Mechanismen der apoptotischen Signaltransduktion: Modulation der Aktivierung von Initiator-Caspasen

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Abkürzungsverzeichnis

$\Delta\Psi_m$	mitochondriales Membranpotential
AICD	***A**ctivation **I**nduced **C**ell **D**eath*
APAF-1	***A**poptotic-**P**rotease **A**ctivating **F**actor-1*
ASC	***A**poptosis-associated **S**peck-like protein containing a **C**ARD*
ATM	***A**taxia **T**elangiectasia **M**utant*
BAD	***B**cl-2 **A**ntagonist of cell **D**eath*
BAK	***B**cl-2 **A**ntagonist **K**iller*
BAX	***B**cl-2 **A**ssociated **X** protein*
Bcl-2	***B**-**C**ell **L**ymphoma-2*
Bcl-XL	***B**-**C**ell **L**ymphoma-**X** **L**arge*
BH	***B**cl-2 **H**omology*
BID	***B**cl-2 **I**nteracting domain **D**eath agonist*
BIM	***B**cl-2 **I**nteracting **M**ediator*
BIR	***B**aculovirus **I**AP **R**epeat*
c-FLIP	***C**ellular-**FL**ICE **I**nhibitory **P**rotein*
CARD	***Ca**spase-**R**ecruiting **D**omain*
CD95L	CD95 ***L**igand*
CDK	***C**yclin-**D**ependent **K**inase*
Chk	***Ch**eckpoint **K**inase*
CrmA	***C**ytokine **R**esponse **M**odifier **A***
CTL	***C**ytotoxic **T**-**L**ymphocyte*
DD	***D**eath **D**omain*
DED	***D**eath **E**ffector **D**omain*
DISC	***D**eath-**I**nducing **S**ignaling **C**omplex*
FADD	***F**as-**A**ssociating **D**eath **D**omain-containing protein*
GPI	***G**lycosyl-**P**hosphatidyl-**I**nsoitol*
HDM2	***H**uman **D**ouble **M**inute **2** protein*
IAP	***I**nhibitor of **A**poptosis **P**rotein*
ICE	***I**nterleukin-1ß-**C**onverting **E**nzyme*
IκBα	***I**nhibitor of NF-**κB**-**α***
IL-1ß	***Inter**leukin-**1ß***
IR	***I**onizing **R**adiation*

MEF	***M**ouse **E**mbryonic **F**ibroblasts*
NALP1	***NA**CHT, **L**eucine-rich-repeat and **P**YD containing protein*
NF-κB	***N**uclear **F**actor of **K**appa light polypeptide gene enhancer in **B**-cells*
PCNA	***P**roliferating **C**ell **N**uclear **A**ntigen*
PIDD	***P**53-**I**nducible protein with a **D**eath **D**omain*
PTP	***P**ermeability **T**ransition **P**ore*
PYD	***Py**rin **D**omain*
RAIDD	***R**ip-**A**ssociated protein with a **D**eath **D**omain*
RB	***R**etino**b**lastoma protein*
RIP	***R**eceptor **I**nteracting **P**rotein*
ROS	***R**eactive **O**xygen **S**pecies*
shRNA	***S**hort **H**airpin* RNA
Smac/DIABLO	***S**econd **M**itochondria-derived **A**ctivator of **C**aspase / **D**irect **IA**P-**B**inding protein with **Lo**w pI*
tBID	***T**runcated BID*
TM	Transmembran
TNF	***T**umor **N**ecrosis **F**actor*
TRAIL	***T**NF-**R**elated **A**poptosis-**I**nducing **L**igand*
XIAP	***X**-chromosome-linked **IAP***

Kapitel 1
Einleitung

1. Einleitung

1.1 Definition von Apoptose

Es gibt mehrere Arten des Zelltodes, die sich durch individuelle morphologische und biochemische Charakteristika unterscheiden (Kroemer et al., 2005). Die häufigste und bisher am besten definierte Zelltodart ist die Apoptose, die 1972 erstmalig beschrieben wurde (Kerr et al., 1972). Apoptose (oder programmierter Zelltod) ist ein in eukaryotischen Zellen festgeschriebenes Suizid-Programm, das zur Eliminierung der betroffenen Zelle führt. Hierbei werden mehrere charakteristische morphologische und biochemische Veränderungen initiiert, die es dem Organismus ermöglichen, unerwünschte Zellen ohne Erzeugung eines inflammatorischen Signals zu beseitigen. Dieser programmierte Zelltod ist für vielzellige Organismen essentiell, da es nicht nur in verschiedenen Entwicklungsstadien, sondern auch im adulten Zustand erforderlich ist, defekte oder entartete Zellen kontrolliert aus dem Zellverband zu entfernen. Nur dadurch kann die zelluläre Homöostase eingehalten und ein Gleichgewicht zwischen proliferierenden und sterbenden Zellen geschaffen werden. Darüber hinaus nimmt die Apoptose eine zentrale Stellung bei der Regulation des Immunsystems ein und wirkt z.B. durch die Eliminierung autoreaktiver sowie nicht mehr benötigter aktivierter T-Zellen Autoimmunerkrankungen entgegen. Außerdem werden entartete oder mit Viren infizierte Zellen durch Apoptose beseitigt und so großer Schaden, wie z.B. eine Tumorbildung, verhindert. Dementsprechend ist eine Deregulation von apoptotischen Prozessen für viele Krankheitsbilder charakteristisch. Tumorzellen entwickeln oftmals während der Tumorgenese eine Resistenz gegenüber bestimmten Apoptose-Stimuli (Brown and Attardi, 2005), da sie andernfalls durch den Organismus eliminiert werden. Bei einer HIV-1-Infektion dagegen ist die Zelltodrate deutlich erhöht, was zu einem massiven Sterben von $CD4^+$-T-Lymphozyten und auf lange Sicht zu einem Verlust des Immunsystems führt (Castedo et al., 2003; Gougeon, 2003). Daher ist die Aufklärung der Signalwege, die zum Zelltod führen, sowie die Erforschung der Möglichkeiten einer zielgerichteten Intervention von enormer Bedeutung für erfolgreiche Therapien zur Krankheitsbekämpfung (Fischer and Schulze-Osthoff, 2005).

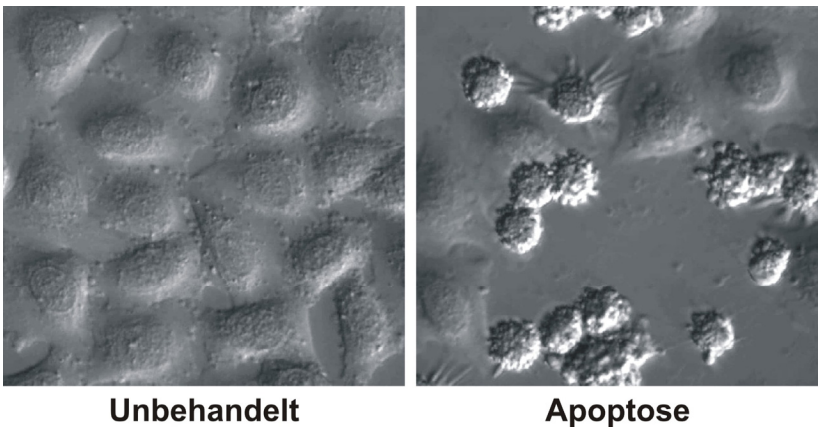

Abbildung 1: Morphologie apoptotischer Zellen

In HeLa-Zellen wurde durch die Zugabe von Actinomycin D (10 µM) gezielt Apoptose induziert. Es ist deutlich das Ablösen und Schrumpfen der Zellen sowie das für die Apoptose so charakteristische „*Blebbing*“ der Zellmembran zu beobachten.

Die Apoptose ist morphologisch charakterisiert durch ein Abrunden und Ablösen der Zellen und eine Reduzierung des Zellvolumens (Pyknose). Der Kern wird dabei fragmentiert und das Chromatin kondensiert, während die nukleäre DNA durch gezielte Spaltung zwischen den Nukleosomen degradiert wird. Im Agarose-Gel kann so ein charakteristisches „*DNA-Laddering*“ beobachtet werden, da die Bruchstücke jeweils ein Vielfaches von ~185 bp groß sind. Die Zellmembran bleibt bis zu den finalen Abschnitten der Apoptose intakt, zeigt aber eine Vesikelbildung („*Blebbing*“) (Abb. 1). Abschließend wird die Zelle fragmentiert und apoptotische Körperchen gebildet, welche durch immunkompetente Phagozyten (Makrophagen, dendritische Zellen) aufgenommen und eliminiert werden. Dabei werden keine intrazellulären Komponenten aus den apoptotischen Körperchen freigesetzt, so dass auch keine Immunantwort ausgelöst wird. Vor der Phagozytose werden so genannte „*find me*“- und „*eat-me*“-Signale ausgelöst, die für eine effiziente Aufnahme und Degradierung der apoptotischen Körperchen sorgen (Lauber et al., 2004).

1.2 Caspasen

Familie der Caspasen

Biochemisch verantwortlich für die meisten charakteristischen Veränderungen einer apoptotischen Zelle sind die Mitglieder einer Enzym-Familie, die Caspasen (Aspartat-spezifische Cysteinyl-Proteasen). Diese Proteasen werden während der Apoptose

kaskadenartig in großen Mengen aktiviert und vollstrecken durch die gezielte Spaltung einer Vielzahl von Substraten das apoptotische Programm (Fischer et al., 2003; Jänicke et al., 1998).

Die heutige Caspase-1 wurde 1992 von zwei Gruppen identifiziert und aufgrund ihrer Funktion, nämlich der aktivierenden Spaltung des bedeutenden Entzündungsmediator Interleukin-1ß (IL-1ß), ICE (***I**nterleukin-1ß-**C**onverting **E**nzyme*) genannt (Cerretti et al., 1992; Thornberry et al., 1992). Über die folgenden Jahre wurden mehrere Proteine mit Sequenzhomologien zu ICE entdeckt, die jetzt die Familie der Caspasen bilden (Degterev et al., 2003; Reed et al., 2004). Insgesamt wurden bis heute Spezies-übergreifend 15 Mitglieder dieser Protein-Familie identifiziert, von denen elf im Menschen exprimiert werden (Abb. 2) (Reed et al., 2003). Diese können entsprechend ihrer Hauptfunktion und über Sequenzhomologien in einen inflammatorischen und einen apoptotischen Zweig eingeteilt werden. Der apoptotische Zweig ist beim Menschen und bei der Maus bis auf das Vorkommen von Caspase-10, die eine hohe Homologie zur Caspase-8 aufweist, identisch (Jänicke et al., 2006).

Die ausschließlich in der Epidermis exprimierte humane Caspase-14 konnte bisher keinem Zweig eindeutig zugeordnet werden. Basierend auf ihrer Lokalisation wird für sie allerdings eine Funktion in der Differenzierung von Keratinozyten angenommen. Außerdem existiert im Menschen noch Caspase-12, die allerdings in Folge zweier Mutationen nur als katalytisch inaktive Variante produziert wird (Fischer et al., 2002; Lamkanfi et al., 2004) und daher nicht in der Liste der aktiven humanen Caspasen vertreten ist. In der Maus hingegen ist Caspase-12 aktiv und wird aufgrund von Sequenzhomologien dem inflammatorischen Zweig zugeordnet, obwohl für sie auch eine Rolle als Initiator-Caspase in der vom Endoplasmatischen Retikulum ausgelösten Apoptose diskutiert wird (Szegezdi et al., 2003).

Struktur der Caspasen

Alle Caspasen besitzen eine große (~p20) und eine kleine Untereinheit (~p10) sowie eine Prodomäne, die allerdings zwischen den Mitgliedern in der Größe variiert. Caspasen werden als inaktive Zymogene exprimiert, die erst durch Spaltung zwischen den beiden Untereinheiten sowie der zusätzlichen Entfernung der Prodomäne aktiviert werden. So wird ein aktives Heterotetramer gebildet, bestehend aus zwei großen sowie zwei kleinen Untereinheiten. Dabei besitzt jedes Heterotetramer zwei katalytische Zentren, die sich jeweils in der großen Untereinheit befinden (Fuentes-Prior and

Salvesen, 2004). Das aktive Zentrum wird durch einen Cysteinyl-Rest gebildet, der spezifisch für alle Caspasen die Spaltung des Substrates an einem Aspartat-Rest katalysiert. Hierbei wird im Substrat eine Tetrapeptidsequenz erkannt, bei der sich das gespaltene Aspartat an Position 1 (P1) befindet. Die Caspasen unterscheiden sich allerdings in ihrer Spezifität für die Aminosäuren in den Positionen P2-P4 und lassen sich nach ihrer optimalen Tetrapeptidsequenz in Gruppen einteilen (Garcia-Calvo et al., 1998; Garcia-Calvo et al., 1999; Thornberry et al., 1997).

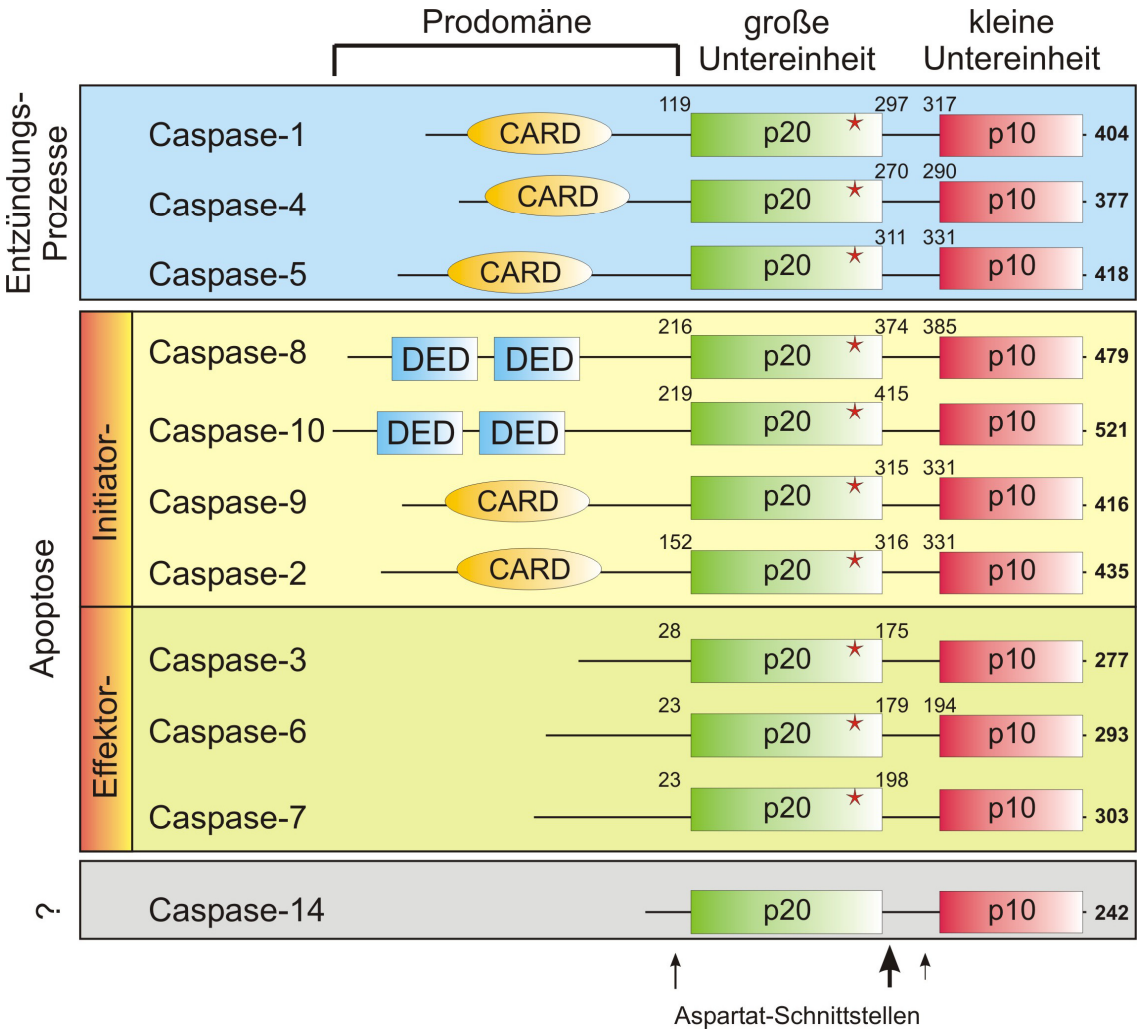

Abbildung 2: Einteilung und struktureller Aufbau der menschlichen Caspasen

Dargestellt sind die humanen Caspasen nach ihren Hauptfunktionen sortiert. Während alle Caspasen eine große (p20) und eine kleine Untereinheit (p10) besitzen, zeichnen sich nur einige Caspasen durch zusätzliche Oligomerisierungs-Domänen (DED und CARD, s.a. Text) in der N-terminal gelegenen Prodomäne aus. Die Spaltstellen der Caspasen sind mit Pfeilen gekennzeichnet, wobei die erste und wichtigste Spaltung mit dem großen Pfeil angedeutet wird. Die Nummern entsprechen der Position des Aspartats im Protein, an dem diese Spaltung stattfindet. Die Position des katalytisch aktiven Cystein-Rests ist mit einem Stern gekennzeichnet. Caspase-14 wird nicht an einem Aspartat-Rest aktiviert, daher ist keine Spaltstelle angegeben. Die fett gedruckten Nummern am Ende entsprechen der Anzahl der Aminosäuren des dargestellten Proteins. Die Größenverhältnisse sind nur schematisch dargestellt.

Der apoptotische Zweig lässt sich nach Funktion und Aufbau der Caspasen in zwei weitere Unterbereiche aufteilen, die Initiator- und die Effektor-Caspasen (Abb. 2). Die Effektor-Caspasen besitzen eine kurze Prodomäne und werden im Verlauf der Apoptose durch Initiator- und andere Effektor-Caspasen kaskadenartig aktiviert. Sie vermitteln durch die gezielte Spaltung definierter Zielproteine die Charakteristika der Apoptose (Fischer et al., 2003).

Im Gegensatz zu den Effektor-Caspasen besitzen Initiator-Caspasen eine längere Prodomäne, in der sich funktionelle Protein-Protein-Interaktionsdomänen befinden. Während sich in der Prodomäne von Caspase-9 und -2 jeweils eine CARD (***Ca**spase-**R**ecruiting **D**omain*) befindet, zeichnen sich die Prodomänen von Caspase-8 und-10 durch das Vorhandensein zweier DEDs (***D**eath **E**ffector **D**omain*) aus. Über diese Domänen können die Initiator-Caspasen in hochmolekulare Aktivierungskomplexe zur Initiierung der Caspasen-Kaskade rekrutiert werden. Diese Komplexe werden als Reaktion auf bestimmte Apoptose-induzierende Stimuli gebildet, die entweder extrinsischer oder intrinsischer Natur sein können.

1.3 Extrinsischer Apoptose-Signalweg

Todesrezeptoren

Die Induktion der Apoptose über so genannte Todesrezeptoren und ihre Liganden wird als extrinsischer Signalweg der Apoptose bezeichnet. Todesrezeptoren sind Mitglieder der TNF (***T**umor **N**ecrosis **F**actor*)-Rezeptor-Superfamilie, die insgesamt aus 29 Rezeptoren und 19 Liganden besteht. Sie sind nicht nur für die Induktion von Zelltod verantwortlich, sondern üben auch wichtige Funktionen in immunologischen, Differenzierungs- und Überlebens-Signalwegen aus (Aggarwal, 2003). Alle Mitglieder der Familie besitzen extrazellulär mehrere Cystein-reiche Domänen, die der Ligandenbindung dienen. Intrazellulär befindet sich bei einigen Rezeptoren, vor allem den Apoptose-vermittelnden Mitgliedern, eine so genannte Todesdomäne (DD, ***D**eath **D**omain*), über die im Zytoplasma nach Rezeptoraktivierung hochmolekulare Signalkomplexe gebildet werden. Nach Bindung des entsprechenden Liganden wird eine Oligomerisierung des Rezeptors induziert, wodurch die intrazellulären DDs der Rezeptoren in der Lage sind, andere DD-besitzende Adapterproteine zu rekrutieren.

Bei dem bisher am besten charakterisierten Todesrezeptor, CD95 (Fas/Apo-1), sowie den beiden Apoptose-induzierenden TRAIL (***T**NF-**R**elated **A**poptosis-*

I**nducing **L**igand*)-Rezeptoren, führt diese Oligomerisierung im Zytoplasma zur Rekrutierung des Adapterproteins FADD (F**as-**A**ssociating **D**eath **D**omain-containing protein*). Über FADD, welches zusätzlich zur DD auch eine DED besitzt, werden durch homophile Interaktionen der DEDs die Initiator-Procaspasen-8 und -10 zum Rezeptor rekrutiert und dort aktiviert. Dieser Signalkomplex wird als DISC (***D**eath-**I**nducing **S**ignaling **C**omplex*) bezeichnet (Debatin and Krammer, 2004).

Zusätzliche Komplexität wird in dieses Signalsystem durch das Vorhandensein von so genannten TRAIL-Abfang (*Decoy*)-Rezeptoren gebracht, die zwar extrazellulär den Liganden binden, aber über keine oder nur eine verkürzte DD verfügen, so dass an diesen Rezeptoren keine DISC-Bildung und Signalweiterleitung möglich ist (Abb. 3) (Ashkenazi and Dixit, 1999; Schulze-Osthoff et al., 1998).

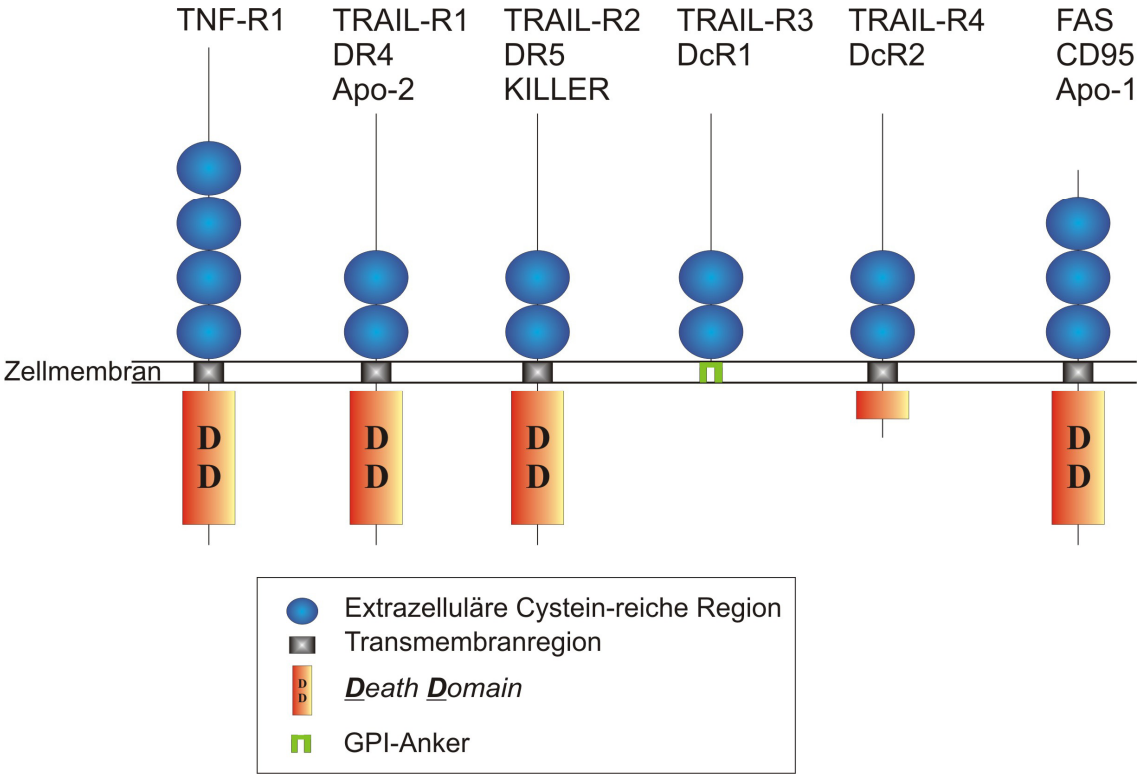

Abbildung 3: Apoptose-induzierende Todesrezeptoren

Schematische Darstellung einiger Mitglieder der TNF-Todesrezeptor-Superfamilie. Zusätzlich sind die dazugehörigen zwei *Decoy*-Rezeptoren (DcR1, DcR2) des TRAIL-Systems abgebildet, denen die funktionelle zytoplasmatische DD fehlt. Einer der Decoy-Rezeptoren ist über einen GPI (***G**lycosyl-**P**hosphatidyl-**I**nsoitol*) -Anker in der Zellmembran verankert und besitzt keine Transmembranregion.

In der CD95-vermittelten Apoptose unterscheidet man Typ I- und Typ II-Zellen, die unterschiedliche Signalwege benutzen (Abb. 4). In Typ I-Zellen wird Procaspase-8 schnell und in großen Mengen am DISC aktiviert. Diese Mengen des aktiven Caspase-8-Heterotetramers können nun direkt die Effektor-Caspase-3 aktivieren, so dass das apoptotische Programm durchgeführt wird. In Typ II-Zellen dagegen werden aus bisher unbekannten Gründen nur geringe Mengen an DISC gebildet. Dies hat zur Folge, dass nur limitierende Caspase-8 Aktivität induziert und damit eine Verstärkung über einen anderen Signalweg benötigt wird. Hierbei spielt das Caspase-8 Substrat BID (***B**cl-2 **I**nteracting domain **D**eath agonist*), ein Mitglied der *BH3-only* Untergruppe der Bcl-2-Familie, eine wichtige Rolle. Nach der Caspase-8 vermittelten Spaltung von BID transloziert dessen C-terminales Fragment tBID (***T**runcated BID*) zu den Mitochondrien und aktiviert dort den intrinsischen Signalweg der Apoptose (s.a. Abschnitt 1.4).

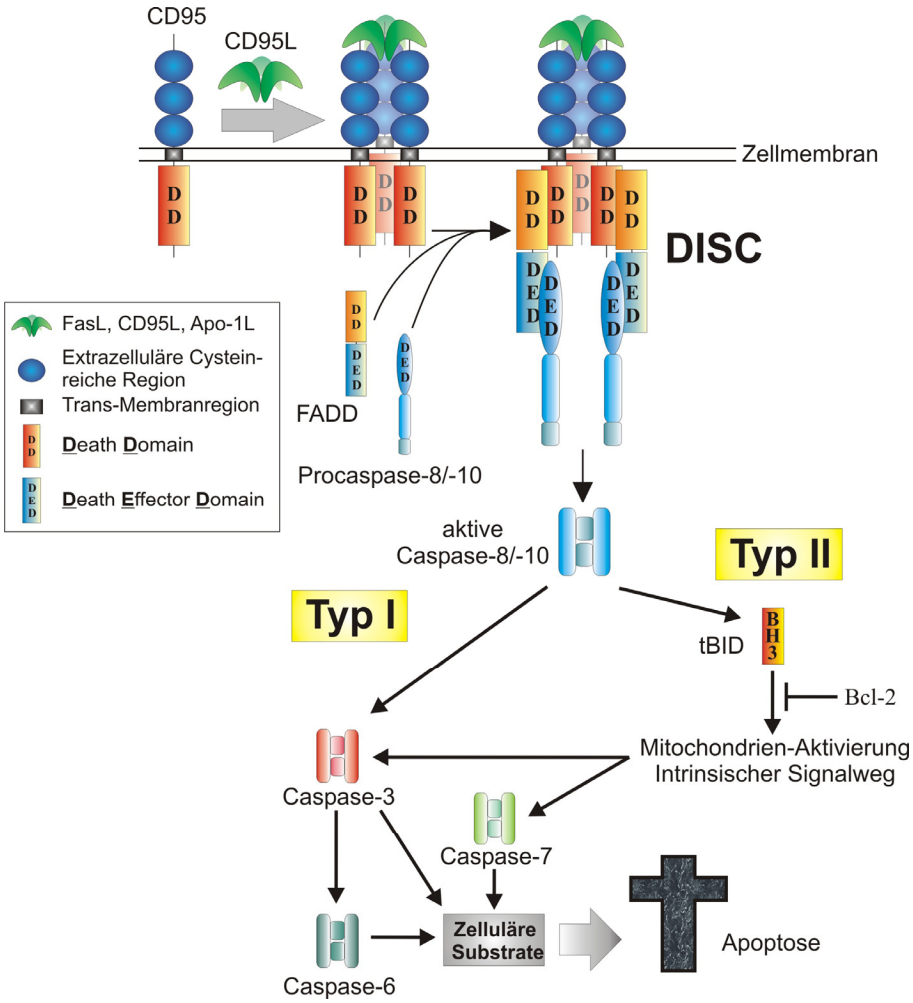

Abbildung 4: Typ I und II der CD95-induzierten Apoptose

Nach der durch den CD95-Liganden (CD95L) induzierten Oligomerisierung des CD95-Rezeptors wird der DISC über die Rekrutierung von FADD und Procaspase-8/-10 gebildet. In Typ I-Zellen wird dadurch genügend Caspase-8/-10 aktiviert, um über eine direkte Prozessierung von Caspase-3 Apoptose auszulösen. Typ II Zellen dagegen benötigen eine Signalverstärkung über die Caspase-8-vermittelte Spaltung von BID, welches den intrinsischen Signalweg aktiviert, der wiederum durch Bcl-2 inhibiert werden kann.

Über diesen Weg werden dann Caspase-3 sowie die restlichen Effektor-Caspasen-6 und -7 aktiviert und Apoptose induziert. Die Unterscheidung zwischen Typ I- und Typ II-Zellen kann mittels Überexpression des anti-apoptotischen Bcl-2-Proteins getroffen werden. Bcl-2 inhibiert tBID an den Mitochondrien und neutralisiert damit die Weiterleitung des apoptotischen Signals in Typ II-Zellen (s.a. Abschnitt 1.4, Die Bcl-2-Proteinfamilie). Auf die Todesrezeptor-vermittelte Apoptose von Typ I-Zellen dagegen hat Bcl-2 keinen Einfluss, da in diesen Zellen Caspase-3 direkt unterhalb des DISCs durch die aktivierten Initiator-Caspasen 8/10 ohne Einbindung des intrinsischen Signalwegs aktiviert wird (Scaffidi et al., 1998).

Der Signalweg über den TNF-Rezeptor ist dagegen weitaus komplexer, da über ihn nicht nur Apoptose- sondern auch Überlebens-Signale übermittelt werden, die hauptsächlich auf eine Aktivierung des Transkriptionsfaktors NF-κB zurückzuführen sind (Wajant et al., 2003). Durch NF-κB wird die Expression anti-apoptotischer Proteine wie z.B. c-FLIP (***FL****ICE* ***I****nhibitory* ***P****rotein*) verstärkt, wodurch die Aktivierung der Procaspase-8 am DISC verhindert wird (s.a. Modulatoren der Initiator-Caspasen-Aktivierung, 1.6). Die apoptotische Caspasen-Kaskade kann demnach nur im Falle einer erfolglosen NF-κB-Aktivierung gestartet werden (Micheau and Tschopp, 2003). Zurzeit existieren zwei Modelle die die Initiierung dieser gegensätzlichen Signalwege durch den TNF-Rezeptor zu erklären versuchen. In beiden Modellen ist ein zellmembranständiger TNF-Rezeptor-Komplex für die NF-κB-Aktivierung verantwortlich. Während allerdings im sequentiellen Komplex I/II Modell (Micheau and Tschopp, 2003) ein Teil des Komplexes vom TNF-Rezeptor dissoziiert und im Zytoplasma über FADD- und Procaspase-8/-10-Rekrutierung das apoptotische Programm initiiert (Komplex II), findet diese apoptotische Signaltransduktion im zweiten Modell (Schneider-Brachert et al., 2004) intrazellulär am endozytierten Rezeptor in TNF-Rezeptosom genannten Vesikeln statt.

Todesrezeptoren in der Tumortherapie

Aufgrund ihrer Apoptose-induzierenden Rolle stellen Todesrezeptoren und ihre Liganden attraktive Ziele für die Entwicklung von Tumortherapien dar. Im Tierversuch zeigte sich allerdings, dass die Aktivierung von CD95- oder TNF-Todesrezeptoren zu massiven Leberschädigungen und schlussendlich zum Tode führte, weswegen eine systemische Anwendung unmöglich ist. Zusätzlich zu den toxischen Nebenwirkungen

(septischer Schock) konnte bei der Anwendung von TNF-α nur eine geringe klinische Effektivität festgestellt werden, wodurch auch diese Behandlungsmöglichkeit ausgeschlossen wurde (Debatin and Krammer, 2004).

TRAIL dagegen besaß in mehreren Studien eine selektive Wirkung gegen Tumorzellen und erzeugte keine systemische Toxizität (Ashkenazi et al., 1999). Durch eine Behandlung mit rekombinantem löslichem TRAIL konnte *in vitro* in einer Vielzahl von Tumorzelllinien, nicht aber in normalen Kontrollzellen, effektiv Apoptose induziert werden. Auch *in vivo* wurden in verschiedenen Xenograft-Maus-Modellen die Tumore durch eine TRAIL-Therapie erfolgreich eliminiert (Almasan and Ashkenazi, 2003; Ashkenazi et al., 1999; LeBlanc and Ashkenazi, 2003; Walczak et al., 1999). Dennoch stellte sich heraus, dass die Mehrheit aller klinisch-relevanten Tumore (>50 %) resistent gegenüber einer Behandlung mit TRAIL ist. Daher ist die Kombination von TRAIL mit anderen Apoptose-induzierenden bzw. –sensitivierenden Agentien in den Fokus weiterer Studien gerückt (Van Geelen et al., 2004) und konnte auch schon in zahlreichen Zellkultur-Systemen erfolgreich getestet werden (Gliniak and Le, 1999; Marini and Belka, 2003).

1.4 Intrinsischer Apoptose-Signalweg

Die Rolle der Mitochondrien

Über mehrere unterschiedliche Signalwege, wie durch DNA-Schädigung, Todesrezeptoren (s. 1.3 und Abb. 4) oder Sauerstoff-Radikale (ROS, ***R****eactive* ***O****xygen* ***S****pecies*), werden die Mitochondrien aktiviert, die daraufhin Cytochrom c freisetzen und die Caspasen-Kaskade initiieren. Dies war eine überraschende Entdeckung, da Cytochrom c eigentlich als Elektronen-Transporter zwischen Komplex III und IV der Atmungskette dient und notwendig für das Überleben der Zelle ist. Gelangt Cytochrom c allerdings in das Zytoplasma, bindet es dort an APAF-1 (***A****poptotic-****P****rotease* ***A****ctivating* ***F****actor-1*), wodurch in Gegenwart von dATP/ATP dessen Heptamerisierung ausgelöst wird (Li et al., 1997; Liu et al., 1996). In dieser heptamerisierten Form kann APAF-1 über seine CARD die Caspase-9 rekrutieren, die so analog zur Caspase-8 im DISC prozessiert und aktiviert wird (s.a. Abschnitt 1.5). An diesem Apoptosom genannten, hochmolekularen Komplex werden nun in definierter Reihenfolge durch Caspase-9 die Effektor-Caspasen-3/-7 direkt und anschließend Caspase-6 indirekt

prozessiert und aktiviert (Slee et al., 1999), welche dann über die gezielte Spaltung ihrer Substrate für den ordnungsgemäßen Ablauf des apoptotischen Programms sorgen (Abb. 5).

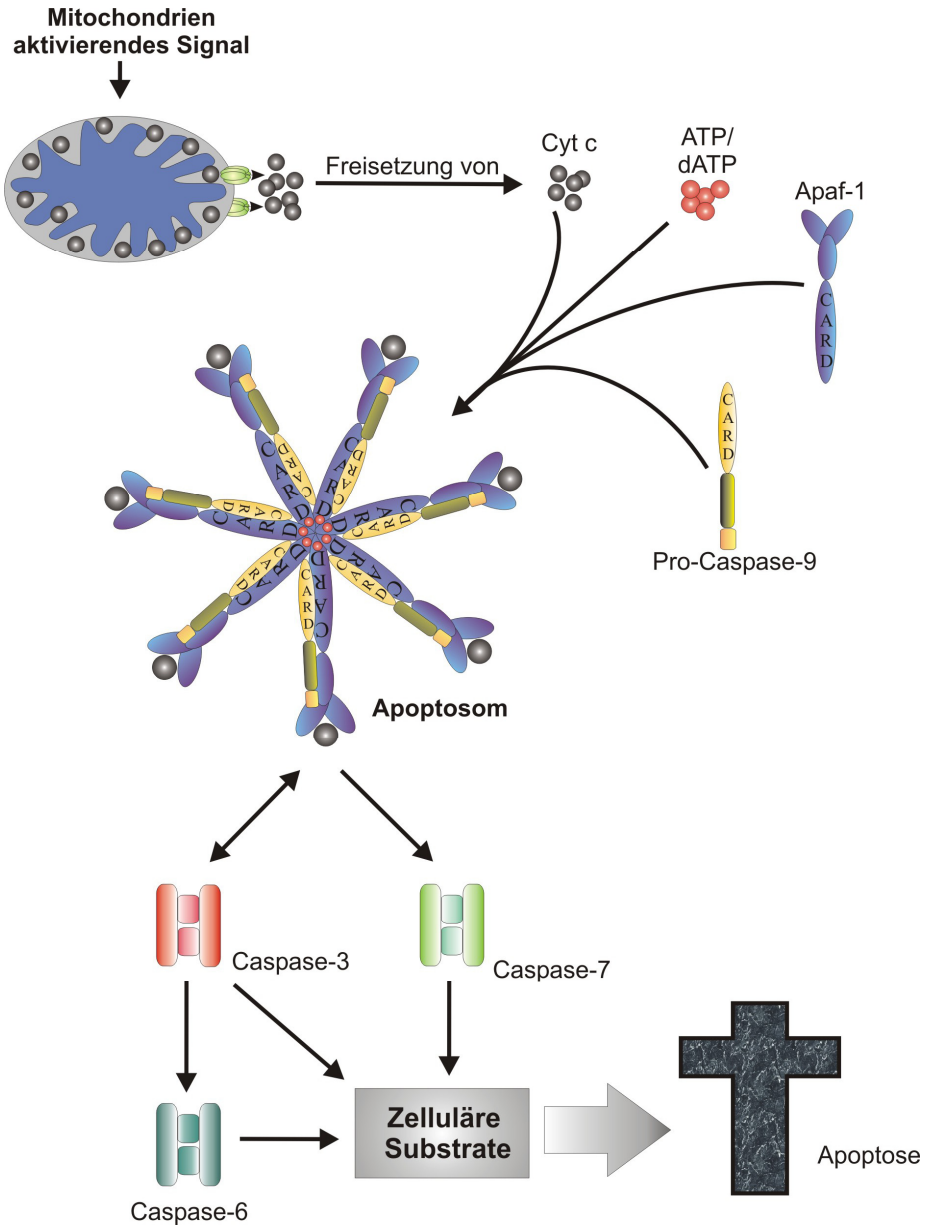

Abbildung 5: Der intrinsische Signalweg der Apoptose

Nach Aktivierung der Mitochondrien wird durch die Freisetzung von Cytochrom C in das Zytoplasma die Bildung des Apoptosoms induziert. Cytochrom C und ATP/dATP binden an APAF-1 und führen zu dessen Heptamerisierung. Dadurch kann Procaspase-9 an die freigelegte CARD von APAF-1 binden und wird aktiviert. Am Apoptosom werden anschließend die Effektor-Caspasen -3 und -7 direkt und Caspase-6 indirekt aktiviert, was letztendlich zur Apoptose führt.

Zusätzlich zur Freisetzung von Cytochrom c und anderen Proteinen (Ekert and Vaux, 2005) bildet sich im Verlauf der Apoptose eine Öffnung an den Mitochondrien (PTP, ***P**ermeability **T**ransition **P**ore*), die sowohl die innere als auch die äußere mitochondriale Membran durchspannt (Newmeyer and Ferguson-Miller, 2003). Eine länger anhaltende Öffnung dieser Pore führt zu einem Ionen-Gleichgewicht zwischen Zytoplasma und mitochondrialer Matrix und damit zu einem Verlust des mitochondrialen Membranpotentials ($\Delta\Psi_m$). Die genaue Zusammensetzung dieser Pore ist ebenso umstritten wie das Signal, woraufhin sie gebildet wird. Ebenso kontrovers wird diskutiert, ob diese Pore überhaupt geformt wird und ob sie essentiell für die korrekte Ausführung der Apoptose ist (Newmeyer and Ferguson-Miller, 2003).

Die Bcl-2 Proteinfamilie: Modulatoren der Mitochondrien-Aktivierung

Der entscheidende Schritt der apoptotischen Aktivierung der Mitochondrien, die Freisetzung von Cytochrom c, muss streng reguliert werden, damit nicht ungewollt die Caspasen-Kaskade initiiert wird. Die wichtigen Entscheidungsträger sind in diesem Fall die Mitglieder der Bcl-2-Proteinfamilie. Der Namensgeber dieser Familie, das anti-apoptotisch wirkende Bcl-2 Proto-Onkogen, wurde 1990 durch eine häufig auftretende chromosomale Translokation in humanen follikulären B-Zell Lymphomen (***B**-**C**ell **L**ymphoma, Bcl*) identifiziert, bei der das Bcl-2 Gen in den Locus der schweren Kette des Immunoglobulins versetzt wird. Diese Fusion führt zu einer stark erhöhten Expression von Bcl-2 und schützt diese Zellen vor der Apoptose-Induktion (Hockenbery et al., 1990).

Bcl-2 besitzt insgesamt vier verschiedene BH-Domänen (***B**cl-2 **H**omology*), von denen in allen Bcl-2 Familienmitgliedern mindestens eine vorhanden ist. Die Familie kann in einen anti- und einen pro-apoptotischen Zweig unterteilt werden, wobei die pro-apoptotischen Mitglieder weiter in die so genannten *BH3-only*- und in die Multidomänen (BH1-3)-Gruppe sortiert werden (Abb. 6) (Daniel et al., 2003; Reed, 2006).

Die bislang am besten charakterisierten Mitglieder des pro-apoptotischen Zweigs sind BAX (***B**cl-2 **A**ssociated **X** protein*) und BAK (***B**cl-2 **A**ntagonist **K**iller*). Beide besitzen die Domänen BH1 bis BH3 sowie eine C-terminale hydrophobe Transmembran-Region (TM). Nach heutigem Verständnis liegen sie in ihrer inaktiven Form als Monomere vor, wobei BAX weitgehend im Zytoplasma lokalisiert ist, während BAK mit den

Mitochondrien assoziiert. Die Aktivierung dieser Proteine ruft eine Konformationsänderung hervor und legt den verborgenen N-Terminus frei, der die Proteine in einer inaktiven, geschlossenen Form gehalten hat. Das aktivierende Signal ermöglicht so der C-Terminal gelegenen Transmembran-Region von BAX/BAK die Insertion in die äußere mitochondriale Membran (Desagher et al., 1999; Griffiths et al., 1999), in der sie mit sich selbst oder auch mit anderen Proteinen oligomerisieren können. Hierdurch bilden sie eine Pore, über die Cytochrom c aus dem periplasmatischen Raum in das Zytoplasma gelangen kann. Die Freisetzung von Cytochrom c und damit die Mitochondrien-vermittelte Apoptose ist in Abwesenheit von BAX und BAK stark beeinträchtigt (Lindsten et al., 2000; Wei et al., 2001). So zeigen z.B. embryonale Maus-Fibroblasten (MEF, ***M**ouse **E**mbryonic **F**ibroblasts*) von BAX/BAK doppel-defizienten Mäusen eine hohe Resistenz gegenüber einer großen Anzahl von Apoptose-induzierenden Stimuli. In diesen Zellen ist keine Aktivierung von Caspasen unterhalb der Mitochondrien detektierbar (Ruiz-Vela et al., 2005). Der Aktivierungsprozess von BAX und BAK wird durch die pro-apoptotischen BH3-only Proteine und die anti-apoptotischen Mitglieder Bcl-2 und Bcl-XL kontrolliert.

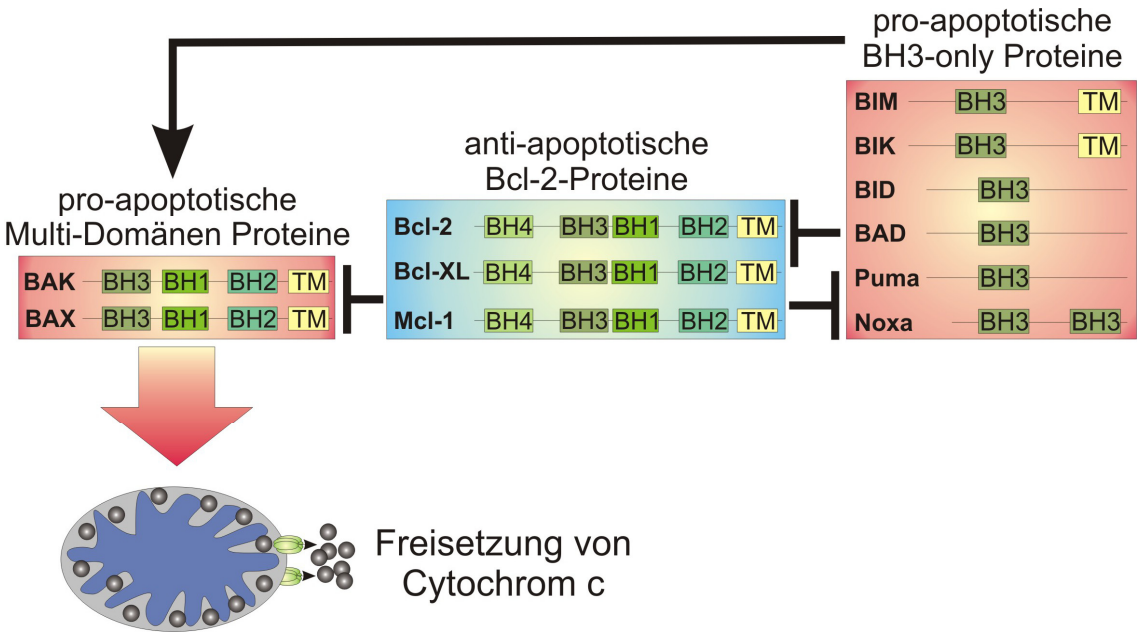

Abbildung 6: Struktur und Funktion der Bcl-2 Familienproteine

Dargestellt sind exemplarisch einige der typischen Mitglieder der Untergruppen der Bcl-2-Familie. Die Untergruppe der pro-apoptotischen Multi-Domänen-Proteine induziert die Freisetzung von Cytochrom c aus den Mitochondrien. Dieser Vorgang wird durch die anti-apoptotischen Bcl-2 Proteine inhibiert. Die *BH3-only* Proteine wiederum assoziieren mit den Bcl-2-Proteinen und heben damit deren anti-apoptotische Funktion auf. Ebenso können sie einen direkten aktivierenden Einfluss auf BAK/BAX ausüben.

Die *BH3-only* Proteine, die das apoptotische Signal zu den Mitochondrien übertragen, werden durch vielfältige Mechanismen reguliert (Strasser, 2005). So können diese Proteine entweder durch proteolytische Spaltung (BID zu tBID), Dephosphorylierung (BAD), mitochondriale Translokation (BIM) oder auch durch p53-induzierte Expression (Puma und Noxa) aktiviert werden (Gross et al., 1999; Reed, 2006). Ihre genaue Wirkungsweise an den Mitochondrien ist hierbei jedoch noch nicht vollständig geklärt. Einerseits induzieren sie über die Bindung an anti-apoptotische Bcl-2 Mitglieder indirekt die pro-apoptotische Wirkung von BAX und BAK. Andererseits können sie auch über eine direkte Interaktion mit BAK und/oder BAX für deren Aktivierung sorgen (Willis and Adams, 2005). Bestätigt scheint bisher nur, dass BAX und/oder BAK absolut notwendig für die pro-apoptotische Wirkung der *BH3-only* Proteine sind (Zong et al., 2001).

Die Antagonisten der Cytochrom c-Freisetzung sind die anti-apoptotischen Bcl-2 Familienmitglieder: Bcl-2, Bcl-XL, und Mcl-1. Diese sind integrale mitochondriale Membranproteine, die an aktiviertes BAX und BAK binden und so die Porenbildung verhindern. Darüber hinaus hemmen sie durch Bindung an aktivierte *BH3-only* Proteine die Signalweiterleitung an BAK/BAX (Cheng et al., 2001).

Der Erfolg einer Apoptose-Induktion hängt demnach von der Stärke des Signals, den Expressionsmengen der pro- und anti-apoptotischen Bcl-2-Familienmitglieder sowie von deren post-translationalen Modifikationen ab. Diesbezüglich konnte gezeigt werden, dass die Transkription der Bcl-2 Familienmitglieder stark von Überlebens- und Apoptose-Signalwegen abhängig ist. So induziert der aktivierte Tumorsuppressor p53 z.B. die Transkription von BAX, Puma und Noxa, wohingegen die Expression von Bcl-2 und Bcl-XL reprimiert wird (Yu and Zhang, 2005). Die Expression von Bcl-XL dagegen wird erhöht durch CD28- oder CD40-vermittelte Überlebens-Signalwege (Boise et al., 1995; Choi et al., 1995) und sowohl Bcl-XL, Bcl-2 als auch das anti-apoptotische Bcl-2 Homolog A1 sind Zielgene von aktiviertem NF-κB (Chen et al., 2000; Viatour et al., 2003; Zong et al., 1999).

1.5 Mechanismen der Initiator-Caspasen-Aktivierung

Initiator-Caspasen: Das "*induced proximity*"-Modell

Im Gegensatz zu Effektor-Caspasen, die durch andere Caspasen prozessiert und damit aktiviert werden, benötigen Initiator-Caspasen andere Aktivierungs-Mechanismen, da sie am Anfang der Caspasen-Kaskade stehen. Dies geschieht nach heutigem Verständnis durch eine Dimerisierung oder auch „*induced proximity*", die durch die Rekrutierung der Initiator-Caspasen in ihre jeweiligen Aktivierungskomplexe zustande kommt (Salvesen and Dixit, 1999). Diese Dimerisierung und die damit verbundene Konformationsänderung generiert ein bereits aktives Initiator-Caspasen-Dimer, welches sich autoproteolytisch spalten kann, um das finale Heterotetramer zu erzeugen. Während die Prozessierung von Effektor-Caspasen für deren katalytische Aktivität absolut erforderlich ist, scheint sie im Falle der Initiator-Caspasen nur für die Stabilität des erzeugten Dimers, nicht aber für dessen Aktivierung verantwortlich zu sein. So zeigen z.B. künstlich dimerisierte, aber unspaltbare Caspase-8- und -9-Punktmutanten eine mit den unveränderten Enzymen vergleichbare Aktivität. Wird allerdings die Oligomerisierung durch einzelne Aminosäure-Austausche beeinträchtigt, so können diese Initiator-Caspasen nicht mehr aktiviert werden (Boatright et al., 2003).
Ähnliche Befunde wurden für die Initiator-Caspasen-2, -10 sowie die murine Caspase-11, einem wichtigen Initiator von Entzündungsprozessen, erhalten (Chang et al., 2003). So wird davon ausgegangen, dass die Aktivierung von Initiator-Caspasen generell nach dem *„induced proximity"*-Modell stattfindet. Aufgrund der Tatsache, dass diese Ergebnisse vorwiegend in künstlichen *in vitro*-Systemen gewonnen wurden, ist es allerdings noch völlig unklar, inwiefern sich dieses Modell auch auf relevante *in vivo*-Situationen übertragen lässt (Shi, 2004).

Caspase-8/-10: Der Todesrezeptorkomplex (DISC)

Der DISC bildet sich nach Aktivierung der Todesrezeptoren an der intrazellulären Seite des Rezeptors (Abb. 7). Über dessen DD wird das Adapterprotein FADD rekrutiert, welches eine DD und eine DED enthält. Procaspase-8 kann anschließend über homophile Interaktionen der DEDs an den DISC binden. Durch die dadurch erreichte Oligomerisierung wird ein aktives Procaspase-8-Dimer erzeugt, in dem sich die einzelnen Procaspasen zunächst intramolekular zwischen den beiden Untereinheiten

spalten. So wird die kleine Untereinheit (p12) sowie ein p43-Fragment generiert, welches aus der Prodomäne und der großen Untereinheit besteht. In diesem Zustand kann kein aktives Heterotetramer in das Zytoplasma gelangen, da es über die DED immer noch an den DISC gebunden ist. Daher erfolgt eine zweite, intermolekulare proteolytische Weiterverarbeitung vom benachbarten Procaspase-8 Molekül, wodurch das finale p20/p12-Heterotetramer entsteht (Lavrik et al., 2003). Obwohl noch nicht eingehend untersucht, wird angenommen, dass Procaspase-10 auf vergleichbare Art und Weise am DISC aktiviert wird.

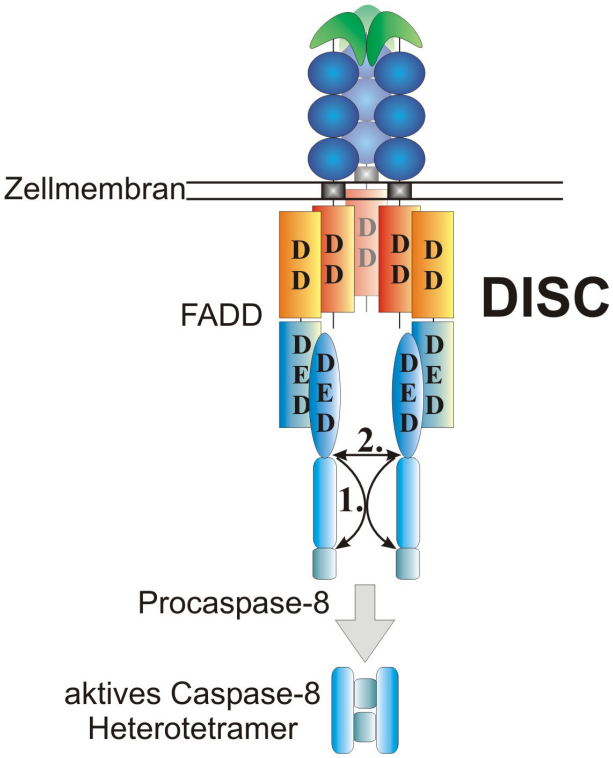

Abbildung 7: Prozessierung von Procaspase-8 am DISC

Proteolytische intra- und intermolekulare Prozessierung von Procaspase-8 Dimeren im DISC. Der erste Spaltungsschritt findet zwischen den beiden Untereinheiten statt. Anschließend wird die Prodomäne entfernt, so dass das finale aktive Heterotetramer in das Zytoplasma freigesetzt werden kann. Für die vollständige Prozessierung werden zwei aktive Procaspase-8 Moleküle benötigt, da die Abspaltung der Prodomäne intermolekular durchgeführt werden muss.

Caspase-9: Das Apoptosom

Im Zytoplasma bindet das aus den Mitochondrien freigesetzte Cytochrom c an APAF-1 und induziert dadurch die Bildung des Apoptosoms. APAF-1 besitzt eine CARD, eine Nukleotidbindungs-Domäne sowie mehrere WD40-*Repeats*. Durch die Bindung von Cytochrom c an die WD40-Domänen wird die Affinität von APAF-1 für dATP oder ATP über das Zehnfache erhöht. Dies induziert eine Konformationsänderung in APAF-1, die zu dessen Heptamerisierung und zur Bildung des Apoptosom führt. Über die nun freigelegte CARD kann Procaspase-9 binden und sich durch die Oligomerisierung autokatalytisch aktivieren (Pop et al., 2006). Das dabei gebildete Apoptosom konnte bereits elektronenmikroskopisch analysiert und die 3D-Struktur ermittelt werden, die basierend auf der Heptamerisierung auch als „*wheel of death*“ bezeichnet wird (Acehan

et al., 2002) (s.a. Abb. 5). Im Gegensatz zur Caspase-8 führt die autokatalytische Aktivität der Caspase-9 nur zu einem Prozessierungsschritt, in dem ein p35 (Prodomäne und große Untereinheit)- sowie ein p12 (kleine Untereinheit)-Fragment generiert wird, so dass aktive Caspase-9 am Apoptosom gebunden bleibt. Obwohl die entstandene aktive Protease nur geringe Aktivität besitzt, kann sie dennoch die zum Apoptosom rekrutierte Effektor-Caspase-3 spalten und aktivieren, da aufgrund der Apoptosom-Bindung ihre Affinität für die Procaspase-3 erheblich erhöht ist (Yin et al., 2006). Im direkten Anschluss an ihre eigene Aktivierung prozessiert Caspase-3 das benachbarte dimerisierte Caspase-9-Molekül. Dabei erkennt und spaltet sie allerdings an einem anderen Aspartat-Rest, so dass ein p35/p10 Caspase-9-Enzym entsteht, welches um ein Vielfaches (~ achtfach) aktiver ist als die zuvor generierte p35/p12-Protease. Ebenso prozessiert Caspase-3 ungespaltene, dimerisierte Procaspase-9 zu einer p37/p10-Form, die über eine ähnlich erhöhte Aktivität verfügt wie die p35/p10-Form (Zou et al., 2003). Caspase-3 wird also für eine effiziente Prozessierung und Aktivierung von Caspase-9 am Apoptosom benötigt (Blanc et al., 2000).

Caspase-2: Das PIDDosom

Von den Caspasen ist Caspase-2 bisher das am schwierigsten zu kategorisierende Mitglied. Einerseits weist die Struktur mit der CARD auf eine Initiator-Caspase hin, die Substratspezifität entspricht jedoch der einer Effektor-Caspase. Weiterhin kann aktive Caspase-2 bis auf ihre eigene Zymogen-Form keine weiteren Caspasen spalten und aktivieren, wodurch eine Rolle an der Spitze der Caspasen-Kaskade unmöglich erscheint. Sie selbst kann allerdings von Caspase-3 und -8 gespalten werden (Van de Craen et al., 1999). Außerdem kann vollständig prozessierte Caspase-2 unabhängig von ihrer katalytischen Aktivität die Freisetzung von Cytochrom c aus den Mitochondrien bewirken, ohne dass hierfür die Poren-formenden Proteine BAK und BAX benötigt werden (Enoksson et al., 2004; Robertson et al., 2004). Dennoch zeigte sich, dass auch die Caspase-2 entsprechend dem „*induced proximity*“-Modell aktiviert wird (Baliga et al., 2004). Sie wird dabei allerdings nicht über ihre CARD zum Apoptosom rekrutiert. Stattdessen wurde als direkter Interaktionspartner das Adapterprotein RAIDD (***R**ip-**A**ssociated protein with a **D**eath **D**omain*) identifiziert, welches sowohl eine CARD als auch eine DD besitzt. Anfänglich wurde angenommen, dass Caspase-2 über RAIDD und RIP (***R**eceptor **I**nteracting **P**rotein*) in den Komplex I des TNF-Rezeptors rekrutiert

und dort aktiviert wird (Ahmad et al., 1997; Duan and Dixit, 1997). Dies konnte aber in einer nachfolgenden Arbeit nicht bestätigt werden (Micheau and Tschopp, 2003). Inzwischen wurde ein anderer Proteinkomplex charakterisiert, der als Caspase-2-Aktivierungsplattform dienen könnte (Tinel and Tschopp, 2004). Das zentrale Protein ist hierbei PIDD (***P**53-**I**nducible protein with a **D**eath **D**omain*), dessen Expression durch den aktivierten Tumorsuppressor p53 (s.a. 1.6, Abschnitt Der Tumorsuppressor p53) erhöht wird und das über seine DD das RAIDD-Protein und damit Caspase-2 binden kann.

Ob und welche Rolle das PIDDosom in der Apoptose-Induktion spielt, ist aber noch völlig ungeklärt. Während einige Arbeitsgruppen in der durch DNA-Schädigung oder Hitzeschock-induzierten (Tu et al., 2006) Apoptose eine Aktivierung der Caspase-2 oberhalb der Mitochondrien beschrieben und demzufolge dieser Caspase eine Initiator-Rolle in diesen Prozessen zuwiesen (Zhivotovsky and Orrenius, 2005), wurden in anderen Studien Caspase-2 unabhängige Mechanismen vorgeschlagen (Milleron and Bratton, 2006). Darüber hinaus kann das PIDDosom auch für eine NF-κB-Aktivierung nach DNA-Schädigung verantwortlich sein, ohne dass Caspase-2 dafür benötigt wird (Janssens et al., 2005). Es ist also durchaus möglich, dass Caspase-2 nur in einer Zelltyp-spezifischen und Stimulus-abhängigen Art und Weise aktiviert wird.

Caspase-1: Das Inflammasom

Analog zu den apoptotischen Caspasen werden auch inflammatorische Initiator-Caspasen durch das „*induced proximity*"-Modell aktiviert. In verschiedenen Formen des Inflammasoms (NALP1 [***NA**CHT, **L**eucine-rich-repeat and **P**YD containing protein*] und NALP2/3 basiert) werden über das ASC-Adapterprotein (***A**poptosis-associated **S**peck-like protein containing a **C**ARD*) Procaspase-1 und -5 rekrutiert und aktiviert (Petrilli et al., 2005). Das Adapterprotein ASC besteht hierbei aus einer CARD zur Bindung von Caspasen und einer PYD (***Py**rin **D**omain*), über die sie mit den NALPs interagiert. Die NALPs reagieren auf so genannte Gefahren-Signale wie z.B. NALP3 auf ein Muramyl-dipeptid, das Bestandteil der bakteriellen Zellwand ist. Die Detektion solcher Moleküle führt damit zur Bildung des Inflammasoms und zur Initiierung einer Entzündungsreaktion, die z.B. durch die von Caspase-1 vermittelte Reifung der Interleukine IL-1ß, IL-18 und IL-33 ausgebildet wird (Ogura et al., 2006). Das Inflammasom ist also essentiell für eine erfolgreiche Immunantwort gegenüber pathogenen Organismen.

1.6 Modulatoren der Initiator-Caspasen-Aktivierung

Da die Aktivierung der Caspasen kaskadenartig stattfindet, ist es sinnvoll die großen Initiierungskomplexe äußerst strikt zu regulieren. Aus diesem Grunde exprimieren die Zellen anti-apoptotische Proteine, die eine ungewollte Initiierung der Caspasen-Kaskade verhindern sollen. Durch eine Veränderung der Expression dieser anti-apoptotischen Proteine wird einer Zelle außerdem die Möglichkeit gegeben, ihre Apoptose-Resistenz bzw. -Sensitivität als Reaktion auf bestimmte Umstände anzupassen. Die bereits beschriebenen anti-apoptotischen Bcl-2 Familien-Mitglieder (s. 1.4) sind verantwortlich für die Kontrolle der apoptotischen Aktivierung der Mitochondrien und die Freisetzung von Cytochrom c. Die Caspasen-Aktivierung der zwei anderen zentralen Initiierungskomplexe der Apoptose, des DISC und des Apoptosoms, werden auch über dementsprechende Modulatoren reguliert.

c-FLIP

c-FLIP (***C****ellular-****FL****ICE* ***I****nhibitory* ***P****rotein*) ist ein Inhibitor der Caspase-8 Aktivierung am DISC (Kataoka, 2005). Das Gen für c-FLIP befindet sich direkt neben den Genen für Caspase-8 und -10 auf dem menschlichen Chromosom 2. Es werden insgesamt elf verschiedene mRNA-Isoformen durch alternatives Splicing gebildet, von denen allerdings bisher nur drei auf Protein-Ebene nachgewiesen werden konnten: FLIP-long (p55), FLIP-short (p27) und FLIP-r (p25). FLIP-long ist sehr homolog zu Caspase-8 und -10 und besitzt zwei DEDs, über die es mit FADD interagieren und in den DISC rekrutiert werden kann. Zusätzlich besitzt FLIP-long eine Caspasen-ähnliche Domäne mit einer großen und kleinen Untereinheit, in der sogar die Spaltstelle zwischen den Untereinheiten vorhanden ist. Im Gegensatz zu Caspase-8/-10 fehlen FLIP-long jedoch essentielle Aminosäuren für eine katalytische Kompetenz, einschließlich des aktiven Cysteinyl-Rest.

FLIP-short und FLIP-r dagegen besitzen nur die N-terminalen DEDs sowie einen jeweils einzigartigen kurzen C-terminalen Rest. Ähnlich wie FLIP-long werden sie über Interaktion mit FADD in den DISC rekrutiert. Für das erst kürzlich entdeckte FLIP-r konnten jedoch bisher keine Unterschiede zu den Eigenschaften von FLIP-short aufgezeigt werden (Golks et al., 2005).

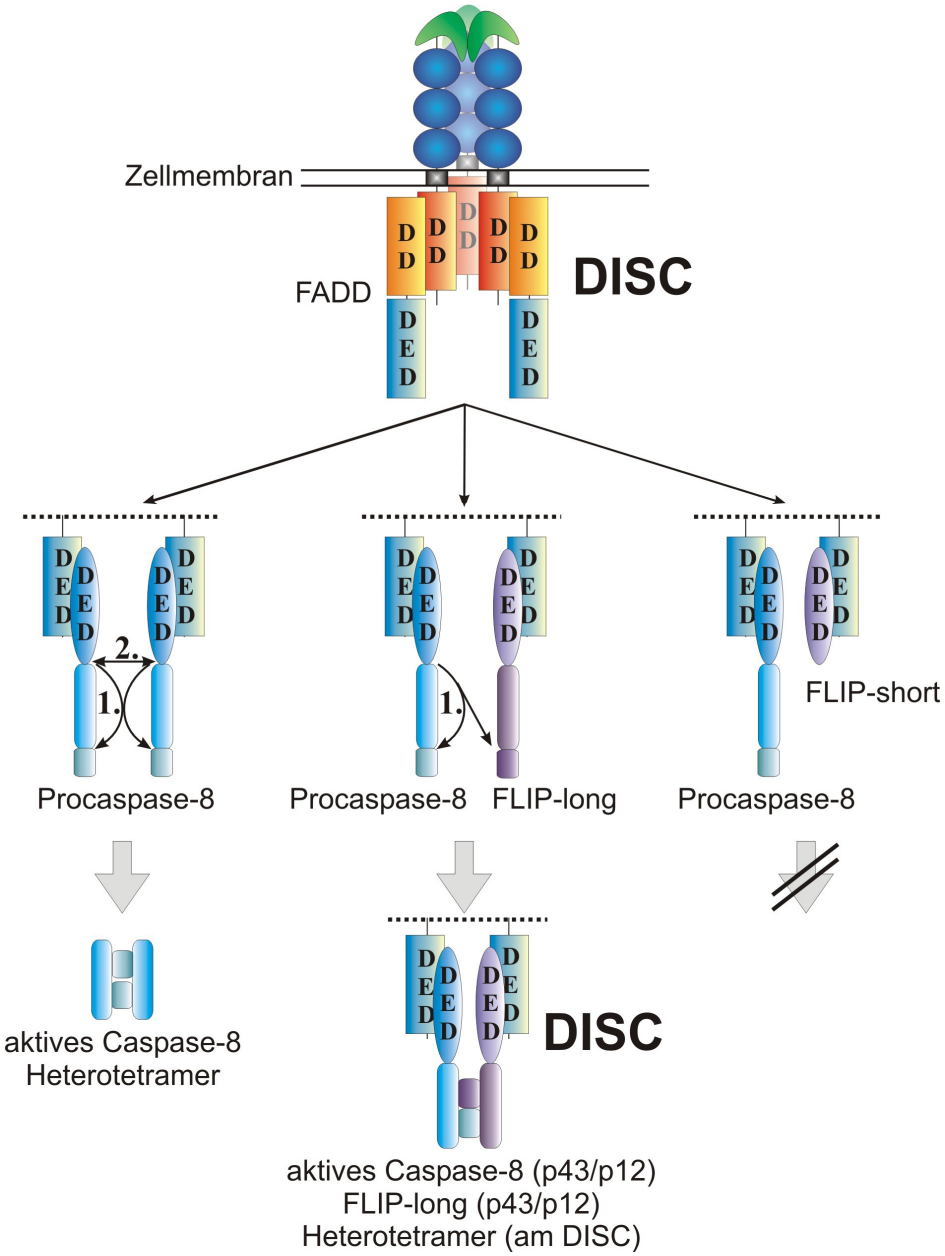

Abbildung 8: Einfluss von c-FLIP auf die Prozessierung der Procaspase-8 im DISC

Bei der DISC-Bildung gibt es durch die Rekrutierung von FLIP-short, FLIP-long und Procaspase-8 mehrere Möglichkeiten der Komplexbildung. Abhängig von den Komponenten der Dimerisierung werden unterschiedliche Caspase-8-Fragmente beobachtet. Diese Dimerisierungen sind von den Expressionsmengen der einzelnen Proteine abhängig. FLIP-short behindert die Prozessierung der Procaspase-8 vollständig, wohingegen FLIP-long zur Generierung eines aktiven Caspase-8/FLIP-long Heterotetramers führt, welches allerdings über seine DED immer noch mit dem DISC assoziiert bleibt. Für FLIP-r wird ein ähnliches Verhalten wie für FLIP-short postuliert.

FLIP-short und FLIP-r sind reine Inhibitoren der Procaspase-8 am DISC. Über ihre DEDs dimerisieren sie mit Procaspase-8, ohne allerdings eine Konformationsänderung und damit eine Aktivierung des benachbarten Procaspase-8-Moleküls hervorzurufen (Abb. 8) (Krueger et al., 2001). Dies liegt daran, dass sie im Gegensatz zu FLIP-long über keine C-terminale Caspase-ähnliche Domäne verfügen. Der Inhibitions-

Mechanismus durch FLIP-long dagegen ist komplexer. Sowohl Procaspase-8-Homodimere als auch Procaspase-8/FLIP-long-Heterodimere weisen die gleiche Aktivität und Substratspezifität auf (Boatright et al., 2004). FLIP-long kann demnach analog zu dem „*induced proximity*"-Modell über seine C-terminale Caspase-ähnliche Domäne ein benachbartes Procaspase-8 Molekül aktivieren (Chang et al., 2002). Dieses prozessiert daraufhin sich selbst intra- sowie das FLIP-long Molekül inter-molekular an der dafür vorgesehen Schnittstelle zwischen den Untereinheiten, was in einer Spaltung von FLIP-long in ein p43- und ein p12-Fragment resultiert. Aufgrund der fehlenden katalytischen Kompetenz von FLIP-long kann dieses aber nicht das benachbarte Procaspase-8 Molekül spalten, weshalb die Prozessierung beim Stande eines Caspase-8 (p43/p12)/ FLIP-long (p43/p12) Heterotetramers endet (Abb. 8) (Krueger et al., 2001). Dieses bleibt im Gegensatz zum vollständig prozessierten Caspase-8-Heterotetramer über seine DEDs mit dem DISC assoziiert und kann damit keine Apoptose induzieren, obwohl es katalytisch aktiv ist.

Es wurde jedoch bereits spekuliert, dass diese ausschließlich lokal stattfindende Aktivierung für nicht-apoptotische Funktionen von Caspase-8 von Bedeutung ist, wie z.B. für die Initiierung anderer Signalwege durch die Spaltung von RIP (***R****eceptor-****I****nteracting* ***P****rotein*), welches auch zum DISC rekrutiert werden kann (Micheau et al., 2002). Auch eine Aktivierung anti-apoptotischer Signalwege durch FLIP-long, z.B. über NF-κB, wurde bereits kontrovers diskutiert (Dohrman et al., 2005; Kataoka and Tschopp, 2004; Kreuz et al., 2004). Zusammenfassend geht man heute davon aus, dass genügend hohe Konzentrationen von FLIP-long und/oder FLIP-short dafür sorgen, dass keine Procaspase-8 Dimere, sondern nur c-FLIP/Procaspase-8-Heterodimere am DISC gebildet werden können und damit keine vollständig prozessierte Caspase-8 ins Zytoplasma gelangen kann (Kataoka, 2005).

Die Expression der c-FLIP-Proteine wird über mehrere verschiedene Mechanismen reguliert. So weisen die c-FLIP-Proteine z.B. eine sehr kurze Halbwertszeit auf, weshalb sie kontinuierlich neu synthetisiert werden müssen. Daher sensitiviert eine Behandlung mit dem Translations-Inhibitor Cycloheximid viele unterschiedliche Zelltypen für die Todesrezeptor-induzierte Apoptose (Fulda et al., 2000). Diese kurze Halbwertszeit liegt an der ständigen Umsetzung der c-FLIP-Proteine durch das Ubiquitin-Proteasom-System. Vor allem FLIP-short wird an seinem einzigartigen C-Terminus ubiquitiniert und proteolytisch degradiert (Poukkula et al., 2005). Zusätzlich wird die Transkription von c-FLIP über mehrere Signalwege reguliert. c-FLIP ist z.B.

ein Zielprotein des NF-κB-Überlebens-Signalweg (Micheau et al., 2001). In frisch aktivierten T-Zellen wird die FLIP-short-Expression nach anti-CD3 Stimulation erhöht und ist verantwortlich für die gesteigerte Resistenz gegenüber CD95-induzierter Apoptose und Aktivierungs-induziertem Zelltod (AICD*, **A**ctivation **I**nduced **C**ell **D**eath*) (Schmitz et al., 2004). Darüber hinaus wird die Aktivität von c-FLIP über posttranslationale Modifikationen wie z.B. Phosphorylierungen reguliert, die sowohl einen positiven als auch einen negativen Effekt auf die c-FLIP-vermittelte Inhibition von Caspase-8 ausüben können (Higuchi et al., 2003; Song et al., 2003; Tourian et al., 2004; Yang et al., 2003).

XIAP

XIAP (***X**-chromosome-linked **IAP***) ist das bisher am besten charakterisierte Mitglied der IAP (***I**nhibitor of **A**poptosis **P**rotein*)-Familie (Salvesen and Duckett, 2002). Alle Mitglieder verfügen über BIR-Domänen (***B**aculovirus **I**AP **R**epeat*), von denen XIAP drei besitzt (BIR1-3), die für die Inhibition von Caspasen essentiell sind. XIAP ist in dieser Familie der effektivste und über mehrere Mechanismen wirkende Apoptose-Inhibitor, dessen Angriffspunkt das Apoptosom ist. XIAP bindet über seine BIR3-Domäne direkt an die abgespaltene kleine Untereinheit von Caspase-9 im Apoptosom und inhibiert damit die enzymatische Aktivität von Caspase-9 (Srinivasula et al., 2001). Dabei ist die Spaltung der Caspase-9 für die XIAP-vermittelte Inhibition essentiell, da hierfür die Aminosäuresequenz direkt hinter der Spaltstelle erkannt und gebunden wird.
Da die dimerisierte Procaspase-9 entsprechend dem „*induced proximity*"-Modell auch ohne Prozessierung aktiv ist, muss gewährleistet sein, dass die Caspasen-Kaskade auch unterhalb dieser Initiator-Caspase kontrolliert werden kann. Dies wird durch eine zusätzliche XIAP-vermittelte Inhibition der Effektor-Caspasen-3 und -7 erreicht. XIAP bindet diese Caspasen an einer konservierten Oberflächenstruktur mittels einer Region direkt N-terminal zur BIR2-Domäne und verhindert so eine Substratbindung an die katalytischen Zentren der Caspasen (Chai et al., 2001; Deveraux et al., 1999; Riedl et al., 2001). Dabei agiert XIAP nicht als Pseudosubstrat für Caspase-3/-7, wie es für die beiden viralen Caspase-Inhibitoren p35 (Baculovirus) und CrmA (***C**ytokine **R**esponse **M**odifier **A***, Orthopox-Virus) beschrieben ist (Stennicke et al., 2002).
Neben diesen direkten inhibitorischen Effekten auf die Caspasen kann XIAP zusätzlich über eine C-terminale RING-Domäne als E3-Ligase agieren (Vaux and Silke, 2005) und

so Caspase-3 für den Abbau durch das Ubiquitin-Proteasom-System markieren (Suzuki et al., 2001). Über diese zwei Mechanismen ist XIAP ein potenter Inhibitor des Apoptosoms und der damit verbundenen Caspasen-Aktivierung. Bemerkenswerterweise besitzt nur XIAP, trotz der hohen Homologie zwischen den IAP-Familienmitgliedern, die benötigten strukturellen Voraussetzungen für eine direkte Inhibition der enzymatischen Aktivität von Caspasen (Eckelman and Salvesen, 2006; Eckelman et al., 2006). Die anti-apoptotische Wirkungsweise der anderen IAP-Familienmitglieder ist voraussichtlich auf einen ausschließlich indirekten Effekt durch Bindung und Ubiquitinierung pro-apoptotischer Proteine wie den Caspasen zurückzuführen. Es ist sogar davon auszugehen, dass die anderen Familienmitglieder ungeachtet ihres Namens (***I****nhibitor of* ***A****poptosis* ***P****roteins*) ihre Hauptfunktion nicht in der Regulation der Apoptose, sondern in anderen Signalwegen (NF-κB, Zellzyklus-Kontrolle) ausüben.
Ähnlich wie die c-FLIP-Proteine wird auch die Expression und Modifikation von XIAP auf mehreren Ebenen kontrolliert. So wird dieses anti-apoptotische Protein nicht nur durch den NF-κB-Überlebens-Signalweg induziert (Stehlik et al., 1998), sondern kann auch aufgrund seiner intrinsischen E3-Ligase-Aktivität auto-ubiquitiniert und damit für den Abbau markiert werden (Yang et al., 2000).

Das Proteasom

Das Proteasom ist ein großer multi-katalytischer Enzymkomplex (26S), der für den Abbau von Ubiquitin-markierten Proteinen verantwortlich ist. Er setzt sich aus einer katalytisch aktiven 20S-Kernuntereinheit und zwei regulatorischen, ATP-abhängigen 19S Untereinheiten zusammen. Die Kernuntereinheit besteht aus sieben weiteren β-Untereinheiten, durch die die verschiedenen proteolytischen (Post-glutamyl peptidyl-, Trypsin- und Chymotrypsin-ähnliche) Aktivitäten ausgeübt werden. Die 19S regulatorischen Untereinheiten sind für die Erkennung ubiquitinierter Proteine verantwortlich und leiten diese zur 20S-Kernuntereinheit weiter, wo sie degradiert und verdaut werden. Da zu den proteasomalen Substraten eine große Anzahl von regulatorischen Schlüsselproteinen, wie z.B. p53, *Cycline*, CDK (***C****yclin-****D****ependent* ***K****inase*)-Inhibitoren oder auch der NF-κB-Inhibitor IκBα (Naujokat and Hoffmann, 2002) gehören, ist das Proteasom an der Regulation vieler zellulärer Signaltransduktions-Prozesse wie Zellzyklus, Differenzierung und natürlich auch Apoptose beteiligt (Jesenberger and Jentsch, 2002). Viele Mitglieder des pro-

apoptotischen Zweiges der Bcl-2 Familie wie BAX, tBID, und BIM werden ebenfalls durch das Ubiquitin-Proteasom-System kontinuierlich degradiert (Nikrad et al., 2005). Aber auch andere pro-apoptotische Moleküle wie der TRAIL-Rezeptor-2, das während der Apoptose aus den Mitochondrien freigesetzte Smac/DIABLO (***S**econd **M**itochondria-derived **A**ctivator of **C**aspase* / ***D**irect **IA**P-**B**inding protein with **Lo**w pI*) (MacFarlane et al., 2002) sowie aktive Caspasen wurden als Zielproteine des Proteasoms identifiziert (Naujokat and Hoffmann, 2002). Das Proteasom wirkt jedoch nicht nur selektiv auf pro-apoptotische Proteine. Auch die Expression der wichtigsten Inhibitoren der Apoptose-Induktion, c-FLIP, Bcl-2 und XIAP, wird durch ständigen Abbau und Neusynthese kontrolliert (Yang and Yu, 2003).

Da das Proteasom einen weit reichenden Einfluss auf wichtige Vorgänge der Zelle wie z.B. den Zellzyklus hat, wurden Proteasom-Inhibitoren zur Bekämpfung von proliferierenden, Apoptose-resistenten Tumoren entwickelt (Richardson et al., 2005; Zavrski et al., 2005). Neben Clasto-Lactacystin (klinische Phase 1) und MG-132 ist Bortezomib (Velcade™, zuvor PS-341) der zur Zeit erfolgreichste Proteasom-Inhibitor (Richardson et al., 2006), der in Europa seit 2005 als Zweittherapie gegen das Multiple Myelom zugelassen ist und sich bereits in klinischen Tests gegen andere Tumorarten befindet (Richardson et al., 2003; Voorhees and Orlowski, 2006). Die exakten Signalwege, über die Proteasom-Inhibitoren den Zelltod bzw. Apoptose auslösen, sind dabei aber noch nicht bekannt. Es ist jedoch davon auszugehen, dass die Stabilisierung von pro-apoptotischen Proteinen zumindest teilweise dafür verantwortlich sein dürfte. Eine weitaus effektivere Krebsbekämpfung wurde mit einer Kombinationstherapie erreicht, in der Proteasom-Inhibitoren zusammen mit anderen Apoptose-induzierenden Agentien verabreicht wurden (Orlowski et al., 2005). So gelang es z.B. Apoptose-resistente Zellen und Xenografts durch Bortezomib gegenüber verschiedenen DNA-Schädigung hervorrufende Stimuli wie z.B. Cisplatin (Cusack et al., 2000), Doxorubicin (Ma et al., 2003) oder auch Bestrahlung (Russo et al., 2001) zu sensitivieren. Die Ursache dieser Sensitivierung liegt dabei vermutlich in der Beeinflussung mehrerer pro- und anti-apoptotischer Mechanismen wie z.B. dem NF-κB-Überlebens-Signalweg. NF-κB wird durch seinen Inhibitor, IκBα, im Zytoplasma komplexiert. Nach einem Aktivierungssignal wird IκBα ubiquitiniert und abgebaut, wodurch NF-κB in den Nukleus translozieren kann, um dort die Transkription von z.B. anti-apoptotischen Genen zu initiieren. Durch Zugabe von Proteasom-Inhibitoren wird IκBα nicht mehr abgebaut und NF-κB trotz stimulierenden Signals nicht aktiviert. Daraus resultiert eine

Reduktion der Expression von anti-apoptotischen Proteinen und demzufolge eine Sensitivierung gegenüber Apoptose-induzierenden Stimuli (Nakanishi and Toi, 2005). Besonders in Bezug auf die selektive zytotoxische Aktivität des Todesrezeptor-Liganden TRAIL (s. 1.2) gegenüber Tumorzellen (Held and Schulze-Osthoff, 2001) erscheint eine Kombinationsbehandlung mit Proteasom-Inhibitoren ein viel versprechender Ansatz für eine Tumortherapie zu sein, die in einigen Zellkultur-Systemen auch schon erfolgreich getestet werden konnte (He et al., 2004; Kim et al., 2004; Wang et al., 1996).

Der Tumorsuppressor p53 und sein Zielprotein p21 $^{WAF/CIP1}$

Der Transkriptionsfaktor und Tumorsuppressor p53 ist das Kernelement einer großen Maschinerie, die auf eine Vielzahl von Stresssignalen (z.B. DNA-Schädigung, Hypoxie, oder reaktive Sauerstoffspezies) unterschiedliche Antworten der Zelle erzeugt (Levine et al., 2006). Hierzu gehört die Induktion der DNA-Reparatur, reversibler und irreversibler (Seneszenz) Zellzyklusarrest sowie anti-angiogene und pro-apoptotische Wirkungen. Anfänglich wurde postuliert, dass es sich bei p53 um ein potentes Onkogen handelt. Es stellte sich jedoch schnell heraus, dass diese Vermutung auf der ursprünglichen Identifizierung einer p53-Mutante basierte, die natürlich die eigentlichen Tumor-suppressiven Wirkungen des Wildtyp-Proteins nicht mehr aufwies.

Im inaktiven Zustand befindet sich p53 in einem zytoplasmatischen Komplex mit der E3-Ligase HDM2 (***H**uman **D**ouble **M**inute **2** protein*), durch die p53 ubiquitiniert und für den proteasomalen Abbau markiert wird. Die Stresssignal-vermittelte Aktivierung von p53 wird über eine ATM (***A**taxia **T**elangiectasia **M**utant*)-Kinase- und Chk1/2 (***Ch**eckpoint **K**inase*)-vermittelte Phosphorylierungskaskade ausgelöst, die in der Dissoziation von HDM2 mündet. Diese Dissoziation führt zur Stabilisierung und Translokation von p53 in den Kern, so dass es seine Funktion als Transkriptionsfaktor ausüben kann. Neben der Phosphorylierung wird p53 bei seiner Aktivierung außerdem zahlreichen anderen Proteinmodifikationen wie z.B. Azetylierung, Ubiquitinierung, Methylierung, Sumoylierung und Neddylierung unterzogen (Lavin and Gueven, 2006). Diese beeinflussen zusätzlich die Entscheidungsprozesse, die in den vielfältigen, zum Teil gegensätzlichen p53-vermittelten Stressantworten münden (Gudkov and Komarova, 2003).

Die Wirkungen von p53 auf die Apoptose sind sowohl indirekter als auch direkter Natur. So induziert p53 die Expression einer Vielzahl von pro-apoptotischen Proteine wie BAX (Miyashita and Reed, 1995), Noxa und Puma (Chipuk et al., 2005), während es die Expression anti-apoptotischer Proteine wie Bcl-2 und Bcl-XL reprimiert und damit die Zellen für die Apoptose-Induktion sensitiviert (Yu and Zhang, 2005). Neben diesen transkriptionellen Aktivitäten wurde allerdings auch eine durch DNA-Schädigung ausgelöste Translokation von p53 zu den Mitochondrien beschrieben. Dort soll es direkt mit den anti-apoptotischen Proteinen Bcl-2 und Bcl-XL interagieren und so zur Aktivierung von BAX/BAK führen (Chipuk et al., 2004; Moll et al., 2005). Allerdings konnte vor kurzem auch eine Stress-induzierte Translokation von p53 zu den Mitochondrien beobachtet werden, die nicht mit einer Apoptose-Induktion korrelierte (Essmann et al., 2005). Die exakte Rolle des mitochondrialen p53-Proteins in Apoptose-Signalwegen bedarf also noch weiterer Aufklärung.

Neben seinen pro-apoptotischen Aktivitäten moduliert p53 auch die Transkription einer zweiten Gruppe von Zielgenen, über die ein Zellzyklusarrest induziert werden kann (u.a. 14-3-3 σ, GADD-45). Hauptverursacher des Zellzyklusarrest ist allerdings das Protein $p21^{WAF/CIP1}$, dessen Expression durch p53 nach DNA-Schädigung stark erhöht wird (el-Deiry et al., 1993). p21 ist ein Mitglied der Cip/Kip Familie der CDK-Inhibitoren, zu denen auch $p27^{KIP1}$ und $p57^{KIP2}$ gehören (Sherr and Roberts, 1999). Im C-Terminus befindet sich eine PCNA (***P****roliferating* ***C****ell* ***N****uclear* ***A****ntigen*)-Bindungsdomäne, mittels derer p21 das PCNA-Protein inhibiert, eine Untereinheit der DNA Polymerase δ. So kann die DNA-Replikation gestoppt werden ohne dabei DNA-Reparatur-Prozesse zu beeinflussen (Gartel and Radhakrishnan, 2005). Über eine N-terminal gelegene CDK-Bindungsdomäne dagegen inhibiert p21 direkt die CDK/*Cyclin*-Aktivität, so dass ein Zellzyklusarrest ausgelöst wird. Diese p21-vermittelte Hemmung der CDKs, welche sonst durch die Phosphorylierung von RB (***R****etino****b****lastoma protein*) die Aktivierung des Transkriptionsfaktors E2F vermitteln, verhindert z.B. die Expression zahlreicher Zellzyklus-relevanter Proteine und somit den Eintritt in die S-Phase. Obwohl auch noch andere, zum großen Teil unverstandene Signalwege im p53-vermittelten Zellzyklusarrest beteiligt sind, kann p21 über die Hemmung verschiedener anderer CDKs Zellen vermutlich in den unterschiedlichsten Zellzyklusphasen arretieren.

Die Komplexität der p53-vermittelten Signalwege wird darüber hinaus durch Hinweise verdeutlicht, dass p21 auch regulierend in Apoptose-Prozesse eingreifen kann (Yu and

Zhang, 2005). So konnte gezeigt werden, dass eine Inhibition der p21-Expression nach DNA-Schädigung, z.B. durch antisense Oligonukleotide, zu einer verstärkten Apoptose-Induktion führt (Tian et al., 2000; Wendt et al., 2006). Von größer Bedeutung war jedoch die Beobachtung, dass in Xenograft-Maus-Modellen Tumore mit intakter p21-Expression selbst nach Bestrahlung zumindest teilweise wieder proliferieren konnten, während p21-defiziente Tumore vollständig eliminiert wurden (Waldman et al., 1997). Obwohl für die p21-vermittelte Apoptose-Modulation verschiedene Modelle diskutiert werden, sind die genauen Mechanismen bis heute noch ungeklärt (Dotto, 2000; Gartel and Radhakrishnan, 2005; Gartel and Tyner, 2002).

Der Tumorsuppressor p53 verfügt also neben seinen bekannteren pro-apoptotischen auch über anti-apoptotische Fähigkeiten. Welche Gruppe von Zielgenen und damit welche Stressantworten induziert werden, ist vermutlich eine Konsequenz des Zusammenspiels der zahlreichen post-translationalen Modifikationen von p53. In diesem komplexen System konnte allerdings noch nicht entschlüsselt werden, welche Modifikationen für welche Stressantworten verantwortlich sind. Aufgrund der gegensätzlichen Antwortmöglichkeiten korreliert ein defizienter oder nicht funktioneller p53-Status einer Zelle nicht immer mit einer Apoptose-Resistenz (Essmann et al., 2004; Lassus et al., 1999) und ist daher Gegenstand intensiver Untersuchungen.

Kapitel 2
Aufgabenstellung

2. Aufgabenstellung

Initiator-Caspasen stehen an der Spitze der apoptotischen Caspasen-Kaskade, deren Aktivierung äußerst strikten Mechanismen unterliegt. Obwohl bedeutende Aspekte der komplexen Natur dieser Kontrollmechanismen in den letzten Jahren entschlüsselt werden konnten, sind besonders die molekularen Zusammenhänge der physiologischen Initiator-Caspasen-Aktivierung noch ungeklärt. Ziel der vorliegenden Arbeit war es deshalb, diese Aktivierungsmechanismen in verschiedenen Apoptose-Systemen zu untersuchen, wobei der Schwerpunkt auf deren Modulierbarkeit durch verschiedene Tumortherapie-Strategien lag. Deshalb sollten dabei insbesondere die momentanen Ansätze der Krebstherapie berücksichtigt werden, die einerseits auf Apoptose-Induktion durch DNA-schädigende Agentien (Chemo- und Strahlentherapie) beruhen und andererseits auf dem Zelltod, der durch Kombinationsbehandlungen (Proteasom-Inhibitoren / Todesrezeptor-Liganden) hervorgerufen wird. Es sollte untersucht werden, ob und wie die verschiedenen Initiator-Caspasen in diesen Signalwegen aktiviert werden und inwieweit ihre Inhibitoren in diese Prozesse eingreifen und sie dadurch modulieren. Die aus diesen Untersuchungen gewonnenen Erkenntnisse können somit zur Optimierung bestehender Tumortherapien herangezogen werden.

Kapitel 3
Ergebnisse und Diskussion der Publikationen

3. Ergebnisse und Diskussion der Publikationen

3.1 Das Proteasom ist für eine effiziente Aktivierung der Initiator-Caspase-8 und damit der Initiierung der Todesrezeptor-vermittelten Apoptose erforderlich

The proteasome is required for rapid initiation of death receptor-induced apoptosis

Dennis Sohn, Gudrun Totzke, Frank Essmann, Klaus Schulze-Osthoff, Bodo Levkau, Reiner U. Jänicke, *Mol Cell Biol*, 2006 Mar; 26(5): 1967-78

Friend or foe? The proteasome in combined cancer therapy

Dennis Sohn, Gudrun Totzke, Klaus Schulze-Osthoff, Reiner U. Jänicke.
Cell Cycle, 2006 Apr; 5(8): 841-5. Epub 2006 Apr 17

Ergebnisse

Eine gezielte und effiziente Aktivierung von Initiator-Caspasen sowie der nachfolgenden Caspasen-Kaskade steht im Fokus von Krebstherapien, die auf Apoptose-induzierenden Wirkstoffen beruhen. Hierbei stellt der Todesrezeptor-Ligand TRAIL aufgrund seiner Caspase-8/-10-aktivierenden Eigenschaften und seiner selektiven Wirkung auf Tumorzellen einen viel versprechenden Ansatz dar. TRAIL-Agonisten sind zwar bereits Gegenstand zahlreicher klinischer Studien (Buchsbaum et al., 2006), jedoch scheint aufgrund häufig spontan auftretender Resistenzen eine Kombinationstherapie mit Chemotherapeutika oder Bestrahlung für eine erfolgreiche Tumorbekämpfung notwendig zu sein (Debatin and Krammer, 2004). Ebenso sind Proteasom-Inhibitoren (Bortezomib) bereits für einige Tumorarten als Zweitbehandlung zugelassen und eignen sich aufgrund ihrer Wirkungsweise auch für eine TRAIL-gestützte Kombinationstherapie (Richardson et al., 2006; Voorhees and Orlowski, 2006). In der Tat konnte in einigen präklinischen Studien eine sensitivierende Wirkung auf die TRAIL-vermittelte Apoptose durch die gleichzeitige Applikation von Proteasom-Inhibitoren erreicht werden (He et al., 2004; Kim et al., 2004). Obwohl die dabei zugrunde liegenden Wirkungsmechanismen nur unzureichend charakterisiert sind, beruht die Sensitivierung vermutlich auf der Stabilisierung von aktivierten Initiator-Caspasen und den von ihnen generierten pro-apoptotischen Proteinen (s.a. 1.6, das Proteasom). Allerdings werden über den proteasomalen Abbau auch anti-apoptotische

Proteine wie z.B. c-FLIP und XIAP reguliert, welche effiziente Inhibitoren der Initiator-Caspasen-Aktivierung sind. Diese anti-apoptotischen Proteine können durch die Behandlung mit proteasomalen Inhibitoren ebenfalls stabilisiert werden und damit dem gewünschten Therapieerfolg entgegen wirken. Der Einfluss dieses Sachverhaltes wurde jedoch in früheren Studien nur unzureichend beachtet und war deshalb Gegenstand der Untersuchungen dieser Arbeit.

Dabei stellte sich in der Tat heraus, dass verschiedene Proteasom-Inhibitoren (MG-132, Clasto-Lactacystein, Calpain-Inhibitor I) mehrere Zelllinien vor der Todesrezeptor-induzierten Apoptose schützten. Diese Protektion korrelierte mit einer wesentlich geringeren Prozessierung und Aktivierung der Initiator-Caspase-8 sowie der Effektor-Caspase-3. Ein optimaler Schutz vor der Apoptose-Induktion wurde dabei durch eine einstündige Vorinkubation mit den Proteasom-Inhibitoren erreicht, während dies bei einer gleichzeitigen Applikation mit dem Todesrezeptor-Liganden nicht mehr gegeben war. Daraus ergab sich, dass das Proteasom besonders in der Induktionsphase der Apoptose unbedingt erforderlich ist, in der es sehr wahrscheinlich durch die Degradierung anti-apoptotischer Proteine zur effizienten Aktivierung von Initiator-Caspasen beiträgt.

Dieser Sachverhalt konnte tatsächlich in nachfolgenden Experimenten bestätigt werden, da der Proteasom-Inhibitor MG-132 zur Stabilisierung der anti-apoptotischen Proteine c-FLIP und XIAP führte. Nur unter diesen Bedingungen, also in Gegenwart von MG-132, wurde FLIP-short in Immunpräzipitations-Experimenten in großen Mengen im DISC detektiert, wo es die Aktivierung der Initiator-Caspase-8 inhibierte. Die Hemmung der Todesrezeptor-induzierten Apoptose durch Proteasom-Inhibitoren wurde dabei noch zusätzlich durch die gleichzeitige Stabilisierung von XIAP verstärkt, so dass die Aktivierung der Caspasen-Kaskade nicht nur über den extrinsischen, sondern auch unterhalb der Mitochondrien im intrinsischen Signalweg blockiert wurde.

Um letztendlich zu beweisen, dass Proteasom-Inhibitoren die Todesrezeptor-induzierte Apoptose tatsächlich über die Stabilisierung dieser anti-apoptotischen Proteine kontrollieren, wurden mehrere Zellklone generiert, in denen die Expression von entweder c-FLIP oder XIAP über eine Vektor-basierte shRNA (***S**hort **H**airpin* RNA)-Transkription stabil supprimiert wurde. Übereinstimmend mit dieser Hypothese konnten die Transfektanten durch Proteasom-Inhibitoren nicht mehr vor der Todesrezeptor-induzierten Apoptose geschützt werden. Im Vergleich zu Wildtyp-Zellen zeigten diese Klone in Gegenwart von MG-132 sogar eine erhöhte Apoptose-Empfindlichkeit

gegenüber Todesrezeptor-Liganden. Aufgrund der Suppression der c-FLIP- und XIAP-Expression kam es nämlich zu einer deutlich beschleunigten Prozessierung und Aktivierung der Initiator-Caspase-8 sowie der Effektor-Caspase-3. Dementsprechend kann davon ausgegangen werden, dass das Proteasom für eine effiziente Aktivierung von Initiator-Caspasen und der nachfolgenden Caspasen-Kaskade unbedingt erforderlich ist

Diskussion

Basierend auf den Ergebnissen dieser Arbeit (Sohn et al., 2006a) postulierten wir erstmalig eine biphasische Rolle des Proteasoms (Sohn et al., 2006b) in der Apoptose-Induktion (Abb. 9). Demnach wird die proteasomale Aktivität zu einem frühen Zeitpunkt, d.h. kurz nach der Todesrezeptoraktivierung, benötigt, um den vorhandenen großen Pool an anti-apoptotischen Proteinen abzubauen und somit die Aktivierung von Initiator-Caspasen sowie die Generierung von weiteren pro-apoptotischen Proteinen zu ermöglichen. Zu einem späteren Zeitpunkt allerdings, während der Effektorphase der Apoptose, werden vom Proteasom auch die entstandenen pro-apoptotischen Proteine abgebaut, wodurch es zu einer Verlangsamung oder auch zu einem Stop des apoptotischen Programms kommen kann. Bemerkenswerterweise werden auch einige Untereinheiten des Proteasoms von Effektor-Caspasen gespalten und dadurch inaktiviert (Adrain et al., 2004; Sun et al., 2004). Demnach sorgen die durch Initiator-Caspasen in großen Mengen aktivierten Effektor-Caspasen für eine Inhibition des Proteasoms, um den reibungslosen Ablauf der finalen Apoptose-Phase zu gewährleisten.

Für eine erfolgreiche Kombinationsbehandlung mit Proteasom-Inhibitoren ergibt sich deshalb aus unseren Ergebnissen, dass der Zeitpunkt der Zugabe dieser Agentien von entscheidender Bedeutung ist. Eine zu frühe bzw. vorzeitige Verabreichung der Proteasom-Inhibitoren würde zu einer Stabilisierung des anti-apoptotischen Protein-Pools führen und damit die Aktivierung der Initiator-Caspasen in Tumorzellen verhindern. Erschwerend kommt dabei die Tatsache hinzu, dass Tumorzellen häufig per se schon eine erhöhte Expression dieser anti-apoptotischen Proteine aufweisen (Mathas et al., 2004; Medema et al., 1999; Tamm et al., 2000), die durch eine zusätzliche Stabilisierung ein unüberwindbares Hindernis für jeden Apoptose-induzierenden Stimulus darstellen würden.

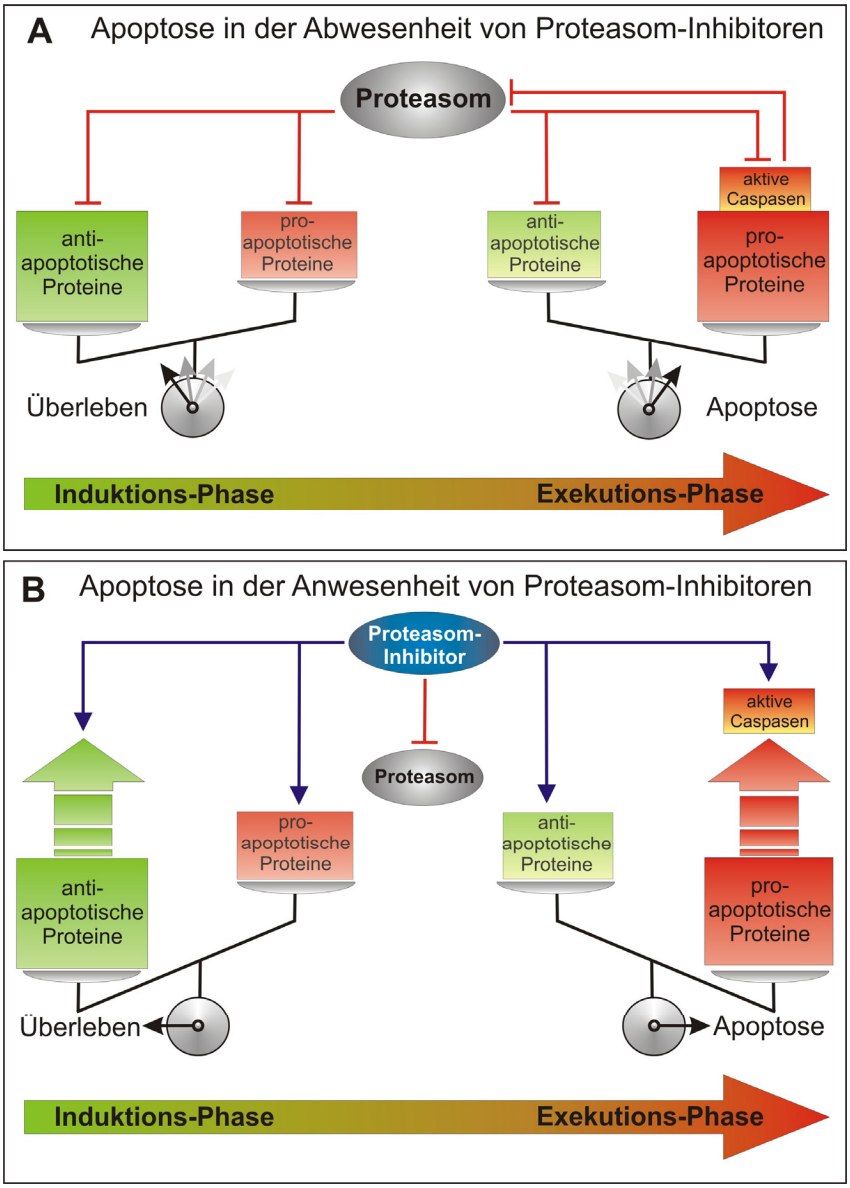

Abbildung 9: Modell für die biphasische Rolle des Proteasoms in der Apoptose

(A) Vor der Apoptose-Induktion sorgt das Proteasom für eine kritische Balance zwischen einem Pool von anti- und pro-apoptotischen Proteinen (dargestellt durch den schwarzen, auf Überleben zeigenden Pfeil). Während der Induktions-Phase der Apoptose erhöht sich der pro-apoptotische Pool durch z.B. aktivierte Caspasen, ein Prozess, der allerdings vom Proteasom-vermittelten Abbau anti-apoptotischer Proteine abhängig ist. Deshalb wird das Proteasom für den Umschwung zur Apoptose hin benötigt. Dies ist durch die sich zur Apoptose bewegenden Pfeile dargestellt. Während der Exekutions-Phase der Apoptose allerdings, nachdem der pro-apoptotische Pool angewachsen ist und die Zelle apoptotisch wird (Pfeil in Richtung Apoptose), behindert das Proteasom durch den Abbau der pro-apoptotischen Proteine die korrekte Durchführung und drückt das Gleichgewicht wieder in Richtung des anti-apoptotischen Pool (Pfeile bewegen sich in Richtung Überleben). Dies wird durch aktive Caspasen kompensiert, die Untereinheiten des Proteasoms spalten und damit inaktivieren.
(B) Durch die Präsenz von Proteasom-Inhibitoren vor der Apoptose-Induktion wird der anti-apoptotische Pool gestärkt. Deshalb werden nach einem Apoptose-Stimulus sehr wenige pro-apoptotische Proteine gebildet, so dass die Zelle eine erhöhte Resistenz gegenüber der Apoptose aufweist (Pfeil in Richtung Überleben). Wird das Proteasom allerdings erst zu einem späteren Zeitpunkt inhibiert, nachdem bereits das Gleichgewicht in Richtung des pro-apoptotischen Pools durch die proteasomale Aktivität gelenkt ist, so sorgt es für den korrekten und irreversiblen Ablauf der Apoptose. Durch ihren drastischen Einfluss auf sowohl den anti- als auch den pro-apoptotischen Pool, verstärken proteasomale Inhibitoren den momentanen Status der Zelle, was zu einem erhöhten Überleben oder Sterben führen kann (d.h., die Pfeile deuten stärker und irreversibler auf Überleben oder Apoptose).

In Übereinstimmung mit unserem Modell konnte auch schon in DNA-Schädigungs-Signalwegen gezeigt werden, dass der Zeitpunkt der Zugabe von proteasomalen Inhibitoren, d.h. vor oder nach dem Apoptose-Stimulus, einen erfolgreichen Ausgang der Behandlung stark beeinflusst (Tabata et al., 2001). Um zunächst den proteasomalen Abbau anti-apoptotischer Proteine zu ermöglichen, sollten Proteasom-Inhibitoren zeitlich nach der Apoptose-Induktion verabreicht werden, um zum richtigen Zeitpunkt die aktivierten Initiator-Caspasen und den daraus entstandenen pro-apoptotischen Pool zu stabilisieren und somit das Schicksal der Zelle in Richtung Apoptose zu lenken (Abb. 9). Eine weitere Option wäre eine zusätzliche Verabreichung von Wirkstoffen, die unabhängig vom Ubiquitin-Proteasom-System die Expression der anti-apoptotischen Inhibitoren der Initiator-Caspasen-Aktivierung verringern (Garber, 2005). Diesbezüglich zeigten die von uns generierten Zellen, in denen die Expression von c-FLIP und XIAP durch spezifische shRNAs supprimiert wurde, eine deutlich schnellere und effizientere Apoptose-Induktion nach einer Kombinationsbehandlung mit proteasomalen Inhibitoren und Todesrezeptor-Liganden.

Für eine auf der Kombinationsbehandlung basierende Tumortherapie ist deshalb nicht nur eine Überprüfung des biochemischen Status der Zelle erforderlich, sondern auch die Zeitpunktbestimmung der Zugabe von Proteasom-Inhibitoren. Die Berücksichtigung dieser komplexen Parameter sollte verhindern, dass eine nicht zeitgerechte Verabreichung von Proteasom-Inhibitoren zu einer verlängerten Lebensdauer des Tumors führt.

3.2 Die Initiator-Caspase-8 wird in der Chemotherapeutika-induzierten Apoptose unabhängig von einer Todesrezeptor-vermittelten Dimerisierung durch proteolytische Spaltung aktiviert

Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis

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Ergebnisse

Mitochondrien werden durch verschiedene exo- und endogene Noxen, wie z.B. nach DNA-Schädigung mittels des Topoisomerase-Inhibitors Etoposid, aktiviert, was zur Freisetzung von Cytochrom c führt. Nach der folgenden Aktivierung der Initiator-Caspase-9 und den Effektor-Caspasen -3, -6 und -7 kann interessanterweise auch eine Prozessierung der Initiator-Caspase-8 in Abwesenheit einer Todesrezeptor-induzierten DISC-Bildung beobachtet werden (Engels et al., 2000; Jones et al., 2001; Wieder et al., 2001). Bisher wurde diese im Western-Blot nachgewiesene Prozessierung als Hinweis auf die Aktivierung von Caspase-8 verstanden. Nach dem kürzlich aufgestellten „*induced proximity"*-Modell allerdings sollte eine proteolytische Spaltung der Initiator-Caspase-8 durch eine andere Caspase in Abwesenheit eines Dimerisierungskomplexes nicht zu einem aktiven Caspase-8-Tetramer, sondern nur zu einem gespaltenen und katalytisch inaktiven Caspase-8-Protein führen (Boatright et al., 2003). Daher wurde in diesem Projekt geprüft, ob die im mitochondrialen Signalweg der Apoptose beobachtete proteolytische Spaltung der Caspase-8 tatsächlich in der Generierung eines aktiven Enzyms resultiert, welche Funktion es in diesem Signalweg übernehmen könnte und ob dieser Prozess tatsächlich unabhängig von einer DISC-Bildung stattfindet.

Diesbezüglich konnte tatsächlich gezeigt werden, dass Caspase-8, die unterhalb der Mitochondrien prozessiert wird, enzymatisch aktiv ist. Für diese Experimente wurde ein *in vivo*-Markierungsverfahren verwendet, welches auf einem Biotin-gekoppelten Pan-Caspase-Inhibitor (Biotin-VAD-FMK) beruht, der ausschließlich durch enzymatisch aktive Caspasen gebunden wird. Diese können nach erfolgter Apoptose-Induktion mittels Streptavidin-Agarose präzipitiert und per Western Blot analysiert werden. Dadurch konnte in Jurkat- und H9-T-Zellen demonstriert werden, dass aktive Caspase-8 nicht nur durch Stimulation von Todesrezeptoren (CD95) sondern auch nach Behandlung mit Chemotherapeutika wie z.B. Etoposid generiert wird. Die Etoposid-

vermittelte Aktivierung der Initiator-Caspase-8 war dabei in der Tat unabhängig von einer Todesrezeptor-induzierten DISC-Bildung, da sie auch in Jurkat T-Zellen stattfand, die defizient für die essentiellen DISC-Komponenten CD95 oder FADD sind. In Bcl-2 überexprimierenden Zellen dagegen, in denen die Aktivierung der Mitochondrien durch dieses anti-apoptotische Protein verhindert wird, konnte nach Etoposid-Behandlung kein aktives Caspase-8 Enzym detektiert werden. Daraus folgt, dass Caspase-8 in der Chemotherapeutika-induzierten Apoptose unterhalb der Mitochondrien sehr wahrscheinlich durch aktivierte Effektor-Caspasen prozessiert und aktiviert wird. Diese Annahme konnte in weiteren *in vitro*-Experimente mit unbehandelten Zellextrakten verschiedener Zelllinien bestätigt werden, in denen die Aktivierung der Mitochondrien durch die gleichzeitige Zugabe von aktiver rekombinanter Caspase-6 und -3 simuliert wurde. Auch hier konnte ein aktives Caspase-8 Enzym beobachtet werden, während dagegen die alleinige Zugabe jeder einzelnen Caspase zu diesen Extrakten nur zu einer unvollständigen Caspase-8-Prozessierung führte. Demnach kann man davon ausgehen, dass die Aktivierung der Caspase-8 *in vivo* aufgrund der hierarchischen mitochondrialen Caspasen-Kaskade sequentiell über Caspase-9, -3 und -6 erfolgt.

Diskussion

Diese Arbeit (Sohn et al., 2005) zeigt, dass die Inititator-Caspase-8 in Abwesenheit einer DISC-Bildung im mitochondrialen Signalweg durch direkte Prozessierung von Effektor-Caspasen aktiviert werden kann. Dies spricht allerdings gegen das „*induced proximity"*-Modell, in dem Initiator-Caspasen durch Dimerisierung und nicht durch Spaltung aktiviert werden. Zudem zeigt diese Aktivierung der Caspase-8, dass sie als Initiator-Caspase in diesem Kontext auch eine wichtige Rolle als Effektor-Caspase annehmen kann. Eines der Hauptsubstrate von Caspase-8 ist nämlich das Protein BID, dessen Caspase-Spaltprodukt tBID zu den Mitochondrien transloziert und diese aktiviert. Im mitochondrialen Signalweg resultiert also eine Aktivierung der Initiator-Caspase-8 durch die Effektor-Caspase-6 und -3 in einer so genannten verstärkenden Rückkopplungsschleife (*feedback amplification loop)* (Abb. 10), da tBID zu einer erhöhten Freisetzung von Cytochrom c und daraus folgend zu einer noch stärkeren Aktivierung der Caspase-9, -3 und -6 führt (Tang et al., 2000).

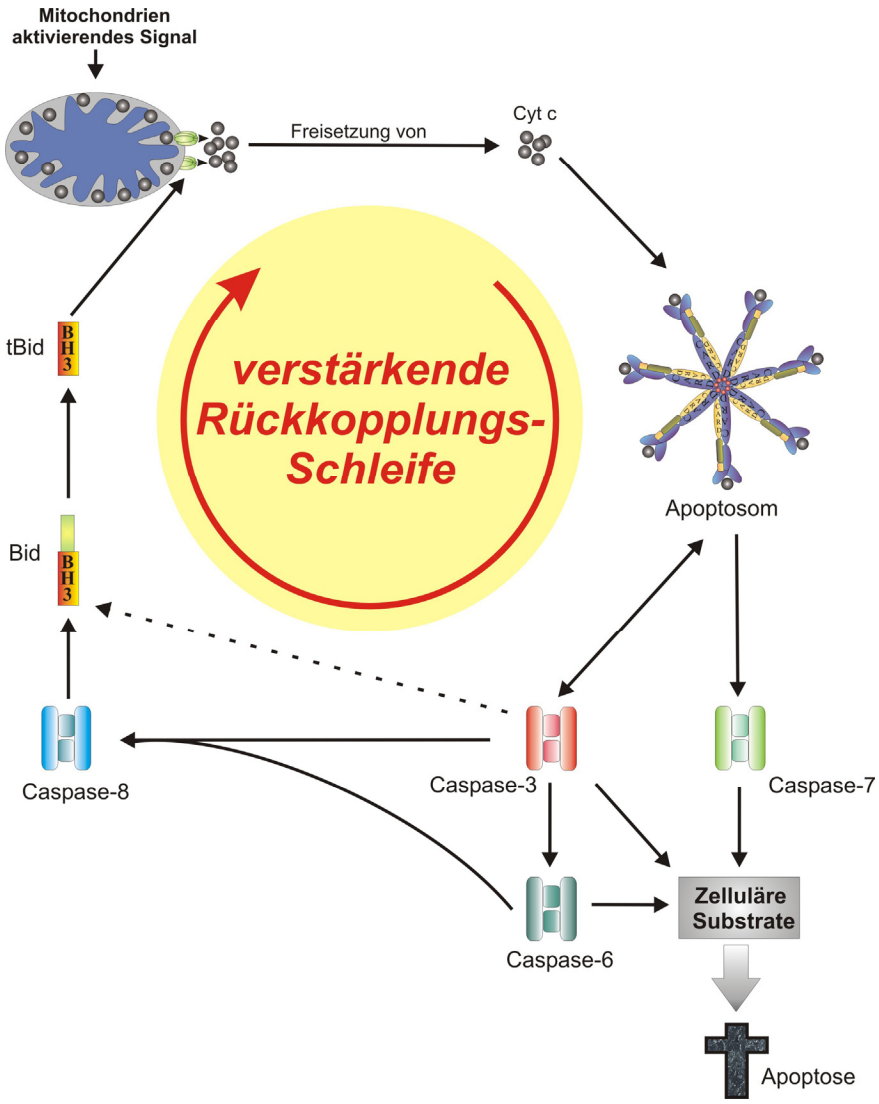

Abbildung 10: Einfluss der Caspase-8 Aktivierung auf den intrinsischen Signalweg

Aktive Caspase-8 nimmt im mitochondrialen Signalweg der Apoptose die Rolle einer Effektor-Caspase ein. Nach der initialen Cytochrom c-Freisetzung wird Caspase-8 durch die Effektor-Caspasen -3 und -6 aktiviert. Die Caspase-8-vermittelte Spaltung von BID führt zur Bildung einer verstärkenden Rückkopplungsschleife, die in einer erhöhten Freisetzung von Cytochrom c resultiert und damit zu einer verstärkten Aktivierung der Effektor-Caspasen. Dadurch wird der korrekte Fortgang der Apoptose gesichert.

In unserem System konnten wir allerdings keinen Unterschied in der Generierung von tBID zwischen parentalen und Caspase-8-defizienten Jurkat-Zellen nach Etoposid-Behandlung feststellen. Dies ist in der Tatsache begründet, dass Jurkat-Zellen große Mengen Caspase-3 exprimieren, die auch BID spalten kann. Caspase-3 weist zwar im Gegensatz zur Caspase-8 eine geringere Affinität zu BID auf, wird aber in Folge eines starken apoptotischen Signals in wesentlich größeren Mengen aktiviert, wodurch es auch zu einer ausreichenden Caspase-3-vermittelten BID-Spaltung kommen kann (Slee et al., 2000). Daher ist die Aktivierung der Caspase-8 besonders bei einem sehr schwachen Apoptose-Signal von großer Bedeutung, da sie schneller und effektiver

durch die oben erwähnte Rückkopplungsschleife (Abb. 10) für gespaltenes BID und somit für weitere Caspasen-Aktivierung sorgt. Darüber hinaus konnte bereits gezeigt werden, dass posttranslationale Modifikationen von BID, z.B. durch Phosphorylierungen, dessen Substratspezifität für Caspase-8 und -3 unterschiedlich beeinflussen können (Degli Esposti et al., 2003; Desagher et al., 2001). Dadurch ist die Möglichkeit gegeben, dass die Caspase-8 selbst im intrinsischen Apoptose-Signalweg von größerer Bedeutung ist als bisher angenommen.

In Übereinstimmung mit unseren Ergebnissen wurde erst kürzlich gezeigt, das Caspase-8 auch durch Granzym B, eine in der CTL (**C***ytotoxic* ***T***-***L****ymphocyte*)-vermittelten Apoptose aktiven Protease, in Abwesenheit eines Dimerisierungs-Stimulus prozessiert und aktiviert wird (Adrain et al., 2005; Murphy et al., 2004). Diese und unsere Arbeit zeigen also, dass die Aktivierungsmechanismen von Initiator-Caspasen noch nicht vollständig aufgeklärt sind und weiterer Untersuchungen bedürfen. Nur dann können Wirkstoffe entwickelt werden, die die Aktivierung der Initiator-Caspase-8 und damit die erfolgreiche Apoptose-Induktion in der Krebstherapie unterstützen.

3.3 Die Aktivierung der Initiator-Caspase-9 in der bestrahlungsinduzierten Apoptose wird durch Cyclin-abhängige Kinasen (CDKs) kontrolliert

p21 blocks irradiation-induced apoptosis downstream of mitochondria by inhibition of cyclin-dependent kinase-mediated caspase-9 activation

Dennis Sohn, Frank Essmann, Klaus Schulze-Osthoff, Reiner U. Jänicke

Cancer Research, 2006 Dec 1; 66(23): 11254-62

Ergebnisse

DNA-Schädigung durch z.B. ionisierende Strahlung führt in der Zelle zunächst zu einem Zellzyklusarrest. Anschließend folgt die Entscheidung, ob die Zelle permanent im Zellzyklus arretiert bleibt und seneszent wird, oder ob sie die Caspasen-Kaskade initiiert und durch Apoptose stirbt. Einer der Entscheidungsträger in diesem Prozess ist das p53-Protein, das nach DNA-Schädigung aktiviert wird und sowohl die Expression von pro-apoptotischen Proteinen wie BAX und Puma steigert als auch über die Induktion von p21 den Zellzyklusarrest auslöst (Yu and Zhang, 2005). Der Tumorsuppressor p53 ist damit auch ein wichtiger Modulator der Aktivierung von Initiator-Caspasen und daraus folgend der Apoptose-Induktion.

Um p53-vermittelte Entscheidungsprozesse und die daraus resultierenden Modulationsmechanismen der Initiator-Caspasen-Aktivierung zu charakterisieren, wurden in dieser Arbeit (Sohn et al., 2006c) bestrahlungsinduzierte Stressantworten in Abhängigkeit von p53 und dessen Zielgen p21 analysiert. Hierbei fanden HCT116 Kolonkarzinomzellen Verwendung, die beide Proteine exprimieren (Wildtyp) sowie Derivate dieser Zelllinie, in denen durch homologe Rekombination entweder das p53- oder das p21-Gen eliminiert wurde (Bunz et al., 1998). Dabei zeigte sich, dass ionisierende Bestrahlung nur in den beiden Kontrollpunkt-defizienten HCT116-Zelllinien die Aktivierung der Initiator-Caspase-9 und der Effektor-Caspase-3 sowie nachfolgend Apoptose auslöste. Wildtyp-Zellen dagegen, in denen Bestrahlung zwar eine effiziente p53-Aktivierung und damit die Expression pro-apoptotischer Proteine wie Bax und Puma induzierte, wurden in einen permanenten Zellzyklusarrest (Seneszenz) ohne eine detektierbare Aktivierung der Caspasen-Kaskade getrieben. Interessanterweise löste die Bestrahlung aber auch in seneszenten Wildtyp-Zellen die Aktivierung der Mitochondrien aus, da sowohl ein Verlust des mitochondrialen Membranpotentials als auch die Freisetzung von Cytochrom c beobachtet werden

konnte. Der nach DNA-Schädigung eingeschlagene apoptotische Signalweg ist also auch in p53-exprimierenden Wildtyp-Zellen bis zu den Mitochondrien intakt und funktionsfähig. Da es aber nur in den beiden Kontrollpunkt-defizienten Zelllinien zur Apoptose-Induktion kam, wird die unterhalb der Mitochondrien stattfindende Aktivierung der Caspasen-Kaskade entweder durch p53 selbst oder durch das p53-induzierte Protein p21 inhibiert. Einzig die Prozessierung der Caspase-2 konnte weder durch die Anwesenheit von p53 noch von p21 beeinträchtigt werden, da sie unabhängig von der bestrahlungsinduzierten Stressantwort (Apoptose oder Seneszenz) in allen drei Zelllinien zu beobachten war. Daraus folgt sowohl, dass die Aktivierung der Caspase-2 in der bestrahlungsinduzierten Apoptose oberhalb der Mitochondrien stattfindet, als auch, dass die Caspasen-Kaskade nicht direkt durch Caspase-2 aktiviert werden kann (s.a. Abschnitt 3.4).

Um den molekularen Mechanismus der p21-vermittelten Bestrahlungsresistenz aufzuklären, wurden Immunpräzipitationsstudien durchgeführt, bei denen allerdings weder eine direkte Interaktion von p21 mit der Initiator-Caspase-9 noch mit der Effektor-Caspase-3 detektiert werden konnte. Darüber hinaus sprach die ausschließlich nukleäre Lokalisation gegen eine direkte Hemmung von zytoplasmatisch lokalisierten Caspasen. Es stellte sich allerdings heraus, dass die CDK-inhibierende Funktion für den p21-vermittelten Schutz vor der bestrahlungsinduzierten Initiator-Caspasen-Aktivierung notwendig ist. So wurden p21-defiziente Zellen nur durch die Expression eines p21-Wildtypproteins vor der bestrahlungsinduzierten Apoptose geschützt, nicht aber durch die Expression einer p21-Mutante, die nicht mehr mit CDKs interagieren kann. Eine Behandlung von p21-defizienten HCT116-Zellen mit dem chemischen CDK-Inhibitor Roscovitin konnte die Induktion der mitochondrialen Caspasen-Kaskade inhibieren. Roscovitin verhinderte dabei allerdings nur die Aktivierung der Caspasen, nicht aber den Verlust des mitochondrialen Membranpotentials. Diese Daten bestätigen unsere Hypothese, dass p21 die bestrahlungsinduzierte Apoptose unterhalb der Mitochondrien über die Hemmung von CDKs blockiert. Diese Inhibition konnte dabei auf eine Apoptosom-hemmende Wirkung zurückgeführt werden, da *in vitro*-Aktivierungs-Experimente zeigten, dass die Zugabe von Cytochrom c zu Extrakten von bestrahlten p21-defizienten Zellen eine weitaus höhere Caspase-Aktivität induzierte als dies in Extrakten von identisch behandelten Wildtyp-Zellen zu beobachten war.

Zusammenfassend kann daraus die Schlussfolgerung gezogen werden, dass p21 die bestrahlungsinduzierte Aktivierung der mitochondrialen Caspasen-Kaskade über die

Inhibition von CDKs verhindert. Daraus ergibt sich auch, dass CDKs über einen noch unbekannten Mechanismus eine effizientere Apoptosom-Formierung und daraus resultierend eine wesentlich erhöhte Aktivierung der Initiator-Caspase-9 vermitteln.

Diskussion

Einige Publikationen berichteten bereits über einen Zusammenhang von CDK-Aktivität und Apoptose. So wurde z.B. festgestellt, dass die Aktivität von CDKs in apoptotischen Zellen ansteigt, und dass deren Inhibition durch dominant-negative Mutanten oder chemische Inhibitoren das Auftreten verschiedener Apoptose-Charakteristika verhindert (Harvey et al., 2000; Zhou et al., 1998). Dabei handelte es sich aber fast ausschließlich um Prozesse, die unterhalb der Aktivierung der Caspasen-Kaskade stattfinden und somit nicht essentiell für deren Initiierung sein können. In der vorliegenden Arbeit (Sohn et al., 2006c) dagegen konnte zum ersten Mal gezeigt werden, dass CDKs auch einen entscheidenden Einfluss auf die Aktivierung der Initiator-Caspase-9 unterhalb der mitochondrialen Freisetzung von Cytochrom c ausüben. Durch diese Resultate können CDKs als essentielle Entscheidungsträger angesehen werden, ohne die eine effektive Stressantwort nicht möglich ist. Die p21-vermittelte Inhibition von CDKs führt also nicht nur zu einem bestrahlungsinduzierten Zellzyklusarrest, sondern über die gleichzeitig stattfindende Hemmung der Caspasen-Kaskade zu einem Schutz vor der Apoptose-Induktion (Abb. 11). Während die Komponenten des p21-abhängigen Zellzyklusarrest bereits relativ gut charakterisiert sind, ist über die Mechanismen und Zielgene, die in einer CDK-abhängigen Aktivierung der Caspasen-Kaskade eine Rolle spielen, noch nichts bekannt. In der Hefe konnten bereits über 200 Substrate der CDKs identifiziert werden (Ubersax et al., 2003), aufgrund technischer Schwierigkeiten gelang dies aber noch nicht im humanen System. Mögliche Kandidaten wären z.B. bekannte Komponenten des Apoptosoms wie APAF-1, Smac/DIABLO oder auch Caspase-9 selbst, aber auch anti-apoptotische Proteine wie XIAP, die durch eine CDK-modulierte Phosphorylierung aktiviert bzw. inhibiert werden könnten. Die Identifizierung Apoptose-relevanter CDK-Zielgene ist deshalb weiterhin ein primäres Forschungsziel unseres Labors.

Ein weiteres durchaus überraschendes Ergebnis dieser Arbeit ist die Rolle des Tumorsuppressor-Proteins p53 in den bestrahlungsinduzierten Stressantworten einer Zelle. Während bisher in unzähligen Publikationen ausgiebig die pro-apoptotischen Eigenschaften von p53 diskutiert wurden (Chipuk and Green, 2006; Michalak et al.,

2005; Moll et al., 2005), weist die vorliegende Arbeit darauf hin, dass p53 über die Induktion von p21 (und sehr wahrscheinlich auch anderen Zielgenen wie z.B. 14-3-3) auch anti-apoptotische Funktionen übernimmt. Damit reihen sich diese Ergebnisse in eine noch geringe, aber stetig wachsende Anzahl von Publikationen ein, die auch anti-apoptotische Wirkungsweisen des p53-Proteins bzw. seiner Zielproteine zeigen (Essmann et al., 2005; Lassus et al., 1999; Wu et al., 2005). Damit rücken auch diese durch p53-induzierten anti-apoptotischen Proteine als mögliche Ziele einer Krebstherapie in den Vordergrund.

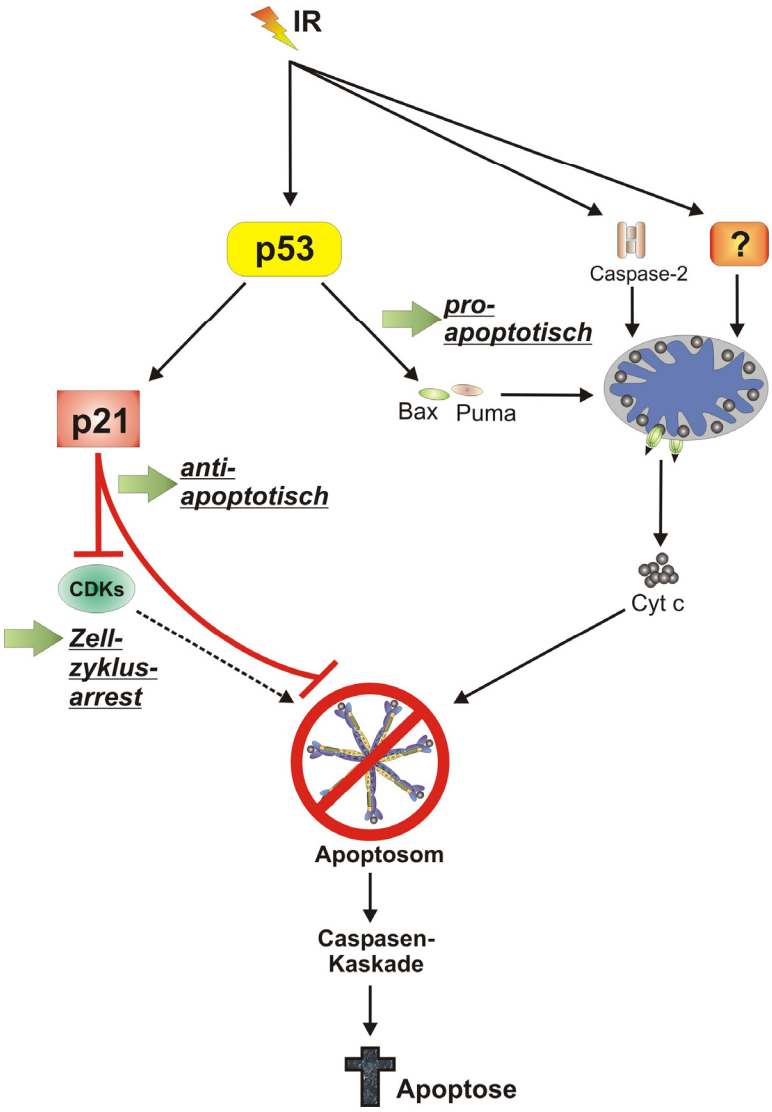

Abbildung 11: Die p53-vermittelten Stressantworten auf ionisierende Bestrahlung

Die Aktivierung von p53 durch DNA-Schädigung setzt mehrere unterschiedliche Prozesse in Gang. Über die Induktion der Bax- bzw. Puma-Expression wirkt p53 pro-apoptotisch, da dies zur Aktivierung der Mitochondrien führt. Die p53-vermittelte Steigerung der Expression von p21 dagegen führt zu einem Zellzyklusarrest. Über die Inhibition von CDKs wirkt p21 und damit der Tumorsuppressor p53 allerdings auch anti-apoptotisch, da aktive CDKs für eine effiziente Aktivierung der Initiator-Caspase-9 im Apoptosom benötigt werden. Eine hohe Induktion der p21-Expression unterdrückt also die pro-apoptotische Stressantwort von p53 und führt zu einer gesteigerten Resistenz gegenüber Apoptose sowie einem Zellyzyklusarrest.

3.4 Die Initiator-Caspase-9 spielt eine essentielle Rolle bei der Aktivierung der Caspase-2 und dem Verlust des mitochondrialen Membranpotentials in der Chemotherapeutika-vermittelten Apoptose

Loss of caspase-9 reveals its essential role for caspase-2 activation and mitochondrial membrane depolarization

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Ergebnisse

Im Gegensatz zur Initiator-Caspase-9, deren Aufgabe als Vermittler des Mitochondrien-gesteuerten Signalwegs der Apoptose klar definiert ist, ist nach heutigem Kenntnisstand die Bedeutung der Caspase-2 für die Apoptose nur äußerst unzureichend charakterisiert. Neben ihrer exakten hierarchischen Stellung ist dabei auch unklar, ob und welche Funktionen die Caspase-2 in apoptotischen Prozessen übernimmt. So gibt es gegensätzliche Berichte, die einerseits eine Rolle als Initiator-Caspase oberhalb der Mitochondrien beschreiben, oder andererseits Caspase-2 unterhalb dieser Organellen als Effektor-Caspase definieren (Enoksson et al., 2004; Robertson et al., 2004; Ruiz-Vela et al., 2005). Während für ihre Rolle als Effektor-Caspase bisher noch keine spezifischen Substrate identifiziert werden konnten, könnte die Caspase-2 oberhalb der Mitochondrien zur Freisetzung von Cytochrom c und/oder dem Verlust des mitochondrialen Membranpotentials beitragen. Dabei ist allerdings noch völlig ungeklärt, wie Caspase-2 diese Ereignisse modulieren könnte und ob zusätzlich andere Caspasen in diese Prozesse eingreifen (Garrido et al., 2006; Newmeyer and Ferguson-Miller, 2003; Ricci et al., 2003).

Zur Beantwortung dieser Fragen wurde in dieser Arbeit (Samraj et al., 2007) eine durch uns identifizierte Initiator-Caspase-9-defiziente Jurkat-Zelllinie (JMR) eingesetzt, die aufgrund dieser Defizienz nicht mehr den intrinsischen Signalweg aktivieren kann und deshalb resistent gegenüber einer Vielzahl von apoptotischen Stimuli ist. Ähnlich wie in Jurkat-Wildtyp-Zellen wurden auch in JMR-Zellen durch verschiedene Chemotherapeutika (Etoposid, Doxorubicin, Staurosporin) typische apoptotische Charakteristika wie die Oligomerisierung von BAK sowie eine partielle Freisetzung von Cytochrom c induziert, was auf einen intakten mitochondrialen Signalweg schließen lies. Aufgrund der Caspase-9-Defizienz wurde in dieser Zelllinie allerdings kein

Apoptosom gebildet, weshalb auch keine Aktivierung der Caspasen-Kaskade stattfand. Im Gegensatz zu den Wildtyp-Zellen konnte in den JMR-Zellen allerdings auch keine Prozessierung und Aktivierung der Caspase-2 detektiert werden. Dies deutete darauf hin, dass die Caspase-2 in der Chemotherapeutika-vermittelten Apoptose, im Gegensatz zur bestrahlungsinduzierten Signalwegen (s.a. Abschnitt 3.3), unterhalb der Mitochondrien über die Initiator-Caspase-9 aktiviert wird und somit in diesem System nicht als Initiator-, sondern sehr wahrscheinlich als Effektor-Caspase fungiert.
Interessanterweise führte die Behandlung der Caspase-9-defizienten JMR-Zellen zwar zu einer partiellen Cytochrom c-Freisetzung, ein Verlust des mitochondrialen Membranpotentials war dagegen nicht zu beobachten. Es scheint also, dass ein nach der erfolgreichen Aktivierung der Caspase-9 stattfindendes Ereignis für diesen Prozess notwendig ist. Diesbezüglich zeigte eine stabil mit Caspase-9 rekonstituierte Zelllinie (JMR/C9) in zahlreichen Experimenten vergleichbare apoptotische Reaktionen auf eine Behandlung mit Chemotherapeutika, wie dies in Jurkat-Wildtyp-Zellen zu beobachten war. Neben der Oligomerisierung von BAK und der Freisetzung von Cytochrom c konnte nun auch wieder eine Aktivierung der Caspasen-Kaskade inklusive der Caspase-2 detektiert werden. Außerdem wiesen diese Zellen einen Verlust des mitochondrialen Membranpotentials auf und starben durch Apoptose. Diese Ergebnisse bestätigen, dass die Prozessierung und Aktivierung der Caspase-2 nach Chemotherapeutika-Behandlung über die Initiator-Caspase-9 unterhalb der Mitochondrien vermittelt wird.

Diskussion

Anhand der in dieser Arbeit (Samraj et al., 2007) identifizierten Caspase-9-defizienten Jurkat-Zelllinie (JMR) gelang es, zeitliche Abfolgen der apoptotischen Signaltransduktion in Bezug auf die Rolle der Caspase-2 aufzuklären. In diesen Zellen konnte keine Aktivierung der Caspase-2 nach DNA-Schädigung durch verschiedene Topoisomerase-Inhibitoren detektiert werden, so dass man in diesem System für die Caspase-2 eine Rolle als Effektor-Caspase in der Caspase-9-abhängigen mitochondrialen Caspasen-Kaskade postulieren kann. Über welche Substrate und auf welche apoptotischen Prozesse sie nach ihrer Aktivierung einwirkt, wird von uns zur Zeit anhand von Zelllinien überprüft, in denen die Expression dieser Protease durch Transfektion mit spezifischen siRNAs supprimiert wird.

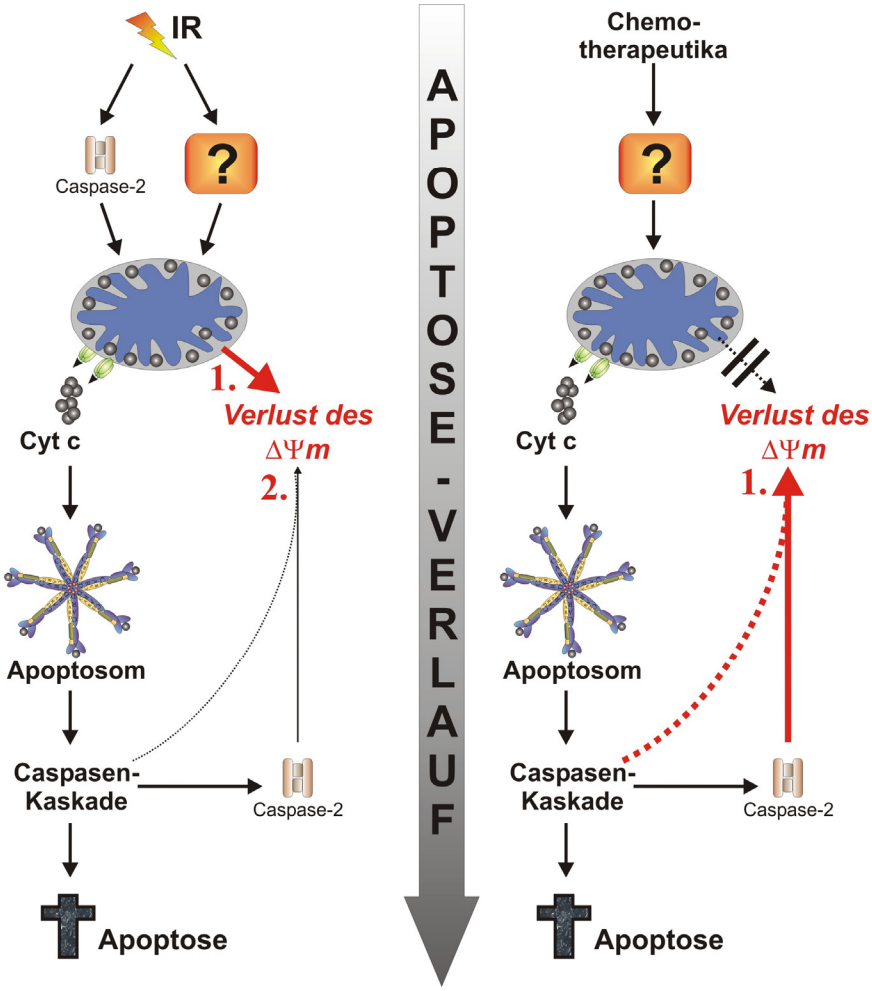

Abbildung 12: Vergleich der apoptotischen Signaltransduktion nach Behandlung mit ionisierender Bestrahlung oder Chemotherapeutika

In der Bestrahlungsinduzierten Apoptose wird die Caspase-2 oberhalb der Mitochondrien aktiviert, während sie in der Chemotherapeutika-ausgelösten Apoptose unterhalb des Apoptosoms in der Caspasen-Kaskade aktiv ist. Dies korreliert mit dem beobachteten Verlust des mitochondrialen Membranpotentials, der in der Bestrahlungsinduzierten Apoptose zeitgleich mit der Freisetzung von Cytochrom c vor der Aktivierung der Caspasen-Kaskade stattfindet (**1.**, rot links). In der Chemotherapeutika-induzierten Apoptose dagegen findet dieser Verlust erst nach der Apoptosom-Bildung statt (**1.**, rot rechts). Dies spricht für einen funktionellen Zusammenhang zwischen der Aktivierung der Caspase-2 und dem Verlust des mitochondriallen Membranpotentials.

Interessanterweise konnte im Gegensatz hierzu in HCT116-Zellen (s. Abschnitt 3.3) eine bestrahlungsinduzierte Prozessierung der Caspase-2 beobachtet werden, die unabhängig von der Aktivierung der Caspase-9 oder -3 oberhalb der Mitochondrien stattfand. Diese auf den ersten Blick widersprüchlich erscheinenden Ergebnisse sind sehr wahrscheinlich darin begründet, dass unterschiedliche Arten der DNA-Schädigung (Chemotherapeutika gegenüber Bestrahlung) auch unterschiedliche Signalwege aktivieren (Jänicke et al., 2001; Norbury and Zhivotovsky, 2004). Daher ist nicht auszuschließen, dass die Caspase-2 in der bestrahlungsinduzierten Apoptose eine

essentielle Initiator-Rolle ausübt, während sie in Chemotherapeutika-vermittelten Signalwegen hauptsächlich als Effektor-Caspase fungiert.

Ein weiterer interessanter Aspekt, der durch diese zwei Arbeiten aufgedeckt wurde, bezieht sich auf den Verlust des mitochondrialen Membranpotentials. Obwohl die Caspasen-Kaskade in beiden Zelllinien nicht aktiviert werden konnte, wurde das mitochondriale Membranpotential in HCT116-Zellen nach Bestrahlung drastisch verringert, während es in JMR-Zellen nach Behandlung mit Topoisomerase-Inhibitoren stabil blieb. Der Verlust des mitochondrialen Membranpotentials korreliert demnach zeitlich mit der Aktivierung der Caspase-2, die in HCT116-Zellen oberhalb und in JMR-Zellen unterhalb der Mitochondrien-Aktivierung stattfindet (Abb. 12). Diese Ergebnisse deuten demnach darauf hin, dass der Verlust des mitochondrialen Membranpotentials nur in Anwesenheit einer prozessierten Caspase-2 stattfindet, die dieses Ereignis aufgrund seiner postulierten Poren-formenden Eigenschaften initiieren könnte. Interessanterweise konnte aber in beiden Zellsystemen eine zumindest partielle Freisetzung von Cytochrom c detektiert werden. Daraus folgt zusätzlich, dass der Verlust des mitochondrialen Membranpotentials und die Cytochrom c-Freisetzung zwei unabhängige Ereignisse darstellen, die nicht immer zwangsläufig gleichzeitig stattfinden müssen (Ricci et al., 2004; Zamzami and Kroemer, 2001; Zamzami et al., 2005). Demnach tragen zusätzlich zu Stimulus-abhängigen Signalwegen sehr wahrscheinlich auch Zelltyp-spezifische Phänomene zur kontrovers diskutierten Rolle der Caspase-2 bei (Zhivotovsky and Orrenius, 2005).

3.5 Abschließende Bemerkungen

In den hier vorgestellten Publikationen konnten einige wichtige Aspekte der Modulation der Initiator-Caspasen-Aktivierung in apoptotischen Signalübertragungswegen geklärt werden (zusammengefasst in Abb. 13). So konnte z.B. in Bezug auf die Initiator-Caspasen-8 und -9 gezeigt werden, dass für deren schnelle und effiziente Aktivierung in der Todesrezeptor-vermittelten Apoptose ein aktives Proteasom eine wichtige Vorraussetzung ist (Abb. 13, Pkt. 1). Da das Proteasom aber auch inhibierend auf apoptotische Prozesse einwirken kann, haben wir ein biphasisches Modell konzipiert, welches von großer Bedeutung für eine auf Proteasom-Inhibitoren basierende Kombinationstherapie sein dürfte.

Weiterhin konnte der Initiator-Caspase-8 im intrinsischen Signalweg der Apoptose eine Rolle als Effektor-Caspase zugewiesen werden, deren Aktivierung auch in Abwesenheit eines DISC stattfindet und deshalb besonders bei schwachen Apoptose-Stimuli von Bedeutung ist (Abb. 13, Pkt. 2). Auch in Bezug auf die Initiator-Caspase-2 konnte in der vorliegenden Arbeit festgestellt werden, dass sie in Abhängigkeit von Zelltyp und Stimulus an verschiedenen Schnittpunkten des intrinsischen Signalwegs aktiviert werden kann und ähnlich wie die Caspase-8 sowohl als Initiator- als auch als Effektor-Caspase fungieren kann (Abb. 13, Pkt. 3 & 4). Inwiefern sie aber für apoptotische Prozesse von Bedeutung ist und welche Funktion sie darin einnimmt, bedarf allerdings weiterer Untersuchungen.

Von besonderer Bedeutung könnten auch unsere neu erworbenen Kenntnisse über eine CDK-abhängige Apoptosom-Formierung sein. Hier konnte erstmalig gezeigt werden, dass die Aktivierung der Initiator-Caspase-9 in bestrahlungsinduzierten Signalwegen durch den CDK-Inhibitor p21 gehemmt wird (Abb. 13, Pkt. 5). Diese Inhibition wird durch eine direkte Interaktion mit CDKs vermittelt, die über noch unbekannte Zielgene und Mechanismen für die Aktivierung der Caspasen-Kaskade benötigt werden. Da die Stress-induzierte p21-Expression von p53 vermittelt wird, ergibt sich hieraus die äußerst interessante Beobachtung, dass auch bedeutende Apoptose-Induktoren wie p53 inhibitorisch in diese Prozesse eingreifen können.

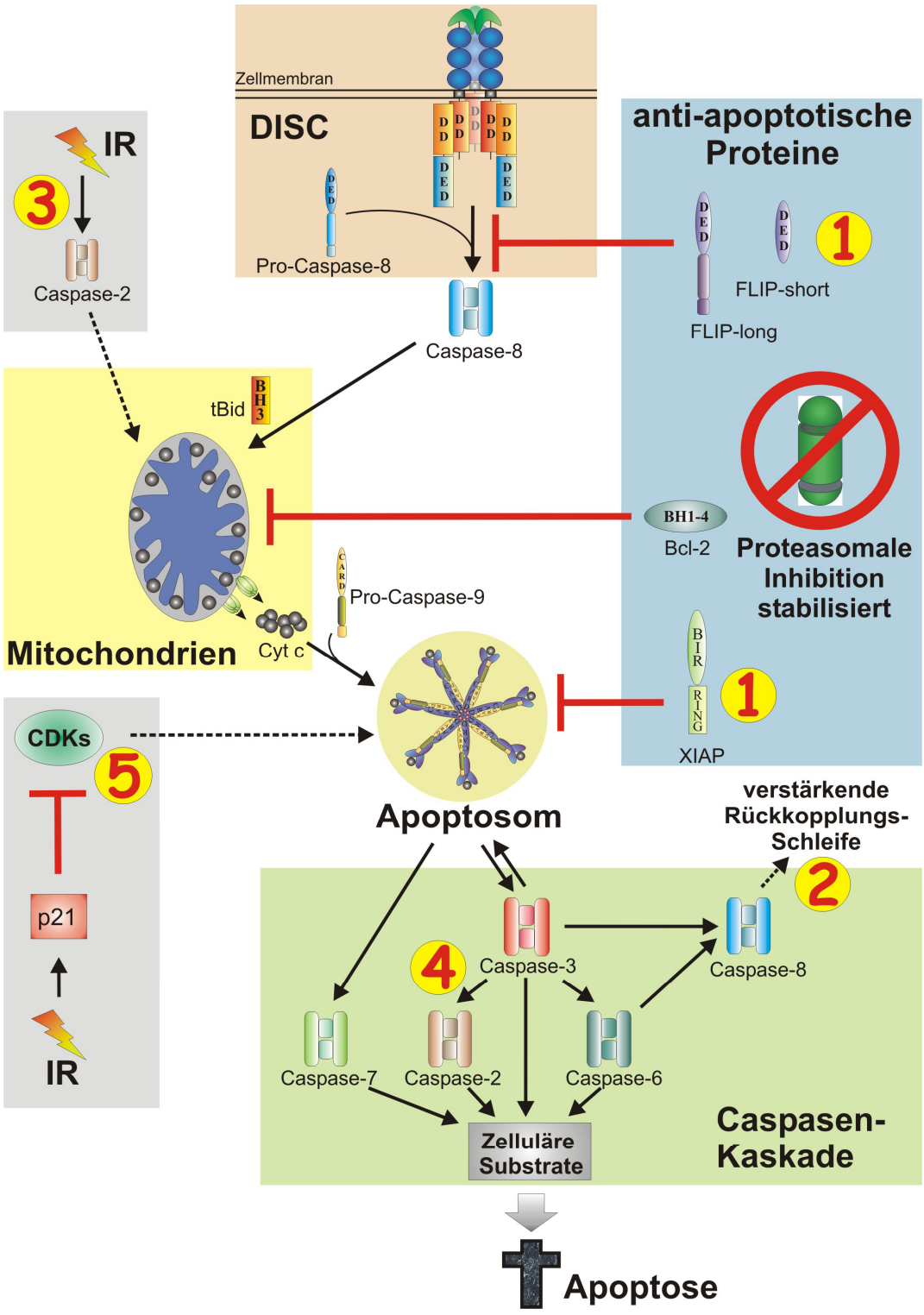

Abbildung 13: Schematische Einordnung der Ergebnisse dieser Arbeit in die apoptotischen Signaltransduktionswege

Dargestellt sind die wichtigsten Schnittpunkte der apoptotischen Signalübertragungswege. Nach Induktion des extrinsischen Signalwegs durch Ligandenbindung-vermittelte Rezeptoroligomerisierung wird die Initiator-Caspase-8 im DISC aktiviert. Über die Caspase-8-vermittelte Spaltung von BID wird das Signal zu den Mitochondrien übertragen und der intrinsische Signalweg der Apoptose aktiviert. Dies führt zur Freisetzung von Cytochrom c und zur Bildung des Apoptosoms, in dem die Initiator-Caspase-9 aktiviert wird. Diese löst dann die mitochondriale Caspasen-Kaskade aus, die letztendlich für die Durchführung der Apoptose durch Spaltung der zellulären Substrate verantwortlich ist. Die in rot gedruckten Zahlen im gelben Kreis verweisen auf die Textstellen, in denen die in dieser Arbeit gewonnen zusätzlichen Erkenntnisse beschrieben werden.

Die in dieser Arbeit gezeigten Ergebnisse erweitern unser Wissen über die apoptotische Signaltransduktion um einige wichtige Aspekte. Die hier gewonnenen Erkenntnisse über die Modulation der Initiator-Caspasen-Aktivierung in verschiedenen Behandlungs-Szenarien könnten als Grundlage einer möglichen Optimierung von bestehenden Krebstherapien Verwendung finden. Deutlich wurde vor allem, dass trotz ihrer bisher ausführlichen Charakterisierung apoptotische Signalwege sehr stark von Zelltyp- und Stimulus-spezifischen Mechanismen beeinflusst werden. Deswegen müssen Tumore sowie die von ihnen beeinflussten Signalwegen gründlich charakterisiert werden, um eine erfolgreiche Krebstherapie durchführen zu können.

Kapitel 4

Zusammenfassung

(Abstract)

Zusammenfassung:

Im Fokus von Krebstherapien steht die gezielte und effiziente Aktivierung von Initiator-Caspasen, die an der Spitze der Caspasen-Kaskade stehen und somit für die Induktion von Apoptose verantwortlich sind. Das Ziel dieser Arbeit war es daher, die molekularen Aktivierungsmechanismen dieser Caspasen in verschiedenen Apoptose-induzierenden Signalwegen zu untersuchen und ihre Modulation über verschiedenen Tumortherapie-Strategien zu charakterisieren.

Hierbei konnte gezeigt werden, dass ein aktives Proteasom für eine schnelle und effiziente Aktivierung der Initiator-Caspase-8, die die Todesrezeptor-induzierte Apoptose vermittelt, sowie der Initiator-Caspase-9, die den mitochondrialen Apoptose-Signalweg induziert, unbedingt benötigt wird. Das Proteasom sorgt durch den Abbau von anti-apoptotischen Inhibitoren der Initiator-Caspasen-Aktivierung wie c-FLIP oder XIAP für eine rasche Initiierung des apoptotischen Programms. In der Effektor-Phase der Apoptose allerdings degradiert das Proteasom auch pro-apoptotische Proteine und wirkt sich damit inhibierend auf die Apoptose aus. Aufgrund dieser Erkenntnisse konnten wir erstmalig ein biphasisches Modell konzipieren, welches von großer Bedeutung für eine auf Proteasom-Inhibitoren und Apoptose-induzierenden Wirkstoffen basierende Kombinationstherapie sein dürfte. Eine zu frühe Verabreichung von Proteasom-Inhibitoren stabilisiert nämlich die bereits vorhandenen anti-apoptotischen Proteine, wodurch eine gesteigerte Apoptose-Resistenz ausgelöst wird. Eine nach der Apoptose-Induktion erfolgende Behandlung allerdings resultiert in einer Stabilisierung der nun generierten pro-apoptotischen Proteine und führt somit zu einer erfolgreichen Tumorbekämpfung.

Weiterhin konnten in dieser Arbeit eine ungewöhnliche Prozessierung und enzymatische Aktivierung der Initiator-Caspase-8 und -2 innerhalb der mitochondrialen Effektor-Caspasen-Kaskade nachgewiesen werden. Diese beiden Caspasen übernehmen in der Chemotherapeutika-vermittelten Apoptose neben ihrer eigentlichen Funktion als Initiator- auch eine zusätzliche Rolle als Effektor-Caspase. Die Aktivierung der Caspase-8 ist hierbei besonders bei einem schwachen Apoptose-Stimulus von großer Bedeutung, da sie über die Spaltung von BID eine zusätzliche Aktivierung der Mitochondrien bewirkt und so die Caspasen-Kaskade verstärkt. Damit diese verstärkende Rückkopplungsschleife nicht unbeabsichtigt induziert wird, werden für eine erfolgreiche und effiziente Aktivierung der Caspase-8 in Abwesenheit eine

Todesrezeptorkomplexes sowohl die Effektor-Caspase-3 als auch die Caspase-6 benötigt.

Außerdem konnte in dieser Arbeit eine interessante Verknüpfung zwischen dem Zellzyklus und der Apoptose-Induktion aufgezeigt werden. Nur in Anwesenheit von aktiven CDK-Komplexen, die für den Ablauf des Zellzyklus verantwortlich sind, findet eine detektierbare Aktivierung der Initiator-Caspase-9 und damit eine Apoptose-Induktion nach einer Behandlung mit γ-Strahlung statt. Die Zellen, die zur Reparatur der DNA-Schäden erfolgreich im Zellzyklus arretieren, in dem sie wegen der Schädigung die Expression des CDK-Inhibitors p21 induzieren, werden so gleichzeitig vor der Apoptose geschützt. Da die Induktion der p21-Expression vom Tumorsuppressor p53 vermittelt wird, ist dies eine der bisher selten beobachteten Fälle, in denen p53 eine anti-apoptotische Rolle spielt.

Die hier veröffentlichten Ergebnisse führen zu einem weit besseren Verständnis der molekularen Aktivierungsmechanismen von Initiator-Caspasen. Zusätzlich zeigen sie neue Möglichkeiten auf, wie diese Aktivierung in verschiedenen Krebstherapie-Ansätzen induziert bzw. erfolgreicher moduliert und effektiver gestaltet werden kann. Allerdings weisen diese Beobachtungen auch darauf hin, dass trotz der immer ausführlicheren Charakterisierung der apoptotischen Signaltransduktion eine erfolgreiche Tumorbekämpfung von der Zelltyp- und Stimulus-spezifischen Beeinflussung dieser Signalwege abhängt.

Abstract:

The main aim of many anti-cancer strategies is the specific and efficient activation of initiator caspases, which are at the apex of the caspase cascade and responsible for the induction of apoptosis. The goal of this thesis was therefore to elucidate the molecular mechanisms of initiator caspase activation in different apoptosis systems and to explore their modulation in different anti-cancer strategies.

One of the studies presented here demonstrates the requirement of proteasomal activity for a rapid and efficient activation of the two initiator caspases -8 and -9 that mediate the death receptor and the mitochondrial pathway of apoptosis, respectively. This conclusion was drawn from experiments showing that the proteasome-mediated degradation of the inhibitors of initiator caspases, c-FLIP and XIAP, is an important prerequisite for the initial steps of apoptosis. At the later stages, however, the proteasome is also responsible for the degradation of pro-apoptotic proteins and thus, counteracts the correct execution of the apoptotic program. We therefore postulate a biphasic model for the influence of the proteasome on apoptosis, which could be of utmost importance for a combinatorial anti-cancer therapy employing proteasomal inhibitors together with apoptosis-inducing agents. This model implies that a premature application of proteasomal inhibitors leads to the stabilization of the inhibitory proteins of initiator caspases and therefore to an increased resistance to apoptosis induction. A more timely application, i.e. at a later time point at which the death stimulus already succeeded in the generation of degradable pro-apoptotic proteins, would surely result in their increased stabilization and hence, to a successful execution of the apoptotic program.

An additional observation in this thesis concerns the discovery of an unusual processing and enzymatic activation of the initiator caspase-8 and -2 during the progression of the mitochondrial effector caspase cascade. During the course of anti-cancer drug-induced apoptosis, these two caspases posses, besides their known function as initiator-caspases, an additional role as effector caspases. Activation of the initiator-caspase-8 in such a manner might be especially important in systems exhibiting a low apoptosis-inducing potential, as the caspase-8-mediated cleavage of BID results in an increased release of cytochrome c out of the mitochondria and therefore, in an amplification of the mitochondrial death pathway. To prevent an accidental induction of this feedback amplification loop, both the effector caspase-3 and -6 are required for an efficient activation of caspase-8 in the absence of a death receptor-induced signaling complex.

Moreover, the results of this thesis indicate an interesting connection between the progression of the cell cycle and the induction of apoptosis. The observation that active CDK complexes, which are the main mediators of cell cycle progression, are required downstream of mitochondrial cytochrome c release for the irradiation-induced activation of the initiator-caspase-9 in the apoptosome adds a new twist to the already complex signaling network following DNA damage. These results not only demonstrate the close connection of events leading to either cell cycle arrest or apoptosis, but also indicate that one and the same molecule, namely the CDK inhibitor p21, has to actively arrest the cell cycle and block apoptosis to allow a successful DNA repair.

Together, the results of this thesis considerably extend our knowledge about the molecular mechanisms of initiator caspase activation. Additionally, they open up new avenues for the design of different anti-cancer strategies that hopefully lead to an increased and more efficient activation of apoptotic program. Beside the ongoing characterization of diverse apoptotic signaling pathways, these results indicate that a successful combat of tumors additionally requires a careful evaluation of cell type- and stimulus-specific events.

Kapitel 5
Referenzen

5. Referenzen

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Kapitel 6
Veröffentlichte Ergebnisse

The proteasome is required for rapid initiation of death receptor-induced apoptosis

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The Proteasome Is Required for Rapid Initiation of Death Receptor-Induced Apoptosis

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Due to their tremendous apoptosis-inducing potential, proteasomal inhibitors (PIs) have recently entered clinical trials. Here we show, however, that various PIs rescued proliferating tumor cells from death receptor-induced apoptosis. This protection correlated with the stabilization of X-linked IAP (XIAP) and c-FLIP and the inhibition of caspase activation. Together with the observation that PIs could not protect cells expressing XIAP or c-FLIP short interfering RNAs (siRNAs) from death receptor-induced apoptosis, our results demonstrate that PIs mediate their protective effect via the stabilization of these antiapoptotic proteins. Furthermore, we show that once these proteins were eliminated, either by long-term treatment with death receptor ligands or by siRNA-mediated suppression, active caspases accumulated to an even larger extent in the presence of PIs. Together, our data support a biphasic role for the proteasome in apoptosis, as they show that its constitutive activity is crucial for the rapid initiation of the death program by eliminating antiapoptotic proteins, whereas at later stages, the proteasome acts in an antiapoptotic manner due to the proteolysis of caspases. Thus, for a successful PI-based tumor therapy, it is crucial to carefully evaluate basal proteasomal activity and the status of antiapoptotic proteins, as their PI-mediated prolonged stability might even cause adverse effects, leading to the survival of a tumor.

Apoptosis is a fundamental process that is essential for normal tissue homeostasis and development (31, 40, 56). Hence, the dysregulation of apoptosis has been implicated in several human diseases, including autoimmune disorders and malignancies. Apoptosis can be instigated via two principal pathways, both of which result in the activation of a class of aspartate-specific cysteine-dependent proteases, called caspases (4), that lead to the demise of the cell via the limited proteolysis of a multitude of cellular substrates (10). In the extrinsic pathway, ligand-induced oligomerization of death receptors, such as TRAIL-R1, TRAIL-R2, or CD95, results in the direct recruitment of the adapter molecule Fas-associated death domain (FADD) and the initiator caspase -8 or -10 that, together, form the death-inducing signaling complex (DISC) (49). Signaling through tumor necrosis factor receptor 1 (TNF-R1), in contrast, appears to be more complex (59) and is proposed to proceed via two sequential complexes, in which the death-inducing signaling complex II containing FADD and an initiator caspase is formed only in the cytosol (39). Nevertheless, despite the fact that procaspase-8 can also be activated independently of DISC formation (51), receptor-triggered dimerization of initiator caspases represents a crucial event in this pathway that results in their autocatalytic processing and activation. In most cell types, activated caspase-8 stimulates the second principal death route, the mitochondrial or intrinsic death pathway, by cleaving Bid, a BH3-only proapoptotic member of the Bcl-2 family (32). The caspase-generated truncated Bid (tBid) fragment promotes the release of the proapoptotic factors cytochrome *c* and Smac from the mitochondria. Once released into the cytoplasm, cytochrome *c* associates with apoptotic protease-activating factor 1 and procaspase-9 to form the apoptosome, which leads to the activation of caspase-9 and the effector caspase-3 (17, 28).

Both death pathways are tightly controlled by multiple mechanisms that efficiently prevent caspase activation (50). For example, the generation of active caspase-8 is blocked by the recruitment of the cellular FLICE-like inhibitory protein (c-FLIP) into the DISC (29). Two isoforms, c-FLIP-long and c-FLIP-short, are major detectable splice variants that have been the subjects of several studies so far. While c-FLIP-short consists of mainly two death effector domains that are required for the interaction with FADD, c-FLIP-long is structurally more closely related to caspase-8 but lacks key residues that are necessary to form an active caspase and is therefore completely devoid of any catalytic activity. Due to their homologies, both c-FLIP isoforms are able to form heterodimers with caspase-8 at the DISC and thereby block the processing and activation of this initiator caspase, which is crucial for the induction of death receptor-induced apoptosis (33, 48). On the other hand, antiapoptotic members of the Bcl-2 family indirectly inhibit caspase-9 activation by preventing the release of proapoptotic factors from the mitochondria. Further complexity is added to the regulatory pathways involved in apoptosis signaling by the inhibitor-of-apoptosis proteins (IAPs) that include X-linked IAP (XIAP), cIAP1, and cIAP2, all of which are potent inhibitors of the active caspase-9, -7, and -3 (6, 47). IAPs are characterized by two distinct motifs, the baculovirus IAP repeats that are crucial for caspase inhibition and a carboxyl-terminal

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RING domain that enables IAPs to catalyze the degradation of both themselves and selected target proteins via the ubiquitin-proteasome pathway (57).

Relative to all the death receptor ligands studied, TRAIL has attracted immense attention due to its ability to preferentially kill tumor cells while leaving most normal cells unharmed in vitro (61) and in vivo (1). However, clinical trials are hampered by the fact that more than 50% of all human cancers are resistant to the cytotoxic activity of TRAIL, demonstrating the necessity for alternative treatment modalities. Promising results were obtained from studies showing that the cytotoxic potential of TRAIL was synergistically enhanced by a combined treatment with chemotherapeutics (16) or radiation (36). Also, the simultaneous treatment with proteasomal inhibitors (PIs), such as bortezomib (PS-341), lactacystin, or MG-132, yielded encouraging results, as all of these compounds synergistically accelerated and enhanced TRAIL- or TNF-induced apoptosis in a variety of tumor cells (11, 19, 30, 60). In view of this success, the proteasome inhibitor bortezomib has recently entered clinical practice as a treatment for multiple myeloma and is also undergoing clinical trials for other types of cancer (45, 58).

The ubiquitin-proteasome pathway plays a central role in the regulation of cell cycle control, transcription, signal transduction, and apoptosis (27). In eukaryotes, it is the major machinery that mediates the targeted degradation of many key regulatory proteins, including p53, cyclins, and cyclin-dependent kinase inhibitors, as well as IκBα (41). Although many diverse mechanisms have been proposed regarding which inhibition of the proteasome sensitizes cells for apoptosis, the precise mode of action remains elusive. The observations that many proapoptotic proteins, such as TRAIL-R2, Smac, Bax, tBid, Bik, and Bim, as well as various caspases, are degraded by the proteasome explain, at least partially, the apoptotic function of proteasomal inhibitors (35, 41, 42). However, several antiapoptotic proteins, such as c-FLIP, Bcl-2, and IAPs, also represent prominent targets of the proteasome (7, 12, 44, 63). Hence, stabilization of these potent caspase antagonists by PIs should confer resistance to apoptotic stimuli; this is a hypothesis that, surprisingly, was not thoroughly investigated before. Therefore, we analyzed such a putative mechanism in more detail in HeLa cells and found that death receptor-induced apoptosis was indeed significantly blocked in the presence of various proteasome inhibitors. The inhibition of apoptosis induction correlated well with the stabilization of XIAP and c-FLIP, and the latter protein was efficiently recruited to the DISC (thereby preventing caspase-8 processing) only in the presence of PIs. The suppression of XIAP and c-FLIP expression by short interfering RNA (siRNA) technology not only abrogated the protective effect of proteasome inhibitors but also accelerated their cytotoxic potential in combination with death receptor ligands. Thus, our data demonstrate a biphasic role for the proteasome in apoptosis in which the degradation of antiapoptotic proteins, such as XIAP and c-FLIP, is necessary for a rapid initiation of the death receptor pathway.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies. HeLa D98 and H21 cells (24), MCF-7/casp-3 cells (26), and the KB cell line were maintained in RPMI 1640, whereas HCT116 cells were cultured in McCoy's medium. Both media were supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from PAA Laboratories, Linz, Austria). The polyclonal goat antibody directed against caspase-3, the polyclonal caspase-8 antibody, and the monoclonal caspase-8 antibody were from R&D Systems (Wiesbaden, Germany), Santa Cruz Biotechnology, and BioCheck (Münster, Germany), respectively. The monoclonal actin antibody, cycloheximide (Chx), and the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin were from Sigma (Deisenhofen, Germany). The monoclonal antibodies against FADD and XIAP were purchased from BD Biosciences (Heidelberg, Germany). From Biomol (Hamburg, Germany) were the fluorogenic substrates DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) for caspase-3, Suc-LLVY-AMC (*N*-succinyl-Leu-Leu-Val-Tyr-AMC) for 20S proteasomal activity, and Z-LRGG-AMC (carbobenzoxy-Leu-Arg-Gly-Gly-AMC) for ubiquitin C-terminal hydrolases, as well as the protease inhibitors MG-132, clasto-lactacystin (CLC), calpain inhibitor I (ALLN), and calpain inhibitor II (ALLM). Human recombinant TNF with a specific activity of 4×10^7 U/mg of protein was obtained from Knoll AG (Ludwigshafen, Germany). The monoclonal c-FLIP antibody (NF6) and the His-tagged Killer-TRAIL preparation were from Alexis (Lausen, Switzerland). If not otherwise indicated, all assays were performed in the presence of cycloheximide (10 μg/ml).

Construction of small interfering RNAs and stable transfection. For the suppression of c-FLIP and XIAP expression, siRNAs were designed using the Dharmacon siDESIGN Center. The selected sense sequences were 5′-GCAGTC TGTTCAAGGAGCA-3′ for c-FLIP and 5′-AATAGTGCCACGCAGTCTA-3′ for XIAP. Complementary oligonucleotides consisting of sense, hairpin loop, and antisense sequences were annealed and ligated into the pSilencer siRNA expression vector according to the manufacturer's instructions (Ambion, United Kingdom). HeLa D98 cells were stably transfected by electroporation using the Bio-Rad gene pulser (500 μF, 160 V). After hygromycin selection, several clones were obtained and the successful reduction of c-FLIP and XIAP expression was controlled by Western blot analyses.

Preparation of cell extracts and Western blotting. Cell extracts were prepared as described previously (26). Briefly, cells were lysed for 30 min at 4°C in lysis buffer A containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM dithiothreitol (DTT), 1% NP-40, and a protease inhibitor cocktail. After removing nuclei and cell debris by centrifugation (10,000 × *g*) at 4°C, protein concentrations were determined with the Bio-Rad protein assay. Subsequently, proteins were separated under reducing conditions on a sodium dodecyl sulfate-polyacrylamide gel and electroblotted to a polyvinylidene difluoride membrane (Amersham, Braunschweig, Germany). Following incubation with the various antibodies, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham).

Measurement of cell death. Cell death determinations were done with the standard TNF cytotoxicity assay (crystal violet assay) that is based on the staining of viable cells (24). Briefly, cells (2×10^5/ml) were seeded into 96-well microtiter plates in 100 μl culture medium. Cells were incubated with the death stimuli for the indicated times at 37°C, and viable cells were stained with 20% methanol containing 0.5% crystal violet and solubilized in 33% acetic acid. The absorbance was measured at an optical density of 590 nm (A_{590}). Percent specific cell death is defined as 100 − (A_{590} of test well × 100/A_{590} of untreated well). Each experiment was performed independently at least three times, and an individual experiment was carried out in triplicate. The percent inhibition of cell death by MG-132 (see Fig. 5 and 8) is defined as 100 − [(percent specific cell death with MG-132)/percent specific cell death without MG-132) × 100]. Please note that negative numbers indicate an increased cell death rate due to the cotreatment with MG-132.

Fluorimetric substrate assays. Caspase-3 activities were expressed as arbitrary units that were determined by the incubation of cell extracts for 2 h with 50 μM of the fluorogenic caspase-3 substrate DEVD-AMC in 200 μl buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% CHAPS {3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate}, and 10 mM DTT. The release of aminomethylcoumarin was measured by fluorometry by using an excitation wavelength of 360 nm and an emission wavelength of 475 nm. For the determination of proteasomal and ubiquitin hydrolase activity, cells were broken in lysis buffer B containing 30 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.2% Triton X-100 by four freeze-thaw cycles. Following centrifugation (10,000 × *g*) at 4°C, the resulting lysates were incubated for 1 h with 100 μM of the fluorogenic substrates LLVY-AMC and LRGG-AMC in 200 μl assay buffer containing 30 mM Tris-HCl (pH 7.6), 10 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, and 0.5 mM DTT. The release of aminomethylcoumarin was measured by fluorometry by using an excitation wavelength of 360 nm and an emission wavelength of 475 nm. To ensure that the observed activity was indeed

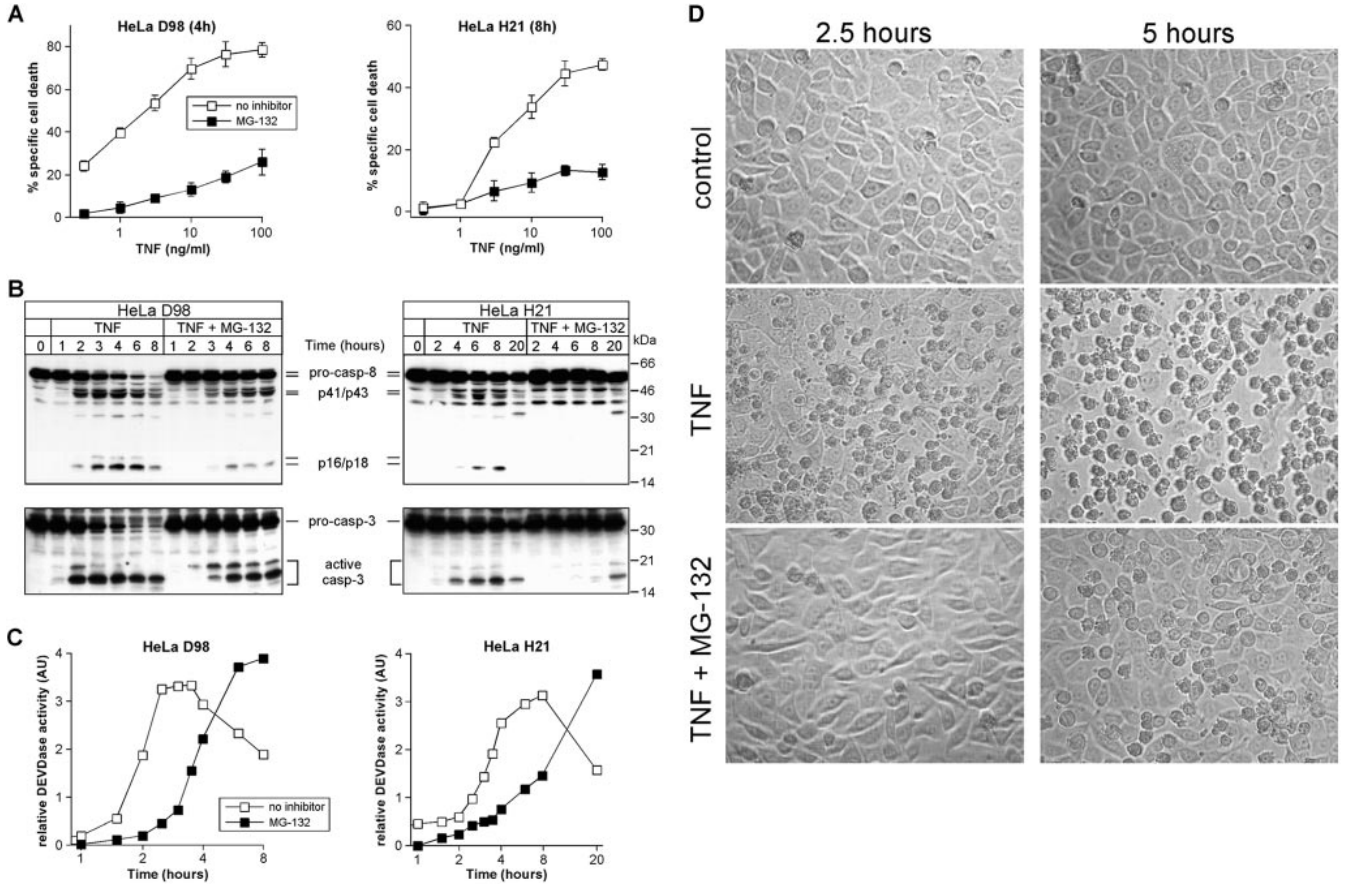

FIG. 1. The proteasomal inhibitor MG-132 protects HeLa cells from TNF-induced apoptosis and caspase activation. (A) HeLa D98 and H21 cells were preincubated for 1 h with or without MG-132 (10 μM), followed by the addition of the indicated concentrations of TNF and Chx. After 4 h (HeLa D98) or 8 h (HeLa H21), the induction of cell death was analyzed by the crystal violet assay. One representative experiment out of at least five performed in triplicate is shown. (B and C) HeLa D98 and H21 cells were pretreated for 1 h with or without MG-132 (10 μM), followed by the addition of TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and analyzed for the processing of caspase-8 and -3 (B) and caspase-3 activity (C). AU, arbitrary units. (D) HeLa D98 cells were either left untreated or treated with TNF (10 ng/ml) and Chx in the absence or presence of a 1-h preincubation with MG-132 (10 μM). Micrographs shown were taken after 2.5 and 5 h.

proteasome derived, the lysates that were analyzed were incubated for 30 min with the fluorogenic LLVY-AMC substrate in the presence of 10 μM MG-132.

Immunoprecipitation. Immunoprecipitation reactions were performed as described previously (51). Briefly, cell extracts were prepared from 1×10^7 cells in 500 μl lysis buffer without DTT. For the precipitation, 1 μg polyclonal caspase-8 antibody and 30 μl protein G-Sepharose (Sigma) were added to the extracts and rotated for 4 h or overnight at 4°C. The Sepharose beads were extensively washed in lysis buffer and then analyzed by Western blotting.

RESULTS

Proteasome inhibition blocks TNF-induced caspase activation and apoptosis. In an attempt to better understand the mode of action of PIs on death receptor-induced apoptosis, we treated two HeLa cell lines that display different apoptosis susceptibilities toward death receptor ligands (24) with increasing concentrations of TNF in the absence or presence of the peptide aldehyde inhibitor MG-132. Surprisingly, we found that MG-132 protected both cell lines very efficiently from TNF-induced apoptosis even when TNF concentrations as high as 100 ng/ml were used (Fig. 1A). This effect not only was determined using the crystal violet assay (Fig. 1A) but also was visualized by micrographs taken from HeLa D98 cells that were incubated for 2.5 or 5 h with TNF in the absence or presence of MG-132 (Fig. 1D). MG-132-mediated protection was caused by the inhibition of caspases, as processing and activity of both caspase-8 and -3 were significantly delayed by this compound in both cell lines (Fig. 1B and C). Although this was clearly evidenced by the diminished generation of active caspase-8 and caspase-3 fragments, the inhibitory effect of MG-132 was most obvious with regard to the unprocessed proforms of both caspases. Depending on the apoptosis susceptibility of the cell line, TNF treatment resulted either in an almost complete loss of both caspase proforms, as evidenced in TNF-sensitive HeLa D98 cells (Fig. 1B, left panel), or in an at least significant reduction of the proforms, as was observed in the more-resistant H21 cell line (Fig. 1B, right panel). In the presence of MG-132, however, TNF induced only a marginal decrease of both proforms in both cell lines, clearly demonstrating that the inhibition of the proteasome blocks TNF-induced caspase processing. Note that protection by MG-132 was much more pronounced in the resistant HeLa H21 cells as the generation of active caspase fragments became almost completely inhibited even after a 20-h incubation with TNF.

These results are consistent with a marked delay in caspase-8 (IETDase) (data not shown) and caspase-3 (DEVDase) (Fig. 1C) activities that were observed in both cell lines and also with the cleavage of α-fodrin into the caspase-3-dependent 120-kDa fragment (25) that was accordingly delayed when the cells were treated with TNF in the presence of MG-132 (data not shown). Interestingly, the incubation of the cells with and without MG-132 in the absence of TNF for up to 20 h did not reveal any significant differences in the amount of procaspase-8 and -3 (data not shown), suggesting that these procaspases are not direct targets of the proteasome. In contrast, after the initial delay in caspase activation, we even observed enhanced caspase-8 and caspase-3 (Fig. 1C) cleavage activities when the cells were treated with TNF in the presence of MG-132. Thus, in contrast to the procaspases, active caspases are degraded by the proteasome, which so far was shown for only caspase-3 (53).

Inhibition of the proteasome transiently blocks TNF-induced apoptosis in a dose- and time-dependent manner. The activity of the peptide aldehyde inhibitor MG-132 appears to be not exclusively specific for the proteasome (9). Therefore, we also performed the experiments in the presence of various other compounds, such as ALLN and ALLM, that are effective and weak inhibitors of the proteasome, respectively, as well as with CLC, which is among the most selective PIs known (9). Except for ALLM, which did not interfere with TNF signaling at any concentration tested, all the other compounds reproducibly blocked TNF-induced apoptosis of both HeLa cell lines in a dose- and time-dependent manner (Fig. 2A and B). The protective effect was of a transient nature, as all of the various PIs efficiently delayed TNF-induced apoptosis of HeLa D98 cells up to 6 h, but they could not protect these cells at later time points (Fig. 2B, left panel). This is consistent with the caspase activation profiles shown in Fig. 1C. On the other hand, more than 80% of HeLa H21 cells survived even a 20-h TNF treatment in the presence of either of these compounds (Fig. 2B, right panel). However, these cells also eventually succumbed to apoptosis, even in the presence of PIs, when they were treated with TNF for up to 36 h (data not shown). Together, these data not only suggest that the protective effect of these inhibitors is indeed caused by their ability to block the 26S proteasome but also demonstrate that the efficiency of this effect critically depends on the strength of the individual apoptotic response. Furthermore, and more intriguingly, they indicate that proteasomal activity might be required for the efficient initiation of the death receptor pathway.

To further analyze the conditions required for a maximal protective effect, we have also investigated the necessity to preincubate the cells with these PIs. Relative to a 1-h preincubation time, the simultaneous addition of the proteasome inhibitors, together with TNF, resulted in a weaker protection, whereas preincubation for more than 1 h did not further increase their protective effect. In contrast, no protection was observed when the PIs were added 1 h or 2 h after TNF treatment (data not shown). Thus, the preincubation of HeLa D98 cells with either MG-132 or CLC for 1 h before the addition of TNF was both necessary and sufficient to achieve optimal protection against the cytotoxic activity of this cytokine (Fig. 2C). Hence, subsequent experiments were conducted following a 1-h preincubation period with the PIs.

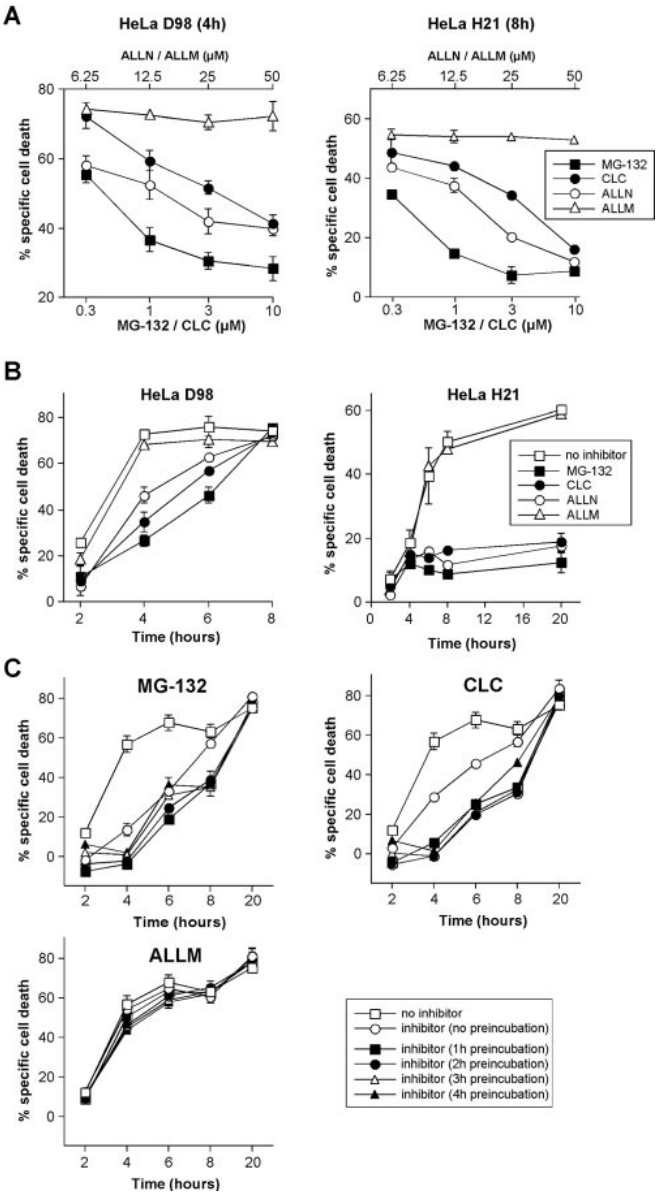

FIG. 2. Dose- and time-dependent protection from TNF-induced apoptosis specifically by proteasomal inhibitors. (A) HeLa D98 and HeLa H21 cells were preincubated with or without (no inhibitor) the indicated concentrations of MG-132, CLC, calpain inhibitor I (ALLN), and the calpain inhibitor II (ALLM), which does not interfere with proteasomal activity before the addition of TNF (10 ng/ml) and Chx. Cell death was assessed after 4 h (HeLa D98) and 8 h (HeLa H21) by the crystal violet assay. (B) HeLa D98 and HeLa H21 cells were preincubated with or without (no inhibitor) MG-132 (10 μM), CLC (10 μM), ALLN (50 μM), or ALLM (50 μM), followed by the addition of TNF (10 ng/ml) and Chx. After the indicated times, cell death was assessed by the crystal violet assay. (C) Before the treatment with TNF (10 ng/ml) and Chx, HeLa D98 cells were preincubated for the indicated times with MG-132 (10 μM), CLC (10 μM), or ALLM (50 μM). After the indicated times, cell death was assessed by the crystal violet assay. One representative experiment out of four performed in triplicate is shown.

Inhibition of the proteasome also blocks TRAIL- and anti-CD95-induced apoptosis and caspase activation. Next we investigated whether the observed effect is restricted to the TNF system or whether PIs would generally protect cells from death

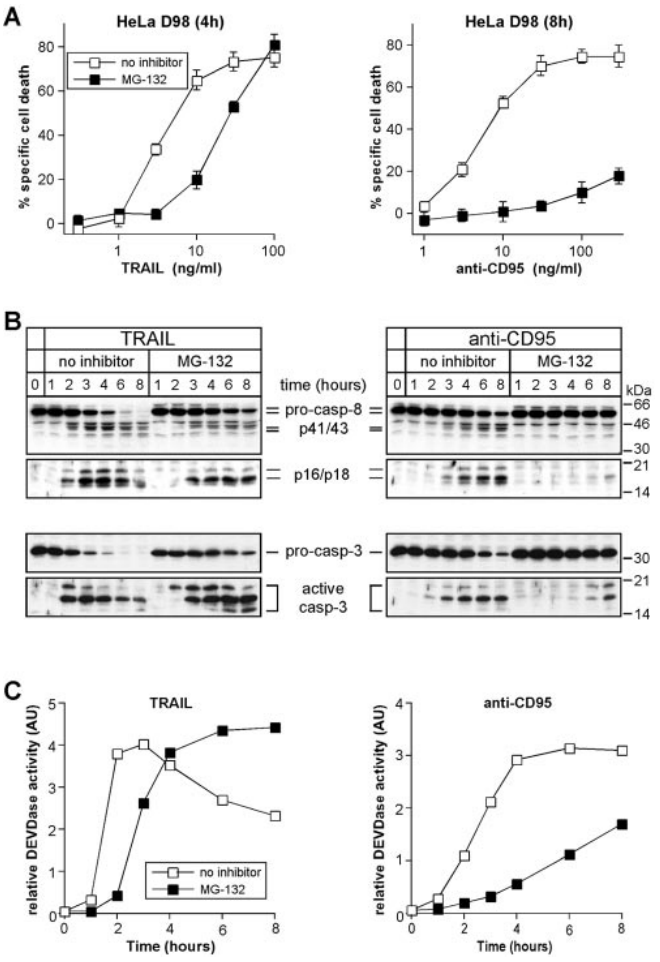

FIG. 3. MG-132 inhibits TRAIL- and CD95-induced apoptosis and caspase activation. (A) Following preincubation for 1 h with or without MG-132 (10 μM), HeLa D98 cells were treated with the indicated concentrations of TRAIL or with the antagonistic CD95 antibody in the presence of Chx. The induction of cell death was analyzed by the crystal violet assay after 4 h (TRAIL) or 8 h (anti-CD95). One representative experiment out of at least five performed in triplicate is shown. (B and C) Following preincubation for 1 h with or without MG-132 (10 μM), HeLa D98 cells were treated with TRAIL (10 ng/ml) or with the antagonistic CD95 antibody (50 ng/ml) in the presence of Chx. After the indicated times, cell extracts were prepared and analyzed for the processing of caspase-8 and -3 (B) and for caspase-3 activity (C). AU, arbitrary units.

receptor-induced apoptosis. To this end, we analyzed TRAIL and CD95 sensitivities of HeLa D98 cells that either were left untreated or were preincubated for 1 h with MG-132. Similar to the results obtained so far with TNF, inhibition of the proteasome by MG-132 also transiently protected the cells against the cytotoxic activities of these two death receptor ligands (Fig. 3A). However, the protection was clearly more pronounced in the CD95 signaling pathway, which induces a significantly slower apoptotic response in these cells than TRAIL. This is reminiscent of the scenario observed with the less sensitive HeLa H21 cells, which are also more efficiently protected from TNF-induced apoptosis by PIs than are the TNF-sensitive HeLa D98 cells. Nevertheless, depending on the dose and time that the two death receptor ligands were applied, the cells also eventually succumbed to apoptosis in the presence of MG-132 (Fig. 3A and data not shown). Similar to TNF-induced apoptosis, MG-132-mediated protection from TRAIL- and anti-CD95-induced killing correlated closely with a significant delay of processing caspase-8 and caspase-3 (Fig. 3B) and the corresponding activities of these caspases (Fig. 3C and data not shown). In addition, MG-132 also significantly delayed TRAIL-induced apoptosis and caspases-8 and -3 activation in HeLa D98 cells in the absence of cycloheximide (data not shown), indicating that the PI-mediated protection also occurs under more physiological conditions, which might be especially relevant for TRAIL-mediated signaling in vivo.

Transient inhibition of death receptor-induced apoptosis and caspase activation by PIs is a common event observed in several cell lines. Next we analyzed whether our findings can also be applied to other experimental cell systems. To this end, we compared caspase-3 (DEVD) activities in several tumor cell lines of various origins that were treated with TNF in the absence or presence of MG-132. Similar to the data obtained with HeLa D98 cells, the inhibition of the proteasome by MG-132 also transiently blocked TNF-induced caspase-3 activation of MCF-7/casp-3 breast carcinoma cells, KB cervical carcinoma cells, and HCT116 colon carcinoma cells (Fig. 4A). Consistent with the transient nature of this effect, we also found that MG-132 efficiently protected the various tumor cell lines from death receptor-induced apoptosis in a short-term (4 h to 8 h) assay but failed to do so following a longer (24 h) stimulation time (Fig. 4B). Similar results were obtained with the lung carcinoma cell line A549, albeit not to the same extent (data not shown). Although MCF-7/casp-3 cells that do not express the CD95 receptor were treated with only TNF and TRAIL, our data clearly show the general protective impact of PIs on death receptor-induced apoptosis.

Proteasomal activity correlates with apoptosis susceptibility. Our experiments performed so far indicate that the proteasome might be required for the initiation of the apoptotic cascade induced by death receptors. Thus, diminished proteasomal activity might be one mechanism contributing to the apoptosis-resistant phenotype of HeLa H21 cells. To investigate this hypothesis, we compared proteasomal activities in cellular extracts of untreated and TNF-stimulated HeLa D98 and H21 cells over a period of 20 h by determining their capabilities to cleave the fluorogenic peptide LLVY-AMC that represents a substrate for the 20S proteasome. Indeed, extracts of untreated HeLa D98 cells reproducibly displayed a proteasomal activity that was on average approximately 1.5-fold higher than that observed in extracts of untreated HeLa H21 cells, a difference that was even further enhanced when the cells were stimulated with TNF (Fig. 5A, left panel). When the cell extracts were analyzed in the presence of MG-132, no LLVY-AMC cleavage could be observed, demonstrating that the cleavage was mediated by the proteasome (data not shown). In contrast, regardless of whether the cells were stimulated with TNF, both cell lines displayed similar ubiquitin C-terminal hydrolase activities, as determined by cleavage of the fluorogenic substrate LRGG-AMC (Fig. 5A, right panel). These results show that apoptosis-sensitive HeLa D98 cells exhibit a higher constitutive proteasomal activity than HeLa H21 cells that are less sensitive toward death receptor-induced cell death. Although the reasons for the differential proteasomal

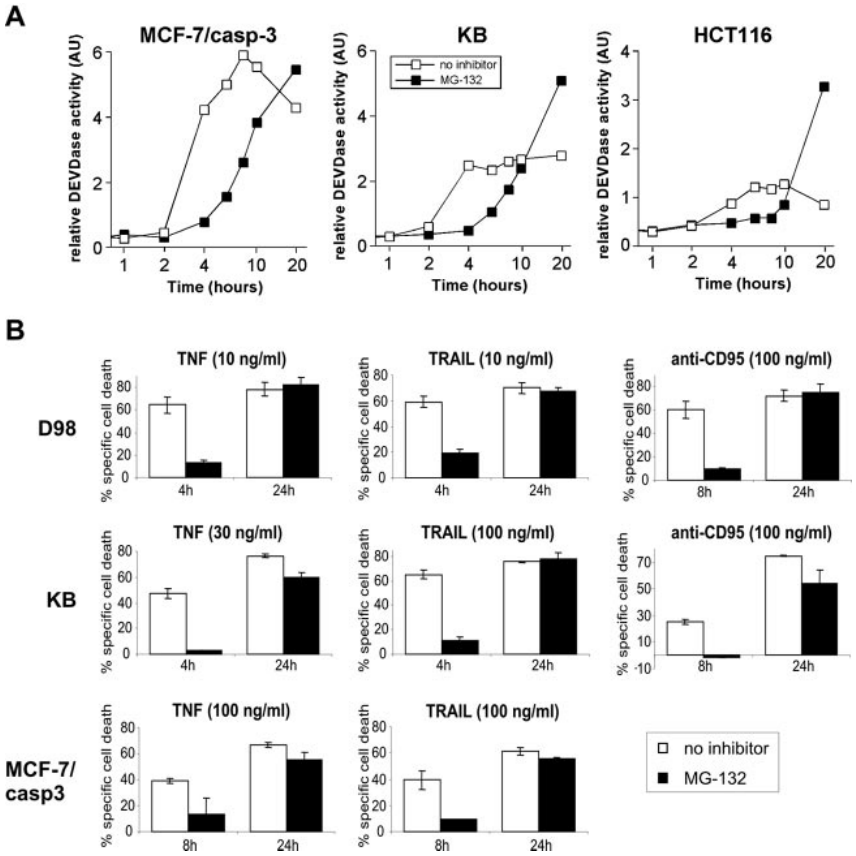

FIG. 4. The proteasomal inhibitor MG-132 transiently protects various cell lines from death receptor-induced apoptosis and caspase activation. (A) MCF-7/casp-3 breast carcinoma cells and KB cervical carcinoma cells, as well as HCT116 colon carcinoma cells, were preincubated for 1 h with or without MG-132 (10 μM), followed by the addition of TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and analyzed for caspase-3 activity. AU, arbitrary units. (B) HeLa D98, MCF-7/casp-3, and KB cells were preincubated for 1 h with or without MG-132 (10 μM), followed by the addition of the indicated concentrations of the death receptor ligands in the presence of Chx. After 4, 8, or 24 h, cell death was analyzed by the crystal violet assay.

activities in these two cell lines are unknown, this finding is in agreement with our data, demonstrating an important role for the proteasome in the initiation of the death receptor program.

In the search for the cellular target(s) that is responsible for the observed PI-mediated resistance toward death receptor-induced apoptosis, we analyzed the expression of several cell cycle and/or apoptosis regulatory proteins. As expected, MG-132 prevented the TNF-induced degradation of the proteasomal targets p21, p27, and cyclin A (41) but had no influence on unrelated proteins, such as RIP and FADD (data not shown). For our study, however, we analyzed the status of XIAP and c-FLIP in more detail because they represent two proteasomal targets that are well-known antiapoptotic proteins which are interfering with the initial stages of caspase activation (12, 44, 47, 63). In addition to their degradation by the proteasome, both proteins are also specifically proteolysed during death receptor-induced apoptosis by caspase-3 and caspase-8, respectively (5, 33). Interestingly, although TNF treatment resulted in comparable amounts of caspase-generated XIAP fragments in both cell lines, a significant reduction of full-length XIAP protein was observed only in HeLa D98 cells (Fig. 5B, left panel). In contrast, the levels of uncleaved XIAP remained almost unchanged during apoptosis of HeLa H21 cells (Fig. 5B, right panel), a finding that is consistent with their lower proteasomal activity (Fig. 5A). Similar results were obtained when we analyzed the status of c-FLIP during apoptosis in these cells. In both cell lines, treatment with TNF induced a rapid proteasome-mediated degradation of c-FLIP-short that was efficiently prevented in the presence of MG-132 (Fig. 5B). In addition to being a proteasomal target, c-FLIP-long was also cleaved within 1 h in both cell lines by DISC-bound caspase-8 generating a p43 fragment. Also, this process was inhibited by MG-132 much more efficiently in HeLa H21 cells, as uncleaved c-FLIP-long remained detectable in these cells up to 8 h, whereas it was cleaved in HeLa D98 cells even in the presence of MG-132 following a 3-h TNF treatment (Fig. 5B). As it is known that c-FLIP-long is only cleaved by caspase-8 when both proteins heterodimerize at the DISC (33, 48), this finding strongly indicates substantially reduced caspase-8 activation at the DISC in both cell lines treated with TNF in the presence of MG-132. Other antiapoptotic proteins, including IAP1, IAP2, and survivin, were not differentially expressed in the two HeLa cell lines, nor were they affected by the individual treatments (TNF in the absence or presence of various PIs) (Fig. 5C) as was observed for XIAP and c-FLIP (Fig. 5B). Together, these data strongly support our hypothesis that the proteasome is an important component required for the rapid initiation of death

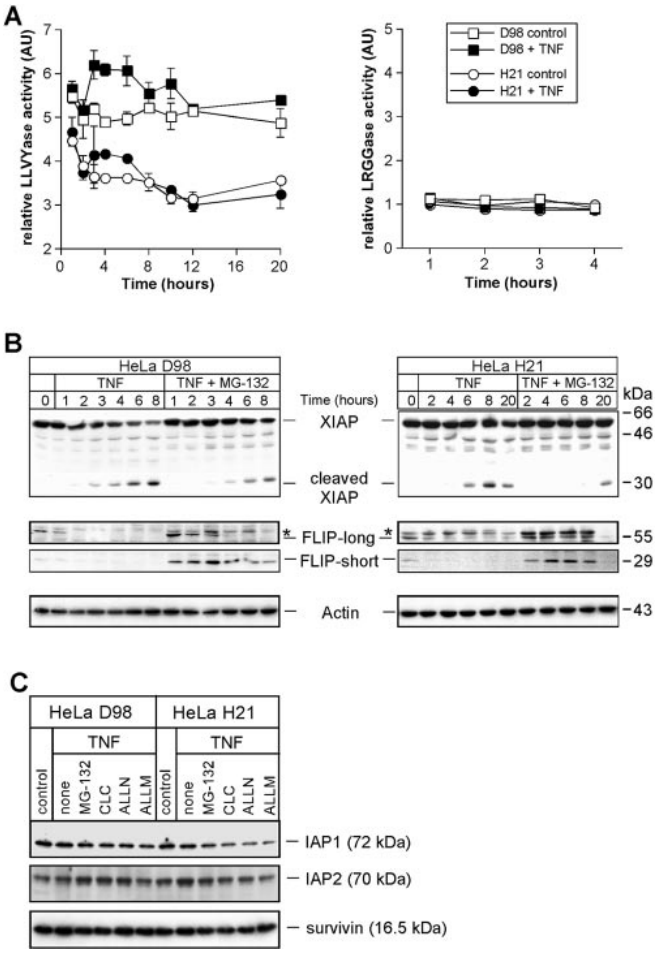

FIG. 5. Higher proteasomal activity in apoptosis-sensitive HeLa D98 cells correlates with accelerated degradation of the antiapoptotic proteins XIAP and c-FLIP-short. (A) HeLa D98 and HeLa H21 cells either were left untreated or were treated with TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and analyzed for proteasomal and ubiquitin C-terminal hydrolase activities using the LLVY-AMC and LRGG-AMC substrates, respectively. AU, arbitrary units. (B) Following preincubation for 1 h with or without MG-132 (10 μM), HeLa D98 and H21 cells either were left untreated or were treated with TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and analyzed by Western blotting for the status of XIAP and c-FLIP expression. The asterisks denote an unspecific band. (C) Following preincubation for 1 h with or without (none) the indicated protease inhibitors, HeLa D98 and H21 cells either were left untreated or were treated with TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and analyzed by Western blotting for the status of the indicated proteins.

receptor-induced cell death via the degradation of antiapoptotic proteins, such as XIAP and c-FLIP.

Inhibition of the proteasome fails to protect XIAP knockdown cells from death receptor-induced apoptosis. If the protective effect of PIs is mediated at least partially by the stabilization of XIAP and c-FLIP, then the suppression of either of these proteins should render TNF-, TRAIL-, or anti-CD95-treated cells unresponsive toward PIs. To test this hypothesis, we first established HeLa D98 clones in which the expression of XIAP was suppressed due to the stable expression of an XIAP siRNA. Several individual clones were obtained, and four that displayed various degrees of XIAP suppression in the absence of any detectable alterations in surface expression of the three death receptors (Fig. 6A and data not shown) were chosen for further analyses. Cytotoxicity assays revealed that, indeed, the inhibition of the proteasome by MG-132 protected only parental HeLa D98 cells from death receptor-induced apoptosis and completely failed to protect the four XIAP siRNA clones from TNF- or TRAIL-induced cell death (Fig. 6B and C). The protective effect of MG-132 was also dramatically reduced when the XIAP siRNA clones were treated with the anti-CD95 antibody, although this protection was never abrogated as efficiently as that in TNF- or in TRAIL-treated cells (Fig. 6D). This is consistent with our observation that the level of PI-mediated protection closely correlates with apoptosis susceptibility of the cells, as it is most pronounced in apoptosis-resistant HeLa H21 cells (Fig. 1) or even in anti-CD95-treated HeLa D98 cells (Fig. 3), which represent the least efficient apoptosis inducer among the three death receptor ligands investigated.

Our finding that suppression of XIAP expression abrogated the protective effect of MG-132 was further verified by Western blot analyses demonstrating that MG-132 was not capable of preventing TNF-induced caspase processing in XIAP siRNA clones as efficiently as in parental HeLa D98 cells (Fig. 7). Whereas the decrease of both caspase-8 and caspase-3 proforms, as well as the generation of the active caspase fragments, was strongly inhibited by MG-132 in D98 cells, no such effect was observed in similarly treated XIAP siRNA clones. Hence, our results suggest that the observed PI-induced protection against death receptor-induced apoptosis is brought about at least partially by stabilizing XIAP protein levels.

Inhibition of the proteasome fails to protect c-FLIP knockdown cells from death receptor-induced apoptosis. With regard to the role of c-FLIP in this process, we observed that MG-132 prevented TNF-induced cleavage of c-FLIP-long in both cell lines (Fig. 5B). As c-FLIP-long is predominantly cleaved by DISC-bound caspase-8, this result suggested that PIs prevent the recruitment of this initiator caspase into the DISC. This hypothesis would also be consistent with our findings that treatment with MG-132 not only led to a massive accumulation of c-FLIP-short (that actively prevents caspase-8 recruitment into the DISC) but also resulted in a substantial inhibition of death receptor ligand-induced caspase-8 processing and activation (Fig. 1 and 3). To verify this scenario, we analyzed caspase-8 immunoprecipitates of HeLa D98 cells that were treated with or without MG-132 for up to 3 h with TNF for the presence of both forms of c-FLIP. Western blot analysis of these precipitates not only verified our previous findings that MG-132 efficiently prevents activation of caspase-8 but also confirmed a successful and efficient caspase-8 precipitation (Fig. 8A). More importantly, however, whereas the adaptor molecule FADD as well as the cleaved p43 fragment of c-FLIP-long was coprecipitated with caspase-8 regardless of whether the TNF treatment occurred in the presence of MG-132, c-FLIP-short was only found in these immunoprecipitates when MG-132 was present during TNF stimulation (Fig. 8B). As both c-FLIP isoforms bind to caspase-8 only within the DISC via interaction with FADD (23), these findings imply that the levels of both c-FLIP isoforms found in the caspase-8 precipitates are constituents of the TNF-R signaling complex

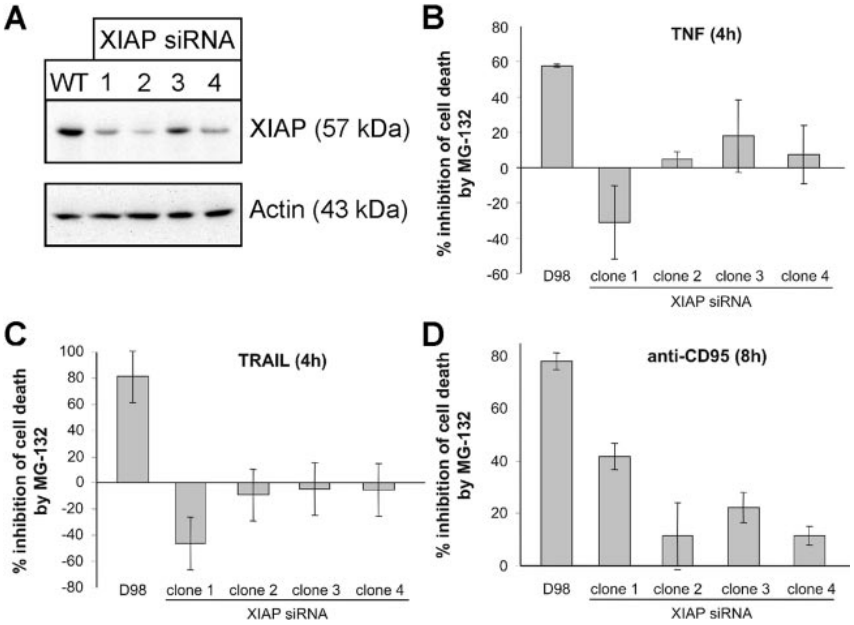

FIG. 6. Inhibition of the proteasome does not protect XIAP knockdown cells from death receptor-induced apoptosis. (A) HeLa D98 cells (wild type [WT]) and four HeLa D98 clones stably transfected with the XIAP siRNA construct were analyzed for XIAP expression. As a control, the blot was reprobed with an actin antibody. (B through D) Following preincubation for 1 h with or without MG-132 (10 μM), HeLa D98 cells and the four XIAP siRNA clones were incubated with 100 ng/ml TNF (B), 30 ng/ml TRAIL (C), or 300 ng/ml of the antagonistic CD95 antibody (D) in the presence of Chx. Cell death was assessed with the crystal violet assay after 4 h (B and C) or 8 h (D). The graphs show percent inhibition of cell death achieved in the presence of MG-132. One representative experiment out of six performed in triplicate is shown.

II (39) and thus explain how MG-132 is able to inhibit caspase-8 activation. MG-132-mediated inhibition of caspase-8 recruitment (due to stabilization and accumulation of c-FLIP-short at the DISC) was also observed with regard to the CD95 DISC or when clasto-lactacystin was used to block TNF-induced cell death (data not shown).

To unambiguously determine the role of c-FLIP in the PI-mediated protection from death receptor-induced apoptosis, we finally generated HeLa D98 transfectants in which c-FLIP expression is suppressed due to the stable expression of a c-FLIP siRNA. As both c-FLIP forms are expressed only very weakly in HeLa D98 cells but accumulate significantly following proteasome inhibition, we analyzed the efficiency of the siRNA-mediated c-FLIP knockdown in the absence and presence of MG-132. As shown in Fig. 9A, the expression of both c-FLIP-long (upper panel) and c-FLIP-short (lower panel) was almost completely suppressed in all three clones examined, whereas a representative D98 clone that was transfected with the empty vector displayed c-FLIP levels comparable to those observed in wild-type D98 cells. All clones, including the vector cells, displayed no obvious alterations in death receptor surface expression (data not shown). Similar to the results obtained with the XIAP siRNA clones (Fig. 6), MG-132 protected only the parental HeLa D98 and vector control cells from death receptor-induced apoptosis but completely failed to do so with regard to the c-FLIP siRNA clones (Fig. 9B through D). In fact, MG-132 treatment even further accelerated and enhanced TNF- and TRAIL-induced apoptosis of the three c-FLIP siRNA clones (Fig. 9B and C). This intriguing finding was substantiated even further when we analyzed TNF-induced caspase processing in these clones. Whereas process-

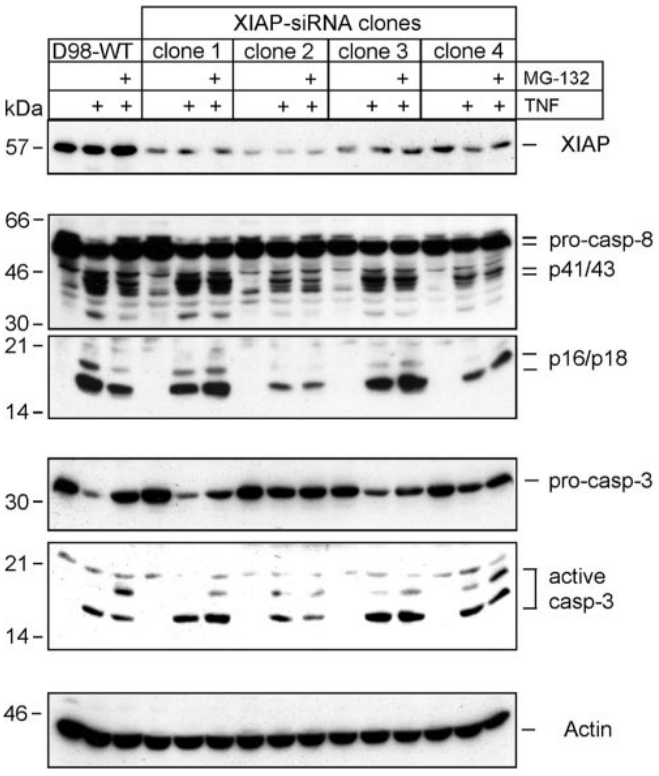

FIG. 7. Proteasome inhibition does not prevent TNF-induced caspase activation in XIAP knockdown cells. Following a 1-h preincubation with or without MG-132 (10 μM), HeLa D98 cells (wild type [WT]), and the four XIAP siRNA clones either were left untreated or were incubated with 10 ng/ml TNF and Chx. After 3 h, cell extracts were prepared and analyzed for the status of XIAP expression and for processing of caspases-8 and -3. The actin blot serves as a loading control. +, with indicated substance.

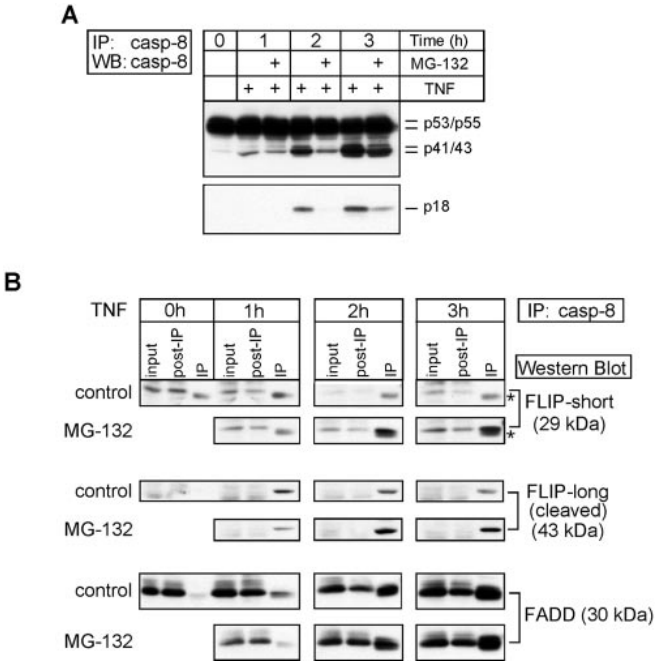

FIG. 8. TNF induces the association of c-FLIP-short with caspase-8 and FADD only in the presence of MG-132. Following a 1-h preincubation with or without MG-132 (10 μM), HeLa D98 cells either were left untreated or were treated with TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and subjected to immunoprecipitation (IP) by using a polyclonal caspase-8 antibody. To verify an efficient immunoprecipitation reaction, Western blot (WB) analysis was performed with a monoclonal caspase-8 antibody (A), whereas the immunoprecipitates were analyzed for the presence of c-FLIP-short, the cleaved p43 fragment of c-FLIP-long, and FADD (B). +, with indicated substance. Asterisks indicate the light chain of the antibody used for precipitation.

ing and thereby activation of caspase-8 and caspase-3 were significantly blocked when HeLa D98 cells were treated with TNF in the presence of MG-132, both caspases were processed much more efficiently and with an accelerated kinetic in similarly treated c-FLIP siRNA clones (Fig. 10). Similar results were obtained when the c-FLIP siRNA clones were treated with TRAIL (data not shown). Interestingly, compared to parental HeLa D98 cells, TNF-induced caspase processing was slightly delayed in the three c-FLIP siRNA clones, which might be explained by a recently proposed hypothesis that c-FLIP-long is required for caspase-8 activation at the DISC (3). Collectively, our data clearly demonstrate that proteasomal inhibitors transiently protect cells from death receptor-induced apoptosis and that they exert this protective effect via stabilization of XIAP and c-FLIP.

DISCUSSION

Two complicated proteolytic systems are involved in the control of cell death: the caspase family of cysteine proteases and the ubiquitin-proteasome degradation system. Whereas the function of caspases is well defined, the exact role of the ubiquitin-proteasome system in apoptosis is far from being elucidated. Several studies showed that the inhibition of the proteasome blocked apoptosis in various settings (18, 20, 46), whereas others demonstrated efficient induction of apoptosis when cells were treated with PIs either alone or in combination with various death-inducing stimuli (8, 11, 19, 30, 60). As the antiapoptotic activities of PIs were described for resting thymocytes and differentiated neuronal cells and the proapoptotic effects were described for various tumor cell lines, it was postulated that the requirement of proteasomal activity for the

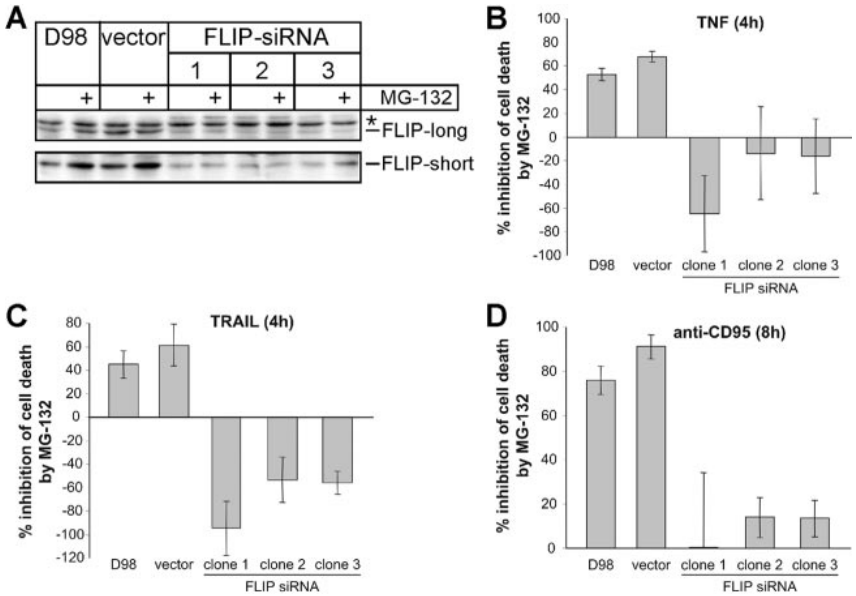

FIG. 9. Inhibition of the proteasome does not protect c-FLIP knockdown cells from death receptor-induced apoptosis. (A) HeLa D98 cells and HeLa D98 clones stably transfected with an empty vector or with the c-FLIP siRNA construct either were left untreated or were incubated for 2 h with 10 μM MG-132 alone and analyzed for c-FLIP-long and c-FLIP-short expression. +, with indicated substance. The upper band marked with an asterisk served as a loading control. (B through D) Following a 1 h preincubation with or without MG-132 (10 μM), HeLa D98 cells and HeLa D98 clones stably transfected with an empty vector or with the c-FLIP siRNA construct were incubated for 4 h with 100 ng/ml TNF (B) or 30 ng/ml TRAIL (C) or for 8 h with 300 ng/ml of the antagonistic CD95 antibody in the presence of Chx (D). Cell death was assessed with the crystal violet assay. The graphs show percent inhibition of cell death achieved in the presence of MG-132. One representative experiment out of four performed in triplicate is shown.

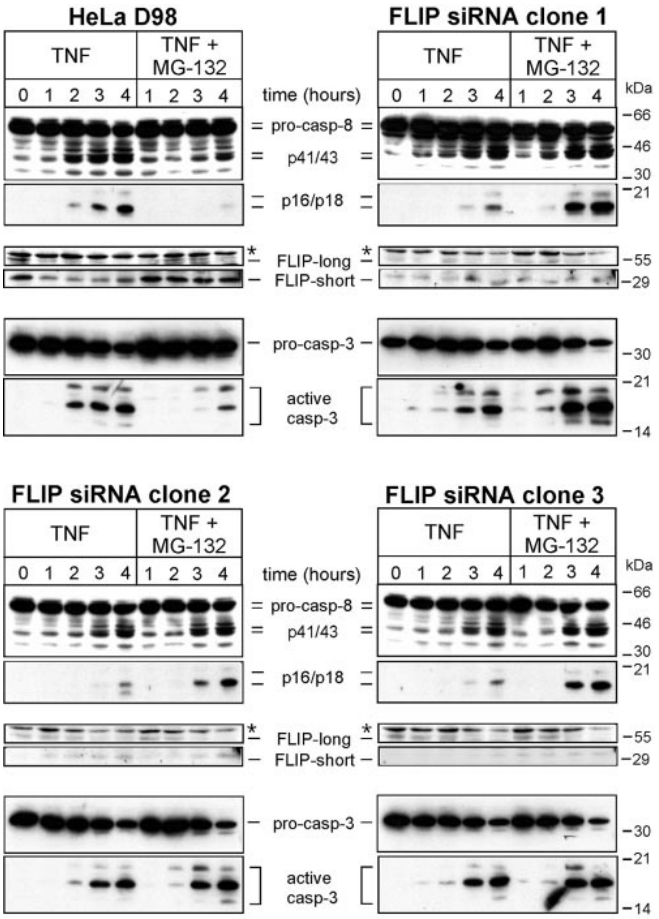

FIG. 10. Proteasome inhibition does not prevent TNF-induced caspase activation in c-FLIP knockdown cells. Following a 1-h preincubation with or without MG-132 (10 μM), HeLa D98 cells and the three c-FLIP siRNA clones either were left untreated or were incubated with 10 ng/ml TNF and Chx. After the indicated times, cell extracts were prepared and analyzed for the presence of c-FLIP and for the processing of caspase-8 and -3. Asterisks indicate an unspecified band.

progression or inhibition of apoptosis critically depends on the proliferative status of the cells (9, 41). In an alternative model, it was suggested that the PI concentrations used determine whether such peptide aldehyde inhibitors function in a pro- or antiapoptotic manner (34). Here, however, we describe an antiapoptotic role of PIs in several proliferating tumor cells that was observed not only at low but also at high PI concentrations. In addition, although at first glance our study appears to be controversial to many other reports, it provides strong evidence that both the pro- and antiapoptotic functions of the proteasome have to be taken into account for efficient PI-based tumor therapy.

Using death receptor-induced apoptosis as a model system, we demonstrated that specifically proteasomal inhibitors, such as clasto-lactacystin, MG-132, or ALLN, but not the calpain and cathepsin inhibitor ALLM efficiently protected cells against TNF-, TRAIL- or CD95-induced apoptosis. This protection correlated well not only with the inhibition of the executioner caspase-3 but also with a marked delay in the processing and activation of caspase-8, the most apical initiator caspase in death receptor signaling. Hence, our results suggest that the PI-mediated protection is caused by inhibition of an early initiation event that most likely involves the stabilization of pre-existing antiapoptotic proteins, such as c-FLIP and XIAP, which are both known to efficiently counteract the initial stages of caspase activation (29, 47). Indeed, both proteins were stabilized by PIs in the two cell lines used. In addition, c-FLIP-short was found to be associated with the DISC only in the presence of PIs, clearly explaining the lack and delay of caspase-8 processing under these conditions. Finally, siRNA-mediated knockdown of either c-FLIP or XIAP expression completely abrogated the protective function of PIs and, in some instances, even reversed this effect, resulting in an increased apoptosis rate. Thus, from these results, it is obvious that PIs exert their antiapoptotic effect via the stabilization of XIAP and c-FLIP. This result is especially important with regard to the fact that many human tumors express high levels of these antiapoptotic proteins, rendering them rather resistant toward apoptosis induction by various agents (21, 37, 38, 47, 55). Based on our results, it is, however, also unlikely that a combined treatment of death receptor ligands (or perhaps drugs; see below) with PIs would result in a more beneficial outcome, as such a treatment might even cause antagonistic effects due to the PI-mediated prolonged survival of these tumors. For an efficient therapy, it is therefore important to thoroughly analyze every individual tumor for the expression levels of XIAP and c-FLIP as well as for the status of other antiapoptotic proteins, such as Bcl-2, that are known to be targets of the proteasome (7, 12, 44, 63). In addition, it is inevitable to also carefully evaluate the biochemical pathways involved, as PIs were recently shown to also inhibit apoptosis induced by retinoic acid, geldanamycin, and staurosporine and even the combined treatment of melanoma cells with TNF and PIs did not show the expected synergism of action (2, 43, 52, 62). The underlying mechanisms causing the resistance to these drugs remained, however, unknown.

Of note, however, is also our observation that although PI treatment significantly delayed caspase activation and cell death of various tumor cell lines, it did not result in a permanent protection from death receptor-induced apoptosis. In fact, when the initial PI-mediated roadblock of caspase processing was eventually overcome, active caspases accumulated to an even larger extent in cells treated with the individual death receptor ligands in the presence rather than in the absence of PIs. Such a scenario was found not only in cells that have been exposed to this treatment for longer terms but also in cells with suppressed c-FLIP or XIAP expressions. These observations clearly demonstrate a biphasic role for the proteasome in death receptor-induced apoptosis in which at first its activity is absolutely essential for the initiation phase by eliminating various roadblocks, including c-FLIP and XIAP. During later stages, however, the proteasome acts in an antiapoptotic manner, as it also promotes the degradation of proapoptotic proteins such as caspases. The proposed biphasic role of the proteasome would also imply that the outcome of a PI-based tumor therapy depends not only on the expression levels of antiapoptotic proteins but also on the time point at which the PIs are applied. With regard to this, we found that the protection was most pronounced when the cells were exposed to MG-132 1 h prior to the death stimulus, whereas the

protection effect was abolished when the inhibitor was added 1 h or 2 h after TNF or TRAIL addition. A similar finding was also reported recently with regard to DNA damage-induced apoptosis (54). In that study, the authors showed that preincubation with MG-132 increased the resistance of human lung carcinoma cells toward etoposide, whereas they observed an increased apoptosis rate when the cells were first exposed to this drug for 30 min, followed by the addition of MG-132.

Besides the expression levels of antiapoptotic proteins and the time point at which the PIs might be applied, our results also provide strong evidence that the level of the intrinsic proteasomal activity represents a crucial factor determining the outcome of a PI-based therapy. In fact, HeLa H21 cells that are less susceptible to death receptor-induced apoptosis displayed a reproducibly lower proteasomal activity than did TNF-sensitive HeLa D98 cells. Together with our observation that TNF did not induce degradation of XIAP as efficiently in HeLa H21 as in HeLa D98 cells, these results are in accordance with our hypothesis that the proteasome is required for an efficient initiation of the death program. In further support of our assumption are also recent findings demonstrating, in vitro and in vivo, that tumors acquired apoptosis resistance by down regulating their proteasomal activity (14, 22). As the proteolytic activity is essential for every proliferating cell, these tumors adapted to the low proteasomal activity by switching to another newly identified proteolytic system, the tripeptidyl peptidase II (15). Due to the hereby altered specificity of cytosolic proteolysis, however, these tumors were unable to efficiently degrade the various IAPs, including XIAP, resulting in rapidly growing tumors in vivo (22).

In summary, our data clearly support a model in which the proteasome plays an important biphasic role in apoptosis. At first, it is essential for the initiation of the cell death program by degrading antiapoptotic proteins but it acts at later stages in an antiapoptotic manner as it also proteolyses proapoptotic proteins. Hence, our data should be taken into serious account when a PI-based tumor treatment is considered in combination with various apoptosis-inducing drugs or death receptor ligands. However, they also encourage attempts to combine such treatments with small molecule inhibitors targeting expression and/or activity of antiapoptotic proteins (13).

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Friend or foe? The proteasome in combined cancer therapy

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Perspective

Friend or Foe?

The Proteasome In Combined Cancer Therapy

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KEY WORDS

proteasome inhibitor, apoptosis, biphasic model, death receptor, caspases, c-FLIP, XIAP, bortezomib, MG-132

ABBREVIATIONS

c-FLIP	cellular FLICE-like inhibitory protein
HSPs	heat shock proteins
IκB	Inhibitor of κB
JNK	c-Jun N-terminal kinase
NF-κb	nuclear factor kappa B
PI	proteasome inhibitor
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
XIAP	X-linked inhibitor of apoptosis protein

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ABSTRACT

The proteasome is a multicatalytic enzyme complex that is responsible for degradation of the vast majority of intracellular proteins. Thus, it is involved in diverse cellular processes such as proliferation, differentiation and apoptosis. Especially its latter function yielded in the development of specific proteasomal inhibitors which have recently entered clinical trials due to their tremendous apoptosis-inducing capability. However, several recent studies including ours provided substantial evidence that a combined treatment of tumors with apoptosis-inducing agents and proteasomal inhibitors might even cause adverse effects leading to a prolonged survival of tumor cells. Based on our model of a biphasic role for the proteasome in apoptosis, we believe that a successful combat of tumors that relies on a combinational therapy with proteasomal inhibitors requires careful evaluation of several critical aspects in order to avoid a friend becoming a foe.

THE PROTEASOME: AN EMERGING TARGET IN CANCER THERAPY

Chemo- and radiotherapy are important treatment modalities for many cancers, but the frequent occurrence of drug- and radiation-resistant tumors constitutes a major clinical problem. Reasonable hopes for a successful cancer therapy circumventing such difficulties were raised in the early eighties with the discovery of the apoptosis-inducing death receptor ligands such as tumor necrosis factor (TNF) or CD95L (Fas/APO1) and later, in the mid nineties, with the identification of the TNF-related apoptosis-inducing ligand (TRAIL).[1] It became, however, soon evident that at least the former two cytokines caused severe systemic side effects and hence, they were mostly dismissed from clinical trials. On the other hand, TRAIL has attracted immense attention due to its ability to preferentially kill tumor cells, while leaving most normal cells unharmed in vitro and in vivo. But even though, many clinical trials are hampered by the fact that more than 50% of all human cancers are resistant to the cytotoxic activity of TRAIL, demonstrating the necessity for alternative treatment modalities. Promising data were obtained from studies showing that the cytotoxic potential of TRAIL was synergistically enhanced by a combined treatment with chemotherapeutic drugs or radiation and even the simultaneous treatment with proteasomal inhibitors (PIs) yielded encouraging results.[2,3]

The proteasome is a multi-catalytic enzyme complex that is present in the nucleus and cytoplasm of all eukaryotic cells. As one of the main players in the rapid degradation of a vast majority of proteins, the proteasome is involved in diverse cellular processes such as cell cycle progression, differentiation and apoptosis.[4,5] Hence, it was no surprise to learn that improper regulation of its expression or its proteolytic activity plays an important role in tumorigenesis. Chronic lymphocytic leukemia (CLL) cells for instance were shown to exhibit a threefold higher proteasomal activity when compared to normal lymphocytes. Also various other neoplastic cells, but not their well differentiated and normally growing counterparts showed an abnormally high expression of proteasomal components including enzymes involved in ubiquitination. In addition, expression of various proteasomal targets such as the tumor suppressor p53, the nuclear factor-kappa B (NF-κB) inhibitor IκB, the cyclin-dependent kinase inhibitor p27 or the pro-apoptotic Bcl-2 family members Bax and Bid was found to be substantially decreased in many tumors correlating with apoptosis resistance and subsequently a poor patient prognosis.[6]

As these findings implied a pivotal role of the proteasome in maintaining survival and proliferation of rapidly growing transformed cells, it was only a logical consequence to study the application of proteasomal inhibitors in search for novel cancer therapies. Indeed, it turned out that many different PIs such as bortezomib (Velcade™, formerly

PS-341), lactacystin or MG-132 were able to specifically induce apoptosis in a variety of tumor cells in vitro and in vivo, whereas normal cells were left unharmed by this treatment.[7-9] Even tumor cells that were shown to be resistant to diverse apoptotic stimuli including chemotherapeutic drugs or radiation could be sensitized to these agents by PIs. Although the mechanisms by which PIs exert their pro-apoptotic function is far from being elucidated, the finding that also apoptosis induction by death receptor ligands was dramatically accelerated in the presence of PIs suggested that these compounds modulate stability of proteins that are common to both, the intrinsic and extrinsic death pathway. Particularly with regard to the selective cytotoxic activity of TRAIL toward tumor cells,[10] a combined treatment with proteasomal inhibitors that apparently display a similar tumor selectivity appears to be a promising approach for an effective cancer therapy. Based on their tremendous anti-tumorigenic potential, PIs such as bortezomib that represents one of the most specific proteasome inhibitors presently known have recently entered clinical practice as a treatment for multiple myeloma and are also undergoing clinical trials for other types of cancer.[6,11,12]

THE PROTEASOME: A DOUBLE-EDGED SWORD FOR TUMOR CELL SURVIVAL?

However, there are always two sides to a coin as various tumor cell lines do not respond to the cytotoxic activity of these compounds and even were shown to be protected from apoptosis induction by PIs under certain circumstances.[13] Hence, the question arose of whether the proteasome behaves as a friend or foe when cells face an apoptotic stimulus. With regard to the latter scenario, we have published very recently that a PI treatment protects several tumor cell lines from death receptor-induced apoptosis.[14] Although our results were to some extent for reasons listed below not completely unforeseeable, they were somehow rather surprising. So far the pro-apoptotic effect of PIs was observed in the majority of cases in neoplastic cells, whereas their anti-apoptotic potential was mainly recognized in resting thymocytes and in differentiated neuronal cells. Thus, the proliferative status of the cell was postulated to be critical for the decision of whether PIs accelerate or inhibit the apoptotic cell death program. Also the PI concentrations applied were suggested to play a role in this process. Our finding that PIs also protect several proliferating tumor cells from apoptosis in a manner independent of the concentrations used shed thereby a new light on the use of these compounds in cancer therapy. More clearly, several circumstances including the cellular context of the tumor have to be taken into serious consideration for a successful combat of cancer.

What are the mechanisms by which PIs exert their pro-apoptotic effects? As the proteasome influences multiple cellular pathways, it is inevitable that also the apoptosis sensitizing activity of PIs is due to diverse events. Early reports claimed the tumor suppressor p53 as being crucially involved in these processes, as expression of a dominant-negative p53 attenuated PI-mediated apoptosis in Rat-1 fibroblasts.[15] However, due to diverse mechanisms, the p53 pathway is known to be inactivated in more than 50% of all human tumors. In addition, several studies provided strong evidence that PIs induce apoptosis regardless of the p53 status of the cell.[16] Thus, these results cast a general doubt on the participation of the p53 pathway in PI-mediated apoptosis.

More likely mechanisms involved in this process include for instance stabilization of pre-existing pro-apoptotic proteins such as the Bcl-2 family members Bax, Bid, Bik and Bim as well as Smac whose participation is essential for the activation of the mitochondrial death pathway.[17-19] In addition, it was shown that also death receptors such as the TRAIL-R2 and CD95 and even active caspases are critical proteasomal targets. As caspases are considered to be the final executioners in several death pathways that lead to the demise of the cell via limited proteolysis of a multitude of cellular substrates,[20] their stabilization explains, at least partially, the pro-apoptotic function of PIs. However, in this context it has to be considered that also several anti-apoptotic proteins such as the cellular FLICE-like inhibitory protein (c-FLIP), and Bcl-2 as well as the inhibitor of apoptosis proteins (IAPs) including X-linked IAP (XIAP) represent prominent targets of the proteasome.[21] Whereas c-FLIP prevents activation of caspase-8, the most apical caspase in death receptor signaling, XIAP was shown to potently inhibit caspases 9, 7 and 3 downstream of the mitochondria. Together with Bcl-2 that prevents loss of the mitochondrial membrane potential, an event closely associated with the onset of the intrinsic mitochondrial death pathway, stabilization of these proteins by PIs surely leads to the establishment of an efficient roadblock for apoptosis. Naturally, such a road block has to be first disposed of before the apoptotic cascade can be launched.

Surprisingly, such considerations were not thoroughly investigated before. In fact, rather unexpected results were obtained in a recent study demonstrating a substantial decrease of c-FLIP protein expression following treatment of tumor cells with PIs.[22] In contrast, the protection against death receptor-induced apoptosis we observed in our study was clearly dependent on the PI-mediated stabilization of c-FLIP and XIAP. Especially the prolonged expression of c-FLIP and its recruitment into the death-inducing signaling complex (DISC) only in PI-treated cells correlated well with inhibition of TNF-, CD95- and TRAIL-induced caspase-8 processing and activation. Also inhibition of the executioner caspase-3 that was observed in the presence of PIs coincided with stabilization of c-FLIP and XIAP, as caspase-3 is not only activated directly by caspase-8, but also indirectly via the caspase-8-mediated cleavage of Bid that then leads to the induction of the mitochondrial death pathway. In support for the theory that stabilization of these two (and most likely also other) anti-apoptotic proteins is responsible for the observed PI-mediated inhibition from death receptor-induced apoptosis were our findings that this protection was almost completely abrogated in cells stably expressing c-FLIP- or XIAP-siRNA. In contrast, in these cells, PI treatment even accelerated the cytotoxic potential of death receptor ligands by stabilizing active caspases.

A BIPHASIC MODE OF PROTEASOME ACTION APPEALS FOR ATTENTION

Based on above-mentioned results, we postulated a biphasic role for the proteasome in apoptosis (Fig. 1A). According to this model, the proteasome is initially required for a rapid initiation of the death program by degrading anti-apoptotic proteins such as c-FLIP and XIAP, whereas at later stages it counteracts an orderly course of the death pathway due to the proteolytic degradation of pro-apoptotic proteins including caspases. Especially the anti-apoptotic role of the proteasome was recognized in early studies leading to many PI-based anti-tumor trials. Also the observation that several proteasomal subunits are cleaved and thereby inactivated by caspases is a finding consistent with our model as this ensures a proper progression of the apoptotic program.[23,24] Curiously, however, the pro-apoptotic role of the proteasome that leads to the elimination of an early road block to apoptosis was not appropriately appreciated previously.

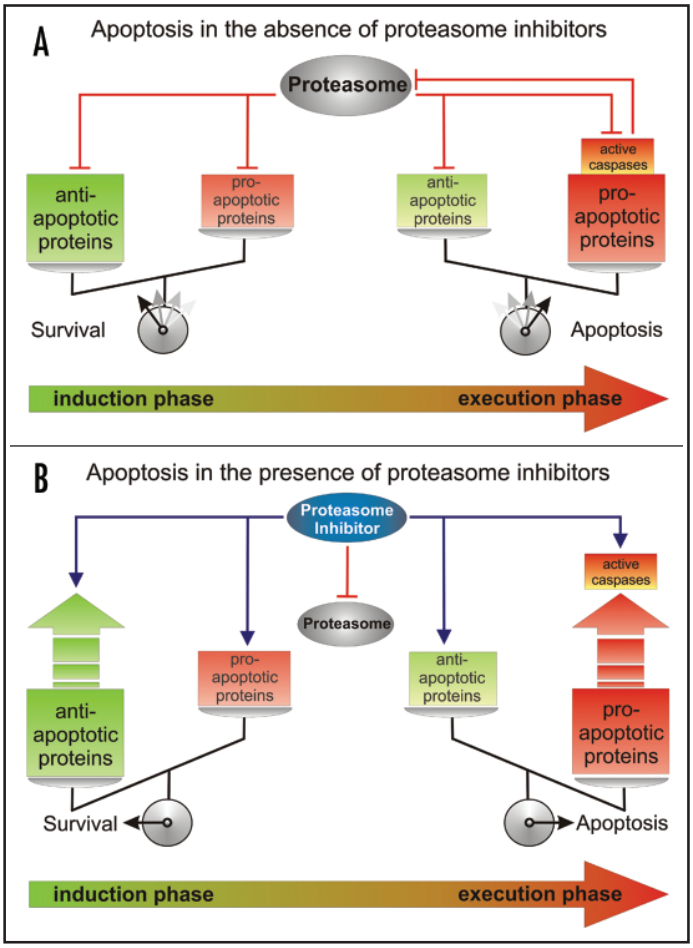

Figure 1. Model of the biphasic role of the proteasome in apoptosis and the influence of proteasome inhibitors. (A) Before the initiation of apoptosis, the proteasome maintains a critical balance between a small and a big pool of pro- and anti-apoptotic proteins, respectively, as indicated by the black arrow on the left pointing toward survival. During the induction phase of apoptosis, however, this balance slowly shifts towards death due to an increase of the pro-apoptotic pool (e.g., active caspases), that critically depends on the proteasome-mediated degradation of anti-apoptotic proteins. This pro-apoptotic role of the proteasome is illustrated by shaded arrows turning to the right, representing the progression of cell death. During the execution phase of apoptosis, the cell has generated a larger pool of pro-apoptotic proteins due to a decreased anti-apoptotic pool (black arrow on the right pointing toward apoptosis). However, both pools are targets for proteasomal degradation, a mechanism that explains the anti-apoptotic role of the proteasome (illustrated by the shaded arrows turning to the left i.e., survival), which can be counteracted by active caspases that cleave and thereby inactivate proteasomal subunits. (B) Inhibition of the proteasome before the apoptotic induction phase results in the generation of an increasing pool of anti-apoptotic proteins as new biosynthesis constantly replenishes this pool that, in addition, is not diminished by proteolysis. Hence, under these conditions that prevent generation of pro-apoptotic proteins the proteasome can not fulfill its pro-apoptotic role leading to the survival of the cell. Inhibition of the proteasome after the apoptotic induction phase, however, clearly results in an increased pro-apoptotic pool as the required proteasomal degradation of the anti-apoptotic pool was achieved afore. Of note, as proteasomal inhibitors dramatically alter levels of both, the pro- and anti-apoptotic pools, survival and apoptosis are also more severely affected in their presence. Thus, in contrast to (A), the black arrows in (B) point directly to either survival or apoptosis.

Such thinking of course entails serious implications that have to be carefully evaluated for a successful cancer therapy. For example, as anti-apoptotic proteins have to be stashed away by the proteasome in order to efficiently activate the apoptotic program, the initial levels of such proteins seem to be very crucial. Hence, it is conceivable that cells expressing low levels of c-FLIP, XIAP or Bcl-2 might show an increased responsiveness toward the sensitizing or cytotoxic activity of PIs than cells overexpressing these proteins. Unfortunately, many human tumors express for varying reasons high levels of such anti-apoptotic proteins,[25,26] implying that a PI treatment would only lead to an even further enhanced resistance (Fig. 1B). Interestingly, and consistent with our hypothesis, PIs were recently shown to inhibit apoptosis in several different models and even the combined treatment of melanoma cells with TNF did not show the expected synergism of action.[27] Although the underlying mechanisms causing the resistance to these agents were not investigated, according to our model, it is tempting to speculate that the stabilization of anti-apoptotic proteins plays a major role in this context.

With regard to this, it is noteworthy that also IκB represents a prominent molecule that is targeted by the proteasome for destruction leading to the activation of NF-κB.[6] NF-κB is a transcription factor that plays an important role in tumorigenesis via transactivation of genes involved in cell proliferation, protection from apoptosis and even in tumor cell invasiveness and angiogenesis. Through association with its inhibitor, IκB, NF-κB is normally sequestered in an inactive state in the cytoplasm and becomes activated upon proteasomal degradation of IκB. This event allows NF-κB to translocate into the nucleus where it mediates transcription e.g., of anti-apoptotic genes including c-FLIP and XIAP. Even though many diverse mechanisms can account for the occurrence of chemo- and radio-resistant tumors, degradation of IκB and subsequent activation of NF-κB surely constitutes a powerful tumorigenic and anti-apoptotic mechanism that is frequently utilized by a variety of tumor cells through upregulation of their intrinsic proteasomal activity. While anti-apoptotic proteins are initially overexpressed under such circumstances, the PI-mediated inhibition of NF-κB most likely prevents new synthesis of these proteins eventually leading to their downregulation and subsequently to an increased apoptosis susceptibility of these tumor cells.[28] Together with the creation of an increasing pool of pro-apoptotic proteins such as active caspases, NF-κB can also be activated by chemotherapeutic drugs or radiation, partially explaining the sensitizing effect of PIs when applied in combination with these agents. However, as many of these anti-apoptotic proteins including c-FLIP and XIAP are also themselves prominent targets of the proteasome, an untimely PI treatment, as discussed below, might also lead in such cases to adverse effects. To make matters even worse, NF-κB can also be activated independently of the proteasome,[29] a finding that, according to our model, suggests a less favorable outcome of a PI-based tumor therapy. This is because in such a scenario PIs stabilize not only the preexisting pool of anti-apoptotic proteins, but in addition contribute also to a prolonged half-life of a new reservoir of these proteins that are continuously generated under the control of NF-κB. Therefore, the expression levels of the various anti-apoptotic proteins as well as the NF-κB status have to be carefully evaluated in order to achieve the desired results in a PI-based tumor therapy.

Several tumors, however, are resistant to the cytotoxic activity of PIs even though they do not rely on NF-κB in order to maintain their tumorigenic potential. In those tumors, other mechanisms must also contribute to the unresponsiveness or even resistance

toward a PI-based cancer treatment. With regard to such a mechanism, we have found in our study that the PI-mediated protection was most pronounced when the cells were exposed to these compounds 1 h prior to the death stimulus. Addition of PIs 1 h or 2 h following treatment with the death receptor ligands failed to protect the cells from apoptosis. A similar finding was also reported recently with regard to DNA damage-induced apoptosis.[30] In this study, the authors showed that preincubation of human lung carcinoma cells with MG-132 resulted in their resistance toward the topoisomerase II inhibitor etoposide, whereas an even increased apoptosis rate was observed when the cells were exposed first to this drug followed by the addition of MG-132. Also treatment of myeloma cells with the anthracycline doxorubicin followed by bortezomib caused a stronger synergistic induction of cell death compared to treatment using the reverse sequence.[31] Together, these and our data suggest that the outcome of a PI-based tumor therapy depends not only critically on the expression levels of anti-apoptotic proteins, but also on the sequence and on the time point at which the PIs are applied (Fig. 1B). Hence, PIs can only be effective in a synergistic manner in cases in which the apoptotic machinery has already been switched on. If they were to be applied before the addition of the death stimulus, they would only create an increasing pool of anti-apoptotic proteins causing the prolonged survival of a tumor. Such a conclusion is highly feasible as the proteasome only maintains the preexisting balance of pro- and anti-apoptotic proteins within a cell, but does not, as a matter of course, preferentially degrade only players on one side of the coin. Thus, it is not the proteasome that is accountable for the critical decision whether a cell lives or dies, but rather other factors such as for example pro-apoptotic conditions that tip the balance towards one side.

Another impressive example for the biphasic role of the proteasome in apoptosis that critically depends on the maintenance of a delicate balance between pro- and anti-apoptotic activities is represented by the family of the heat shock proteins (HSPs) and by the c-Jun N-terminal kinase (JNK) pathway.[6,32] Members of the HSP family are markedly upregulated upon PI treatment, and, based on experiments in which the introduction of HSP antisense oligonucleotides potentiated the pro-apoptotic activity of MG-132, were postulated to mediate resistance to apoptosis. The pro-apoptotic JNK pathway, on the other hand, was shown to contribute to PI-mediated apoptosis, as it was substantially activated by this treatment and, furthermore, JNK inhibition efficiently abrogated cell death induced by PIs. Interestingly, JNK activation can be blocked by HSP-72 and preincubation of cells with PIs resulted in accumulation of HSP-72 and resistance toward JNK-mediated apoptosis induced by heat shock and ethanol treatment.[33] Thus, consistent with our model, the balance between these opposite actions determines the fate of the cell. In this context, it is noteworthy that also death receptor ligands are potent activators of the JNK pathway which is implicated to be involved in TNF-induced apoptosis. In agreement with their protective role in this death pathway, treatment of the cells with PIs strongly suppressed the TNF-induced phosphorylation of JNK (Jänicke RU, unpublished results) implying that besides the stabilization of c-FLIP and XIAP also this mechanism might contribute to the observed resistance. Whether HSPs are involved in this process is currently unknown, but under thorough investigation.

Finally, based on the outline given above, our study and many others clearly point to the fact that, besides the factors mentioned so far, also the level of the intrinsic proteasomal activity represents a crucial factor that influences the outcome of a PI-based tumor therapy. As mentioned, several tumors display an increased expression and activity of proteasomal components when compared to their normally growing counterparts. Such an elevated proteasomal activity not only implies continuous degradation of several anti-proliferative proteins, but also an uninterrupted new synthesis of anti-apoptotic proteins via activation of NF-κB. Remarkably, although such conditions are clearly beneficial for the outgrowth of a tumor, a lowered proteasomal activity as it was also observed in several tumors[34,35] does not necessarily lead to an increased apoptosis susceptibility of these cells. In fact, also such a scenario is consistent with our model as anti-apoptotic proteins are not continuously degraded resulting in an increased resistance toward apoptosis induction. As the proteolytic activity is essential for every proliferating cell, it was shown that these tumors adapted to their lowered proteasomal activity by switching to another newly identified proteolytic system, the tripeptidyl peptidase II.[36] Due to the hereby altered specificity of cytosolic proteolysis, however, anti-apoptotic proteins such as XIAP were not as efficiently degraded, resulting in rapidly growing tumors in vivo. In agreement with these studies, we could show that apoptosis-resistant cells displayed a lower proteasomal activity than their more susceptible counterparts correlating with a hampered XIAP and c-FLIP degradation. As death receptor ligands were able to induce an upregulation of the proteasomal activity only in apoptosis-sensitive cells, our observations clearly point to the fact that the proteasome is required for rapid initiation of the death program.

CONCLUSIONS

In summary, current data support a model in which the proteasome plays an important biphasic role in apoptosis. At first, it is essential for the initiation of the cell death program by degrading anti-apoptotic proteins, but acts at later stages in an anti-apoptotic manner, as it also proteolyses pro-apoptotic proteins (Fig. 1A). These findings imply that at these later stages inhibition of the proteasome by either PIs or active caspases will surely lead to an acceleration of the apoptotic process, whereas an untimely early PI application might even cause adverse effects leading to a growth advantage for the tumor (Fig. 1B). Thus, our model is consistent with many studies demonstrating the tremendous apoptotic potential of these compounds. At the same time, however, they also sound a serious note of caution as several aspects have to be taken into account when a PI-based tumor treatment is considered in combination with various apoptosis-inducing drugs or death receptor ligands. These include not only thorough elucidation of the status of anti-apoptotic proteins and the intrinsic proteasomal activity, but also, and most importantly, a proper timing of a PI application. On the other hand, our study especially encourages attempts of a combined PI treatment with small molecule inhibitors that target expression and/or activity of anti-apoptotic proteins.[37]

In a recent review it was stated that there is yet no clear answer to the question of whether the proteasome behaves as a killer or a savior when the cell faces an apoptotic stimulus.[38] We believe that we have now provided well-founded evidence that the proteasome represents both, friend and foe and that its friendly face is a valuable component for any combined cancer therapy that, however, has to be disposed when the time is right.

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Caspase-8 Can Be Activated by Interchain Proteolysis without Receptor-triggered Dimerization during Drug-induced Apoptosis*

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Proteases of the caspase family are thought to be activated by proteolytic processing of their inactive zymogens. However, although proteolytic cleavage is sufficient for executioner caspases, a different mechanism has been recently proposed for initiator caspases, such as caspase-8, which are believed to be activated by proximity-induced dimerization. According to this model, dimerization rather than proteolytic processing is considered as the critical event for caspase-8 activation. Such a mechanism would suggest that in the absence of a dimerization platform such as the death-inducing signaling complex, caspase-8 proteolytic cleavage would result in an inactive enzyme. As several studies have described caspase-8 cleavage during mitochondrial apoptosis, we now investigated whether caspase-8 becomes indeed catalytically active in this pathway. Using an *in vivo* affinity labeling approach, we demonstrate that caspase-8 is activated in etoposide-treated cells *in vivo* in the absence of the receptor-induced death-inducing signaling complex formation. Furthermore, we show that both caspase-3 and -6 are required for the efficient activation of caspase-8. Our data therefore indicate that interchain cleavage of caspase-8 in the mitochondrial pathway is sufficient to produce an active enzyme even in the absence of receptor-driven procaspase-8 dimerization.

Caspases, a family of aspartate-specific cysteine proteases, play an essential role in the signal transduction and effector processes of apoptosis (1–5). In healthy cells, caspases exist as inactive proforms (zymogens) consisting of a prodomain followed by two catalytically active subunits that are separated by a short linker domain. Upon activation, the prodomain is removed, and the linker domain is cleaved off resulting in the generation of the mature caspase, an active heterotetramer formed out of two large p20 and two small p10 subunits (6–7). Based on their order in cell death pathways, caspases can be divided into initiator and downstream effector caspases. Effector caspases, such as caspase-3, -6, and -7, generally contain only a small prodomain and cleave diverse cellular substrates (8). The initiator caspases such as caspase-8, -9, and -10, in contrast, contain a long prodomain that is used to recruit the enzymes to high molecular weight activation platforms, including the apoptosome in the mitochondrial pathway or the death-inducing signaling complex (DISC)[1] in the death receptor pathway (9–12).

Caspase-8 is a key mediator of apoptotic signals triggered by death receptors such as CD95, TNF-R1, and TRAIL-R1/TRAIL-R2. In the case of the TRAIL receptors and CD95, caspase-8 is directly recruited into the DISC by the adapter protein FADD (11, 12). Signaling through TNF-R1, in contrast, appears to be more complex and to proceed via two sequential complexes (13, 14). It was suggested that following TNF stimulation a first complex is formed at the cell membrane, which contains TNF-R1, the adapter protein TRADD, and the NF-κB-activating signaling components RIP and TRAF2. Then, complex I leaves the receptor, and a second complex is formed in the cytosol, which recruits FADD and caspase-8 and induces cell death.

In contrast to the executioner caspases that are found in the cytosol as inactive dimers, the initiator caspases are present as inactive monomers. Although executioner caspases are activated by direct proteolytic cleavage, initiator caspases are thought to require more complex activation events. Initially, receptor-driven oligomerization of caspase-8 was thought to activate this enzyme through facilitating the autoproteolysis of procaspase-8 molecules (15). Recent studies, however, have proposed that dimerization rather than interchain proteolysis is the critical event for activation of initiator caspases. It was suggested that dimerization is sufficient and a prerequisite for the activation of caspase-8 and -9, whereas proteolytic processing merely stabilizes the active caspase dimers (16, 17). In agreement with this hypothesis, it was shown that chemically induced dimerization of a non-cleaveable caspase-8 mutant yielded in an active procaspase-8 dimer that exhibited the same cleavage activity as the wild-type mature heterotetramer *in vitro* (17–19). However, although the current experimental observations would support this model, most of the evidence was gathered in artificial systems that might not reflect the correct *in vivo* situation (20).

Various studies have proposed that caspase-8 is not only activated in the death receptor pathway, but can be also activated independently of death receptors, for instance during genotoxic stress-induced apoptosis (21–31) or by direct cleavage through other proteases including the CTL protease granzyme B and human immunodeficiency virus type 1 protease (32, 33). During stress-induced apoptosis triggered by anticancer drugs or ionizing irradiation, it was proposed that caspase-8 activation is mediated in a postmitochondrial event by the prior cleavage through caspase-6 (29). In all these studies, the proteolysis of caspase-8 has been considered sufficient evidence for the activation of caspase-8 that was proposed to

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[1] The abbreviations used are: DISC, death-inducing signaling complex; TNF, tumor necrosis factor; fmk, fluoromethyl ketone; AMC, aminomethylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

trigger a feedback amplification loop through the cleavage of Bid (23, 25, 28). Hence, in such a death receptor-independent activation pathway, caspase-8 was proposed to act as an executioner caspase. These studies, however, could not distinguish whether caspase-8 processing resulted in the gain of enzymatic activity or merely reflected a substrate cleavage event. Moreover, activation through interchain cleavage would be incompatible with the recent proximity-induced dimerization model, which should result in a processed but catalytically inactive caspase-8 monomer.

To address the question whether caspase-8 becomes indeed catalytically active in the mitochondrial death pathway, we now employed an *in vivo* affinity labeling approach using the biotinylated caspase-inhibitor biotin-VAD-fmk that only detects active caspases. We demonstrated that caspase-8 is indeed activated independently of death receptors and that both caspase-6 and -3 are required for the generation of the mature caspase-8 enzyme.

MATERIALS AND METHODS

Cell Lines and Reagents—All cell lines including MCF-7 breast carcinoma cells, the leukemic T cell lines Jurkat and H9, and human B lymphoblastoid SKW6.4 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml (all from PAA Laboratories, Linz, Austria). The CD95-resistant Jurkat subline, Jurkat-R, was generated by continuous culture in the presence of anti-CD95 mAb (1 μg/ml, BioCheck, Münster, Germany) for 6 months (22). FADD- and caspase-8-deficient Jurkat cells as well as Jurkat cells overexpressing Bcl-2 were kindly provided by J. Blenis (Harvard Medical School, Boston, MA) and H. Walczak (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively. Biotin-VAD-fmk (biotin-Val-Ala-Asp-(OMe)-fluoromethyl ketone) and biotin-DEVD-CHO (biotin-Asp-Glu-Val-Asp-aldehyde) were purchased from ICN (Eschwege, Germany) and Calbiochem. The caspase-3 substrate DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) and the caspase-8 substrate IETD-AMC (*N*-acetyl-Ile-Glu-Thr-Asp-aminomethylcoumarin) were from Biomol (Hamburg, Germany). Etoposide was obtained from Sigma. The following antibodies were used for Western blotting or immunoprecipitation: goat antibodies recognizing caspase-3 or Bid (R&D Systems, Wiesbaden, Germany), mouse anti-caspase-8 (clone 12F5, BioCheck), mouse anti-caspase-8 (clone C15, Alexis, Lausen, Switzerland), and goat anti-caspase-8 (Santa Cruz Biotechnology). For immunoblot analysis, horseradish peroxidase-coupled secondary antibodies against mouse or goat IgG were purchased from Bio-Rad and Calbiochem.

Measurement of Apoptosis—For the determination of apoptosis, 5×10^4 cells/well were seeded in microtiter plates and treated for the indicated time with anti-CD95 mAb or etoposide. The leakage of fragmented DNA from apoptotic nuclei was measured by the method of Nicoletti *et al.* (34). 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 analyzed by flow cytometry. Nuclei to the left of the 2N peak containing hypodiploid DNA were considered apoptotic. Flow cytometric analyses were performed on a FACSCalibur (BD Biosciences) using CellQuest analysis software.

In Vivo Labeling of Active Caspases—To label the active site of caspases, 2×10^7 cells were incubated following apoptosis induction for an additional hour with 10 μM biotin-VAD-fmk. Cells were harvested by centrifugation and extracted in 500 μl of lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol) containing the protease inhibitors aprotinin, leupeptin, pepstatin (each 2 μg/ml), and 1 mM phenylmethylsulfonyl fluoride. The biotinylated proteins were captured on 30 μl of streptavidin-conjugated agarose beads (Calbiochem). After overnight rotation at 4 °C the agarose beads were extensively washed in lysis buffer containing 0.5% Nonidet P-40. The biotinylated proteins were eluted from the beads by the addition of 60 μl of SDS-sample buffer and incubation at 95 °C for 10 min. 25 μg of the cell extracts or 25 μl of the eluted biotinylated proteins were used for SDS-PAGE and subsequent Western blot analysis.

Immunoprecipitation and Fluorometric Cleavage Assay—After apoptosis induction, cell extracts were prepared from 1×10^7 cells in 500 μl of lysis buffer without dithiothreitol. For the precipitation, 2 μg of antibody (anti-caspase-8, anti-caspase-3) and 30 μl of protein G-Sepharose (Sigma) were added to the extracts and rotated for 4 h or overnight at 4 °C. The Sepharose beads were extensively washed in lysis buffer and then used for fluorometric caspase assays or analyzed by SDS-PAGE and Western blotting. For the fluorometric cleavage assay, 50 μg of the cell extracts or the beads were suspended in 200 μl of substrate buffer (50 mM HEPES, pH 7.3, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol) supplemented with 50 μM IETD- or DEVD-AMC. The reaction was incubated at 37 °C for 3 h and measured in a Lambda Fluro 320 Plus fluorometer (Biotek, Bad Friedrichshall, Germany).

In Vitro Cleavage and Affinity Labeling of Caspase-8—Recombinant His-tagged caspase-3 was generated as described (35). His-tagged caspase-6 was produced by the transformation of *Escherichia coli* BL21 gold with the pET23[casp6] plasmid (35) and induction of protein expression with isopropyl 1-thio-β-D-galactopyranoside. One unit of caspase-6 was defined as the cleavage of 1 μmol of IETD-AMC/min at 37 °C. Cytosolic extracts from 1×10^7 wild-type or FADD-deficient Jurkat cells, MCF-7 or MCF-7/casp-3 cells, were prepared in 200 μl of lysis buffer and diluted with 600 μl of substrate buffer containing 10 mM dithiothreitol. The cleavage reaction was incubated at 37 °C after addition of the recombinant caspases at the indicated amounts. 25-μg aliquots of the reaction mixture were then taken in 30-min intervals and analyzed by SDS-PAGE and subsequent Western blot analysis. For affinity labeling of caspase-8 following *in vitro* caspase cleavage, the non-methylated agent biotin-DEVD-CHO was used. Caspase-8 from Jurkat cell lysates was cleaved with caspase-3 and -6 for 2 h as described above. After the removal of the His-tagged recombinant caspases with nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany), the supernatant was incubated for 1 h with biotin-DEVD-CHO. Biotinylated proteins were captured with streptavidin-conjugated agarose beads and used for immunoblotting with anti-caspase-8 antibodies.

RESULTS AND DISCUSSION

Treatment of Jurkat Cells with Anti-CD95 or Etoposide Results in Caspase-8 Processing—To examine whether the caspase-8 processing that was observed following anti-cancer drug-induced apoptosis also results in the generation of catalytically active caspase-8 molecules, we employed the Jurkat T-cell line that is a commonly used and established cellular model system for the elucidation of apoptotic pathways. First, we determined the time-dependent induction of apoptosis using the anti-cancer drug etoposide and as a control the agonistic CD95 antibody. As expected, both agents efficiently induced apoptosis of Jurkat wild-type cells, whereas only etoposide but not anti-CD95 was able to trigger apoptosis in Jurkat-R cells, a subclone that had lost CD95 expression, or in FADD-deficient Jurkat cells (Fig. 1*A*). Etoposide-induced cell death proceeded with similar kinetics in all Jurkat subclones confirming previous reports (22, 23) and indicating that etoposide-induced cell death proceeds independently of death receptors. Apoptosis induction in Jurkat wild-type cells (Fig. 1*B*) as well as in the CD95-resistant counterparts (see Fig. 3) was accompanied by the processing of caspase-8 and -3. Interestingly, whereas triggering of the death receptor pathway by the agonistic CD95 antibody resulted in the appearance of both a p16 and a p18 caspase-8 fragment, etoposide treatment produced only the p16 subunit (Fig. 1*B*). Although it was shown that caspase-8 processing in Jurkat cells that are classified as so-called type II cells results first in the generation of the p16 fragment before the p18 subunit appears (36), the exact reason for the lack of the p18 caspase-8 fragment in etoposide-treated cells is presently unknown. It is, however, tempting to speculate that the appearance of the p16 fragment is closely associated with activation of the mitochondrial pathway, whereas the generation of the p18 fragment requires DISC formation.

Immunoprecipitation of Caspase-8 and -3—Having established the conditions for anti-CD95- and etoposide-induced apoptosis and caspase processing, we wanted to assess whether proteolytic processing of caspase-8 in etoposide-treated cells produced the catalytically active enzyme. Therefore, we first analyzed the cleavage of the preferred caspase-8 substrate, IETD-AMC, in extracts of anti-CD95-treated Jurkat cells. As expected, those extracts displayed a significant increase in

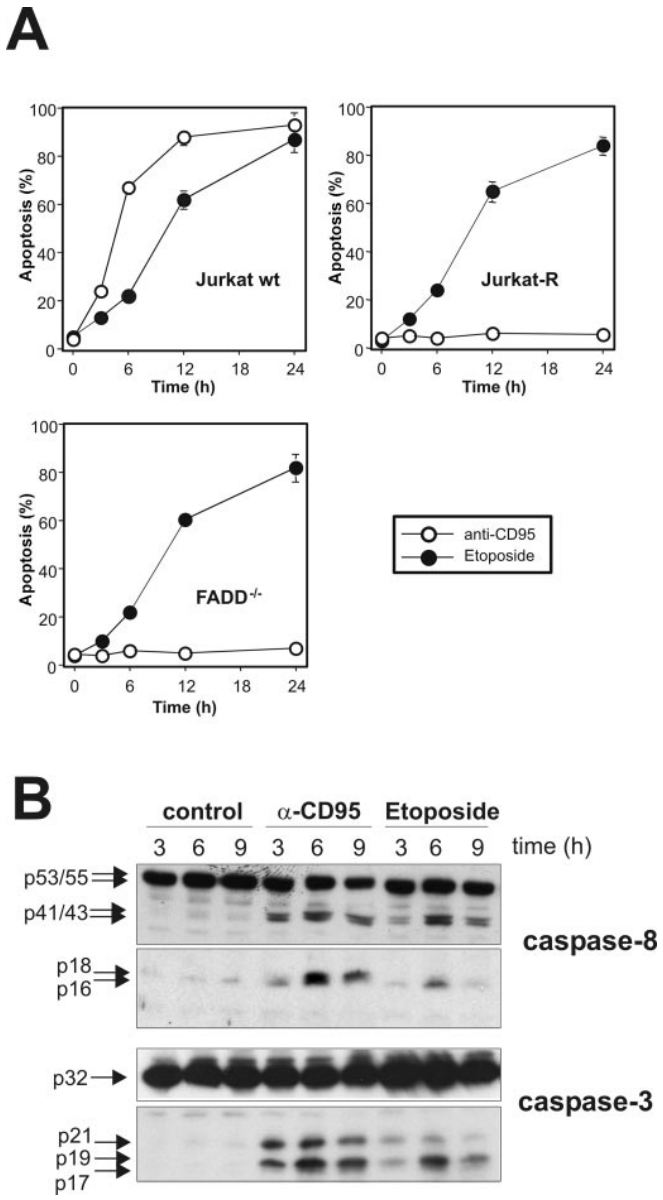

FIG. 1. **Caspase activation during apoptosis induction.** Induction of apoptosis and caspase processing by anti-CD95 and etoposide. *A*, apoptosis induction was analyzed in Jurkat wild-type (*wt*) cells, CD95-deficient Jurkat-R cells, as well as in a FADD-deficient Jurkat subclone. Cells were treated for the indicated times with anti-CD95 (1 μg/ml) or etoposide (100 μM) before induction of apoptosis was assessed by propidium iodide staining of hypodiploid nuclei and flow cytometry. *B*, Western blot analysis demonstrating processing of caspase-8 and -3 in Jurkat wild-type cells that were either left untreated or incubated with anti-CD95 (1 μg/ml) or etoposide (100 μM). After the indicated incubation times cell extracts were prepared, and 25 μg of protein was analyzed by Western blotting. The procaspases and the processed fragments of caspase-8 and -3 are marked with *arrows*.

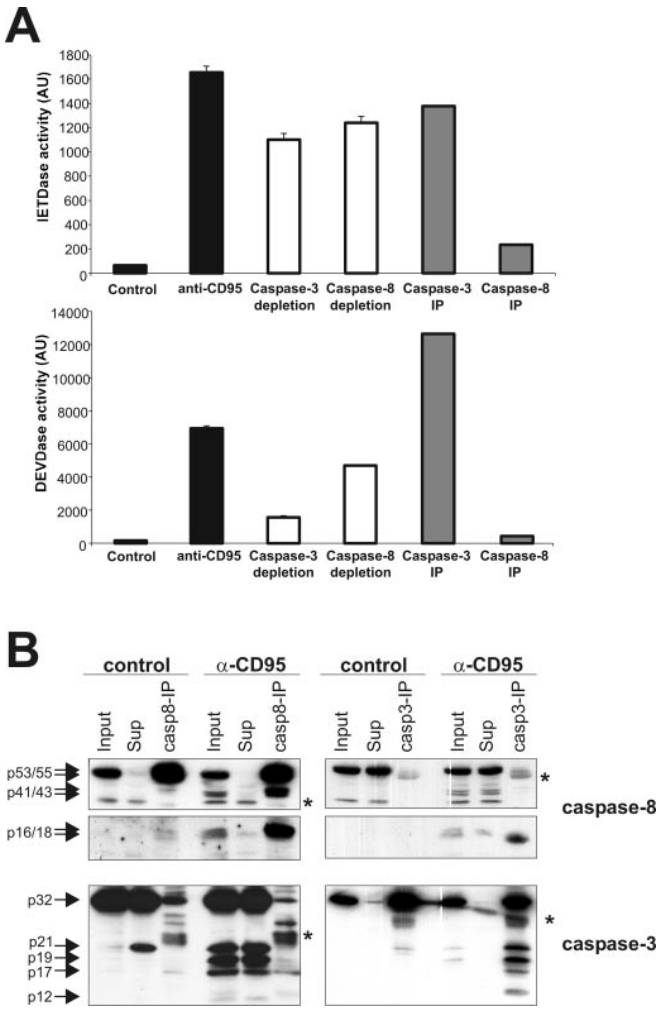

FIG. 2. **Effect of caspase-8 and -3 immunoprecipitation and depletion on proteolytic activity.** Jurkat cells were left untreated or stimulated with anti-CD95 (1 μg/ml) and were then extracted after 5 h of incubation. The extracts were either left untreated or immunoprecipitated with caspase-3 or -8 antibodies. *A*, the immunoprecipitates (*IP*) as well as aliquots of the cell extracts before and after immunodepletion were assayed for caspase-8-like IETDase and caspase-3-like DEVDase activity in fluorometric cleavage assays. Caspase activities are given in arbitrary units (*AU*). *B*, as a control for efficient immunodepletion and precipitation, aliquots of the cell lysates before (*input*) and after (*sup*) precipitation as well as the precipitates were subjected to Western blot analysis and probed for caspase-8 and -3. The proforms of the caspases and the proteolytic fragments are indicated by *arrows*. *Asterisks* denote nonspecific protein bands.

cleavage activity of both IETD-AMC and DEVD-AMC, the preferred caspase-3 substrates (Fig. 2*A*). However, although the DEVD-AMC cleavage activity was substantially reduced following caspase-3 immunodepletion, indicating that caspase-3 is indeed the main DEVD cleaving caspase, depletion of caspase-8 resulted only in a slight reduction of IETD-AMC cleavage activity (Fig. 2*A*). In addition, the depletion of caspase-3 had a reproducibly even stronger impact on IETD-AMC cleavage activity than the depletion of caspase-8 itself. This is probably because of the fact that caspase-3 can efficiently cleave IETD-AMC as well as to our observation that the active p16/p18 caspase-8 fragment was co-precipitated with caspase-3 (Fig. 2*B*). On the other hand, no caspase-3 was detected in immunoprecipitates of caspase-8 (Fig. 2*B*). Together, these results indicate that measurement of the IETD-cleavage activity in whole apoptotic cell extracts does not allow us to exactly determine the contribution of caspase-8 as also other caspases, such as caspase-3 and -6, are able to cleave this substrate (37). Although these caspases do not cleave IETD-AMC as efficiently as caspase-8, they are activated in considerably larger amounts and have a higher specific activity and therefore cleave a substantial proportion of the IETD-AMC substrate (38).

To more directly assess the catalytic competence of caspase-8 in apoptotic cells, caspase-8 and as a control also caspase-3 were immunoprecipitated from untreated and anti-CD95-treated Jurkat cells and tested directly in the substrate cleavage assay. Surprisingly, whereas immunoprecipitated caspase-3 exhibited significant DEVD-AMC as well as IETD-

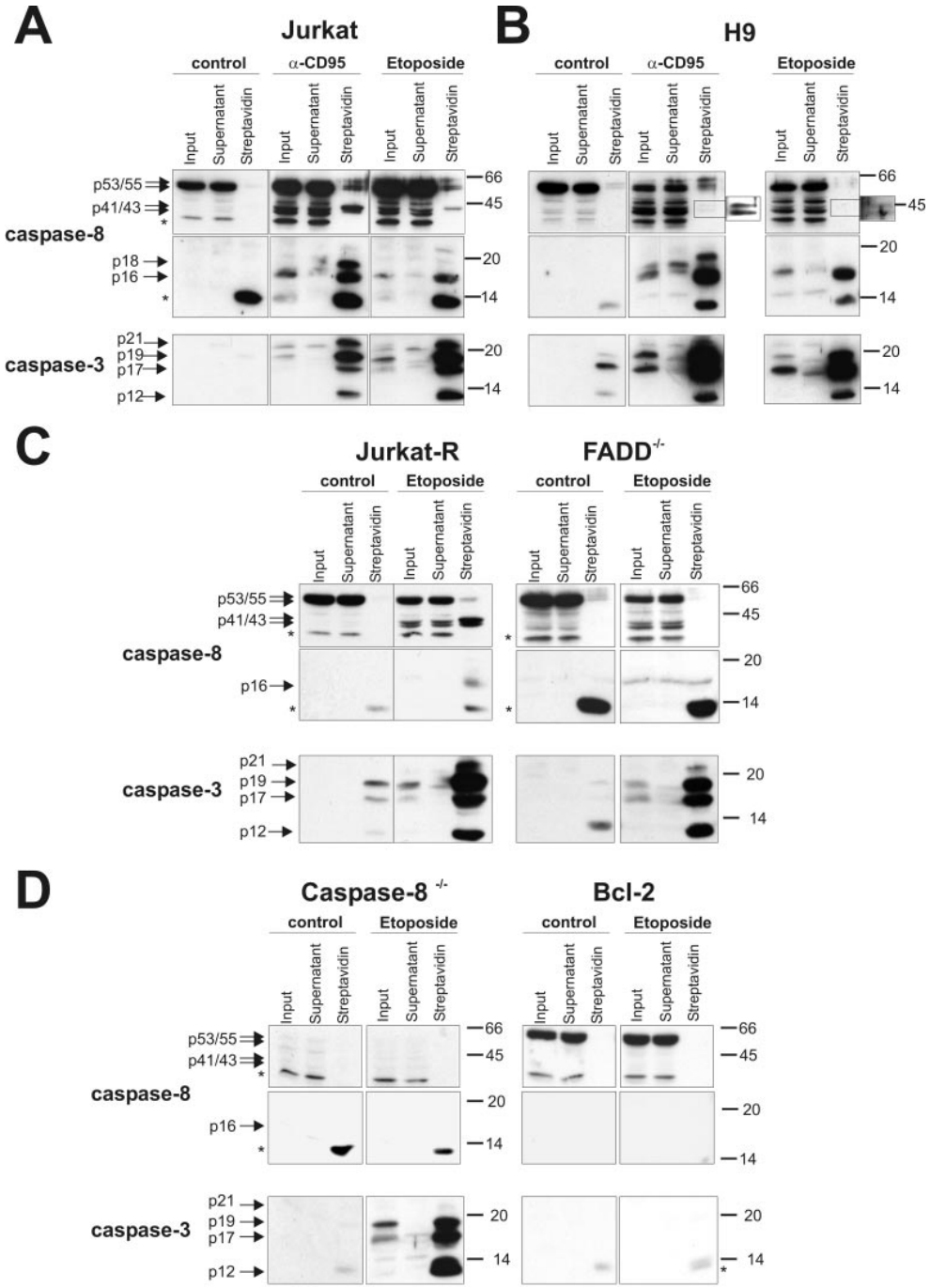

FIG. 3. ***In vivo* peptide affinity labeling of active caspases during apoptosis.** Jurkat wild-type cells (type II, *A*), H9 cells (type I, *B*) as well as Jurkat subclones deficient in CD95 (Jurkat-R), FADD (*C*), or caspase-8 (*D*), or Jurkat cells overexpressing Bcl-2 (*D*) were left untreated or stimulated with anti-CD95 (1 μg/ml, 5 h) or etoposide (100 μM, 9 h). After the incubation period, active caspases were labeled *in vivo* by the addition of biotin-VAD(OMe)-fmk. Cell extracts were prepared (*input lane*), and the biotinylated proteins were captured with streptavidin-agarose beads (*streptavidin lane*). The streptavidin beads and 25 μg of protein from the remaining supernatant and input were separated by SDS-PAGE and assayed for the activation of caspase-8 and -3 by Western blotting. For the detection of the streptavidin-captured intermediate p41/43 form of caspase-8 from H9 cells, a longer exposure time of the film was required, which is shown in a separate section of the gel. *Asterisks* denote nonspecific protein bands that are detected by the caspase-8 antibody also in caspase-8-deficient Jurkat cells (*D*).

AMC cleavage activity when compared with the cleavage activity obtained from whole apoptotic cell extracts, immunoprecipitated caspase-8 was only slightly more active than untreated control extract (Fig. 2*A*). The failure of immunoprecipitated caspase-8 to convincingly cleave IETD-AMC was not because of an inefficient precipitation, as both antibodies used almost completely precipitated the full-length caspase zymogens as well as the proteolytically processed fragments of caspase-8 and -3, respectively (Fig. 2*B*). Also the general procedure of the immunoprecipitation technique does not seem to inhibit caspase activity as, firstly, caspase-3 precipitates were fully active (Fig. 2*A*), and, secondly, addition of either the caspase-8 antibody or protein G-Sepharose to cell extracts of anti-CD95-stimulated Jurkat cells changed neither IETD-AMC nor DEVD-AMC cleavage activity (data not shown). Even when we used two additional monoclonal or polyclonal antibodies that successfully precipitated caspase-8, we were not able to detect significant IETDase activity in these precipitates (data

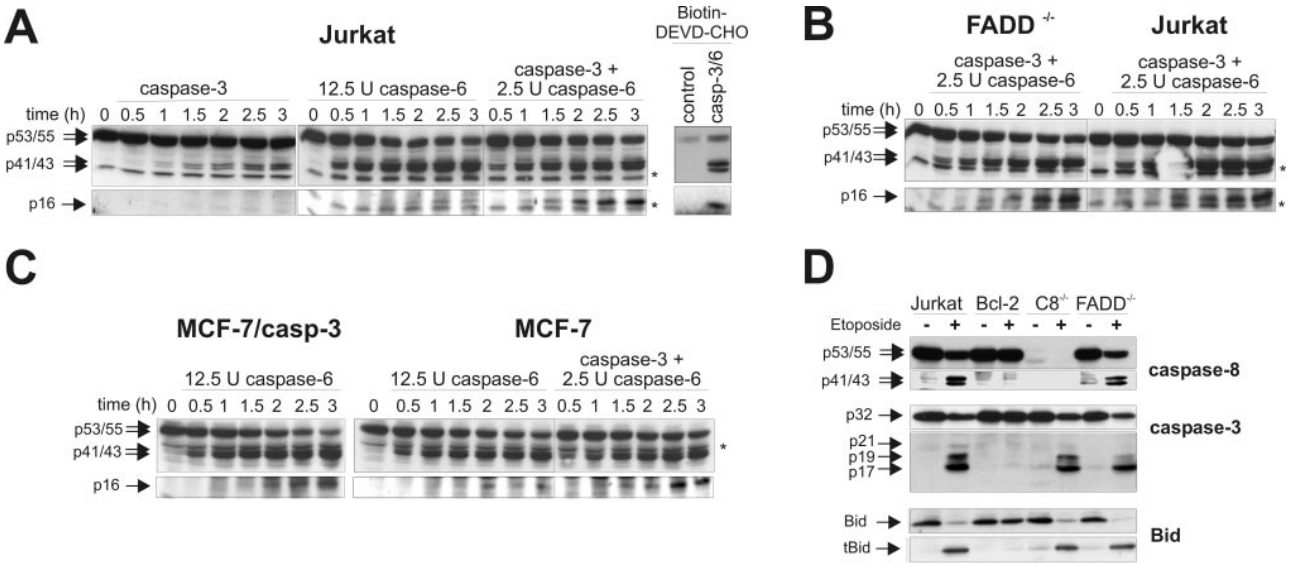

FIG. 4. **Caspase-3 and -6 are required for the efficient activation of caspase-8.** Extracts from unstimulated Jurkat wild-type cells (*A*), FADD-deficient Jurkat cells (*B*), or MCF-7 and MCF-7/casp-3 cells (*C*) were incubated at 37 °C with recombinant caspase-3 (2 μg) and/or the indicated units (*U*) of caspase-6. During the incubation, aliquots of 25 μg of protein were taken from the reaction mixtures and analyzed for the proteolytic processing of caspase-8 and the generation of its p16 subunit by immunoblot analysis. *Asterisks* denote nonspecific protein bands. Aliquots of the Jurkat cell extracts incubated for 2 h in the presence or absence of both caspase-3 and -6 were also used for affinity labeling with the non-methylated reagent biotin-DEVD-CHO, confirming that the *in vitro* cleaved caspase-8 becomes proteolytically active (*A, right panel). D*, Bcl-2 prevents processing of caspase-8, -3, and Bid. The indicated cell lines were treated for 15 h with etoposide (100 μM), and the cell extracts were analyzed for the processing of caspase-8, -3, and Bid.

not shown). Our observation is consistent with findings from another group[2] but in slight contrast to a very recent report (39). These authors demonstrated IETDase activity of precipitated caspase-8; however, only precipitated caspase-8 from untreated and apoptotic cell extracts were compared without relating these activities to the activity of the complete apoptotic lysates. Nevertheless, based on our results, the immunoprecipitation technique does not seem to be suitable for the determination of whether caspase-8 is active or not, at least in our hands. It appears that this technique captures either only inactive subunits or that caspase-8 is rendered inactive during the precipitation procedure, perhaps through an induced conformational change or due to the failure to precipitate other components that might be required for its activity.

Etoposide-generated Caspase-8 Fragments Are Active—To circumvent the problems using the immunoprecipitation procedure, we employed an alternative approach by labeling apoptotic cells *in vivo* with the biotinylated caspase inhibitor VAD-fmk (biotin-VAD-fmk). This inhibitor easily penetrates cell membranes and only binds active caspases that can then be captured using streptavidin-conjugated-agarose beads. Jurkat cells were first treated with either the anti-CD95 antibody or with etoposide followed by incubation for an additional hour in the presence of biotin-VAD-fmk. The detection of the active caspase-3 fragments (p21/p19/p17/p12) induced by both death stimuli served as a positive control for the induction of apoptosis as well as for the efficient labeling and isolation procedure of the active caspases (Fig. 3*A*, *lower panels*). No active caspase-3 subunits were pulled down from untreated control cells, validating the specificity of this technique. Most importantly, biotin-VAD-fmk-labeled caspase-8 fragments were present in extracts of anti-CD95-treated as well as in etoposide-treated Jurkat cells indicating that both death stimuli generate active caspase-8 (Fig. 3*A*, *upper* and *middle panels*). Consistent with our results shown in Fig. 1, etoposide treatment resulted only in the generation of the p16 caspase-8 fragment, whereas both the p16 and the p18 fragments could be detected in cells treated with the agonistic CD95 antibody (Fig. 3*A*, *middle panels*). Similar results were also obtained in type 1 cells such as H9 (Fig. 3*B*) or SKW cells (data not shown) as well as in CD95- or FADD-deficient Jurkat cells (Fig. 3*C*), supporting our hypothesis that only the generation of the active p18 caspase-8 fragment requires DISC formation. Also the p41/p43 caspase-8 fragments that were generated by both death stimuli were, at least partially, labeled by biotin-VAD-fmk suggesting that also these intermediate forms represent active caspase-8 (Fig. 3, *upper panels*). This finding is in agreement with several reports demonstrating that active initiator caspases are already generated via dimerization or oligomerization events even in the absence of complete processing of their proforms (16–19). However, considering that the input lanes contain approximately only 5% of the protein amount used for the pull downs in the respective streptavidin-captured lanes, very little active caspase-8 p41/p43 fragments could be detected. This is further emphasized by the observation that the relative amounts of the p41/p43 caspase-8 fragments before (input) and after (supernatant) the pull down did not differ significantly from each other, demonstrating that the majority of the caspase-8 intermediate fragments are not active regardless of whether they were generated following DISC or apoptosome formation (Fig. 3, *upper panels*).

Biotin-VAD-fmk-labeled and hence active caspase-8 fragments were also detected in etoposide-treated CD95- and FADD-deficient Jurkat cell lines (Fig. 3*C*) but not in Jurkat cells overexpressing Bcl-2 or caspase-8-deficient Jurkat cells (Fig. 3*D*) These results not only verify the specificity of our pull-down experiments but also demonstrate clearly that caspase-8 can be activated via the mitochondrial death pathway independently from death receptor signaling and DISC formation. In addition, these results also argue against an involvement of FADD in the mitochondrial pathway acting perhaps as a scaffolding protein in a secondary activation complex, as it was suggested for apoptosis induction through TNF receptor-1 (14).

[2] M. E. Peter, personal communication.

Both Caspase-3 and -6 Are Required to Generate the p16 Caspase-8 Fragment—So far our data provide strong evidence that direct interchain proteolysis of caspase-8 most likely in the absence of a death receptor-mediated dimerization platform is sufficient to produce active caspase-8. In the mitochondrial pathway, caspase-8 is believed to be proteolytically processed and thereby activated by caspase-6 (29, 40, 41). However, all of these reports could only detect the p41/p43 intermediate caspase-8 fragments but failed to convincingly demonstrate the presence of the p16/p18 caspase-8 fragments following incubation with caspase-6. In contrast, another report concluded that caspase-6, at least *in vitro*, does not activate caspase-8, because the caspase-6-generated p41/p43 caspase-8 fragments were not labeled by biotin-VAD-fmk (16). Based on our present results demonstrating that only the p16/p18 caspase-8 fragments were successfully labeled by biotin-VAD-fmk, whereas the p41/p43 intermediate fragments of caspase-8 were only labeled very weakly, we next analyzed the caspases that are responsible for the generation of the large p16 subunit of caspase-8. For this purpose, we incubated cell extracts from untreated Jurkat cells with either caspase-6 or -3 alone or with a combination of both. Remarkably, although either caspase alone was able to proteolytically process caspase-8 to yield the p41/p43 intermediate fragments, the p16 caspase-8 fragment was only very barely detectable (in the case of caspase-6) or not at all (in the case of caspase-3) (Fig. 4*A*). When both caspase-6 and -3, however, were added to Jurkat cell extract, an efficient generation of the p16 caspase-8 subunit could be observed suggesting that both caspases are required for the activation of caspase-8 *in vitro* (Fig. 4*A*). Similar results were obtained with extracts of FADD-deficient Jurkat cells (Fig. 4*B*). Moreover, incubation of the cell extracts with caspase-3 and -6 not only resulted in the correct processing but also in the generation of caspase-8 fragments that could be affinity-labeled with the biotinylated caspase substrate (Fig. 4*A*, *right panel*).

The hypothesis that both caspase-3 and -6 are required for the activation of caspase-8 was verified by employing MCF-7 cells that harbor a functional deletion in the *CASP-3* gene and hence are deficient for caspase-3 protein expression (42). Similar to the results described above (Fig. 4, *A* and *B*), the p16 caspase-8 fragment was generated in MCF-7 cell extracts in the presence of both recombinant caspases (caspase-6 and -3), whereas caspase-6 alone only generated the p41/p43 fragments (Fig. 4*C*). On the other hand, caspase-6 alone was sufficient to produce the p16 caspase-8 fragment in extracts of MCF-7 cells stably expressing caspase-3 further supporting our hypothesis that both caspase-3 and -6 are required.

Finally, we analyzed the hierarchical caspase activation profile in etoposide-induced apoptosis and found that the processing of caspases as well as the cleavage of Bid were induced in Jurkat and FADD-deficient Jurkat cells but were completely abrogated in Jurkat cells overexpressing Bcl-2 (Fig. 4*D*). Consistent with this we also observed that streptavidin-captured biotin-VAD-fmk did not recover any active caspase-8 or -3 fragments from these cells (Fig. 3*D*). Together these data not only demonstrate that the etoposide-induced activation of caspase-8 depends entirely on caspases that are activated in a postmitochondrial event such as caspase-3 and -6 but thereby also argue against an involvement of caspase-2 that was recently implicated as an initiator caspase acting upstream of mitochondria in drug-induced apoptosis (43).

In Jurkat cells deficient of caspase-8 Bid cleavage occurred to an almost similar extent as in wild-type cells (Fig. 4*D*). This observation is mostly likely because of the fact that in comparison to caspase-8, Jurkat cells abundantly express caspase-3, which was shown to efficiently cleave Bid (25, 40). Interestingly, the susceptibility of Bid to cleavage by caspase-8 and -3 appears to be cell-type specific and differentially regulated by posttranslational modifications (44, 45). Thus, even though postmitochondrial activation of caspase-8 did not strongly affect Bid cleavage in our system, it might constitute a mitochondrial amplification loop by augmenting Bid cleavage and cytochrome *c* release in particular in response to a weak apoptotic signal. Indeed, such a positive postmitochondrial feedback loop has been recently proposed for several cell types in studies investigating the proteolytic processing of caspase-8 during death receptor-independent apoptosis (28, 40, 46–48).

Together our data demonstrated that interchain processing of caspase-8 in the absence of DISC formation, a process that occurs during the mitochondrial death pathway *in vivo*, is indeed sufficient to produce active caspase-8 enzyme. Thus, it is valid to conclude that the appearance of caspase-8 fragments observed in numerous death receptor-independent apoptosis systems correlates with its activity. During the preparation of our paper, Murphy *et al.* (39) showed that direct proteolysis of caspase-8 by the CTL protease granzyme B produces an active enzyme *in vitro* that displays proteolytic activity toward synthetic as well as natural caspase-8 substrates. Our results are not only supportive but further extend this finding as we show that active caspase-8 is generated during etoposide-induced apoptosis *in vivo*. Furthermore, we show that both executioner caspases, caspase-3 and -6 are required to process and thereby activate caspase-8 *in vitro*, supporting the idea that a similar process occurs also *in vivo* during the course of the mitochondrial death pathway. As the p41/p43 caspase-8 fragments were much more efficiently generated in the presence of caspase-6, it seems plausible that caspase-6 preferentially cleaves the linker domain between the large and small subunit of caspase-8, whereas caspase-3 removes preferentially the prodomain. Although our data do not reveal whether the observed caspase-8 fragments following etoposide treatment *in vivo* are active as a dimer or monomer, the observation that substrate binding promotes the formation of caspase-8 dimers supports the former view (17). Finally, our results argue against an involvement of the DISC adapter protein FADD further supporting our view of a death receptor-independent activation of caspase-8 via the caspase-3- and -6-mediated interchain proteolysis.

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p21 blocks irradiation-induced apoptosis downstream of mitochondria by inhibition of cyclin-dependent kinase-mediated caspase-9 activation

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p21 Blocks Irradiation-Induced Apoptosis Downstream of Mitochondria by Inhibition of Cyclin-Dependent Kinase–Mediated Caspase-9 Activation

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Abstract

The role of the cyclin-dependent kinase (CDK) inhibitor p21 as a mediator of p53-induced growth arrest is well established. In addition, recent data provide strong evidence for new emerging functions of p21, including a role as a modulator of apoptosis. The mechanisms, however, by which p21 interferes with the death machinery, especially following ionizing radiation (IR), are largely unknown. Here, we report that IR induced caspase-9 and caspase-3 activation and subsequent apoptosis only in p21-deficient colon carcinoma cells, whereas similar treated wild-type cells were permanently arrested in the G_2-M phase, correlating with the induction of cellular senescence. Interestingly, activation of the mitochondrial pathway, including caspase-2 processing, depolarization of the outer mitochondrial membrane, and cytochrome *c* release, was achieved by IR in both cell lines, indicating that p21 inhibits an event downstream of mitochondria but preceding caspase-9 activation. IR-induced p21 protein expression was restricted to the nucleus, and no evidence for a mitochondrial or cytoplasmic association was found. In addition, p21 did neither interact with caspase-3 or caspase-9, suggesting that these events are not required for the observed protection. Consistent with this assumption, we found that CDK inhibitors potently abrogated IR-induced caspase processing and activation without affecting mitochondrial events. In addition, *in vitro* caspase activation assays yielded higher caspase-3 activities in extracts of irradiated p21-deficient cells compared with extracts of similar treated wild-type cells. Thus, our results strongly indicate that p21 protects cells from IR-induced apoptosis by suppression of CDK activity that seems to be required for activation of the caspase cascade downstream of the mitochondria. (Cancer Res 2006; 66(23): 11254-62)

Introduction

When proliferating cells encounter a genotoxic stress induced for instance by ionizing radiation (IR) or chemotherapeutic drugs, the cell cycle must be arrested immediately to ensure DNA integrity. This event is usually followed by the decision of whether the cells remain arrested in the cell cycle, initiate DNA repair, or execute the apoptotic program. One of the main players involved in this process is the tumor suppressor protein p53 that is known to accomplish these tasks via transcription-dependent and transcription-independent events (1). Whereas cell cycle arrest induced by p53 is mediated by only a few proteins, including the cyclin-dependent kinase (CDK) inhibitor p21, numerous candidates are known to be involved in p53-dependent apoptosis. Among them, the proapoptotic members of the Bcl-2 family (Bax, Noxa, and Puma) are certainly the most important players (2). They instigate the intrinsic or mitochondrial death pathway either directly via disruption of the outer mitochondrial membrane or indirectly by sequestering antiapoptotic proteins, such as Bcl-2 and Bcl-x_L (3). Recently, it was also shown that p53 itself translocates to mitochondria during genotoxic stress where it activates this pathway via binding to these antiapoptotic proteins (4). Although such a mechanism could nicely explain its transcription-independent proapoptotic activity, p53 was also found associated with mitochondria in the absence of apoptosis (5). Nevertheless, disruption of the outer mitochondrial membrane results in the release of cytochrome *c* that together with apoptosis-activating factor 1 (APAF-1) and caspase-9 forms a high molecular weight complex, the apoptosome. Oligomerization of caspase-9 in the apoptosome then leads to its autocatalytic processing and subsequent activation of caspase-3 that is known to cleave the majority of cellular substrates during apoptosis (6).

Thus, p53-dependent apoptosis is well documented and appreciated as a valuable facet of its tumor suppressive activities. Intriguingly, p53 was also shown to possess antiapoptotic capabilities, as several cells lacking functional p53 are under certain circumstances even more sensitive to apoptosis than their p53-proficient counterparts (7). With regard to this, we have also shown recently that IR induced a decrease in the mitochondrial membrane potential only in MCF-7 breast carcinoma cells stably expressing a p53 small interfering RNA, suggesting that the presence of a wild-type p53 contributes to the radioresistant phenotype of these cells (8). Although the mechanisms responsible for the antiapoptotic effect of p53 are far from being elucidated, accumulating evidence suggests the involvement of p21 in this process. Inhibition of DNA damage–induced p21 expression for instance rendered MCF-7 cells sensitive toward IR-induced apoptosis in a caspase-3-dependent manner, whereas overexpression of p21 reverted this effect (9). In addition, wild-type HCT116 cells were efficiently chemosensitized and radiosensitized by either homologous disruption of the *p21* gene or by p21 antisense oligonucleotides both *in vitro* and in tumor xenografts in nude mice (10, 11). Moreover, whereas p21-proficient tumors underwent regrowth following treatment with γ-radiation, a significant fraction of p21-deficient tumors were completely eradicated (12). Although p21 was shown in the following years to confer resistance to a variety of apoptotic stimuli, little is known about the underlying mechanisms (13, 14).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Many diverse signaling pathways have been proposed to be involved in the p21-mediated protection from apoptosis, including inhibition of the apoptosis signal-regulating kinase 1 (ASK1) and c-Jun NH_2-terminal kinase (JNK), and even the direct binding and inhibition of caspase-3 was described (15–17). Interestingly, it was shown that phosphorylation of p21 by the Akt kinase not only leads to its stabilization but also to a redistribution of p21 into the cytoplasm, thereby contributing to the prosurvival activities of both Akt and p21 (18–20). In other studies, cytoplasmic localization of p21 was forced by removal of the two nuclear localization sequences at the COOH terminus either by the caspase-3-mediated cleavage at Asp^{112} or following deletion of its COOH-terminal amino acids (21, 22). These truncations abrogated the survival function of p21 and correlated with increased CDK activities. Elevated CDK activities were also observed in several other apoptosis systems, and inhibition of CDK activity prevented several manifestations of apoptotic death, including chromatin condensation (23, 24). Thus, these data provided a causal link between the activity of these cell cycle regulatory kinases and the execution of the cell death program. With occasional exceptions (25), however, CDKs became only activated in a caspase-dependent manner, and activation of caspases was unimpaired in cells in which CDK activity was inhibited. In contrast, CDK inhibition was also shown to induce caspase activation and cell death, a phenomenon that is presently explored in phase II clinical trials (26). Based especially on the lack of information on possible CDK targets, it is still very much unresolved whether these cell cycle regulatory kinases are indeed required for induction of apoptosis, and if so, at which step they interact with the apoptotic machinery.

To further delineate the mechanisms by which p21 confers resistance, we compared apoptosis susceptibilities of checkpoint-deficient ($p53^{-/-}$ and $p21^{-/-}$) HCT116 cells with their wild-type counterparts toward IR treatment. We found the p21 protein in IR-resistant HCT116 wild-type cells almost exclusively localized in the nucleus, and no evidence was detected for an association of p21 with caspase-3 or caspase-9 that would explain its protective role. Interestingly, although IR induced processing of caspase-2 and activation of the mitochondria and cytochrome *c* release in all three cell lines, including the wild-type cells, the post-mitochondrial caspase-9 and caspase-3 were only activated in p21- and p53-deficient cells. Together with our observation that the specific CDK inhibitor roscovitine blocked IR-induced post-mitochondrial caspase activation without affecting the loss of the mitochondrial membrane potential, our data suggest that p21 protects cells via inhibition of CDKs that are required downstream of the mitochondria for an efficient activation of the caspase cascade.

Materials and Methods

Cell lines, reagents, and antibodies. HCT116 wild-type cells and their checkpoint-deficient variants were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated FCS, 10 mmol/L glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (PAA Laboratories, Linz, Austria). The pan-caspase inhibitory peptide Q-VD-OPH (Q-Val-Asp-CH_2-O-Ph) and the caspase-2 and caspase-3 inhibitory peptides z-VDVAD-fmk (z-Val-Asp-Val-Ala-Asp-fluoromethylketone) and z-DEVD-fmk (z-Asp-Glu-Val-Asp-fluoromethylketone) were from MP Biomedicals (Irvine, CA). The fluorogenic caspase-3 and caspase-9 substrates DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) and LEHD-AMC (*N*-acetyl-Leu-Glu-His-Asp-aminomethylcoumarin) were from Biomol (Hamburg, Germany). The polyclonal antibodies against caspase-3 and caspase-9 were from R&D Systems (Wiesbaden, Germany) and from Cell Signaling Technology (Danvers, MA), respectively. The rat caspase-2 monoclonal antibody (mAb) as well as the CDK inhibitors roscovitine and olomoucine were from Alexis Biochemicals (Lausen, Switzerland). The p53 mAb (Ab-6) was from Calbiochem (Bad Soden, Germany), whereas the mAbs recognizing cytochrome *c*, TOM20, poly(ADP-ribose) polymerase (PARP), and p21 and the polyclonal antibodies directed against the high mobility group 1 (HMG1) protein were from BD Biosciences (Heidelberg, Germany). The mAbs towards Bcl-2 and Bax were from Novocastra Laboratories (Newcastle, United Kingdom) and Trevigen (Gaithersburg, MD), respectively. The mAb recognizing the proliferating cell nuclear antigen and the polyclonal antibody to CDK2 was from Santa Cruz (Heidelberg, Germany). The actin mAb, the nuclear stain 4′,6-diamidino-2-phenylindole, and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin were from Sigma (Deisenhofen, Germany). Peroxidase-labeled secondary antibodies were from Promega GmbH (Mannheim, Germany), and the secondary chicken anti-mouse antibody coupled to Alexa Fluor 488 that was used for the immunofluorescence studies were from Molecular Probes (Mobitech, Göttingen, Germany).

Treatment of cells and measurement of cell death. Cells were exposed to IR (usually 20 Gy) using a Gammacell 1000 Elite (Nordion International, Inc., Fleurus, Belgium). Cell death was assessed by microscopic examination or by determination of the lactate dehydrogenase (LDH) activity in supernatants of 10^5 cells according to the protocol of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The values obtained are given in arbitrary units. These supernatants were also analyzed on SDS-polyacrylamide gels for the death-associated release of HMG1.

Senescence-associated β-galactosidase staining and immunofluorescence microscopy. Staining for β-galactosidase activity was done as described (8). Pictures were taken on an Axiovert135 microscope (Zeiss, Jena, Germany) with an Apochromat ×20 objective using OpenLab software (Improvision, Tübingen, Germany). Immunofluorescence staining was done as described (5). Pictures were taken using a Zeiss LSM 510 Meta equipped with a ×40 oil immersion objective.

Preparation of cell extracts, subcellular fractionation, and Western blotting. Total cell extracts were prepared in high-salt lysis buffer containing 1% NP40, 20 mmol/L HEPES, 2 mmol/L PMSF, 350 mmol/L NaCl, 20% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 0.5 mmol/L DTT, and protease inhibitors. The preparation of highly purified mitochondrial M2 fractions by a discontinuous 1.2 to 1.6 mol/L sucrose gradient was done as described (5). Protein concentrations were determined with the Bio-Rad protein assay. Subsequently, proteins were separated on SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes (Amersham, Braunschweig, Germany). Following antibody incubation, the proteins were visualized by enhanced chemiluminescent staining using enhanced chemiluminescence reagents (Amersham Biosciences, Freiburg, Germany).

Determination of the mitochondrial transmembrane potential and cytochrome *c* release by fluorescence-activated cell sorting analyses. The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analyzed by flow cytometry using the $\Delta\Psi_m$-specific stain TMRE (Molecular Probes) as described (8). For the measurement of mitochondrial cytochrome *c* release (27), 10^5 cells were suspended in 250 μL permeabilization buffer (50 μg/mL digitonin, 2 mmol/L EDTA, 100 mmol/L KCl) in PBS and fixed for 20 minutes at room temperature by addition of 300 μL of 8% formaldehyde followed by three PBS washes. Cells were incubated for 1 hour in 300 μL blocking buffer (3% bovine serum albumin, 0.05% saponin in PBS) at 4°C followed by the addition of 1.5 μL of the cytochrome *c* antibody and overnight incubation at 4°C. Samples were washed thrice, and secondary phycoerythrin-conjugated goat anti-mouse antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 hour at room temperature in the dark. Cells were washed and resuspended in 200 μL PBS, and cytochrome *c* staining was determined by flow cytometric analysis on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) using the FL2-histogram profile and the CellQuest software.

Immunoprecipitation. Cell extracts were prepared from 1×10^7 cells in 500 μL lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40] containing protease inhibitors. For the precipitation, the protein

concentrations were adjusted, and 2 μg antibody and 30 μL protein G-Sepharose (Sigma) were added to the extracts and rotated overnight at 4°C. The Sepharose beads were washed in lysis buffer and analyzed by SDS-PAGE and Western blotting.

***In vitro* caspase activation assay and fluorometric determination of caspase activity.** For *in vitro* activation of caspases, cells were resuspended in low salt buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L EDTA] containing protease inhibitors and 1 mmol/L DTT. After incubation for 15 minutes at 4°C, cells were disrupted by 30 passages through a 20-gauge needle. Cell extracts were cleared twice by centrifugation (10,000 × *g*) at 4°C for 15 minutes each and adjusted to an equal protein concentration. *In vitro* activation reactions were started by adding 10 mmol/L DTT, 2 mmol/L dATP, 1 mmol/L $MgCl_2$ in the presence of 3.5 μmol/L cytochrome *c* (from horse heart; Sigma) to 200 μg aliquots in 75 μL and incubation at 37°C. Caspase-3 and caspase-9 activities were assessed after 60 minutes using 50 μg aliquots of each reaction in the fluorometric DEVD and LEHD cleavage assays as described (28). Student's *t* test was employed for statistical analysis.

Results

IR induces apoptosis only in checkpoint-deficient HCT116 cells. To delineate the underlying mechanisms by which the CDK inhibitor p21 exerts its antiapoptotic effect, we first exposed wild-type HCT116 cells and their checkpoint-deficient ($p53^{-/-}$ and $p21^{-/-}$) counterparts to a single dose of 20 Gy γ-irradiation and assessed cell death induction by several means. Remarkably, during a period of 7 days after IR, that represents time points not considered in previous studies, only marginal signs of cell death were detected in wild-type cells. These cells did not release a substantial amount of either the enzyme LDH or the HMG1 protein into their supernatants (Fig. 1*A* and *B*). Instead, they entered a permanent cell cycle arrest in the G_2-M phase that correlated with the induction of cellular senescence as measured by staining of the senescence-associated β-galactosidase activity (Fig. 1*C*). In contrast, IR treatment clearly induced death of the two checkpoint-deficient HCT116 cell lines as evidenced by a significant time-dependent release of LDH and HMG1 into their supernatants (Fig. 1*A* and *B*) in the absence of any detectable β-galactosidase activity (Fig. 1*C*; data not shown). Hence, expression of p53 and p21 (see Fig. 5) critically influences the outcome of an IR treatment.

The IR-induced cell death was most likely mediated via apoptosis because the death-associated release of LDH and HMG1 could be efficiently blocked by the pan-caspase inhibitory

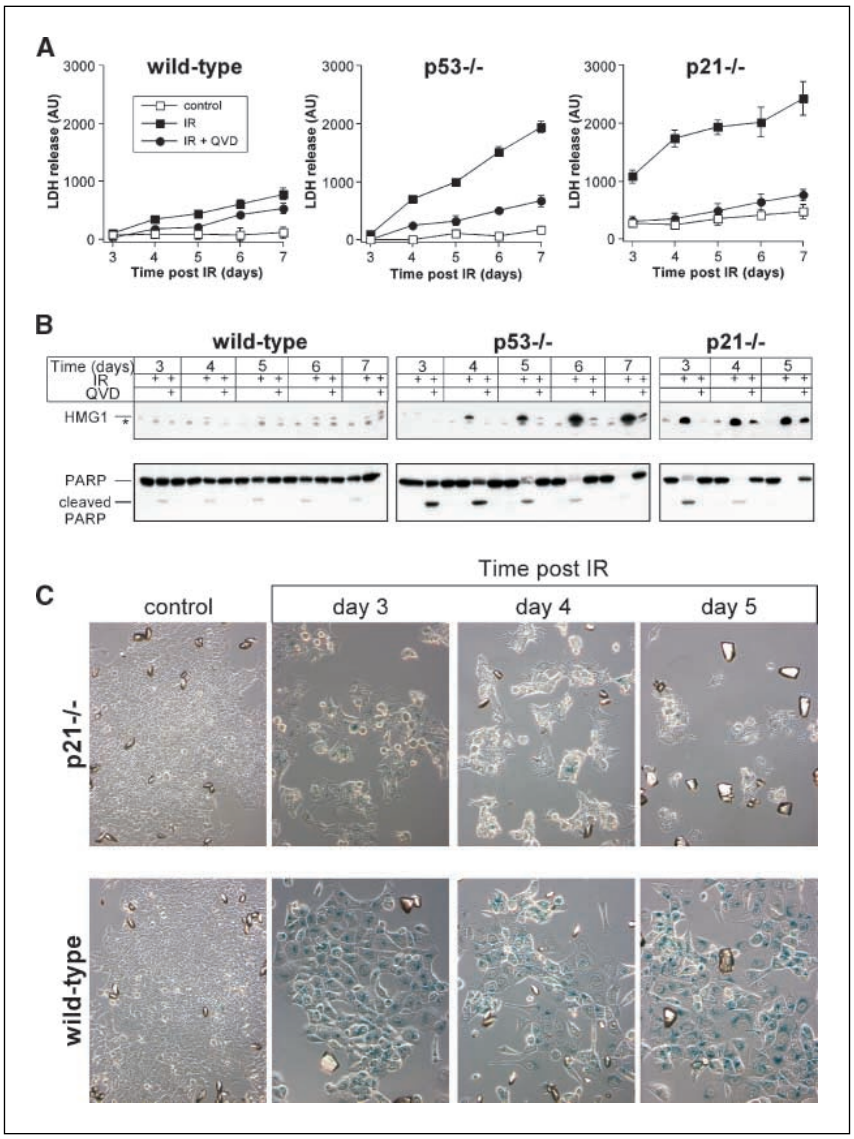

Figure 1. IR induces apoptosis of checkpoint-deficient HCT116 cells but not of their wild-type counterpart. Wild-type and checkpoint-deficient cells were either left untreated or were exposed to IR in the absence or presence of the pan-caspase inhibitory peptide QVD (10 μmol/L). After the indicated days, cell death was determined by the release of LDH (*A*) and HMG1 (*B*) into the supernatant, or by cleavage of PARP that represents a typical caspase substrate. QVD was added at days 0, 3, and 5. *Points,* mean of three independent experiments; *bars,* SD. *, band of unknown origin. *C,* IR treatment results in the p21-dependent induction of cellular senescence. Wild-type and p21-deficient cells were either left untreated or were exposed to IR and analyzed after the indicated days for the induction of cellular senescence by staining of the senescence-associated β-galactosidase activity. One representative experiment out of two.

peptide Q-VD-OPH (QVD; Fig. 1). In addition, cleavage of PARP that was predominantly observed in irradiated p21- and p53-deficient cells was inhibitable by QVD, implying that caspases were responsible for the observed cell deaths (Fig. 1*B, bottom*). Indeed, IR induced the processing of caspase-9 and caspase-3 in both checkpoint-deficient cell lines, as indicated by the decrease of the procaspase-9 protein and the appearance of the active caspase-3 subunits (Fig. 2*A*). In addition, activation of both caspases was confirmed by using the fluorogenic DEVD and LEHD substrate cleavage assays that measure caspase-3 and caspase-9 enzymatic activities, respectively (Fig. 2*B*). Interestingly, although neither caspase-3 nor caspase-9 were found to be processed or activated in IR-treated HCT116 wild-type cells, confirming their resistance to this treatment, procaspase-2 was processed in all three cell lines to a similar extent (Fig. 2*A, top*). Thus, our results show that only the two checkpoint-deficient cell lines undergo apoptosis following exposure to IR and suggest that caspase-2 is unable to directly activate caspase-9 and caspase-3. More importantly, as caspase-2 is considered to act upstream of the mitochondria in many apoptotic systems (29), our results indicate that the apoptosis resistance of wild-type cells toward IR might be caused by a p21-mediated event located at or downstream of the mitochondria.

Expression of p21 does not interfere with the IR-induced activation of mitochondria. To characterize such an event, we first asked at which step p21 interacts with the apoptotic cascade. Therefore, we exposed wild-type and p21-deficient cells to IR and analyzed their mitochondrial membrane potential ($\Delta\Psi_m$). Interestingly, loss of $\Delta\Psi_m$, an event that is closely associated with the onset of apoptosis (30), was not only observed in p21-deficient cells

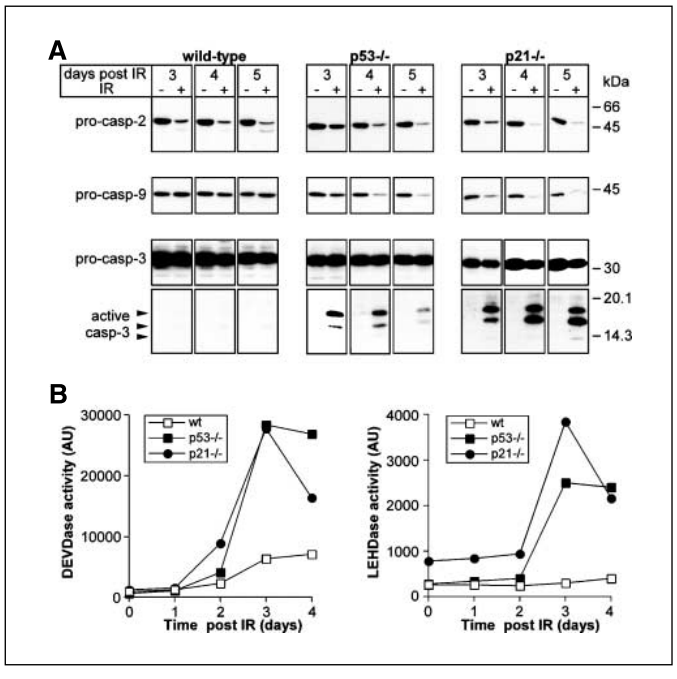

Figure 2. Caspase processing and activation in irradiated HCT116 cell lines. *A,* extracts of cells that were either left untreated or exposed to IR were analyzed by Western blotting after the indicated days for the processing of the indicated caspases. The arrows indicate active caspase-3 subunits. *B,* extracts of cells that were either left untreated or exposed to IR were analyzed after the indicated days for caspase-3 (DEVDase) and caspase-9 (LEHDase) enzymatic activities. Note that caspase-9 and caspase-3 were only processed and activated in checkpoint-deficient cells, whereas caspase-2 was processed in all three cell lines. One representative experiment out of three.

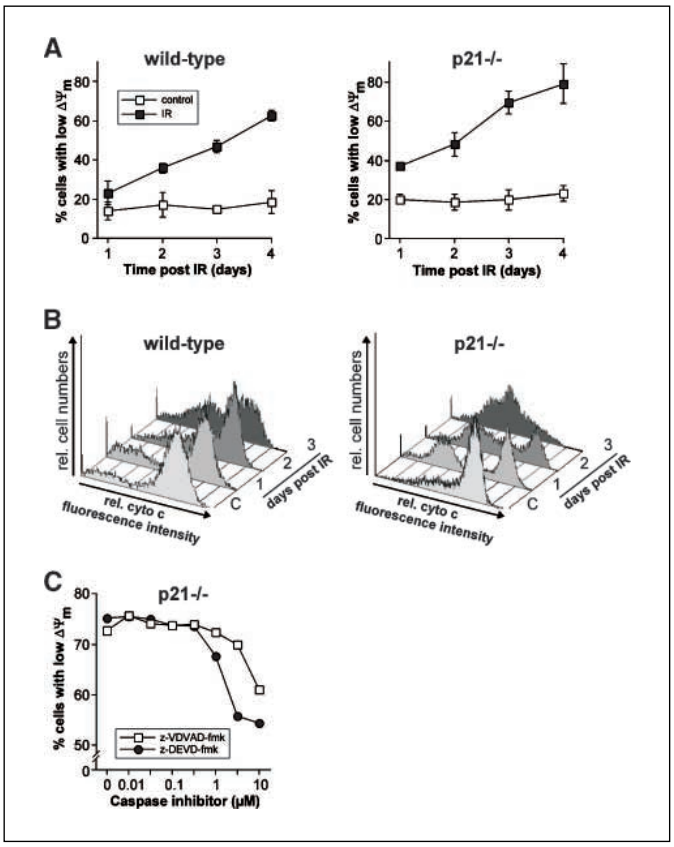

Figure 3. IR activates also the mitochondria in apoptosis-resistant HCT116 wild-type cells. *A,* measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of untreated (*control*) and irradiated (*IR*) cells after the indicated times. *Points,* mean of three independent experiments; *bars,* SD. *B,* measurement of cytochrome c release from the mitochondria of untreated (*C*) and irradiated cells after the indicated times. One representative experiment out of three. *C,* measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of p21-deficient cells 3 days after irradiation in the absence or presence of the indicated caspase inhibitory peptides. One representative experiment out of two.

but also to an almost similar extent in their IR-resistant wild-type counterparts (Fig. 3*A*). Furthermore, mitochondrial cytochrome *c* release occurred in a time-dependent manner also in wild-type cells, although not as efficiently as observed in p21-deficient cells (Fig. 3*B*). This is most likely due to the fact that activation of mitochondria is, at least partially, amplified in a caspase-dependent manner (31), an event unlikely to be achieved in wild-type cells due to the failure of IR to activate caspase-9 and caspase-3. Consistent with this, we found indeed that the IR-induced loss of $\Delta\Psi_m$ in p21-deficient cells could be partially rescued by the caspase inhibitory peptides VDVAD-fmk and DEVD-fmk that block caspase-2 and caspase-3, respectively (Fig. 3*C*). Thus, together with our previous data showing only activation of caspase-2, but not of caspase-9 or caspase-3 in irradiated HCT116 wild-type cells, these results clearly narrow down the interference of p21 with the mitochondrial death pathway to an event that follows cytochrome *c* release but precedes activation of caspase-9.

IR-induced p21 protein does not bind caspase-3 and caspase-9 and is not associated with mitochondria or localized in the cytoplasm. Previously, p21 was reported to bind to procaspase-3, thereby preventing its activation (17). The same group further postulated that mitochondria are essential for the procaspase-3/p21 complex formation (32). Intriguingly, this would

perfectly match the series of events in our aforementioned hypothesis, and the obvious lack of such an event in p21-deficient cells might indeed contribute to their increased sensitivity. Therefore, we analyzed whether p21 is associated with caspase-3 in irradiated HCT116 wild-type cells. We immunoprecipitated caspase-3 from extracts of untreated cells and from cells 1 day after IR under conditions that allow coprecipitation of associated proteins, as we have successfully shown previously (33). However, although caspase-3 was almost completely precipitated from extracts of control and irradiated cells, p21 was not detected in these precipitates (Fig. 4*A*). In addition, the reverse approach (i.e., the immunoprecipitation of p21) did not yield in the coprecipitation of caspase-3 or caspase-9 (Fig. 4*B*). The faint caspase-3 signal detected in the precipitates of irradiated cells does not represent caspase-3 specifically coprecipitated with p21, as it was also present in control reactions that were done in the absence of the p21 antibody (data not shown). The p21 interacting target CDK2 in contrast, was successfully coprecipitated under these conditions, thus validating our approach. Similar results were obtained when immunoprecipitations were done with extracts of cells prepared 2 days after IR (data not shown). Thus, these data argue against a direct association of p21 with caspases in HCT116 wild-type cells and, hence, do not explain the antiapoptotic activity of p21.

As mitochondria were postulated to be essential for the procaspase-3/p21 complex formation (32), we also examined a possible association of p21 with these organelles. However, p21 expression induced by either IR or the chemotherapeutic drug camptothecin was exclusively found in nuclei-containing total cellular extracts but not in highly purified mitochondrial M2 fractions (Fig. 4*C*) that were previously shown to contain p53 protein (5). In addition, and consistent with these results, immunofluorescence studies also showed that p21 expression was almost exclusively confined to the nuclei of irradiated HCT116 wild-type cells that displayed a dramatic increase in size due to this treatment (Fig. 4*D*).

Analysis of apoptosis-relevant proteins in wild-type and checkpoint-deficient HCT116 cells. Next, we compared p53, Bax, and Bcl-2 expression in these cell lines, as it was postulated that the sensitivity of p21-deficient HCT116 cells to drug-induced apoptosis is caused by an increased expression of p53 and the subsequent modification of the Bax/Bcl-2 ratio (34). We found indeed higher protein levels of p53 and Bax in p21-deficient cells when compared with their wild-type counterparts (Fig. 5). Consistent with higher p53 expression, Bcl-2 levels were also found to be reproducibly lower in p21-deficient cells, as this antiapoptotic protein is transcriptional repressed by p53 (Fig. 5; ref. 35). However, we noticed that $p53^{-/-}$ cells that also lack detectable levels of p21 do undergo IR-induced apoptosis (Figs. 1 and 2), although they display significant lower Bax and higher Bcl-2 protein expression when compared with wild-type cells (Fig. 5). Thus, besides a hyperfunctional p53 (34, 36), additional mechanisms must be involved in the increased IR sensitivity of p21-deficient cells.

CDK inhibitor roscovitine blocks IR-induced caspase activation but has no influence on the mitochondrial membrane potential. As we could neither verify a cytoplasmic localization of p21 nor its binding to caspase-3 or caspase-9, which would have argued for a direct effect of p21, we hypothesized that its protective

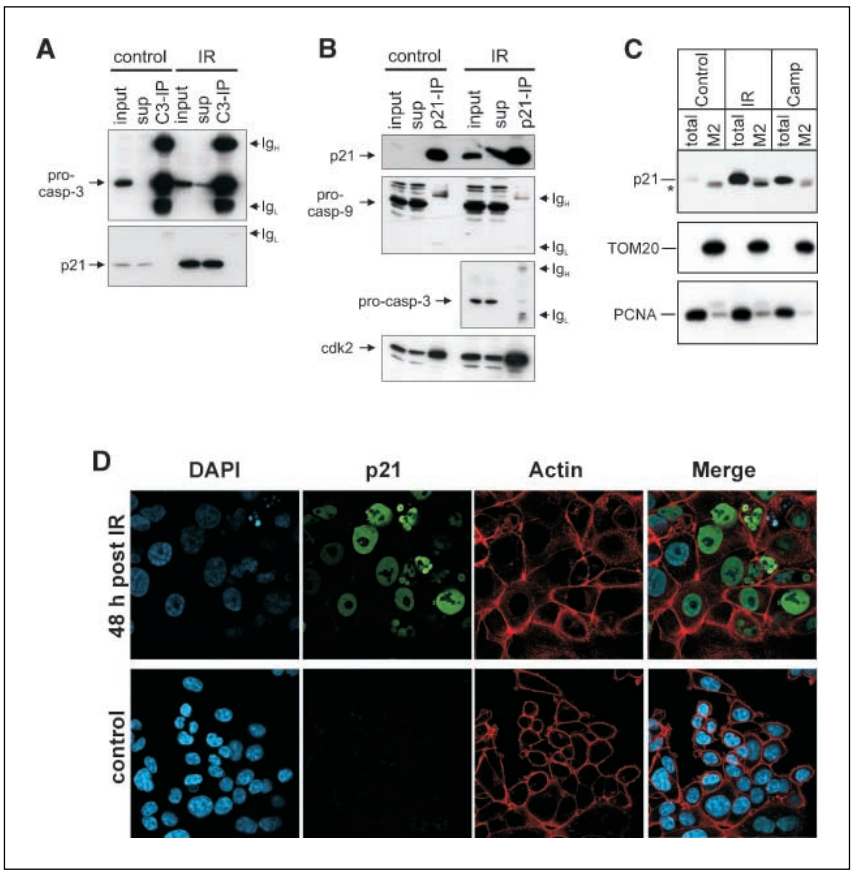

Figure 4. p21 does not associate with caspase-3 or caspase-9. Extracts of wild-type cells that were either left untreated or exposed to IR were subjected to Western blot analysis either before (*input*) or after (*sup*) immunoprecipitation with the caspase-3 antibody (*A*) or the p21 antibody (*B*). The immunoprecipitates for caspase-3 and p21 are labeled as C3-IP and p21-IP, respectively. As a control for the p21 immunoprecipitation, the presence of cdk2 was confirmed by Western blot. One representative experiment out of two. *C* and *D,* p21 is localized in the nucleus but not in the cytoplasm or mitochondria. *C,* wild-type cells were either left untreated or exposed to IR or 1 μmol/L camptothecin (*camp*). After 48 hours, total cell extracts or highly purified mitochondrial fractions (M2) were prepared and analyzed by Western blotting for the presence of p21 (*top*). *, band of unknown origin. The composition and purity of the fractions were confirmed by probing the membrane with antibodies recognizing Tom20 and PCNA that represent mitochondrial and nuclear markers, respectively. *D,* immunofluorescence staining of p21 in untreated wild-type cells (control) or in cells 48 hours following IR. Cells were also stained with the nuclear dye 4′,6-diamidino-2-phenylindole (*DAPI*) and phalloidin-TRITC that labels actin. Note the almost exclusive nuclear localization of p21 and the dramatic increase in size of irradiated cells.

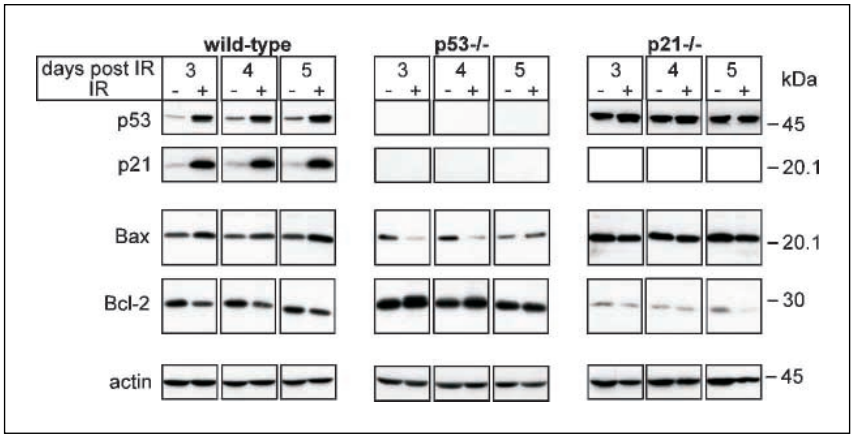

Figure 5. No correlation between p53, Bax, and Bcl-2 expression and IR-induced apoptosis. Extracts of cells that were either left untreated or exposed to IR were analyzed by Western blotting after the indicated days for the status of the indicated proteins. One representative experiment out of two.

function is due to a nuclear event, most likely mediated indirectly via inhibition of CDKs. We first attempted to confirm this by using a cellular system described by Cayrol et al. (37), in which DLD1 carcinoma cells were engineered to stably express either p21 wild-type protein or a p21 mutant deficient for CDK interaction (p21-CDK$^-$), both of which were placed under the control of a tetracycline-regulated promoter (Tet off). Only the expression of p21 wild-type protein partially rescued the cells from IR-induced apoptosis, whereas DLD1 cells expressing the p21-CDK$^-$ mutant protein were as sensitive to this treatment as uninduced (Tet on) p21-deficient control cells (Supplementary Fig. S1). Although these results are in agreement with our hypothesis, the observed effect was not as pronounced as in the HCT116 cell system. Western blot analysis revealed that this was most likely due to the down-regulation of both p21 proteins in DLD1 cells following IR (Supplementary Fig. S1), which is in sharp contrast to the results obtained with the HCT116 wild-type cells (Fig. 5).

To further explore the role of CDKs in irradiation-induced apoptosis, we exposed p21-deficient HCT cells to IR in the presence of roscovitine that is one of the most specific pharmacologic CDK inhibitors (38). Roscovitine efficiently prevented IR-induced caspase-9 and caspase-3 activation in a time- and dose-dependent manner, as shown by the absence of the active caspase subunits in the presence of this inhibitor (Fig. 6*A* and *B*). Inhibition of caspase-3 activity by roscovitine was further confirmed using the DEVD substrate cleavage assay (Fig. 6*C*). Similar results were obtained when the experiments were done in the presence of the CDK inhibitor olomoucine (data not shown), indicating that activated CDKs are essential for IR-induced apoptosis. Interestingly, and in agreement with our hypothesis that p21 functions downstream of mitochondria, caspase inhibition by roscovitine was achieved without affecting the IR-induced loss of $\Delta\Psi_m$ (Fig. 6*D*). This was further supported by our finding that activation of the pre-mitochondrial acting caspase-2 was also not affected by roscovitine (Fig. 6*A*). Together, these data provide strong evidence that suppression of CDK activity represents an event that is critically involved in the protective function of p21. In addition, the results suggest that CDKs interfere with the death pathway downstream of the mitochondria but upstream of caspase-9 activation.

Therefore, we speculated that active CDKs must positively interfere with apoptosome formation. As a reasonable approach to examine such a scenario, we did gel filtration analyses to compare apoptosome formation in irradiated p21-deficient and wild-type HCT116 cells. However, all our attempts using this approach failed, as we were unable to detect an active apoptosome complex. This is most likely due to the inhomogeneity of the irradiated cells that at any given time point after IR form an active apoptosome only in a fraction of the cells, which seems to be too little for a successful visualization by gel filtration and subsequent Western blot analyses.

Therefore, we used an alternative procedure (i.e., an *in vitro* caspase activation assay) that reflects, at least to a certain extent, their capabilities to form an apoptosome. For this purpose, we harvested the cells 24 hours after IR, as this represents a time point at which all extracts showed comparable caspase-3 activities in the absence of cytochrome *c* (no activation; Fig. 6*E*). In the presence of cytochrome *c* (activation), however, we found a significantly higher caspase-3 activity in extracts of irradiated compared with untreated p21-deficient cells. This difference was not observed between extracts of untreated and irradiated p21-proficient wild-type cells. Together, these data show that the intrinsic death pathway, although efficiently instigated by IR, requires additional signals that are only provided in the absence of p21, most likely by active CDKs, to successfully complete the caspase activation cascade downstream of the mitochondria.

Discussion

Although p21 was originally identified as a mediator of p53-induced growth arrest, new functions of p21 emerged recently, including its role as a modulator of apoptosis. However, the mechanisms by which p21 interferes with the death-inducing signaling cascade are still very elusive. Based on recent evidence, p21 could be involved in the modulation of apoptosis at three different levels (13). First, p21 expression was shown to induce genes for secreted proteins with antiapoptotic activities (39). It remains to be tested, however, whether this effect extends beyond the human fibrosarcoma cells in which it was originally described.

Second, by direct binding and inhibition of molecules known to be involved in the apoptotic process, such as caspase-3, JNK, or ASK1 (15–17). To perform those tasks, p21 has to be relocalized to the cytoplasm, which might be achieved either by the caspase-3-mediated cleavage or by its phosphorylation by the kinase Akt (19, 21). In the present report, however, we were not able to document such a cytoplasmic localization of p21 that was strictly confined to the nucleus following exposure of the cells to IR. Consistent with this, also the previously proposed mitochondrial

association of p21 that apparently is required for its binding to procaspase-3 (32) could not be observed in our system. Particularly, a direct binding of p21 to caspase-3 would have been a very attractive model that could have explained the failure of IR to induce apoptosis in HCT116 wild-type cells despite the occurrence of a recently described proapoptotic event (i.e., the translocation of p53 to mitochondria; refs. 4, 5). However, in our hands, neither caspase-3 nor caspase-9 were found to be associated with p21 in IR-treated cells. This suggests that the observed protection by p21 is most likely mediated via other events not involving a cytoplasmic localization or a direct interaction with proapoptotic proteins, such as caspases.

Finally, p21 was postulated to modulate apoptotic processes via inhibition of CDKs. Evidence for such a mechanism was not only obtained by reports showing increased CDK activities in apoptotic cells (23, 24, 40) but also from studies in which the protective function of p21 could be abrogated by deletion of its NH_2-terminal region required for CDK binding (41). However, although inhibition of CDKs by either dominant-negative mutants or pharmacologic inhibitors prevented the generation of several apoptotic characteristics, in most cases, the CDK function was localized downstream of the caspase cascade. In contrast to these studies, we report here for the first time an involvement of CDKs in IR-induced apoptosis acting downstream of the mitochondria, but upstream of caspase-9 activation. These conclusions were drawn from our observation that IR treatment not only resulted in the activation of the mitochondria including cytochrome *c* release in apoptosis-sensitive checkpoint-deficient cells but also in resistant HCT116 wild-type cells in which IR failed to activate caspase-9 and caspase-3. These data were further substantiated by our finding that the selective CDK1/CDK2 inhibitor roscovitine efficiently prevented IR-induced caspase-9 and caspase-3 processing and activation in p21-deficient cells but had no effect on caspase-2 processing or the loss of $\Delta\Psi_m$ induced by this treatment. Inhibition of apoptosis by CDK inhibitors, such as roscovitine or structurally related compounds, was also observed in several other cellular systems; the underlying events that were affected by these treatments, however, were not further investigated (42–44).

In contrast to our study, it was reported previously that only p21-deficient HCT116 cells, but not wild-type cells, displayed extensive $\Delta\Psi_m$ loss, cytochrome *c* release, and caspase activation following treatment with the DNA-damaging agent Adriamycin (45). The exact reason for the discrepancy to our study is presently unknown but is most likely due to stimulus-dependent signaling events (46).

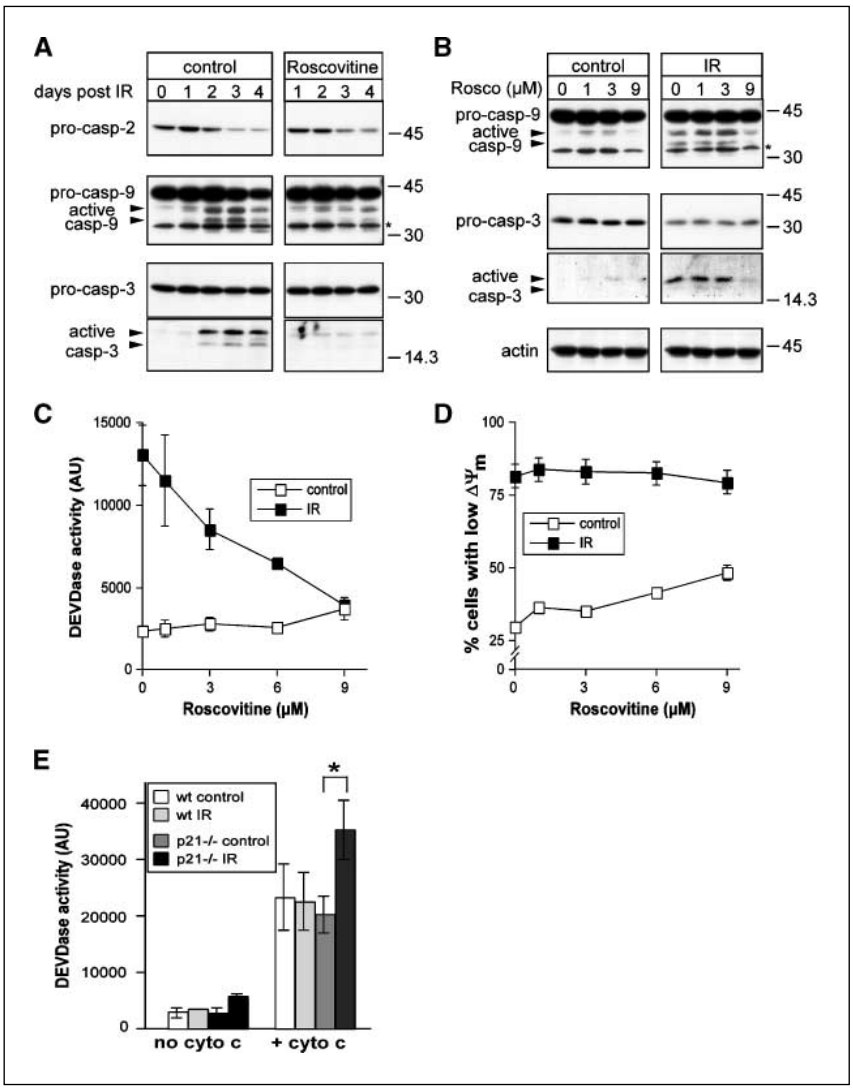

Figure 6. The CDK inhibitor roscovitine prevents processing and activation of caspases downstream of the mitochondria. *A,* Western blot analyses for the status of the indicated caspases in p21-deficient cells that were irradiated and incubated for the indicated times in the absence (control) or presence of 10 μmol/L roscovitine. *B,* Western blot analyses for the status of the indicated caspases in p21-deficient cells that were irradiated and incubated for 3 days in the absence (control) or presence of the indicated roscovitine concentrations. Blots shown in (*A*) and (*B*) are a representative experiment out of two. *Arrows,* active caspases; *, band of unknown origin. *C,* measurement of the caspase-3 (DEVDase) enzymatic activities in extracts of untreated and irradiated p21-deficient cells that were incubated for 2 days after IR in the absence or presence of the indicated concentrations of roscovitine. *D,* measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of p21-deficient cells incubated for 3 days after irradiation in the absence or presence of the indicated concentrations of roscovitine. *E, in vitro* caspase activation assay. Extracts of untreated and irradiated p21-deficient cells were prepared 1 day after IR and were incubated for 1 hour in the absence or presence of cytochrome *c*/dATP and subsequently analyzed for caspase-3 (DEVDase) enzymatic activities. *Points/columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$ (Student's *t* test).

In further support for this assumption is our finding that caspase-2 is activated by IR also in resistant wild-type cells in the absence of active caspase-9 or caspase-3. Together with the fact that the IR-induced $\Delta\Psi_m$ loss could be partially blocked by z-VDVAD-fmk, a caspase-2-specific inhibitory peptide, our data argue for a pre-mitochondrial role of caspase-2 in IR signaling. In contrast, in drug-induced apoptosis, caspase-2 activation was mainly detected downstream of the mitochondria (47), an observation that might explain the lack of mitochondrial activation in Adriamycin-treated HCT116 wild-type cells. However, whether caspase-2 plays a role upstream or downstream of mitochondria is still controversially discussed (29).

What are the specific CDK-mediated events required for apoptosis induction? Although a recent screen of a yeast proteomic library succeeded in the identification of ~200 CDK substrates (48), only a few human CDK targets have been characterized thus far. This is probably due to the lack of appropriate proteomic libraries and also to the difficulty to identify phosphorylated substrates in a large mixture of proteins. Nevertheless, one of the few CDK targets identified is the proapoptotic Bcl-2 family member BAD that becomes a mitochondrial activator upon phosphorylation by CDK1 (49). However, as we have located the putative CDK-mediated event required for caspase-9 activation downstream of mitochondria, it is unlikely that the phosphorylation of BAD at Ser^{128} plays a role in this system.

Especially with regard to our observation that CDKs act in a post-mitochondrial event, more likely candidates influenced either directly or indirectly by CDK activity might be proteins known to be involved in apoptosome formation, such as APAF-1, the antiapoptotic X-linked inhibitor of apoptosis protein (XIAP), and even caspase-9 itself. In favor of this assumption are our *in vitro* caspase activation experiments in which the formation of the apoptosome is initiated artificially by the addition of cytochrome *c*. Using this assay, we observed consistently a higher inducible caspase-3 activity in extracts of irradiated p21-deficient cells than in extracts of similar treated wild-type cells, suggesting the requirement for a coactivation event that only occurs in the absence of p21. Unfortunately, comparative gel filtration analyses of irradiated cells that would have yielded further insights into the composition of the apoptosome failed. In addition, Western blot analyses for the status of several apoptosome-related proteins, including XIAP and caspase-9, did not reveal any differences with regard to their expression and their migration pattern that would have indicated post-translational modifications (data not shown). Nevertheless, phosphorylation of XIAP and caspase-9 by Akt or other kinases that rendered these cells resistant to apoptosis induction was shown in previous studies (50–52). Furthermore, overexpression of XIAP and/or Akt seems to be a common mechanism to block activation of the caspase cascade downstream of the mitochondria (53), and inhibition of their expression and/or function was recently postulated to overcome chemoresistance in cells expressing wild-type p53 (54). Besides its role as a prosurvival kinase, Akt is also known to control cell cycle progression via stabilization of p21. As pharmacologic CDK inhibitors were shown to modulate Akt activity (26) and possibly also activities of other kinases, these results suggest a complex interaction network between these proteins. Hence, it is tempting to speculate that CDKs interfere with the apoptotic machinery in a similar manner, perhaps by inactivating Akt or other related kinases. This hypothesis is currently under thorough investigation in our laboratory.

In summary, we have shown that the p21-mediated protection against IR-induced apoptosis is most likely caused by inhibition of CDKs that are required for the activation of the caspase cascade downstream of mitochondria. Within this context, it is inevitable to mention that CDK inhibitors such as roscovitine have also recently entered clinical trials not only due to their antiproliferative function but also because of their strong proapoptotic effects that were, however, only observed when higher drug concentrations were used than those applied in the present study. As the outcome of such a therapeutic treatment critically depends on the inhibitor concentrations used, it is absolutely necessary to carefully evaluate the biochemical pathways involved to avoid adverse effects.

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Loss of caspase-9 reveals its essential role for caspase-2 activation and mitochondrial membrane depolarization

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Loss of Caspase-9 Reveals Its Essential Role for Caspase-2 Activation and Mitochondrial Membrane Depolarization

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Caspase-9 plays an important role in apoptosis induced by genotoxic stress. Irradiation and anticancer drugs trigger mitochondrial outer membrane permeabilization, resulting in cytochrome c release and caspase-9 activation. Two highly contentious issues, however, remain: It is unclear whether the loss of the mitochondrial membrane potential $\Delta\Psi_M$ contributes to cytochrome c release and whether caspases are involved. Moreover, an unresolved question is whether caspase-2 functions as an initiator in genotoxic stress-induced apoptosis. In the present study, we have identified a mutant Jurkat T-cell line that is deficient in caspase-9 and resistant to apoptosis. Anticancer drugs, however, could activate proapoptotic Bcl-2 proteins and cytochrome c release, similarly as in caspase-9–proficient cells. Interestingly, despite these alterations, the cells retained $\Delta\Psi_M$. Furthermore, processing and enzyme activity of caspase-2 were not observed in the absence of caspase-9. Reconstitution of caspase-9 expression restored not only apoptosis but also the loss of $\Delta\Psi_M$ and caspase-2 activity. Thus, we provide genetic evidence that caspase-9 is indispensable for drug-induced apoptosis in cancer cells. Moreover, loss of $\Delta\Psi_M$ can be functionally separated from cytochrome c release. Caspase-9 is not only required for $\Delta\Psi_M$ loss but also for caspase-2 activation, suggesting that these two events are downstream of the apoptosome.

INTRODUCTION

Cell death can occur via distinct biochemical pathways and morphological alterations, one of which is apoptosis (Leist and Jaattela, 2001). Cell death by genotoxic drugs is usually mediated by the intrinsic mitochondrial apoptotic pathway, which is regulated by pro- and anti-apoptotic proteins of the Bcl-2 family (Cory *et al.,* 2003). Activation of the proapoptotic family members Bax and Bak leads to mitochondrial outer membrane permeabilization (MOMP) and release of apoptogenic factors from the mitochondrial intermembrane space, including cytochrome c (Wang, 2001).

Another characteristic event observed during cell death is the loss of the mitochondrial transmembrane potential $\Delta\Psi_M$, the electrochemical proton gradient generated by the respiratory chain (Newmeyer and Ferguson-Miller, 2003). It is currently unclear how the loss of $\Delta\Psi_M$ contributes to the apoptotic process. Breakdown of $\Delta\Psi_M$ could be caused by opening of the permeability transition pore, which is a large protein channel spanning the outer and inner mitochondrial membrane, during a process called mitochondrial permeability transition (Zamzami and Kroemer, 2001). A second model suggests that only Bcl-2 family proteins are necessary for MOMP and cytochrome c release and that the loss of $\Delta\Psi_M$ is a downstream and caspase-dependent phenomenon (Martinou and Green, 2001; Ricci *et al.,* 2004). Currently, there is major controversy over whether the loss of $\Delta\Psi_M$ is coupled to and required for MOMP and cytochrome c release or whether loss of $\Delta\Psi_M$ is a downstream and caspase-dependent phenomenon.

Caspases are aspartate-specific cysteine proteases that are synthesized in cells as inactive zymogens containing a prodomain and a large and a small subunit (Fuentes-Prior and Salvesen, 2004). The active enzyme is composed of a heterotetramer formed by two large and two small subunits. Caspases can be divided functionally into initiator and executioner caspases (Fuentes-Prior and Salvesen, 2004). Initiator caspases, such as caspase-8, -9, and -10, are characterized by a long prodomain, containing protein–protein interaction motifs. These interaction motifs allow dimerization of the initiator caspases, which is sufficient for initial enzyme activity and autoproteolytic cleavage (Boatright *et al.,* 2003; Donepudi *et al.,* 2003; Baliga *et al.,* 2004). Activation of executioner caspases occurs by cleavage between the subunits. On processing, initiator caspases become fully active and activate downstream executioner caspases, such as caspase-3, -6, and -7, which then cleave key substrates, leading to apoptotic cell death (Fischer *et al.,* 2003).

The dimerization and activation of the initiator caspases occurs at multiprotein complexes. In death receptor pathway, caspases-8 and -10 are activated at a death-inducing signaling complex (DISC) formed upon ligand binding (Peter and Krammer, 2003). In the mitochondrial pathway caspase-9 serves as an initiator caspase. When cytochrome c is released from the mitochondrial intermembrane space, it binds to Apaf-1, leading to the recruitment and activation of caspase-9 in a high-molecular-weight complex called the apoptosome (Wang, 2001).

Similarly to caspase-8, -9, and -10, caspase-2 is characterized by a long prodomain and is thus often regarded as a bona fide initiator caspase. The cleavage specificity of caspase-2, however, is more related to effector caspases

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Abbreviations used: $\Delta\Psi_M$, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeabilization; PTP, permeability transition pore.

(Thornberry *et al.*, 1997), making it difficult to assign a function of caspase-2 as a regulatory or downstream protease. Recent reports demonstrated that caspase-2 might act as an apical protease in stress- or death receptor–mediated apoptosis (Lassus *et al.*, 2002; Robertson *et al.*, 2002; Tinel and Tschopp, 2004; Wagner *et al.*, 2004; Werner *et al.*, 2004). Moreover, it was suggested that caspase-2 is required for MOMP and the release of cytochrome c in response to DNA-damaging agents (Guo *et al.*, 2002; Robertson *et al.*, 2002; Enoksson *et al.*, 2004). Other reports suggested that caspase-2 is activated downstream of Bax and Bak and cannot bypass the apoptosome (O'Reilly *et al.*, 2002; Ruiz-Vela *et al.*, 2005). Thus, there is conflicting evidence of whether caspase-2 functions as an initiator or effector during apoptosis. Moreover, elucidation of the functional role of caspase-2 has been obscured by the lack of an overt phenotype of caspase-2 knockout mice (Bergeron *et al.*, 1998; O'Reilly *et al.*, 2002).

Here we describe the identification of a caspase-9–deficient Jurkat clone that is resistant to induction of apoptosis by genotoxic agents and activates virtually no caspase in response to DNA damage. Analysis of mitochondrial events showed that caspase-9–deficient cells released cytochrome c upon DNA damage but, interestingly, retained their mitochondrial membrane potential $\Delta\Psi_M$. When caspase-9 was reconstituted, both apoptosis and loss of $\Delta\Psi_M$ were restored, clearly suggesting that cytochrome c release and $\Delta\Psi_M$ breakdown are separate and consecutive events, with the latter being caspase-dependent. Finally, we demonstrate that during genotoxic stress caspase-2 processing as well as acquisition of its catalytic activity is dependent on caspase-9.

MATERIALS AND METHODS

Cell Lines and Reagents

All cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U 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. Anti-Bid, anti-caspase-3 mAb, and polyclonal anti-caspase-9 were purchased from R&D Systems (Wiesbaden, Germany) and New England BioLabs (Beverly, MA). Bax and cytochrome c monoclonal antibodies were obtained from PharMingen (Hamburg, Germany). The conformation-specific anti-Bak mAb was from Oncogene (La Jolla, CA). Monoclonal antibodies against β-actin and Bcl-2 as well as antisera against c-IAP1 and c-IAP2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Bim mAb was from Alexis (Grünwald, Germany) and polyclonal anti-Bcl-x_L from Transduction Laboratory (Heidelberg, Germany). The anti-caspase-8 mAb was obtained from Biocheck (Münster, Germany), and antibodies against caspase-2 were from Alexis and Santa Cruz Biotechnology. The anti-Apaf-1 polyclonal antibody was from Chemicon (Temecula, CA). Secondary antibodies, anti-mouse IgG, and anti-rabbit IgG coupled to horseradish peroxidase were purchased from Promega (Mannheim, Germany). Anti-goat IgG coupled to horseradish peroxidase was purchased from Molecular Probes (Karlsruhe, Germany) and biotin-VAD-fmk (biotin-Val-Ala-Asp-[OMe]-fluoromethylketone) from ICN (Eschwege, Germany). Staurosporine, etoposide, doxorubicin, daunorubicin, and propidium iodide (PI) were obtained from Sigma (Deisenhofen, Germany). CD95L was a gift of Dr. Harald Wajant.

Reverse Transcriptase-PCR

RNA was isolated from cells using a total RNA isolation kit (Qiagen, Hilden, Germany). Reverse transcriptase (RT) reaction and PCR were performed using the titanium one-step RT-PCR kit (BD Biosciences, Heidelberg, Germany). For standardization, each RT sample was PCR-amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The 3′ and 5′ primers used for amplification were (5′-ATG GAC GAA GCG GAT CGG) and (5′-CCC TGG CCT TAT GAT GTT-3′) for caspase-9, and (5′-GTG GAA GGA CTC ATG ACC ACA G-3′) and (5′-CTG GTG CTC AGT GTA GCC CAG-3′) for GAPDH.

Stable Expression of Caspase-9

Caspase-9–deficient Jurkat cells were electroporated with a GenePulser II (Bio-Rad, Munich, Germany) in a 0.4-cm cuvette at 250 V and 950 μF with 20 μg of DNA in 200 μl per transfection and selected for G418 resistance. The N-terminally Flag-tagged procaspase-9 construct was kindly provided by G. Salvesen.

Flow Cytometric Analyses

Cells (1×10^6 per assay) were stimulated for the indicated time in a 24-well plate with 100 μM etoposide, 1 μM doxorubicin, 1 μM daunorubicin, or 2.5 μM staurosporine or were left untreated. Apoptosis was assessed by measurement of DNA fragmentation as described previously (Schmitz *et al.*, 2004). Briefly, apoptotic nuclei were prepared in a hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, 50 μg/ml PI) and analyzed by flow cytometry. Nuclei to the left of the 2N peak, containing hypodiploid DNA, were considered apoptotic. PI uptake (2 μg/ml) into nonfixed cells was evaluated by flow cytometric analyses with the FSC/FL2 profile (Wesselborg *et al.*, 1999). Flow cytometric analysis of cytochrome c release was carried out as published previously (Waterhouse and Trapani, 2003). For measurement of the mitochondrial membrane potential, cells were incubated with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; 5 μg/ml; FL-1; Molecular Probes) for 20 min at room temperature in the dark, followed by analysis in a flow cytometer (FACScalibur, BD Biosciences). For flow cytometric analysis of Bak conformational change, cells were fixed in PBS/0.5% paraformaldehyde on ice for 30 min and subsequently washed three times in PBS/1% FCS. Staining with conformation-specific anti-Bak and isotype-matched control antibody was performed with a 1:50 dilution of the respective antibody in 50 μl staining buffer (PBS, 1% FCS, 50 μg/ml digitonin). Subsequently, cells were washed and incubated for 30 min in 50 μl staining buffer containing 0.1 μg Alexafluor 488–labeled chicken anti-mouse IgG.

Immunoblotting

Cells were lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF, and 1 μg/ml each leupeptin, antipain, chymostatin, and pepstatin A) for 15 min on ice and centrifuged (15 min, 14,000 $\times$ *g*). For Western blot analysis postnuclear supernatants, equivalent to 1×10^6 cells or 30 μg of protein, were loaded on a SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Freiburg, Germany). The membrane was blocked with 5% BSA in Tris-buffered saline (TBS)/0.2% Tween for 2 h and incubated overnight with the primary antibodies at 4°C. Membranes were washed four times with TBS/0.02% Triton X-100 and incubated with the respective peroxidase-conjugated secondary antibody for 1 h. After extensive washing, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Bioscience).

Caspase Activity Assay

Fluorogenic caspase assays were performed as described previously (Sohn *et al.*, 2005), using total cell extracts from cells treated with 2.5 μM staurosporine or 100 μM etoposide. Caspase-2-, -3-, and -9-like activities were measured using their respective substrates VDVAD-AMC, DEVD-AMC, and LEHD-AMC (Biomol, Hamburg, Germany). For the cleavage assays, 50 μg of the cell extracts was dissolved in 200 μl substrate buffer containing 50 mM HEPES, pH 7.3, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 50 μM substrate. The reaction was incubated at 37°C and measured in triplicates in a Lambda Fluro 320 Plus fluorimeter (Biotek, Bad Fridrichshall, Germany).

In Vivo Labeling of Active Caspases

To label the active site of caspases, 1×10^7 cells were incubated after apoptosis induction for an additional hour with 10 μM biotin-VAD-fmk. Cells were harvested by centrifugation and extracted in 500 μl lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DTT) containing 2 μg/ml the protease inhibitors aprotinin, leupeptin, pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The biotinylated proteins were captured on 30 μl streptavidin-conjugated agarose beads (Calbiochem, Bad Soden, Germany). After overnight rotation at 4°C the agarose beads were extensively washed in lysis buffer containing 0.5% NP-40. The biotinylated proteins were eluted from the beads by addition of 60 μl SDS-sample buffer and incubation at 95°C for 10 min. Cell extracts, 25 μg, or the eluted biotinylated proteins, 25 μl, were used for SDS-PAGE and subsequent Western blot analysis.

Transmission Electron Microscopy

Transmission electron microscopy was performed as described previously (Schwerk and Schulze-Osthoff, 2005). Briefly, treated and untreated J16 and JMR cells were harvested, washed with PBS, and fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. Cells were further processed, embedded, and prepared using standard methods. Electron micrographs were taken using a Zeiss 902 electron microscope (Jena, Germany).

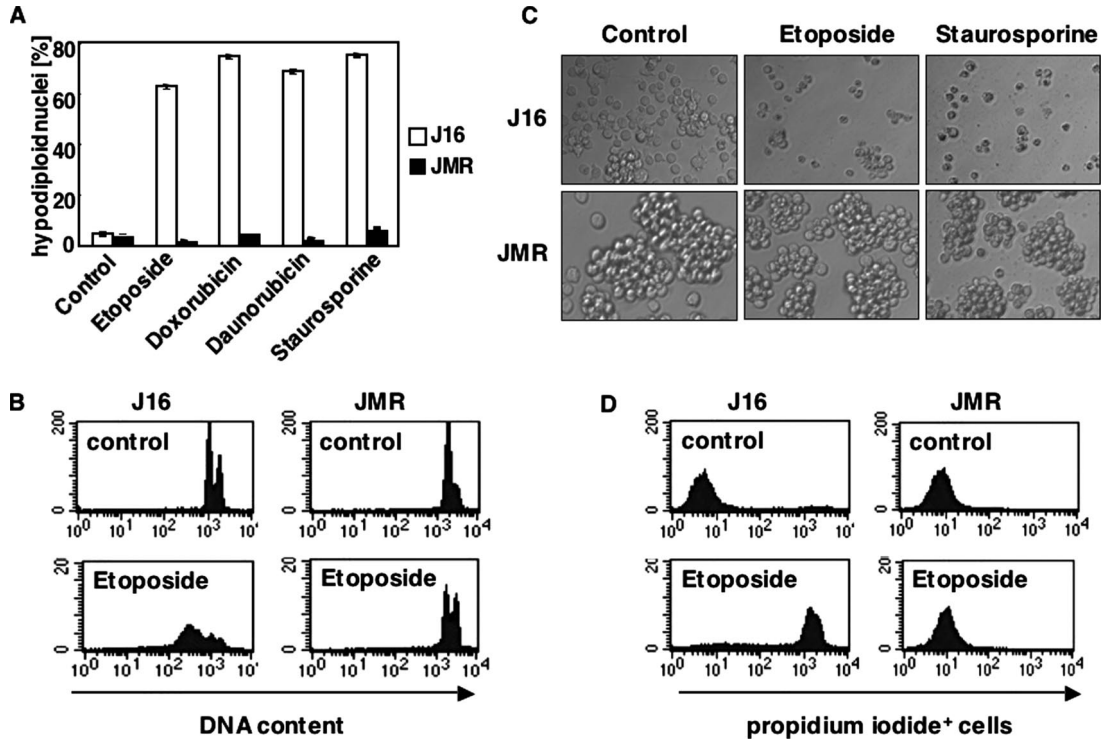

Figure 1. JMR cells are apoptosis-resistant to various inducers of genotoxic stress. (A) Induction of apoptosis: J16 and JMR cells were treated with 100 μM etoposide, 1 μM doxorubicin, 1 μM daunorubicin, or 2.5 μM staurosporine. After 24 h apoptosis was quantified by flow cytometric measurement of DNA fragmentation. The results show the means ± SD of triplicate samples and are representative of one of five independent experiments. (B) Measurement of DNA content: J16 and JMR cells were treated with 2.5 μM staurosporine for 24 h or left untreated. Subsequently, cells were analyzed for the formation of hypodiploid DNA by PI staining. (C) Light microscopic pictures of J16 and JMR cells that were treated with etoposide or staurosporine for 48 and 24 h, respectively. (D) Detection of plasma membrane damage: J16 and JMR cells were treated with 100 μM etoposide for 24 h or left untreated and then stained with PI. Flow cytometric analysis was carried out based on dye exclusion.

RESULTS

JMR Cells Are Resistant to Genotoxic Drugs

Jurkat cells are commonly used in signal transduction research of T-cells and were originally established from the peripheral blood of a 14-y-old boy with acute lymphoblastic leukemia (Schneider *et al.*, 1977). Jurkat cells are known to have a high degree of clonal heterogeneity (Parson *et al.*, 2005), and various subclones have been generated that are deficient in the expression of certain signaling molecules (Abraham and Weiss, 2004). We screened several clones of Jurkat cells obtained from ATCC (Manassas, VA) and other laboratories for their apoptosis sensitivity toward anticancer drugs. To this end, cells were treated for 20 h with 100 μM etoposide, 1 μM doxorubicin, 1 μM daunorubicin, or 2.5 μM staurosporine. Subsequent flow cytometric analyses identified a clone, designated JMR, which was completely resistant to apoptosis induction (Figure 1A). In contrast to the sensitive J16 Jurkat cells, JMR cells did not reveal apoptotic DNA fragmentation, but were resistant to all drugs tested (Figure 1A). However, JMR cells did sense noxious stimuli, as etoposide treatment resulted in accumulation of G2-arrested cells (Figure 1B).

In addition to DNA fragmentation, cells were analyzed by light microscopy for morphological alterations. J16 cells treated with etoposide or staurosporine showed typical apoptotic morphological changes, such as cell shrinkage, nuclear condensation, and membrane blebbing (Figure 1C). In contrast, JMR cells retained a normal morphology upon drug treatment, as did untreated cells, indicating that JMR cells are resistant to genotoxic stress. To exclude necrosis as an alternative response, both cell lines were analyzed for membrane damage by the uptake of PI. In contrast to J16 cells, JMR cells excluded PI even after prolonged exposure to etoposide (Figure 1D).

JMR Cells Express Normal Levels of Bcl-2 and IAP Family Proteins, but Are Devoid of Caspase-9

Bcl-2 proteins are the major regulators of the mitochondrial apoptotic pathway, and their deregulated expression might result in altered sensitivity toward genotoxic drugs. Therefore, immunoblot analyses were carried out for the proapoptotic multidomain Bcl-2 family members Bak and Bax as well as for the anti-apoptotic proteins Bcl-2 and Bcl-x_L. As shown in Figure 2A, JMR cells and J16 cells expressed equivalent amounts of Bcl-2 and Bcl-x_L. Moreover, there were no significant differences in the expression levels of Bax and Bak between the two Jurkat cell clones. Another subgroup within the Bcl-2 family represents the proapoptotic BH3-only proteins, e.g., Bim and Bid. Western blot analyses showed no alterations in the expression of Bim and Bid in JMR cells, compared with J16 cells (Figure 2A). Thus, differences in the expression levels of Bcl-2 family proteins are presumably not responsible for the apoptosis-resistant phenotype of JMR cells.

We compared also the expression levels of inhibitor of apoptosis proteins (IAPs), which directly bind to and thereby inhibit active caspases (Deveraux and Reed, 1999). The expression of cIAP1, cIAP2, and XIAP did not differ

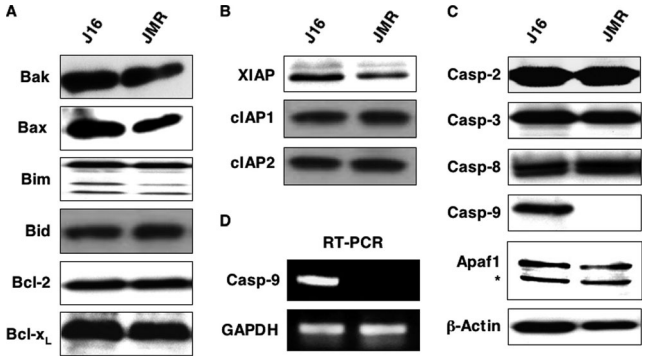

Figure 2. JMR cells are deficient of caspase-9. Total cell extracts of J16 and JMR cells were prepared and analyzed by Western blotting for (A) the Bcl-2 family members Bak, Bax, Bim, Bid, Bcl-2 and Bcl-x_L, for (B) the IAP family members cIAP1, cIAP2, and XIAP, and (C) for caspase-2, -3, -8, and -9 and Apaf-1. Equal protein loading of the blots was confirmed by staining for β-actin. (D) RNA was extracted from J16 and JMR cells and subjected to RT-PCR using oligonucleotide primers specific for caspase-9. The PCR products were separated on agarose gels and visualized by ethidium bromide staining. A GAPDH PCR product that was amplified in parallel served as a loading control.

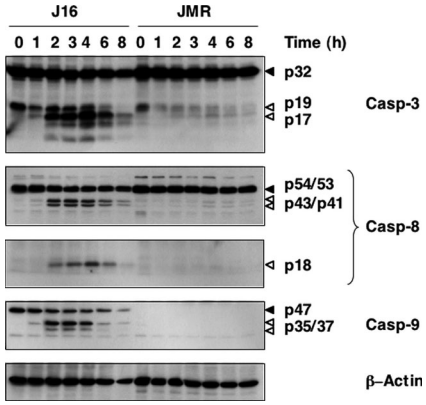

Figure 3. Caspase activation is severely impaired in JMR cells. J16 and JMR cells were stimulated with 2.5 μM staurosporine for the indicated times. Total cell extracts were separated by SDS-PAGE and blotted for caspase-3 (top panel), -8 (middle panel), and -9 (bottom panel). Closed and open arrowheads, the zymogens and the cleaved forms of the individual caspases, respectively. Blots were probed for β-actin as a loading control.

between the two Jurkat clones (Figure 2B), indicating that the resistance of JMR cells was not due to up-regulation of IAPs. Another molecular hallmark of apoptosis is the activation of caspases. Caspase-2 and -3 as well caspase-8 were expressed at comparable levels in J16 and JMR cells. Striking, however, was the fact that expression of caspase-9, the essential initiator caspase in the mitochondrial pathway, was completely absent in JMR cells (Figure 2C). In contrast, Apaf-1, another essential component of the apoptosome, was equally expressed in J16 and JMR cells (Figure 2C). RT-PCR analysis showed that JMR cells were not only devoid of the caspase-9 protein, but also of its mRNA expression (Figure 2D). Therefore, resistance of JMR cells to apoptosis correlates with a genetic deficiency of caspase-9.

Staurosporine-mediated Caspase Activation Is Severely Impaired in JMR Cells

To investigate whether caspase-9 deficiency affects the activation of other caspases, J16 and JMR cells were treated with staurosporine for different time periods. Subsequently, immunoblot analyses were carried out for caspase-3, -8, and -9. A strong activation of all three caspases was observed in J16 cells within 2 h of staurosporine treatment, as shown by the appearance of the respective cleavage fragments of the individual caspases (Figure 3). In contrast, no proteolytic processing of caspase-3 or -8 was seen in caspase-9–deficient JMR cells (Figure 3). Caspase activation was also absent in JMR cells when measured by fluorescent substrate cleavage assays (see below). Taken together, these results suggest that Jurkat cells depend on caspase-9 for the execution of apoptosis induced by stimulation of the intrinsic death pathway.

Although staurosporine-treated JMR cells appeared morphologically normal as assessed by light microscopy (Figure 1C) and did not show any signs of necrosis, we wanted to investigate whether the cellular ultrastructure was affected by drug treatment. To this end, J16 and JMR cells were treated overnight with staurosporine and subjected to transmission electron microscopy. J16 cells were completely dismantled and showed highly condensed chromatin (Figure 4). JMR cells, in contrast, appeared normal except that staurosporine-treated cells contained slightly enlarged nuclei (Figure 4). Importantly, no vacuoles were observed suggesting that caspase-9–deficient JMR cells do not switch to autophagy upon staurosporine treatment.

Upstream Mitochondrial Apoptotic Events Are Not Altered in JMR Cells

Because activation of the caspase cascade was impaired in JMR cells, we investigated whether events further upstream

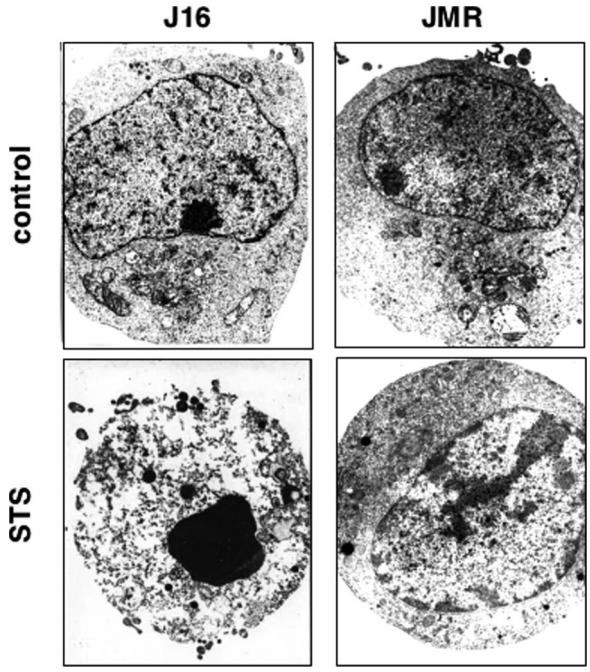

Figure 4. Preserved ultrastructure of JMR cells after drug treatment. J16 and JMR cells were treated for 24 h with 2.5 μM staurosporine or left untreated. Subsequently, cells were analyzed by electron microscopy. Note the pronounced chromatin condensation in staurosporine-stimulated J16 cells that is completely absent in JMR cells.

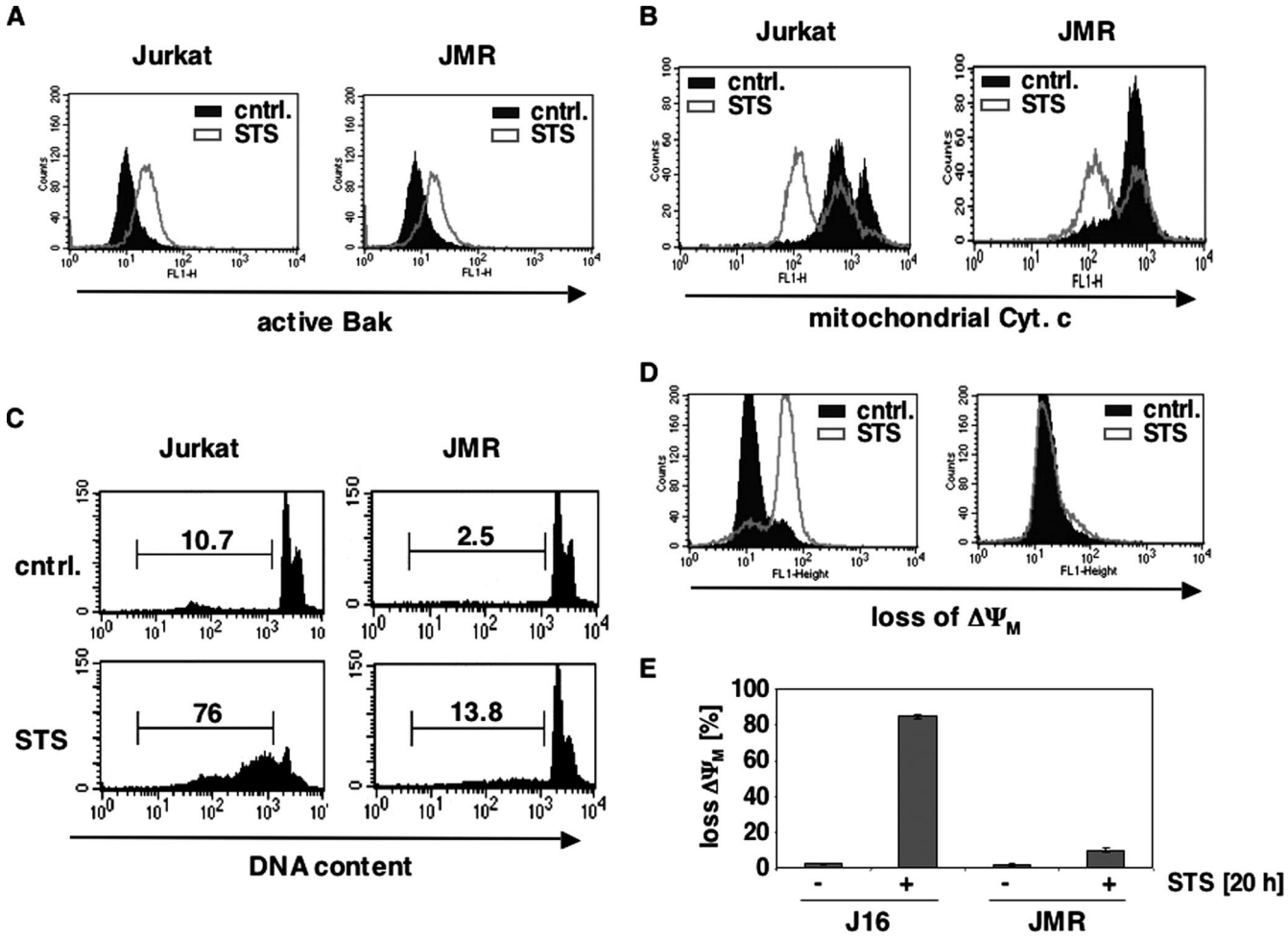

Figure 5. Mitochondrial membrane permeabilization, but not Bak activation or cytochrome c release requires caspase-9. (A) Measurement of Bak activation: J16 and JMR cells were treated for 2 h with 2.5 μM staurosporine (open histograms) or left untreated (filled histograms). Subsequently, cells were permeabilized with digitonin, incubated with a conformation-specific Bak antibody, and analyzed by flow cytometry. No staining was detected with an isotype-matched control antibody. (B) Detection of cytochrome c release: Cells were stimulated as described in A and analyzed for cytochrome c release in a flow cytometer. Filled histograms, the staining of the untreated cells; open histograms, the results of the staurosporine-treated cells. (C) Measurement of DNA fragmentation: J16 and JMR cells were treated in parallel with 2.5 μM staurosporine for 24 h and monitored for the formation of hypodiploid DNA. (D) Measurement of $\Delta\Psi_M$ loss: Cells were treated as in A, stained with JC1 and analyzed for the loss of the $\Delta\Psi_M$ by flow cytometry. (E) J16 and JMR cells were treated for 20 h with 2.5 μM staurosporine and $\Delta\Psi_M$ was measured as in D.

in the mitochondrial pathway were also affected by caspase-9 deficiency. Mitochondrial apoptosis is regulated by the multidomain Bcl-2 proteins Bax or Bak, which undergo a N-terminal conformational change, resulting in their homo-oligomerization and the subsequent release of pro-apoptotic proteins from the mitochondrial intermembrane space (Cory *et al.*, 2003). The conformational change and activation of Bak can be analyzed with specific antibodies against their normally occluded N-terminus. Flow cytometric analysis with such antibodies revealed that upon staurosporine treatment Bak was activated not only in J16 cells, but also in JMR cells (Figure 5A). Next, we analyzed whether activation of Bak leads to permeabilization of mitochondria and cytochrome c release. As assessed by flow cytometry, J16 cells as well as JMR cells released cytochrome c within 2 h upon treatment with staurosporine (Figure 5B). Therefore, apoptotic events upstream of the apoptosome proceed normally in JMR cells. To validate that JMR cells were resistant to staurosporine treatment in this experiment, cells were incubated for an additional 6 h to monitor DNA fragmentation. Indeed, despite the fact that mitochondria were permeabilized, JMR cells did not exhibit significant DNA fragmentation (Figure 5C).

$\Delta\Psi_M$ of JMR Cells Is Not Altered during Genotoxic Stress

In addition to the release of proapoptotic proteins from the intermembrane space, the loss of $\Delta\Psi_M$ is a typical and early event during apoptosis (Zamzami *et al.*, 1995, 1996). Disruption of $\Delta\Psi_M$ suggests that the integrity of the inner mitochondrial membrane is affected. Therefore, we measured $\Delta\Psi_M$ as an additional parameter for mitochondrial integrity. To this end, J16 and JMR cells were stimulated with staurosporine for 2 h and then stained with the $\Delta\Psi_M$-sensitive dye JC-1. As assessed by flow cytometry, a rapid loss of $\Delta\Psi_M$ could be observed in J16 cells upon stimulation with staurosporine. In contrast, $\Delta\Psi_M$ did not decrease in JMR cells (Figure 5D). Even after prolonged treatment with staurosporine or etoposide, the $\Delta\Psi_M$ remained intact in JMR cells (Figure 5E). Thus, caspase-9 deficiency seems to uncouple

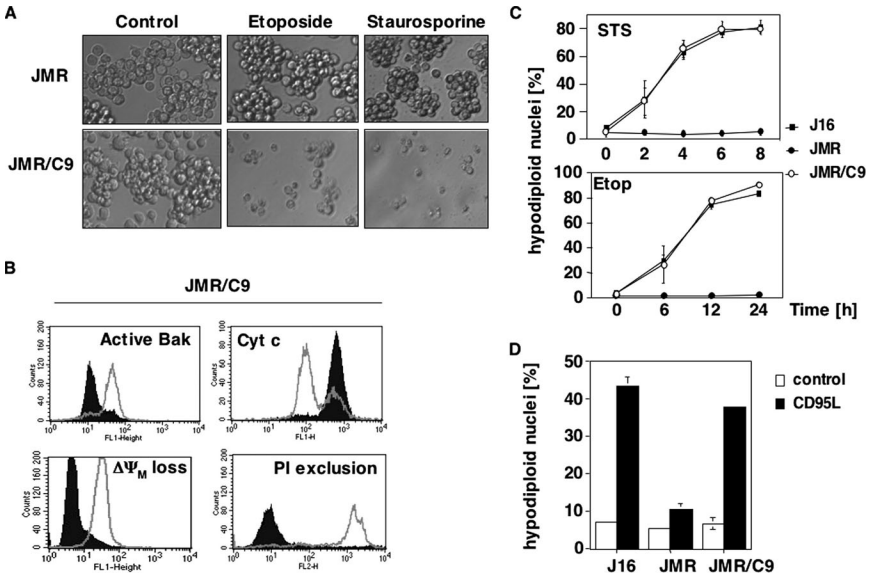

Figure 6. Reexpression of caspase-9 in JMR cells restores apoptosis and $\Delta\Psi_M$ loss. (A) JMR cells and caspase-9–reconstituted JMR/C9 cells were treated with 2.5 μM staurosporine or 100 μM etoposide for 24 and 48 h, respectively. Light microscopic pictures were taken at a 200-fold magnification. (B) Caspase-9–reconstituted JMR/C9 cells were treated with 2.5 μM staurosporine for 2 h (open histograms) or left untreated (closed histograms) and analyzed for Bak activation (top left panel), cytochrome c release (top right panel), mitochondrial membrane potential (bottom left panel) and PI exclusion (bottom right panel). The analyses were performed by flow cytometry as described in Figure 4. (C) J16, JMR, and JMR/C9 cells were treated with 2.5 μM staurosporine or 100 μM etoposide for the indicated time points. Apoptosis was assessed by measurement of DNA fragmentation and flow cytometry. The results show the means ± SEM of two independent experiments. (D) J16, JMR, and JMR/C9 cells were treated for 16 h with 2 ng/ml CD95L (■) or left untreated (□). Apoptosis was measured as in C.

cytochrome c release and loss of $\Delta\Psi_M$ as two separate events in apoptosis.

Stable Transfection of Caspase-9 Restores Sensitivity for Genotoxic Stress in JMR Cells

To verify that the resistance to anticancer drugs in JMR cells was due to the absence of caspase-9, JMR cells were stably transfected with a caspase-9 expression construct. When treated with etoposide or staurosporine, caspase-9–reconstituted JMR/C9 cells showed cell shrinkage, nuclear condensation, membrane blebbing, and eventual demise comparable to J16 cells (Figure 6A). On incubation with staurosporine for 2 h, caspase-9–reconstituted JMR cells displayed Bak activation, cytochrome c release and, importantly, also showed a rapid loss of $\Delta\Psi_M$ (Figure 6B), similar to J16 cells (see Figure 5). After 8 h of treatment the majority of caspase-9–retransfected cells became PI-positive and underwent cell death (Figure 6B). Flow cytometric analyses of DNA fragmentation also indicated that apoptosis proceeded with comparable kinetics in J16 and JMR/C9 cells, while the parental JMR cells showed no signs of DNA fragmentation upon treatment with staurosporine or etoposide (Figure 6C).

In addition, we investigated whether the absence of caspase-9 had any influence on death receptor-mediated apoptosis. To this end, we treated J16, JMR, and JMR/C9 cells with CD95L. Interestingly, CD95L-induced apoptosis was severely impaired in JMR cells (Figure 6D), which is in consistent with the fact that Jurkat cells are type II cells (Scaffidi *et al.*, 1998).

To analyze caspase activation in J16, JMR, and JMR/C9 cells, fluorescent substrate cleavage assays were performed with extracts from cells treated with either staurosporine or etoposide for different periods of time. Caspase-3-like (DEVDase) activity was observed in lysates from J16 and JMR/C9 cells after etoposide as well as after staurosporine treatment (Figure 7A). This was also true for caspase-9-like (LEHDase) activities. Even after prolonged stimulation with staurosporine or etoposide, JMR cells showed caspase activities for both substrates that were even lower than the background levels in unstimulated J16 and JMR/C9 cells (Figure 7A). Activation of caspases in response to etoposide or staurosporine treatment was also tested by immunoblotting. Western blot analysis showed proteolytic activation of caspase-9 in JMR/C9 cells upon etoposide treatment. The cleaved forms, p37 and p35, appeared with kinetics similar to those of J16 cells (Figure 7B). In addition, caspase-3 activation, as shown by the appearance of its processed p19 and p17 fragments, was similar in J16 and JMR/C9 cells, but absent in JMR cells (Figure 7B). Activation of caspase-9 and -3 was observed also in JMR/C9 cells, but not in parental JMR cells when treated with staurosporine (Figure 7C). Thus, the presence of caspase-9 is essential for activation of downstream effector caspases such as caspase-3.

Activation of Caspase-2 Is Dependent on Downstream Caspase Activation

Recently, an initiator role has also been suggested for caspase-2 in stress-induced apoptosis (Lassus *et al.*, 2002; Robertson *et al.*, 2002), whereas other reports proposed that caspase-2 acts downstream of mitochondria. Because caspase-9–deficient cells represent an ideal system to analyze whether caspase-2 activation occurs upstream or downstream of apoptosome function, we treated J16, JMR, and JMR/C9 cells with etoposide and analyzed caspase-2 processing. Procaspase-2 was processed into the p32 intermediate and the p19 active fragments in J16 and JMR/C9 cells, but not in the parental JMR cell line (Figure 8A). Additionally, analysis of caspase-2 cleavage activity using the fluorometric substrate VDVAD-AMC, confirmed the lack of caspase-2 activation in JMR cells, but revealed significant caspase-2 activity in J16 and JMR/C9 cells during etoposide-induced apoptosis (Figure 8B).

Recent investigations suggested that dimerization, but not proteolytic cleavage is an initial event for the activation of caspase-2 and other initiator caspases (Baliga *et al.*, 2004). To examine whether caspase-2 was activated in the absence of proteolytic cleavage, we used an in vivo affinity labeling approach, using the biotinylated caspase inhibitor biotin-VAD-fmk that detects only active caspases. J16, JMR, and JMR/C9 cells were treated with etoposide for 6 h and incubated the cells with biotin-VAD-fmk for an additional hour. The affinity-labeled caspases were then precipitated with streptavidin agarose beads and immunoblotted for caspase-2 (Figure 8C). A moderate labeling of full-length caspase-2, which contains a low proteolytic activity compared with the processed caspase-2 (Baliga *et al.*, 2004), was detected in un-

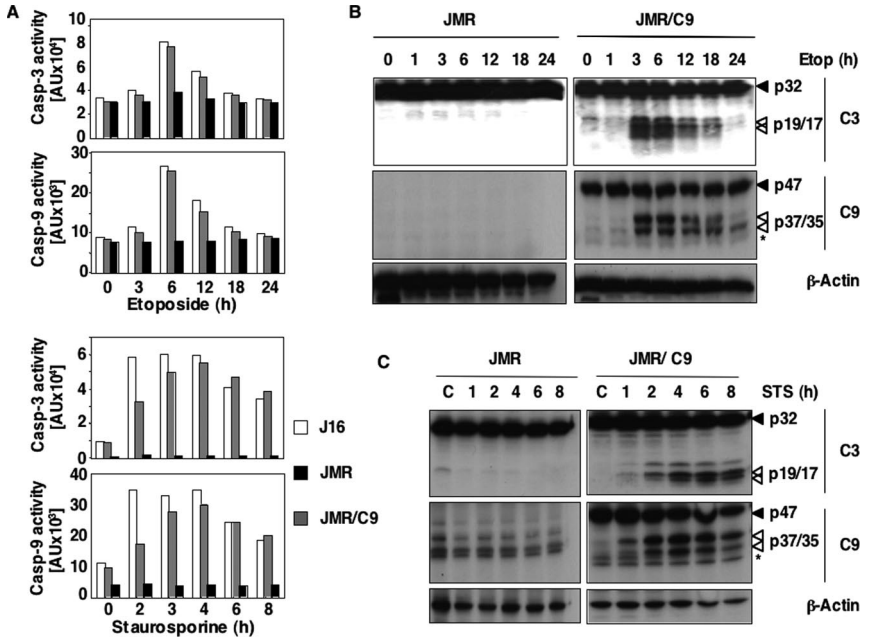

Figure 7. Reconstitution of caspase-9 restores caspase processing and activation in JMR cells. (A) J16 (□), JMR cells (■), and caspase-9–reconstituted JMR/C9 cells (▨) were treated for the indicated time with 100 μM etoposide (top panels) or 2.5 μM staurosporine (bottom panels). Cellular extracts were then subjected to fluorescence-based enzyme assays for caspase-3-like activities with the substrate DEVD-amc, and for caspase-9-like activities with the substrate LEHD-amc. (B) JMR and JMR/C9 cells were treated with 100 μM etoposide for the indicated time points. Total cell extracts were separated by SDS-PAGE and immunoblotted for caspase-3 (top panel) and -9 (middle panel). (C) JMR and JMR/C9 cells were treated with 2.5 μM staurosporine for the indicated times and blotted for caspase-3 (top panel) and -9 (middle panel). The asterisk denotes a nonspecific protein that was detected by the antibody. Blots were probed for β-actin as a loading control (bottom panels in B and C). Closed and open arrowheads, the zymogen forms and cleaved forms of the indicated caspases, respectively.

stimulated J16, JMR/C9, and JMR cells and might be explained by spontaneous dimerization of caspase-2 during the experimental procedure (Tinel and Tschopp, 2004). Biotin-VAD-fmk–labeled unprocessed procaspase-2 was observed in etoposide-treated JMR cells as well. However, the amount of biotin-VAD-fmk–labeled caspase-2 was similar in untreated and etoposide-treated JMR cells, suggesting that this caspase-2 activation was not related to genotoxic stress. Importantly, biotin-VAD-fmk–labeled and hence fully active caspase-2 fragments were detected in etoposide-treated J16 and JMR/C9 cells, but not in untreated cells, as shown by appearance of the precipitated p19 fragment (Figure 8C). No labeling of active caspase-2 cleavage fragments was observed in caspase-9–deficient JMR cells, indicating that caspase-2 was not activated in the cells (Figure 8C). In agreement with our previous findings, active caspase-3 fragments (p21/p19/p17/p12) were detected in precipitates from etoposide-treated J16 and JMR/C9 cells, but not in the parental JMR cells. Next, we asked whether caspase-2 could be activated in JMR cells by other inducers of cytotoxic stress. To this end, J16 and JMR cells were treated with doxorubicin and staurosporine (Figure 8D). The active p19 fragment of caspase-2 was observed in caspase-9–proficient J16 cells but not in caspase-9–deficient JMR cells upon doxorubicin and

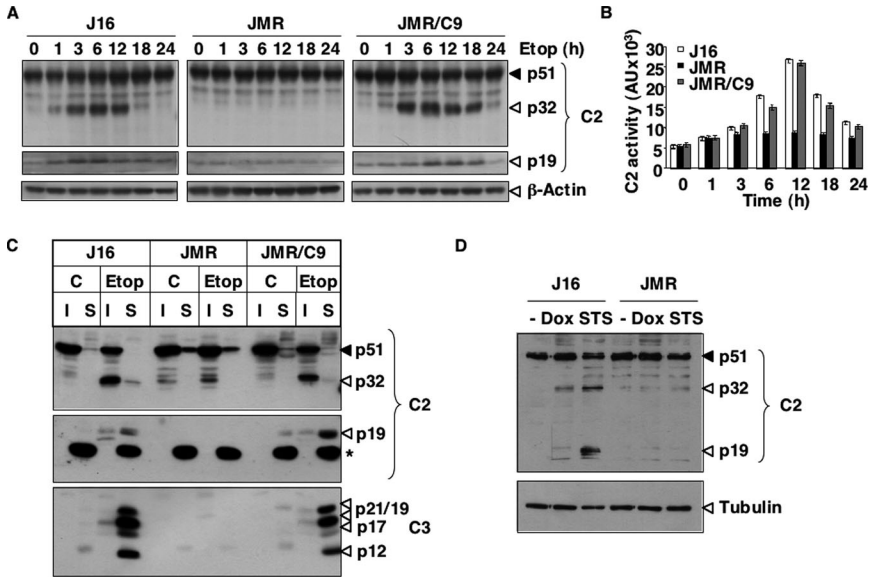

Figure 8. Genotoxic stress-induced caspase-2 processing and activation is dependent on caspase-9. (A) Proteolytic processing of caspase-2: J16, JMR, and JMR/C9 cells were treated with 100 μM etoposide for the indicated time, before cells extracts were prepared and immunoblotted for caspase-2. Arrowheads indicate the proform and the p32 and p19 proteolytic fragments of caspase-2. β-actin served as a loading control. (B) Detection of caspase-2-like activity. Cells were treated with etoposide for the indicated time, and caspase-2 activity was measured in a fluorimeter by incubating the cell extracts with the substrate VDVAD-amc. The results show the means ± SD of triplicate samples and are representative of one of more than two independent experiments. (C) In vivo peptide affinity labeling of active caspases: Each, 1×10^7 J16, JMR, and JMR/C9 cells were left untreated (C) or incubated with 100 μM etoposide (Etop) for 6 h. After the incubation, active caspases were labeled in vivo by the addition of biotin-VAD-fmk for 1 h. Cell extracts were prepared and the biotinylated proteins were captured with streptavidin agarose beads. The streptavidin beads (S) and 25 μg protein from the input material (I) were analyzed by SDS-PAGE and assayed for the activation of caspase-2 and -3 by Western blotting. Closed and open arrowheads, the zymogens and active forms of the caspases, respectively. The asterisk denotes a nonspecific protein that was detected by the caspase-2 antibody. (D) J16 and JMR cells were treated for 8 h with 1 μM doxorubicin or 1 μM staurosporine or were left untreated. Cellular lysates were analyzed by Western blot with a caspase-2–specific antibody. Anti-tubulin was used as a loading control.

staurosporine treatment. These results therefore clearly demonstrate that caspase-2 is not activated in the absence of caspase-9, but rather requires the apoptosome pathway for genotoxic stress-induced apoptosis.

DISCUSSION

In this study, we identified a Jurkat T-cell line deficient in caspase-9 expression that is, to our knowledge, the first caspase-9–lacking tumor cell line and thus represents an excellent functional complementation to the previously described caspase-8-deficient Jurkat cells (Juo *et al.*, 1998). Short tandem repeat DNA profiling verified that the caspase-9–deficient JMR cells were derived from Jurkat cells (data not shown). Using JMR cells we show that caspase-9 is indispensable for genotoxic stress-induced apoptosis. This is consistent with the initial characterization of fibroblasts or embryonic stem cells from caspase-9 and Apaf-1 null mice that are resistant to a broad range of cytotoxic agents (Cecconi *et al.*, 1998; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998; Soengas *et al.*, 1999). Nevertheless, subsequent studies hinted at the possibility that additional, context-specific pathways eventually exist that could operate in the absence of either caspase-9 or Apaf-1. Caspase-9 activation was found to be uncoupled from Apaf-1 in Sendai-virus induced apoptosis (Bitzer *et al.*, 2002). Furthermore, Apaf-1–deficient fibroblasts but not myoblasts were protected from apoptosis induced by cytotoxic drugs or E2F1 overexpression (Ho *et al.*, 2004). Marsden *et al.* (2002) detected marginal caspase-7 activation in caspase-9– and Apaf-1–deficient thymocytes and speculated that caspase-7 activation might be due to an alternative intrinsic pathway that bypasses the apoptosome. Although different Apaf-1–related molecules exist, the identity of such alternative pathways remains unknown. The caspase-9–deficient Jurkat line JMR will certainly prove to be useful for further analysis of apoptosis signaling pathways in the absence of apoptosome function.

We used JMR cells to address two highly contentious issues of apoptosis regulation. First, we show that, unlike cytochrome c release, loss of $\Delta\Psi_M$ is dependent on caspase activation in vivo. The loss of caspase-9 allowed us to clearly separate cytochrome c release from the decrease of $\Delta\Psi_M$, suggesting that loss of $\Delta\Psi_M$ and mitochondrial outer membrane permeabilization (MOMP) are two independent and consecutive events. Although it is well established that anti-apoptotic Bcl-2 members control MOMP, there is a major controversy between the interrelationship of MOMP and loss of $\Delta\Psi_M$. Several reports suggested that both events are coupled and that even cytochrome c release is dependent on the loss of $\Delta\Psi_M$ (Zamzami *et al.*, 1995; Zamzami and Kroemer, 2001). Also the role of caspases in both processes has been unclear. It was reported that both, cytochrome c release and mitochondrial depolarization, depend on caspase activation (Marsden *et al.*, 2002, 2004), whereas cytochrome c release was also demonstrated to occur earlier than caspase activation and mitochondrial membrane depolarization (Bossy-Wetzel *et al.*, 1998). Interestingly, blocking caspase activity by zVAD-fmk prevented loss of $\Delta\Psi_M$, but not cytochrome c release. In fact it was shown that this disruption of mitochondrial function might be caused by the caspase-3–mediated cleavage of a 75-kDa subunit of complex I in the electron transport chain (Ricci *et al.*, 2003, 2004).

The opposing data have led to two models of mitochondrial permeabilization during apoptosis. In one model, MOMP has been suggested to occur by the opening of the permeability transition pore (PTP), a channel spanning the outer and the inner mitochondrial membrane. This PTP model suggests that the release of cytochrome c and the loss of $\Delta\Psi_M$ are coupled events (Zamzami and Kroemer, 2001). The other model implies that only the outer mitochondrial membrane is permeabilized by proapoptotic Bcl-2 proteins, whereas mitochondrial membrane depolarization is a secondary event. It should be noted that most of these studies were done in cell-free systems or in permeabilized cells. Our data strongly argue against the PTP model and support a model in which the permeabilization of the outer membrane is sufficient for cytochrome c release without the requirement of additional events, including the loss of $\Delta\Psi_M$. Moreover, because cytochrome c release occurred with similar kinetics in JMR and J16 cells, our data also argue against a caspase-dependent amplification loop of cytochrome c release. In agreement with our results, it was shown that in granzyme B–induced apoptosis a transient loss of $\Delta\Psi_M$ could be regenerated by zVAD-fmk, despite the fact that cytochrome c had been released into the cytosol (Waterhouse *et al.*, 2006).

We further used caspase-9–deficient JMR cells to investigate the role of caspase-2 in genotoxic stress-induced apoptosis. Some reports had suggested that caspase-2 plays a crucial role in DNA-damage-induced apoptosis (Lassus *et al.*, 2002; Robertson *et al.*, 2000). Moreover, a recent study showed that caspase-2 is recruited to and activated at the CD95 DISC (Lavrik *et al.*, 2006). Supportive for an initiator function is the sequence of caspase-2, which contains a CARD motif that might assist dimerization of caspase-2 upon interaction with different adapter proteins including RAIDD or others (Read *et al.*, 2002; Baliga *et al.*, 2004; Zhivotovsky and Orrenius, 2005). Caspase-2 has also been suggested to mediate the function of p53, which transcriptionally activates the death domain protein PIDD (Tinel and Tschopp, 2004). Up-regulated PIDD, together with RAIDD or RIP1, can form a multiprotein complex, called the PIDDosome, which activates either caspase-2 or transcription factor NF-κB (Tinel and Tschopp, 2004; Janssens *et al.*, 2005). Biochemical studies using RNAi or antisense strategies placed caspase-2 upstream of mitochondria and cytochrome c release (Lassus *et al.*, 2002; Robertson *et al.*, 2002; Lin *et al.*, 2004). In line with this, it was shown that recombinant caspase-2 was able to release cytochrome c in a Bcl-2-independent manner in permeabilized cells (Enoksson *et al.*, 2004; Robertson *et al.*, 2004). However, it cannot be excluded that recombinant caspase-2 activates other caspases in permeabilized cells or, by its ability to activate Bid, mediates cytochrome c release. Surprisingly, although one study showed that chemically inactivated caspase-2 still released cytochrome c (Robertson *et al.*, 2004), this was not observed with genetically inactivated caspase-2 (Enoksson *et al.*, 2004). Therefore, it remains unclear whether caspase-2 is directly involved in cytochrome c release. It is noteworthy that most studies suggesting a requirement of caspase-2 in stress-induced apoptosis used a single and identical small-interfering (si) RNA sequence (Lassus *et al.*, 2002; Lin *et al.*, 2004, 2005). A recent correction to one of these studies, however, pointed out that other siRNA sequences that reduced caspase-2 levels in a similar manner in the same cell type failed to influence cell death induced by genotoxic stress (Lassus *et al.*, 2004).

Unlike caspase-8 or -9, caspase-2 obviously does not directly cleave another mammalian caspase, aside from its own precursor (Ricci *et al.*, 2003). However, procaspase-2 is efficiently cleaved by caspase-3 in vitro (Slee *et al.*, 1999), and in many experimental systems caspase-2 cleavage is detected downstream of caspase-3. For instance, it was shown that caspase-2 processing depends on caspase-3 and -9 during UV- and TNF-α–induced apoptosis (Paroni *et al.*, 2001). Moreover, expression of dominant-negative caspase-9 inhib-

ited caspase-2 activation upon stimuli of the intrinsic, but not the extrinsic pathway (Werner *et al.*, 2004). In line with this, no caspase-2 processing has been observed in irradiated thymocytes from Apaf-1 or caspase-9 knockout mice (O'Reilly *et al.*, 2002) or from Bax/Bak double-deficient fibroblasts (Ruiz-Vela *et al.*, 2005). Although most of these previous studies investigated caspase-2 processing as a measure of its activation, our experiments using peptide affinity labeling clearly suggest that caspase-2 also fails to acquire catalytic activity in the absence of caspase-9. Interestingly, a recent study (Tu *et al.*, 2006) failed to observe activation of caspase-2 in several settings where it had been implicated, including genotoxic stress. In contrast, caspase-2 was identified as a specific initiator caspase for heat-shock–induced apoptosis, in which it mediated MOMP and caspase-3 activation in a strictly Bid-dependent manner (Bonzon *et al.*, 2006; Tu *et al.*, 2006).

Thus, our data demonstrate that caspase-2 cannot bypass the apoptosome, but depends on caspase-9. Certainly, we cannot exclude the possibility that the role of caspase-2 in genotoxic stress-induced apoptosis might be cell type– or stimulus–specific; however, studies proposing an initiator role of caspase-2 were also performed in Jurkat cells using the same apoptotic stimuli as in our study (Robertson *et al.*, 2002; Lin *et al.*, 2004; Tinel and Tschopp, 2004). It might be argued that a minor amount of caspase-2 activation is sufficient to trigger the caspase cascade. We consider this possibility unlikely, because overexpression of caspase-2 is generally required to induce cell death (Baliga *et al.*, 2004). Recently, it was reported that caspase-2 is an activator of NF-κB and p38 kinase (Lamkanfi *et al.*, 2005), suggesting that caspase-2 might act as a proinflammatory rather than as an apoptotic caspase. This assumption would not only be consistent with the lack of an apoptotic phenotype in caspase-2 null mice (Bergeron *et al.*, 1998), but also with the sequence of caspase-2 that is more closely related to the proinflammatory than to the proapoptotic initiator caspases (Lamkanfi *et al.*, 2002).

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Kapitel 7
Anhang

7. Anhang

7.1 Lebenslauf

Dennis Sohn	geboren am 05.05.1977 in Düsseldorf
1983-1987	Besuch der Städt. Gemeinschaftsgrundschule Lennéstrasse Düsseldorf
1987-1996	Besuch des Leibniz-Gymnasium in Düsseldorf Abschluss Abitur
1996-1997	Zivildienst in der MNR-Klinik der Universitätskliniken Düsseldorf.
1997-2003	Studium der Biochemie an der Ruhr-Universität Bochum
2000	Vordiplom der Biochemie
2002-2003	Diplomarbeit in der Abteilung für Medizinische Mikrobiologie (Prof S. Gatermann) der Ruhr-Universität-Bochum, Thema: „Komplementation einer autolysin-defekten Mutante von *Staphylococcus saprophyticus*“
2003	Diplom der Biochemie
2003-2007	Doktorarbeit am Institut für Molekulare Medizin (Prof. Klaus Schulze-Osthoff) an der Heinrich-Heine-Universität-Düsseldorf, Thema: „Mechanismen der apoptotischen Signaltransduktion: Modulation der Aktivierung von Initiator-Caspasen“

7.2 Publikationsliste

Sohn D, Schulze-Osthoff K, Jänicke RU. Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. *J Biol Chem*, 2005 Feb 18; 280(7):5267-73. Epub 2004 Dec 15

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Jänicke RU, **Sohn D**, Totzke G, Schulze-Osthoff K. Caspase-10 in mouse or not? *Science*, 2006 Jun 30; 312(5782):1874

Sohn D, Essmann F, Schulze-Osthoff K, Jänicke RU. p21 blocks irradiation-induced apoptosis downstream of mitochondria by inhibition of cyclin-dependent kinase-mediated caspase-9 activation. *Cancer Research*, 2006 Dec 1; 66(23):11254-62

Samraj AK, **Sohn D**, Schulze-Osthoff K, Schmitz I. Loss of caspase-9 reveals its essential role for caspase-2 activation and mitochondrial membrane depolarization. *Mol Biol Cell*, 2007 Jan; 18(1):84-93. Epub 2006 Nov 1

Poster:

Sohn D, Totzke G, Schulze-Osthoff K, Jänicke RU. Proteasomale Aktivität ist für eine effiziente Initiierung der Todesrezeptor-vermittelten Apoptose erforderlich.
2. Platz bei der Posterpreisvergabe am "Tag des wissenschaftlichen Nachwuchs" der Heinrich-Heine Universität Düsseldorf, 24./25.06.2005

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14th Euroconference on Apoptosis (European Cell Death Organization), Sardinia, Italy "Death or survival? Fate in Sardinia", 30.09. - 04.10.2006

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der hier vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den

Dennis Sohn