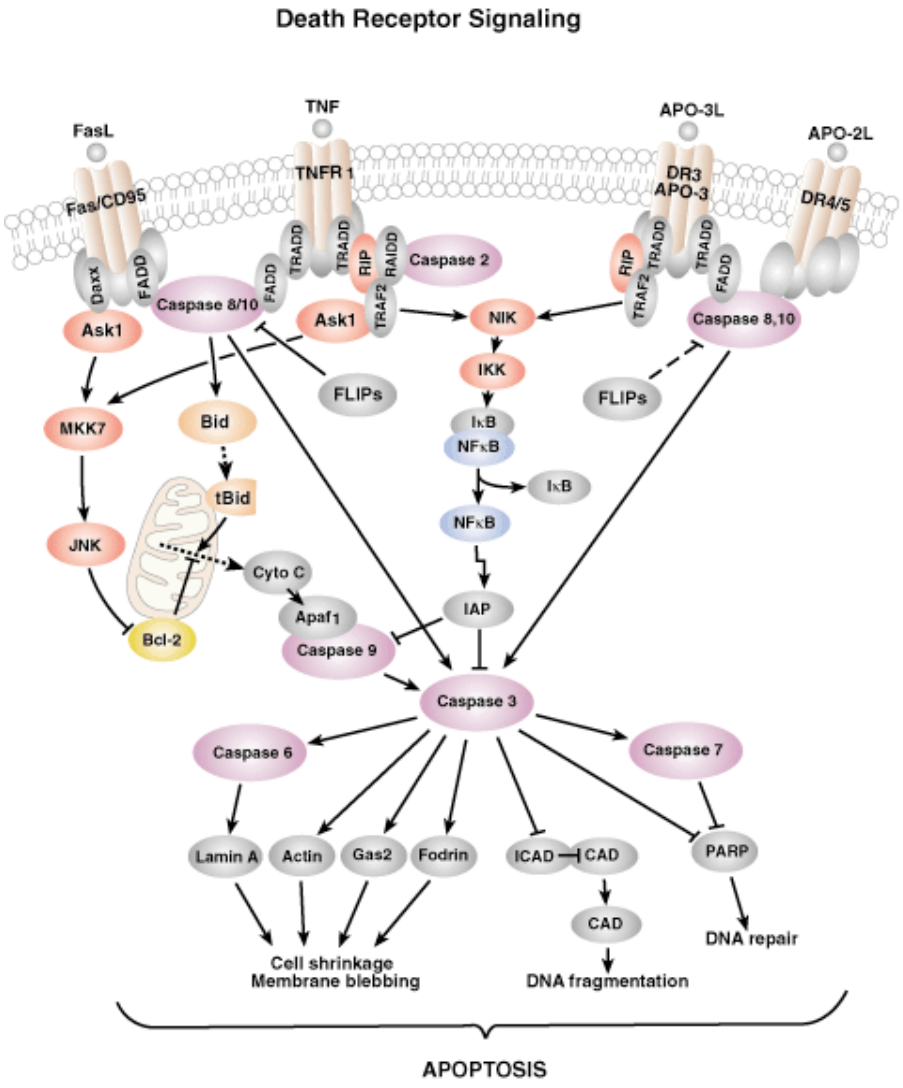

Figure 5: Receptor-mediated apoptosis. Death receptor ligands initiate signalling via receptor oligomerization, recruitment of specialized adaptor proteins and activation of caspase cascades. CD95L (Fas ligand) binding induces CD95 trimerization and recruits initiator caspase-8 via the adapter protein FADD. Caspase-8 then oligomerizes and is activated via autocatalysis. Activated caspase-8 stimulates apoptosis via two parallel cascades: it directly cleaves and activates caspase-3, and it cleaves Bid (a Bcl-2 family protein). Truncated Bid (tBid) translocates to mitochondria, inducing cytochrome c release, which sequentially activates caspases-9 and -3. TNF and Apo-3L can deliver pro- or anti-apoptotic signals. TNFR1 and DR-3 promote apoptosis via the adaptor proteins TRADD/FADD and the activation of caspase-8 (Baker and Reddy, 1998; Budihardjo et al., 1999).

1.4.2 The intrinsic pathway: mitochondria

The intrinsic or the mitochondrial apoptotic pathway integrates stress-induced signals, induced for instance by DNA damage, radiation or growth factor withdrawal. The essentials of this apoptotic pathway reside in the intermembrane space of mitochondria and are triggered by the translocation of pro-apoptotic Bcl-2 family members to mitochondria. It is thought that proapoptotic Bcl-2 proteins induce the formation of a pore in the outer mitochondrial membrane, which results in the release of various proapoptotic factors. In addition, the opening of the mitochondrial permeability transition (MPT) pore is suggested to be essential for the release of apoptogenic proteins from the mitochondrial intermembrane space (Vander Heiden et al., 2000). The adenine nucleotide translocase (ANT), the voltage dependent anion channel (VDAC) and cyclophilin D are the basic units of this pore complex that is located between the inner and outer mitochondrial membrane (Crompton, 1999). Pore opening can lead to matrix swelling, outer membrane rupture and the release of certain mitochondrial proteins.

An early event in the intrinsic apoptotic pathway is the translocation of cytochrome c from mitochondria into the cytosol, which is inhibited by anti-apoptotic Bcl-2 proteins (Figure-6). In the cytosol, cytochrome c interacts with Apaf-1 (apoptotic protease-activating factor-1), the mammalian homologue of the *C. elegans* cell death regulator CED-4. A second cofactor required for Apaf-1 function is dATP/ATP. Binding of these two components presumably leads to a conformational change in Apaf-1 and exposes the caspase recruitment domain (CARD). In particular, caspase-9 is recruited and activated by Apaf-1 (Li et al., 1997). The mitochondrial release of cytochrome c results in the oligomerization of Apaf-1 and the subsequent binding of caspase-9 into a high-molecular weight complex. This complex, called the apoptosome, supports the catalytic activation of caspase-9, which further cleaves and activates effector caspases, thereby resulting in the subsequent degradation of cellular death substrates.

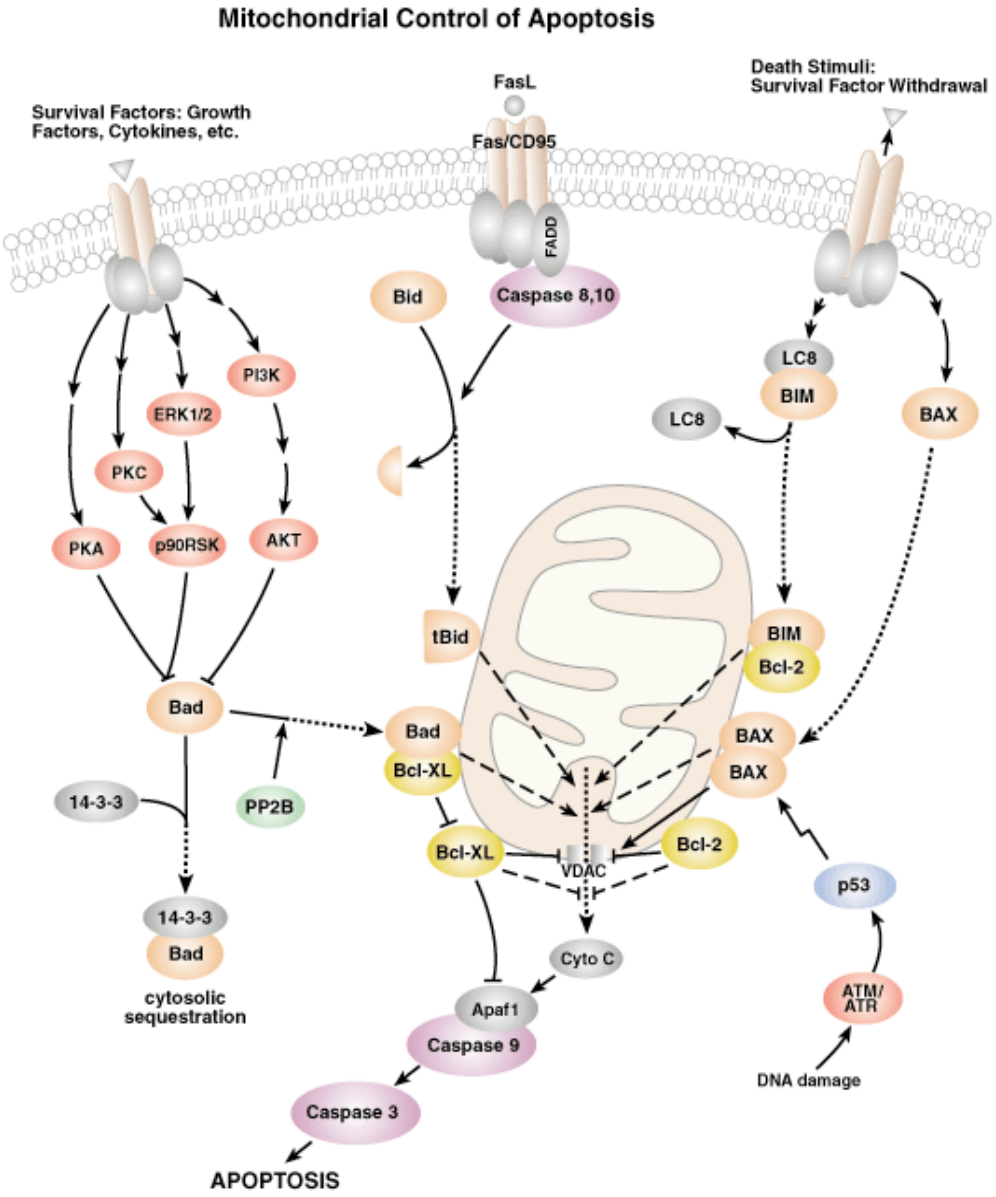

Figure 6: Mitochondrial control of apoptosis. The Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability transition and the release of cytochrome c. The anti-apoptotic proteins Bcl-2 and Bcl-x_L reside in the outer mitochondrial membrane and inhibit cytochrome c release. The pro-apoptotic Bcl-2 proteins Bad, Bid, Bax and Bim reside in the cytosol but translocate to mitochondria following death signalling where they promote the release of cytochrome c. Bad translocates to mitochondria and forms a pro-apoptotic complex with Bcl-x_L. Cytosolic Bid is cleaved by caspase-8 into tBid, which translocates to mitochondria. Following DNA damage, activated p53 induces the transcription of Bax. Released cytochrome c binds Apaf-1 and forms an activation complex with caspase-9. Bcl-x_L, Bcl-2 and Bax apparently influence the voltage-dependent anion channel (VDAC), which can control cytochrome c release (Baker and Reddy, 1998; Budihardjo et al., 1999).

In addition to cytochrome c, several other mitochondrial proteins with proapoptotic activity can also be released into the cytosol. These include apoptosis-inducing factor (AIF), endonuclease G, and Smac (second mitochondria-derived activator of caspases) which is also called DIABLO (direct IAP binding protein with low PI) (Jesenberger and Jentsch, 2002). AIF and endonuclease G probably function by exerting effects in the nucleus, whereas Smac/DIABLO sequesters caspase inhibitors away from the active caspases.

It is important to note that the mitochondrial and death receptor pathways of apoptosis can also interfere with each other. In some cell types, death receptor-mediated caspase-8 activation is insufficient to induce cell death, but requires the amplification by the mitochondrial pathway. In this context, caspase-8 can induce the cleavage of the Bcl-2 family member Bid. Upon cleavage by caspase-8, the truncated Bid translocates to the mitochondria where it induces the release of cytochrome c.

1.5 The Bcl-2 family: key regulators of apoptosis

Proteins of the Bcl-2 family are the core components of the intrinsic mitochondrial apoptotic pathway. All members of the Bcl-2 family contain one to four conserved sequence motifs, called the Bcl-2 homology (BH1-4) domains (Figure-7). Members of this family can be subdivided into pro- and anti-apoptotic proteins. Although the precise mechanisms, by which they regulate apoptosis, are still unclear, the stochiometric balance of pro- and antiapoptotic proteins of this family seems to be crucial (Oltvai and Korsmeyer, 1994). High concentrations of the antiapoptotic proteins can inhibit the mitochondrial apoptotic pathway. Besides the prototype member, Bcl-2, other antiapoptotic proteins include Bcl-x_L (Boise et al., 1993), Bcl-w (Gibson et al., 1996), A1/Bfl-1 (Choi et al., 1995; Lin et al., 1996), Mcl-1 (Kozopas et al., 1993) and Boo/Diva (Inohara et al., 1998; Song et al., 1999).

As tumor radiotherapy as well as most chemotherapeutic agents use this pathway to kill cancer cells, overexpression of antiapoptotic Bcl-2 family members confers resistance to treatment. Inhibition of apoptosis by overexpressed Bcl-2 leads to accumulation of otherwise dying cells and promotes thereby tumor formation.

Elevated Bcl-2 levels are frequently found in B-cell malignancies and are often caused by the translocation t(14;18)(q32;q21), which brings the bcl-2 gene under the transcriptional control of the highly active immunoglobulin heavy chain promoter. Similarly, transgenic mice overexpressing Bcl-2 display a phenotype that is associated with the accumulation of mature B-cells. Bcl-2 overexpression is not limited only to malignancies of B-lymphocytes, but is also frequently found in very different types of human cancers of both haematological and non-haematological origin. In these diseases, Bcl-2 overexpression mostly correlates with poor prognosis.

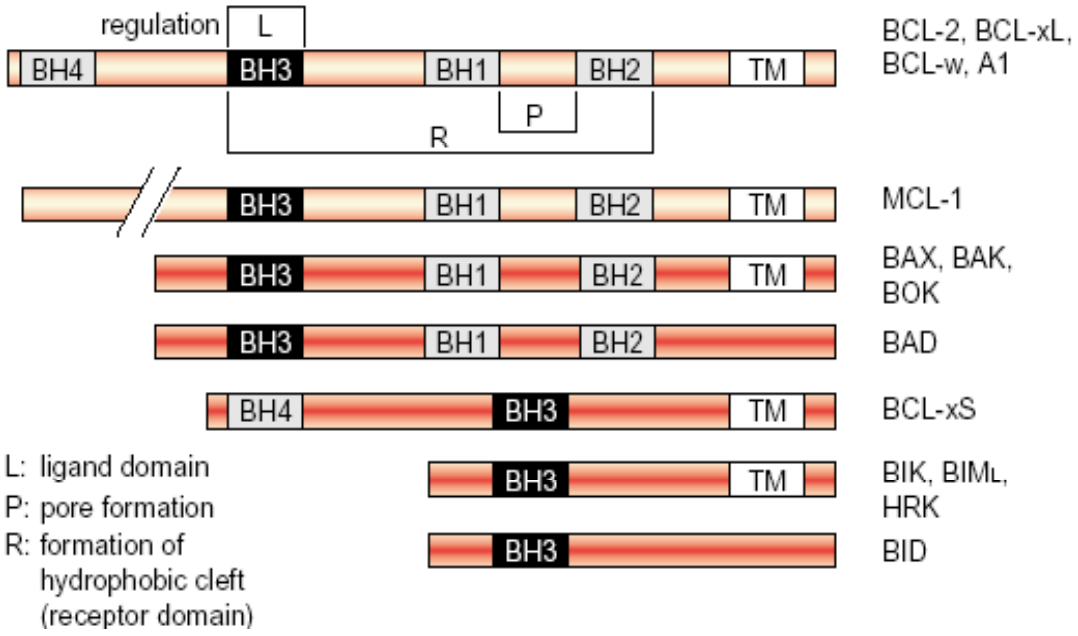

Figure 7: Structural comparison of members of the Bcl-2 protein family. The Bcl-2 family can be divided into functional subgroups comprising anti- and pro-apoptotic proteins. The anti-apoptotic group includes Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1, which contain four BH domains. Pro-apoptotic Bcl-2 proteins include the Bax- and the BH3-only subfamilies. The Bax family is comprised of Bax, Bak and Bok, which have sequence similarity to the anti-apoptotic Bcl-2 members. The other pro-apoptotic members, including for instance Bid, Bim and Bik, have one single BH3 domain. BH-stands for Bcl-2 homology domain and TM stands for transmembrane domain.

Among the proapoptotic members of the Bcl-2 family two groups of molecules are distinguished. A first group comprises the Bax family of multidomain proapoptotic Bcl-2 proteins with two to three BH domains, such as Bax (Oltvai et al., 1993), Bcl-

xs (splice variant of Bcl-x) (Boise et al., 1993), and Bak (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995). The second subfamily comprises the so-called BH3-only proteins, which contain only a single BH3 domain (Puthalakath and Strasser, 2002). Members of this subfamily include Bad (Yang et al., 1995), Bik/Nbk (Boyd et al., 1995; Han et al., 1996), Bid (Wang et al., 1996), Hrk/DP5 (Inohara et al., 1997), Bim/Bod (Hsu et al., 1998; O'Connor et al., 1998) and Blk (Hegde et al., 1998).

Some BH3-only proteins selectively interact with antiapoptotic Bcl-2 family members, whereas others also interact with proapoptotic members. BH3-only proteins have been proposed to be allosteric regulators of the Bcl-2 proteins and serve as sensors and effectors of apoptotic signalling. The current thought is that BH3-only proteins require at least one Bax-type partner to induce cell death. Both Bax and Bak undergo a conformational change in response to apoptotic stimuli, which mediates their assembly into homomultimers with channel-forming properties in the mitochondrial membrane, resulting in cytochrome c release. This conformational change and multimerization of Bak or Bax is inducible by BH3-only proteins and inhibitable by Bcl-2.

Many Bcl-2 proteins are characterized by a conserved C-terminal transmembrane domain, which helps the proteins to localize to the outer mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum (Chen-Levy and Cleary, 1990; Krajewski et al., 1993; Krajewski et al., 1994; Lithgow et al., 1994). Several studies revealed that Bcl-2, Bcl-x_L, A1, Mcl-1, Bax and Bak are localized in the cytosolic site of the membranes (Hockenbery et al., 1993; Borner et al., 1994; Krajewski et al., 1994; Zha et al., 1996).

Comparative studies in cell lines and transgenic mice have shown that apoptotic pathways that can be inhibited by Bcl-2 can also be inhibited by Bcl-x_L, Bcl-w, A1 and Mcl-1. Similarly, the death stimuli that are insensitive to Bcl-2 are also insensitive to its homologues (Gibson et al., 1996; Lin et al., 1996; Zhou et al., 1998). It has been shown that Bcl-x_L transgene expression was able to substitute for Bcl-2 in lymphocytes of Bcl-2 deficient mice (Chao et al., 1995). The existence of several genes encoding proteins with similar functions allows the expression of anti-apoptotic regulators in tissue- and stimulus-specific manner. This idea has

been supported by the findings that the expression of several Bcl-2 proteins is inducible and regulated by extracellular signals. For instance, A1 and Mcl-1 can be stimulated by colony stimulating factors (Boise et al., 1995; Chao et al., 1998; Karsan et al., 1996) and Bcl-x_L is inducible by mitogens (Boise et al., 1995).

The activity of different pro-apoptotic family members, in particular BH3-only proteins, is controlled by distinct post-translational modifications. The activity of Bad can be regulated by Akt- or PKA-mediated phosphorylation (Zha et al., 1996; Datta et al., 1997; del Peso et al., 1997). Bid is activated through caspase-mediated proteolysis (Li et al., 1998; Luo et al., 1998). Bim is bound to the dynein light chain and sequestered to microtubuli. Upon apoptosis stimulation, Bim is released from the dynein motor complex and neutralizes anti-apoptotic Bcl-2 (Puthalakath et al., 1999). BH3-only proteins have therefore been proposed to serve as sensors and effectors of apoptotic signalling.

1.6 Inhibitors of apoptosis (IAPs)

Caspase activation is central to apoptotic pathways. Studies of the regulation of caspase activity have revealed highly complex mechanisms that control the decision between cell survival and cell death. A number of proteins that can prevent cell death through caspase inhibition have been identified. Among these, inhibitor of apoptosis proteins (IAPs) are a major control point in the execution of cell death.

IAPs comprise a family of caspase-inhibiting proteins characterized by a shared conserved sequence region, termed the baculoviral IAP repeat (BIR) domain (Salvesen and Duckett, 2002). Currently eight endogenous IAPs are known in the human system, all of which inhibit apoptosis. These include XIAP, cIAP1, cIAP2, ILP-2, ML-IAP, NIAP, survivin, and BRUCE. The main physiological roles of IAPs seem to be: (1) the establishment of a threshold under which caspases are kept at bay, and (2) providing a pool of active caspases which can rapidly execute death after release. Growing evidence also suggests the participation of IAP proteins in other cellular functions apart from inhibiting caspases, including protein

degradation, cell cycle control and signal transduction (Deveraux and Reed, 1999).

The most thoroughly characterized member of the IAP family is X-linked inhibitor of apoptosis (XIAP), which inhibits caspase-3, -7 and -9. XIAP contains three separate BIR domains. BIR2 and the linker to the N-terminal BIR1 domain are responsible for binding active caspase-3 and -7. XIAP potently inhibits these caspases by masking their substrate binding site (Huang et al., 2001; Riedl et al., 2001). In contrast, inhibition of caspase-9 solely depends on the BIR3 domain and flanking regions (Deveraux et al., 1999; Sun et al., 2000). Cleavage of caspase-9 results in the exposition of an IAP-binding motif (IBM) that fits into a hydrophobic pocket of the BIR3 domain. Homologous domains have been discovered in Smac (second mitochondria-derived activator of caspases) and the serine protease Omi/HtrA2. SMAC and Omi/HtrA2 are nuclear encoded proteins residing in mitochondria. After induction of the intrinsic apoptotic pathway both proteins are released into the cytosol. Most interestingly with respect to therapeutic intervention is the fact that the IBMs of SMAC and Omi/HtrA2, termed the „knob“, also fit into the hydrophobic groove of XIAP. Therefore, SMAC is able to replace and release caspase-9 from the XIAP-inhibitory complex.

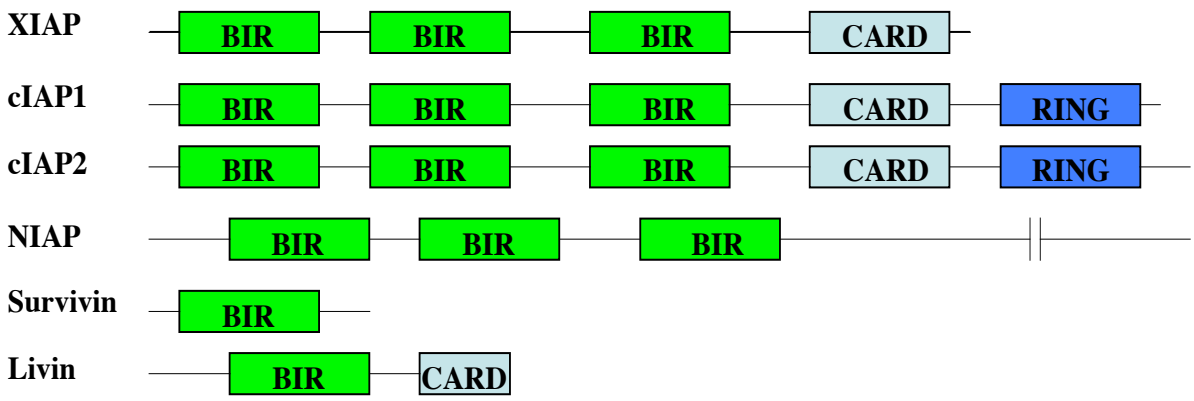

Figure 8: Structural comparison of IAPs. Inhibitor of apoptosis proteins (IAPs) of mammalian origin. IAPs are characterized based on the presence of baculovirus IAP repeat domain (BIR) that inactivates the active caspases. IAPs such as cIAP1 and cIAP2 also contain a RING domain that leads to the RING-dependent ubiquitination and proteasomal degradation of caspases. Based on Reed 2000.

Some IAP members contain, in addition to one or three BIR domains, also a RING domain associated with ubiquitin E3 ligase activity. The first evidence for a possible ubiquitin-conjugation activity in IAP came from the study of BRUCE (BIR-containing ubiquitin-conjugating enzyme). Other IAP members with ubiquitin ligase include cIAP1 and cIAP2. RING proteins can mediate the degradation of other proteins and themselves via the proteasome pathway. The RING-containing IAPs use their ubiquitination properties to subject caspase-3 and -7 to proteasomal degradation (LeBlanc, 2003). Thus, activation of the ubiquitin pathway provides another mechanism, by which members of the IAP exert antiapoptotic properties.

1.7 Immune system and apoptosis

The immune system is a society of interacting cells consisting of T- and B-lymphocytes, natural killer (NK) cells, macrophages, granulocytes and other antigen-presenting cells (APCs). The cellular components of the immune system develop from bone marrow-derived precursor cells. B-lymphocytes, NK cells and macrophages mature in bone marrow and in fetal liver, whereas T-lymphocytes mature mostly in the thymus. Apoptosis in the immune system is a fundamental process regulating lymphocyte maturation, receptor repertoire selection and homeostasis. Thus, death by apoptosis is an essential event for lymphocytes in the context of cell growth and differentiation.

1.7.1 General overview of apoptosis in a T-cell's life

The T-cell repertoire is shaped in the thymus by apoptosis and survival signals. Only 2-3% of the T-cells leave the thymus from the initially generated thymocytes. Lymphoid progenitors, which have developed from hematopoetic stem cells in the bone marrow, migrate to the thymus to complete their maturation to functional T-cells. In the thymus, T-cells develop their specific T-cell markers, including the T-cell receptor (TCR), CD3, CD4 or CD8 and CD2. When progenitor cells begin to express CD2 but have not yet rearranged the TCR genes, they are double-negative (DN) for CD4 and CD8 (Figure-9). These double-negative cells, which express the adhesion molecule CD44 and the □-chain of the IL-2 receptor (CD25),

start rearranging their chain at the stage of DN3 and DN4. Afterwards the cells undergo a period of proliferation, and begin to express both CD4 and CD8, thereby becoming double-positive (DP) T-cells. At this stage, the cells rearrange the chain of the TCR. Those T-cells that fail to rearrange their TCR genes productively cannot be stimulated by the MHC-peptide complex and die. Thymocytes that have passed pre-TCR selection mature further by positive and negative selection, which leads to the mature single positive $CD4^+$ MHC class II and $CD8^+$ MHC class I restricted cells. Single-positive cells will leave the thymus to generate the peripheral T-cell pool. Still after all these events, deletion of peripheral T-cells by apoptosis can occur, when T-cells are insufficiently stimulated by the growth signals . This step is very crucial to eliminate the auto-reactive cells, which might lead to autoimmune disorders. After successful activation, peripheral T-cells can also differentiate into memory cells, which are relatively apoptosis-resistant.

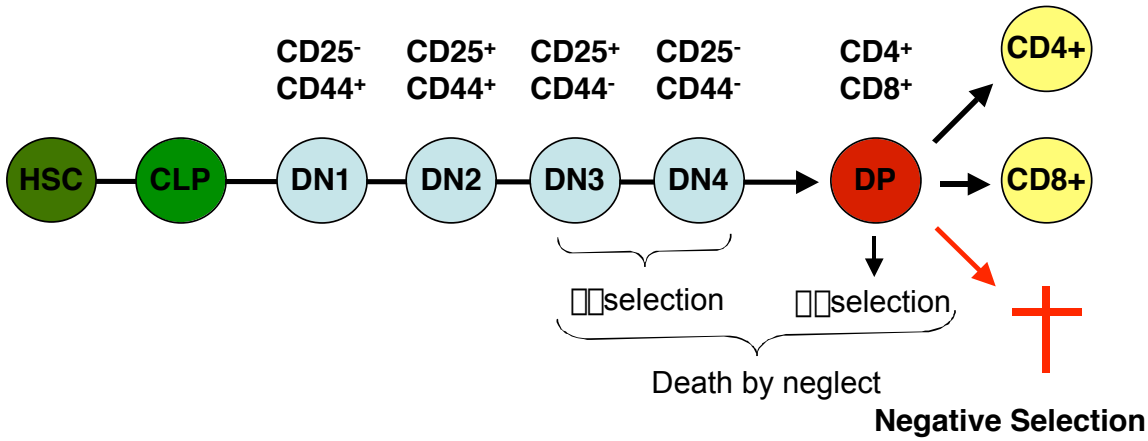

Figure 9: Schematic representation of T-cell development. Lymphoid progenitor cells are derived from hematopoetic stem cells (HSC). Pre T-lymphocytes are shaped in the thymus by rearranging the T-cell receptor (TCR) genes. Since the progenitor cells do not express CD4 and CD8 markers, they are called double-negative (DN). These DN cells undergo a period of proliferation to rearrange the TCR genes (DN1 to DN4). At the stage of DN3 and DN4, the ß-chain of the TCR is rearranged. The -chain of the TCR rearranges at the double positive (DP) stage. Defective peripheral T-lymphocytes are removed by negative selection (Starr et al., 2003).

1.7.2 The T-cell receptor activation

The TCR is composed of six different polypeptide chains (Figure-10). The antigen specificity is dictated by the clonotypic TCR □ and □ chains, which arise from the process of genetic rearrangement and results in a huge number of receptor variants. The □, □heterodimer directly binds to the peptide MHC-complex. The TCR □, □□associated CD3 chains are the signalling subunits of the TCR complex, which exist in three dimers (Tonegawa, 1983; Alarcon et al., 2003). Each of them contains immunoreceptor tyrosine-based activation motifs (ITAMs), which are rapidly phosphorylated by the Src-family kinase Lck following TCR stimulation. This event is required for initiating TCR signalling (Tonegawa, 1983; Pitcher and van Oers, 2003).

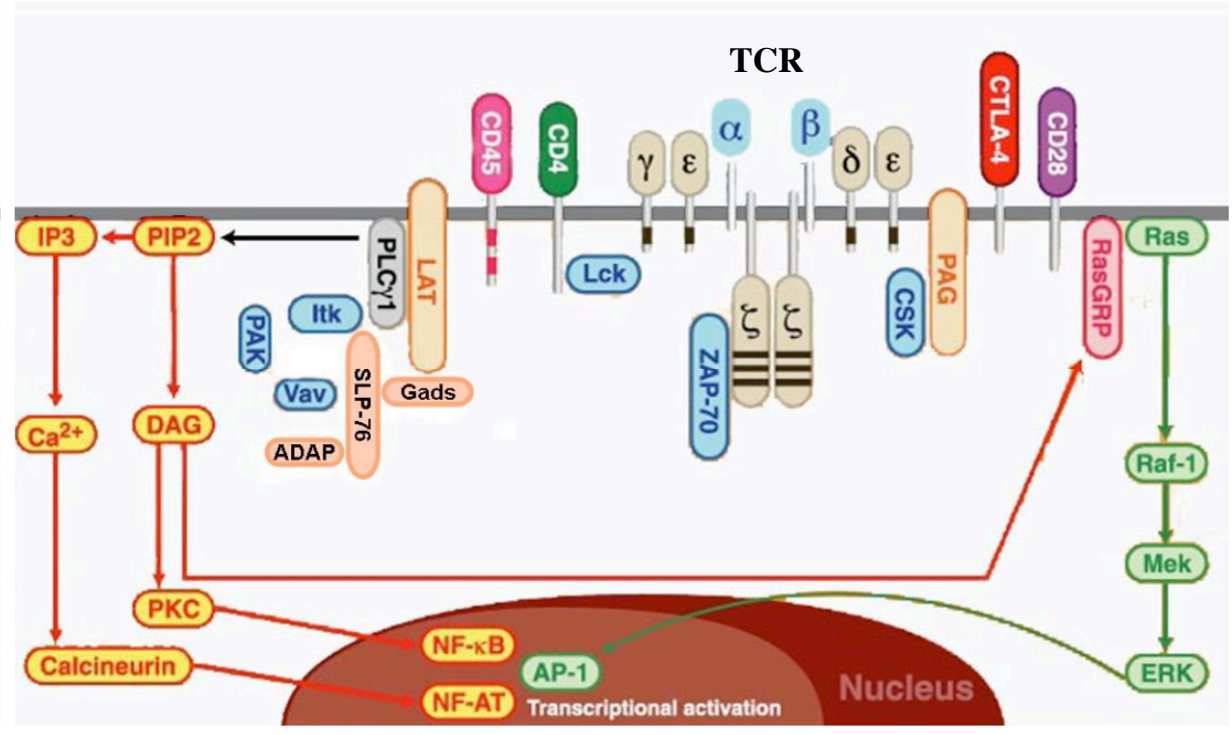

Figure 10. T-cell receptor signalling events leading to the activation of transcription factors. An overview of some of the key signalling events linking the binding of peptide-MHC to the T-cell antigen receptor (TCR/CD3) and the CD4 co-stimulatory receptor with the activation of downstream transcriptional activators. Stimulation leads to the TCR clustering and subsequent activation of Lck. Activated Lck leads to the phosphorylation of ITAMs, which creates binding sites for ZAP-70, a cytosolic tyrosine kinase that in turn activates LAT and PLC□1. As a result various signalling cascades are triggered resulting in the activation of transcription factors (Klas et al., 1993; Wange and Huang, 2004).

There are multiple mechanisms acting in concert to block unwanted spontaneous TCR signalling. The first is the physical sequestration of Lck away from the TCR by the differential partitioning of Lck and the TCR into lipid rafts (DosReis and Shevach, 1998; Cherukuri et al., 2001; Horejsi, 2003). Lck, by being myristoylated or palmitoylated, constitutively partitions to the lipid rafts, while the unstimulated TCR is largely excluded from this membrane fraction.

Following T-cell stimulation, there is an increased translocation of TCR to the lipid rafts and a sequestration of negative regulatory elements. This event results in the activation of the Src kinases Lck and Fyn. The kinases then phosphorylate specialized motifs (ITAMs) in the signal-transducing CD3 subunits of the TCR complex. Phosphorylation of ITAMs creates docking sites for another tyrosine kinase, ZAP-70. As a result of ZAP-70 recruitment, the TCR is effectively endowed with tyrosine kinase function, resulting in phosphorylation of additional proteins, including Vav-1, LAT and SLP-76 (Klas et al., 1993; Wange and Huang, 2004). The latter two substrates play a pivotal role in TCR signalling. The phosphorylated LAT and SLP-76 act as linker/adaptors and play a key role in building up a higher order multi-molecular signalling complex, also called the signalosome (Leithauser et al., 1993; Medema et al., 1997; Lindquist et al., 2003). In concert, these proteins trigger the activation of PLCγ1, and the subsequent hydrolysis of phosphatidylinositol to generate diacylglycerol (DAG) and inositoltriphosphate (IP_3), which act as second messengers to induce calcium release as well as the activation of the protein kinase C (PKC) and Ras pathways (Miller and Berg, 2002; Takesono et al., 2002). LAT and SLP-76 signalling complexes also support the activation of various mediators like the MAP kinases (p38, JNK and ERK) and G-proteins (Ras, Rac and Cdc-42). Moreover, TCR signalling induces formation of the immunological synapse, which is involved in cytoskeleton reorganization and activation of important transcription factors including NF-κB and NFAT (Tanaka et al., 1996; Lee et al., 2003).

1.8 Tyrosine kinases and Lck

As mentioned above, protein tyrosine kinases such as Lck and Fyn play an integral role in the activation of T-cells through various immunoreceptor molecules, such as the T-cell receptor (TCR)/CD3 complex. Besides serine and threonine kinases, tyrosine kinases participate in almost all signal transduction networks. Actually, the major molecular principle behind the signal transduction mechanisms is represented by specific protein associations, which are mostly regulated by phosphorylation and dephosphorylation reactions. The balance between phosphorylation and dephosphorylation normally determines the intracellular signal transduction.

Tyrosine kinases catalyse the transfer of phosphate from ATP to a tyrosine residue of a specific protein target. The major categories of tyrosine kinases are divided into two groups, receptor and nonreceptor tyrosine kinases. All receptor kinases share a similar structure: they contain a ligand-binding extracellular region, hydrophobic transmembrane domain and a cytoplasmic region. At present there are nineteen receptor kinases classified. Nonreceptor tyrosine kinases are intermediate conductors of diverse intracellular signalling pathways. Many of them are associated with transmembrane receptors, such as hormone, cytokine, growth factor receptors. Nonreceptor tyrosine kinases are generally activated by means of association of receptors with their extracellular ligands. According to their structural and functional properties, they are divided into eight classes and differ in size from 50-150 kDa (Bolen, 1993; Taniguchi, 1995).

Non-receptor protein tyrosine kinases exert crucial roles in a wide variety of cellular processes, including cell growth, differentiation, adhesion apoptosis and gene transcription. In many cases, tyrosine kinases have been implicated in oncogenesis. Protein tyrosine kinases normally function as tightly regulated switches in the signal transduction network of the cell. If these switches become stuck in the “on” position, they have the potential to induce oncogenic transformation.

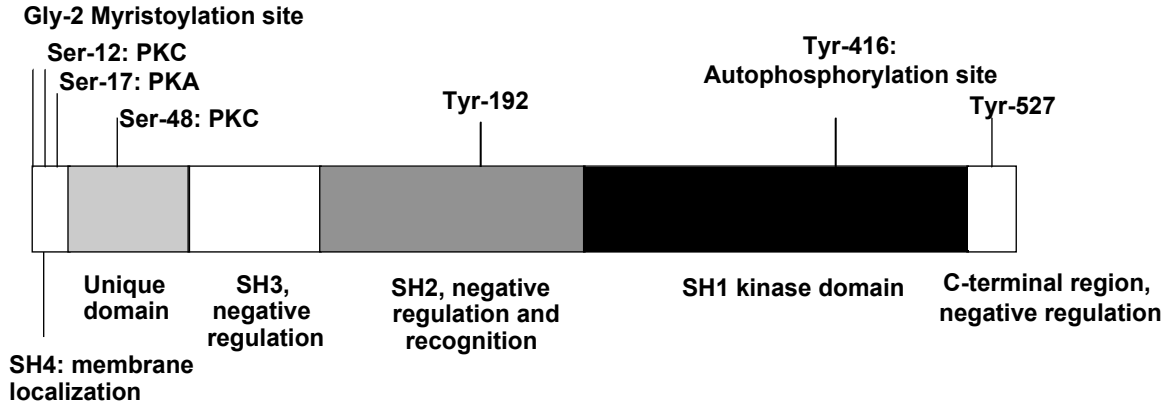

Figure 11: Domain structure of Src proteins. Proteins of the Src family comprise six distinct functional domains including the Src homology domain 4 (SH4), a unique domain, the SH3 domain and SH2 domains, a catalytic domain (SH1) and a C-terminal regulatory region. Different phosphorylation sites are indicated. Based on Bolen, 1993.

Members of the Src family are typical representatives of nonreceptor tyrosine kinases. Src, the prototype member of this family, was the first oncogene and the first tyrosine kinase to be discovered. Mutations in the *Src* gene render the kinase constitutively active and thereby induce cellular transformation. The members of the Src family are divided into two classes: tyrosine kinases with a broad tissue expression range (Fyn, Yes) and those with limited expression (Fgr, Lyn, Hck, Lck, Blk, Yrc). Src kinases range from 52-62 kDa and comprise six distinct functional domains (Figure-11), including the N-terminal Src homology domain 4 (SH4), a unique domain, the SH3 and SH2 domains, a catalytic domain (SH1) and negative regulatory region at the C-terminus.

1.9 Aim of this study: the role of Lck in apoptosis

Recent studies indicate that protein tyrosine kinases of the Src family do not only play a role in cell activation and proliferation, but might be also involved in apoptosis. Several Src kinases are activated by different forms of stress, which could eventually lead to apoptosis. For instance, the B-cell receptor-associated Src-like kinases Btk and Lyn have been implicated in regulation of apoptosis in B-

cells in response to radiation or anticancer drugs. Lyn-deficient cells also undergo a delayed apoptosis in response to growth factor deprivation. Furthermore, studies using Lck-deficient T-cell lines have suggested that Lck plays a role in transducing signals leading to apoptosis in response to ionizing irradiation, ceramide and HIV-Tat exposure (Manna and Aggarwal, 2000; Manna et al., 2000; Belka et al., 2003). The mechanism, how activation of Lck or related protein kinases contributes to apoptosis in certain situations, while inducing proliferation in others, is completely unknown. The present study therefore aimed at elucidating the role of Lck and its involvement in anticancer drug induced apoptosis.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Substance	Supplier
Acrylamide	Carl Roth GmbH, Karlsruhe, Germany
Agarose	BioRad, München, Germany
Ammoniumperoxodisulfate (APS)	Carl Roth GmbH
Ampicillin	Carl Roth GmbH
Aprotinin	Sigma, München, Germany
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Carl Roth GmbH
Coomassie Brilliant Blue R-250	Carl Roth GmbH
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Digitonin	Sigma
Ethanol	Carl Roth GmbH
Ethylendiamine tetra acetic acid (EDTA)	Carl Roth GmbH
Ethlyleneglycol-bis-(β-aminoethyl ether)N, N, N', N'-tetra acetic acid (EGTA)	Carl Roth GmbH
Fetal calf serum	Life Technologies GmbH
Formaldehyde	Carl Roth GmbH
Glutathione sepharose 4B	Amersham Pharmacia
L-Glutamine	Life Technologies GmbH
N-[2-hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid] (HEPES)	Carl Roth GmbH
Magnesium chloride	Carl Roth GmbH
Methanol	Carl Roth GmbH
Na-Lauroyl-sarcosine (Sarkosyl)	Sigma
N, N', N' –Tertamethylethylenediamine (TEMED)	Carl Roth GmbH
Non-fat dry milk powder	Applichem, Darmstadt, Germany

Nonidet P-40 (NP-40)	Calbiochem, La Jolla, USA
Phosphate-buffered saline (PBS)	PAA, Linz, Austria
Penicillin/Streptomycin	Life Technologies GmbH
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Ponceau-S	Sigma
Poly (dI/dC)	Amersham Pharmacia
Potassium chloride	Carl Roth GmbH
Propidium iodide (PI)	Carl Roth GmbH
Protease inhibitor cocktail (25x)	Roche, Mannheim, Germany
Protein-A sepharose	Sigma
RNase A	Carl Roth GmbH
RPMI-1640	PAA Laboratories
Sodium chloride	Carl Roth GmbH
Sodium dodecylsulfate (SDS)	Carl Roth GmbH
Sucrose	Carl Roth GmbH
Tris-(hydroxymethyl)-amino-methane (Tris)	Carl Roth GmbH
Triton X-100	Carl Roth GmbH
Trypsin	PAA Laboratories
Tween-20	Carl Roth GmbH

2.2 Cell lines

Cell line	Reference	Description
JCaM1	(Goldsmith and Weiss, 1987)	Jurkat variant deficient for Lck
JCaM1/Lck	(Goldsmith and Weiss, 1987)	JCaM1 retransfected with Lck
JCaM2	(Finco et al., 1998)	Jurkat variant deficient for LAT
JCaM2/LAT	(Finco et al., 1998)	JCaM2 retransfected with LAT
P116	(Williams et al., 1998)	Jurkat variant deficient for ZAP-70
J.γ1	(Irvin et al., 2000)	Jurkat variant deficient for PLC-γ1
J.γ1/PLCG	(Irvin et al., 2000)	J.γ1 retransfected with PLC-γ1
J16	(Boesen-de Cock et al., 1999)	Jurkat wild-type variant
J16/Bcl-2	(Rudner, 2001)	J16 transfected with Bcl-2

2.3 Antibodies

2.3.1 Primary antibodies

Antibody	Description	Supplier
Actin	Monoclonal mouse	Santa Cruz, California, USA
Akt	Polyclonal rabbit	Cell , California, USA
Bax	Monoclonal mouse	Pharmingen, Hamburg, Germany
Bax-NT	Polyclonal rabbit	Upstate, Lake Placid, NY, USA
Bak	Monoclonal mouse	Oncogene, USA
Bcl-2	Monoclonal mouse	Santa Cruz
Bcl-x	Monoclonal mouse	Santa Cruz
Bim	Monoclonal mouse	Santa Cruz
Bid	Monoclonal mouse	Santa Cruz
Caspase-3	Monoclonal mouse	R&D Systems, Wiesbaden, Germany
Caspase-8	Monoclonal mouse	BioCheck, Münster, Germany
Caspase-9	Polyclonal rabbit	Cell
CD95	Monoclonal mouse	BioCheck
Cytochrome c	Monoclonal mouse	Pharmingen
Lck	Monoclonal mouse	Santa Cruz
c-Myc	Monoclonal mouse	Santa Cruz
c-IAP1	Polyclonal rabbit	Santa Cruz
c-IAP2	Polyclonal rabbit	Santa Cruz
JNK	Polyclonal rabbit	Cell
Phospho JNK	Polyclonal rabbit	Cell
PARP	Polyclonal rabbit	Cell
P38 MAPK	Polyclonal rabbit	Cell
P44/42(ERK)	Polyclonal rabbit	Cell
Phospho p38	Polyclonal rabbit	Cell
Phospho-p44/42	Polyclonal rabbit	Cell

2.3.2 Secondary antibodies

Secondary antibodies, anti-mouse IgG and anti-rabbit IgG coupled to horseradish peroxidase were purchased from Promega (Mannheim, Germany). Anti-goat IgG coupled to horse radish peroxidase was purchased from Molecular Probes (Karlsruhe, Germany).

2.4 Total RNA isolation

For isolation of total RNA, $1x10^7$ cells per condition were plated. Treated and untreated cells were harvested by centrifugation and washed with ice-cold PBS. For RNA extraction the Total RNA isolation kit (Qiagen) was used. Washed cells were suspended in 600 µl RLT buffer (provided in the kit) and homogenized by passing them three times through a sterile 26g needle. Subsequently, 600 µl of 70% ethanol was added to the homogenate. The total lysate was loaded on an RNeasy mini-column and centrifuged at 8000 *g* for 1 min. The flow-through was discarded, 700 µl of buffer RW1 (provided in the kit) was added to the column and centrifuged at 8000 *g* for 1 min. The column was washed by adding 500 µl of RPE buffer twice and centrifuged at 8000 *g* for 1 min. The washed columns were centrifuged at 8000 *g* for 2 min at RT to remove the residual buffer. Column-bound RNA was eluted with 50 µl RNase-free water, followed by a spin at 8000 *g* for 1min.

The integrity of the RNA was checked on formaldehyde-agarose gels for the presence of 18S and 28S RNA. The formaldehyde-agarose gel was cast as follows: 1.2 g of agarose was added to 88.8 ml of DEPC-H_2O and boiled to melt the agarose. Before casting the gel, the agarose was cooled to 50°C and 10 ml of 10x HEPES and 1.2 ml formaldehyde were added. RNA samples for electrophoresis were prepared as explained below: 2.5 µl 10x HEPES, 10 µl formamide, 4.4 µl formaldehyde, 2 µl loading buffer and 0.2 µl ethidiumbromide were added per sample. 25 µl RNA was added to the reaction mix which was heated at 60°C for 5 min and subsequently cooled on ice. Then, the samples were loaded on the formaldehyde agarose gel and electrophoresed at 170 V in 1x HEPES buffer. The integrity-confirmed RNA samples were subsequently

quantified in a photometer (GeneQuant, Biorad) at A_{260} and stored at -80 °C for further analysis.

2.5 Reverse transcriptase-polymerase chain reaction (RT-PCR)

To investigate the gene expression levels of Bcl-2 family members from the purified total RNA, TITANIUM™ One-Step RT-PCR Kit (BD Biosciences, Heidelberg, Germany) was used. As recommended by the manufacturer 0.5 µg of total RNA per reaction was used. All reagents used in this experiment were provided by the manufacturer. The RT-PCR master mix was prepared as explained below:

5.0 µl	10 x One-Step Buffer
1.0 µl	50 x dNTP mix
0.5 µl	RNase inhibitor (40units/µl)
25.0 µl	thermo stabilizing agent
10.0 µl	GC-Melt™
1.0 µl	Oligo (dT) primer
1.0 µl	50x RT-TITANIUM™ enzyme mix

To the mix 0.5 µg total RNA and primers (45 µM forward and reverse primers) were added and the volume was adjusted to 50 µl with RNase-free water. Reverse transcription (RT) was carried out for 1 h at 50°C. The PCR reaction was carried out in a PCR machine (PE Biosystems) at the following conditions:

94°C	5 min	
	94°C 30 sec	30 cycles
	65°C 30 sec	
	68°C 1 min	
68°C	2 min	

Primers

Bak	Forward 5'-GAA GAT CTG CTT CGG GGC AAG GCC CAG GT-3' Reverse 5'-AAG GAT CCT CAT GAT TTG AAG AAT CTT CG-3'
Bax	Forward 5'-GAA GAT CTG ACG GGT CCG GGG AGC AGC CC-3' Reverse 5'-AAG GAT CCT CAG CCC ATC TTC TTC CAG AT-3'
Bcl-2	Forward 5'-ATG GCG CAC GCT GGG AGA AC-3' Reverse 5'-CCA GCC TCC GTT ATC CTG GATC-3'
Bcl-x	Forward 5'-ATG TCT CAG AGC AAC CCG GAG-3' Reverse 5'-TTT CCG ACT GAA GAG TGA GCC C-3'
GAPDH	Forward 5'-GTG GAA GGA CTC ATG ACC ACA G-3' Reverse 5'-CTG GTG CTC AGT GTA GCC CAG -3'

The mRNA expression levels of the above mentioned Bcl-2 members were monitored by RT-PCR using sense and anti-sense primers from MWG Biotech. For standardization purposes, each RT sample was subjected to PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 20 µl of each PCR reaction were loaded on an ethidium bromide stained agarose gel. A X-174-RF DNA Hae III digest was used as a marker.

2.6. Electrocompetent cell preparation

Electrocompetent bacteria (*E. coli* BL21 gold, Invitrogen) were first plated on a LB agar plate (10.0 g trypton, 5.0 g yeast extract, 10.0 g NaCl and 15.0 g agar per liter) and incubated at 37°C overnight. Single colonies were picked from the agar plate and inoculated in 5 ml LB medium (10.0 g trypton, 5.0 g yeast extract and 10.0 g NaCl per liter) and grown in a bacterial shaker overnight at 37°C. 400 ml of LB medium was inoculated with the overnight culture and grown in a bacterial shaker at 37°C until an optical density (OD_{600}) of 0.3 was reached. The bacterial suspension was then cooled on ice for 10 min and centrifuged at 6000 rpm in Sorvall GS3 rotor for 15 min at 4°C. The centrifuged bacteria were resuspended in 10 ml of 1 mM HEPES pH 7.0, and the volume was adjusted to 400 ml with the same buffer. After another centrifugation step, the pellet was resuspended in 10 ml

of ice-cold, sterile, 10% glycerol and centrifuged in Sorvall SS34 rotor for 15 min at 4°C. Finally, the resulting bacterial pellet was resuspended in 0.8 ml of sterile 10% glycerol and devided into aliquots of 80 µl. The aliquots were immediately transferred to –80°C and stored for further use.

2.7 Electroporation

For transformation 10 ng of purified plasmid containing the gene of interest (e.g. pGEX-2T-tBid) was directly added to a freshly thawed aliquot of competent cells. Before electroporation the electroporation cuvette was cooled on ice. The competent cells, mixed with DNA, were transferred to the cooled cuvette and electroporated (5 msec, 2.5 kV) in a micropulser (Biorad). Immediately after electroporation cells were suspended in 1 ml of warm LB medium and incubated at 37 °C for 1 h with shaking. 10-200 µl from the culture was plated on a LB agar plate and the transformed bacteria were selected based on the antibiotic resistance.

2.8 Plasmid preparation

Mini-preparation

From the agar plate single colonies were picked and inoculated in 2 ml LB medium with the respective antibiotic and incubated in a bacterial shaker at 37°C overnight. The cultures were then transferred to 2 ml Eppendorf tubes and centrifuged at 6000 rpm for 5 min (Biofuge fresco, Heraeus). Pellets were resuspended in 300 µl GTE buffer (250 mM Tris-HCl pH 8.0, 100 mM EDTA, 500 mM glucose, 0.5 mg/ml RNase). The suspension was lysed in 600 µl lysis buffer (0.2 M NaOH, 1% SDS) at room temperature for 5 min. To the lysate 450 µl of ice-cold 3 M Na-acetate, pH 4.8, was added and incubated on ice for 5 min. The precipitate was removed by centrifugation at 13000 rpm for 15 min at 4°C. The supernatant was transferred to another 2 ml tube and 700 µl of isopropanol was added to precipitate the DNA. Precipitated DNA was pelleted by centrifugation at 13000 rpm for 20 min at 4°C. The resulting DNA pellet was washed in 70% ethanol for 5 min at RT and dried at

the air. The pellet was resuspended in 40 µl of ddH_2O, and the intactness of the plasmid was checked on an ethidium bromide-stained agarose gel.

Maxi-preparation

From agar plate a single bacterial colony was picked, inoculated in 2 ml LB medium and grown in a bacterial shaker for 8h at 37°C. From the pre-inoculum 150 ml of LB medium was inoculated and grown in a bacterial shaker overnight at 37°C. The culture was then centrifuged in a Sorvall GS3 rotor at 5000 rpm for 15 min at 4°C. Plasmid purification was carried out by using the HiSpeed™ plasmid purification kit (Qiagen). For transfection purpose the DNA was further concentrated with 0.3 M Na-acetate and 2 volumes of absolute alcohol for 5 min at room temperature, followed by a centrifugation step for 15 min at 13000 rpm. The resulting pellet was washed with 70% ethanol, air-dried and suspended in 100-300 µl TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The DNA concentration was estimated in Gene Quant II photometer (Pharmacia Biotech). The DNA was aliquoted and stored at -20°C.

2.9 Purification of GST-tagged proteins

The following buffers were used for the purification of glutathione-S-transferase (GST) fusion proteins:

Lysis Buffer A

0.5% (v / v)	Triton X-100
2.0 mM	DTT
0.5 mM	PMSF
3.0 µg/ml	Aprotinin, Pepstatin, Leupeptin

Elution Buffer B

100 mM	NaCl
50 mM	Tris-HCl pH 8.0
20 mM	Glutathione (GSH)

Dialysis Buffer C	
200 mM	NaCl
50 mM	Tris-HCl pH 8.0
1 mM	DTT

A single bacterial colony that was transformed with a plasmid encoding the gene of interest (e.g. GST-tBid) was picked up from the agar plate, inoculated in 5 ml LB medium and grown in a bacterial shaker overnight at 37°C. The 5 ml preculture was inoculated in 1000 ml of LB medium and incubated in a bacterial shaker at 37°C until an OD_{600} of 0.6 was reached. Then, the culture was induced with 1 mM IPTG for protein expression and grown at 37°C for 4 h. The bacteria were harvested by centrifugation in a Sorvall GS3 rotor at 5000 rpm for 15 min at 4°C. The resulting pellet was washed in ice-cold PBS and resuspended in 10 ml lysis buffer A. To the suspension 1 mg/ml lysozyme was added and slowly stirred on a magnetic stirrer for 30 min on ice. Sarcosyl was then added to the lysate at a final concentration of 1% (w / v) and sonicated for 6x30 sec (Sonoplus HD 2070, Cycle 9, TT13; Bandelin, Berlin, Germany) for complete lysis. The clear lysate was centrifuged at 20,000 rpm for 30 min in Sorvall SS34 rotor at 4°C**.** In parallel, 25 ml of GSH-Sepharose fast flow material was packed in a XK-16 column (Amersham Bioscience, Uppsala, Sweden) and equilibrated with PBS containing 2 mM DTT. The clear supernatant obtained after centrifugation was passed through the column at a flow rate of 2 ml/min. Then, the column was washed with PBS containing 2 mM DTT, until the flow-through was devoid of protein. The column-bound GST-tagged protein was eluted with elution buffer B in 0.5 ml fractions. Protein fractions were analysed for the presence of protein by loading 20 µl from each fraction on a SDS-gel. The relevant protein fractions were then pooled and dialysed overnight against dialysis buffer C at 4°C, followed by a short dialysis for 4 h two times. The purified protein was distributed into aliquots, snap-frozen in liquid nitrogen and stored at –80 °C.

2.10 Cell culture

All cell lines used in this work were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units of penicillin/ml, and 0.1 mg streptomycin/ml (PAA Laboratories, Linz, Austria). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere and maintained in the logarithmic phase.

2.11 Treatment of cells and measurement of cell death

Approximately $3x10^4$ cells were seeded per condition in a 12-well plate and stimulated for various time points with the following stimuli: anti-CD95 (1 µg/ml), chemotherapeutic agents such as etoposide (5 µg/ml), doxorubicin (1 µg/ml), daunorubicin (1µg/ml), mitomycin C (5 µg/ml) and protein kinase inhibitor staurosporine (2.5 µM). After the specified time points, cells were harvested by centrifugation at 500 *g* for 5 min. The cells were then lysed in hypotonic lysis buffer for further analysis. The leakage of fragmented DNA from apoptotic nuclei was measured by the method of Nicoletti et al. (1991). Briefly, apoptotic nuclei were prepared by lysing cells in a hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, 50 µg/ml propidium iodide) and subsequently analysed by flow cytometry. Nuclei to the left of the 2N peak containing hypodiploid DNA were considered as apoptotic. Cell death was also evaluated by the uptake of propidium iodide (2 µg/ml) into nonfixed cells and subsequent flow cytometric analyses with the FSC/FL2 profile (Wesselborg et al., 1999). All flow cytometric analyses were performed on a FACScalibur (Becton Dickinson, Heidelberg, Germany) using CellQuest analysis software.

In order to determine the involvement of various signalling pathways, specific inhibitors were preincubated with the cells for 1 h at the following concentrations: JNK inhibitor II (Calbiochem, 1 µM), MAP kinase inhibitor UO126 (Calbiochem, 10 µM), p38 inhibitor SB203580 (Cell Signalling, 1 µM) and the Src kinase inhibitor herbimycin A (Calbiochem, 1 µM). All inhibitors were used at concentrations that did not elicit any cytotoxic response on their own. To determine the level of apoptosis, 3 x 10^4 cells per well were seeded and pre-incubated with the

abovementioned inhibitors and treated for the indicated time with anti-CD95 or the chemotherapeutic agents.

2.12 Preparation of cell extracts

2.12.1 Total cell extract

High-salt lysis buffer

350 mM	NaCl
20 mM	HEPES pH 7.9
1.0 mM	$MgCl_2$
0.5 mM	EDTA
0.1 mM	EGTA
0.5 mM	DTT
1.0%	NP-40
20%	glycerol
1x	protease inhibitor cocktail

After drug treatment cells ($3x10^5$/condition) were harvested by centrifugation at 300 *g* for 5 min at 4°C and washed in ice-cold PBS. The resuspended cells were directly mixed with high-salt lysis buffer with an appropriate volume (100 µl) and incubated on ice for 10 min. For complete lysis samples were shortly vortexed. Lysates were then centrifuged at 10,000 *g* for 15 min at 4 °C. The resulting supernatants, considered as total cell extract, were stored at -20°C for further experiments. Shortly before use, protein concentrations of the samples were determined with the Bradford reagent (Biorad, Munich, Germany).

2.12.2 Cytosolic and mitochondrial extracts

Subcellular fractionation was carried out in order to determine the localization of candidate proteins in the cell and also to determine their translocation upon stimulation with anti-cancer drugs. The following buffer was used for the fractionation:

Low-salt lysis buffer

10 mM	HEPES pH 7.9
10 mM	KCl
1.0 mM	$MgCl_2$
0.1 mM	EDTA
0.1 mM	EGTA
0.5 mM	DTT
2.0 mM	PMSF
1 x	protease inhibitor cocktail

After drug treatment, cells ($3x10^5$ cells per condition) were harvested by centrifugation at 300 *g* for 5 min at 4°C. Cells were washed in PBS, suspended in low-salt lysis buffer (100 µl) and incubated on ice for 15 min. After incubation, cells were passed three times through a 26 g needle. Nuclei and intact cells from the lysates were removed by centrifugation at 500 *g* for 10 min at 4°C. The resulting supernatants were centrifuged at 10,000 *g* for 30 min at 4°C to obtain the cytosolic fraction. The resulting pellet containing crude mitochondria was lysed in high-salt lysis buffer and vortexed to extract the mitochondrial protein. Lysates were then centrifuged at 10000 *g* for 15 min, and the resulting supernatant was used as mitochondrial extract. Both mitochondrial and cytosolic extracts were then stored at -20°C for further investigation.

2.13 Isolation of intact mitochondria

In order to determine the ability of certain proapoptotic factors to induce mitochondrial permeabilization or cytochromce c release *in vitro*, intact and fully functional mitochondria were used. The following procedure was employed to isolate intact mitochondria.

Buffer A

250 mM	sucrose
50 mM	Tris-HCl pH 7.4
1.0 mM	EDTA
1.0 mM	DTT
1x	protease inhibitor cocktail
50 µg/ml	digitonin

Buffer B	
250 mM	sucrose
50 mM	Tris-HCl pH 7.4
1.0 mM	EGTA
1.0 mM	DTT
1x	protease inhibitor cocktail

Buffer C	
220 mM	mannitol
68 mM	sucrose
10 mM	HEPES pH 7.4
2.0 mM	NaCl
2.5 mM	KH_2PO_4
0.5 mM	EGTA
2.0 mM	$MgCl_2$
4.2 mM	sodium succinate
1.0 mM	DTT
1x	protease inhibitor cocktail

To obtain intact mitochondria for *in vitro* cytochrome c release assays, $1x10^7$ cells harvested and washed in PBS. the cells were then permeablized in buffer A and immediately passed three times through 26 g needle. Intact cells, debris and nuclei were removed by centrifugation at 500 *g* for 10 min at 4°C. The resulting supernatants containing mitochondria and other cellular compartments were further centrifuged at 10,000 *g* for 30 min at 4°C. The resulting crude mitochondrial pellets were washed three times with buffer B to remove lysosomal and microsomal contamination. The crude mitochondrial fraction was then resuspended in buffer C and used for analysis of cytochrome c release.

2.14 SDS-Polyacrylamide Electrophoresis (SDS-PAGE)

2.14.1 Gel casting

Based on the molecular weight of protein the percentage of the acrylamide gel was decided. The composition for various percentage of the polyacrylamide gels is

given below. The acrylamide stock solution contained acrylamide:bisacrylamide in a ratio of 29:1.

Stacking Gel	40% Acrylamide (ml)	0.5 M Tris-HCl (ml)	H_2O (ml)	10% SDS (µl)	TEMED (µl)	10% APS (µl)
4%	0.375	0.380	2.185	30	3	30
Separation Gel	40% Acrylamide (ml)	0.5 M Tris-HCl (ml)	H_2O (ml)	10% SDS (µl)	TEMED (µl)	10% APS (µl)
8%	2.0	2.5	5.3	100	8	100
10%	2.5	2.5	4.7	100	8	100
12%	3.0	2.5	4.3	100	8	100
16%	4.0	2.5	3.3	100	8	100

The following buffers were use for SDS-PAGE:

Sample buffer: 250 mM Tris-HCl pH 6.8, 40 % (v / v), glycerol, 8.2% (w / v) SDS, 400 µg/ml bromophenol blue, 4% (v / v) β-mercaptoethanol.

Electrophoresis buffer: 25 mM Tris, 250 mM glycine, 0.1% SDS.

2.14.2 Electrophoresis and blotting

Before electrophoresis, equal amounts of protein (30 µg) were mixed with sample buffer and denatured at 95°C for 5 min. Samples were shortly centrifuged and loaded on the SDS-gel. After loading, gels were electrophoresed at constant voltage (50 V) in order to stack the samples. When the samples had entered the separating gel, it was run at 100 V until the dye front reached the bottom. Gels were carefully removed from the plates and equilibrated in wet blot transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol). A polyvinylidene difluoride (PVDF) membrane, cut to the size of the gel, was activated in methanol for a few seconds and equilibrated in the transfer buffer. The gel was sandwiched between the PVDF membrane and Whatman blotting paper,

which were equilibrated with the transfer buffer. Care was taken to avoid air pockets between membrane and gel. The sandwiched gel was placed in special transfer module provided by the manufacturer (Biorad, Munich, Germany). The tank was filled with transfer buffer and electro-blotted at constant current (50 mA) overnight. After complete transfer, the membranes were blocked in 5% non-fat milk powder prepared in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 2 h at room temperature. The membrane was then washed with TBST buffer for 5 min and incubated with the primary antibody against the protein of interest overnight. Blots probed with primary antibody were washed in TBST three times at 5 min interval. Then, the membranes were incubated with their respective HRP conjugated antibodies for 1 h at room temperature. Blots were extensively washed with TBST buffer to remove unbound antibodies. ECL solution (Amersham Bioscience, Buckinghamshire, UK) was used to activate the conjugates for chemiluminescence. The blots were immediately exposed to photosensitive films (Amersham Bioscience) and developed.

2.15. Flow cytometric analysis of Bak and Bax conformational change

At the indicated times after drug treatment, cells ($1x10^6$ per condition) were harvested by centrifugation at 300 *g* for 5 min at 4°C, washed with PBS, and fixed in 1 ml PBS containing 0.5 % paraformaldehyde on ice for 30 min. Cells were then washed three times in PBS containing 1% FCS. Staining with conformation-specific antibodies against Bax, Bak and isotype-matched control antibodies was performed by incubating cells in an 1:50 dilution of the respective antibody in 50 µl staining buffer (PBS, 1% FCS, 50 µg/ml digitonin). Digitonin was used to permeabilize the plasma membrane. Then, cells were washed three times with PBS, resuspended in 50 µl staining buffer containing 0.1 µg Alexafluor 488-labeled chicken anti-mouse or FITC-coupled goat anti-rabbit IgG antiserum (Molecular Probes) and incubated on ice for 30 min in the dark. After three washing steps in PBS, conformational changes of Bak and Bax were immediately measured with logarithmic amplification in the FL-1 channel of a flow cytometer.

2. 16 *In-vitro* cytochrome c release assay

For *in vitro* experiments $1x10^7$ JCaM or JCaM/Lck cells were taken and the mitochondria were purified as mentioned previously. Mitochondria suspended in buffer C were immediately used for experiments and incubated in 50 µl volume with GST-tBid (1 µg) at 37°C for 30 min. Then, the samples were centrifuged at 10,000 *g* for 20 min. The resulting supernatant and mitochondrial pellets were collected separately, mixed with 4x SDS-loading buffer, and analyzed by SDS-PAGE and immunoblotted for cytochrome c release.

2.17 In vitro kinase assays

For the kinase assay $2x10^6$ Lck-proficient and deficient JCaM cells were seeded per condition. The cells were stimulated with etoposide (5 µg/ml) for various time points as indicated. As a positive control cells were stimulated with anti-CD3 antibodies for the same time points.

IP-Lysis buffer	
30 mM	Tris/HCl, pH 7.5
150 mM	NaCl
1.0 mM	PMSF
1.0%	NP-40
10%	glycerol
1x	protease inhibitor cocktail

To measure Lck activity, autophosphorylation assays were performed. The stimulated cells were harvested by centrifugation at 300 *g* for 5 min and washed with cold PBS. The pellets were suspended in IP-lysis buffer and incubated on ice for 30 min. The lysates were centrifuged 10,000 *g* for 30 min, and the resulting supernatant was used for further experiments. Simultaneously, 3 µg of Lck antibody was coupled to Protein A-sepharose (50% suspension, Sigma) beads by incubation for 1 h at 4 °C. Unbound antibody was removed by washing the beads with IP-lysis buffer. The Lck immunoprecipitates were washed twice in lysis buffer, followed by two washes in wash buffer (20 mM Tris-HCl pH 7.4, 0.5 M LiCl, 0.4

mM sodium vanadate) and a single wash in distilled H_2O. The immobilized Lck was resuspended at 37 °C in Lck kinase buffer containing 20 mM Tris-HCl pH 7.4, 10 mM $MnCl_2$, 0.01 mM ATP and 10 µCi of □[^{32}P]-ATP. After the indicated times, aliquots were withdrawn and transferred to microcentrifuge tubes containing ice-cold lysis buffer plus 2 mM EDTA, and 10 mM ATP. The samples were washed twice with lysis buffer and resuspended in SDS sample buffer. Samples were loaded on a 15% SDS-PAGE and separated under reducing conditions. Subsequently, the gels were dried in a gel drier (Biorad) and exposed on an X-ray film overnight at -80°C.

2.18 Immunoprecipitation of the death-inducing signalling complex (DISC)

Lysis buffer

30 mM	Tris/HCl, pH 7.5
150 mM	NaCl
1.0 mM	PMSF
1.0 %	Triton X-100
10 %	glycerol
1x	Protease inhibitors

Lck-proficient and deficient JCaM cells were adjusted to a concentration of 1x10^7 cells per 5 ml RPMI medium and stimulated with 1 □g/ml anti-CD95 for 15 min. After incubation cells were harvested by centrifugation at 300 *g* for 5 min and washed with cold PBS in order to stop stimulation. Cell pellets were resuspended in 750 □l lysis buffer and incubated for 15 min on ice. The samples were spun at 10000*g* for 15 min at 4°C. The resulting supernatant was added to 30 □l of a 50% Protein A-sepharose suspension. Samples were incubated on a rotating mixer for at least 3 h at 4°C. After incubation beads were spun down at 6000 rpm for 1 min at 4°C. Lysates were removed and the beads were washed 4-5 times in lysis buffer. Then, the beads were resuspended in reducing SDS sample buffer and boiled for 3 min at 95°C. As a negative control, the same amount of cells (1x10^7) was lysed without anti-CD95 stimulation. The lysate was supplemented

with 1 µg anti-CD95 and immunoprecipitation was performed with Protein A-sepharose as described above. Samples eluted from the beads were loaded on 12% SDS-gels and separated under reducing conditions. The separated proteins from the gel were transferred onto a PVDF membrane and probed against caspase-8, FADD, CD95 and Lck.

2.19 Electrophoretic mobility shift assay

In order to investigate the DNA-binding activity of the transcription factors NF-κB and NF-AT, electrophoretic mobility shift assays (EMSAs) were performed.

2.19.1 Cell extracts

Both Lck-proficient and deficient JCaM cells were seeded in equal numbers (10^6 cells/well) and stimulated with etoposide (5 µg/ml) for various time points. As a positive control cells were stimulated with TNF (10 ng/ml). After the specified incubation period cells were harvested and washed with ice-cold PBS. Washed samples were lysed in 50 µl of Totex buffer (1 % NP-40, 20 mM HEPES, 2 mM PMSF pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT and 3 µg/ml of each aprotinin, leupeptin, and pepstatin A) on ice for 20 min. Lysed samples were then centrifuged at 10,000 *g* for 30 min at 4°C, and the supernatants were used for further experiments.

2.19.2 Casting Gel

Electrophoretic mobility shift assays were run in 4% acrylamide gels under non-denaturing conditions. The composition of the gel is given below:

Gel composition	
10.5 ml	Acrylamide mix
7.5 ml	5 x TBE
0.5 ml	APS
75 µl	TEMED
56.5 ml	H_2O

5xTBE (45 nM Tris-borate, 2 mM EDTA)

2.19.3 Labelling oligonucleotides

Gel shift assays were performed for the transcription factors NF-κB and NF-AT. The following oligonucleotides were used:

NF-κB	5'-AGCTTCAGAGGGGATTTCCGAGAGG-3'
NF-AT	5'-TCGACAAAGAGGAAAATTTGTTTCATACAGAAG-3'

The oligonucleotides were labelled with radioactive [^{32}P]-ATP in the following reaction mixture:

6.0 µl	5x T4-polynucleotide kinase (PNK) buffer
1.5 µl	Oligonucleotides (for NF-κB or NF-AT, 2.65 pmols)
5.0 µl	[^{32}P]-ATP(45mCi:10pmol)
2.0 µl	T4-PNK (10 U/ml)
18 µl	ddH_2O

After incubation at 37°C for 30 min, the reaction mix was mixed with STE buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1mM EDTA) to terminate the reaction. Non-incorporated nucleotides were removed by loading the reaction mixture on a P-10 gelfiltration column (BioRad). After centrifugation of the columns at 3000 rpm for 2 min, the purified and radiolabelled oligonucleotides were used for gel shift assays.

Binding reaction mixture

2-4 µl	Cell extract
4.0 µl	5x binding buffer
2.0 µg	poly(dI-dC)
2.0 µg	BSA
[^{32}P]	10,000-150,00 c.p.m of the oligonucleotide

The total volume was adjusted to 20 µl.

5x Binding buffer

20 mM	HEPES pH 7.5
50 mM	KCl
2.5 mM	$MgCl_2$
1.0 mM	DTT
20%	Ficoll

Equal amounts of the cell extracts (about 10 µg crude protein; determined by the Bio-Rad assay kit) were incubated with the [^{32}P]-labeled NF-κB and NF-AT-specific oligonucleotides in binding buffer. After 20 min of binding reaction at room temperature samples were loaded on a 4 % non-denaturing polyacrylamide gel and electrophoresed in 0.5 x TBE buffer pH 8.0. Gels were carefully removed by placing a Whatman filter paper on the gel, dried in a gel dryer and exposed to an X-ray film overnight at –80°C.

3. RESULTS

3.1 Deficiency of the Lck tyrosine kinase results in resistance to apoptosis induced by different anticancer drugs

T cells express primarily two members of the Src family of tyrosine kinases, Lck and Fyn, both of which have been implicated in various biological processes. To examine the role of Lck in apoptosis signalling, JCaM1.6 cells were employed. This cell line is a genetic variant of Jurkat T-cells and deficient in p56lck protein due to a deletion in exon 7 in the p56lck mRNA encoding the ATP binding site (Straus and Weiss, 1992). As revealed by immunoblot analysis, an antibody against the N-terminus of Lck detected small amounts of a 49 kD splice product in JCaM1 cells (Fig. 12), whereas an antibody against C-terminus did not show any immunoreactivity (data not shown). Both the wild-type Jurkat cells and the Lck-transfected JCaM1 cells expressed similar amounts of the wild-type tyrosine kinase at a molecular weight of 56 kD.

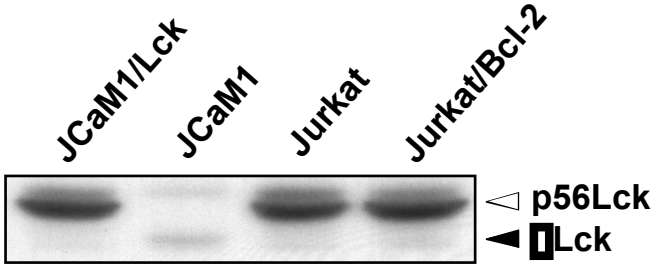

Figure 12: JCaM1 cells do not express full-length Lck. Total cell extracts from unstimulated Jurkat wild-type, Jurkat/Bcl-2, JCaM1 and JCaM1/Lck cells were prepared by lysing the cells in high-salt lysis buffer. Equal amounts (30 µg) of protein were separated on a SDS-PAGE and blotted on a PVDF membrane. Western blot analysis with an antibody against the C-terminus of Lck revealed that JCaM1 cells express only low amount of a truncated version of Lck (filled arrowhead). Retransfection of wild-type Lck (open arrowhead) restored the expression levels equivalent to the parental Jurkat cells.

Analysis of apoptosis showed that, in contrast to wild-type Jurkat cells, JCaM1 cells were almost completely resistant against apoptosis induced by the topoisomerase II inhibitor etoposide, even at high drug concentrations and after prolonged incubation for 48 h (Fig. 13). Apoptosis resistance of JCaM1 cells was not only observed after treatment of cells with a topoisomerase inhibitor, but also after incubation with unrelated anticancer drugs including doxorubicin,

daunorubicin and mitomycin C. Interestingly, reexpression of Lck in JCaM1 cells fully overcame drug resistance and reconferred apoptosis sensitivty in response to all chemotherapeutic agents.

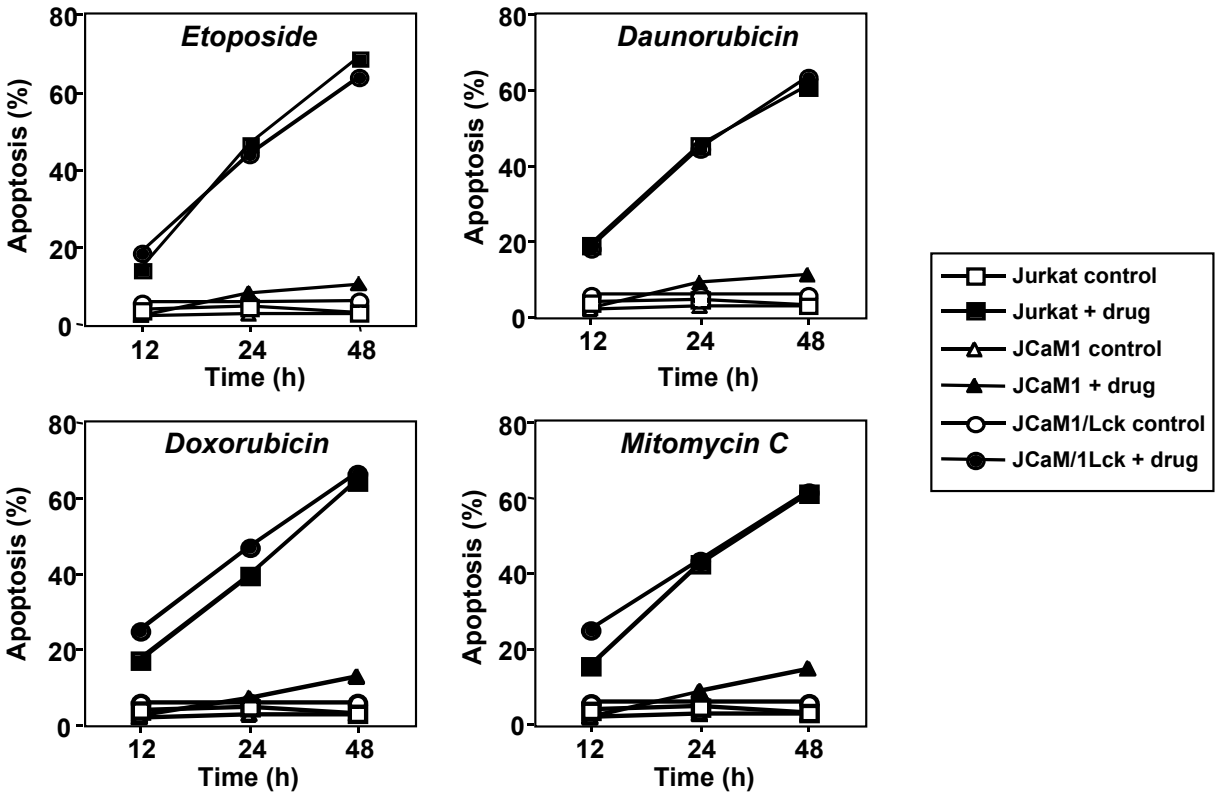

Figure 13: Lck-deficient JCaM1 cells are resistant against drug-induced apoptosis. Parental Jurkat cells as well as Lck-deficient and proficient JCaM1 cells ($3x10^4$ per condition) were either left untreated or incubated with etoposide (5 µg/ml), daunorubicin (1 µg/ml), doxorubicin (1 µg/ml) or mitomycin C (5 µg/ml). After the indicated times cells were harvested and suspended in hypotonic lysis buffer containing 50 µg/ml propidium iodide. Lysed cells were measured for the presence of hypodiploid nuclei by flow cytometry. The analysis shows that Lck-deficient JCaM1 cells are resistant for anti-cancer drugs. Lck retransfected JCaM1 cells remained as sensitive as wild-type Jurkat cells.

3.2 CD95-mediated apoptosis is a delayed event in Lck-deficient JCaM1 cells

JCaM1 cells were also resistant against apoptosis induced by the protein kinase inhibitor staurosporine, a classical inducer of the mitochondrial apoptosis pathway, and again reexpression of Lck sensitized the cells to apoptosis (Fig. 14). The resistance of JCaM1 cells was in a similar range as in Jurkat cells overexpressing

Bcl-2. In order to find out whether the absence of Lck modulates apoptosis upon ligation of death receptors such as CD95, both Lck-proficient and deficient JCaM1 cell lines were stimulated with anti-CD95. However, regardless of the Lck status, both Lck-deficient and proficient cells were equally sensitive to death receptor CD95 ligation when measured after 24 h (Fig. 14, right panel); a slight protection was only seen at early time points (Fig 14, left panel). Similarly, CD95-mediated apoptosis was only delayed but not prevented in Bcl-2 overexpressing Jurkat cells. The selective protection contributed by Lck deficiency against drug- and staurosporine-triggered cell death, but not against CD95-mediated apoptosis therefore indicated that Lck interfered with the mitochondrial pathway of apoptosis.

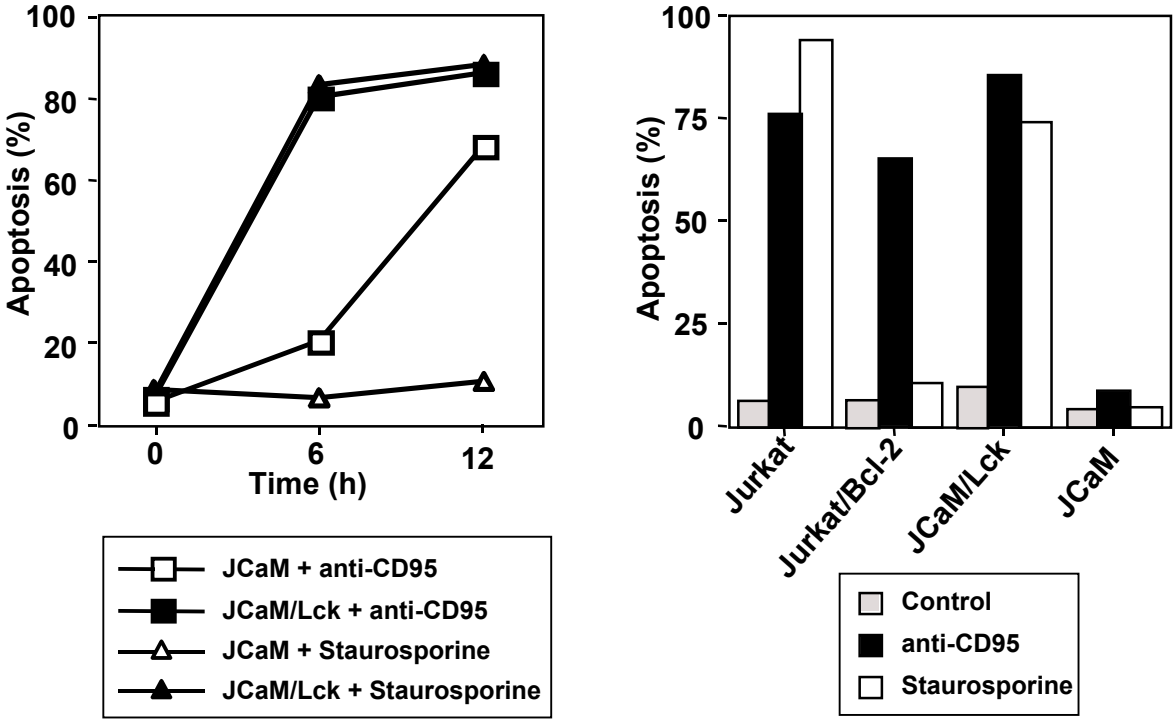

Figure 14: Lck interferes with the mitochondrial apoptotic pathway. Wild-type and Bcl-2 overexpressing Jurkat cells as well as Lck-deficient and proficient JCaM1 cells ($3x10^4$ per condition) were stimulated with staurosporine (2.5 µM) or anti-CD95 moAb (1 µg/ml). After different time points (left panel) or 24 h (right panel) apoptosis was determined by measurement of hypodiploid DNA. Comparison of CD95- and staurosporine-mediated apoptosis in Jurkat wild-type, Jurkat-Bcl-2, JCaM1 and JCaM1/Lck cells revealed that early apoptosis was slightly attenuated in JCaM1 cells, whereas Lck deficiency did not protect against CD95 at later time points. This suggests that Lck is primarily interfering with the mitochondrial but not with the death receptor apoptotic pathway. The data show mean values of triplicate measurements from one representative out of three experiments.

3.3 Lck deficiency interferes with the mitochondrial apoptotic cascade

The mitochondrial apoptosis pathway is primarily triggered by the release of cytochrome c from mitochondria, which together with dATP and Apaf-1 binds to procaspase-9 to form the apoptosome. Upon formation of the apoptosome, procaspase-9 is autoproteolytically processed resulting in the activation of downstream caspases such as caspase-3, -6 and -7. To determine in more detail the reason for the inability of Lck-deficient cells to undergo apoptosis, both Lck-proficient and deficient cells were treated with etoposide for various time points and the release of cytochrome c from mitochondria, activation of caspase-3 and caspase 9 were monitored by Western blot analysis. In Lck-expressing cells, but not in Lck-deficient cells etoposide treatment resulted in the marked proteolytic activation of caspase-3 within 4 to 5 h, as determined by the proteolytic processing from its 32 kD precursor into the 17 kD active subunit (Fig. 15).

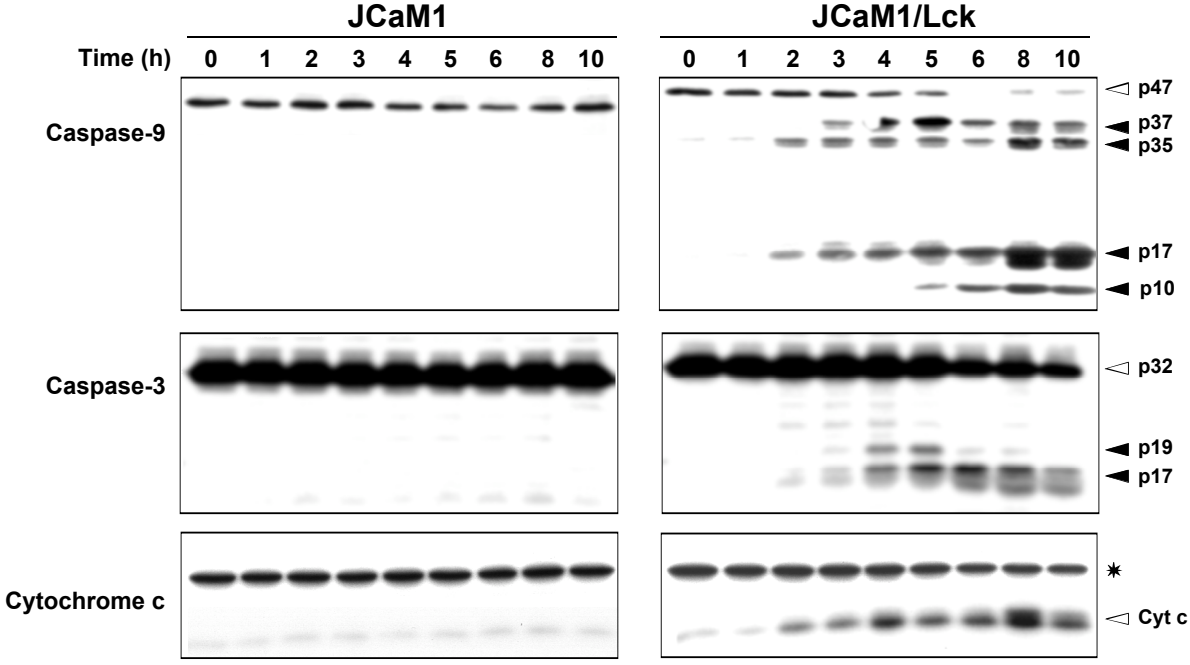

Figure 15: Lck is required for cytochrome c release and caspase activation. Lck-proficient and deficient JCaM1 cells were treated for the indicated time points with etoposide (5 µg/ml). Cellular proteins were separated by SDS-PAGE, and the proteolytic processing of caspase-9 *(upper panel)* and caspase-3 *(middle panel)* was detected by immunoblotting. Open arrowheads indicate the uncleaved and closed arrowheads the cleaved forms of the indicated proteins. For measurement of cytochrome c release *(lower panel)* cells were homogenized and the S10 fraction depleted of mitochondria was analyzed by SDS-PAGE. An unspecific protein band that served as a control for equal protein loading is indicated with an asterisk.

The cleavage of caspase-3 was preceded by the activation of caspase-9 that was only observed in Lck-expressing but not in Lck-deficient JCaM1 cells (Fig. 15). Caspase-9 is first autoproteolytically cleaved into a 37 kD intermediate fragment, followed by an appearance of a caspase-3-generated p35 subunit that is further processed to the active subunits (Srinivasula et al., 1998). Almost coincident with the activation of caspase-9 was the mitochondrial release of cytochrome c that again was exclusively seen in the presence of Lck.

The mitochondrial pathway can be interconnected with the death receptor pathway by the Bcl-2 protein Bid (Li et al., 1998; Luo et al., 1998). Upon cleavage by caspase-8, Bid translocates to mitochondria triggering the release of cytochrome c and subsequent caspase-9 activation. In JCAM1/Lck but not in JCaM1 cells the truncated p15 form of Bid appears within 4 h after etoposide treatment. In addition, cleavage of caspase-8 processing into the p18 subunit was observed only in Lck-expressing cells. In comparison to cytochrome c release and caspase-9 processing, the cleavage of Bid and caspase-8 was delayed (Fig. 16), indicating that it occurred as a postmitochondrial event, as recently also suggested in other studies (Engels et al., 2000; Tang et al., 2000; Wieder et al., 2001).

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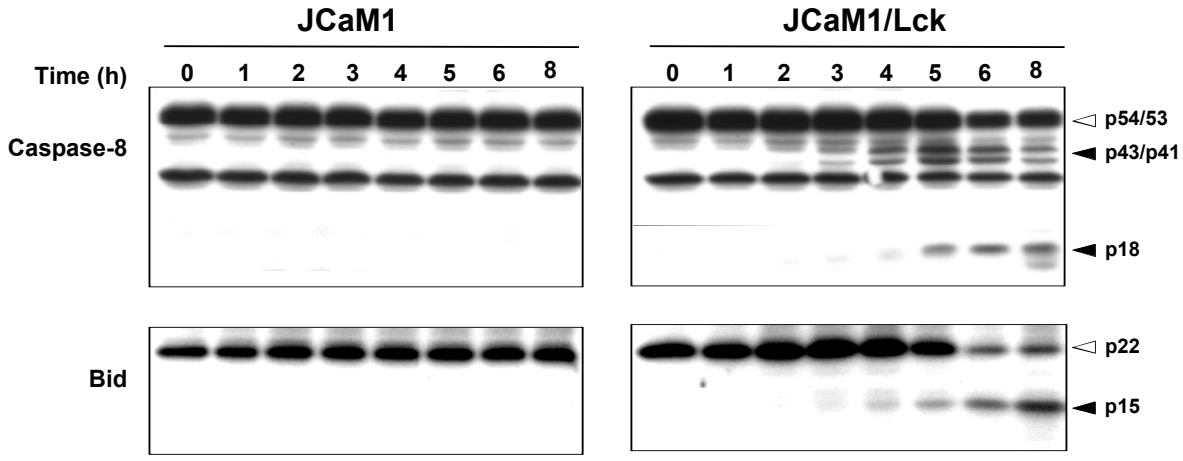

Figure 16: Caspase-8 and Bid cleavage in the presence and absence of Lck. JCaM1 and JCaM1/Lck cells were treated for the indicated time points with etoposide (5 µg/ml). Western blot analysis of Lck-proficient and deficient JCaM1 cell extracts shows that Lck-reexpressing cells activate caspase-8 independently of CD95 ligation as a secondary postmitochondrial event during drug mediated apoptosis. Lck-deficient JCaM1 cells failed to activate caspase-8 or Bid cleavage upon treatment with etoposide.

3.4 The proapoptotic effect of Lck is independent of T-cell receptor signalling

In the next experiments, the specificity of the proapoptotic effect of Lck and its downstream signalling was investigated. As mentioned in the introduction, upon T-cell stimulation, Lck phosphorylates several signalling mediators, most importantly the ZAP-70 tyrosine kinase. ZAP-70 then phosphorylates key adapter proteins, including linker for activation of T-cells (LAT), which ultimately promotes the activation of downstream signalling pathways. Loss of any of the downstream partners such as ZAP-70 or LAT leads to disruption of TCR signalling and activation (Williams et al., 1998; Finco and Weiss, 1998). Thus, if the drug-mediated apoptosis is dependent on these events, Jurkat cells that are deficient for LAT and ZAP-70 should be able to resist apoptosis induction by anti-cancer drugs.

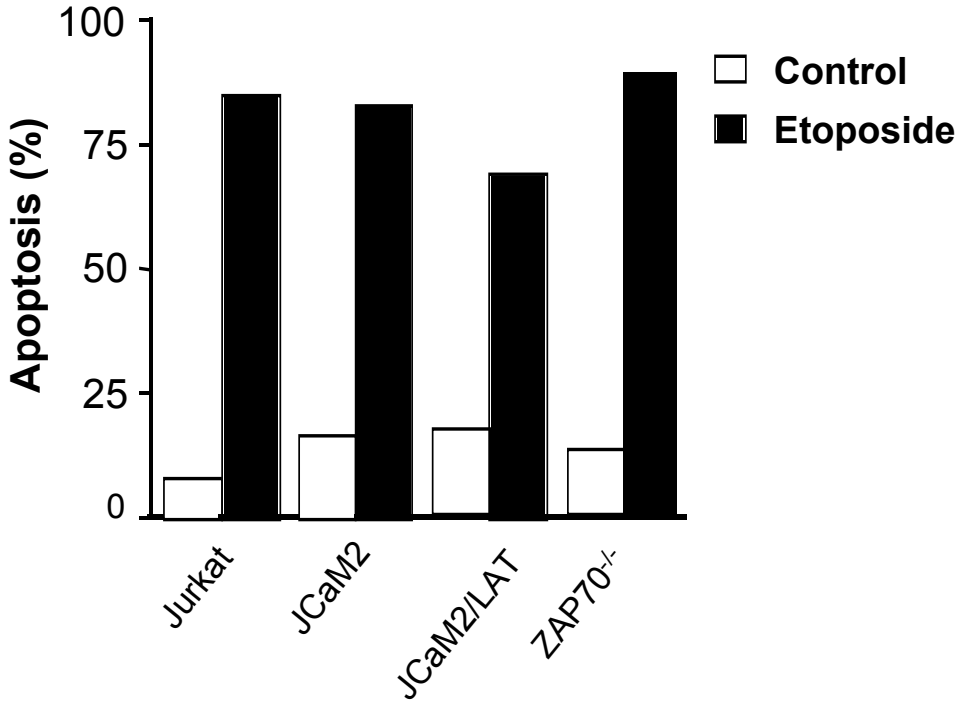

Figure 17: Lck-mediated apoptosis is independent of T-cell signalling. Jurkat cells deficient for LAT and ZAP-70 were stimulated with etoposide for 20 h. After stimulation cells were harvested and suspended in hypotonic lysis buffer containing propidium iodide and analysed for apoptotic cells by flow cytometry. The analysis shows that Jurkat cells that are deficient for LAT and ZAP-70 remain as sensitive as the wild-type Jurkat cells upon etoposide treatment. The results show the mean of three experiments measured in triplicates.

To analyze the assumption, Jurkat cells that are deficient for LAT and ZAP-70 were treated with etoposide along with the Lck-deficient counterparts. Interestingly, cells deficient for LAT underwent apoptosis to similar extent as their LAT-transfected counterparts or JCaM1/Lck cells (Fig. 17). Neither was apoptosis sensitivity impaired in ZAP-70-deficient Jurkat cells, suggesting that these downstream mediators of Lck are not involved in the apoptosis sensitization.

3.5 Apoptosis induced by Lck is independent of its myristoylation and membrane sequestration

It is well known that membrane targeting of Lck is crucial for T-cell receptor signalling, whereas a defect in the myristoylation property of Lck results in the disruption of its membrane sequestration and a consequent blockade of T-cell signalling. In order to establish whether membrane sequestration and myristoylation of Lck are required for stress-mediated apoptosis, JCaM A3 cells stably expressing a myristoylation-deficient form of Lck with a mutation at the Ser-6 position were used. In these cells, Lck cannot be sequestered to the plasma membrane (Yasuda et al., 2000). JCaM1 cells expressing a myristoylation-deficient form Lck were treated with etoposide along with wild-type Jurkat and wild-type Lck-expressing JCaM1 cells. Interestingly, the myristoylation-deficient Lck expressing JCaM1 cells did not show any significant difference in apoptosis in comparison to the wild type Lck expressing cells (Fig. 18). In addition, no significant difference in apoptosis was observed in JCaM1 A3 cells upon CD95 ligation. Thus, these results indicate that Lck myristoylation, which is essential for T cell stimulation, was not required for apoptosis sensitization.

3.6 Lck is activated upon etoposide treatment

Since drug-induced apoptosis is independent of TCR signalling and plasma membrane sequestration of Lck, the involvement of Lck itself was investigated by measuring its phosphorylation activity. To this end, Lck was immunoprecipitated from etoposide-treated and untreated cells and subjected to autophosphorylation assays using radioactive ATP. Treatment of JCaM1/Lck cells with etoposide

resulted in the activation of Lck as measured by its autophoshorylation (Fig. 19). In comparison to T-cell receptor stimulation by anti-CD3, the activation of Lck by etoposide was slightly delayed but clearly visible.

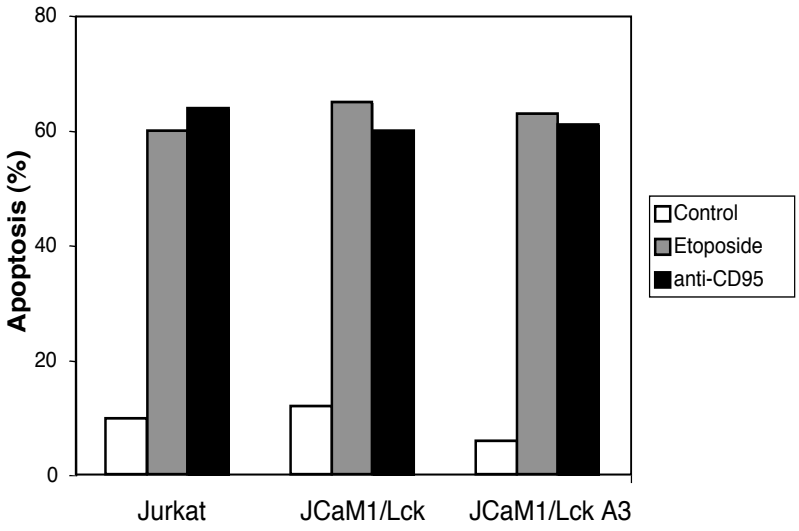

Figure 18: Apoptosis mediated by Lck is independent of Lck myristoylation and membrane sequestration. Jurkat cells expressing wild type Lck and the JCaM1 A3 cell line were stimulated with anti-CD95 or etoposide for 8 and 20 h, respectively. Cells were harvested, lysed in hypotonic lysis buffer and subsequently analysed for the presence of hypodiploid nuclei by flow cytometry. JCaM1 cells expressing a myristoylation-deficient Lck mutant (JCaM1 A3) were sensitized for both anti-CD95 and etoposide. No significant difference between JCaM1/Lck and JCaM1 A3 cells observed.

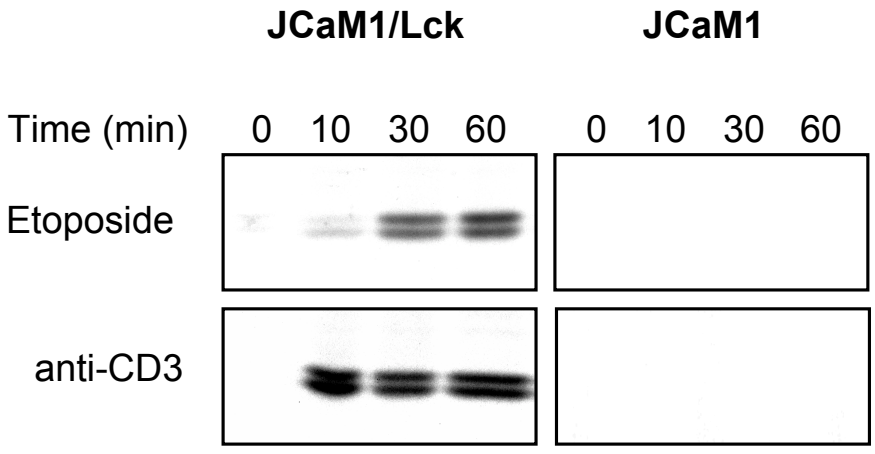

Figure 19: Etoposide treatment activates Lck. Lck-proficient and deficient JCaM1 cells were stimulated with etoposide or anti-CD3. Autophosphorylation analysis revealed that Lck activation upon etoposide stimulation occurred within 30 min and was slightly delayed in comparison to anti-CD3 treatment.

3.7 Activation of mitogen-activated protein kinases rather a consequence than a cause of Lck-mediated apoptosis

Several recent reports demonstrated that MAP (mitogen-activated protein) kinases interfere with mitochondrial apoptosis signalling. MAP kinases can target different Bcl-2 proteins and in most cases promote their apoptotic activity (Wada and Penninger, 2004). Since activation of these downstream mediators has been reported to occur following stimulation of Lck, their potential involvement in Lck-mediated apoptosis was investigated. As determined with phosphospecific antibodies, p38 was activated within 3-4 h of etoposide incubation in Lck-expressing JCaM1 cells but not in the Lck-deficient counterparts (Fig. 20). Within a similar time course activation of ERK1/2 was observed only in the presence of Lck. JNK activation was detected slightly earlier, and activation was only seen in the Lck-expressing cells. The constitutive expression of either kinase did not differ in the two cell lines.

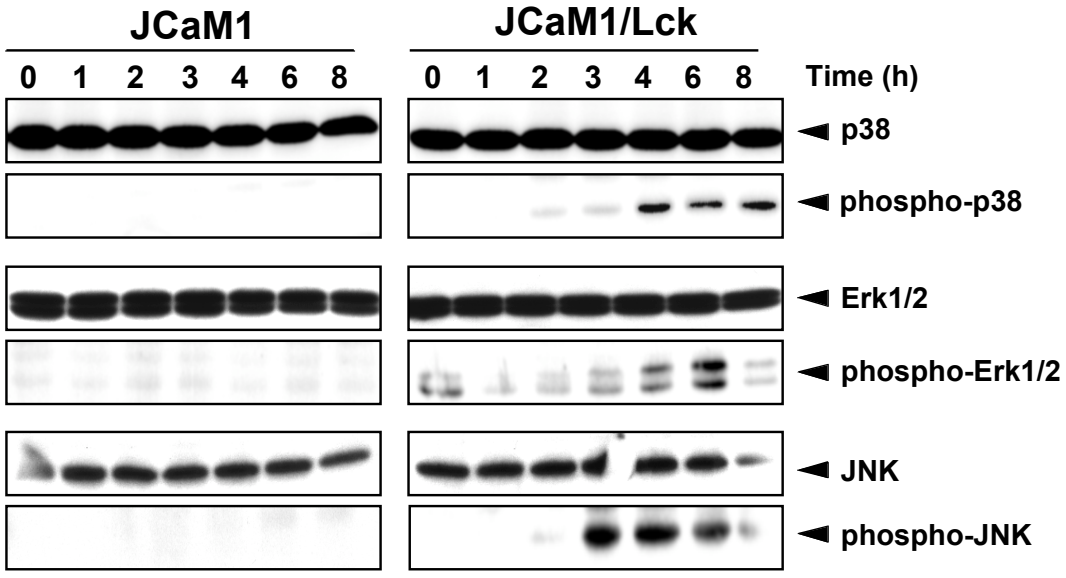

Figure 20: Mitogen-activated protein kinases are activated in the presence of Lck. Lck-deficient and proficient JCaM1 cells were stimulated with 5 µg/ml etoposide for different time points. Equal amounts of protein were separated by SDS-PAGE and immunoblotted against the indicated MAPK members. Analysis of cell extracts shows that MAP kinase family members were not activated in the Lck-deficient cells, whereas restoration of Lck triggered p38, ERK1/2 and JNK activation upon etoposide treatment.

Since the MAP kinases were activated upon etoposide stimulation, their functional role in apoptosis induction was investigated by using specific pharmacological kinase inhibitors. To this end, SB203580 was employed as an inhibitor of p38, UO126 as inhibitor of the ERK1/2 pathway, and SP600125 (JNK II) as a JNK-specific inhibitor. Lck-proficient JCaM1 cells were preincubated with the indicated concentrations (Fig. 21) of the inhibitors and treated with etoposide. Surprisingly, as assessed by flow cytometric analysis, inhibition of any of these MAP kinase cascades did not influence the apoptosis induced by etoposide. Thus, this suggested that MAP kinase cascades were at least not directly involved in apoptosis induction by etoposide.

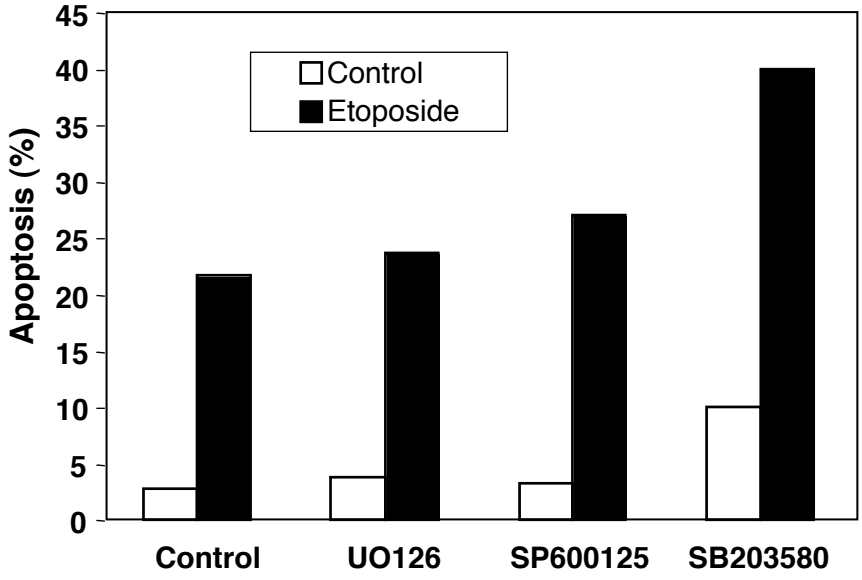

Figure 21: Lck-mediated apoptosis is independent of MAP kinases. Lck-reconstituted JCaM1 cells ($2x10^4$ cells) were either left untreated or preincubated for 1 h with the ERK inhibitor U0126 (10 µM), the JNK inhibitor SP600125 (1 µM), or the p38 inhibitor SB203580 (1 µM). Cells were then further stimulated with etoposide for 12 h. After incubation apoptosis was assessed by measurement of hypodiploid nuclei in a flow cytometer. The data represent the results from one out of three similar experiments. The analyses show that inhibition of p38, ERK1/2 and JNK did not influence apoptosis induced by etoposide.

3.8 Survival kinase Akt and proto-oncogene c-Myc are not involved in Lck-mediated apoptosis

It is well documented that growth factors can promote cell survival by activating phosphatidylinositide-3'-OH kinase and its downstream target, the serine-

threonine kinase Akt. Akt activation results in the phosphorylation and inactivation of the Bcl-2 family member Bad, thereby suppressing apoptosis and promoting cell survival (Datta et al., 1999). Treatment of both JCaM1 cell lines with etoposide did not affect the activation of Akt, as measured with phosphorylation-specific antibodies against Tyr-326 (Fig. 22). In Lck-expressing cells even a slightly higher activation of Akt was found which, however, would trigger a survival instead of a proapoptotic signal. The product of the c-myc proto-oncogene is an important positive regulator of cell growth and proliferation. However, deregulated expression of the c-Myc can lead to apoptosis under certain physiological and stress conditions (Pelengaris et al., 2002). Western blot analyses of both Lck-proficient and deficient JCaM1 cells showed that there was no deregulation of c-Myc expression upon etoposide stimulation. Both Lck-deficient and proficient JCaM1 cells expressed the same level of c-Myc (Fig. 22).

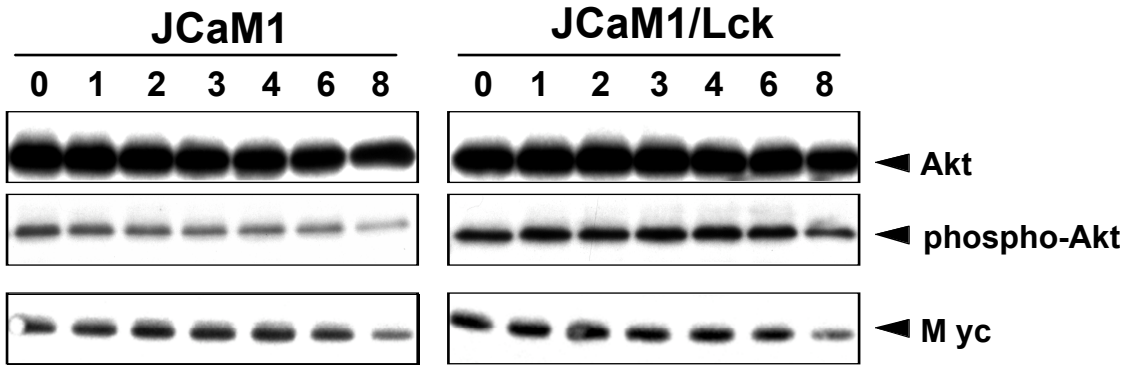

Figure 22: Survival kinase Akt/PKB or proto-oncogene Myc are not involved in etoposide induced apoptosis. Lck-expressing and deficient JCaM1 cells were stimulated with etoposide for various time points. Western blot analysis of the cell extracts showed no significant change in the phosphorylation of Akt upon stimulation. A slight decrease in the phospho-Akt in the Lck-proficient cells in latter time point was observed. Furthermore, no alterations in expression of the proto-oncogene c-Myc were detected.

3.9 Activation of transcription factors NF-κB and NF-AT

NF-κB is a transcription factor that plays an evolutionarily conserved and critical role in the triggering and coordination of immune responses. The wide variety of genes regulated by NF-κB includes those encoding cytokines, chemokines, adhesion molecules and others. Furthermore, NF-κB is central for the overall

immune responses through its ability to activate genes coding for regulators of apoptosis and cell proliferation, such as c-IAPs, A1, Bcl-x_L, CD95 ligand, c-Myc, and cyclin D1 that are critical for apoptotic processes (Kuracharzak et al., 2003). Nuclear factor of activated T cells (NF-AT) is a family of four related transcription factors also implicated in cytokine and early response gene expression in activated lymphocytes (Hogan et al., 2003).

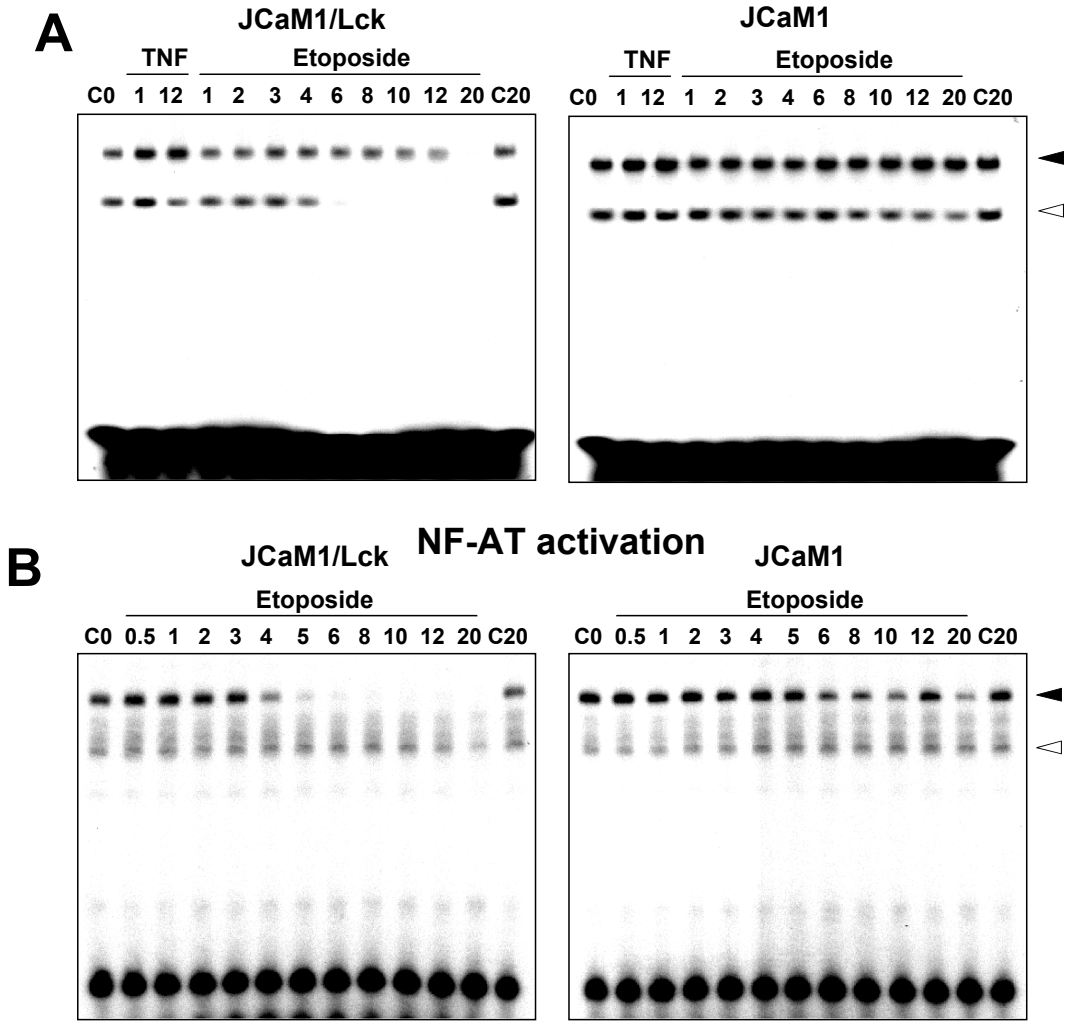

Figure 23: Activation of NF-□B and NF-AT during Lck-mediated apoptosis. Lck-proficient and deficient JCaM1 cells (1 x 10^6 cells per condition) were treated with etoposide or TNF for various time points. Total cell extracts were then prepared and incubated with the ^{32}P-labelled NF-□B and NF-AT-specific oligonucleotides. Electrophoretic mobility shift assays reveal no significant change in the DNA-binding activity of NF-□B and NF-AT, whereas in Lck-proficient cells degradation in both transcription factor levels was observed. The filled arrowhead indicates the position of the specific protein/DNA complex, the open arrowhead an unspecific DNA binding. C0: control before cell stimulation, C20: control after 20 hours.

Gel shift analyses were performed to investigate the activation of transcription factors NF-AT and NF-□B in the presence and absence of Lck during etoposide treatment. In contrast to classical proinflammatory stimuli, etoposide treatment did not induce significant activation of the transcription factors (Fig. 23). In Lck-expressing JCaM1 cells degradation of both transcription factors was observed, which can be attributed to the ongoing apoptotic process.

3.10 Lck specifically controls Bak expression

The mitochondrial pathway is essentially controlled by the ratio of pro- and anti-apoptotic Bcl-2 proteins. Since Lck deficiency resulted a defect in apoptosis probably upstream of cytochrome c release, expression levels of several proapoptotic Bcl-2 family members such as Bax, Bak, Bad and Bim and antiapoptotic proteins such as Bcl-2, Bcl-x_L were investigated. Unexpectedly, apoptosis-resistant JCaM1 cells revealed not increased but even slightly reduced levels of Bcl-2 and Bcl-x_L compared to parental Jurkat and Lck-transfected JCaM1 cells (Fig. 24). This might concur with observation that Lck stimulates the expression of these antiapoptotic molecules. Jurkat cells and their Lck-deficient counterparts expressed similar levels of Bax. Striking, however, was the observation that expression of the Bax-related proapoptotic Bcl-2 member Bak was almost completely absent in JCaM1 cells, whereas expression of Lck resulted in expression levels of Bak similar to wild-type Jurkat cells (Fig. 26.).

In addition, the expression levels of inhibitor of apoptosis proteins (IAPs) in the different Jurkat cell lines were compared. Members of the IAP family are known to directly bind to and inhibit active caspases (Salvesen and Duckett, 2002). However, expression of neither c-IAP1, c-IAP2 nor XIAP differed in the individual cells (Fig. 24), indicating that these apoptosis inhibitors were presumably not responsible for the apoptosis resistant-phenotype of Lck-deficient cells.

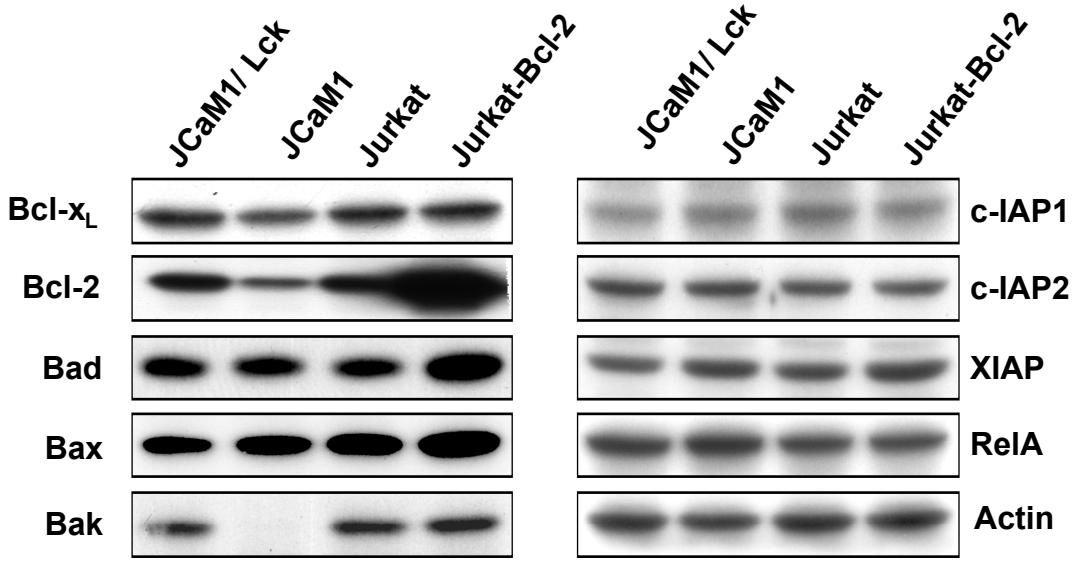

Figure 24: Lck-deficient JCaM1 cells do not express the Bcl-2 family member Bak. The expression status of Bcl-2, IAP family proteins and NF-□B RelA in the different Jurkat cell lines was investigated by Western blot analysis. Total cell extracts of JCaM1, JCaM1/Lck, parental Jurkat cells and cells overexpressing Bcl-2 were immunoblotted with antibodies against the antiapoptotic proteins Bcl-x_L and Bcl-2 as well as against the proapoptotic family members Bad, Bax and Bak *(left panels).* Cell extracts were analyzed in parallel for the expression of the IAP family members c-IAP1, c-IAP2 and XIAP as well as RelA *(right panels)*. Immunoblotting against actin served as a control for equal protein loading.

3.11 Subcellular localization of Bcl-2 family members in JCaM1 cells

In the following experiments, the subcellular localization of the Bcl-2 proteins in the course of etoposide treatment was investigated. Therefore, cytosolic and mitochondrial fractions of JCaM1 cells and their Lck-expressing counterparts were prepared. In support with the previous experiments (Fig. 15), cytochrome c was only released from mitochondria of Lck-expressing, but not of Lck-deficient cells. Both Bcl-2 and Bcl-x_L were mainly found in the mitochondrial fraction and remained relatively constant during etoposide treatment (Fig. 25). Also Bax was mainly found at the mitochondria, even in untreated cells, although compared to other Bcl-2 members both the cytosolic and mitochondrial Bax levels were relatively low. Furthermore, the 33 kD form of Bim appeared to be expressed at relatively low levels in the cytosol and was not in the mitochondria of all Jurkat cell lines, although one cannot exclude that this finding might be due to a weak affinity

of the antibodies used. Importantly, fractionation in cytosolic and mitochondrial extracts confirmed that Bak was strikingly absent in JCaM1 cells, whereas considerable levels of Bak were detected in mitochondria from JCaM1/Lck cells (Fig 25). In line with the previous data, during drug treatment Bak was not translocated from the cytosol to mitochondria but rather constitutively found in the mitochondrial compartment.

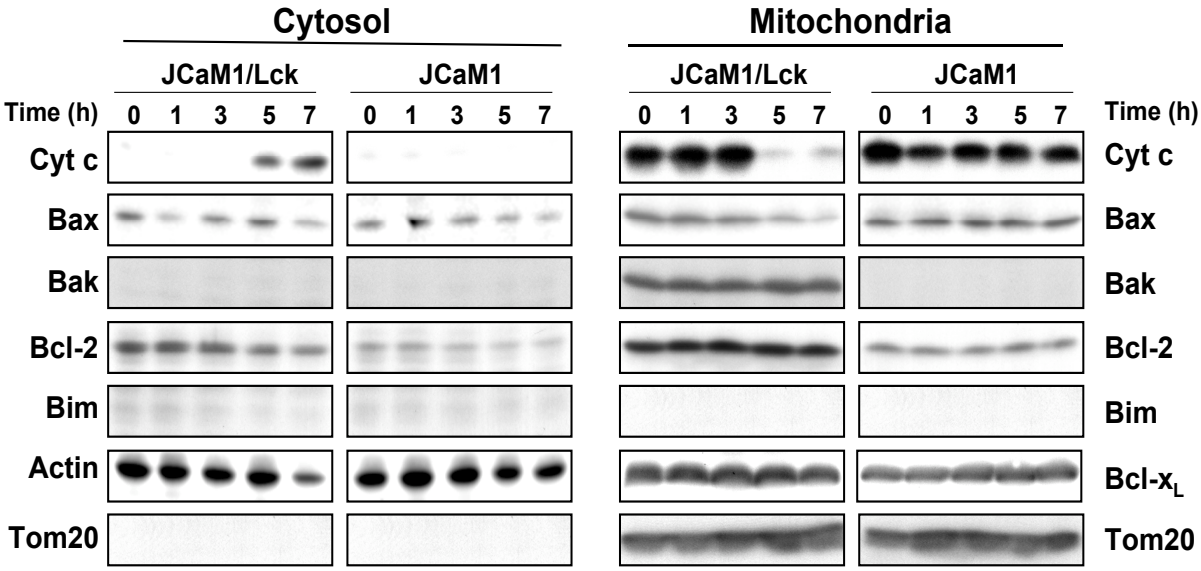

Figure 25: Subcellular localization of cytochrome c and Bcl-2 proteins during etoposide-induced apoptosis. JCaM1/Lck and JCaM1 cells were treated for the indicated times with etoposide and fractionated in a cytosolic and a heavy-membrane fraction containing mitochondria. The fractions were immunoblotted against different pro- and antiapoptotic Bcl-2 proteins. Actin served as control for equal protein loading of cytosolic extracts. Fractions were reprobed with an antibody against the outer mitochondrial membrane marker Tom20. Note that expression of Bak is completely absent in JCaM1 cells, whereas the expression levels of the other Bcl-2 protein are not altered in the presence or absence of Lck.

3.12 The absence of Lck transcriptionally downregulates the Bcl-2 family member Bak

To investigate whether Lck regulates Bak at the transcriptional level, RT-PCR analysis of Bak mRNA expression was performed. As shown in Figure 26, Bak-specific transcripts were almost absent in JCaM1 cells, whereas Bak mRNA was

expressed in the parental Jurkat and Lck-transfected cells. The expression levels of the antiapoptotic Bcl-2 family members Bcl-x and Bcl-2 itself were not altered.

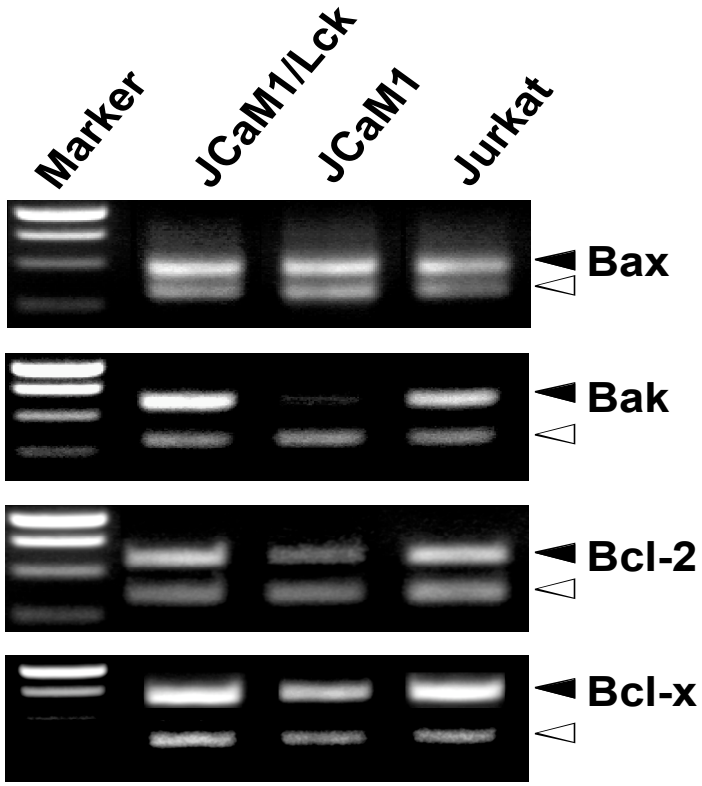

Figure 26: JCaM1 cells do not express Bak mRNA. Transcriptional expression of different Bcl-2 family members in Lck-deficient and proficient JCaM1 cells and parental Jurkat cells was analyzed by reverse transcription-PCR using oligonucleotide primers specific for Bax, Bak, Bcl-2 and Bcl-x. The PCR products were separated on agarose gels and visualized by ethidium bromide staining. The closed arrowheads indicate the position of the relevant PCR products, the open arrowheads a GAPDH PCR product that was amplified in parallel.

In order to substantiate whether any of these Bcl-2 family members is upregulated upon treatment of cells with anti-cancer drugs or a T cell receptor stimulus, JCaM1 cells and their Lck-expressing counterpart were stimulated with etoposide and phytohemagglutinin (PHA) for various time points. From the isolated total RNA RT-PCR analysis were then performed for Bax, Bak, Bcl-2 and Bcl-x-from the isolated total RNA. The mRNA expression pattern for any of these Bcl-2 members did not change, when JCaM1 and JCaM1/Lck cells were stimulated with either etoposide or PHA (Fig. 27). Stimulation of the cells did also not affect the expression levels of other pro- and antiapoptotic Bcl-2 proteins (Fig. 27). Together, these data show

that absence of Lck is required for efficient Bak expression at the transcriptional level.

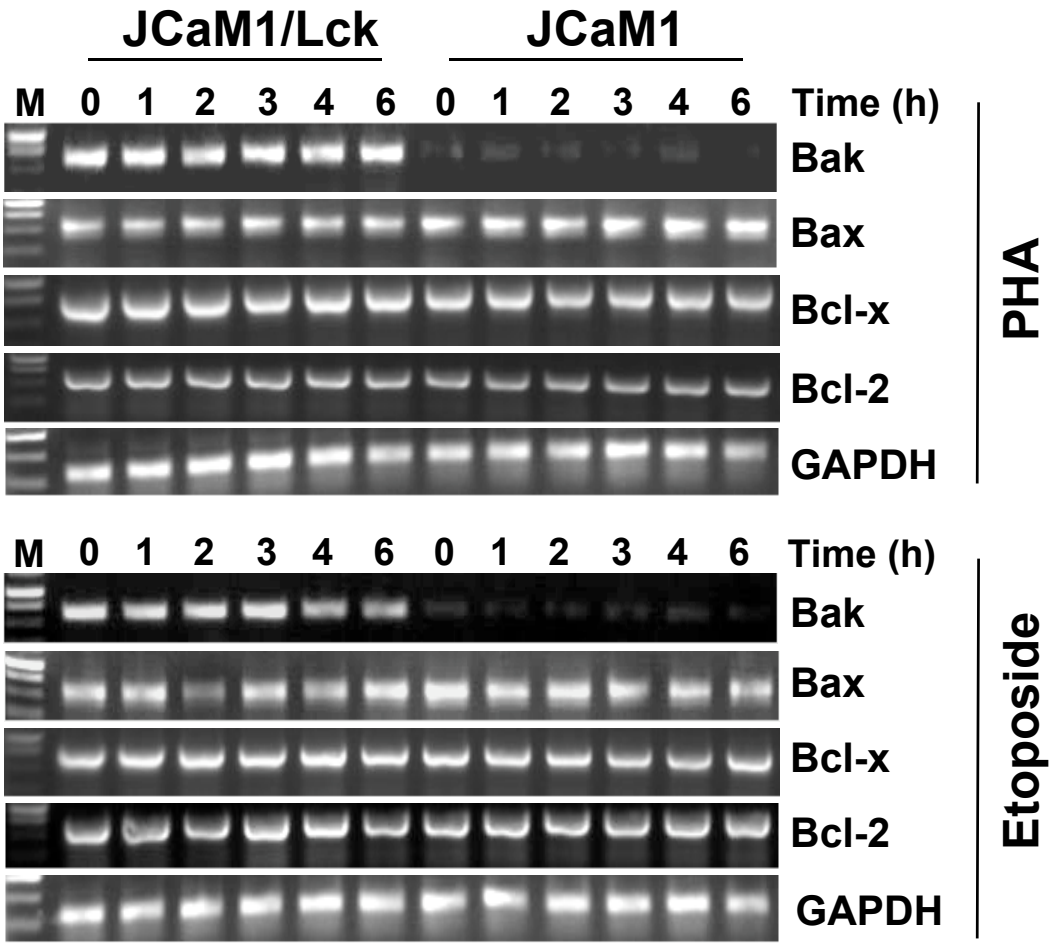

Figure 27: Bcl-2 family members mRNA levels during cell stimulation. Lck-proficient and deficient JCaM1 cells were stimulated with etoposide (5 µg/ml) or phytohemagglutinin (PHA, 50 µg/ml) for different time points, before RNA was extracted and subjected to RT-PCR analysis. 500 ng of total RNA was used for RT-PCR analysis. The results show that there is no change in the levels of anti- and pro- apoptotic Bcl-2 family members during cell stimulation, whereas Bak mRNA is almost completely absent in Lck-deficient cells.

3.13 Bak undergoes a conformational change in Lck-expressing cells

Several lines of evidence suggests that a Bcl-2 controlled and irreversible commitment to cell death is an event occurring before the so-called execution phase of apoptosis, which is largely regulated by caspases. Proteins of the Bcl-2 family have been found to interact with each other to set a survival threshold for the cell. Important for this threshold are the Bcl-2 multidomain proteins Bak or Bak. Both Bax and Bak undergo a conformational change in response to apoptotic

stimuli, which mediates exposure of their occluded N-terminus (Desagher et al., 1999). This conformational change mediates the assembly of both proteins into homomultimers with channel-forming properties in the mitochondrial membrane, which finally results in cytochrome c release (Wei et al., 2000). The conformational change of Bak or Bax is inducible by BH3-only proteins and inhibitable by Bcl-2.

The conformational change of Bak and Bax can be analyzed using conformation-specific antibodies against their normally occluded N-terminus. FACS analysis with such antibodies showed that in JCaM1 cells no conformational change of Bak was observed, and a slight activation of Bax occurred in response to etoposide treatment (Fig. 28A). In contrast, JCaM1/Lck cells showed a pronounced conformation change of Bak upon apoptosis induction (Fig. 28B). Interestingly, however, under these conditions JCaM1/Lck also displayed a stronger activation of Bax compared to the Bax-deficient JCaM1 cells. This result suggested a degree of functional cooperation of Bax with Bak regarding its conformational activation.

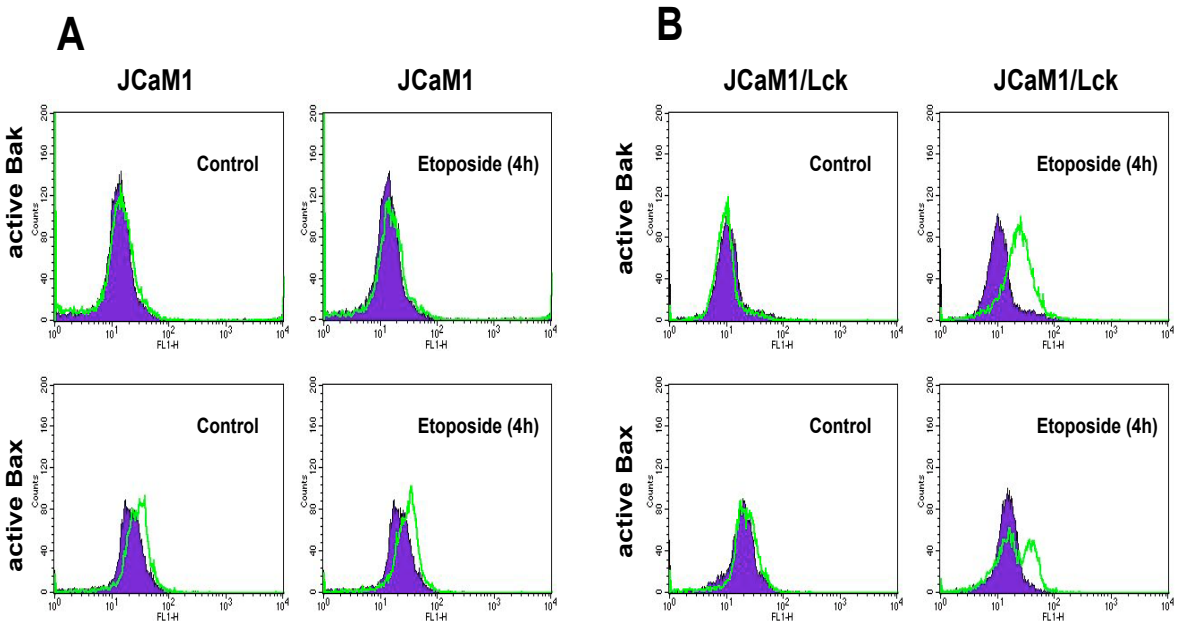

Figure 28: Etoposide induces a Bax and Bak conformational change in JCaM1/Lck cells. Lck-deficient (A) and proficient JCaM1 cells (B) were stimulated with etoposide for 4 h. After incubation cells were harvested, fixed in paraformaldehyde and permeabilized with digitonin. Cells were then stained with conformation-specific antibodies against Bak and Bax (solid lines) or with isotype-matched control antibodies (filled histograms). After incubation with fluorescent secondary antibodies cells were analysed by flow cytometry. (A) Etoposide induces a slight Bax conformational change in Lck-deficient JCaM1 cells. (B) In contrast, in Lck-reconstituted JCaM1 cells both Bak and Bax undergo a pronounced conformational change.

3.14 Ectopic expression of Bcl-2 abrogates a Bak conformational change

It is well documented that the antiapoptotic protein Bcl-2 interferes with the mitochondrial apoptotic pathway. Therefore, the effect of Bcl-2 on the conformational change of Bak during genotoxic stress was investigated. To this end, Jurkat cells overexpressing Bcl-2 were treated with etoposide and analyzed for the proapoptotic conformational change of Bak by flow cytometry. The analyses revealed that the ectopic expression of Bcl-2 completely abrogated the proapoptotic conformational change of Bak, which is a key step for mitochondrial membrane permeabilization (Fig. 29).

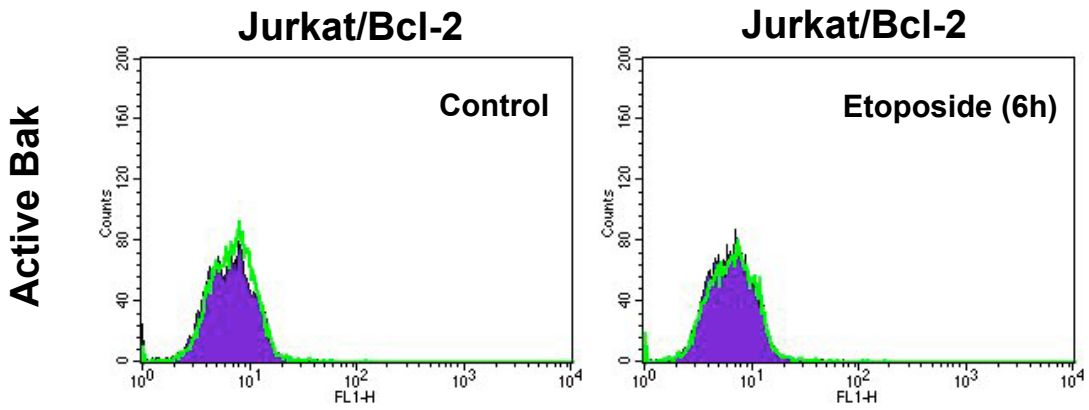

Figure 29: The conformational change of Bak is blocked by ectopic expression of Bcl-2. Jurkat Bcl-2-overexpressing cells were stimulated with etoposide. After 6 h, cells were harvested and stained with the conformation-specific antibody against Bak. The filled histograms show the staining with isotype-matched control antibodies, the green line indicates the specific staining for the active form of Bak. Flow cytometric analysis revealed that expression of Bcl-2 blocked the Bak conformational change.

3.15 Truncated Bid induces a Bak conformational change only in mitochondria from JCaM1/Lck cells

It has been described that caspase-8 cleaves Bid to tBid, which translocates to mitochondria and induces the oligomerization of Bax and Bak, resulting in the release of cytochrome c (Li et al., 1998; Luo et al., 1998). It has been suggested that the BH3 domain of Bid interacts with Bak and induces an “open” conformation of Bak, thereby exposing its N-terminus. This open (active) conformation of Bak potently induces the assembly of Bak proteins into homooligomers with channel-forming activity. To further substantiate a role of Bak activation in Lck-mediated

apoptosis, Lck-expressing JCaM1 cells were permeabilized and treated with recombinant tBid, representing the proapoptotic caspase-8-generated form of Bid. Addition of exogenous tBid rapidly induced the proapoptotic conformational change of Bak, but not of Bax *in vitro* (Fig. 29).

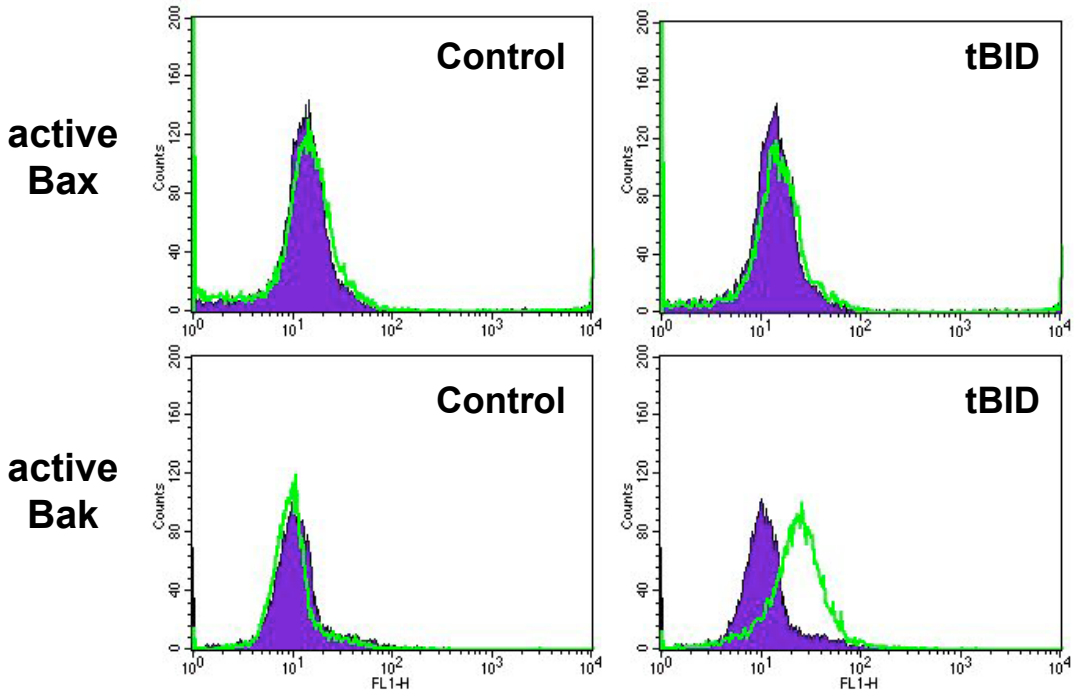

Figure 30: tBid induces a conformational change of Bak. Lck-proficient JCaM1 cells were incubated for 30 min in PBS containing 2 µM recombinant tBid and digitonin. Thereafter, cells were fixed in paraformaldehyde and incubated with conformation-specific antibodies for Bak and Bax. Samples were then stained with the respective secondary antibodies conjugated to Alexafluor488 and analysed by flow cytometry. The analysis shows that tBid induces a conformational change of Bak, but not of Bax.

3.16 Recombinant tBid induces cytochrome c release only in Bak-expressing mitochondria *in vitro*

To substantiate these findings, further experiments were performed with isolated mitochondria. Subcellular fractionation of intact mitochondria was carried out for both Lck-deficient and Lck-reexpressing JCaM1 cells. Mitochondria from both cell lines were treated with recombinant tBid for 30 min *in vitro*. Western blot analysis revealed that mitochondria from Lck-expressing cells responded to tBid treatment and released cytochrome c, whereas mitochondria from Lck-deficient cells did not

release cytochrome c upon tBid incubation (Fig. 31). This observation is strongly in line with the proapoptotic conformational change of Bak. Thus, these results confirm that the defect in apoptosis displayed by the Lck-deficient cells is located at the mitochondria and mediated by Bak deficiency.

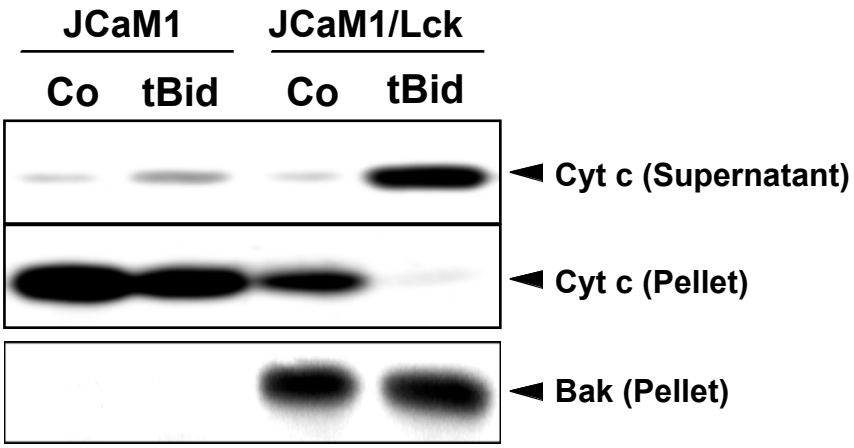

Figure 31: tBid induces cytochrome c release from mitochondria of JCaM1/Lck but not of JCaM1 cells. Intact mitochondria were isolated from the Lck-deficient and proficient JCaM1 cells and incubated with 2 µM recombinant tBid for 30 min. Samples were then centrifuged, and both the supernatant and the pellet were analyzed for cytochrome c by Western blot analysis. The blots were reprobed with anti-Bak confirming the absence of Bak in mitochondria from JCaM1 cells.

3.17 SH2 and SH3 domains are required for the expression of Bak

It has been reported that the Src homology domains SH2 and SH3 of Lck can trigger cell signalling independently of the kinase domain (Straus and Chan, 1996; Denny and Kaufman, 1999). In order to investigate the contribution of the SH2, SH3 and the kinase domains for the expression of Bak, an Lck expression construct lacking the kinase domain was generated (Fig. 32). JCaM1 cells were transiently transfected with expression constructs encoding either wild-type Lck or the kinase-deleted mutant. Western blot analysis of the cell extracts showed that Lck-deficient cells transfected with full-length Lck plasmid restored Bak expression as expected (Fig. 33). Surprisingly, JCaM1 cells transfected with the kinase mutant were also able to restore Bak protein expression. This suggested that the

Src homology domains of Lck were sufficient for the expression of the proapoptotic Bcl-2 member Bak, whereas the kinase domain was not required.

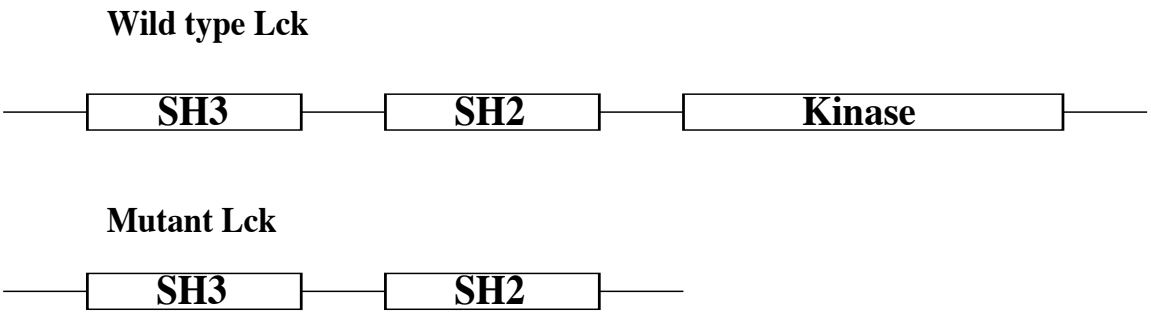

Figure 32: Schematic representation of the wild-type and kinase-mutant Lck constructs. The cDNA of full-length wild-type Lck and a kinase domain-deleted version were cloned at HindIII and NotI restriction sites in the mammalian expression vector pRK5 under the control of the CMV promoter.

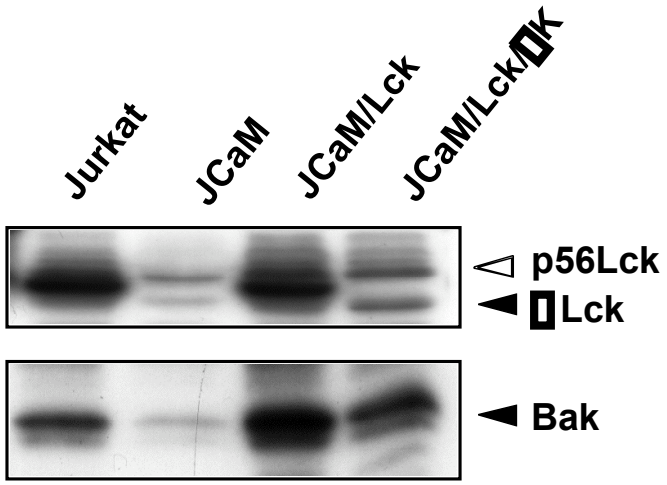

Figure 33: Effect of kinase-deleted Lck on Bak expression. Lck-deficient JCaM1 cells were transiently transfected (10^7 cells per transfection) with 5 □g of the kinase-deficient mutant Lck□K. Western blot analysis of the total cell extracts showed that the SH2/SH3 domains of Lck were sufficient to restore the expression of Bak in JCaM1cells.

3.18 Herbimycin A blocks Lck-mediated apoptosis induced by anticancer drugs

In order to substantiate the importance of Lck for genotoxic stress-induced apoptosis, also a pharmacological inhibitor of Lck, herbimycin A, was used. Herbimycin A is known to interfere with Lck by promoting its proteasomal

degradation and has been widely used to study the signalling mechanisms mediated by Lck (Sepp-Lorenzino et al., 1995). JCaM1 cells proficient for Lck were preincubated with herbimycin A and then treated with etoposide for an incubation period of additional 20 hours. Lck-proficient JCaM1 cells, which were preincubated with herbimycin A, were protected and only about 30% of the cells underwent from apoptosis in response to etoposide treatment (Fig. 34). In contrast, more than 85% of cell death was observed in the absence of the inhibitor.

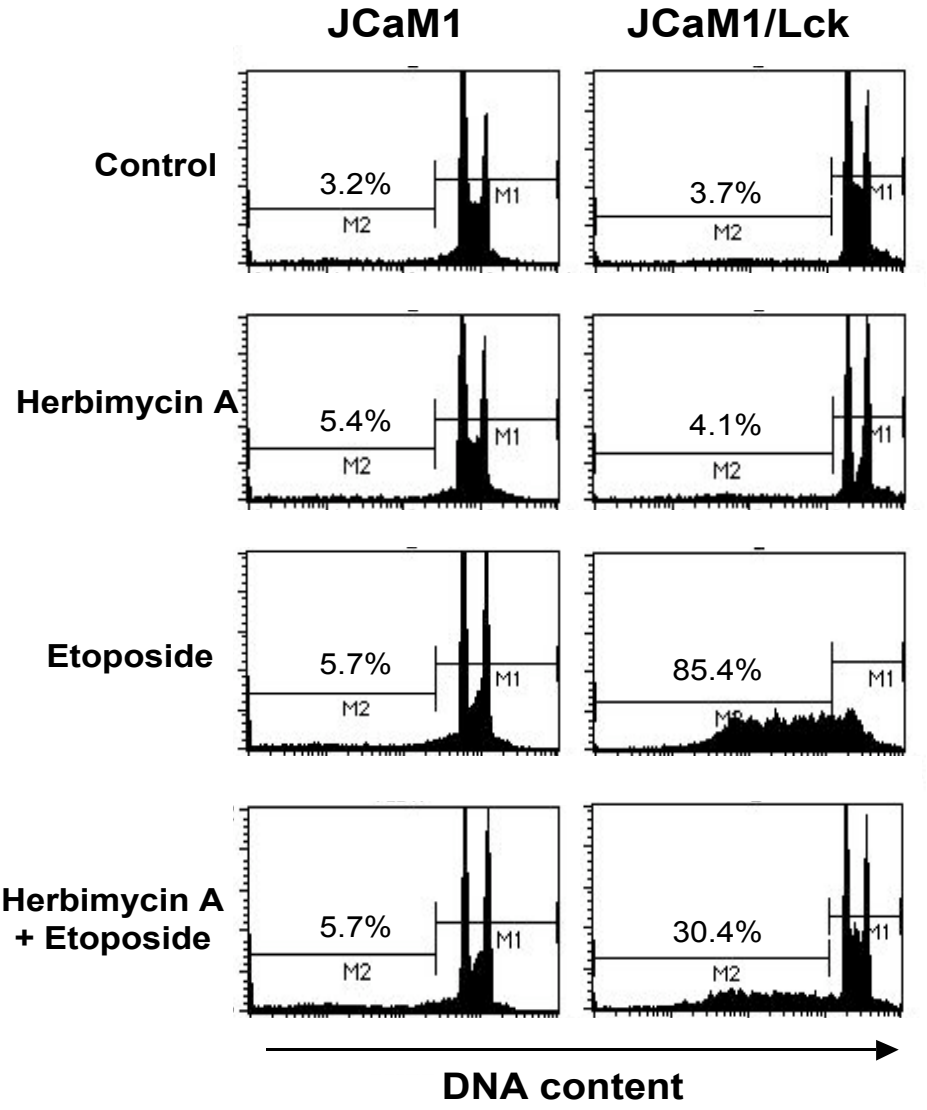

Figure 34: Herbimycin A inhibits Lck-mediated apoptosis. JCaM1 cells proficient and deficient for Lck ($3x10^4$ per condition) were preincubated with herbimycin A for 1 h and then stimulated with etoposide. After 20 h cells were harvested and suspended in a hypotonic buffer containing 50 µg/ml propidium iodide. Flow cytometric analysis for hypodiploid nuclei showed that JCaM1/Lck cells preincubated with herbimycin A became resistant against etoposide-induced apoptosis.

Since herbimycin A protected JCaM1/Lck cells against apoptosis, the effect of the Lck inhibitor on the conformational change of Bak was also investigated. Following preincubation with herbimycin A, cells were stimulated with etoposide. Flow cytometric analysis of the Bak status with the conformation-specific antibody revealed that herbimycin A blocked the conformational change induced upon etoposide treatment (Fig. 35).

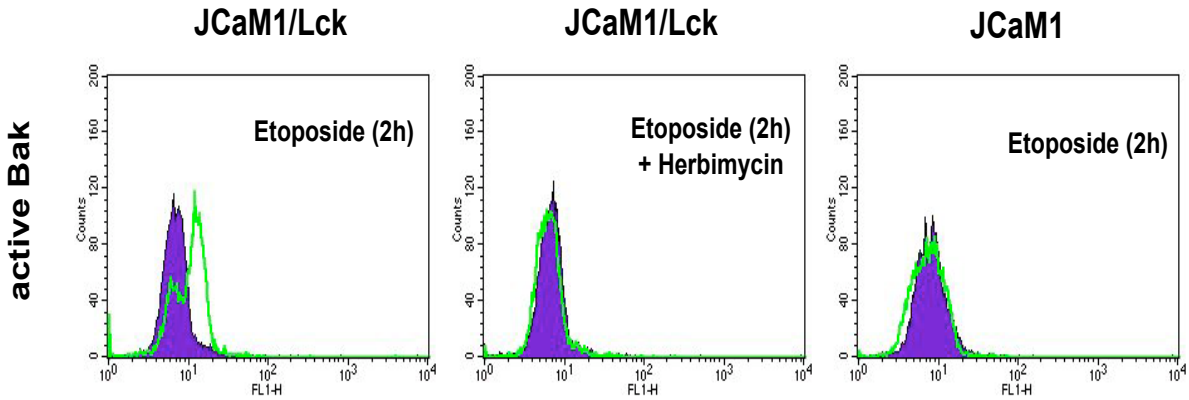

Figure 35: Herbimycin A blocks the proapoptotic conformational change of Bak. Lck-deficient and proficient JCaM1 cells (10^6 cells per condition) were preincubated with herbimycin A for 1 h and then stimulated with etoposide. After 2 h cells were harvested, fixed in paraformaldehyde and stained with the Bak conformation-specific antibody. Flow cytometric analysis showed that herbimycin A strongly prevented the etoposide-induced conformational change of Bak.

3.19 Role for Lck in death receptor-mediated apoptosis

Based on the usage of different CD95 downstream signalling pathways, cells can be divided in two types (Scaffidi et al., 1999). So-called type I cells recruit and activate large amounts of caspase-8 in the death-inducing signalling complex (DISC), which leads to the rapid activation of caspase-3. In contrast, in type II cell only a weak DISC formation is found, which is insufficient to directly induce apoptosis. In type II cells induction of apoptosis rather requires a mitochondrial amplification mechanism of the initial DISC signal that is regulated by capase-8-mediated cleavage of Bid. Following translocation to mitochondria, cleaved Bid triggers cytochrome c release and subsequent caspase-9 activation. Also Jurkat T-cells require such as an amplification loop to undergo efficient apoptosis.

In the previous experiments (Fig. 16), it was observed that apoptosis induced by anti-CD95 cells was less pronounced in Lck-deficient JCaM1 cells as compared to Lck-transfected cells. To further investigate this finding, CD95-mediated apoptosis was analyzed in a time course experiment. Incubation of Lck-proficient and deficient JCaM1 cells as well as wild-type Jurkat cells (J16) with agonistic anti-CD95 antibodies revealed that CD95-mediated apoptosis was strongly reduced at early time points in the absence of Lck (Fig. 36). However, at later time points (12 hours) also JCaM1 cells underwent efficient apoptosis. Therefore, it was conceivable that the delay of anti-CD95-induced apoptosis in JCaM1 cells could be due to a defective amplification loop caused by the absence of Bak.

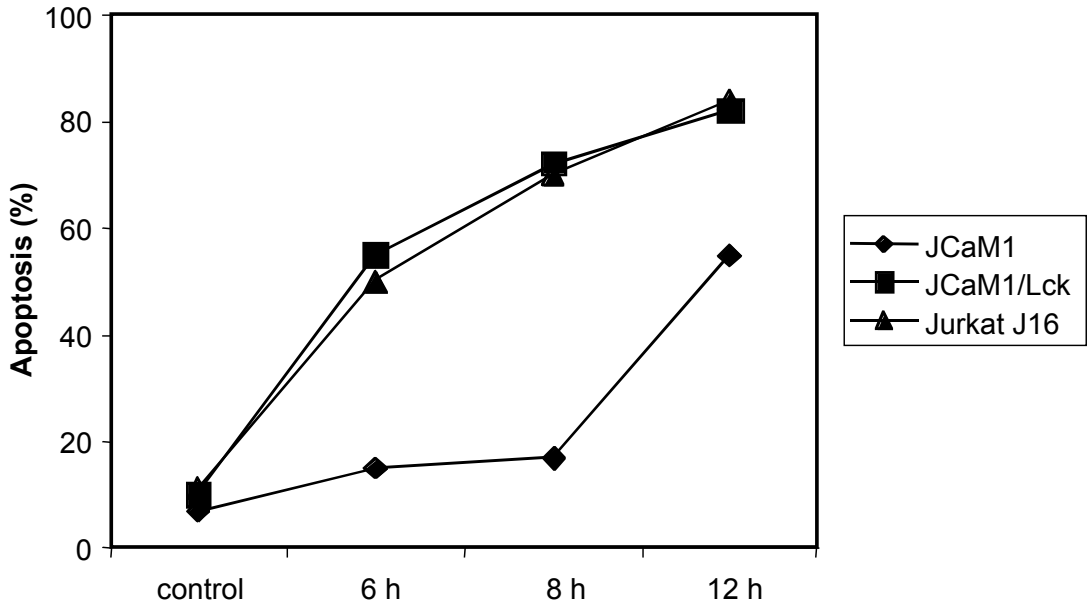

Figure 36: Lck deficiency results in delayed anti-CD95-induced apoptosis. Parental Jurkat (J16) cells as well as Lck-proficient and deficient JCaM1 cells ($3x10^4$ cells) were stimulated with anti-CD95 (500 ng/ml) for different time points. Cells were harvested and suspended in hypotonic lysis buffer containing 50 µg/ml propidium iodide. Cells were then measured for hypodiploid nuclei as an apoptotic marker in a flow cytometer. The analysis showed that anti-CD95 treatment did not induce apoptosis as rapidly in the absence of Lck as in the presence of the kinase.

To investigate whether the absence of a mitochondrial amplification loop was responsible for the delay in apoptosis upon CD95 ligation, also a Jurkat cell line (J.g1) was employed that was deficient for caspase-9. Because of the absence of caspase-9, the mitochondrial apoptotic pathway cannot be executed in these cells.

Upon CD95 ligation J.g1 cells underwent apoptosis much more rapidly and efficiently as compared to the JCaM1 cells, which was in particular observed at early time of CD95 stimulation (Fig. 37). Thus, this suggested that the delay of CD95-mediated apoptosis in JCaM1 cells might not be caused only by the absence of a mitochondrial and caspase-9-dependent amplification loop, but also by additional mechanisms.

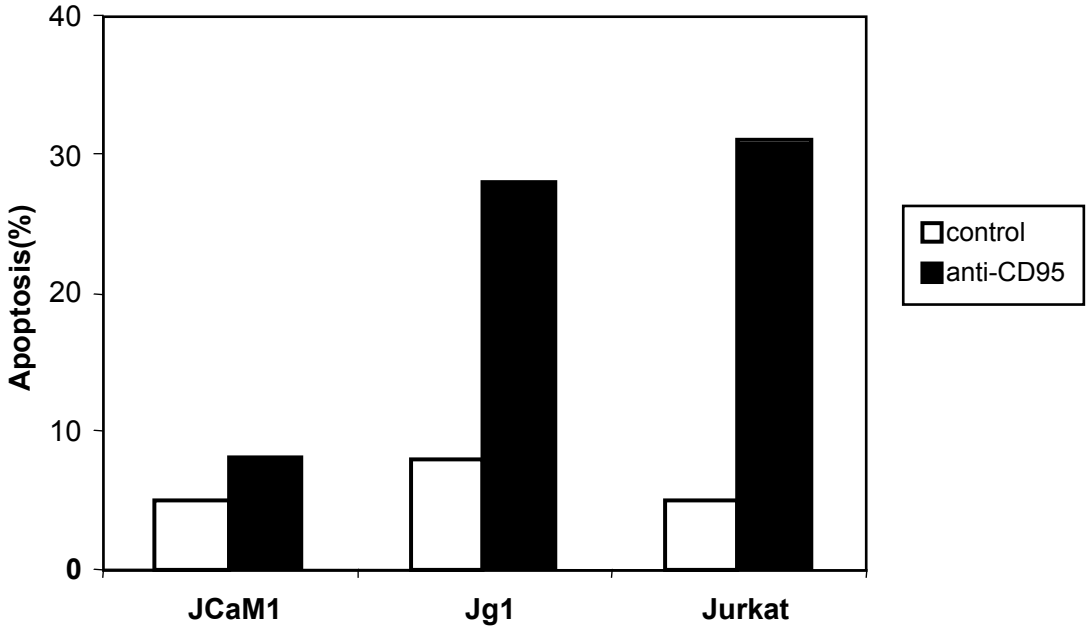

Figure 37: Comparison of CD95-mediated apoptosis in Jurkat cells lacking either Lck or caspase-9. Lck-deficient JCaM1 cells, caspase-9-deficient Jg1 cells and Jurkat wild-type cells were treated with anti-CD95. After 6 h apoptosis was assessed by flow cytometric measurement of hypodiploid nuclei. The analysis revealed that apoptosis was even more strongly reduced in JCaM1 cells as compared to caspase-9-deficient Jg1 cells, suggesting that a defective mitochondrial pathway was not the only reason for the delay of CD95-mediated apoptosis caused by the absence of Lck.

3.20 The absence of Lck delays CD95-mediated caspase activation

To investigate the role of Lck in CD95-mediated caspase activation, Lck-proficient and deficient JCaM1 cell were stimulated with anti-CD95 for various time points. Total cell extracts were then assessed for the activation of caspase-8, caspase-3, caspase-9 as well as Bid cleavage by Western blot analysis. As shown in Figure 38, caspase-8 processing occurred much faster in the Lck-proficient cell than in the deficient JCaM1 cells. Within two hours of stimulation caspase-8 was cleaved

into the p43/41 intermediate cleavage fragments, whereas in Lck-deficient cells caspase-8 cleavage did not occur earlier than after 6 hours of CD95 stimulation. Approximately at the same times points, subsequent cleavage to the p18 active subunit of caspase-8 was observed. As a result of delayed caspase-8 activation, Bid was cleaved only after 8 hours of stimulation in Lck-deficient JCaM1 cells (Fig. 38). In Lck-proficient cells caspase-9 was processed as soon as the cleaved form of Bid appeared, while in Lck-deficient cells no processing of caspase-9 was observed. As a consequence of delayed caspase-8 and caspase-9 activation, proteolytic cleavage of executioner caspase-3 was also delayed (Fig. 38). Together, these data suggest that Lck is involved in the acceleration of caspase activation.

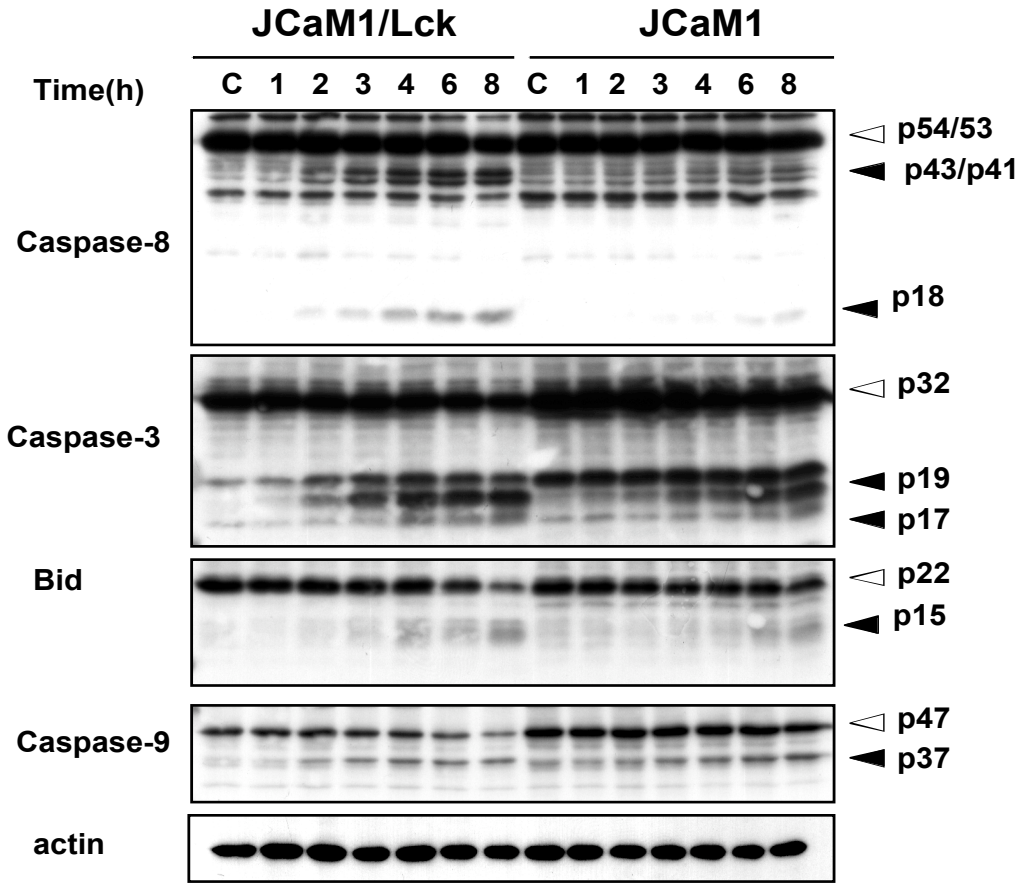

Figure 38: The absence of Lck delays caspase activation upon CD95 ligation. Both Lck-proficient and deficient JCaM1 cells ($6x10^4$) were stimulated with anti-CD95 for different time points. Western blot analysis of the cell extracts showed that in Lck-deficient cells caspase-8 activation was strongly reduced in comparison to the Lck-expressing cells. Furthermore, no proteolytic cleavage of caspase-9 was observed. As a result, activation of executioner casase-3 and Bid cleavage were also delayed in Lck-deficient cells.

3.21 Lck associates with the death-inducing signalling complex

Because CD95-mediated apoptosis and caspase activation was impaired in Lck-deficient cells, it was investigated whether Lck interferes with the DISC formation. Triggering of CD95 results in receptor trimerization and a rapid recruitment of FADD and caspase-8 into the DISC, which can be co-immunoprecipitated with CD95 antibodies. Stimulation of cells with anti-CD95 for 15 min resulted in the recruitment of caspase-8 into the DISC, which was much more pronounced in the absence than in the presence of Lck (Fig. 39). In contrast to caspase-8, considerable amounts of FADD seemed to be recruited to the DISC in both cell lines. Interestingly, precipitation of the DISC with anti-CD95 also co-immunoprecipitated the Lck protein, suggesting that the kinase was associated with the DISC. The recruitment of Lck into the DISC was clearly more pronounced following CD95 stimulation. Thus, these results indicate that the enhancement of caspase-8 activation by Lck might be caused by a direct recruitment of Lck to the vicinity of the death receptor.

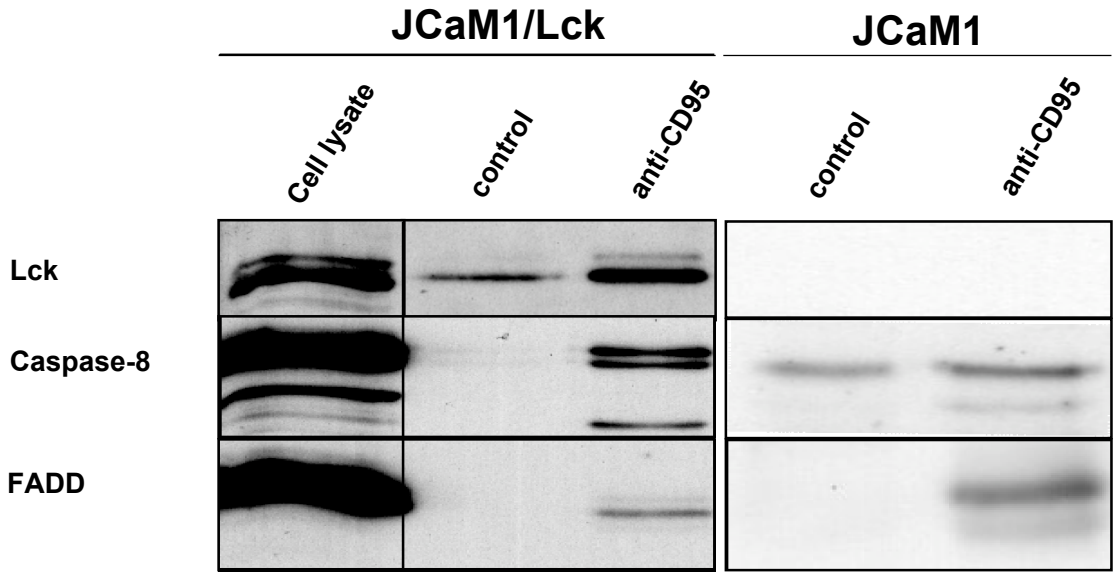

Figure 39: Lck is associated with the DISC upon CD95 ligation. Lck-proficient and deficient JCaM1 cells ($1x10^7$) were stimulated with anti-CD95 for 15 min. Thereafter, the DISC was immunoprecipitated by incubating the cell lysate with anti-CD95 and protein A-sepharose beads. After incubation beads were washed and incubated in SDS loading buffer. Western blot analysis shows an increased incorporation of Lck into the DISC along with caspase-8 and FADD in response to CD95 stimulation.

3.22 Bak promotor analysis

In order to get an idea of how Lck might regulate the proapoptotic Bcl-2 family member Bak, a promoter analysis of the Bak gene was carried out. The human Bak gene is located on chromosome 6; 6p21.3 (Herberg et al., 1998). However, no functional promotor studies of the Bak gene have yet been reported. The functional organization of promotors is known to consist of individual transcription factor binding sites that are often combined in functional promotor modules. In order to investigate the Bak promoter, 4000 nucleotides upstream of the transcriptional start site of the Bak gene were analysed for potential transcription factor binding sites using an online programme (www.genomatix.de).

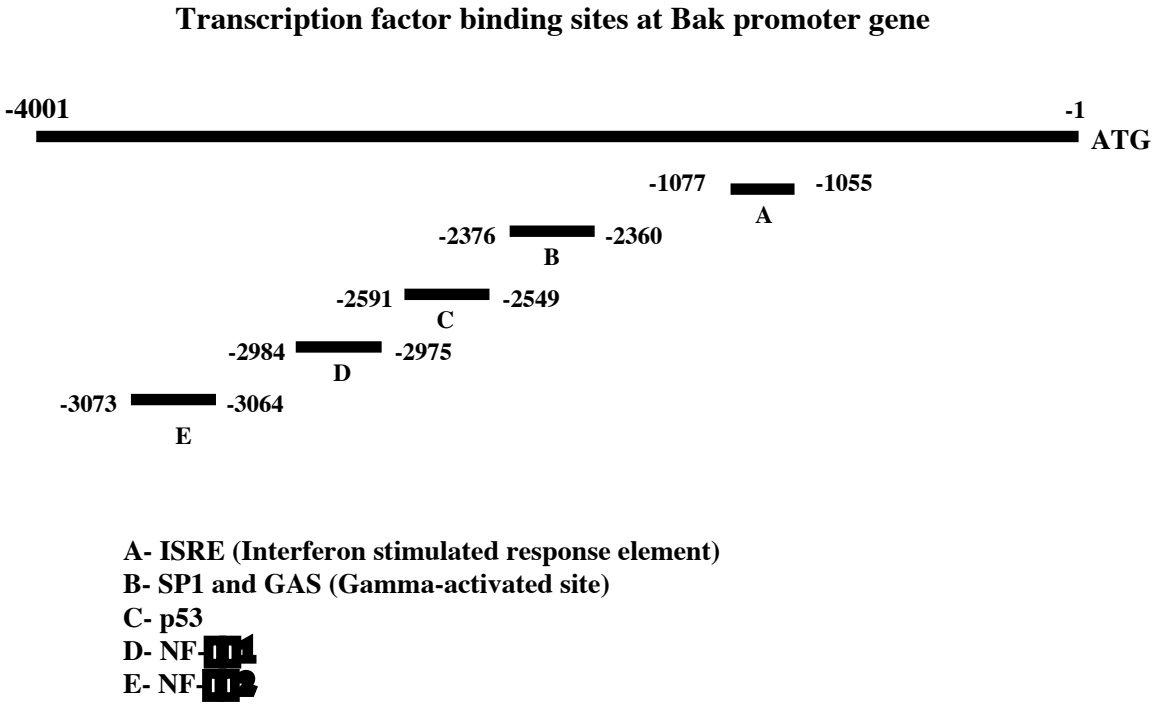

Figure 40: Bak promotor and potential transcription factor binding sites. 4000 basepairs upstream of the transcriptional start site of the Bak gene were analysed for potential transcription factor binding sites. The analysis indicated the following potential binding sites: ISRE (interferon stimulated response element), SP1 and GAS (gamma-interferon activated site), p53, NF-κB1 and NF-κB2.

The computer analysis revealed no TATA-box, but putative binding sites for transcription factors NF-κB, SP1 and p53. Moreover, two interferon-responsive

sites, namely an ISRE (interferon-stimulated response element), and a GAS (gamma interferon-activated site) motif were present that could bind IRF (interferon-regulatory factor) and STAT (signal transducer and activator of transcription) family members. The latter sites are of particular interest, as they might be at least partially responsible for the induction of Bak expression in response to interferon treatment (Ossina et al., 1997) as well as for apoptosis resistance conferred by targeted gene deletion of certain IRF and STAT family members (Tamura et al., 1995; Battle and Frank, 2002). Future studies will therefore investigate the functional role of these transcriptional elements in Lck-mediated Bak expression.

4. DISCUSSION

4.1 T-cell signalling and apoptosis - Implications for Lck

Lck is a protein tyrosine kinase, which belongs to the Src family. It plays a key role in T-lymphocyte activation and differentiation. Lck is required for the adequate propagation of T-cell receptor signalling. After stimulation of the T-cell receptor complex by the respective antigen presented by an MHC molecule, Lck becomes activated and phosphorylates the critical components of TCR complex. These phosphorylation events trigger various downstream signalling events including the activation of transcription factors NF-AT, NF-□B and AP1, calcium mobilization and the activation of the mitogenic kinases ERK1 and ERK2 (Straus, 1992; Kabouridis, 1997; Samelson, 2002). Recently, it has been shown that Lck is also involved in apoptosis induced by HIV-tat, a transactivating protein of HIV-1, and ceramide, a sphingosine-based lipid signalling molecule. Upon exposure to HIV-tat or ceramide, Lck-deficient Jurkat cells (JCaM1) showed a strong resistance against apoptosis. In contrast, reconstitution of JCaM1 cells with Lck reversed the resistance for apoptosis triggered by HIV-tat and ceramide (Manna and Aggarwal, 2000; Manna et al., 2000). It has also been shown that Lck plays a crucial role in mediating apoptosis in response to ionizing radiation. Belka and colleagues demonstrated that the presence of Lck is important for the activation of the mitochondrial apoptotic pathway and CD95 independent caspase-8 activation in response to ionizing radiation (Belka, 1999; Belka, 2003). In Lck-deficient JCaM1 cells, neither caspases were activated nor was the mitochondrial potential lost upon irradiation. Interestingly, Lck-proficient JCaM1 cells restored apoptosis by activating the caspase cascade, which was preceded by loss of mitochondrial membrane potential.

The role for Lck in anti-cancer drug mediated apoptosis remains unclear. In order to address this question, the present study investigated the role of Lck in apoptosis induced by various anti-cancer drugs. The present experiments could help to understand the modulation of apoptosis by Lck as well as the molecular mechanisms of apoptosis resistance conferred by absence of the kinase. For the experiments different anti-cancer drugs were used: etoposide, a topoisomerase II

inhibitor that induces DNA strand breaks and blocks cell cycle progression at the S and G2 phase, daunorubicin and doxorubicin, both of which induce DNA damage and inhibit RNA polymerase, and mitomycin C, which inhibits DNA synthesis. Interestingly, upon treatment with these drugs, Lck-deficient JCaM1 cells showed a high amount of resistance, which was even evident when the cells were treated with the drugs for prolonged incubation times (48 hours). In contrast, transfection of the cells with wild-type Lck restored the sensitivity for apoptosis.

In addition to the anti-cancer drugs, the impact of Lck deficiency upon anti-CD95 treatment was assessed. Unlike anti-cancer drugs, prolonged (12 hours) anti-CD95 treatment sensitized Lck-deficient JCaM1 cells for apoptosis, suggesting that Lck plays a major role in mitochondrial apoptosis, but less so for the death-receptor mediated pathway. To confirm this assumption, Lck-deficient cells were treated with staurosporine, a broad protein kinase inhibitor and classical activator of mitochondrial apoptotic cascade. JCaM1 cells, upon staurosporine treatment, showed a strong apoptotic resistance, similarly as observed for anti-cancer drugs, whereas sensitivity for apoptosis was restored in JCaM1/Lck cells. Ectopic expression of Bcl-2, an anti-apoptotic inhibitor of the mitochondrial apoptotic pathway, completely abrogated the drug-mediated apoptosis. Collectively, these data suggested that Lck is involved in the regulation of the mitochondrial apoptotic pathway.

4.2 Role for Lck in the mitochondrial apoptotic cascade

The activation of the mitochondrial apoptotic cascade is initiated by cellular stress such as ionizing and UV irradiation and genotoxic drugs. The mitochondrial death pathway that is activated anti-cancer drugs has been well described in several publications (Green, 1998; Gross et al., 1999; Huang and Strasser, 2000; Li et al., 1997). It is mediated by the opening of a large channel, most probably the mitochondrial permeability transition pore (MPTP) (Reed, 1997), which leads to the release of cytochrome c. Once released into the cytosol, cytochrome c binds to APAF-1 (apoptotic protease-activating factor-1). Upon additional binding of dATP, cytochrome c induces a conformational change in APAF-1, which results in its

oligomerization (Qin et al., 1999). The conformational change of APAF-1 exposes its CARD motif and thereby allows the recruitment procaspase-9 into a high-molecular weight complex, called the apoptosome. Subsequently, procaspase-9 is activated by dimerization and further autoproteolytic processing (Zou et al., 1999). Once activated, caspase-9 processes and thereby activates the predominant effector caspase-3. Besides caspase-3, caspase-6 and caspase-7 are also activated (Bratton et al., 2000).

In order to investigate the influence of Lck on the mitochondrial apoptotic cascade, both Lck-deficient and -proficient cells were treated with etoposide for various time points and analysed for cytochrome c release, caspase-9 and caspase-3 activation. Interestingly, this analysis revealed that JCaM1 cells neither released cytochrome c nor activated caspases. In contrary, a rapid release of cytochrome c was observed in Lck-proficient JCaM1 cells, which was followed by an immediate caspase-9 activation. The intermediate cleavage fragments of caspase-9 (p37/p35) started to appear readily after cytochrome c release. In addition, the caspase-3 processed p17 fragment of caspase-9 product also appeared as soon as the executioner caspase-3 was activated, suggesting that the mitochondrial apoptotic cascade was the first one to be activated.

In addition, the status of caspase-8 activation and Bid cleavage upon etoposide treatment was explored. Analysis of JCaM1/Lck cell extracts, however, revealed that caspase-8 activation was a secondary event, which started at least three hours after the activation of the mitochondrial cascade. As a consequence of caspase-8 activation, Bid was cleaved to tBid, which in turn activates pro-apoptotic Bcl-2 family members present on the mitochondrial membrane. In contrast, cells deficient for Lck neither activated caspase-8 nor cleaved Bid. Collectively, these data show that the absence of Lck rendered the cells highly resistant for various anti-cancer drugs by blocking the activation of the mitochondrial apoptotic cascade.

4.3 Kinase cascades and their role in Lck-mediated apoptosis

In addition to mitogenic signals transmitted by survival factors, cells are subject to anti-proliferative signals induced by certain cytokines as well as by environmental stress. Examples of stress conditions that counteract proliferation signals include heat, UV light, ionizing irradiation and genotoxic drugs (Caffrey et al., 1999). It is clear that cells constantly have to monitor the environment for stresses that signal growth arrest or cell death. Integration of these noxious signals results in different cellular responses that contribute to cellular proliferation, survival and death. Phosphorylation of cellular components, a common post-translational modification, is employed for this purpose. Phosphate can be reversibly added to serine, threonine, tyrosine or histidine residues of proteins as well as to hydroxyl groups of sugars. A cross-talk between individual kinase cascades appears to integrate conflicting exogenous stimuli (Gjertsen and Doskeland, 1995).

4.3.1 The mitogen-activated protein kinases (MAPK)

Among the many signalling pathways that respond to stress, mitogen-activated protein kinase (MAPK) family members have been implicated as major mediators. Three subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAP kinases (Caffrey et al., 1999; Widmann et al., 1999). MAP kinases transduce signals from the cell membrane to the nucleus in response to various stimuli including cellular stress (Chang and Karin, 2001). It has been proposed that JNK activation triggers apoptosis in response to many types of cellular stress, including UV and gamma irradiation. Gene disruption studies revealed that JNK is essential for UV-stimulated apoptosis which is mediated by the mitochondrial pathway (Tournier et al., 2000). In the absence of JNK, cytochrome c was not released in response to UV radiation. It has been shown that JNK is also crucial for Bax- and Bak-mediated permeabilization of mitochondria (Lei et al., 2002; Xia et al., 1995). Recently, it was demonstrated that JNK phosphorylates the BH3-only protein Bim, which in turn induces a conformational change in Bax to release cytochrome c (Cheng et al., 2001; Lei and Davis, 2003; Zong et al., 2001).

In the present study, investigations were carried out in order to determine the role of JNK in Lck-mediated apoptosis. Both Lck-deficient and -proficient JCaM1 cells were stimulated with etoposide for various time points, and the cell extracts were analysed for the activation of JNK with phospho-specific antibodies. Interestingly, Lck-deficient JCaM1 cells did not activate JNK even to its slightest level. In contrast, Lck-expressing JCaM1 cells activated JNK. However, a careful analysis of the blots revealed that mitochondrial permeabilization preceded JNK activation, suggesting a relegate role for JNK. Nevertheless, a JNK-specific inhibitor was used to evaluate the importance of JNK activation for apoptosis. Lck-proficient JCaM1 cells were pre-incubated with the JNK inhibitor and challenged with etoposide for the induction of apoptosis. Flow cytometric analysis revealed that there was no significant difference in apoptosis between cells that were treated with the JNK inhibitor or untreated cells, indicating that JNK has no crucial role in Lck-mediated apoptosis induced by anticancer drugs.

Another important member of MAP kinase family is p38, which is activated by various stress stimuli that also activate JNK. It has been shown that p38 MAPK is involved in NGF deprivation-induced cell death (Xia et al., 1995), developmental neuronal cell death (Aloyz et al., 1998) and apoptosis induced by different cytotoxic agents (Kawasaki et al., 1997; Yang et al., 1997). Several stimuli that activate caspases also upregulate p38 MAPK, so it is conceivable that p38 MAPK contributes to the activation of caspases. Ghatan et al. reported that administration of p38 MAPK inhibitors blocked Bax translocation to mitochondria and partially suppressed caspase activation in neuronal cells (Ghatan et al., 2000). A recent report suggested that cantharidin, a compound used in cancer treatment, upregulated p38 in U937 cells. Inhibition of p38 MAPK blocked apoptosis and increased a significant amount cell survival (Huh et al., 2004).

Western blot analysis revealed that both JCaM1 and JCaM1/Lck cells had the same expression levels of MAPK. An early activation of p38 MAPK was observed upon etoposide stimulation in Lck-proficient JCaM1 cells, but not in the Lck-deficient counterparts, suggesting a possible involvement of p38 in Lck-mediated apoptosis. However, the fact that inhibition of p38 by a pharmacological inhibitor did not affect etoposide-induced apoptosis in JCaM1/Lck cells indicated that p38

activation was rather a consequence than a cause of etoposide-induced apoptosis.

The third member of the canonical MAPK family is ERK; it is centred on multiple signal transduction pathways to accomplish a variety of cellular functions. ERK might also function in response to DNA damage, as its activation was observed in response to cisplatin treatment in ovarian cancer cells (Cui et al., 2000). Recently, Tang et al. reported that ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53 (Tang et al., 2002). In the present experiments, Lck-expressing JCaM1 cells displayed an up-regulation of ERK activation in response to etoposide stimulation. In a time course experiment, ERK activation coincided with mitochondrial membrane permeabilization and the beginning of caspase activation, suggesting that ERK might play a role in induction of apoptosis. A functional role of ERK activation was assessed by blocking its activation with a specific inhibitor. However, flow cytometric analysis of apoptosis revealed that inhibition of ERK activation did not influence the ongoing apoptosis process, suggesting an ERK-independent role for Lck in mediating apoptosis. Taken together, these results indicate that neither JNK nor p38 or ERK were involved in drug-induced apoptosis and the sensitizing effect of Lck. MAP kinase activation rather appears to be secondary event that is functionally not linked to apoptosis induction.

4.4 Implications for Akt and c-Myc in Lck-mediated apoptosis

In addition to various stress-activated protein kinases, the role of survival kinases such as Akt was investigated. Akt, also called PKB, is one of the most important survival kinases. A number of oncogenes and tumor suppressor genes have been found to influence cancer progression by regulating Akt (Aoki et al., 1998; Chang et al., 1997). An important upstream component is phosphatidylinositol-3-kinase (PI3K) that phosphorylates and activates Akt. Therefore, oncoproteins that are known to increase PI3K activity may also require Akt to promote oncogenesis. Akt plays an important role in protection against apoptosis. Akt phosphorylates and inactivates proapoptotic molecules such Forkhead transcription factor FKHRL1,

which functions as a transcriptional regulator of CD95 ligand expression. Another important apoptosis mediator found to be phosphorylated and inactivated by Akt is the proapoptotic Bcl-2 protein Bad (Juin et al., 1999). Bad was originally identified based on its ability to bind to anti-apoptotic Bcl-2 family members, such as Bcl-x_L (Yang et al., 1995). Akt has been recently also shown to block death induced by the Bcl-2 family members Bax and Bid (Kennedy et al., 1999). In the present study, both Lck-proficient and deficient JCaM1 cells were stimulated with etoposide for the analysis of the activation status of Akt. Western blot analysis revealed that both JCaM1 and JCaM1/Lck cells had a basal constitutive amount of Akt activation, which might help to keep Bad phosphorylated and sequestered to Bcl-x_L. Nevertheless, the apoptosis-sensitive, Lck-expressing JCaM1 cells had even slightly increased levels of active Akt, which is supposed to contribute to the survival rather than to cell death. Thus, this suggested that Akt might not play a crucial role in the differential apoptosis sensitivity of the two Jurkat cell lines.

The proto-oncogene c-Myc also plays an important role in apoptosis. It promotes proliferation in the presence of growth factors, but appears to promote apoptosis under starvation conditions or other forms of stress (Evan and Littlewood, 1998). More often, the response to c-Myc induction is specified by a second signal. For instance c-Myc and Bcl2 together are proliferative, while c-Myc along with p53 induces apoptosis (Evan and Vousden, 2001). Enforced c-Myc expression has been shown to be associated with apoptosis in a wide variety of cell types including fibroblasts, B-cells, T-cell hybridomas and breast cancer cells (Davidoff and Mendelow, 1993; Kang et al., 1996). Similarly, amplification of proto-oncogenes like c-Myc has been reported to occur in a variety of tumors and is relatively common in carcinomas and sarcomas. Normally, the expression of c-Myc has a survival value for cells unless they are exposed to environmental stress. Sensitization of cells to apoptosis by c-Myc is associated with the induction of several pro-apoptotic target genes such as p53, Bax and CD95L (Brunner et al., 2000; Zindy et al., 1998). Similarly, c-Myc expression induces the release of cytochrome c, loss of the mitochondrial membrane potential and downregulation of survival mediators such as Bcl-2 and NF-□B (Hotti et al., 2000).

Therefore, investigations were carried out to find out whether etoposide stimulation of cells induces alterations in the expression level of c-Myc. Western blot analysis revealed that both Lck-proficient and -deficient JCaM1 cells did not show any changes in their expression levels of c-Myc upon etoposide treatment. A slight decrease in the levels of c-Myc was observed in JCaM1/Lck cells after 6 hours. Since the apoptotic process, however, started much earlier, the loss of c-Myc expression could be attributed to the ongoing apoptotic process and a concomitant protein degradation. The results therefore indicated that changes in the expression levels of c-Myc were presumably not functionally involved in Lck-mediated sensitization to drug-induced apoptosis.

4.5 Role of TCR signalling in genotoxic stress-induced apoptosis

The TCR is a multi-subunit immune-recognition receptor. An important signalling event after TCR engagement is the phosphorylation of ITAM tyrosine residues by the non-receptor Src-like tyrosine kinases Lck and Fyn (Langlet et al., 2000). Phosphorylated ITAMs function as recognition motifs for the binding of ZAP-70, which then becomes activated and phosphorylates LAT. LAT is a transmembrane protein that couples the TCR to several pathways and provides signals for proliferation, differentiation and apoptosis. It is well known that Lck is the first kinase phosphorylated upon TCR engagement (Wange and Huang, 2004; Wange and Samelson, 1996). In agreement with this observation, kinase assays revealed that Lck activation largely preceded activation of the apoptotic cascade upon etoposide stimulation, thus suggesting a possible involvement of the T-cell signalling in Lck-mediated apoptosis. It could be therefore assumed that, if the apoptotic process is dependent on a T-cell signalling event, any block of T-cell signalling downstream of Lck should also result in a cessation of apoptosis, as observed in Lck-deficient cells. To investigate this possibility, two Jurkat cell lines that were deficient for either LAT or ZAP-70 were used for further investigations. Flow cytometric analysis of the LAT- and ZAP-70-deficient Jurkat cells did not show any significant differences in apoptosis sensitivity as compared to the Jurkat wild-type cells or to the LAT- and ZAP-70-retransfected counterparts. These

observations suggested that etoposide-induced apoptosis was independent of TCR signalling. It further indicated that TCR components like LAT and ZAP-70 do not play a crucial role in drug-induced apoptosis. Thus, despite being a crucial T-cell signalling component, Lck presumably directly regulates drug-mediated apoptosis independently of other TCR signalling components.

4.6 Lck sequestration and its influence on cell death

Lck is normally sequestered towards the cytosolic side of the plasma membrane. It is constitutively associated with the cytoplasmic portions of the CD4 and CD8 surface receptors and thereby regulates TCR signalling pathways. Lck interacts with the inner leaflet of the plasma membrane via its hydrophobic myristoylated N-terminus (Veillette, 1988; Resh, 1994). A recent study revealed that a defect in the myristoylation property of Lck leads to a complete block of its membrane sequestration and results in a cytosolic localization of Lck (Yasuda et al. 2000). As a consequence of the deficient myristoylation of the kinase, Lck-mediated TCR signalling event was severely impaired. To elaborate the importance of membrane sequestration of Lck for drug-induced apoptosis, a JCaM1 cell line reconstituted with myristoylation-deficient Lck was employed and treated with etoposide. Flow cytometric analysis showed that the membrane targeting of Lck did not exert any influence on apoptosis in comparison to the wild-type Lck expressing JCaM1 cells. Collectively, these data suggest that membrane sequestration of Lck was not required for the sensitizing effect of Lck in apoptosis.

4.7 Lck and its relation to Bcl-2 family proteins

The Bcl-2 family members are key regulators of apoptosis and play a critical role in development and tissue homeostasis. Bcl-2 proteins are divided into two groups, those that promote survival and those that promote cell death in response to a wide variety of cytotoxic insults. Bcl-x_L and Bcl-2 themselves are pro-survival members, whereas Bax, Bak and Bok are pro-apoptotic proteins. Since the pro-apoptotic members promote apoptosis by targeting mitochondria, they are often called as gatekeepers of mitochondria. Both pro and anti-apoptotic Bcl-2 family

members titrate each other's function, which suggests that their relative concentration acts as a rheostat for the apoptosis programme (Oltvai et al., 1993).

4.7.1 The anti-apoptotic Bcl-2 proteins

It is well known that anti-apoptotic family members mostly bind to or interact with pro-apoptotic members and sequester them to an inactive conformation. The phenotypic behaviour of Lck-deficient JCaM1 cells for various anti-cancer drugs indeed resembled the Jurkat cells that ectopically overexpress Bcl-2. This led to the assumption that the absence of Lck has left anti-apopotic machinery “turned-on”. In order to elaborate this hypothesis, both the protein and mRNA expression levels of Bcl-2 family members (Bcl-x and Bcl-2) were investigated under stimulated and unstimulated conditions. Analysis of the total RNA and cell extracts from JCaM1, JCaM1/Lck, wild-type Jurkat cells and its Bcl-2 overexpressing counterparts, however, revealed that none of the above mentioned candidates was upregulated, neither transcriptionally nor translationally. Therefore, the absence of Lck did not “turn-on” any anti-apoptotic mechanism in Jurkat T-cells.

4.7.2 The pro-apoptotic Bcl-2 proteins

The pro-apoptotic Bcl-2 members (e.g. Bax and Bak) bind to the outer membrane of mitochondria in response to various cytotoxic insults. Upon activation, they homo-oligomerize and form a channel, through which cytochrome c is released. This event then leads to the activation of the caspase-9 cascade and final cell death. Recent data in knockout mice revealed that both Bax and Bak are important to induce mitochondrial dysfunction. In the absence of both Bcl-2 proteins, all cytotoxic stimuli were unable to induce apoptosis (Wei et al., 2001). This recent evidence suggested that the resistance of JCaM1 cells against anti-cancer drugs might be due to the downregulation or absence of pro-apoptotic Bcl-2 family members. To address this assumption, cell extracts of Lck-deficient and proficient JCaM1 cells were investigated by Western blot analysis and checked for the status of the pro-apoptotic Bcl-2 members Bax and Bak. The analysis revealed that in the absence and presence of Lck JCaM1 cells expressed equal amounts of

Bax. Surprisingly, however, Lck-deficient JCaM1 cells were completely devoid of the pro-apoptotic Bcl-2 member Bak. Reconstitution of wild-type Lck restored the expression levels of Bak to a level comparable as in the wild-type Jurkat cells. Furthermore, analysis of mRNA expression levels for these pro-apoptotic Bcl-2 members revealed that the absence of Lck not only downregulated Bak translationally, but also transcriptionally. Thus, these results suggested that the resistance mediated by the absence of Lck was due to the absence of Bak at the mitochondria. The experimental data also lead the conclusion that the non-receptor tyrosine kinase Lck is involved the transcriptional regulation of Bak. These results are consistent with the observation that Bak deficiency provides resistance for various anti-cancer drugs in Jurkat T-cells (Wang et al., 2001).

Recent knockout studies performed in mouse embryonic fibroblasts suggested that Bax and Bak might play redundant roles in mitochondrial dysfunction. Thus, apoptosis was not compromised in single knockout cells lacking either Bak or Bax (Wei, 2001; Lindsten, 2000). Similar studies further revealed that Bak knockout mice were developmentally normal. Also mice with Bax deficiency displayed limited phenotypic abnormalities. However, mice lacking both Bak and Bax had severe phenotypic abnormalities, and most mice lacking both genes died perinatally (Knudson et al., 1995; Wei et al., 2000). These observations strongly suggested that Bax and Bak play a redundant role in the development of mammalian cells.

So far, a redundant function of Bax and Bak, however, has not yet clearly been demonstrated in human tumour cells. Even though JCaM1 cells did express Bax protein to a similar amount as wild-type Jurkat cells, mitochondrial damage and caspase activation were not observed in these cells upon etoposide treatment. This suggested therefore the existence of non-redundant functions for Bax and Bak in tumour cells. In support of this idea, a recent study revealed that mutations in Bax conferred protection against apoptosis in cancer cells (Eguchi, 2000; Kondo, 2000). Other studies using Bax-deficient cells established an important role of Bax for the sensitivity of tumour cells for anticancer drug-induced apoptosis (Eguchi, 2000; Kondo, 2000). It has also been reported that gastric and colorectal tumour cells have reduced Bak protein levels that cause resistance against non-

steroidal anti-inflammatory drugs (Zhang et al., 2000). Finally, a downregulation of Bak expression has been linked to the development of breast cancer. For instance, it was demonstrated that in transformants of MCF-7, a breast carcinoma cell line, the stable expression of Bak resulted in an impaired proliferation capability (Eguchi et al., 2000). In contrast, the stable introduction of a Bak antisense vector enhanced colony formation, indicating a possible role for Bak in tumour formation. Thus, it is conceivable that, in contrast to the studies analyzing mouse development, Bak and Bak might play non-redundant functions in human tumour cells. In line with these data, restoration of Bak expression by Lck transfection reinstated apoptosis in JCaM1 cells upon etoposide stimulation. The results therefore also indicated an absolute requirement of Bak for drug-induced apoptosis in human leukaemic T-cells.

As mentioned above, Bax and Bak undergo a conformational change in response to various apoptotic stimuli, which mediates the exposure of their occluded N-terminus (Desagher et al., 1999; Griffiths et al., 1999). Moreover, the conformational change leads to the assembly of Bak and Bax into homomultimers with presumed channel-forming properties in the mitochondrial membrane, resulting in cytochrome c release (Wei et al, 2000). In order to investigate the conformational status of Bak and Bax in Lck-proficient and deficient JCaM1 cells, FACS analyses were employed using conformation-specific antibodies against the N-terminus of Bax and Bak. Upon anti-cancer drug treatment both pro-apoptotic Bcl-2 members underwent a conformational change in JCaM1/Lck cells. Furthermore, time course experiments revealed that the conformational change of Bax was preceded by a conformational change of Bak. Interestingly, JCaM1 cells did not show a conformational change upon etoposide stimulation. This finding might be explained by the possibility that either the absence of Bak hindered the conformational change of the Bax, or that the Bax protein is not functional in JCaM1 cells. A functional deficiency of Bax, however, can be easily ruled out, because Bax resume a conformational change upon stimulation of JCaM1/Lck cells. These results therefore rather indicate that a Bax conformational change is dependent on the pro-apoptotic conformational change of Bak, suggesting a pivotal role of Bak for activation of the mitochondrial pathway.

Since ectopic expression of Bcl-2 protected against anticancer drug-mediated apoptosis, similar investigations were performed in Jurkat cells overexpressing Bcl-2. It is known that Bcl-2 inhibits mitochondrial membrane permeabilization and cytochrome c release by interacting with the pro-apoptotic Bcl-2 family members Bax and Bak. In the present experiments, ectopic expression of Bcl-2 completely abrogated the conformational change of Bak upon etoposide stimulation. Bcl-2 therefore interfered with an early step of mitochondrial alterations and thereby prevented cytochrome c release.

The impact of the absence of Bak for the apoptosis resistance of JCaM1 cells was further substantiated by additional *in vitro* and *in vivo* experiments. To this end, Lck-proficient and deficient JCaM1 cells were permeabilized with digitonin and then treated with recombinant tBid, a classical activator of Bak and Bax. Subsequent analysis revealed that Bax alone was unable to undergo a conformational change in the presence of tBid *in vivo*, whereas Bak readily resumed its pro-apoptotic conformational status. This suggested that Bax is somehow dependent on Bak to change its conformational status. To verify these findings, isolated mitochondria from Lck-deficient and proficient JCaM1 cells were treated with tBid *in vitro*. The experiments revealed that in the absence of Bak, tBid was not able to permeabilize mitochondria and to trigger cytochrome c release. Thus, the results confirm that the presence of Bak on mitochondria is essential for drug-mediated apoptosis in human leukaemic Jurkat T-cells. These results are supported by a recent report suggesting a requirement of Bak in tBid-induced cytochrome c release in hepatocytes (Wei et al., 2000).

Collectively, the present results represent the first observation that Lck, a non-receptor tyrosine kinase, is involved in the regulation of a pro-apoptotic Bcl-2 family member, suchas Bak. In addition, unlike in murine models, the data point to non-redundant functions of Bax and Bak. *In vitro* experiments revealed that there is an absolute requirement for Bak for the permeabilization of mitochondria to release cytochrome c. In addition, it was observed that the pro-apoptotic conformational change of Bax is dependent on the prior conformational change of Bak, suggesting a kind of cooperative action of these two pro-apoptotic Bcl-2 members.

4.8 Lck domains that regulate Bak expression and apoptosis

It has been suggested that the Src homology (SH)-2 and –3 domains of Lck might have additional regulatory functions independently of Lck's kinase activity. Recent studies for instance revealed that a mutation in the SH2 domain of Lck completely inactivated TCR signalling (Straus, 1996), whereas mutations in the SH3 domain indicated a crucial role of the SH3 domain for the activation of MAPK signalling (Denny, 1999). Recently, Hur and colleagues provided evidence that Lck is involved in apoptosis induced by rosmarinic acid, an anti-inflammatory plant metabolite (Hur et al., 2004). The researchers also demonstrated that the SH2 and -3 domains of Lck mediated rosmarinic acid-induced apoptosis independently of its kinase activity.

The importance of the SH2, SH3 and the kinase domains of Lck was investigated by transiently transfecting JCaM1 cells with expression vectors encoding either wild-type Lck or a kinase-deleted Lck mutant containing only the SH2 and SH3 domain. The subsequent analysis revealed that the SH2 and SH3 domains of Lck were indeed sufficient for the induction of Bak expression. Furthermore, treatment of JCaM1/Lck cells with PP-2A, a Lck kinase inhibitor, did not confer resistance to etoposide-induced apoptosis (data not shown). In contrast, incubation of cells with herbimycin A, a reagent promoting the proteosomal degradation of the Lck protein, not only blocked the pro-apoptotic conformational change of Bak, but also very efficiently protected the cells from apoptosis. Taken together, these data suggested that the SH2 and SH3 domains are sufficient for the expression pro-apoptotic protein Bak, whereas the kinase domain of Lck is not required for the sensitizing effect to apoptosis.

4.9 Bak promoter analysis

As the investigation revealed that Lck is involved in the restoration of Bak protein expression, it raised the intriguing question of how Lck might be regulating Bak expression. Therefore, an analysis of potential transcription factor binding sites of the upstream region (4 kb from the translational start site) of the Bak gene was carried out. The sequence analysis unveiled the following transcription factor

binding sites: ISRE (interferon-stimulated response element), GAS (gamma interferon-activated site), which bind IRF and STAT family members, as well as binding sites for the transcription factors p53, NF-kB1 and NF-kB2. It has been reported that Bak expression is regulated by interferon (IFN)-g (Ossina et al., 1997). The study demonstrated the human colon adenocarcinoma cell line HT-29, which is largely resistant against apoptosis mediated by CD95, TNF and the anti-cancer drugs cis-platin and adriamycin, expresses very low constitutive Bak levels. In contrast, pre-treatment of HT-29 cells with IFN-g sensitized the cells to apoptosis, an effect that was associated with the transcriptional induction of the Bak gene. Although the functional elements of the Bak promoter have not been explored so far, these results are inline with the Bak promoter analysis, indicating at least two binding sites for IFN-regulated transcription factors. The presence of the IFN-responsive ISRE and GAS elements in the Bak promoter could also explain the apoptosis resistance conferred by targeted gene deletion of certain IRF and STAT family members (Tamura et al., 1995; Battle and Frank, 2002). Current studies are therefore investigating the functional role of these transcriptional elements in Lck-mediated Bak expression.

4.10 Receptor-mediated apoptosis- perspectives for Lck

Death receptors induce apoptosis by recruitment of the adaptor protein FADD and the initiator caspase-8 to the vicinity of the receptor. At the DISC, caspase-8 then oligomerizes leading to its activation via autocatalysis. Activated caspase-8 in turn propagates the apoptotic signal via two downstream cascades: first, it can directly cleave and activate caspase-3; second, it can cleave and thereby activate the proapoptotic Bcl-2 protein Bid, which then translocates to mitochondria. Bid cleavage subsequently induces cytochrome c release, resulting in the sequential activation of caspase-9 and -3.

A potential role of Lck in death receptor-mediated apoptosis is controversial. One study suggested that Lck is major mediator of apoptosis induced by death receptors, as in the absence of Lck no cell death was observed upon CD95 stimulation (Schlottmann, 1996). Another study, in contrast, proposed that anti-

CD95 mediated apoptosis is independent of Lck in Jurkat leukaemic T-cells (Schraven, 1995). Interestingly, both observations provide adequate evidence in support of their hypotheses.

To investigate a role of Lck in CD95-mediated apoptosis, JCaM1 cells were treated for different time points with agonistic anti-CD95 antibodies. Flow cytometric analysis revealed that JCaM1 cells deficient for Lck cells did indeed resist anti-CD95-induced apoptosis for up to 8 hours, whereas 80% of the Lck-proficient JCaM1 and wild-type Jurkat cells underwent cell death at the same time point. Prolonged incubation with anti-CD95 (12 hours), however, sensitized the Lck-deficient JCaM1 cells as well, reaching similar levels of apoptosis in the presence and absence of Lck. From this experiment, the reason for the two contradictory observations became apparent, because Schlottmann et al. (1996) exposed the cells to anti-CD95 for a shorter time period (6 hours), whereas Schraven and Peter (1995) incubated JCaM1 cells for 18 hours. Thus, it appears that the absence of Lck causes a delay in CD95-mediated apoptosis, but not a complete resistance as observed for the mitochondrial apoptotic pathway.

Further investigations were carried out to understand the influence of Lck deficiency on caspase activation upon anti-CD95 treatment. Western blot analysis of extracts from JCaM1 cells stimulated with anti-CD95 revealed that caspase-8 activation was significantly delayed in comparison to Lck-expressing JCaM1 cells. The intermediate cleavage forms of caspase-8 (p43/41) and the active p18 subunit appeared considerably later in JCaM1 cells than in JCaM1/Lck cells. As a consequence, activation of effector caspase-3 was also significantly delayed. Moreover, caspase-8 mediated Bid cleavage that links the death receptor with the mitochondrial pathway occurred. These experiments clearly explain that the absence of Lck contributes to the delayed activation of the caspase cascades.

As mentioned above, an initial event of receptor-mediated apoptosis is the formation of a high-molecular weight protein complex, designated as the death-inducing signalling complex (DISC), which consist of FADD and caspase-8. As the caspase-8 activation was delayed in the absence of Lck during CD95-mediated apoptosis, it was conceivable that Lck was somehow involved in DISC formation. In order to investigate this idea, co-immunoprecipitation experiments of the DISC

were performed following stimulation of JCaM1/Lck cells with anti-CD95. Analysis of the DISC composition revealed that indeed a considerable amount of Lck could be co-immunoprecipitated with the DISC, suggesting that Lck might be an additional component of the DISC.

Recent reports suggest that also membrane rafts play an important role in the initiation of CD95 mediated apoptosis. Rafts are tightly packed membrane microdomains composed of sphingolipds and cholesterol as well as of different lipid-anchored and transmembrane proteins (Simons and Ikonen, 1997). It was shown that lipid rafts play an important role in cell signalling, in particular through the organization of surface receptors, signalling enzymes and adaptor molecules into membrane complexes at specific sites. Raft association appears to be essential for the initiation of signalling from a number of receptors, especially in immune cells. Studies in mouse thymocytes provided evidence that CD95 is recruited into rafts upon receptor stimulation. Apart from CD95 also on other members of the DISC, such as FADD and caspase-8, were found in rafts (Hueber et al., 2002). In addition, it was demonstrated that Src family of kinases including Lck have a raft affinity upon activation (Langlet et al., 2000). The experiments presented in this study also suggest that, together with the DISC, Lck appears to be recruited in membrane rafts. Although further experiments are required to demonstrate a functional role of Lck in the DISC and lipid rafts, it is conceivable that such a raft recruitment of Lck could facilitate the activation of caspase-8. In conclusion, these data demonstrate that, even though Lck has certainly a major impact on mitochondrial apoptosis by regulating Bak expression, other mechanisms in addition may account for a sensitizing effect of Lck, in particular in the context of death receptor-mediated cell death.

5. Summary

Protein tyrosine kinases of the Src family have been implicated in key biological processes including cell growth, differentiation, transformation and gene transcription. In T-lymphocytes, protein tyrosine kinases play an integral role in the activation of cells through various immunoreceptors, and in particular the lymphoid-specific Src kinase Lck is essential for T-cell receptor (TCR) signalling. A few recent studies indicated that tyrosine kinases might not only play a role in proliferation, but also in apoptosis. The mechanism of how activation of Src kinases contributes to apoptosis in certain situations, while inducing proliferation in others, is completely unknown. The present study therefore investigated the role of Lck for apoptosis of human leukaemic T-cells in response to various anticancer drugs.

It was found that Lck-deficient T-cells were completely resistant to anticancer drugs, which trigger a mitochondria-regulated pathway of apoptosis. In contrast, the apoptosis sensitivity to death receptors was only impaired, but not fully abrogated in the absence of Lck. Reexpression of Lck in the Lck-deficient Jurkat T-cells JCaM1, interestingly, restored the sensitivity to anticancer drug-induced apoptosis. Furthermore, JCaM1 cells stably transfected with Lck underwent apoptosis and induced the mitochondrial release of cytochrome c as well as caspase activation. This suggested that Lck plays a crucial role for the activation of the mitochondrial apoptosis pathway.

To determine in more detail the reason for the inability of Lck-deficient cells to undergo apoptosis, several pathways were studied. The proapoptotic effect of Lck was independent of the TCR downstream pathway, and several regulators of the TCR (e.g. ZAP-70, LAT) were not involved in Lck's proapoptotic effect. The proapoptotic effect of Lck did also not require its myristoylation, which normally targets Lck to the plasma membrane.

To investigate the mechanisms of Lck-mediated sensitization to apoptosis, the activation of other mediators that have been implicated in apoptosis was explored. Upon treatment with the anticancer drug etoposide, Lck-deficient and proficient cells did not differ in their activation profile of the transcription factors NF-□B and

NFAT. Moreover, neither the stress-activated MAP kinases ERK, JNK or p38 nor the survival kinase Akt seemed to be involved in the apoptosis resistance mediated by Lck deficiency.

A detailed analysis of different apoptotic regulators, however, revealed that the proapoptotic effect of Lck was essentially mediated by the Bcl-2 protein Bak. Expression of Bak was completely absent at the mRNA and protein level in Lck-deficient cells, while, importantly, reexpression of Lck transcriptionally triggered Bak expression and conferred sensitivity to apoptosis. Furthermore, upon etoposide treatment, Lck-proficient but not the deficient cells showed a proapoptotic conformational change of Bak. To further substantiate these findings, mitochondria of permeabilized cells were treated with recombinant tBid, a classical activator of the mitochondrial pathway. Addition of exogenous tBid protein rapidly induced the conformational change of Bak *in vitro*, which was associated with the mitochondrial release of cytochrome c. The translocation of cytochrome c by tBid was specifically observed in JCaM1/Lck cells, but virtually absent in mitochondria from Lck-deficient cells. These data therefore confirmed that the defect of the apoptosis-resistant and Lck-deficient cells was located at the mitochondria and mediated by Bak deficiency.

In summary, these results do not only demonstrate a sentinel role of Lck in drug resistance, but also delineate a hitherto unknown pathway of Src kinases in regulation of Bcl-2 proteins, which might be involved in chemoresistance. Although mutations in the Bak gene have been detected in some cancers, Bak deficiency as a mechanism for chemoresistance has not yet been addressed. The resistance to anticancer drugs suggests that the deficiency of Lck and a consequent failure to express Bak participates in the regulation of mitochondrial apoptosis in leukaemic cancer cells.

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