Characterisation of Nuclear Events in Apoptosis by a Comprehensive Proteome Approach

Dissertation

zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften des Fachbereichs für Biologie der Universität Konstanz

vorgelegt von
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Tag der mündlichen Prüfung: 20.12.2004
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DANKSAGUNG

Die vorliegende Arbeit wurde im Zeitraum von August 2001 bis Juli 2004 unter der Leitung von Herrn Prof. Dr. Alexander Bürkle am Lehrstuhl für Molekulare Toxikologie im Fachbereich Biologie an der Universität Konstanz angefertigt. Die Proteomanalysen wurden zwischen Oktober 2002 und September 2003 in der Arbeitsgruppe für Proteinanalytik von Herrn Prof. Dr. Friedrich Lottspeich am Max-Plank-Institut für Biochemie in Martinsried durchgeführt.

Ich danke Herrn Prof. Dr. Alexander Bürkle für die herzliche Aufnahme in seine Arbeitsgruppe, für die Möglichkeit dieses Projekt an seinem Lehrstuhl durchführen zu können, für seine wissenschaftliche Unterstützung, die Einführung in viele interessante Bereiche der Toxikologie sowie für die Übernahme des Erstgutachtens.

Ganz besonders herzlich möchte ich mich bei Frau Dr. Elisa Ferrando-May für die Anregung zu dieser Arbeit, die exzellente Betreuung, die sehr engagierten wissenschaftlichen Diskussionen sowie für die insgesamt sehr gute und überaus motivierende Zusammenarbeit während der letzten drei Jahre bedanken.

Herrn Prof. Dr. Friedrich Lottspeich und Herrn Dr. Josef Kellermann danke ich dafür, daß ich herzlich in der Abteilung Proteinanalytik am Max-Plank-Institut für Biochemie in Martinsried aufgenommen wurde und dort unter hervorragenden Arbeitsbedingungen entscheidende Proteomanalysen durchführen konnte.

Ein besonderer Dank auch an Herrn Prof. Dr. Peter Macheroux für die Bereitschaft, das Zweitgutachten zu übernehmen.

Den ehemaligen und derzeitigen Mitgliedern der Arbeitgruppe Lottspeich möchte ich allgemein für die herzliche Aufnahme in die Gruppe danken. Mein spezieller Dank geht an Alexander Schmidt für die Einführung und Hilfe mit dem ICPL-Labelling, Monika Zobawa für die Durchführung von unzähligen MS-Analysen und Heidemarie Groß für die Einweisung in alle Tricks und Tips der 2D-Gel-Elektrophorese.

Ebenso möchte ich mich bei allen früheren und gegenwärtigen Mitgliedern des Lehrstuhls Bürkle für die hervorragenden Arbeitsbedingungen bedanken. Hierbei geht mein spezieller Dank an das kleine, aber sehr durchsetzungsstarke „Haus I-Team“. Bei Daniela Herrmann
möchte ich mich für die ausgezeichnete technische Unterstützung bedanken und bei Patricia Grote für die stets anregenden, meist Kaffee und Schokolade untermauerten, Diskussionen.

Bei Tina Baur, Tine Brabec, Conny Ciosto, Katharina Hüttner, Sebastian Röhrig, Frank Schwöbel, Verena Tautorat und Tina Wünsch möchte ich mich dafür bedanken, daß die letzten drei Jahre trotz vieler Arbeit auch viel Freude bereiteten.

Meinem Freund Jürgen danke ich für seine permanente Unterstützung, ohne die diese Arbeit nicht gelungen wäre und für seine aktive Beteiligung an der Minimierung der Reisekosten unserer Arbeitsgruppe nach München.

Nicht zuletzt möchte ich meiner Familie für die ständige Unterstützung jeglicher Art während meiner gesamten Ausbildung danken.
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1 ZUSAMMENFASSUNG

Apoptose, die kontrollierte Form des Zelltodes, spielt eine zentrale Rolle in der Embryonalentwicklung, der Organ-Homöostase und zahlreichen Erkrankungen. Obwohl das Schrumpfen und die Fragmentierung des Zellkerns zu den auffälligsten morphologischen Veränderungen in der apoptotischen Zelle gehören, ist wenig über die Mechanismen, die hierzu führen, bekannt.

Ziel dieser Arbeit war es, mit Hilfe eines neuartigen, auf Massenspektrometrie beruhenden Proteomansatzes eine umfassende Analyse der apoptose-bedingten Veränderungen der Proteine des Zellkerns durchzuführen und diese anschließend biochemisch zu charakterisieren.


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zusätzlich die positive Ladung der Lysine und führt so zu einer Verschiebung des isoelektrischen Punktes in den sauren Bereich. Dies ermöglichte eine verbesserte Trennung basischer Proteine (< pI 10) während der anschließenden 2D-Gel-Elektrophorese.


Im dritten Teil der Arbeit wurden einige Kandidaten biochemisch charakterisiert, woraus folgende Teilergebnisse abgeleitet werden konnten:


4. Weder eine Translokation aus dem Kern noch eine proteolytische Spaltung konnte für das Proto-Onkogen DEK in der Apoptose beobachtet werden. Vielmehr nimmt im Laufe der Apoptose die Bindungsaffinität von DEK für DNA ab, was wahrscheinlich auf eine Veränderung des Phosphorylierungsstatus des Proteins zurückzuführen ist. Außerdem ist diese apoptose-spezifische Veränderung der Bindungsaffinität abhängig von Caspasen und der Protein Kinase CK2.
2 SUMMARY

Apoptosis is a controlled process of cell demise which plays an essential role in development, organ homeostasis and disease.

Shrinkage and fragmentation of the nucleus are among the most striking morphological features of cell death by apoptosis, but little is known about the underlying mechanisms.

The objective of the present study was to perform a comprehensive analysis of the nuclear proteome during apoptosis using a mass spectrometry based analysis platform. Subsequently, the candidate proteins should be characterised biochemically.

The work was divided into three major parts. In the first part, a cell-free apoptosis reaction consisting of isolated nuclei and cytosolic extracts had to be established. First, CD95-L induced apoptosis in Jurkat T-cells was characterised. Then, cytosolic extracts from control cells as well as from cells undergoing CD95-L induced apoptosis were isolated using the detergent digitonin. These extracts were free from nuclear, mitochondrial and ER-proteins. Nuclei in high-purity were prepared from mouse liver. The successful induction of cell-free apoptosis was monitored by the caspase-dependent cleavage of the nuclear proteins PARP-1 and lamin B as well as by oligonucleosomal DNA fragmentation.

In the second part, a method had to be established which was suitable for the relative quantification of the highly positive charged nuclear proteins. The method applied here is named Isotope Coded Protein Label (ICPL) and is based upon the differential isotopic labelling of all free amino groups, at lysines and at the N-terminus, followed by the identification and quantification of peptides using mass spectrometry. The proteins were labelled with two different isoforms of the reagent nicotinoyloxy-succinimide, a light (4 hydrogen, 4H) and a deuterated, heavy form (4 deuteriums, 4D). The chemical reaction of free amino groups with nicotinoyloxy-succinimide leads to their modification with nicotinic acid resulting in a neutralisation of the positively charged lysines and thus shifting the isoelectric point towards the acidic area. This shift improved the separation of nuclear alkaline proteins (> pl 10) in the following 2D-gel-electrophoresis that followed.

In a high-throughput approach, nuclei from cell-free control reactions were labelled with the light ICPL-reagent (H4) and nuclei from apoptotic reactions were reacted with the heavy
reagent (D4) before both samples were combined. Then, the complexity of this protein mixture was reduced by 2D-gel-electrophoresis. The most prominent 384 spots were excised from the stained gels, digested with trypsin and analysed by mass spectrometry. Three independent sets of experiments, consisting of freshly isolated components were performed. This led to the identification of 13 nuclear proteins the level of which were reproducibly altered between the control and apoptosis reaction.

These identified proteins can be classified mainly into two groups of protein: proteins involved in chromatin organisation and architecture (HMG B1/B2, DEK, HCC-1, Histone H1.0/H1.2/H4) and proteins involved in RNA-transport and -metabolism (hnRNP A2/B1, hnRNP C1/C2, U2 snRNPA’). Three further proteins were identified which did not belong to either group: hsp 70, lamin B2 and PP1α.

In the third part of this work, some of the identified proteins were characterised using biochemical and immunochemical methods, leading to the following conclusions:

1) Neither HMGB1 nor HMGB2 are proteolytically processed in apoptosis. In addition, no translocation between the nucleus and the cytoplasm takes place. Most probably, both proteins are post-translationally modified in the course of apoptosis.

2) Proteolysis of hnRNP A2/B1 was not observed in apoptosis. A translocation of hnRNP A2/B1 out of the nucleus explains most likely constant reduction of its protein level observed by the proteome approach.

3) No proteolytic cleavage of hnRNP C1/C2 was detected in apoptosis, whereas a translocation from the nucleus to the cytoplasm was observed. Additionally, post-translational modification may influence this translocation process.

4) Neither translocation from the nucleus nor proteolytic cleavage was observed for the proto-oncogene DEK. Nevertheless, the binding affinity of DEK toward DNA changed during apoptosis. This effect is dependent on caspases and on the protein kinase CK2 and is most likely due to changes of the phosphorylation status of DEK.
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3 INTRODUCTION

3.1 Apoptosis: definition and general concepts

Cell death is an important event in all multicellular organisms throughout the plant and animal kingdom. In the past three decades it has become clear that cell death can contribute to both health and disease. Cell death has been categorised mainly into two classes: Apoptosis, which allows the elimination of a single cell without lysis and therefore without further damage of the surrounding tissue; and necrosis, which is primarily defined by leakage of cellular constituents with the subsequent induction of inflammation.

Apoptosis, the physiological form of cell death, is essential for the appropriate development, differentiation and function of multicellular organisms. To ensure structural and functional tissue homoeostasis, superfluous, damaged and potentially harmful cells have to be deleted from the surrounding healthy cell tissue.

In embryonic development, a programme of proliferation and cell death is carried out to ensure correct cellular patterning and organogenesis. Examples are: the formation of free and independent digits by massive cell death in the interdigital mesenchymal tissues; the development of the brain during which half of the neurons that are initially created will die in later stages when the adult brain is formed; and the development of the reproductive organs. In adult tissue, apoptosis is also an indispensable event for the “steady-state” turnover in many tissues, the intestine and the skin to name two of them. Furthermore, a cell-selective death is fundamental for the development, regulation, and function of the immune system, including the elimination of self-reactive thymocytes, negative selection of B- and T-lymphocytes and cell killing by cytotoxic T-lymphocytes (Jacobson et al., 1997). Last but not least, cells that have sustained irreparable genotoxic damage are also deleted by apoptosis in order to minimise genetic lesions.

In a human body about 100,000 cells are produced every second by mitosis and a similar number die by apoptosis. Over a life span, over 99.9 % of the cells undergo the same fate (Vaux and Korsmeyer, 1999).

Besides this physiological role, apoptosis can also occur in pathological situations in a wide variety of diseases. Defects of apoptosis can result in cancer, autoimmune diseases and
spreading of viral infections, while neurodegenerative disorders like Parkinson’s, Alzheimer’s and Huntington’s disease, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis (Mattson et al., 2000; Reed, 2002).

The term apoptosis, first introduced in 1972, is derived from the term “ptosis” meaning “falling off” and refers to the morphological feature of formation of “apoptotic bodies”. This analogy emphasises that this type of cell death is an integral and necessary part of the life cycle of multicellular organisms. In this first description, apoptosis was characterised as a programmed i.e. genetically determined form of cell death that was found to follow a conserved morphological pattern in various tissues and cell types from yeast and the nematode Caenorhabditis elegans to mammals (Ameisen, 2002; Kerr et al., 1972; Metzstein et al., 1996). Recent reports illustrate the involvement of programmed cell death in the life cycle of unicellular eukaryotes and even of prokaryotes (Ameisen, 2002).

In general, the process of apoptosis can be divided into three distinct stages: commitment, in which the cell, having received a potentially lethal stimulus, becomes irreversibly committed to death; execution, during which the major structural changes occur; and clearance, when cellular remnants are removed by phagocytosis.

The identification of the proteins involved in the execution and regulation of this process revealed an unusual class of cysteine proteases, now termed caspases (for cysteine-dependent aspartate-specific proteases). The activation of these caspases is followed in the execution phase by the coordinated degradation of structural and functional components, which is followed by morphological changes like cell shrinkage, detachment from neighbouring cells and chromatin condensation (Oberhammer et al., 1993; Wyllie et al., 1980). The so-called apoptotic bodies are formed. They can easily be engulfed and removed by surrounding phagocytic cells, thus avoiding the escape of potentially infectious, or immunogenic, genetic material (Fadok and Henson, 1998).

In contrast to apoptosis, necrosis is always the outcome of severe acute injury. The term necrosis was first been used in a tissue-related context in pathology. It describes the release of intracellular constituents into the extracellular milieu, which may potentially invoke an inflammatory reaction with local cellular infiltration, vascular damage, edema, and further injury to the surrounding tissue following massive non-physiological cell lysis. However, this definition also includes the secondary lysis of primary apoptotic cells when there is a lack of
enough phagocytic activity to remove all apoptotic cells rapidly, e.g. after massive apoptosis in acute liver failure after poisoning (Leist and Nicotera, 1997).

## 3.2 Apoptosis signalling

Apoptosis is a highly regulated and efficient cell death programme which requires the interplay of a multitude of factors. The components of the apoptotic signalling network are genetically encoded. They are considered to be usually in place and ready to be activated by a death inducing stimulus (Weil et al., 1996).

### 3.2.1 The role of caspases

Caspases were first implicated as components of the cell death machinery by studies conducted on the nematode worm *Caenorhabditis elegans*, and shortly thereafter the homology of the worm ced-3 protease to the mammalian interleukin-1β-converting enzyme (ICE) was discovered (Degterev et al., 2003; Yuan et al., 1993). Since then 14 related ICE-like proteases have been identified and partially characterised (for a review see (Degterev et al., 2003; Grutter, 2000; Takahashi, 1999)). Caspases are specific proteases which have a definite requirement for cleavage after aspartic acid residues in proteins. Recognition of at least four amino acids N-terminal of the cleavage site is also required for efficient catalysis. Caspases prefer different tetrapeptide recognition motifs, which explains the diversity of their biological function (Thornberry et al., 1997). A large body of evidence indicates that caspases play a central role in apoptosis. Genetic (Gagliardini et al., 1994; Kuida et al., 1996) and pharmacological (MacFarlane et al., 1997; Slee et al., 1999) inhibition of caspases have been shown to suppress apoptotic cell death. Furthermore, apoptosis is inhibited by mutations in the caspase catalytic site (Kumar et al., 1994; Miura et al., 1993) or by ectopic expression of caspase antisense DNA (Miura et al., 1993). Finally, overexpression of caspases is a strong inducer of apoptosis in cell culture (Miura et al., 1993). The caspase family can be grouped into two classes, depending on their point of entry into the apoptotic cascade. There are the initiator (or upstream) caspases (such as caspase-2, -8, -9 and -10) and the effector (or downstream) caspases (like caspase-3, -6, -7).
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Based on studies with caspase-deficient cells or caspase inhibitors it is generally assumed that execution of caspases play a major role in the formation of typical morphological and biochemical changes during apoptosis, mainly through the proteolytical cleavage of various cellular targets (Cohen, 1997; Nicholson and Thornberry, 1997). Amongst many divergent caspase substrates are proteins from different cellular compartments which are involved in cell structure, signalling, cell cycle control and DNA repair. Many caspase substrates are just cleaved as bystanders, because they happen to contain a caspase cleavage site in their sequence. Several targets, however, have a discrete function in propagation of the cell death process. Many structural and regulatory proteins are inactivated by caspases, while other substrates can be activated. To date, close to 300 proteins have been found to undergo caspase-dependent cleavage during apoptosis, but in most cases, the relationship of their cleavage to apoptosis execution is still unknown. Some caspase substrates act as key players for morphological changes in apoptosis. Several caspase substrates also proceed as transducers and amplifiers that determine the apoptotic threshold and cell fate (Fischer et al., 2003).

3.2.2 Mechanisms of caspase activation

All caspases are synthesised as inactive zymogens composed of a variable N-terminus prodomain, one large (~ 20 kDa), and one small subunit (~ 10 kDa) joined by a small interdomain linker.

Caspase prodomains can vary in sequence and size with caspase-3, -6, and -7 having the shortest and caspase-8, -9, and -10 the longest prodomains. Prodomains have been shown to mediate dimerization and promote auto-processing of pro-enzyme molecules (Srinivasula et al., 1998) as well as to carry nuclear localisation signals (Baliga et al., 2003; Colussi et al., 1998).

Recent data demonstrated that caspase activation mechanisms vary for initiator and effector caspases. Upon apoptotic induction the latent monomeric zymogens of initiator caspases are activated at a multiprotein complex. Activation of the zymogen monomers occurs only by a dimerization process, no cleavage of the pro-caspase is necessary for activation. Rather, if occurring, the cleavage might be necessary for stabilisation of the activated caspase dimers (Boatright et al., 2003). In contrast to the initiator caspases, the effector caspases latently exist as preformed zymogen dimers. They are activated by proteolysis within their interdomain linker regions which can be carried out by initiator caspases or by other specific proteases.
This cleavage results in a tetrmeric complex, thus facilitating the formation of the active site (for review see (Boatright and Salvesen, 2003)). Activation in both cases occurs via the translocation of the activation loop from one monomer to the other. For initiator caspases this is possible by the dimerisation event, and in effector caspases prior proteolysis of the dimers must occur.

Four major caspase-dependent pathways of apoptosis have been identified which involve the activation of four different apical caspases: the endoplasmic reticulum stress pathway attributed to activation of caspase-12 in rodents (Nakagawa et al., 2000) and caspase-4 in humans (Hitomi et al., 2004), the DNA damage pathway which activates caspase-2 upstream of mitochondria (Guo et al., 2002; Robertson et al., 2002), the mitochondrial pathway leading to the activation of caspase-9 and downstream cleavage of executioner caspases (Green and Reed, 1998), and the death receptor pathway involving caspase-8 and caspase-10 (for review see (Chen and Wang, 2002)). The latter two pathways will be described in the following.

### 3.2.2.1 The mitochondrial pathway

A number of cellular damages, like heat shock, various cytotoxic drugs, DNA-damaging agents and death domain receptor signalling, attack mitochondria, thereby inducing the permeabilisation of mitochondrial membranes and the release of mitochondrial proteins.

The molecular mechanisms leading to mitochondrial damage are still being discussed, it is, however, widely accepted that they are dependent on the interaction of pro- and anti-apoptotic members of the Bcl-2 family of proteins. The initial model proposes a Bax- and Bak-dependent opening of a large, poorly understood protein complex, the permeability transition pore (PTP), resulting in the release of cytochrome c (van Loo et al., 2002a).

However, recent studies have demonstrated that the actual pore-forming effector molecules are the pro-apoptotic Bcl-2 family members themselves. Bax and Bak form tetrameric outer membrane channels which could mediate the release of the apoptogenic factors. They can be activated either transcriptionally or by conformational change induced by cleavage or binding to another activated Bcl-2 like protein, such as Bad, tBid, Bim, Noxa or PUMA. On the other hand, anti-apoptotic members such as Bcl-2 and Bcl-X\textsubscript{L} oppose the permeability of mitochondrial membranes most probably by hetero-dimerisation with Bax-like proteins (Cheng et al., 2001; Danial and Korsmeyer, 2004; Jaattela, 2002; Jiang and Wang, 2004).

The diverse pathways of apoptosis often display cross-talk to the mitochondrial pathway. The most prominent linker protein between the death receptor pathway and mitochondria is Bid, a
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pro-apoptotic member of the Bcl-2 protein family. Cleavage of Bid by death receptor activated initiator caspases results in the truncated form tBid, which translocates to mitochondria, promoting there the Bax- and Bak-dependent release of pro-apoptotic proteins, such as cytochrome c, Smac/Diablo, Omi/HtrA2 and AIF (apoptosis-inducing factor) by mitochondrial outer membrane permeabilisation. These proteins promote the formation of the caspase-9-activating protein complex, the apoptosome, consisting of cytochrome c, Apaf-1 (apoptotic protease activating factor 1), dATP and pro-caspase-9. Finally, active caspase-9 processes the executioner caspase-3, thereby completing the apoptotic cascade.

3.2.2.2 The death receptor pathway via the CD95/CD95-L system

The death receptor CD95 (also named APO-1 and Fas) is a member of the tumour necrosis factor (TNF) and nerve growth factor (NGF) receptor superfamily, which includes among others TNF-R1, DR-3, TRAIL-R1, and TRAIL-R2 (Schulze-Osthoff et al., 1998). CD95 is a widely expressed glycosylated cell surface protein of 45 to 52 kDa. It is a type I transmembrane protein that possesses characteristic cysteine-rich repeats in its extracellular domains and can also occur in a soluble form (Oehm et al., 1992). It is activated by its natural ligand, the CD95 ligand (CD95-L), which is a type II transmembrane protein (Sartorius et al., 2001). The membrane-bound form of the ligand is the active form and can induce apoptosis (Hohlbaum et al., 2000; Schneider et al., 1998; Suda et al., 1997).

The primary function of CD95 is to trigger apoptosis. Although expressed in a variety of tissues and cell lines, its predominant physiological role lies in the regulation of the immune system, where it contributes to lymphocyte maturation, receptor repertoire selection and homeostasis. The CD95 receptor/ligand system represents one of the best studied apoptotic signalling systems (Sartorius et al., 2001).

CD95 consists of an extracellular ligand binding domain, a transmembrane domain, and the cytoplasmic domain including the so-called death domain (DD) (Nagata, 1999). Binding of the ligand to CD95 results in trimerisation of the receptor. The clustering of the individual receptor molecules brings the cytoplasmic DDs into close proximity, whereafter the adaptor molecule FADD/MORT1, which also contains a DD, is recruited to the receptor (Boldin et al., 1996; Muzio et al., 1996). This process in turn attracts the cytosolic protein pro-caspase-8 and -10 to FADD and results in the formation of the death-inducing signalling complex (DISC) (Boldin et al., 1996; Kischkel et al., 1995; Muzio et al., 1996) (Fig. 1A). Complex
formation results in a proximity-driven dimerisation of the initiator caspases-8 and -10, leading to their activation (Donepudi et al., 2003; Shi, 2004).

The signalling response to CD95 induced DISC formation has been shown to vary between cell types. In type I cells, the amount of active caspase-8 is sufficient to directly activate other downstream members of the caspase family, whose action on defined substrates paves the way to the execution phase of apoptosis (Cohen, 1997; Krammer, 2000) (Fig. 1B). In type II cells, proper activation of effector caspases by CD95 depends on an amplification loop mediated by Bid and comprising the mitochondrial pathway (see above), thus resulting in the activation of caspase-9 and subsequently processing of the executioner caspase-3. This in turn activates caspase-8 outside the CD95-DISC, thereby completing a positive feedback loop (Fig. 1B).

Figure 1: The CD95 signalling pathway (adapted from (Igney and Krammer, 2002)).

(A) Ligation of CD95 results in DISC assembly and the release of caspase-8 (caspase-10) subunits. (B) In type I cells, the strong caspase-8 signal directly activates executioner caspases. In type II cells, the mitochondrial release of apoptogenic factors initiates the caspase cascade downstream of mitochondria.
3.2.3 Caspase-independent apoptosis

Although caspases are widely accepted to be the key executors of apoptosis, several studies have shown that apoptosis can still occur when caspases are inhibited (Berndt et al., 1998; Deas et al., 1998; Dumont et al., 2000; Quignon et al., 1998). Nuclear apoptotic changes still occur when the activity of the caspase-dependent nuclease CAD is specifically blocked (Sakahira et al., 1999). This indicates that there must be caspase-independent pathways also directed at the nucleus of cells dying by apoptosis.

It was shown in genetic studies that one of these caspase-independent pathways involves AIF, which initiates chromatin condensation and high molecular weight DNA fragmentation in the absence of caspase activity (Susin et al., 1999b). The mechanisms underlying AIF’s function in the nucleus are still unclear. Since AIF itself does not possess an intrinsic DNase activity, DNA fragmentation probably requires interaction with a yet unknown nuclear DNase. Recently an additional factor, associated with mitochondria, was reported to induce cell death independently from caspases, when overexpressed. This is the AIF homolog AMID (AIF-homologous mitochondrion-associated inducer of cell death) (Wu et al., 2002a) also known as PRG3 (Ohiro et al., 2002).

Experiments using an in vitro system reported phospholipase A$_2$ (PLA$_2$) to be responsible for nuclear shrinkage in the process of caspase-independent, Apaf-1-independent, and Bcl-2-insensitive cell death (Shinzawa and Tsujimoto, 2003).

Proteins such as tBID, BIM and BAD, all belonging to the BH3-domain only protein-family, have been shown to promote caspase activation and apoptosis in many studies. Genetic studies in Apaf-1$^{-/}$ mouse embryonic fibroblasts demonstrated that they can also induce cell death with apoptotic features independently of Apaf-1 and downstream caspases. Activation was not detected for effector caspases-2, -3, -6, or -7, nor could cell death be blocked by the pan-caspase inhibitor zVAD-fmk (Cheng et al., 2001).

Further studies show the involvement of other proteases, such as calpains (Squier and Cohen, 1996), cathepsins (Stoka et al., 2001), and serine proteases (reviewed in (Egger et al., 2003)) in caspase-independent cell death pathways.
3.2.4 Nuclear apoptosis

Among the most prominent morphological features of apoptosis are changes occurring within the nucleus. These include nuclear fragmentation, chromatin condensation and aggregation along the nuclear periphery in a crescent-shaped pattern. Molecular characterisation of apoptotic chromatin reveals the ordered degradation of DNA first into large fragments of 300-50 kbp (Oberhammer et al., 1993) and subsequently into smaller fragments that are monomers and multimers of 180 bases (Wyllie, 1980). However, cleavage of chromatin into nucleosomal fragments does not occur in all cell types and can be inhibited without blocking other features of apoptosis (Jacobson et al., 1994; Schulze-Osthoff et al., 1994). The nucleosomes are then exposed at the surface of blebs and apoptotic bodies become accessible for the interaction with receptors (Radic et al., 2004).

Concomitantly with chromatin condensation, the nuclear ultrastructure is altered. The disruption of the structural framework of the nucleus is mainly achieved by the cysteine protease family of caspases which cleave a variety of nuclear substrates including scaffold proteins like the lamins (Rao et al., 1996b) and the scaffold-attachment factor (SAF)-A (Göhring et al., 1997), DNA repair proteins, like PARP-1 (Gu et al., 1995; Lazebnik et al., 1994) and the filamentous nucleoporin Tpr (Ferrando-May et al., 2001). Recent data from our group show that the ordered dismantling of the nuclear pore complex in apoptosis is a caspase-dependent process (Patre et al., 2004). Caspases can also activate factors which contribute to the condensation and degradation of the chromatin like apoptotic chromatin condensation-inducing factor Acinus (Sahara et al., 1999) and caspase-dependent DNase CAD/DFF40 (Enari et al., 1998; Liu et al., 1997).

Whereas the pathways leading to the activation of the cytosolic caspase cascade in response to extracellular apoptotic signals are well characterised (Earnshaw et al., 1999), the mechanisms which lead to caspase activity in the nucleus are still unclear. Caspases may be activated in the cytosol and then translocate to the nucleus. This has been demonstrated for caspase-3, the main executioner caspase in apoptosis, which enters the nucleus coincidently with the appearance of chromatin condensation (Faleiro and Lazebnik, 2000). Alternatively, the proenzymes may be directly activated within the nucleus. In tumour necrosis factor-α (TNF)-treated HeLa cells, the proform of caspase-1 translocates into the nucleus shortly after stimulation and is then processed to the active form. The nucleus has also been shown to harbour precursors of caspase-2 and caspase-9 (Colussi et al., 1998; Zhivotovsky et al., 1999). However, nuclear targets for these caspases have not yet been identified. These evidences
suggest that, while cytosolic caspase-3 may be recruited to the nucleus at the final stage of nuclear execution, activation of nuclear caspases may proceed in parallel and also independently from the caspase cascade in the cytoplasm.

The nuclear protein encoded by the N5 gene (p84N5) has two prominent features. First, it acts as an activator of a G2/M cell cycle checkpoint prior to cell death (Doostzadeh-Cizeron et al., 2001) and second, it harbours a death domain like those found in the tumour necrosis factor 1 (TNFR-1) superfamily of death receptors, which is unique among nuclear proteins (Doostzadeh-Cizeron et al., 1999). Overexpression of p84N5 induces apoptosis, while dominant interfering death domain mutants of p84N5 compromise the apoptotic response to ionising radiation. In analogy to TNF-R1, p84N5 may act as a nuclear death receptor, recruiting adaptor molecules to a complex that ultimately leads to caspase activation and apoptosis. In line with these findings, Rich et al. recently postulated the existence of a nuclear apoptosome whose molecular composition and regulation remain to be discovered (Rich et al., 2000).

### 3.2.4.1 Protein modifications in nuclear apoptosis

Post-translational modifications of proteins have long been recognized as a way to regulate structure and function of proteins, also in the nucleus. One prominent protein group within the nucleus is the family of histones, which can be altered predominantly at the N-terminal tail by acetylation, methylation, phosphorylation, poly(ADP-ribosylation) and ubiquitinylation. Independent groups have reported on histone hyperphosphorylation in the course of apoptosis, namely histone H3 (Waring et al., 1997), H2B and H4 (Ajiro, 2000), and H2AX (Rogakou et al., 2000). From cell-free systems, the importance of H2B phosphorylation in the N-terminal tail for chromatin condensation is known (de la Barre et al., 2001). Recently, the phosphorylation at H2B’s serine 14 by the caspase-3 activated Mst-1 kinase was shown (Cheung et al., 2003). This Ser14 phosphorylation is not found during mitosis and is suggested to be a unique “death” marker for chromatin condensation in apoptosis (Wang et al., 2004). Contrary to these indications on hypophosphorylation of histones in apoptosis, one group reported the dephosphorylation of some histone H1 subtypes (Kratzmeier et al., 2000). Early in apoptosis enhanced poly(ADP-ribosylation) of histone H1.0 could be demonstrated, facilitating the internucleosomal DNA fragmentation by increasing the susceptibility of chromatin to endonuclease activity (Yoon et al., 1996). A rapid and extensive deubiquitination of nucleosomal H2A occurs in Jurkat T-cells undergoing apoptosis initiated

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by different apoptotic stimuli (Mimnaugh et al., 2001). Underacetylation of histones in the course of apoptosis has been observed, which triggers tight binding of the high mobility group protein B1 (HMGB1) to chromatin (Scaffidi et al., 2002). Other nuclear proteins beside the histones are also affected by post-translational modifications in apoptosis. Examples are the hyperphosphorylation of the chromatin-bound replication A middle subunit (RPA32) and of the lamins during apoptosis (Shimizu et al., 1998; Treuner et al., 1999). The HMGA1a protein undergoes dynamic changes in the phosphorylation status: in early apoptotic stages it is first hyperphosphorylated and then, later dephosphorylated in condensed chromatin of apoptotic bodies (Diana et al., 2001). Additionally, methylation of HMGA1a during apoptosis was reported (Sgarra et al., 2003).

3.2.4.2 Protein translocation in nuclear apoptosis

The subcellular localisation of many proteins changes in the course of apoptosis influencing their biological function. The translocations mainly occur between the nucleus, the mitochondrion and the cytoplasm. Proteins are translocated from one compartment to the other in order to perform their specific function in apoptosis.

Bcl-2 family members translocate at the early onset of apoptosis, as described above, from the cytoplasm to the mitochondria (see 3.2.2.1). However, as an early event in apoptosis, protein release from mitochondria followed by translocation to different cellular compartments was demonstrated to result from mitochondrial dysfunction. Irreversible alterations of mitochondrial ultrastructures lead to the passive release of mitochondrial proteins (Single et al., 1998; Susin et al., 1996). Proapoptotic proteins that are released from the mitochondrial intermembrane space into the cytoplasm include cytochrome c (Liu et al., 1996), caspases (Susin et al., 1999a), AIF (Susin et al., 1996), the endonuclease G (endoG) (Li et al., 2001), Smac/Diablo (Adrain et al., 2001) and the serine protease Omi/HtrA2 (van Loo et al., 2002b). Cytochrome c release links mitochondrial death sensors to caspase activation. On the other hand, released AIF translocates to the nucleus and causes partial chromatin condensation and high molecular weight DNA fragmentation in a caspase-independent manner (Daugas et al., 2000; Susin et al., 2000; Susin et al., 1999b; Zamzami et al., 1996). Additionally, AIF suffices to cause other apoptotic changes such as phosphatidylserine exposure when microinjected in healthy cells (for review see (Daugas et al., 2000)).
EndoG, a mitochondrion-specific nuclease, translocates to the nucleus during apoptosis. Once released from mitochondria, endoG cleaves chromatin DNA into nucleosomal fragments in a caspase-dependent manner (Li et al., 2001).

There are several factors translocated from the nucleus to the mitochondria to potentiate the cellular response following apoptosis induction. After DNA damage, the first nuclear factors translocated to mitochondria were identified as a PARP-1-dependent, signal inducing caspase-independent AIF release (Hong et al., 2004; Yu et al., 2002), most likely PAR (V.L. Dawson, personal communication), and histone H1.2, initiating a cytochrome c release (Konishi et al., 2003). TPA induction causes the protein translocation of the nuclear protein Nur77 to the mitochondria resulting in the release of cytochrome c (Wu et al., 2002b).

The tumour suppressor protein p53 induces apoptosis by targeting gene regulation and transcription-independent signalling. After irradiation, p53 is released from the nucleus and directly induces permeabilisation of the outer mitochondrial membrane by forming complexes with the Bcl2-family of proteins, resulting again in cytochrome c release (Chipuk et al., 2004; Mihara et al., 2003). The histone deacetylase 4 (HDAC4), an important regulator of gene expression, varies its subcellular localisation from nuclear to cytosolic depending on the cell type and status. In apoptosis, upon cleavage by caspase-2 and -3, fragments of HDAC4 are generated which localise differentially: the amino-terminal fragment remains in the nucleus and the C-terminal fragment translocates to the cytoplasm (Paroni et al., 2004). The N-terminal fragment induces apoptosis itself by activating the mitochondrial pathway.

BARD1 is a protein found in both compartments, inside the nucleus and in the cytoplasm. Upon an apoptotic stimulus this distribution is shifted, the nuclear fraction translocates to the cytoplasm (Jefford et al., 2004).

Further proteins with a different origin than the mitochondria, were also described to translocate into the nucleus upon an apoptotic stimulus. GAPDH is a multifunctional protein with glycolytic and non-glycolytic functions, including pro-apoptotic activities. In early apoptosis, translocation is triggered from the cytoplasm into the nucleus (Shashidharan et al., 1999), however the function of GAPDH within the apoptotic nuclei is not clear yet. One further protein translocated from the cytoplasm to the nucleus early in apoptosis is the programmed cell death 5 (PDCD5) protein. The appearance of PDCD5 in the nuclei of apoptotic cells precedes the characteristic apoptotic features, such as the externalisation of phosphatidylserine and fragmentation of chromosomal DNA (Chen et al., 2001).
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Different studies describe the release of different members of the heterogeneous ribonucleoprotein family (hnRNPs) from the nucleus into the cytoplasm (Biggiogera et al., 1997; Pinol-Roma and Dreyfuss, 1993; Thiede et al., 2002). The majority of pro-caspases have been localised in the cytoplasm or the intermembranous space of mitochondria. So far only one caspase, caspase-2, was found to be permanently present within the nucleus. This localisation depends on the prodomain, which harbours a NLS-sequence (Baliga et al., 2003). It is still controversial whether procaspase-3 is constitutive localised within the nucleus (An et al., 2003; Ramuz et al., 2003). Still, most executioner caspases have to be translocated to the nucleus to ensure nuclear apoptosis. A possible mechanism might be disruption of the nucleo-cytoplasmic barrier which increases the diameter of the nuclear pore complex to allow caspases to enter the nuclear interior passively (Faleiro and Lazebnik, 2000; Ferrando-May et al., 2001; Roehrig et al., 2003).

3.3 Cell-free systems for the study of apoptosis

Cell-free systems were originally developed for the study of cell cycle regulated events, such as mitosis (Lohka and Masui, 1983), DNA replication (Blow and Laskey, 1986), nuclear assembly (Newport, 1987), chromatin condensation (Wood and Earnshaw, 1990) and the cell cycle itself (Murray and Kirschner, 1989). In principle, these systems made it possible to mimic and manipulate the natural process using purified components in a test tube. Complex cellular functions can thus be dissected into individual steps. One main experimental advantage is the easy manipulation of these systems, since there are no functional membrane barriers which need to be overcome. Recently, the cell-free technique has been applied to the study of apoptosis. A typical cell-free apoptosis reaction consists of cytosolic extracts derived from cells committed to undergo apoptosis and purified nuclei isolated from healthy cells (Takahashi and Earnshaw, 1997). In such a system, it is then possible to follow morphological changes and molecular events that are induced inside the nucleus by the active cytosolic extracts.

Cell-free systems have contributed to the identification and characterisation of several factors involved in DNA fragmentation and condensation during apoptosis, like CAD/DFF40, Acinus, AIF and Endo G (Enari et al., 1998; Li et al., 2001; Liu et al., 1997; Sahara et al., 1999; Susin et al., 1999b). They have also been pivotal to the discovery of major components of the apoptotic signalling cascade, like cytochrome c (Liu et al., 1996), Apaf-1 (Li et al., 1997; Zou et al., 1997) and Smac/Diablo (Du et al., 2000; Verhagen et al., 2000). In all these
studies, the molecular identification of apoptotic factors was achieved by the classical biochemical method of cell fractionation and stepwise chromatographic purification of subcellular fractions. An alternative strategy, which has not been described so far, combines the cell-free technique with the powerful analytical tools of proteomics in order to obtain a more comprehensive information of the processes occurring during apoptosis in one specific subcellular compartment, like the nucleus.

3.4 Comparative proteomics: An overview

Cellular dynamics is often addressed through the study of gene expression and the quantification of expressed messenger ribonucleic acid (mRNA) expression (Ramanathan et al., 2001). Nevertheless, the correlation between mRNA expression and the presence of specific functional proteins is poor, because the final steps in the synthesis of functional proteins occur by post-translational modifications (Gygi et al., 1999b). The measurement of the expressed proteins is a more direct way to study cellular dynamics (Regnier et al., 2002).

The proteome is defined as the PROTEin complement expressed by a genOME in dependence on the environment and time (Wilkins et al., 1996). While the genome is static, the proteome continually changes in response to external and internal events. Proteomics aims at quantifying the expression levels of the complete protein complement in a cell at any given time. It is mostly based upon the comparison of different protein profiles in a defined biological system under different conditions.

3.4.1 Relative protein quantification by 2D-gel-electrophoresis

Proteomic research was initially focussed on 2D-gel-electrophoresis as the technique for protein separation first introduced by Klose and O'Farrell in 1972 (Klose, 1975; O'Farrell, 1975).

A theoretical distribution of protein spots of mouse proteins in one 2DE-gel is demonstrated in figure 2. Experimentally, optimal resolution on 2DE-gels is only achieved for proteins ranging from pI 3 to pI 7 and a mass between 10 and 150 kDa. Resolution of more basic proteins requires optimisation (Olsson et al., 2002). Additional separation of numerous proteins, such as hydrophobic membrane proteins, is hardly possible by 2DE-gels (Herbert, 1999; Molloy et al., 1998).
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Figure 2: Theoretical spot distribution in 2DE-gels.
The isoelectric points of all mouse proteins contained in the NCBInr-database were plotted versus their molecular weight using the software GPMAW 6.0 (Lighthouse data, Odense, Denmark).

So far most quantitative approaches were based upon comparison of protein levels in different samples, representing different proteomes, after separation and visualisation by 2D-gel-electrophoresis. The spots are detected, quantified by comparing their position and intensity, and finally identified by mass spectrometric analysis. Whereas the technique is mature and robust, several conceptual and technical considerations limit its general utility (Griffin and Aebersold, 2001). Recently, the usage of fluorophores and difference gel-electrophoresis (DIGE) increased the reproducibility and accuracy of quantification (Patton, 2002). However there are still limitations. The general difficulty is, that 2D-PAGE selects against specific classes of proteins, such as membrane proteins, very large and very small proteins, and extremely acidic and basic proteins (Gygi et al., 1999a). In addition, a study using unfractionated soluble proteins from yeast (Saccharomyces cerevisiae) whole cell lysate demonstrated that even with maximal sample loading and extended electrophoretic separation, low-abundance proteins, which constitute nearly half of the yeast proteome, were systematically excluded. It was also shown in this study that up to six proteins were identified in a 2DE-gel spanning only one pH unit (Gygi et al., 2000). More than one protein will thus localise to one single spot and contribute to spot intensity. Although proteins can be identified by the classical method of comparing 2DE-gels, quantitation of protein levels from spot intensities becomes difficult, since changes in intensity may arise from any proteins present within a spot.
3.4.2 Mass spectrometry-based protein quantification

To overcome the limitations of comparative 2D-gel-electrophoresis, automated tandem mass spectrometric approaches based on stable isotope labelling of proteins or peptides were described (Gygi et al., 1999a). Chemically identical but mass-differentiated stable isotope tags were introduced into the proteins of at least two sample mixtures. The combined mixtures were then processed and analysed by mass spectrometry, so that each analyte from one sample represents a quantitative standard for a chemically identical analyte from the other sample, making relative quantification possible.

The most prominent among quantitative mass spectrometric approaches is the isotope-coded affinity tag (ICAT)-platform. The ICAT reagent consists of a thiol specific group, an eightfold deuterated linker, and a biotin affinity tag. It covalently reacts with each cysteiny l residue. Two different samples are derivatised \textit{in vitro} with two different ICAT reagents; heavy (deuterated) and light (normal). The samples are combined, fractionated if necessary, proteolysed and affinity isolated via the biotin tag. The peptides are further separated by liquid chromatography and are finally analysed by tandem mass spectrometry. Since isotopes have identical physico-chemical properties, the only difference in chemical behaviour is a 8 kDa mass differences for each labelled cysteine in the peptide (Gygi et al., 1999a).

However, the ICAT reagent has disadvantages due to the relatively low abundance of cysteines in proteins. Proteins that do not contain any cysteines are transparent to this method and generally relatively low sequence coverage of the identified proteins is achieved (Tao and Aebersold, 2003).

To overcome this limitation, further isotopic \textit{in vitro}-labelling approaches have been proposed. In one attempt, N-terminal isotopic labelling of each peptide in a protein digest was described (Chakraboty and Regnier, 2002; Munchbach et al., 2000). In another study, acetylation of primary amines at the N-terminus and lysines was reported (Ji et al., 2000). Recently, a novel approach based on the isotopic labelling of all free amino groups (lysines and N-terminus) in proteins was developed, named the isotope coded protein label (ICPL) (Schmidt et al., 2004). The workflow for ICPL labelling is shown in Fig. 3A. Here, the proteins of two different samples are derivatised with a light (no isotope) or a heavy (4 deuterium) N-nicotinoyloxy-succinimide (Fig. 3B). After the combination of both samples the complexity of the mixture can be reduced either chromatographically or electrophoretically. Identification and quantification are performed by automated high throughput tandem mass spectrometry. In this system, peptides of identical sequence derived from both differentially
labelled samples appear as doublets in the acquired MS-spectra and relative abundance can be determined by comparing the ion intensities of the sister peptide pairs.

There are some advantages of this method compared to other types of isotopic label: firstly, nicotinoylated peptides show a striking increase in relative ion intensity in MS-spectra compared to acetylated and native peptides (A. Schmidt, personal communication); secondly, a higher sequence coverage allows more reliable identification and quantification, and the possibility to detect protein modifications is increased. The system is excellent for quantification: the experimental variability lies between 0.3 and 7.0 % as shown by Schmidt.
(Schmidt et al., 2004). Finally, it is well suited for the study of basic proteins due to the fact that lysines, which together with arginines contribute positive charges, are neutralised by the derivatisation with N-nicotinoyloxy-succinimide.

### 3.4.3 Proteomic techniques for the study of apoptosis

Several studies have already focused on apoptosis-related protein alterations in cells undergoing apoptosis induced by various stimuli. These studies followed the classical proteome analysis strategy, consisting in the separation by 2DE-gel-electrophoresis is combined with image analysis for relative quantification.

The first study was performed with whole cell lysates from a Burkitt lymphoma cell line induced to undergo apoptosis by treatment with an anti-IgM antibody (Brockstedt et al., 1998). Although the majority of proteins are not affected in apoptosis, 12 apoptosis-associated protein changes were identified, among them were the cleavage products of hnRNP A1 and C1/C2, the new arising C-terminal fragment of nucleolin, the shift of 60 S acidic ribosomal protein P0 (L10E) towards the neutral pH, the decrease of one dUTPase, the DUT-N, and the increase in heterochromatin protein 1 homologue alpha (HP1α). Also, the RNA polymerase B transcription factor 3 (BTF3) was predominantly altered (Brockstedt et al., 1999).

Other studies mainly identified cytosolic proteins in the colon cancer cells HT-29 treated with butyrate (Tan et al., 2002), and in ionizing radiation-induced apoptosis in prostate epithelial tumour cells (Prasad et al., 1999). Further, several cytoplasmic, mitochondrial and peroxisomal peroxiredoxins are modified as the major cellular response to oxidative stress in Jurkat T-cells (Rabilloud et al., 2002).

Radiation-induced alterations in mouse fibrosarcoma L929 cells lead to alterations of diverse sets of proteins, which affect various events induced by radiation, such as antioxidant reactions, cell cycle proliferation, DNA repair mechanism, and apoptosis in general (Szkanderova et al., 2003). Nuclear proteins identified here are the transcription factors nucleoside diphosphate kinase B (NDK B) and metal-response element-binding transcription factor 2 (MTF2), the components of the protein synthesis apparatus 40S ribosomal protein SA and initiation factor 5A (eIF5A), and one component of the proteasome.

Two approaches analysed protein changes in whole cell extracts of Jurkat T-cells stimulated to undergo apoptosis with the CD95 ligand. Metabolic labelling with $[^{32}P]$orthophosphate in the first study identified proteins which were phosphorylated (eIF1β, IκB-α, several heat
shock proteins, myosin light chain, and rho GDI 1) and proteins which were dephosphorylated (calreticulin, endoplasm, kinesin heavy chain, nuclear autoantigen SP100, and vimentin) in apoptosis (Gerner et al., 2000). Furthermore caspase degradation products were identified, including the nuclear proteins eIF4G, importin-β3 and the protein phosphatase 2A (PP2A).

In a similar approach but in the presence of the translation inhibitor cycloheximide, mainly RNA-binding proteins and proteins involved in splicing processes were identified as targets of CD95 induced apoptosis (Thiede et al., 2001).

To improve the sensitivity of 2D-gel-electrophoresis these authors performed cell fractionation of apoptotic prior to gel-electrophoresis. Translocation from the cytosolic to the nuclear fraction was shown for the T-complex protein 1, alpha subunit (TCP-1α) in Jurkat T-cells undergoing CD95-induced apoptosis (Gerner et al., 2000).

Fractionation studies in various cell types implicated the involvement of nuclear matrix proteins, factors associated with chromatin as well as DNA replication and repair proteins in various models of apoptosis, like CD95-treated Jurkat T-cells, UV-light treated U937 cells and also staurosporine-treated HeLa cells (Gerner et al., 2002). In Jurkat T-cells stimulated with CD95-L and subsequently fractionised, caspase-dependent alterations of heterogenous ribonuclear proteins were observed. Cleavage of hnRNP A1, A2/B1, C1/C2 and K as well as the translocation of several hnRNPs (A1, A2/B1, A3, C1/C2, D and K) from the nucleus to the cytoplasm and/or mitochondria was predicted from 2D-gel-electrophoretic data. Additionally, the translocation of Rho guanine nucleotide from the nucleus to different cellular compartments was predicted (Thiede et al., 2002).

However, most studies still bear experimental disadvantages. The main pitfall is the fractionation of apoptotic cells: due to the fragility of intracellular membranes it is difficult to obtain clean fractions and avoid cross-contaminations. Further difficulties concern the reproducibility of 2DE-gels and the quantification of silver stained gels. For these reasons, a recent study addressing the release of apoptotic factors from mitochondria upon opening of the permeability transition pore complex (PTPC) employed a novel approach. In a cell-free system mouse liver mitochondria were treated with the PTPC-opening agent atractyloside and the mitochondrial supernatant was investigated by non-comparative mass spectrometry. Proteins identified in the supernatant were shown to have their origin in different mitochondrial subcompartments: the intermembrane space, the matrix and the inner membrane (Patterson et al., 2000).
4 OBJECTIVES OF THE THESIS

The shrinkage and fragmentation of the nucleus are among the most striking morphological changes of cell death by apoptosis. Over the last years, there has been growing evidence that nuclear events are crucial steps in the process of apoptosis. Proteins have been identified which directly or indirectly play a role in the process of chromatin fragmentation. Their activity is controlled either by proteolytic cleavage, by post-translational modification or by protein translocation.

The objective of the present study was to contribute to the understanding of the mechanisms leading to apoptotic nuclear execution by identifying early changes of the protein complement of the nucleus associated with apoptosis. This was achieved by combining a cell-free apoptosis system with a proteomic analysis platform.

The present study was initiated with the following aims:

- To find and characterise an apoptotic system suited for the isolation of cytosolic extracts.
- To find and establish purification procedures for both components of the cell-free reaction: the cytosolic extracts and the isolated nuclei.
- To establish and to validate a cell-free apoptosis reaction with the help of classical hallmarks of apoptosis.
- To find and to apply an appropriate proteomic analysis platform for the identification and relative quantitation of alterations of nuclear protein levels in apoptosis.
- To characterise the identified candidate proteins with biochemical methods.
5 MATERIALS AND METHODS

5.1 Materials

5.1.1 Technical devices

**Centrifuges:** Biofuge fresco and Megafuge 1.0 R (Heraeus Instruments, Hanau, Germany).

**Confocal microscope system:** Zeiss LSM 510 Meta (Zeiss, Oberkochen, Germany). **Digital camera:** Hamamatsu Digital Camera C 4742-95, (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany). **Electrophoresis chambers:** Hoefer MiniVE Vertical Electrophoresis System (Amersham Biosciences Europe GmbH, Freiburg, Germany).

**Electrophoretic transfer cell:** Trans-blot cell (Biorad, Munich, Germany), Hoefer MiniVE Vertical Electrophoresis System blot module (Amersham Biosciences Europe GmbH, Freiburg, Germany). **ELISA-Reader:** SLT Spektra (SLT Labinstruments, Crailsheim, Germany).

**Film material:** GBX Developer and Fixer twin pack (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), Fuji Medical X-ray Film (Fuji Photo Film, Düsseldorf, Germany). **Fluorimeter:** Microplate Fluorescence Reader FL 600 (Deelux Labortechnik, Gödenstorf, Germany).

**Gradient gels:** Novex Precast 4-20 % glycine gels (Novex Electrophoresis GmbH, Frankfurt/Main, Germany). **iCycler:** iCycler iQ Real-Time PCR Detection System (Biorad, Munich, Germany).

**Image reader:** Luminescent Image Analyzer LAS-1000 CH, acquisition software Image Reader LAS-1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan), and Advanced Image Data Analyzer (AIDA) software (Raytest Isotopenmessgeräte GmbH, Straubehardt, Germany).

**Imaging camera:** Dage-72 CCD camera (Dage-MTI, Michigan City, IN, USA). **Incubator:** Model BB 6220 (Heraeus Instruments, Hanau, Germany). **Luminometer:** 1250 and Display 1250 (Wallac-ADL GmbH, Freiburg, Germany). **Mass spectrometer:** 4700 Proteomics Discovery System (Applied Biosystems, Foster City, USA). **Membrane:** Nitrocellulose Hybond™ ECL™ (Amersham-Buchler GmbH & Co. KG, Braunschweig, Germany).

**Microscopes:** Fluorescent microscope Axiovert 25 (Zeiss, Oberkochen, Germany). **Pipettes:** Eppendorf (Eppendorf-Netheler-Hitz GmbH, Hamburg, Germany), Gilson (Abimed, Langenfeld, Germany). **Scanner:** Sharp JX-
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330, colour image scanner (Sharp, Corporation, Japan), FLA-3000, fluorescent image scanner (Fujifilm, Japan). **Spectrophotometric analyser**: Eppendorf ACP 5040 (Eppendorf-Netheler-Hitz GmbH, Hamburg, Germany). **Spotcutter**: ProteomWorks Spot Cutter (Biorad, Munich, Germany). **Symbiot**: SymBiot I® Sample Workstation XVI (Applied Biosystems, Foster City, USA). **Thermomixer**: Eppendorf Thermomixer (Eppendorf-Netheler-Hitz GmbH, Hamburg, Germany). **2D-gel-electrophoresis**: First dimension: Multiphor II electrophoresis unit (Pharmacia & Pfizer GmbH, Karlsruhe, Germany); second dimension: Ettan™ DALTtWelve Large Vertical System, Ettan™ DALTsix Large Vertical System (Amersham Biosciences Europe GmbH, Freiburg, Germany). **Ultracentrifuges**: Beckman, TL-100 Ultracentrifuge; Beckman Coulter, Optima™ LE-80-K (Beckman Coulter, Fullerton, Canada).

5.1.2 Chemicals

**Amersham Biosciences Europe GmbH, Freiburg, Germany**: immobiline™ dry strip pH 4-7, pH 3-10 and pH 6-11, 24 cm; pharmalyte 3-10.

**Applied Biosystems**: Trifluoracetic Acid, protein sequencing grade


**Bender & Hobein GmbH, Heidelberg, Germany**: Pierce BCA protein assay reagent.

**BioRad Laboratories GmbH, Munich, Germany**: biotinylated SDS-Page standards, immobilized pH gradients pH 3-6. Bio-Rad protein assay, Bio-Rad protein assay standard 1, 1,2-Bis(dimethylamino)ethane (TEMED, for 2DE-gels), tris-(hydroxymethyl)-aminomethan (Tris, for 2DE-gels).

**Biomol, Hamburg, Germany**: Asp-Glu-Val-Asp-aminotrifluoromethylcoumarine (DEVD-afc).

**Boeringer Mannheim, Mannheim, Germany**: DNaseI.

**Bruker**: α-cyano-4-hydroxycinnamic acid (HCCA).

**Calbiochem-Novabiochem GmbH, Schwalbach, Germany**: calphostin C from cladosporium cladosporioides, formaldehyde (for 2DE-gels), N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone (Q-VD-Oph Non-O-methylated).

**BD Biosciences Clontech, Heidelberg, Germany**: RNase A.

**MPI Fermentas, St. Leon-Rot, Germany**: M-MuLV reverse transcriptase, prestained protein molecular weight marker, ribonuclease inhibitor.
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Merck, Darmstadt, Germany: acetonitrile, β-mercaptoethanol, dithiothreitol (DTT), formaldehyde, potassium-hexacyanoferrate (K₃Fe(CN)₆), sodium-thiosulfate, sucrose, sucrose for density gradient centrifugation, urea.

Molecular Probes Europe BV, Leiden, Netherlands: Hoechst 33342, SYTOX green.

New England Biolabs, Beverly, USA: protein λ-phosphatase.

Pierce, Rockford, USA: super signal west pico chemiluminescent substrate.

Polyscience Inc. Warrington, USA: aquapolymount.

Roche, Germany: complete protease inhibitor mix, trypsin sequencing grade, trypsin inhibitor from soybeans.

Roth GmbH & Co., Karlsruhe, Germany: acetic acid glacial, ethanol, glycine (for 2DE-gels), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), ponceau S, rotiphoress gel 30, sodium chloride, Tris.

Serva, Heidelberg, Germany: acrylamid-bis (37.5:1) 30 % (w/v) (for 2DE-gels), ammonium persulfate, coomassie brilliant blue G250, glycerol (for 2DE-gels), paraformaldehyde, silicone oil (for 2DE-gels), sodium dodecylsulfate (SDS), 1,2-Bis(dimethylamino)ethane (TEMED).

Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany: 7-amino-4-trifluoromethyl-coumarin (AFC), Adenosin 5′-Triphosphat (ATP), bovine serum albumin (BSA), 3-[(3-cloamidopropyl)-dimethylammonio]-propanesulfate (CHAPS), cycloheximide (CHX), cytochalasin B, digitonin, creatine phosphokinase, dimethylsulfoxide (DMSO), ethidium bromide, ethylenediamine tetraacetic acid (EDTA), ethylglycol-bis(β-aminoethylether) tetraacetic acid (EGTA), glycine, glycerol, Gö6983, jodacetamide, normal goat serum, ocdacic acid, phosphocreatine, proteinase K, tiourea, tris-(hydroxymethyl)-aminomethan (Tris), Triton X-100, trypan blue 0.4 %, Tween 20.

4,5,6,7-tetrabromobenzotriazole (TBB) and K25 was a kind gift from Dr. L.A. Pinna (University of Padua, Italy).

RuBPS was a kind gift from Dr. J. Kellermann (MPI of Biochemistry, Munich, Germany).

All other reagents not further specified were from Fluka (Buchs, Germany), Merck (Darmstadt, Germany), Riedel-de-Haen (Seelze, Germany), Roth GmbH & Co. (Karlsruhe, Germany, Serva (Heidelberg, Gemany) or Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).
5.1.3 Antibodies

**BD Bioscience, San Jose, USA**: monoclonal anti-DEK.

**Cell Signalling Technology Inc., Beverly, USA**: monoclonal ant-caspase-8 (clone 1C12).

**Chemicon, Temecula, USA**: monoclonal anti-actin (clone C4).

**DAKO AIS, Glastrup, Denmark**: Peroxidase conjugated goat anti-mouse Immunoglobulins, Peroxidase conjugated goat anti-rabbit Immunoglobulins.

**Molecular Probes Europe BV, Leiden, Netherlands**: Alexa™ 488-conjugated anti-mouse IgG antibody, Alexa™ 488-conjugated anti-rabbit IgG antibody, Alexa™ 546-conjugated anti-rabbit IgG antibody.

**Pharmingen, San Diego, USA**: monoclonal anti-cytochrome c antibody for western blot (clone 7H8.2C12), polyclonal anti-HMG1, polyclonal anit-HMG2.

**Santa Cruz Biotechnology Inc., Santa Cruz, USA**: polyclonal anti-Lamin B.

**Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany**: anti-goat IgG peroxidase conjugated.

**Stressgen, Victoria, Canada**: polyclonal anti-PDI.

**Upstate Biotechnology, Waltham, USA**: polyclonal anti-caspase-3 (#06-735).

Anti-DEK monospecific antibody was a kind gift from Dr. C. Gruss (University of Konstanz, Germany). Anti-hnRNP A1/A2/B1 was generously provided by Dr. J. Toutant, University of Sherbrooke, Canada). Anti-hnRNP A1 (clone 4B10) and C1/C2 (clone 4F4) were a kind gift from Dr. G. Dryfuss, University of Pennsylvania, USA). Anti-PARP-1 antibodies, LIMA4 and CII-10, were kindly provided by Dr. A. Bürkle (University of Konstanz, Germany). Anti-polyclonal UBF antibody (H-300) was generously provided by Dr. A. Lamond (University of Dundee, Great Britain).

5.1.4 Kits

**QuickPrep Total RNA Extraction Kit**: Amersham Biosciences Europe GmbH, Freiburg, Germany.

**iQ™ SYBR® Green Supermix Kit**: Biorad, Munich, Germany.
5.2 Animals

The strain C3HeB/FeJ was kindly provided for further breeding by Dr. J. Backers (Institute of experimental Genetic, GSF, national research centre for environment and health, Munich, Germany).

Specific pathogen-free male C3HeB/FeJ (approximately 25 g, from the in-house animal breeding station of the University of Konstanz, Germany) were maintained under controlled conditions (22 °C and 55 % humidity, constant day/night cycle of 12 h) and fed a standard laboratory chow. All animals received humane care in compliance with the NIH guidelines as well as with the regulatory requirements in Germany.

5.3 Cells

Jurkat human T-cell lymphoma clone E6-1 were obtained from the American tissue culture collection (ATCC No. TIB-152, Rockville, MD, USA).

Human cervix carcinoma cells (HeLa 229) were kindly provided by the Karolinska Institute in Stockholm, Sweden.

N2A neuroblastoma cells stably transfected with the plasmid of CD95-L were kindly provided by Prof. Weller (Tübingen, Germany).

5.3.1 Cell culture material

RPMI-1640 medium (containing stabilized glutamine) and fetal calf serum were purchased from Biochrom KG (Berlin, Germany). RPMI-1640 medium without glucose and without glutamine, Dulbecco’s modified Eagle medium with high and also low glutamine, penicillin and streptomycin, and glutamine were bought from Gibco BRL Life Technologies (Eggenstein, Germany). G418 was purchased from PAA Laboratories GmbH (Linz, Austria).

Cell culture flasks and plates were obtained from Costar GmbH (Bad Homburg, Germany) and Greiner GmbH (Frickenhausen, Germany).
5.4 Methods

5.4.1 Cell Culture Experiments

5.4.1.1 Culturing of various cell lines

All media were supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were grown in a humidified atmosphere at 5% CO₂ and 37 °C.

Jurkat human T cells clone E6-1 were grown in suspension in RPMI-1640 medium.

Human cervix carcinoma cells (HeLa 229) were cultured in DMEM (Dulbecco’s modified Eagle medium). Both Jurkat and HeLa cells were passaged routinely every two to three days in a ratio of 1:5 or 1:10, respectively.

N2A neuroblastoma cells stably transfected with murine CD95 ligand cDNA were cultured in DMEM additionally supplemented with 200 µg/ml G418 as selection marker for transfection. CD95 ligand was obtained as culture supernatant after incubation for 48 h with serum-free DMEM Media (Rensing-Ehl et al., 1995).

5.4.1.2 Viability assays

To distinguish normal, apoptotic, and necrotic cells, cultures were stained with a combination of the fluorescent chromatin dyes Hoechst 33342 (500 ng/ml; membrane permeant, stains all nuclei) and SYTOX (500 µM, membrane impermeant, stains nuclei of lysed cells) by adding the dye solution (100 x in DMSO) 10 min prior to investigations. Using a fluorescence microscope and lenses providing 400 x final magnification, cells with condensed or fragmented nuclei were scored as apoptotic; lysed cells with non-condensed nuclei were scored as necrotic. For each data point >100 cells were scored in at least 3 different microscopic fields.
5.4.1.3 Immunocytochemistry

For immunocytochemistry HeLa 229 cells were seeded in 12-well plates containing glass coverslips at a density of 1.5 x 10^5 cells/ml. After 12-16 h incubation (i.e. 50-60 % confluency) the cells were stimulated to undergo apoptosis by supplementing 300 ng/ml TRAIL for 30, 60 or 120 min to the media. All substances were dissolved in phosphate-buffered saline (PBS). After respective time points the cells were fixed with 4 % paraformaldehyde for 15 min, the reactive aldehyde groups were blocked for 10 min with 50 mM ammonium chloride. Then, if not quoted differently, the cells were permeabilised in 0.3 % Triton X-100 for 3 min, non-specific binding sites were blocked in 1 % bovine serum for 60 min. Antibodies were supplemented with 10 % serum from the animal in which the primary antibody had been generated to reduce cross reactions, and antibody incubation of primary antibodies was performed for 60 min in a humidified chamber at room temperature. The secondary antibody was labelled either with Alexa™ 488 (\( \lambda_{ex} = 495 \text{ nm}, \lambda_{em} = 519 \text{ nm} \)) or Alexa 546 (\( \lambda_{ex} = 556 \text{ nm}, \lambda_{em} = 573 \text{ nm} \)) and was employed at a dilution of 1:400 for 30 min. After the incubations with specific antibodies all cells were stained for 10 min with 100 ng/ml Hoechst-33342. Stained HeLa cells were mounted either in aquapolymount and dried at room temperature over night or in Vectashield and directly imaged by confocal microscopy using the Meta LSM 510 from Zeiss.

To monitor alterations of protein distributions in apoptosis, HeLa cells were stained with the following antibodies:

**DEK**: Blocking was performed in 3 % bovine serum. Permeabilised cells were incubated at 37 °C for 60 min either with the affinity-purified rabbit antibody (1:5000, gift from C. Gruss) or the mouse monoclonal antibody (1:500, BD Bioscience).

**UBF**: The rabbit polyclonal antibody was used in a dilution of 1:100 (gift from A. Lamond).

**heterogenous ribonucleoprotein A1/A2/B1 (hnRNP A1/A2/B1)**: A rabbit polyclonal serum was used in a dilution of 1:5000 (gift from J. Toutant).

**heterogenous ribonucleoprotein A1 (hnRNP A1)**: The cells were permeabilised in cold acetone at -20 °C for 3 min. The mouse monoclonal antibody 4B10 was used at a dilution of 1:1000 (gift from G. Dryfuss).

**heterogenous ribonucleoprotein C1/C2 (hnRNP C1/C2)**: The cells were permeabilised in cold acetone at -20 °C for 3 min. The mouse monoclonal antibody 4F4 was used at a dilution of 1:1000 (gift from G. Dryfuss).
**high mobility group protein B1 (HMG B1):** The polyclonal rabbit antibody was used at a dilution of 1:1600 (Pharmingen).

**high mobility group protein B2 (HMG B2):** The cells were permeabilised in cold acetone at -20 °C for 3 min. The rabbit polyclonal antibody was used at a dilution of 1:1600 (Pharmingen).

5.4.1.4 Preparation of whole cell extracts for immunoblot

Cells were lysed in RIPA-buffer (150 mM NaCl, 50 mM Tris, 1 % IGEPAL, 0.25 % sodium deoxycholate, 1 mM EGTA), which was supplemented with proteases inhibitor (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 mM iodoacetate, 1 mM iodoacetamide, 40 µM leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin). Alternatively cells were lysed in boiling SDS-buffer (50 mM Tris/HCl pH 8.0, 0.5 % SDS, 1 mM DTT) for 10 min at 95 °C. Cell debiris and solubilised proteins were separated by centrifugation at 14,000 x rpm for 10 min at 4 °C. The supernatant was recovered, snap frozen and stored at -80 °C.

5.4.1.5 In vitro dephosphorylation of whole cell lysates

Jurkat T-cells were lysed in cell lysis buffer A (0.4 % NP-40, 1 % Triton, 50 mM Tris pH 7.5, 50 mM NaCl) supplemented with complete protease inhibitor mix (Roche, Germany) and 10 nM okadaic acid (Sigma, Germany). The viscosity was reduced by shearing DNA with a needle (size 18, 0.45 x 25 mm), until the protein solution became clear. Cell debris were pelleted at 14,000 x rpm for 10 min at 4 °C and the supernatant was snap frozen and stored at -80 °C. The remaining pellet was lysed in boiling SDS-buffer (50 mM Tris/HCl pH 8.0, 0.5 % SDS, 1 mM DTT) for 10 min at 95 °C and contained insoluble nuclear proteins. Dephosphorylation was carried out using the protein λ-phosphatase according to manufacturer’s protocol. In brief, 500 µg of protein extract were dephosphorylated in a total volume of 80 µl (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.01 % Brij 35 and 2 mM MnCl₂) using 1000 U λ-phosphatase for 30 min at 30 °C.
5.4.1.6 Determination of protein content

The amount of protein in cell lysates was determined by using the BCA (bicinchoninic acid) method of Pierce. On a 96 well microtiter plate, 5 µl of lysates were incubated with 95 µl of a 1:51 mixture of reagents A and B (provided by the supplier). After 30 min at 37 °C, the colour reaction, due to the reduction of Cu$^{2+}$ to Cu$^{+}$ by peptide bounds and other compounds of proteins, was analysed at 550 nm in an ELISA reader. Calibration was performed by using BSA in concentrations of 0.2-2 mg/ml.

5.4.1.7 Isolation of a cytosolic fraction with digitonin

The release of cytochrome c from mitochondria was analyzed in cytosolic fractions isolated by selective digitonin permeabilisation of the plasma membrane. The experiment was carried out at room temperature. Briefly, 4 x 10$^6$ cells were harvested, pelleted (1000 x g, 3 min), and resuspended in 250 µl phosphate buffered saline (2 mM NaH$_2$PO$_4$, 16 mM Na$_2$HPO$_4$, 150 mM NaCl, pH 7.4). Then, plasma membrane lysis was performed by adding 250 µl of a digitonin solution (130 µg/ml in sucrose (500 mM)) to the cell suspension under vigorous vortexing for 10 s and further incubation for 30 s. The cytosolic fractions were quickly isolated and removed from organelles and cell debris by centrifugation at 14 000 x g for 60 s at 4 °C. Protein in the supernatant was precipitated with 5 % trichloric acid over night at -20 °C, followed by centrifugation step at 14.000 x rpm and 4 °C for 30 min. Precipitated protein was dissolved in sample buffer (6 M urea, 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol, 60 mM Tris, pH 6.8) by sonification.

5.4.2 SDS polyacrylamide gel electrophoresis and Western blot

In the general procedure, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970). All samples were supplemented with 0.1 x volume of sample buffer (584 mM Tris, pH 6.8, 17 % SDS, 60 % glycerine and 10 % β-mercaptoethanol), and loaded in equal volumes. After focusing of the proteins in the stacking gel at 60 V, the separating gel run with a constant voltage of 120 V. After electrophoresis was completed, six layers of Whatman 3MM paper, one nitrocellulose membrane all the size of the gel, and 5-6 packing sponges, were soaked in blotting-buffer (20 % methanol, 25 mM Tris, 192 mM glycine, 0.1 % SDS). The separating gel was
equilibrated for 5 min in blotting buffer. Assembly of the transfer stack was started with 2-3 packing sponges on the black cathode side. Next filter papers, the gel and the membrane were added to the stack and air bubbles between the layers were removed by rolling a pipette across the stack. Completion of the assembly was reached by adding 3 packing sponges onto the filter membranes. The proteins were transferred at 350 mA for 2 h. Cooling of the blotting apparatus is performed by filling the tank with chilled blotting buffer (recyclable).

After blotting, transfer and loading of protein was controlled by staining the membrane with Ponceau red (0.2 % Ponceau S, 5 % acetic acid). Then, membranes were destained in TBST (150 mM NaCl, 50 mM Tris/HCl pH 8, 0.05 % Tween 20), and incubated in TBST plus 5 % non-fat dry milk for 1 h to block unspecific binding of the antibody. Then the membrane was incubated with the primary antibody diluted in 5 % milk powder in TBST for 1 h at room temperature or over night at 4 °C. Non-bound antibody was removed by washing the membrane thoroughly in TBST. Next, the secondary antibody conjugated to HRP diluted in 5 % milkpowder/TBST was added, and in parallel the biotinylated marker was treated with streptavidin (1:5000 in TBST). After another washing step, HRP activity was detected using the Supersignal solution (Pierce) and exposed to x-ray films or recorded in a chemiluminescence image analyser.

Detailed protocols for immunodetection of:

**PARP-1:** 30 µg/lane were loaded on a 12 % gel. The primary antibodies against murine PARP-1, LIMA4 (1:1000), and the mouse hybridoma supernatant CII-10 (1:10) against human PARP-1 were used (gift from A. Bürkle).

**caspase-3 (proform and active fragments):** 40 µg protein/lane were loaded on a 4-20 % gel. The monoclonal rabbit primary antibody was used at a 1:1000 dilution (Upstate Biotechnology).

**caspase-8:** 40 µg protein/lane were loaded on a 4-20 % gel. The monoclonal mouse antibody against caspase-8 was used in a 1:1000 dilution (Cell Signalling).

**cytochrome c:** For the detection of cytochrome c, 30 µl of dissolved cytosolic fraction (corresponding to 2 x 10^6 cells) were resolved on a 12 % gel. The monoclonal mouse antibody against cytochrome c was used in a dilution of 1.5 µg/ml (Pharmingen).

**protein disulfide isomerase (PDI):** 40 µg/lane protein were loaded on a 12 % gel. Primary polyclonal rabbit antibody against PDI was used in a dilution of 1:2000 (Stressgen).
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**DEK:** 40-200 µg/lane were loaded on a 10 % gel. The affinity purified rabbit antibody was used in a dilution 1:5000 (gift from C. Gruss) and the commercial monoclonal mouse antibody was used in a 1:1000 dilution (BD Bioscience).

**heterogenous ribonucleoprotein A1/A2/B1 (hnRNP A1/A2/B1):** For *in vitro* samples 50 µg/lane (corresponding to 1.2 x 10^6 theoretical cell equivalents) were loaded on a 10 % gel. The rabbit polyclonal serum was used in a dilution of 1:5000 (gift from J. Toutant).

**heterogenous ribonucleoprotein A1 (hnRNP A1):** For *in vitro* samples 25-50 µg/lane (corresponding to 1.2 x 10^6 – 2.4 x 10^6 theoretical cells equivalents) were loaded on a 10 % gel. A mouse monoclonal antibody 4B10 was diluted 1:1000 (gift from G. Dryfuss).

**heterogenous ribonucleoprotein C1/C2 (hnRNP C1/C2):** For *in vitro* samples 50 µg/lane (corresponding to 1.2 x 10^6 theoretical cells equivalents) were loaded on a 10 % gel. The mouse monoclonal antibody 4F4 was used in a dilution of 1:1000 (gift from G. Dryfuss).

**high mobility group protein B1 (HMG B1):** For *in vitro* samples 50 µg/lane (corresponding to 1.2 x 10^6 theoretical cells equivalents) were loaded on a 12 % gel. The polyclonal rabbit antibody was used in a dilution of 1:500 (Pharmingen).

**high mobility group protein B2 (HMG B2):** For resolution of *in vitro* samples 50 µg/lane (corresponding to 1.2 x 10^6 theoretical cells equivalents) were loaded on a 12 % gel. The rabbit polyclonal antibody was used in a dilution of 1:500 (Pharmingen).

**actin:** Actin was used as a loading control for all Western blot analysis. The membranes were reprobed with a primary antibody against actin (1:10.000) (Chemicon).

### 5.4.3 DEVD-afc cleavage activity assay

Caspase activity was determined by measuring the cleavage of the fluorogenic substrate DEVD-aminotrifluoromethylcoumarine (-afc) either in isolated cytosolic fractions (supplemented with 1 mM EGTA and 1mM AEBSF) or in whole cell extracts after lysis in caspase lysis buffer (25 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 0.5 % Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotenin, 1 mM AEBSF). The fluorimetric assay was performed in microtiter plates with sample volumes corresponding to a total protein amount of 5 µg in a total volume of 100 µl. DEVD-afc cleavage was followed in reaction buffer (50 mM HEPES, 10 mM dithiothreitol, 1 % sucrose, 0.1 % CHAPS) with a substrate concentration of 40 µM. Free afc was detected over a period of 20 min at 37 °C with
λ<sub>ex</sub> = 390 nm and λ<sub>em</sub> = 505 nm in a microtiter plate fluorimeter. Absolute activity was calibrated with afc-standard solutions.

5.4.4 Spectrophotometric enzyme activity assays

Activity of β-hexosaminidase, a specific marker for lysosomes, was measured in microtiter plates. 10 µg of protein of isolated cytosolic fractions were combined with reaction buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, pH 5.0) to a total volume of 100 µl. Determination of the substrate turnover (100 µM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide), was performed at 37 °C for 30 min in intervals of 5 min at 344/460 nm.

LDH as cytosolic marker in digitonin lysates was determined according to Bergmeyer (Bergmeyer, 1984). Measurements were performed in an Eppendorf ACP 5040 Analyser. The reduction of NAD to NADH was followed at 340 nm.

5.4.5 Preparation of cytosolic extracts

5.4.5.1 Cell lysis with digitonin

For induction of apoptosis Jurkat T-cells were incubated in serum-free medium at a density of 5 x 10<sup>6</sup> cells/ml with 2.5 % CD95L-containing supernatant. At defined time points, the cells were harvested by centrifugation at 4 °C and 1200 x rpm, carefully washed twice with cold phosphate buffered saline (2 mM NaH<sub>2</sub>PO<sub>4</sub>, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 7.4) and once with cold transport buffer (20 mM HEPES pH 7.3, 110 mM K-acetate, 5 mM Na-acetate, 2 mM Mg-acetate, 0.5 mM EGTA, 250 mM sucrose) supplemented with 1 mM DTT and protease inhibitors (complete protease inhibitor, Roche, Germany). The cells were then resuspended in a small volume of transport buffer and transferred to an Eppendorf tube. The cells were pelleted for 3 min at 700 x g and 4 °C. For lysis the volume of the packed cell pellet was estimated and 0.5 volumes of transport buffer and this buffer containing 2.4 mg/ml digitonin (60 µg/5 x 10<sup>7</sup> cells) were added. Cells were lysed under vigorous vortexing for 10 s, followed by incubation on ice for 3 min. Organelles and cell debris were pelleted by centrifugation at 18.000 x g for 15 min at 4 °C. The supernatant was snap-frozen and stored at -80°C.
Cytosolic extracts from untreated cells were prepared in parallel using the identical procedure.

### 5.4.5.2 Hypotonic cell lysis

For induction of apoptosis Jurkat T-cells were incubated in serum-free medium at a density of 5 x 10^6 cells/ml with 2.5 % CD95L-containing supernatant. At defined time points, the cells were harvested by centrifugation at 4 °C and 1200 x rpm, carefully washed twice with cold phosphate buffered saline (2 mM NaH₂PO₄, 16 mM Na₂HPO₄, 150 mM NaCl, pH 7.4) and once with 5 ml hypotonic buffer supplemented with sucrose (10 mM Hepes pH 7.5, 5 mM KCl, 15 mM MgCl₂, 1 mM DTT, 250 mM sucrose, protease inhibitors (complete protease inhibitor, Roche, Germany)). Afterwards the cells were resuspended in 10 ml hypotonic buffer without sucrose and pelleted by centrifugation at 1200 x rpm. The supernatant was discarded and the cells were allowed to swell on ice for 10 min. The lysis was achieved by homogenising the cells in a glass homogenizer with 12 strokes of a tight-fitting pestle and the lysates was incubated on ice for further 30 min. Organelles and cell debris were pelleted by centrifugation at 18.000 x g for 20 min at 4 °C. The supernatant was snap-frozen and stored at -80 °C.

### 5.4.6 Permeability assay

The permeability assay was performed as described previously (Roehrig et al., 2003). In brief, HeLa 229 cells were seeded in 12-well plates containing glass coverslips at a density of 1.5 x 10^5 cells/ml. After 12-16 h incubation (i.e. 50-60 % confluency) the coverslips were transferred to a 6-well plate on ice, washed with 5 ml cold PBS and then incubated in 5 ml permeabilisation buffer (20 mM HEPES-NaOH pH 7.5, 110 mM potassium acetate, 5 mM magnesium diacetate, 0.5 mM EGTA, 250 mM sucrose) containing 40 µg/ml digitonin for 4-5 min. After removal of the digitonin solution the coverslips were washed three times with cold permeabilisation buffer for 1, 5 and 10 min, respectively and briefly equilibrated in transport buffer (20 mM HEPES-NaOH pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium diacetate, 0.5 mM EGTA, 250 mM sucrose). Next, the coverslips were placed in a humidified chamber and incubated for 7 min with 30 µl of a mix containing cytosolic extract (corresponding to 300-400 µg of total protein) plus 1.5 µl of an energy-regenerating system (1 mM HEPES-NaOH pH 7.3, 0.5 mM ATP, 0.5 mM GTP, 12.5 mM glucose, 10 mM phosphocreatine, 0.3 U/ml creatine kinase) in transport buffer. Then 10 µl of
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a 0.6 mg/ml dextran solution in transport buffer was added and homogeneously distributed. After another 7 min the coverslips were mounted in a coverslip holder and imaged by confocal microscopy. Images were taken using a Leica TCS confocal microscope with a 40 x oil immersion objective at constant pinhole and laser power settings. The size of the scanned area was 250 µm x 250 µm. Four randomly distributed, non-overlapping fields were scanned per coverslip during a maximum time period of 5 min. Images obtained with the confocal microscope (512 x 512 pixel) were analysed using the NPA software (Roehrig et al., 2003).

5.4.7 Isolation of nuclei

5.4.7.1 Isolation of mouse liver nuclei

The separation of nuclei was performed following the protocol of Blobel and Potter (Blobel and Potter, 1966) modified by Cordes (Cordes, 1992). Mice were sacrificed by cervical dislocation and the liver was removed. All separation steps were carried out at 4 °C. 2 -30 g of liver tissue were homogenised in 50 - 100 ml ice-cold buffer A (15 % Sucrose, 1 mM Tris/HCl pH 7.2, 70 mM KCl, 2 mM MgCl₂), first by cutting into small pieces, then by pressure through a narrow metal sieve using a metal stamp, and finally by douncing in a glass homogenizer with a loose-fitting pestle. After filtration through gaze, the homogenate was centrifuged for 10 min at 4 °C and 1000 x g. The pellet was resuspended in 38 ml buffer A, dounced and mixed with 85 ml (112.5 g) of a 92 % sucrose solution (92 % sucrose, 10 mM Tris/HCl pH 7.2, 2 mM MgCl₂). This 70 % sucrose suspension was then transferred on top of pre-cooled 72 % sucrose (72 % sucrose, 10 mM Tris/HCl pH 7.2, 2 mM MgCl₂) cushions previously prepared in polyallomer centrifuge tubes. The separation of cellular components was achieved by ultracentrifugation in a SW27 rotor at 16.000 x rpm (40.000 x g) at 4 °C for 60 min. The pelleted nuclei were resuspended in 20 - 30 ml buffer A, dounced and centrifuged again for 10 min at 4 °C and 1000 x g. Finally, the nuclei were counted, snap-frozen and stored in buffer A supplemented with 20 % glycerin at -80 °C. 1 mM DTT and 30 µg/ml trypsin-inhibitor were added to all buffers before usage.
5.4.7.2 Isolation nuclei from culture cells

A total of 2 x 10^8 Jurkat T-cells were incubated with 10 µM cytochalasin B for 30 min at 37 °C. 

The cells were harvested by centrifugation at 4 °C and 1200 x rpm, washed twice in phosphate buffered saline (2 mM NaH_2PO_4, 16 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) and once in 20 ml NB buffer (10 mM Pipes pH 7.0, 10 mM KCl, 2mM MgCl2, 1mM DTT, protease inhibitors (complete protease inhibitor, Roche, Germany)). The pellet was resuspended in 7 ml NB buffer supplemented with 10 µM cytochalasin B and incubated for 30 min on ice. The lysis was performed by homogenising the cells in a glass homogenizer with 10 strokes of a loose-fitting pestle. 1 ml of this lysate was then transferred onto 5 ml of a 30 % sucrose cushion (solved in NB buffer) in 50 ml vials and pelleted for 10 min at 500 x g and 4 °C. The pellet, containing the nuclei, was resuspended in 5 ml NB buffer and the nuclei were counted. After a final washing step in NB, the nuclei were resuspended in NSB buffer (10 mM Pipes pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 50 % glycerin, complete inhibitor mix) at a density of 1 x 10^8/ml, snap-frozen and stored at -80 °C.

5.4.8 In vitro apoptosis assay

The reaction was carried out in extract dilution buffer (EDP) containing 10 mM Hepes pH 7.0, 50 mM NaCl, 2 mM MgCl_2 and 5 mM EGTA. In all experiments, cytosol was supplemented with an adenosine triphosphate (ATP) regenerating system consisting of 10 mM phosphocreatine, 2 mM ATP, 1 mM DTT and 15-20 mU/mg creatine phosphokinase. Cell-free apoptosis was performed by adding 4 mg of cytosolic extract gained by digitonin lysis to 1 x 10^8 isolated mouse liver nuclei, the final volume of the reaction accounted 600 µl. When using nuclei derived from cell culture 1.5 mg of cytosolic extract gained by hypotonic lysis were added to 2.5 x 10^7 isolated nuclei in a total volume of the reaction mix of 200 µl. The mixture was incubated at 37 °C for 10 min by shaking at 300 x rpm.

The mouse liver nuclei were recovered by centrifugation through a sucrose cushion (15 % and 40 % sucrose in 10 mM Tris pH 7.2) at 10.000 x g and 4 °C for 2 min. The pelleted mouse liver nuclei were resuspended in different buffers depending on their further processing either in buffer A (15 % Sucrose, 1 mM Tris/HCl pH 7.2, 70 mM KCl, 2 mM MgCl_2) and examined for typical apoptotic changes or directly solubilised for proteomic analysis (see 5.5).
The nuclei from cell culture were recovered by centrifugation through a 30 % sucrose in NB buffer (10 mM Pipes pH 7.0, 10 mM KCl, 2mM MgCl₂) cushion at 500 x g and 4 °C for 5 min. The pelleted nuclei were resuspended in in cell lysis buffer A (0.4 % NP-40, 1 % Triton, 50 mM NaCl, 50 mM Tris pH 7.5) for Western blot analysis of DEK in apoptosis.

5.4.9 Conventional agarose gel-electrophoresis (CAGE)

To determine DNA degradation into oligonucleosomal fragments, nuclei (3 – 5 x 10⁵) were briefly pelleted at 1500 rpm, 4 °C, and 10 min. The supernatant was carefully removed and the nuclei were dissolved in 200 µl of DNA lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl). Then, proteinase K was added to a final concentration of 100 µg/ml and the lysed nuclei were incubated for 2-4 h at 37 °C. The DNA was precipitated by adding 200 µl of isopropanol at room temperature and incubated over night at 4 °C. The DNA was pelleted at 14,000 x g for 30 min at 4 °C. The pellet was washed once in 70 % ethanol and resuspended in 10 µl of TE (10 mM Tris/HCl pH 7.5, 1 mM EDTA). 1 µl of 10 mg/ml RNase was added and the samples were incubated at 37 °C for 30 min. After addition of 10 µl of DNA sample buffer (50 % sucrose, 0.1 mM EDTA, 0.1 % BPB, pH 7.0), the samples were analysed on a 1-1.5 % agarose/TAE (40 mM Tris, 1mM EDTA pH 8.0, 3.75 % (v/v) glacial acetic acid) gel and run at a constant voltage of 80 V in TAE buffer. The gels were stained by the DNA-intercalating dyes SybrGreen for 10 min, shortly washed in water and visualised by UV trans-illumination.

5.4.10 Isolation and analysis of RNA

5.4.10.1 Isolation of total RNA

For all RNA experiments, RNase-free plastic material, glassware heated for 8 h at 180 °C and DEPC-H₂O (0.1 % diethylpyrocarbonate in MilliQ water, autoclaved) were used. RNA was isolated from Jurkat T-cells (5x10⁶ cells) using the QuickPrep Total RNA Extraction Kit (Amersham Bioscience) following the manufacturer’s protocol. The final pellet was dissolved in 60 µl DEPC-H₂O and the concentration was determined spectrophotometrically at 260 nm. Contaminating DNA was removed by DNasel treatment, using the following protocol. 2 µg
total RNA was mixed with 10 U DNaseI in reaction buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl and 2 mM MgCl$_2$ in DEPC-H$_2$O) and incubated for 15 min at room temperature. To inactivate DNase, the sample was heated for 15 min to 65 °C. Samples were stored in aliquots at -80 °C.

### 5.4.10.2 First strand cDNA synthesis via reverse transcriptase

Reverse transcription of mRNA to a complementary DNA single strand (cDNA), which was used as a DNA template for PCR reactions, was performed using the M-MuLV (moloney murine leukemia virus) reverse transcriptase. 1 µg of total RNA was incubated with 0.5 µg oligo(dT) added to a total volume of 11 µl with DEPC-H$_2$O, the annealing of primer to RNA was performed for 5 min at 70 °C. Next reaction buffer (50 mM Tris/HCl pH 8.3, 50 mM KCl, 5 mM MgCl$_2$, 10 mM DTT), deoxynucleotides (dNTP’s), RNase Inhibitor (20 units) and DEPC-H$_2$O were added to a volume of 19 µl and incubated for 5 min at 37 °C. Finally the reverse transcriptase was added (40 units) and transcription was performed at 37 °C. The reaction was stopped after 60 min by increasing the temperature to 70 °C for 10 min. The cDNA was either directly used for PCR reaction or stored at -20 °C.

### 5.4.10.3 Quantitative polymerase chain reaction (Q-PCR)

Relative quantification of mRNA based on the relative expression of a target gene versus one reference gene, in this experiments the housekeeping gene actin, was performed in the course of apoptosis. Real-time PCR was realised using the iQ™ SYBR® Green Supermix Kit (BioRad) with two sets of primers, one specific for actin and one for DEK.

**Primers:**

- **actin for:** 5’-CTAGAAGCATTGCGGTGGACCAT-3’
- **actin rev:** 5’-GTGGCCGGGACCTGACTGAC-3’
- **DEK for:** 5’-GAGGAAGAGGACGGACGACGAGG-3’
- **DEK rev:** 5’-GTTAGCCTTCTTGGCATTCCAGA-3’

One PCR reaction consisted of:

- 10 µl iQ™ SYBR® Green Supermix
- 10 µmol (1 µl) of each primer
- 2 µl of DNA template (0.01 - 10 ng)
- ad 20 µl with bidestilled H$_2$O
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First, for the estimation of the PCR efficiency of each set of primers, a standard curve was pipetted with a cDNA template isolated from non-stimulated control Jurkat T-cells. The amount of DNA varied from 0.01 to 10 ng in a 10-fold dilution series and each dilution was run in triplicates. Plotting of each threshold cycle (CT) against the corresponding logarithm of the cDNA dilution revealed a slope of which the PCR efficiency was automatically calculated by the programme iCycler (Biorad).

This DNA template served as the control for further evaluation and was run in parallel to the samples of interest from the apoptotic time course.

The standard protocol for amplification was the following:

- **fast start** 95 °C 3 min
- **amplification** 50 cycles
  - 95 °C 30 s
  - 60 °C 45 s
  - 72 °C 1 min
- **melt curve** 55 to 95 °C in steps of 0.5 °C
- **cool down to 20 °C**

For relative quantification, 1 ng of cDNA template from each sample was analysed in triplicates and in parallel with both sets of primers. Then the CT value of each sample for each set of primers was estimated.

The relative quantification of the target gene DEK in comparison to the reference gene actin was determined using the mathematical model established by Pfaffl (Pfaffl, 2001), which takes into account the PCR-efficiencies of the primers:

\[
\text{ratio} = \left( \frac{E_{\text{target}}^{\Delta CT_{\text{target}} \text{(control-sample)}}}{E_{\text{ref}}^{\Delta CT_{\text{ref}} \text{(control-sample)}}} \right)
\]

Here, the relative expression ratio of DEK (target) is calculated in comparison to actin (reference). This equation takes account of: (1) the determined PCR-efficiencies (E) for each set of primers, and (2) the CT deviation (mean of triplicates) of the unknown sample (from the apoptotic time course) and the control cDNA (the same as used for determination of PCR-efficiency).
5.5 Proteomic experiments

5.5.1 Determination of protein content (Bradford)

Reduction compounds, which are present in high amounts in solubilisation buffers for proteomic experiments, interfere with protein determination methods based on the reduction of Cu$^{2+}$ to Cu$^+$ such as the BCA method (5.4.1.6). Therefore, a modified Bradford method (Ramagli, 1999) which is only mildly affected by basic reagents, such as urea, was used for all protein determinations in proteomic experiments.

A fresh mixture of 0.1 N HCl in bidestilled H$_2$O was prepared and 10 µl were added to each sample. Bidestilled water was added to a final volume of 100 µl. The commercial Biorad protein assay-dye was diluted 1:4 in bidestilled H$_2$O, filtered through a round paper filter and 1.725 µl were applied to each sample. After gentle vortexing the samples were transferred to cuvets and the colour reaction was measured after 5 min at 595 nm. The calibration was performed using ovalbumin at concentrations between 2.5 to 25 µg.

5.5.2 Isotope code protein labelling (ICPL)

The protocol for isotope code protein labelling was established by Schmidt et al. (Schmidt et al., 2004). N-nicotinoyloxy-succinimide (Nic-NHS) was a kind gift of A. Schmidt and was prepared following the protocol of F. Hausch (Hausch and Jaschke, 2001).

To differentially label all free amino groups - lysines and the N-terminal amino group - of nuclear proteins, the recovered nuclei from the cell-free apoptotic reaction (5.4.8) were directly dissolved in a guanidine-hydrochloride solution (6 M guanidine-hydrochloride, 0.1 M HEPES, pH 8.5). After heavy vortexing the samples were shaken vigorously for 15 min at RT, sonified, shaken again for 30 min and finally centrifuged at 100.000 x g at 4 °C for 30 min. The supernatant was taken at a concentration of 5 mg/ml and the pH was checked to be exactly 8.5 before proceeding with the labelling experiment.

If not stated differently, all chemicals were dissolved in 0.1 M HEPES, the pH was adjusted to 8.5 with NaOH and all reactions were carried out at 25 °C. Firstly, disulfide bonds were reduced with 5 mM tris(2-carboxyethyl)phosphine (TCPE) at 60 °C for 30 min. To exclude
disturbing oxidations the samples in the reaction tube were overlayed with argon. After cooling down, the free cysteins were alkylated with 0.4 µM iodoacetamide. The reaction was allowed to proceed for 30 min in the dark. Excess of iodoacetamide was quenched by adding 0.5 µM N-acetyl-cysteine for 15 min.

For nicotinoylation, the samples to be compared were incubated with 20 mM N-nicotinoyl-NHS containing either the heavy (d4) or the light (d0) isoform after their surface lamination in the reaction tube with argon for 2 h at pH 8.3. Then, 115 mM hydroxyamine, pH 8.3 was added for 20 min to destroy the remaining reagents and equal aliquots of both samples were combined. Esters, which also form during the labelling procedure, were hydrolysed by raising the pH to 11-12 for 20 min.

5.5.3 Two-dimensional gel electrophoresis

This method, first introduced in 1975 by O'Farrell and Klose (Klose, 1975; O'Farrell, 1975), separates proteins according to the physico-chemical parameters charge (isoelectric point) in the fist dimension and size (molecular weight) in the second dimension. The protocol used here for 2DE-gel-electrophoresis is based on the work of Klose and Kobalz (Klose and Kobalz, 1995) and combines isoelectric focusing (IEF) using carrier ampholytes in the first dimension with SDS-PAGE in the second dimension.

5.5.3.1 Sample preparation

Nuclei recovered from the cell-free apoptotic reaction were directly dissolved in rehydration buffer (8 M urea, 2 M thiourea, 4 % chaps, 20 mM TRIS and 2 % pharmalyte 3-10).

ICPL labelled nuclear proteins were dialysed (cut off 8 kD) against 8 M urea, 2 M thiourea and 20 mM TRIS overnight at 4 °C. Before submitting those to two-dimensional gel electrophoresis 2 % pharmalyte 3-10 and 0.5 % bromphenolblue were added.

5.5.3.2 First dimension (Isoelectric focussing, IEF)

Ready made immobilized pH gradients were rehydrated directly with sample solution overnight. A total of up to 1 mg protein dissolved in 600 µl rehydration buffer was pipetted
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into the grooves of the reswelling tray. The IPG strips were then inserted (gel side down) into the grooves without trapping air bubbles and covered with a 3 ml silicone oil.

The IPG strips rehydrated with sample solution were put directly (gel side up) inside a strip aligner on the kerosene covered cooling plate of a Multiphor II electrophoresis unit. Electrode paper strips, soaked with deionized water were placed on the ends of the IPG strips, and then the strips were covered completely with silicone oil. The IEF was carried out at 20 °C. Depending on the samples, isoelectric focussing was performed in a stepwise fashion at 65 kVh for the non-labelled samples (2 h at 150 V, 2 h at 300 V, 1.5 h at 600 V, 1 h at 1200 V, 1 h at 2400 V, 17 h at 3500 V) and 80 kVh for the ICPL labelled samples (1 h at 300 V, increase every 15 min by 300 V until 5000 V, 15 h at 5000 V). Current and power settings were limited to 1 mA and 5 W.

Prior to the second dimension, the IPG strips were equilibrated for 2 x 10 min with gentle shaking in 10 ml of a solution containing Tris/HCl buffer (50 mM, pH 8.8), 6 M urea, 30 % (w/v) glycerol and 4 % (w/v) SDS and a trace of bromophenol blue. DTT (1 % w/v) was added to the fist, and iodoacetamide (4 % w/v) was added in the second equilibration step.

5.5.3.3 Second dimension

The second dimension was performed on an Ettan Dalt II system. The gel thickness was 1 mm. The gel cells were assembled according the manufacturer and a degased acrylamide solution of 13 % (5 % glycerol, 375 mM Tris/HCl pH 8.8, 0.1 % SDS, 13 % acrylamide, 0,005 % TEMED and 0.05 % APS) was poured avoiding air bubbles. Each gel was overlayed with 2 ml water saturated butanol and the gel solution was allowed to polymerise for 4 h. Next, the n-butanol was exchanged against bidestilled water and gels were stored at least overnight, but not longer than one week at 4 °C.

The IPG strips were placed between the glassplates on top of and in close contact to the SDS-gel, and then the strips were sealed with 0.5 % (w/v) agarose in electrode buffer (25 mM Tris, 192 mM glycine and 0.1 % w/v SDS).

After the setting of the agarose, all gels were transferred to the gel chamber and were run simultaneously overnight in one gel tank at 20 °C. Electrophoresis was carried out for 1 h at a constant current of 5 mA per gel, and then increased to 10 mA until the colour front reached the lower end of the gel.
5.5.3.4 Gel staining procedures

Silver staining
After electrophoresis the gels were stained according to a method of Shevchenko et al. (Shevchenko et al., 1996) which was slightly modified. This method is specific and reversible and therefore allows preparative analysis of the samples.
The gels were transferred into large plastic containers and were agitated for 1 h in fixation solution (40 % ethanol, 10 % acetic acid). Then the gels were washed twice for 20 min in 30 % ethanol, followed by a 20 min rinsing step in bidestilled water. For sensitisation the gels were covered for 1 min with 0.02 % sodium thiosulfate pentahydrate and rinsed twice for 20 s in bidestilled water. The staining was performed by shaking the gels 20 min in an ice-cold aqueous silver nitrate solution (0.2 % silver nitrate / 0.025 % formaldehyde). Then the gels were rinsed shortly in bidestilled water and developed by intense shaking in an aqueous developing solution (3 % sodium carbonate/ 0.05 % formaldehyde) until the desired staining intensity was reached (1-5 min). The development process was stopped with 0.5 % glycin.
The silver stained 2DE images were scanned with a colour image scanner.

SYPRO Ruby staining
SYPRO Ruby is a highly sensitive stain with a linear quantitation range over three orders of magnitude (Lopez et al., 2000). For improved relative quantification, gels were stained according to a modified SYPRO Ruby staining method. The gels were transferred after electrophoresis into large plastic containers and were agitated for 4 h in fixation solution (30 % ethanol, 10 % acetic acid). Then the gels were washed 4 times in 20 % ethanol. The staining was performed with 500 ml of an aqueous SYPRO Ruby solution (20 % ethanol, 15 µl RuBPS/l) in light protected boxes overnight. The background was destained for 2 h in a solution containing 10 % methanol and 6 % acetic acid. Gels were rinsed in bidestilled water before images were scanned with a fluorescent image scanner.

5.5.4 Enzymatic cleavage of gel-separated proteins
For protein characterisation, the samples were digested directly within the polyacrylamide matrix. The protein spots were cut with an automatic spot cutter (Biorad) with a diameter of 1 mm and were placed directly into Acro-Prep™ 384 well filter membrane plates (Pall).
Destaining of the silver stained proteins was performed after incubation with 60 % acetonitrile and lyophilisation with the chemical reducer potassium ferricyanide (15 mM) and the complexing agent sodium thiosulfate (50 mM) for 5 min. Then gel pieces were rinsed in bidestilled water. The gel spots were treated with 100 % acetonitrile for 5 min, lyophilised and rehydrated with 50 mM Tris/HCl pH 8.5. This procedure was repeated twice and the final rehydration was performed with the protease solution (0.05 mg trypsin in 25 mM Tris/HCl pH 8.5). The digestion was performed at 30 °C overnight.

The elution of cleaved protein fragments was performed with acidic and organic solvents by passive diffusion assisted by centrifugal forces. Each centrifugation step was performed at 2000 x rpm for 2 min. The digestion buffer was collected in a new 384 well plate. The first elution step was carried out with 60 % acetonitrile and the second step with a solution containing 80 % acetonitrile and 0.5 % TFA. Depending on the size of the gel spots the second step was repeated twice.

The eluates were dried in a Speed Vac (Eppendorf). The 384 well plates were either stored at -80 °C or the peptides were dissolved for mass spectrometry in 50 % acetonitrile and 0.2 % TFA.

5.5.5 Mass spectrometry

0.4 µL of the sample solution were applied to the MALDI target, followed by 0.4 µL of the matrix solution (2.5 mg α-cyano-4-hydroxycinnamic acid (HCCA) / ml). Both solutions were then mixed on the target and allowed to air dry before the sample was transferred to the vacuum chamber of the mass spectrometer. Mass spectra were acquired using a Proteomics Analyser 4700 (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems). Mass analysis was performed using positive reflector mode with a deflection cut-off range with a mass/charge ratio of 800. 2500 shots were accumulated to produce a single MALDI-TOF spectrum. Subsequently, high energy MALDI-TOF/TOF collision induced dissociation (CID) spectra were recorded on selected ions from the same sample spot, again 2500 shots were carried out for each spectra.
5.5.6 Data processing

The modified proteins were identified screening the SWISS-PROT-database using an in house version of MASCOT from the Max Plank Institute of Biochemistry (Perkins et al., 1999). Peptide quantification was performed with the Peakpicker software (Applied Biosystems), which automatically calculates the peptide pair ratios by comparing their relative signal intensities. For combining the peptide mass fingerprint (PMF) and peptide fragmentation results the GPS explorer was used (Applied Biosystems).

Finally, all determined ratios of isotopic pairs for one identified protein spot generated by the PeakPicker programme were manually combined with the results of the identification of protein spots from the GPS explorer. Here, all identified peptides, singlets and doublets, either identified by PMF or peptide fragmentation were taken into account. However, only proteins, of which 2 or more peptides were differentially labelled within one single MS spectrum, were taken for further analysis. Subsequently, all ratios determined for one individual protein were averaged and the standard deviation (SD) was calculated. The change was considered to be significant, if the differences in ion intensities between ion pairs were greater than twice the SD.

Finally, the average experimental variability for each labelling reaction was estimated. This was performed by averaging the ratios of the isotopic pair of all peptides detected in one experiment by the PeakPicker software and setting this value to 100 %.
6 RESULTS

6.1 Outline of the project

The objective of this project was to identify nuclear proteins potentially involved in apoptosis signalling. The strategy consisted in the comprehensive detection of nuclear proteins that were altered as a consequence of apoptosis induction. The tools of choice were the large-scale analytical methods developed for proteomics research. The main challenges consisted in finding a highly reproducible and synchronous system for the induction of apoptosis and the separation of the nuclear subproteome. It was therefore decided to induce apoptotic changes in the nucleus in a cell-free reaction consisting of purified mouse liver nuclei and cytosolic extracts isolated from cells treated to undergo apoptosis. Treated nuclei were recovered and submitted to a proteomic platform. The main advantages of this approach were the reduced complexity of the samples, as compared to whole-cell extracts (the specific enrichment of nuclear proteins) and the possibility to produce apoptotic nuclei always starting from identical components, thereby minimising the experimental variations.

In the first part of the project, an appropriate apoptosis model, suited for the isolation of apoptotic extracts had to be identified in the literature and then evaluated experimentally. Further, an extraction method had to be selected which would avoid the disruption of cellular organelles and thus the contamination of extracts with non-cytosolic components. A procedure for the isolation of pure cell nuclei had to be devised also. The second step consisted in the set-up and optimisation of a cell-free apoptosis reaction, which would recapitulate all major features of nuclear apoptosis. Finally, a proteomic analysis platform suited for the study of nuclear proteins had to be identified and applied to the investigation of apoptotic nuclei. The experimental outline is illustrated in Fig. 4.
Figure 4: Flowchart representation of the individual steps of the project.
6.2 Characterisation of CD95-induced apoptosis in Jurkat T-cells

The death receptor CD95 has an important regulative role in the immune system and has been characterised in great detail (Sartorius et al., 2001). Jurkat T-cells triggered to undergo apoptosis by its cognate ligand (CD95-L) represent one of the best understood models of apoptosis (see introduction).

CD95-L was obtained as culture supernatant of neuroblastoma cells transfected with murine CD95-L cDNA (Rensing-Ehl et al., 1995). The supernatant was added to Jurkat T-cells at a concentration of 2.5 % (v/v) and resulted in a time-dependent increase in cells undergoing morphological features of apoptosis (Fig. 5A). After 50 min, first alterations in chromatin structure were observed, resulting in a maximum of 75 % apoptotic cells at 3 h after apoptosis induction. This was paralleled by an increase of the activity of caspase-3 related proteases starting after 45 min from stimulation (Fig. 5A).

The signal pathway of CD95-dependent apoptosis was further characterised by immunoblot analysis of several known intermediates. Cleavage of the upstream caspase, caspase-8, which is processed directly at the death receptor was detected by the appearance of the small subunit p18 and occurred 40 min after apoptosis induction (Fig. 5B). A downstream target of caspase-8 is Bid, a pro-apoptotic member of the Bcl-2 family of proteins. Bid is known to be cleaved by caspase-8 into a truncated form (tBid), which was detected 45 min after apoptosis induction (Fig. 5B). The truncated form of Bid affects the mitochondria resulting in cytochrome c release. This was detected in the cytosol of Jurkat T-cells treated with CD95-L concomitantly to Bid cleavage (Fig. 5B). Cytochrome c itself promotes formation of the apoptosome leading to activation of the effector caspase-3 (Chen and Wang, 2002). Processing of caspase-3 occurs via the generation of an inactive p20 intermediate which is subsequently processed primarily to its active p19-form or its fully mature p17-form (Sun et al., 2002). Formation of all three fragments was observed: p20 starting at 40 min, p19 at 45 min after apoptosis induction and p17 occurring in late apoptotic samples. In parallel to p19 appearance, activation of caspase-3 related caspases, including caspase-7 was also observed (Fig. 5A, DEVDase activity). Both the apoptotic cascade as well as the nuclear morphological changes were abrogated by addition of the broad spectrum caspase inhibitor zVAD-fmk (20 µM), confirming the caspase dependence of this apoptotic model.
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Figure 5: Characterisation of CD95-L induced apoptosis in Jurkat T-cell.

Jurkat T-cells were incubated with CD95-L (2.5 %). (A) At the indicated time points, cells were stained with Hoechst 33342 and the apoptosis rate was measured by scoring cells with condensed chromatin. Caspase activity (DEVD-afc cleavage) was measured in whole cell lysates. (B) Characterisation of the CD95-L dependent apoptotic pathway by immunoblot. For activation of caspase-8, caspase-3 and Bid, whole cell extracts were probed with antibodies specific for the p18 fragment of caspase-8, for Bid/tBid and for the p17, p19, p20 fragments of caspase-3. For determination of cytochrome c release from mitochondria, cytosolic fractions were isolated by selective lysis of the plasma membrane with digitonin and immunoblot analysis was performed with an antibody specific for cytochrome c. As a loading control, the blot was reprobed with an actin-specific antibody.

In order to investigate events occurring in the nucleus upstream and downstream of caspase activity, cytosolic extracts for in vitro reactions were isolated at different time points after apoptosis induction. To elucidate nuclear events preceding caspase activation, an early time point, 30 min after induction, was chosen. As an intermediate stage of apoptosis, when
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caspases are not yet fully processed, the 60 min time point was selected. At this time point, cells were committed to apoptosis, but no obvious apoptosis-associated morphological changes had occurred so far. For endpoint analysis, cells were treated with the apoptotic stimulus for 3 h prior to the preparation of cytosolic extracts.

6.3 Cell-free system for nuclear apoptosis

The development of a cell-free system for the induction of nuclear apoptosis was performed in three stages: (1) Isolation of cytosolic extracts. These extracts were derived from either untreated or CD95-L treated Jurkat T-cells. (2) Purification of cell nuclei. Mouse liver was used as the source of intact cell nuclei. (3) Validation of a cell-free apoptosis system. The isolated nuclei were combined with apoptotic cytosolic extracts and subsequently analysed for the occurrence of typical apoptotic changes.

6.3.1 Isolation of cytosolic extracts

The first step was to isolate cytosolic extracts from Jurkat T-cells without compromising the integrity of intracellular membranes. Mitochondria, the endoplasmic reticulum, lysosomes and nuclei are known to contain several apoptogenic factors, such as cytochrome c, AIF and cathepsins (for reviews see (Jaattela et al., 2003; van Gurp et al., 2003)). Disruption of these organelles during cell lysis would result in the contamination of the extracts with these factors which may artefactually influence the cell-free apoptosis system.

Digitonin was used as a mild detergent for the selective lysis of the plasma membrane. This detergent strongly binds cholesterol, which is more enriched in plasma membranes than in other cellular membranes. Selective cell permeabilisation with digitonin has already been employed for the study of cytochrome c release from mitochondria (Mackall et al., 1979). Using digitonin, at 60 µg per 5x10^7 cells, it was possible to retrieve 80 % of the cellular activity of lactate dehydrogenase (LDH) (Fig. 6A), which is a typical cytosolic enzyme. This result proves that this extraction method preserves the activity of cytosolic proteins.
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Figure 6: Analysis of the isolated cytosolic fraction and enzymatic activity of cytosolic proteins.

Jurkat T-cells were selectively lysed using digitonin as indicated. (A) Spectrometric determination of the activity of lactate dehydrogenase (LDH) and β-hexosaminidase (HexB). The respective enzyme activities present in whole cell lysates obtained by triton-X 100 (0.1 % (v/v)) extraction was defined as 100 %. (B) Characterisation of the purity of cytosolic fractions by immunoblot. The whole cell lysates were gained by repeated freeze-thaw cycles. The blots were probed with antibodies specific for PARP-1, cytochrome c and PDI. As a loading control, the blot was reprobed with an actin-specific antibody.

In order to check the integrity of various cellular organelles, Western blot analysis of cytosolic extracts isolated with increasing amounts of digitonin was performed. Poly(ADP-ribose)polymerase-1 (PARP-1) was used as nuclear marker, protein disulfide isomerase (PDI) as a marker for the endoplasmatic reticulum and cytochrome c as an indicator for the integrity of mitochondria. The activity of the lysosomal protein β-hexosaminidase (HexB) was measured in parallel.
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In the course of these experiments it became clear that the composition of the lysis buffer is critical. Only using a buffer which was adjusted to the intracellular ion concentration and which was supplemented with sucrose (transport buffer: 20 mM HEPES-NaOH pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium diacetate, 0.5 mM EGTA, 250 mM sucrose), it was possible to retain the integrity of cellular organelles. By using this buffer none of the marker proteins was released from its compartment at digitonin concentrations up to 200 µg/5 x 10^7 cells (Fig. 6A and 6B).

For the preparation of cytosolic extracts to be used in the in vitro reaction, digitonin was diluted in transport buffer at a concentration at which a substantial amount of LDH could be retrieved, but none of the marker proteins was released from the corresponding intracellular compartment. The effect of digitonin turned out to depend strongly on the cell density. The optimal concentration of digitonin was determined to be 60 µg per 5 x 10^7 cells.

Since the cytosolic extracts should be combined with isolated nuclei in the cell-free apoptosis reaction, it was important to exclude that digitonin present in these extracts had an effect on the integrity of the nuclear envelope. To this end, a nuclear permeability assay was performed (Roehrig et al., 2003).

In this assay Hela cells grown on coverslips are treated with low concentrations of digitonin which permeabilise the plasma membrane but leave the nuclear envelope intact. Subsequently these semipermeabilised cells are incubated with cytosolic extracts in the presence of fluorescently labelled 70 kDa dextran. After a short incubation time the cells are imaged by confocal microscopy. In healthy cells with an intact nuclear membrane the images show the nuclei as dark circles surrounded by the fluorescent marker that can freely diffuse through the plasma membrane. Disruption of the integrity of the nuclear envelope abolishes the capacity to exclude the fluorescent dextran, leading to an increase in the nuclear fluorescence signal. This effect can be quantified with the help of a custom designed image evaluation software called NPA (nuclear permeabilisation assay) (Roehrig et al., 2003). The software recognises the cell nuclei in the confocal images, calculates the mean greyscale value of the nuclear fluorescence and corrects it for the mean greyscale value of the background.

To measure whether the digitonin present in the cytosolic extracts had an effect on the nuclear envelope, this assay was performed using the extracts either undiluted or in a 1:4 dilution in transport buffer. The actual concentration of cytosolic extracts in the in vitro apoptosis reaction corresponds approximately to a dilution of 1:3 in the NPA assay. The results are
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shown in Fig. 7 and Table 1 and revealed no difference in nuclear membrane permeability between the undiluted and diluted cytosolic extracts gained by digitonin lysis. Consequently, these cytosolic extracts did not have any effect on the integrity of nuclear membranes.

Figure 7: Effects of digitonin on the integrity of the nuclear envelope.
HeLa cells were grown on glass coverslips in 12-well plates to 60% confluency. Plasma membranes were selectively permeabilised with digitonin. Cytosolic extracts isolated by digitonin lysis were applied to the cells in the presence of 70 kDa dextran and images were recorded at a confocal microscope (magnification 40x). (A) Confocal images of semipermeabilised HeLa cells incubated with cytosolic extracts which were diluted 1:4 in transport buffer. (B) Confocal images of semipermeabilised HeLa cells which were incubated with undiluted cytosolic extracts.
Table 1: Comparison of the fluorescence intensity of the nucleoplasm in nuclei exposed to diluted and undiluted cytosolic extracts gained by digitonin lysis.

<table>
<thead>
<tr>
<th></th>
<th>accepted nuclei</th>
<th>x/BG*</th>
<th>standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>diluted extracts</td>
<td>91</td>
<td>0.2243</td>
<td>0.0195</td>
</tr>
<tr>
<td>undiluted extracts</td>
<td>141</td>
<td>0.2228</td>
<td>0.0202</td>
</tr>
</tbody>
</table>

* the corrected fluorescence intensity of the nucleoplasm.

Images (Fig. 7) were analysed by the NPA software. First, the software detects the grey value of areas corresponding to the cytoplasm and defines remaining area as background. Then it accepts darker regions as nuclei, if their size is above their minimal threshold. Those nuclei are checked for the relation between their size and their form and for the presence of surrounding cytoplasm. Finally, the software calculates the mean greyscale value of the accepted nuclei [x] and divides it by the mean greyscale value of the background [BG]. Each data set represents the average of 4 images. The experiment was performed in triplicate with similar results.

6.3.2 Purification of mouse liver nuclei

The next step for establishing the desired in vitro system for nuclear apoptosis was to find an appropriate source for the isolation of nuclei as well as a suitable purification protocol. Previous observations had shown that nuclei isolated from in cultured cells were often contaminated with remnants of the endoplasmic reticulum and mitochondria (E. Ferrando-May, personal communication). It was therefore decided to isolate nuclei from mouse liver following a protocol first established by Blobel and Potter (Blobel and Potter, 1966) and modified by Cordes (Cordes, 1992). First, liver tissue was thoroughly homogenized, by rough cutting with scissors followed by douncing with a glass homogenizer. Then, several washing steps were performed. Pure nuclei were obtained by a centrifugation step through a sucrose gradient. Fig. 8A shows a clear change in the relative protein composition between the samples before (lane 2) and after (lane 3) this centrifugation step through the sucrose cushion. This went along with the enrichment of the major nuclear proteins, the histones, migrating between 14 and 20 kDa. A phase contrast microscopic image of isolated nuclei (Fig. 8B) shows that no remnants of the cellular cytoskeleton or other associated cellular fragments are present. The clear appearance of several nuclear components, like the nucleoli (indicated by arrows) and the nuclear envelope, demonstrates the high purity of the nuclear preparation.
6.3.3 In vitro reaction

Having successfully isolated the individual components needed for the in vitro reaction, the next step was to test whether the typical nuclear changes occurring in apoptosis, such as rippled nuclear contours, margination of chromatin and formation of nuclear bodies (Ruchaud et al., 2002), were inducible under cell-free conditions.

A typical in vitro reaction consisted of cytosolic extracts and isolated nuclei in a ratio corresponding approximately to their proportional amount in intact cells. The incubation of nuclei with cytosolic extracts was performed for 10 min, in the presence of an ATP-regenerating system (see Materials and Methods). Thereafter, nuclei were recovered by a brief centrifugation step through a sucrose gradient.

In general, the process of apoptosis in a cell-free reaction can be followed by staining the nuclei and observing chromatin changes under the microscope. Therefore, liver nuclei were incubated with either control or apoptotic cytosolic extracts, and stained with the chromatin
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dye Hoechst. In nuclei incubated with cytosolic extracts derived from Jurkat T-cells exposed to CD95-L for 120 min, DNA loss, margination of nuclear chromatin and clumping of the chromatin was observed (Fig. 9A).
Biochemical endpoints of apoptosis, namely the cleavage of caspase substrates and the oligonucleosomal DNA fragmentation were also monitored in the cell-free assay.
Isolated nuclei were incubated with intermediate and late apoptotic extracts, gained from cells incubated for 60 min or 120 min with CD95-L. Poly(ADP-ribose)polymerase-1 (PARP-1) and lamin B, two classical caspase substrates, were cleaved in intermediated and late stage cell-free apoptosis reaction (Fig. 9B). DNA fragmentation was detected in nuclei incubated only with late apoptotic cytosolic extracts (Fig. 9C). Both caspase-dependent proteolysis and DNA fragmentation are highly specific apoptotic changes which do not occur spontaneously but are dependent on the active extracts which are supplemented to the nuclei (see Fig. 9).
The experiments show that the cell-free system is suited for the study of nuclear apoptotic changes in vitro. Depending on the input of active extracts, early, intermediate or late apoptotic changes can be detected in the nucleus.
The cytosolic extracts employed in the following proteomic analysis were obtained either prior to caspase activation at 30 min after induction of apoptosis by CD95-L, or shortly after the appearance of active caspases, 60 min after CD95-L treatment.
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Figure 9: Characterisation of the in vitro nuclear apoptosis reaction.

Isolated mouse liver nuclei were incubated with cytosolic extracts gained from Jurkat T-cells at different apoptotic stages, as indicated. (A) Epifluorescence images of liver nuclei incubated with control or apoptotic cytosolic extracts. After staining with the DNA-dye Hoechst 33342 morphological changes of the in vitro nuclear apoptosis reaction were monitored (exposure time 10 ms, magnification 63x). (B) Characterisation of in vitro nuclear protein cleavage by Western blot analysis. Nuclei were recovered, 40 µg of protein were loaded on a SDS-PAGE and blotted onto a nitrocellulose membrane. Blots were probed with antibodies specific for PARP-1 and lamin B. (C) Characterisation of in vitro induced nuclear DNA fragmentation by agarose gel electrophoresis. DNA was purified from nuclei recovered after the cell-free reaction and resolved on a 1.5 % agarose gel. The gel was stained with the DNA-intercalating dye SybrGreen and visualised by UV transillumination.

A further main issue for the development of an in vitro apoptosis system concerned the reproducibility of the experimental system. The high sensitivity with which proteomic methods can detect protein changes require that the experimental variations are kept to a minimum.

Therefore the reproducibility of the in vitro system was tested. Four cell-free reactions were carried out, each one of them consisting of cytosolic extracts and purified mouse liver nuclei isolated independently. For this experiment, the cytosolic extracts were obtained from non-apoptotic control cells. After the reaction, nuclei were recovered, solubilised in rehydration buffer (see Materials and Methods) and submitted to 2D-gel-electrophoresis. The relative
intensity of the spots was determined by the comparison of SyproRuby stained gels using the programme Proteomweaver 2.0 (Definions, Munich). At least two separate gels were run per each cell-free reaction, thus resulting in a total of 10 gels. The relative comparison of spot intensities was carried out for the complete area of each 2DE-gel. First, all spots were detected (settings of Proteomweaver: intensity limit 2000, contrast limit 20 and manual noise estimate 70; statistical filters: average from 0.03 to 4, frequency from 2 to 2/3 dependent on the number of gels per reaction) and an average gel of all gels of the same cell-free reaction was created by the software. Matrixes for each gel (single and average gels) were compiled automatically by the programme. Then matrixes between different experimental groups (different cell-free reactions) were matched. To minimise the signal-noise ratio, only spots which were present in at least two gels from each experiment were taken into account for further analysis, resulting in a total number of 333 spots. Finally, these spots were compared with respect to their intensities. A comparison of two average gels is illustrated in the scatter plot in Fig. 10B. The ratio in spot intensities for the majority of spots (303 spots) lay in the range between 0.5 and 2. Only 30 spots showed a higher variation of their intensities. Comparison of spot intensities between all 10 gels led to a variability of 30.02 %, without any manual correction of spot detection in the Proteomweaver programme. This variation lies within the accepted range of tolerance for 2DE-gel-electrophoresis (see discussion).

For a clearer demonstration of the high reproducibility between different cell-free reactions, the corresponding small area compromising approximately 20 protein spots was enlarged in each gel and is shown in Fig. 10A.
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Figure 10: Analysis of the reproducibility of *in vitro* reactions.
Nuclei from 4 independent sets of *in vitro* control reactions were resolved by 2DE-gel-electrophoresis and spot intensities were compared using the Proteomweaver software 2.0 from Definitions. (A) Enlargement of an identical area of the gels. (B) Scatter plot of spot intensity distributions obtained from the analysis of two average gels created from the gels of two independent cell-free reactions.
6.4 Proteomic analysis of apoptotic cell nuclei

The initial experiments aimed at the comparison of the protein complement of apoptotic and control nuclei were based on differential 2D-gel-electrophoresis. A good resolution of protein spots is critical for a computer-based comparison of 2DE-gels and thus the identification of differentially expressed or modified proteins. Due to the extreme alkaline nature of many nuclear proteins, such as the histones with pI values between 10 and 12, it was necessary to resolve proteins up to a pH of 11. This proved to be a problem from the beginning. A typical resolution on IPGs from pH 3-10 and pH 6-11 is shown in Fig. 11, demonstrating that proteins beyond the pH of 9 are not properly separated.

Different parameters were varied in the attempt to improve the penetration in the gel and the resolution of alkaline proteins, for example sample loading onto the IPG strips (ingel rehydration, cup loading), amount of DTT, DeStreak reagent (Amersham Biosciences) instead of DTT, total Vh during isoelectric focussing procedure, and precipitation of proteins prior to the isoelectric focussing. No condition could be identified which led to a reproducible improvement of protein resolution in the basic area at pH values higher than 8.

Figure 11: Resolution of nuclear proteins by 2D-gel-electrophoresis.

Isolated mouse liver nuclei were resolved in RHL and submitted to 2D-gel-electrophoresis. (A) Isoelectric focussing on a linear broad range IPG strip (24 cm, pI 3 – pI 10). 800 µg of protein were loaded. After the second dimension (13 % SDS-PAGE) proteins were visualised by silver staining. (B) Isoelectric focussing of an alkaline narrow range linear IPG strip (18 cm, pI 6 – pI 11). 400 µg of protein were loaded. After the second dimension proteins were visualised by silver staining.
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Due to the problems encountered with the separation of basic nuclear proteins on 2D-gel-electrophoresis, a different analysis system was selected, mainly based on mass spectrometry thereby circumventing the need for extensive 2DE-gel-electrophoresis was chosen. In this system both the protein identification and quantification are carried out by mass spectrometry. The method used is called the isotope coded protein label (ICPL). Based on differential labelling it is possible to identify and quantify the proteins in one single mass spectrometric step. N-nicotinoyloxy-succinimide was used for the labelling of all free amino groups, namely at the N-terminus and at lysines. The guanidinium group of arginines is not reactive under these conditions. This ICPL-label exists in a deuterium free (light) or deuterium containing (heavy) version. The heavy isoform contains four deuterium atoms (see introduction for detailed information).

Since peptides of identical sequence, when containing ICPL-modified amino groups, differ in mass, they appear as doublets in acquired MS-spectra. The mass shift between the ion intensity peaks within one sister peptide pair corresponds exactly to a multiple of 4 kDa, reflecting the mass difference between the heavy and light ICPL reagent and depending on the number of amino groups labelled in the peptide.

The principle of the method and the complete workflow is shown in Fig. 4. Two nuclear protein mixtures which were obtained from two distinct in vitro reactions, in this case from a control and an apoptotic reaction, were processed. First, each reaction was reduced and alkylated to denature the proteins and to ensure easier access to free amino groups for the isotopic labelling reaction. Subsequently, the protein mixtures were derivatised with either the deuterium free (light) form of the ICPL-reagent or the deuterium containing (heavy) form. The differentially modified protein mixtures were combined and 2DE-gel-electrophoresis as the method of choice for reducing the complexity of the sample was performed. After excision of spots and digestion with trypsin a high throughput tandem mass spectrometry (MS/MS) step was carried out (Schmidt et al., 2004).

From the ratios of the ion intensities of sister peptide pairs, the relative abundance of their parent proteins in the original samples could be determined. Finally, proteins were identified by peptide mass fingerprint (PMF) and additional collision-induced dissociation (CID) of single peptides followed by correlation with sequence databases using highly sophisticated search algorithms.
6.4.1 ICPL labelling of basic nuclear proteins

As discussed by Schmidt et al. (Schmidt et al., 2004) the modification of the basic amino groups with the ICPL reagent reduces the isoelectric point of a protein and consequently shifts the position of the corresponding spot in 2DE-gel towards more acidic regions.

To test the suitability of the method for the analysis of a complex mixture of nuclear proteins, a pilot experiment was performed. In this experiment, two identical samples containing purified untreated nuclei were submitted to the ICPL procedure. As predicted, 2DE-gel-electrophoresis revealed a good resolution of nuclear proteins. Most nuclear proteins were resolved in the pI range between 3 and 5 (Fig. 12A). Different spots were excised and after in-gel digestion with trypsin were analysed by MALDI-TOF mass spectrometry. Since all amino groups are blocked at lysines by the ICPL modification, the enzyme only cleaves after arginine residues. A ratio between the light and heavy ICPL-modified peptides of approximately 1:1 was observed within each spectrum. This indicated that there was the same abundance of differentially labelled proteins, as expected for a 1:1 mixture of the two samples, and thus demonstrates the reliability of this labelling method. One identified nuclear protein, indicated with the arrow in Fig. 12A, is presented below. Histone H1.0 is an abundant nuclear protein with a free amino group content of 29 % (55 lysines and the N-terminus). Hence, it is strongly shifted after isotopic labelling from the theoretical pI 10.9 to pI 5.5 (Fig. 12A). Different peptides, always with the same ratio of light to heavy labelled isoforms of 1:1, were detected in the MS-spectra (Fig. 12B). The ratio of the ion intensity of differentially labelled proteins is unchanged, as expected for a 1:1 mixture of identical samples.

Two main experimental advantages were demonstrated in this control experiment. Firstly, ICPL allows for an accurate quantitative comparison between two protein populations, and secondly, the separation of alkaline proteins by 2DE-gel-electrophoresis is highly improved allowing their recovery and analysis by mass spectrometric analysis.
Figure 12: ICPL labelling of nuclear proteins.

(A) 2DE-gel-electrophoresis of ICPL-modified isolated mouse liver nuclei. Nuclear proteins derived from two identical samples were isotopically labelled with the light (d0) or heavy (d4) variant of N-nicotinoyloxy-succinimide, mixed and resolved on a 2DE-gel. (B) Mass spectra of one major spot picked and digested with trypsin. Peptides were reconstituted for mass spectrometry in 50 % acetonitrile and 0.2 % TFA. 0.4 µl of sample solution was applied on the MALDI target and mixed with an equal volume of the matrix solution (α-cyano-4-hydroxycinnamic acid (HCCA)). The expanded view of MALDI-TOF-MS of two isotope labelled peptide pairs revealing the doublet composition of each peptide is shown at the bottom.
6.4.2 Determination of protein changes by the ICPL technique

Three different cell-free apoptotic reactions were performed: (1) control reaction, (2) early stage apoptosis reaction, and (3) intermediate stage apoptosis reaction. The nuclei were recovered and labelled with the ICPL reagent as described above. Modification of control nuclei was always performed using the light ICPL variant, while apoptotic nuclei were labelled with the heavy reagent. The control sample was combined with one apoptotic reaction before performing the 2D-gel-electrophoresis. Two different experimental approaches were performed: (1) control nuclei were compared to intermediate stage apoptotic nuclei, which were exposed to cytosolic extracts gained from Jurkat T-cells 60 min after apoptosis induction, (2) control nuclei were related to nuclei in the early stage of apoptosis, which were incubated with cytosolic extracts from cells induced to undergo cell death for 30 min. A high throughput screen of both experiments was carried out: first, automatic picking of all detectable spots was performed, then in-gel digestion with trypsin, followed by an automated tandem mass spectrometry analysis (MALDI-TOF and MALDI-TOF/TOF). Ion intensities were calculated and differentially labelled peptides were detected using the PeakPicker software (Applied Biosystems). Finally the ion intensities within the doublets were compared.

In Fig. 13, an example is illustrated in which relative changes in ion intensities were detected. In both early and intermediate stage apoptotic nuclei peptide mass fingerprinting identified the corresponding spot as the heterogeneous ribonucleoprotein C (hnRNP C) (Fig. 13A). This was confirmed by MALDI-TOF/TOF analysis of the precursor peptide with a molecular weight of 1470.6 kDa (Fig. 13B). The enlargement of the MALDI-TOF spectra demonstrates the ICPL-modification of two lysines within the amino acid sequence of this peptide, thus resulting in a shift of 8 kDa in the mass spectrum. Comparison of ion intensities from control to early apoptotic proteins did not show any differences, whereas the intermediate stage apoptosis reaction yielded a decrease of the intensities of the peptides derived from apoptotic nuclei (Fig. 13A).

For statistical analysis, the ratios of the isotopic pairs of all peptides belonging to the hnRNP C protein were calculated from the peak area in the MALDI-MS spectrum and averaged. The observed decrease of hnRNP C in this intermediate apoptotic reaction amounted to 1:0.41.

To estimate the variability of the labelling procedure, mainly caused by the unequal combination of differentially isotopic labelled samples, the ratios of the isotopic pair of all
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peptides detected in one experiment were averaged and this value was set to 100%. For the experiment shown in Fig. 13, the average of the peptide ratios was 0.973. The ratio of 1:0.41 observed for hnRNP C in intermediate apoptotic reactions corresponded to a relative reduction in protein level of 56% as a specific apoptotic nuclear protein change.

Additionally, the significance of the results was examined by calculating the difference between the experimental variability and the average ratio of all peptides belonging to one protein. If this value was more than the double of its standard deviation, the result was judged to be significant.
Figure 13: Analysis of time dependent changes of hnRNP C in apoptotic samples.

Nuclei were recovered from control, early stage or intermediate stage apoptotic cell-free reaction. Nuclear proteins were differentially labelled with the light (control) or heavy (apoptotic) isoform of the ICPL reagent. The labelled nuclear proteins from control reaction were mixed with the labelled proteins from either early or intermediate stage reactions and resolved by 2D-gel-electrophoresis. Identical spots were picked from both gels, digested with trypsin and analysed by mass spectrometry. (A) MALDI-TOF mass spectra of ICPL modified hnRNP C1/C2 at early and intermediate stage apoptosis reactions in comparison to the control protein. (B) MALDI-TOF/TOF mass spectra were recorded for peptide and protein identification.
6.4.3 Identification of nuclear proteins altered during apoptosis

Cell-free reactions performed with early and intermediate stage apoptotic extracts were processed as described above and separated on 2DE-gels. For each gel, the most prominent spots were excised and transferred to a 384 well plate. The spots were processed and submitted to an automated MS-analysis. Peptide pairs which showed differences in ion intensities between control and apoptosis and peptides which were present only in one sample were selected using the PeakPicker software. Then the sequence of those peptides was identified by an additional automated MALDI-TOF/TOF.

The whole procedure was repeated twice, starting from cell-free reactions which consisted of an independent batch of nuclei and cytosolic extracts. The spots which had been identified in the first run were selectively excised and analysed.

The processing, identification, quantitation and statistical analysis were performed as described above. The experimental variability was estimated for each labelling reaction independently.

Finally, 13 candidates were identified which showed reproducible alterations in their protein levels in at least 2 out of 3 independent experiments. The relative abundance of apoptotic to control nuclear proteins as well as their positions on a master 2DE-gel are summarised in Fig. 14.

Using the ICPL method, it was possible to resolve time-dependent changes of nuclear protein levels in apoptosis (Fig. 13A and Fig. 14), thus enabling differentiation between early and intermediate apoptotic events in the nuclear proteome. Some of the identified nuclear proteins were altered only in intermediate stage apoptotic reactions. These were: DEK, lamin B2, phosphoprotein phosphatase 1alpha (PP1α), U2 small nuclear ribonucleoprotein A' (U2 snRNP A’), nuclear protein Hcc-1 and high mobility group proteins B1 (HMGB1) and B2 (HMGB2). Other proteins identified were modified both in intermediate as well as in early apoptotic reactions. These were: heat shock protein 70 (hsp70), heterogeneous ribonucleoproteins A2/B1 (hnRNP A2/B1) and C1/C2 (hnRNP C1/C2), and the histones H1.0, H1.2, and H4.

In general, three different types of changes were observed, (1) either the protein level was always increased, (2) or always decreased, or (3) the protein level was found to be decreased...
and increased, depending on the location of the spot in the 2DE-gel. These data are summarised in table 2. Additionally, the molecular weight, the theoretical isoelectric point before and after the labelling reaction and the according accession number in the SwissProt database are listed for each protein.

Some proteins were identified in various spots. These were DEK, HMGB2, lamin B2, hsp70, HCC-1 and the hnRNPs.
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Figure 14: Identification of nuclear proteins altered in apoptosis.

Nuclei were coincubated with control, early stage or intermediate stage apoptotic extracts. Solubilisation, labelling and digestion procedure were performed as described above. The results from all experiments were collected and are shown in one 2DE-gel. The numbers in the 2DE-gel and on the corresponding diagrams indicate the identified protein. The letters mark different spots belonging to one single protein. If a letter occurs more than once this indicates that the protein was identified in different independent experiments. The percentage of up- and down-regulation was corrected for the average experimental variability calculated for each labelling reaction. The relative increase or decrease of protein level is expressed in % change in comparison to the control reaction. Early stage (white bar) and intermediate stage (black bar) apoptotic changes are shown in the same bar chart for each protein.

Table 2: Synopsis of observed relative changes in protein level.

<table>
<thead>
<tr>
<th>proteins identified</th>
<th>MW [kDa]</th>
<th>theoretical pl before labelling a)</th>
<th>theoretical pl after labelling b)</th>
<th>swissprot accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>decrease in protein level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. hsp 70</td>
<td>70.2</td>
<td>5.53</td>
<td>4.20</td>
<td>P17879</td>
</tr>
<tr>
<td>3. Lamin B2</td>
<td>67.0</td>
<td>5.44</td>
<td>4.60</td>
<td>P21619</td>
</tr>
<tr>
<td>4. PP1α</td>
<td>37.5</td>
<td>5.94</td>
<td>4.37</td>
<td>P62137</td>
</tr>
<tr>
<td>5. hnRNP A2/B1</td>
<td>35.9</td>
<td>8.67</td>
<td>4.96</td>
<td>O88569</td>
</tr>
<tr>
<td>9. U2 snRNP A’</td>
<td>28.3</td>
<td>8.71</td>
<td>4.35</td>
<td>P57784</td>
</tr>
<tr>
<td>11. HMG B1</td>
<td>24.7</td>
<td>5.62</td>
<td>3.71</td>
<td>P07155</td>
</tr>
<tr>
<td>13. hnRNP C</td>
<td>34.2</td>
<td>4.91</td>
<td>3.94</td>
<td>Q9Z204</td>
</tr>
<tr>
<td>increase in protein level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Histone H4</td>
<td>11.2</td>
<td>11.36</td>
<td>11.27</td>
<td>P02304</td>
</tr>
<tr>
<td>10. HMG B2</td>
<td>24.0</td>
<td>7.05</td>
<td>3.87</td>
<td>P30681</td>
</tr>
<tr>
<td>increase and decrease in protein level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. DEK</td>
<td>43.1</td>
<td>6.51</td>
<td>3.72</td>
<td>Q7TNV0</td>
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<tr>
<td>6. Histone H1.0</td>
<td>20.7</td>
<td>10.90</td>
<td>5.51</td>
<td>P10922</td>
</tr>
<tr>
<td>7. Histone H1.2</td>
<td>21.1</td>
<td>11.00</td>
<td>4.55</td>
<td>P15864</td>
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<tr>
<td>12. nuclear protein Hcc-1</td>
<td>23.5</td>
<td>6.29</td>
<td>4.19</td>
<td>Q9D1J3</td>
</tr>
</tbody>
</table>

a) The pl of each protein was calculated using the algorithm of SwissProt’s programme compute MW/pl.
b) The pls of the completely labelled proteins were estimated after eliminating all lysine residues from the calculation.
6.5 Biochemical characterisation of the identified proteins

The changes in protein levels detected by the proteome analysis may be attributed to three different mechanisms: (1) degradation by apoptosis-specific proteases, (2) post-translational modification, as well as (3) protein translocation between the cytosol and the nucleus. This translocation could be due either to an unspecific loss/uptake of proteins from the nucleus in the *in vitro* reaction or to a translocation process.

To explore these possibilities and gain information about the processes underlying the observed changes in protein levels, a detailed investigation of selected candidates, mainly DNA-binding and mRNA-splicing proteins, was performed. Biochemical experiments were conducted with the components of the cell-free apoptosis reaction as well as in intact cells, the latter to explore the significance of the observed changes *in vivo*.

6.5.1 High mobility group proteins B1 and B2 (HMGB1/B2)

The proteome analysis of apoptotic nuclei revealed changes in protein levels for both, HMGB1 and HMGB2 proteins. The level of HMGB1 was found to be decreased in all proteomic experiments, being detected in only one single spot, whereas the level of HMGB2 was always increased and this was determined for three independent spots. Both proteins were running at the expected positions corresponding to their molecular weight in the 2DE-gels.

High mobility group proteins are DNA binding proteins predominantly localised within the nucleus (Bonaldi et al., 2003), although they have also been described to occur in the cytosol (Mosevitsky et al., 1989). In the nucleus, HMGB proteins bind in the minor groove of DNA and induce a sharp bend within one superhelical turn. This action is known to promote the interaction of transcription factors (for review see (Langst and Becker, 2004)). Although early reports proposed a possible nucleocytoplasmic shuttling of HMGB1 and B2 (Isackson et al., 1980), the cytosolic function of these proteins still remains unclear. Recent findings show that in addition to its role in the nucleus, HMGB1 mediates inflammation when secreted, as a consequence of cellular damage and necrosis (Scaffidi et al., 2002).

To investigate the specificity of the changes in HMGB protein levels observed in the proteomic analysis and to check for apoptosis-specific proteolysis, Western blot analysis of
the components of the cell-free apoptosis reaction prior and after their coincubation was performed. Subsequently, to verify the behaviour of HMGB1/B2 during apoptosis in intact cells, HeLa cells were induced to undergo apoptosis by treatment with TRAIL. TRAIL binds to and triggers apoptosis via the death receptors TRAIL-R1 and TRAIL-R2 similarly to CD95-L to which HeLa cells, however, are insensitive. To study whether apoptosis induction is associated with HMGB1 and B2 translocation, immunocytochemical studies of TRAIL-stimulated HeLa cells with antibodies specific for HMGB1 and B2 were performed.

Western blot analysis of cytosolic extracts and nuclei before and after the cell-free apoptotic reaction were performed with an antibody specific for HMGB1 (Fig.15A). This revealed in all samples examined a single specific band. The intensity of the HMGB1-specific signal did not change in the course of apoptosis. This result shows that no proteolysis of HMGB1 occurs during apoptosis and indicates additionally that no translocation of HMGB1 in or out of the nucleus occurs in the cell-free apoptosis reaction.

To study the distribution of HMGB1 at the cellular level, immunostainings of control cells and of cells stimulated to undergo apoptosis were performed (Fig. 15B). The nuclear distribution can be clearly seen in control cells. In the course of apoptosis, this distribution did not change and the protein remained strictly confined to the nucleus.

Since neither proteolysis nor protein translocation of HMGB1 could be detected by two independent experimental methods, Western blot analysis (Fig. 15A) and immunostainings (Fig. 15B), post-translational modifications would be a possible explanation for the results of the proteome analysis of apoptotic nuclei are. Further experiments, which were beyond the scope of this work, are needed to explore this possibility.
Figure 15: Analysis of HMGB1 in cell-free reactions and intact cells.

(A) Western blot analysis of isolated mouse liver nuclei and cytosolic extracts, before and after the cell-free apoptosis reaction. $1 \times 10^6$ nuclei and cytosol extracted from $2 \times 10^6$ cells were loaded on a 12 % Laemmli gel and blotted onto nitrocellulose membranes. The blot was probed with antibodies specific for HMGB1. As a loading control for cytosolic proteins, the blot was reprobed with an actin-specific antibody.

(B) Immunocytochemical staining of HeLa cells with HMGB1-specific antibodies. HeLa cells were treated with TRAIL [300 ng/ml]. At the indicated time points, cells were fixed with 4 % paraformaldehyde and immunocytochemical staining was performed. Chromatin was stained with H-33342. The figure shows representative confocal images. The scale bar corresponds to 10 µm.
Similarly to HMGB1, Western blot analysis of cytosolic extracts and nuclei before and after the cell-free apoptosis reaction was performed with an antibody specific for HMGB2 (Fig. 16A). Two variants of HMGB2 were detected: the first one with a molecular weight of 19 kDa is present predominantly in nuclear samples; the second one with a molecular weight of 28 kDa is mainly detected in the cytosolic samples. No additional cleavage band was detected, thus implying that HMGB2 is not proteolytically processed in the course of apoptosis. The cytosolic 28 kDa form was present also in the nuclei recovered after the in vitro reaction; nuclei from late stage apoptotic reactions showed slightly higher signal intensity compared to the other time points. In contrast, the nuclear signal only appeared in cytosols recovered from late stage apoptotic reactions, indicating that there might be an exchange of HMGB2 between the two components of the cell-free reaction.

The results from the proteome analysis, in which the level of HMGB2 was constantly increased in three independent spots running at molecular weights ranging from approximately 24 to 28 kDa, might thus be explained by a translocation of HMGB2 from the late apoptotic extracts to the nucleus. However, possible post-translational modifications leading to the separation into distinct spots on 2DE-gels cannot be excluded and need further analysis.

In order to examine the protein localisation in living cells, an immunocytochemical analysis of HMGB2 was performed, as described above. Confocal images of untreated HeLa cells show that HMGB2 is predominantly located in the nucleus, but to a minor extent is also present in the cytosol (Fig. 16B). In cells treated to undergo apoptosis, this HMGB2 specific staining was not changed.

From these experiments it is possible to draw the following conclusion: First, the HMGB2 protein is not proteolytically processed in the course of apoptosis. Second, translocation of the protein might occur in late stage cell-free apoptotic reactions, as demonstrated by Western blot analysis (Fig. 16A). Whether this occurs also in cells undergoing apoptosis is still unclear. The third possible explanation, post-translational modifications occurring in the course of apoptosis, needs further experimental investigation.
Figure 16: Analysis of HMGB2 in cell-free reactions and intact cells.

(A) Western blot analysis of isolated mouse liver nuclei and cytosolic extracts, before and after the cell-free apoptosis reaction. $1 \times 10^6$ nuclei and cytosol extracted from $2 \times 10^6$ cells were loaded on a 10 % Laemmli gel and blotted onto nitrocellulose membranes. The blot was probed with antibodies specific for HMGB2. As a loading control, the Ponceau staining of the same blot was used (not shown). (B) Immunocytochemical staining of HeLa cells with HMGB2-specific antibodies. HeLa cells were treated with TRAIL [300 ng/ml]. At the indicated time points, cells were fixed with 4 % paraformaldehyde and immunocytochemical staining was performed. Chromatin was stained with H-33342. The figure shows representative confocal images. The scale bar corresponds to 10 µm.
6.5.2 Heterogeneous ribonucleoproteins (hnRNPs)

In the proteome analysis two major hnRNPs were found to be modified, the hnRNP A2/B1 and hnRNP C1/C2. Protein levels were always decreased in both the early and the intermediate stage apoptotic reactions.

A total of about 20 major proteins belong to the group of hnRNPs. They are all associated with RNA polymerase II transcripts in the nucleus and have important roles in the biogenesis of RNA (Kim et al., 2000; Nakielny et al., 1997). Several hnRNPs are known to shuttle between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss, 1993). In some hnRNPs, such as hnRNP A1, A2/B1 and C1/C2, putative caspase cleavage sites were reported (Brockstedt et al., 1998; Thiede et al., 2001; Thiede et al., 2002; Waterhouse et al., 1996).

6.5.2.1 Heterogeneous ribonucleoproteins A1 and A2/B1 (hnRNP A1 and A2/B1)

The proteomic analysis revealed that the hnRNP A/B splicing variants A2/B1 were altered in apoptotic nuclei.

To test changes due to apoptosis in intact cells, HeLa cells were stimulated with TRAIL. Immunocytochemistry was performed with two antibodies, one raised against a peptide common to all hnRNP A/B splicing variants (KEDTEEHHLRDYFE) and the other specific for hnRNP A1 (clone 4B10, immunogen: full length native protein partially purified) to at least one specific hnRNP A isoform. An antibody specific for hnRNP A2/B1 was not available. The specimens were imaged at the confocal microscope, and the protein distribution within the cells was analysed by plotting distribution profiles specific for hnRNP A (green line) isoforms and for chromatin (red line). The general anti-hnRNP A/B antibody in untreated control cells revealed a dominant nuclear staining with a clear omission of the nucleoli. Thirty minutes after induction of apoptosis, an increase of the cytosolic hnRNP A/B signal could be detected, which was accompanied by the formation of foci in the cytosol (Fig. 17 upper column), appearing as additional peaks outside of the chromatin stained region in the intensity profile. In addition, at a later apoptotic stage the clear exclusion from the nucleoli in control cells vanished. None of theses changes in protein distribution could be detected using the monoclonal antibody specific for hnRNP A1, clone 4B10 (Fig. 17 lower column). Here, omission of nucleolar structures as well as the ring staining along the nuclear envelope was not changed after apoptosis induction.
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These observations suggest that the change in protein distribution detected inside the cell is specific for the hnRNP A2/B1 protein, but not for hnRNP A1.
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Figure 17: Heterogeneous ribonucleoprotein A2/B1 form granular structures in the cytosol upon apoptosis induction.

HeLa cells were treated with TRAIL [300 ng/ml]. At the indicated time points, cells were fixed with 4% paraformaldehyde and immunocytochemical staining with antibodies specific for hnRNP A1/A2 and B1 (upper panel, scale bar = 10 µm) and specific for hnRNP A1 (lower panel, scale bar = 20 µm) were performed. Chromatin was stained with H-33342. The figure shows representative confocal images. The localisation of hnRNP A1/A2/B1 and hnRNP A1 inside the cell is illustrated by comparing its distribution profile to the one of Hoechst, and is demonstrated for one representative cell.
Figure 18: Characterisation of hnRNP A2/B1 and A1 distribution in the cell-free reaction.

(A) Characterisation of hnRNP A1/A2/B1 distribution in isolated nuclei and apoptotic extracts by immunoblot. 1 x 10⁶ nuclei and cytosol extracted from 1 x 10⁶ cells were analysed by Western blotting using antibodies specific for hnRNP A1/A2/B1. (B) 5 x 10⁵ nuclei and cytosol extracted from 5 x 10⁵ cells were analysed by immunoblotting. The blot was probed with an antibody specific for hnRNP A1 (clone 4B10). (C) Characterisation of hnRNP A1 distribution in nuclei and cytosolic extracts recovered from the in vitro reaction by Western blot analysis. The blot was probed with antibodies specific for hnRNP A1 (clone 4B10). (D) Characterisation of hnRNP A1/A2/B1 distribution in nuclei and cytosolic extracts recovered from the cell-free apoptosis reaction by Western blot analysis. The cell-free apoptosis reaction was performed in the presence or absence of WGA [200 µg/ml] and zVAD [20 µM]. Western blot analysis was performed of 1 x 10⁶ nuclei and cytosol extracted from 2 x 10⁶ cells using antibodies specific for hnRNP A1/A2/B1. As a loading control for cytosolic samples, the blot was reprobed with an actin-specific antibody.
In the next step, changes in protein distribution during the cell-free apoptotic reaction were examined by Western blot analysis using both hnRNP A antibodies. Examination of cytosolic extracts before the *in vitro* reaction by probing a Western blot with an antibody specific for hnRNPA1 revealed a time dependent increase of the cytosolic concentration of hnRNP A1 in the course of apoptosis (Fig. 18A). hnRNP A1 was reported to be cleaved during apoptosis into three fragments with molecular weights of 32, 29 and 16 kDa (Brockstedt et al., 1998). A cleavage band of hnRNP A1 at 29 kDa in late apoptotic extracts was detected (Fig. 18A).

Further, the hnRNPA1 content of the cytosolic extract before and after the *in vitro* reaction was compared. As shown in Fig. 18B the hnRNP A1-specific increase observed in the apoptotic extract during apoptosis (Fig. 18A) could be detected in nuclear and cytosolic compartments after the cell-free reaction. The observation implies that during the cell-free incubation hnRNP A1 equilibrated between the cytosol and the nucleus to a certain degree. Surprisingly, the apoptosis specific cleavage band could only be observed in late stage apoptotic extracts before and after the cell-free reaction but not in the nuclear fraction. Most likely this fragment exits the nucleus during apoptosis induction.

The same set of experiments was repeated using the general anti-hnRNP A antibody. First, the occurrence of hnRNP A variants in isolated nuclei as well as in control and apoptotic cytosolic extracts prior to the *in vitro* reaction was examined (Fig. 18C). Three hnRNP-specific signals were detected in nuclei corresponding to the three splicing isoforms hnRNP B1, A2 and A1 (Patry et al., 2003). In control and apoptotic extracts gained after 30 and 60 min after CD95-L treatment, two bands were detected migrating at a smaller molecular weight than hnRNP A1. These bands (indicated by asterisks) could not be assigned to any of the known hnRNP A variants. Since it is known that hnRNP A proteins are extensively modified by phosphorylation and poly(ADP-ribosyl)ation, these bands may correspond to post-translationally modified hnRNP A1 and A2 proteins. These bands were absent in late apoptotic extracts (180 min TRAIL). The signal corresponding to hnRNP A2 was detected in all cytosolic samples, with a significant increase 180 min after apoptosis induction.

In Fig. 18D nuclei and cytosolic extracts were analysed for the occurrence of hnRNP A variants after having been submitted to the cell-free apoptotic reaction. The hnRNP A2- and B1-specific signals were present in cytosolic samples recovered after the *in vitro* reaction, although they were absent in the same extracts prior to the reaction (compare Fig. 18D with 18C). This implies that a certain amount hnRNPs A2 and B1 equilibrate between nucleus and
RESULTS

cytosol during the cell-free incubation. In cytosolic extracts recovered from late stage apoptotic reactions a significant increase of hnRNP A1 was observed. This increase was partially inhibited by the pan caspase inhibitor zVAD-fmk. Nuclei recovered from late stage apoptotic reactions show only the specific signals for hnRNP A2 and B1. These results are in accordance with the current knowledge that hnRNP variants shuttle between the nucleus and the cytoplasm to function as transporters for mRNA molecules out of the nucleus. The only way to traverse the nuclear membrane is through the nuclear pore complex, a macromolecular complex spanning the nuclear envelope. Small molecules (< 40 kDa) pass the pore by passive diffusion. For bigger molecules a complex and tightly regulated active transport process is necessary.

To evaluate if active transport between the cytosol and nucleus is important for the increase in hnRNP A1 observed in late stage apoptotic extracts recovered after the in vitro reaction, a cell-free apoptosis reaction in the presence of WGA (wheat germ agglutinin) was performed. WGA blocks specifically the nuclear pore complex and is a commonly used inhibitor of active nuclear transport. Isolated mouse liver nuclei were incubated prior to the in vitro reaction with WGA for 10 min on ice. Two effects on the localisation of hnRNP A variants in apoptotic reactions but none in control reactions were observed and are shown in Fig. 18D. Firstly, hnRNP A1 was retained in late apoptotic nuclei when nuclear transport is blocked; secondly, the hnRNP A-specific signal which was observed in Fig. 18C (labelled by asterisks) was detectable in nuclei incubated with WGA.

Together, the effect of WGA on the distribution of hnRNP A1 in the cell-free apoptosis reaction (Fig. 18D) and the formation of hnRNP A2/B1 specific granular structures within the cytosol observed in immunocytochemical studies (Fig. 17) strongly suggest that the hnRNP A/B variants leave the nucleus during apoptosis.

6.5.2.2 Heterogeneous ribonucleoprotein C1/C2 (hnRNP C1/C2)

Similarly to hnRNP A2/B1, a decrease in the level of hnRNP C1/C2 protein was observed in the proteome analysis.

To investigate possible changes in the distribution of the protein between nucleus and cytosol, Western blot analysis of isolated nuclei and cytosolic extracts before and after the in vitro apoptosis reaction was performed. Investigation of cytosolic extracts prior to the cell-free reaction revealed an increase in the hnRNP C1/C2-specific signal in these extracts in the
course of apoptosis (Fig. 19A). While hnRNP C2 was visible at low levels already in extracts from untreated cells and increased 60 min after apoptosis induction, the C1-specific signal appeared only in samples from late apoptotic cells. In native liver nuclei both hnRNPs C1 and C2 were detected. Interestingly, the C1 protein migrated at a lower molecular weight in isolated mouse liver nuclei than in cytosolic extracts from Jurkat T-cells. No cleavage band of hnRNP C1 or C2 could be detected in the apoptotic cytosolic extracts.

The fact that an increase in the hnRNP C1/C2-specific signal was detected in cytosolic fractions after apoptosis induction is suggestive of a translocation of hnRNP C1/C2 from the nucleus to the cytoplasm in apoptosis. Comparison of the components of the cell-free reaction showed that hnRNP C1/C2 equilibrates between the nucleus and the cytosol during the incubation period leading to an increase of hnRNP C1/C2 in the recovered nuclei (Fig. 19B). This is most likely due to the artificial conditions of the cell-free reaction.

Figure 19: Characterisation of hnRNP C1/C2 distribution in the cell free reaction.

(A) Characterisation of hnRNP C1/C2 distribution in isolated nuclei and apoptotic extracts by Western blot analysis. 1x 10^6 nuclei and cytosol extracted from 2x10^6 cells were loaded on a 10 % Laemmli gel and blotted onto a nitrocellulose membrane. The blot was probed with antibodies specific for hnRNP C1/C2. (B) Characterisation of hnRNP C1/C2 distribution in nuclei and cytosolic extracts recovered from the cell-free apoptosis reaction by immunoblot. Each lane corresponds to 1x10^6 nuclei or cytosol extracted from 1 x 10^6 cells. The blots were probed with hnRNP C1/C2-specific antibodies. As a loading control for cytosolic samples, the blot was reprobed with an actin-specific antibody.
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Figure 20: Analysis of hnRNP C1/C2 distribution in intact cells during apoptosis. HeLa cells were treated with TRAIL [300 ng/ml]. At the indicated time points, cells were fixed with 4 % paraformaldehyde and immunocytochemical staining with antibodies specific for hnRNP C1/C2 were performed. Chromatin was stained with H-33342. The figure shows representative confocal images (scale bar = 10 µm). The localisation of hnRNP C1/C2 inside the cell is illustrated by comparing its distribution profile to the one of Hoechst, and is demonstrated for one representative cell.

Immunocytochemical staining of untreated HeLa cells with anti-hnRNP C1/C2 revealed a distinct localisation of the protein within the nucleus with a clear omission of nucleoli (Fig. 20). In HeLa cells stimulated for 30 min with TRAIL, in addition to a dominant nuclear staining, comparable to the control staining, the formation of hnRNP C1/C2 positive dots in the cytosol was observed. In general, a hnRNP C1/C2-specific staining was apparent in the cytosol of all apoptotic cells. This was confirmed by the protein distribution profile, which showed a significant appearance of peaks corresponding to hnRNP C1/C2 (green line) in the area outside the chromatin stained region (red line). Similarly, as observed for hnRNP A/B in
Fig. 19, the obvious exclusion of nucleoli seemed to vanish later in the course of apoptosis (60 min TRAIL).
These images indicate that there might be a translocation of hnRNP C1/C2 from the nucleus to the cytoplasm upon apoptosis induction.

6.5.3 The proto-oncogene DEK

In the proteomic analysis of apoptotic nuclei DEK was found in two distinct spots with a shift in the isoelectric points while the molecular weight of the protein was unchanged. The positions of the spots indicate that DEK might undergo post-translational modifications in the course of apoptosis.

DEK was discovered in 1992 as one part of the fusion protein DEK-Can in patients suffering from acute myeloid leukaemia (mutations in tumours). Since then it was considered to be a proto-oncogene. Additionally, DEK was found to be involved in many diseases (see discussion) and the level of DEK was observed to be increased in cells derived from a variety of tumour lineages.

DEK is a DNA binding protein which recognises DNA structures rather than DNA sequences and is thus believed to play a role in the regulation of chromatin architecture (Waldmann et al., 2003). It is known that DEK is a phosphoprotein, and its activity might possibly be regulated by phosphorylation (Fornerod et al., 1995). Phosphorylation sites for CK2 were determined in vitro and possible PKC phosphorylation sites were predicted. It was additionally shown that the phosphorylation by CK2 peaks in the G1 phase of the cell cycle and influences the DEK/DNA interaction (Kappes et al., 2004a).

In spite of the number of clinical observations which were made, the biological function of DEK so far remains uncertain (Sitwala et al., 2003).

In order to investigate how DEK is affected by the induction of apoptosis, different experimental strategies were chosen. Immunostaining of DEK in control as well as in apoptotic cells was performed and Western blot analysis was carried out to investigate the protein distribution and to explore possible post-translational modifications.

In all following studies two distinct antibodies were used, a commercially available monoclonal antibody, clone 2 (BD Bioscience) and an affinity purified rabbit antiserum (kind gift from F. Kappes, Konstanz). The antibodies recognise different epitopes on the DEK
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protein. The monoclonal antibody is directed against an epitope in the N-terminal region (amino acids 19-169) whereas the affinity purified serum was raised by immunizing rabbit with the entire recombinant DEK polypeptide and probably recognizes a conformational epitope (Kappes et al., 2004a).

6.5.3.1 Characterisation of DEK

Immunostaining of untreated HeLa cells, with the mentioned anti-DEK antibodies shows that DEK localises exclusively to the nucleus with a punctuate pattern which is characteristic for DNA binding proteins (Fig. 21).

However, differences in the nuclear staining pattern were observed depending on the antibody used. The affinity purified antiserum labelled all cells homogenously. In contrast, the intensity of the signal obtained with the monoclonal antibody varied strongly within the cell population (Fig. 21). Confocal images of HeLa cells stained with the purified serum revealed structures of similar intensity all over the nuclear region. In contrast, the monoclonal antibody detected in most cells bright signals with a precise omission of the nucleolus; others had a staining pattern similar to that obtained with the affinity purified serum. Independently of the antibody used, in some cells dots appeared in the nucleolus, here demonstrated with the affinity purified serum (further discussed in 6.5.3.2).

These observations led to the assumption that the antibodies may detect different forms of the DEK protein.
Figure 21: Immunocytochemical characterisation of DEK.

Untreated HeLa cells were fixed with 4 % paraformaldehyde and immunocytochemical staining with two different DEK-specific antibodies (the monoclonal DEK-specific antibody and the affinity purified antiserum) were performed. Chromatin was counterstained with Hoechst-33342. The figure shows representative confocal images (scale bar = 10 µm).

Although the calculated molecular weight of DEK is 43 kDa, the protein migrated in Western blots at an apparent molecular weight of approximately 60 kDa. As it is known that DEK is phosphorylated \textit{in vitro} and \textit{in vivo}, the question whether this unexpected migration behaviour could be the result of phosphorylation was addressed.

To this end, total cell lysates were prepared from Jurkat T-cells using cell lysis buffer A and were treated with $\lambda$-protein phosphatase which removes phosphate groups from proteins.
RESULTS

λ-phosphatase-treated and -untreated samples were then analysed in parallel by Western blot (Fig. 22).

This in vitro dephosphorylation assay was performed with Jurkat T-cell lysates gained from control and late apoptotic cells. As shown in Fig. 22, the DEK-specific signal at 60 kDa was increased in lysates obtained from apoptotic cells. In samples treated with the phosphatase, an additional band was detected with the affinity purified serum running at a molecular weight of 43 kDa. This band was not detectable with the monoclonal antibody. Treatment with λ-protein phosphatase moreover led to a significant decrease of the DEK-specific signal at 60 kDa. These results indicate that the monoclonal antibody is unable to detect the dephosphorylated form of DEK while being specific for the highly phosphorylated form. This corroborated the hypothesis that the different staining pattern observed in the immunostaining might reflect differently modified species of DEK.

![Characterisation of DEK-antibodies](image)

**Figure 22: Characterisation of DEK-antibodies.**

Characterisation of DEK-specific antibodies by Western blot analysis. Jurkat T-cells were treated with 2.5 % CD95-L. At indicated time points proteins were solubilised in a cell lysis buffer A (0.4 % NP-40, 1 % Triton, 50 mM NaCl, 50 mM Tris pH 7.5). Then 400 µg of protein lysate were treated with 800 Units of λ-protein phosphatase for 30 min at 30 °C. 200 µg of lysate treated or untreated with phosphatase were loaded onto a 10 % SDS-PAGE and proteins were transferred onto a nitrocellulose membrane. The blot was probed with DEK-specific antibodies: either the affinity purified antiserum or the monoclonal DEK-specific antibody. As a loading control, the blot was reprobed with an actin-specific antibody.
6.5.3.2 Subcellular distribution of DEK

In order to investigate the distribution of the DEK protein in the course of apoptosis, immunocytochemical analysis on HeLa cells treated with TRAIL using the monoclonal antibody against DEK were performed (Fig. 23).

Early in apoptosis, when no morphological chromatin changes occur, no difference of DEK protein distribution could be observed. As soon as chromatin condensed and the cell nucleus began to shrink the intensity of the DEK specific signal in the nucleus became weaker and the punctuate staining pattern, which was characteristic of DNA binding proteins, disappeared (after 60 min TRAIL induction), although the signal remained strictly limited to the nucleus. Similar results were obtained with the anti-DEK affinity purified serum (data not shown). These data suggest that the interaction between DEK and DNA was weakened in the course of apoptosis.

Looking at the DEK-specific staining in Fig. 21, in addition to a clear staining of the nucleoplasm, some brighter dots located inside the nucleolus were visible. These structures were labelled both by the monoclonal anti-DEK antibody and the affinity purified antiserum. Major structures, known to be localised inside the nucleolus, are the fibrillary centres, the sites where the rRNA gene clusters are located and where RNA polymerase I (Pol I) accumulates. In addition to Pol I, transcription of ribosomal genes requires the trans-activating factor UBF (upstream binding factor), which tethers the Pol I to the ribosomal gene promoter complex (Panov et al., 2001).

The question whether the DEK-specific nucleolar dots represent fibrillary centres was addressed by performing a double-immunostaining with antibodies specific for DEK and UBF. The results of the co-immunostaining of DEK and UBF are summarised in Fig. 24. UBF (red) localised in the nucleolus in large foci. DEK (green) was again predominantly located in the nucleoplasm, additional showing bright signals inside the nucleus. However, merging both confocal images, no co-localisation of DEK and UBF could be detected. So far, excluding that DEK may interact with fibrillar centres, the significance and function of the intranucleolar foci remain unclear.
RESULTS

Figure 23: Immunocytochemical characterisation of DEK during apoptosis.

HeLa cells were treated with TRAIL [300 ng/ml]. At indicated time points, cells were fixed with 4% paraformaldehyde in HBS (145 mM NaCl, 3 mM KCl, 10 mM HEPES, 3 mM CaCl$_2$, 8 mM glucose, 2 mM MgCl$_2$, pH 8.3) and immunocytochemical staining with the DEK-specific monoclonal antibodies were performed. Chromatin was stained with H-33342. The figure shows representative confocal images (scale bar = 10 µm).
Untreated HeLa cells were fixed with 4% paraformaldehyde and used for an immunocytochemical co-staining of DEK and UBF. First the mouse monoclonal antibody specific for DEK and subsequently the rabbit polyclonal antibody specific for UBF was incubated. Chromatin was stained with H-33342. The figure shows representative confocal images (scale bar = 10 µm).

**6.5.3.3 DEK mRNA and protein levels are not upregulated during apoptosis**

In the dephosphorylation experiment shown above (Fig. 22) an increase in the amount of DEK protein in apoptotic cells compared to untreated cells was observed. It was thus investigated whether DEK expression was upregulated in the course of apoptosis.

First, DEK mRNA levels in cells undergoing apoptosis were analysed. Absolute or relative quantification of mRNA can be achieved by real-time RT-PCR. Total RNA from Jurkat T-cells exposed to CD95-L for different time periods was isolated and reverse transcription was performed. Then, the relative mRNA level of DEK-cDNA was compared with that of the housekeeping gene actin by real-time RT-PCR. Actin served as a reference gene whose expression is stable in cells undergoing apoptosis (Fig. 25B). Amplification with both primer pairs, for actin and DEK, revealed one specific product (melt curve analysis, Fig. 25A).

PCR efficiencies for each set of primers were determined by amplifying a 10-fold dilution series of control mRNA with a known concentration. Then threshold cycles (CT) for triplicates of the samples were plotted against the logarithm of the concentration of each cDNA dilution from the sample. The PCR efficiency was derived from the slope, and automatically calculated by the programme iCycler (Biorad, Munich) with the corresponding
correlation coefficient. The absolute CT values, for DEK and actin did not change in the course of apoptosis (Fig. 25B, Table 3).

Figure 25: Determination of DEK-mRNA levels during apoptosis.
Real-time PCR analysis was performed using the iCycler from Biorad. Jurkat T-cells were treated with 2.5 % CD95-L and total RNA was isolated using the QuickPrep Total Extraction Kit from Amersham. First conversion from RNA into cDNA by the reverse transcriptase was performed. Then 1 ng of cDNA was amplified using the SybrGreen Supermix (Biorad) and 50 cycles of PCR-amplification were started, triplicates were measured of each sample. (A) Melt curves of amplification products. After amplification cycles, melt curve of the PCR-products were monitored by increasing the temperature in steps of 0.5 °C. (B) Absolute CT values of DEK and actin were compared for each apoptotic samples and are unchanged in apoptosis. Data represent means ± SD from triplicate determinations.

Table 3: Calculated mRNA ratio of DEK/actin.

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean CT&lt;sub&gt;actin&lt;/sub&gt;</th>
<th>Mean CT&lt;sub&gt;DEK&lt;/sub&gt;</th>
<th>Ratio</th>
<th>± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.97</td>
<td>30.73</td>
<td>0.985</td>
<td>0.00085</td>
</tr>
<tr>
<td>30</td>
<td>19.93</td>
<td>30.27</td>
<td>0.988</td>
<td>0.00113</td>
</tr>
<tr>
<td>60</td>
<td>22.20</td>
<td>31.63</td>
<td>0.992</td>
<td>0.00571</td>
</tr>
<tr>
<td>180</td>
<td>21.27</td>
<td>31.70</td>
<td>0.985</td>
<td>0.00117</td>
</tr>
</tbody>
</table>

a) Time [min] after apoptosis induction.
b) Mean of three values.
c) The ratio was measured using a mathematical model for relative quantification in real-time PCR of Pfaffl (Pfaffl, 2001).
In a second step the question whether DEK mRNA translation was increased in apoptosis was addressed. To this end, protein translation was inhibited with cycloheximide before inducing apoptosis. Western blot analysis of whole cell extracts showed that the DEK-specific signals in lysates from cells treated with or without cycloheximide were comparable in intensities (Fig. 26), indicating that translation of DEK-mRNA did not increase during apoptosis. Altogether, these data do not support an upregulation of DEK gene expression during apoptosis. Looking for a different reason for the increase in the DEK-specific signal observed here and in the dephosphorylation experiments (see Fig. 22), the composition of the buffer used for the preparation of extracts was considered.

**Figure 26: Characterisation of translational regulation of DEK in apoptosis by immunoblot.**
Jurkat T-cells were preincubated with 5 µg/ml cycloheximide (CHX) for 30 min. Then the cells were treated with 2.5 % CD95-L. At indicated time points proteins were solubilised in cell lysis buffer A (0.4 % NP-40, 1 % Triton, 50 mM NaCl, 50 mM Tris pH 7.5). Then 200 µg of protein were loaded per lane onto a 10 % SDS-PAGE. Immunoblotting was carried out using DEK-specific antibodies: the affinity purified antiserum and the monoclonal antibody. As a loading control, the blot was reprobed with an actin-specific antibody.

### 6.5.3.4 DEK is released from DNA during apoptosis

It is known that chromatin associated proteins elute completely from DNA only under high salt conditions. The DEK protein was described to elute in a minor portion at 100 mM and the main portion at 250 mM salt concentrations (Kappes et al., 2001). Furthermore, it was described that the affinity of DEK to chromatin is affected by its phosphorylation status. Phosphorylation diminishes the affinity of DEK for chromatin, whereas dephosphorylation tightens the interaction (Kappes et al., 2004a).

To test if the increase of DEK in apoptotic extracts (Fig. 22) was due to changes of the interaction between DEK and DNA, two different extraction procedures were compared: in
the first one cells were extracted under mild conditions on ice, using a low salt buffer which could release from the nucleus proteins binding weakly, if at all, to DNA. With the second method all proteins were solubilised in the presence of SDS at 95 °C.

Western blot analyses were performed and DEK was detected with both available antibodies. In apoptotic samples prepared by SDS-lysis no difference in DEK abundance can be detected, demonstrating that the total level of DEK in apoptosis is not changed. Contrarily, in the material gained by low salt extraction a time-dependent increase in DEK signal can be observed during apoptosis. In parallel, SDS-solubilisation of the pellet from the low salt extraction showed a decrease of DEK in the insoluble protein fraction (Fig. 27A). These changes were detected with both antibodies, although they were clearer with the monoclonal antibody, again supporting the hypothesis that the two antibodies recognise different DEK-species. The increase of DEK could be first observed 90 min after apoptosis induction with CD95-L. At this point about 35 % of cells showed condensed chromatin and caspase activity was readily detectable (Fig. 27B). DEK release from DNA is caspase-dependent, because the increase of DEK in low salt extracts was blocked by the pan-caspase inhibitor zVAD-fmk (Fig. 25C).

These data support the previous observation that apoptotic HeLa cells lose their DEK-specific characteristic punctuate staining pattern in the course of apoptosis (Fig. 23).
RESULTS

Figure 27: Analysis of different nuclear pools of DEK by low salt extraction.
Jurkat T-cells were treated with 2.5 % CD95-L. (A) Characterisation of DEK release from DNA during apoptosis by Western blot analysis. At indicated time points proteins were solubilised in the low salt containing lysis buffer A and in parallel in hot SDS-lysis buffer. The pellet from the low salt extraction was additionally lysed in hot SDS buffer. Then 200 µg of protein solubilised in cell lysis buffer and 50 µg of protein lysed in SDS were loaded per lane onto a 10 % SDS-PAGE. Immunoblotting was carried out using either the DEK-specific monoclonal antibody or the DEK-specific affinity purified antiserum. As a loading control, the blots were reprobed with an actin-specific antibody. (B) In parallel the cell viability rate and the enzymatic caspase activity were determined. The apoptosis rate was measured by scoring cells with condensed chromatin after H-33342 staining. The enzymatic caspase activity (DEVD-afc cleavage) was measured in whole cell lysates. (C) Characterisation of caspase-dependence of the DEK release from DNA during apoptosis by Western blot analysis. Jurkat T-cells were treated with 2.5 % CD95-L in the presence or absence of 20 µM zVAD. Lysis was carried out in the low salt extraction buffer and immunoblot was performed using the DEK-specific monoclonal antibody. As a loading control, the blots were reprobed with an actin-specific antibody.
6.5.3.5 The DNA release of DEK is prevented by CK2- and caspase-inhibitors

It is known that DEK is phosphorylated *in vitro* and *in vivo* mainly by CK2, and with lower affinity also by PKC (Kappes et al., 2004a). To test possible effects of these kinases on the apoptotic release of DEK from DNA, apoptosis was induced in the presence of different kinase inhibitors. Gö6983 and calphostin C are general inhibitors of all different forms of PKC, whereas TBB is a specific inhibitor of CK2.

Western blot analysis of low salt extracts from Jurkat T-cell untreated or stimulated to undergo apoptosis showed that the PKC inhibitors did not have any effect on the DNA-binding affinity of DEK, while treatment with TBB totally abolished the release of DEK (Fig. 28A). Additionally, the PKC inhibitors hardly affected caspase activation and condensation of the nuclei, whereas TBB led to a marked reduction of the number of cells with typical apoptotic morphology (Fig. 28B). A closer look at the effect of TBB revealed that, even in the absence of an apoptotic stimulus, 40 % ± 6 % of the cell nuclei appeared to be round and shrunken. Including these cells in the determination of the rate of apoptosis resulted in 65 % ± 8.5 % of cells with an altered nuclear morphology. Thus TBB seems to affect cell viability on its own, but does not trigger classical apoptosis. TBB has been shown to interfere with many signalling pathways (Ruzzene et al., 2002). The effect on DEK release from DNA in intact cells in this experiment might therefore be indirect. To clarify the influence of the CK2-inhibitor TBB, cell-free apoptosis reaction were performed next.
RESULTS

Figure 28: Characterisation of the effect of kinases on DEK-chromatin association during apoptosis in intact cells.

Jurkat T-cells were preincubated with Gö6983 and Calphostin C for 30 min and with TBB for 2 h. Then the cells were treated with 2.5 % CD95-L. (A) Characterisation of the influence of kinase inhibitors on the release of DEK from DNA during apoptosis by Western blot analysis. After indicated time points proteins were solubilised in a low salt extraction buffer. Then, 200 µg of protein were loaded per lane on a 10 % SDS-PAGE. Immunoblotting was carried out using the DEK-specific monoclonal antibody. As a loading control, the blots were reprobed with an actin-specific antibody. (B) In parallel, the kinase influence on cell viability and enzymatic caspase activity was determined. The apoptosis rate was measured by scoring cells with condensed chromatin after H-33342 staining, and enzymatic caspase activity (DEVD-afc cleavage) was measured in whole cell lysates.
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To clarify the influence of the protein kinases PKC and CK2 on the release of DEK from DNA in the course of apoptosis, cell-free apoptosis reactions were performed consisting of cytosolic extracts, which had been pre-treated for 10 min with the kinase-specific inhibitors, and of isolated Jurkat T-cell nuclei. In parallel, in vitro apoptosis reactions were performed in the presence of the broad spectrum caspase inhibitors zVAD-fmk and Q-VD-OPh to estimate the influence of caspases on the release of DEK from DNA during cell death. After the reaction, nuclei were recovered and solubilised first with the low salt containing cell lysis buffer A. The remaining pellet was dissolved in boiling SDS-lysis buffer.

Western blot analysis of recovered nuclei confirmed that DEK is released from DNA also under cell-free conditions (Fig. 29). The PKC-specific inhibitors, calphostin C and Gö6983, did not abolish this DEK-release from DNA. In contrast, pre-incubation of cytosolic extracts with the CK2-specific inhibitors TBB and K25 showed a significant decrease of the apoptosis-specific DEK-release from chromatin. Similar to the observation with CK2 inhibitors, the caspase-inhibitors zVAD-fmk and Q-VD-OPh also decreased the amount of soluble DEK in nuclei incubated with apoptotic cytosolic extracts.

These observations showed that the affinity of DEK to chromatin is changed during apoptosis in a caspase- and CK2-dependent manner. Whether these factors act directly on DEK or on an unknown protein linking DEK to DNA is still unclear and needs further experimental effort.

To monitor apoptosis in the presence of the specific inhibitors for PKC, CK2 and caspases in parallel to the release of DEK from chromatin, Western blot analysis were performed to detect the caspase-dependent cleavage of PARP-1. To this end, the pellets of the low salt extraction procedure were solubilised in boiling SDS lysis buffer and Western blot analysis was performed (Fig. 29). In the nuclei recovered from cell-free reactions in the presence and absence of kinase inhibitors, either PKC-specific (Gö6983, calphostin C) or CK2-specific (TBB, K25), the cleavage band at 89 kDa was clearly visible. The intensity of the cleavage band was significantly reduced but was still detectable in nuclei which were incubated with cytosolic extracts pretreated with caspase inhibitors (zVAD-fmk, Q-VD-OPh). The apoptosis specific cleavage of PARP-1 has been attributed by caspase-3, -7, and -9 (Earnshaw et al., 1999), which are efficiently inhibited by the broad range inhibitors. The cleavage of PARP-1 during the in vitro apoptosis reaction in the presence of caspase inhibition indicates that there might be an additional indirect activation and processing of caspases inside the nucleus.
RESULTS

Figure 29: Characterisation of the effect of kinases and caspases on DEK-chromatin association during apoptosis in cell-free apoptotic reactions by western blot analysis.

Isolated Jurkat T-cell nuclei were incubated with extracts gained from Jurkat T-cells at different apoptotic stages, as indicated by hypotonic extraction in the absence or presence of 1 µM G66983, 10 nM Calphostin C, 20 µM TBB, 5 µM K25, 20 µM zVAD and 2.5 µM Q-VD-OPh. Nuclei were recovered and solubilised in low salt containing cell lysis buffer A. The remaining pellet was lysed in boiling SDS-lysis buffer. Then 150 µg of protein solubilised in cell lysis buffer A and 50 µg of protein lysed in hot SDS-lysis buffer were loaded per lane onto a 10 % SDS-PAGE. Immunoblotting was carried out either using the DEK-specific monoclonal antibody for the blot loaded with the samples in cell lysis buffer A (exposure time 5 min) or using the PARP-1-specific antibody for the blot loaded with samples in SDS-lysis buffer. As a loading control for the DEK-blot, the Ponceau staining of the same blot was used.
7 DISCUSSION

The shrinkage and fragmentation of the nucleus (also called pyknosis and karyorrhexis) are among the most striking morphological changes of cell death by apoptosis (Zamzami and Kroemer, 1999).

Whereas much is known about the signal cascades involved in the cytoplasmic execution of apoptosis primarily due to caspase activation, the processes leading to nuclear apoptosis are less explored. Proteins have been identified which play a role in the process of chromatin fragmentation, such as the endonucleases CAD/DFF40 (Enari et al., 1998; Liu et al., 1997) and EndoG (Li et al., 2001), as well as factors promoting chromatin condensation like AIF (Susin et al., 1996) and Acinus (Sahara et al., 1999). Also factors modifying chromatin associated proteins, like histones, during apoptosis were described (Cheung et al., 2003). Over the last years first indications of a genetically encoded apoptosis signalling machinery inside the nucleus, such as the first nuclear protein containing a death domain, p84N5, (Doostzadeh-Cizeron et al., 1999) and a possible nuclear analogue to the cytotoxic caspase-activating protein complex, the apoptosome, have emerged (Rich et al., 2000).

The aim of this work was to identify proteins involved in nuclear apoptosis and gain further evidences on the existence and composition of a nuclear apoptotic signalling cascade. Firstly, a model for cell-free apoptosis was established, followed by a comprehensive proteomic screen for the analysis of nuclear protein alterations in apoptosis, and finally, identified candidates were characterised by biochemical and immunochemical methods. The mass spectrometry-based analysis identified 13 nuclear proteins which are altered in CD95-L-induced apoptosis. Some of these proteins had been previously described to be involved in apoptotic signalling and others have not been related specifically to apoptosis, so far.

7.1 In vitro apoptotic system

Previous studies aimed at the comprehensive characterisation of the cellular proteome in apoptosis followed different approaches and identified protein alterations in whole cell extracts gained from apoptotic cells. Various cell lines were exposed to different apoptotic
stimuli and changes in protein expression were estimated using comparative 2DE. The major contributions are outlined in the following.

The group of B. Wittmann-Lieberg identified apoptotic protein alterations in whole cell extracts gained from human Burkitt lymphoma cells (Brockstedt et al., 1999; Brockstedt et al., 1998). Similar studies in whole cell protein extracts were performed using Jurkat T-cells treated with anti-CD95 antibody, staurosporine, camphothecin and oligomycin (Gerner et al., 2000; Thiede et al., 2001), or submitted to oxidative stress using glucose oxidase (Rabilloud et al., 2002). In these studies highly abundant proteins from the mitochondria and the nucleus were shown to be altered in apoptosis (see introduction for more details).

Two strategies for increasing the sensitivity of 2DE-gel-electrophoresis to allow for the detection of minor proteins were proposed, so far. In the work by Brockstedt et al. the cells were sorted magnetically, in order to compensate for the lack of synchronicity of the cell death process (Brockstedt et al., 1998). Other studies attempted to decrease the complexity of the sample prior to proteomic characterisation by separating the cells after CD95-L treatment into distinct fractions (the nucleus on the one side and the mitochondria, cytosol and membrane on the other side) (Thiede et al., 2002) or by isolating the nuclear matrix (Gerner et al., 2002). However, fractionation of apoptotic cells bears several difficulties, which will be discussed later in detail.

In this work the problem of sensitivity of 2DE-gels was overcome by using a highly synchronised apoptotic system in combination with cellular fractionation. A suitable approach consists in triggering nuclear apoptosis in a cell-free system by treating isolated mouse liver nuclei with cytosolic extracts derived either from control or apoptotic cells. Such cell-free reactions were originally established in the early eighties for the study of cell cycle events (Blow and Laskey, 1986; Lohka and Masui, 1983; Murray and Kirschner, 1989; Newport, 1987). More recently, in vitro systems were employed for the identification and characterisation of factors involved in apoptotic signalling such as cytochrome c (Liu et al., 1996), Apaf-1 (Li et al., 1997; Zou et al., 1997) and Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), as well as factors responsible for DNA condensation and fragmentation, like CAD/DFF40, Acinus, AIF and Endo G (Enari et al., 1998; Li et al., 2001; Sahara et al., 1999; Susin et al., 1999b).

Basically two methods for the preparation of cytosolic extracts for in vitro reactions are discussed in the literature. In the first one, the cells are lysed by repeated freeze-thaw cycles, as performed in studies on apoptotic chromatin condensation (Lazebnik et al., 1993) and the requirement of caspase-6 for this morphological change (Ruchaud et al., 2002).
DISCUSSION

An alternative lysis method to selectively gain cytosolic extracts uses hypotonic extraction buffers. These buffers lead to cellular swelling and therefore an increased yield in cytosolic material when disrupting the plasma membranes by mechanical shear stress as compared to freeze-thaw lysis (Ellerby et al., 1997; Martin et al., 1995; Newmeyer et al., 1994). However, this technique has the disadvantages of incomplete cell dissociation on the one hand, and, if the mechanical stress exerted by the pestle strokes is excessive, the disruption of cell nuclei on the other hand (Mackall et al., 1979).

In order to circumvent these problems, a method based on the detergent digitonin, which is known to perforate the plasma membrane while leaving the intracellular membranes intact, was chosen (Mackall et al., 1979). Over the last years, studies on the role of mitochondria in apoptosis were performed using buffers containing digitonin (Duan et al., 2003; Hajek et al., 2001; Waterhouse et al., 2001).

In this project a method was established which uses digitonin at low concentrations in a sucrose containing buffer to selectively gain cytosolic extracts free from mitochondrial and ER proteins as well as from nuclear contaminations (Fig. 6).

Some of the published cell-free apoptosis systems employed nuclei isolated from cultured cells as substrates (Ellerby et al., 1997; Lazebnik et al., 1993; Martin et al., 1995; Ruchaud et al., 2002). Previous work in the laboratory had shown that it is very difficult to obtain nuclei from cells in culture without carrying over contaminations from the cytoskeleton, the mitochondria and the ER. For this reason, most groups working with in vitro reactions employed nuclei isolated from tissues, mostly mouse (Enari et al., 1995; Zhao et al., 2001), rat (Newmeyer et al., 1994) or hamster liver (Liu et al., 1996).

Cytosolic extracts for cell-free apoptotic reactions are typically derived from cells committed to apoptosis (Takahashi and Earnshaw, 1997). The CD95 ligand/receptor system is one of the best understood apoptotic signalling systems, mainly because of its predominant physiological function in the regulation of the immune system (Sartorius et al., 2001). Consequently this system was chosen for the development of a cell-free apoptosis reaction. The analysis of the kinetics of apoptosis illustrates the fast onset of cell death after CD95-L treatment (Fig. 5). Caspase processing, protein degradation and cytochrome c release from mitochondria all start around 40 to 45 min after apoptosis induction. An increase in cells showing morphological changes like chromatin condensation was observed shortly after.

It was demonstrated before that isolated nuclei exposed to apoptotic extracts showed morphological changes characteristic for apoptosis, and oligonucleosomal fragmentation of
DISCUSSION

DNA (Enari et al., 1995). Degradation of specific caspase substrates, such as lamin A (Zhao et al., 2001), PARP, fodrin and PKC-δ (Ellerby et al., 1997) was observed as a result of caspase processing in the course of cell-free apoptosis. These processes could be inhibited by the anti-apoptotic protein Bcl-2 (Enari et al., 1995).

In the present work morphological and biochemical hallmarks of apoptosis were induced in isolated mouse liver nuclei incubated for a short period of time (10 min) with apoptotic extracts gained from Jurkat T-cells undergoing CD95-dependent apoptosis. As shown in Fig. 9 apoptotic DNA-loss, margination of nuclear chromatin and clumping of the chromatin was observed. These morphological changes were accompanied by the high molecular weight fragmentation of DNA and the caspase-dependent degradation of nuclear proteins, such as PARP-1 and lamin B.

7.2 Proteome analysis

For the detection of a maximum number of proteins altered as a consequence of apoptosis induction in the cell-free reaction, a high throughput proteomic platform was established. Depending on the type, cells may express up to 20,000 different proteins (Celis and Gromov, 1999). So far, most studies relied on the high resolution of 2D-gel-electrophoresis, which, under proper circumstances, can resolve up to 4000 protein components (Ji et al., 2000).

Most commonly, 2DE-gels are used for determining the proteome of cells and even to compare the protein pattern of cells exposed to different conditions. Moreover, the characteristic spot distribution on 2DE-gels of one cellular system yields information on the purity and the reproducibility of sample preparation.

To test the reproducibility of the cell-free reaction, four reactions were carried out consisting of independently isolated mouse liver nuclei and non-stimulated cytosolic extracts. The nuclear proteins were resolved, submitted to 2DE-gel-electrophoresis and analysed for their spot intensity distribution using the Proteomweaver 2.0 software (Definitions, Munich) (Fig. 10). In the pI range of 4 to 7 the standard deviation of the spot intensities between independent samples amounted to approximately 30 %.

The average standard deviation for 2DE-gel-electrophoresis was estimated by Blomberg et al. by comparing the resolution of one single solubilised sample separated by 2DE-gel-electrophoresis in three different laboratories. They were all using the same instruments, the same IPG-strips and same standard protocols. In this test, an average standard deviation of 34.5 % was calculated for protein quantitation between all three laboratories. This represents a
value slightly higher than the intralaboratory variation, ranging from 20 to 28 % (Blomberg et al., 1995). These values only include variations in sample separation, but not in sample preparation, which is one of the most critical steps for 2DE-gel-electrophoresis (Rabilloud, 1996). This implies that the variation of 30 % observed in this work between 2DE-gels of nuclei recovered from different cell-free reaction lies within the tolerance of the method.

### 7.2.1 Limits of comparative 2D-gel-electrophoresis

There are limitations to the resolution of protein mixtures by 2DE-gel-electrophoresis which are not only due to the high complexity of eukaryotic cells and affect the comparison of expression patterns. At the protein level this complexity is defined by the number of proteins expressed by a single cell, the broad variation of isoelectric point, hydrophobicity, and molecular weight values (Jung et al., 2000) as well as the broad dynamic range of cellular expression levels which may by as great as seven to eight orders of magnitude (Anderson and Anderson, 1998). Many nuclear proteins are of extreme basic nature, and therefore difficult or partially impossible to resolve in 2D-gel-electrophoresis.

In 2D-gel-electrophoresis, streaking in the focusing dimension is a conspicuous problem in the region corresponding to pH values higher than ~7. It is connected to the disappearance of the reducing agent (most commonly, DTT) from the basic part of the IPG strip, followed by an oxidation of the thiol groups of cysteins, resulting in inter and intra chain S-S bonds (Olsson et al., 2002). A further reason for the poor resolution is the loss of basic proteins by the cathodic drift of carrier ampholyte focusing in the first dimension (Görg et al., 1997).

Over the last five years IPG strips have been developed in the basic range from 3-12, 6-12, and 6-11. However, so far optimal conditions for sample preparation and gel running in the alkaline region have not been sufficiently characterised.

As demonstrated in Fig. 9, it was possible to gain an acceptable, but not optimal resolution in gels ranging from pH 3-10, but the detection of distinct spots was not possible using IPG strips in the range of 6-11. Different attempts to optimise the procedure did not result in any improvement. These were: protein precipitation with TCA/acetone prior to rehydration, cup loading instead of in-gel rehydration, and increasing amounts of DTT. Even running the first dimension in presence of the reducing agent hydroethyldisulphide (HED), better known as DeStreak rehydration solution (Amersham Bioscience), instead of DTT could not improve the resolution of the gels.
The relative changes in concentration of specific proteins are generally measured by comparing the difference in staining intensities between two gels. Usually master-gels built from an average of at least three gels from the control trail versus the average of gels from an experimental trail are compared. Depending on the staining procedure, the quantification is more or less reliable. Most widely used are various silver staining protocols and fluorescent based stainings using the SYPRO Ruby protein stain. Both methods are suited for subsequent MS-analysis, but SYPRO Ruby provides a more linear correlation between protein amount and staining intensity and presents, in contrast to silver staining, a one step staining procedure with higher experimental reproducibility (Berggren et al., 2000; Krieg et al., 2003). However the determination of small amounts of protein in 2DE-gels remains difficult and comparison of their intensities bears a high level of uncertainty (Ji et al., 2000).

An improvement in resolution and consequently quantitation is achieved by reducing the isoelectric focusing range down to one pH unit. This results in the detection of far more proteins, but still up to six distinct proteins can be identified within one single faint spot (Gygi et al., 2000). Although identification of more than one protein within one MS-spectrum is possible, intensity differences between spots on different gels cannot be compared, because they could be the result of changes in any of the proteins within the spot.

7.2.2 Mass spectrometric quantification via ICPL labelling

Mass spectrometry based proteomics is the method of choice to overcome the limitations of 2D-gel-electrophoresis. In general, all mass spectrometry based methods for relative protein quantitation rely on the differential labelling of the samples of interest, prior to their mixing in equal amounts and the combination of them. This is then followed by a separation step, either chromatographic or gel-based, and the final identification and relative quantification by mass spectrometry.

In the project an approach called the isotope coded protein label (ICPL) technique was chosen which bears some basic advantages for the analysis of extreme alkaline nuclear proteins. This method is based on the differential labelling of all free amino groups, at lysines and at the N-terminus of proteins, with a nicotinic acid tag containing either no isotopes (light) or four deuteriums atoms (heavy). This reaction bears many advantages for the covalent labelling, as it is fast and selective; it is quantitative with no side reactions, it results in stable products with stable isotope incorporation, and introduces a small molecule (Schmidt et al., 2004; Hausch and Jaschke, 2001). Further advantages are the increase of ion intensities in mass
spectra compared to unmodified peptides (A. Schmidt, unpublished data) and the relatively high sequence coverage, due to the high abundance of free lysines in proteins. All together this can lead to a more reliable identification and quantitation of proteins (Schmidt et al., 2004) and increases the possibility to identify protein modifications (Mann and Jensen, 2003). After covalent modification of all lysines and the N-terminus, the positive charges of labelled amino acids are neutralised and the pI of alkaline proteins is shifted to the acidic region. This simplifies the separation and further analysis of extreme alkaline proteins, as illustrated in Fig. 12. There it was possible to resolve nuclear histones after ICPL-modification by conventional 2D-gel-electrophoresis. As summarised in Table 2, the isoelectric point of ICPL-labelled proteins could be shifted up to 6 units to the acidic range. In the case of the histone H1.2, the pI shifted from 11.0 to 4.55.

Using differential labelling methods prior to protein separation by 2D-gel-electrophoresis, protein overlays in one single spot were no longer limiting the identification and quantification by mass spectrometry. Due to the facts that mass spectrometric quantitation is based on the relative comparison of ion intensities of each single sister peptide pair and the identification is routinely carried out by fragmentising all visible precursor ions, singlets and doublets, more than one protein can be identified and quantified within one MS-spectrum.

7.3 Nuclear protein alterations in apoptosis

The analysis of apoptosis-induced changes of the nuclear proteome resulted in the identification of 13 candidates. Only proteins identified in at least two independent sets of experiments were taken into account, and were submitted to statistical analysis. As shown in Fig. 13 it was possible to follow the time-dependency of apoptotic nuclear protein changes. Some candidates were detected in nuclei exposed to early apoptotic extracts (30 min), while the majority was identified in intermediate stage (60 min) apoptotic nuclei.
7.3.1 Classification and characterisation of identified proteins

The proteins identified can be classified into two groups: proteins involved in chromatin organisation and architecture and abundant nuclear proteins participating in RNA transport and metabolism. In addition, three proteins were identified which could not be assigned to either group (Table 4).

Table 4: Classification of proteins identified by ICPL analysis of apoptotic nuclei.

<table>
<thead>
<tr>
<th>Chromatin organisation and architecture</th>
<th>RNA transport and metabolism</th>
<th>others</th>
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</thead>
<tbody>
<tr>
<td>HMG B1/B2</td>
<td>hnRNP A2/B1</td>
<td>hsp 70</td>
</tr>
<tr>
<td>DEK</td>
<td>hnRNP C1/C2</td>
<td>lamin B2</td>
</tr>
<tr>
<td>nuclear protein Hcc-1</td>
<td>U2 snRNP A1</td>
<td>PP1α</td>
</tr>
<tr>
<td>histone H1.0/H1.2/H4</td>
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Lamin B2

Lamin B2, a nuclear envelope protein, is one of the first identified caspase substrates. Once it is cleaved by caspase-3 into two fragments sized 24 kDa and 43 kDa, nuclear lamina disassembly takes place (Rao et al., 1996a). In 2DE-gels of differentially labelled samples, two separate spots were observed migrating at the same molecular weight, but with distinct isoelectric points which were indicative of different phosphorylation forms of the lamin protein. Phosphorylation of lamin is known to occur in the course of apoptosis (Shimizu et al., 1998). In addition, both spots migrating at a molecular weight corresponding to the uncleaved form were significantly reduced in their protein levels in the intermediated apoptotic samples compared to control reactions (Fig. 14). Considering the kinetics of apoptosis in Jurkat T-cells (Fig. 5), caspase activity was already present 60 minutes after CD95-L treatment and lamin B degradation was shown to take place in the cell-free reaction (Fig. 9B), indicating that decrease in spot intensity might result from caspase-mediated proteolysis.
Heat shock protein 70

Although the heat shock protein 70 (hsp 70) is known to be involved in the stress response in many cells, its role in apoptosis is not as clear as that of the lamins. Albeit hsp 70 is mainly located in the cytoplasm and mitochondria (Sreedhar and Csermely, 2004), in all native nuclear preparations an abundant form to be expressed within the nucleus was observed and this with different isoelectric points, most probably representing different modified forms of the protein. Translocation of most hsp proteins to the nucleus has been described after stress, where they have a major role in protecting the cells from DNA damage induced by various damaging agents (Samali and Orrenius, 1998). The observation that also in control nuclei high levels of hsp 70 are present could be due to stress induced by the preparation procedure. Nevertheless the constitutive high level of nuclear hsp 70 was significantly decreased in all apoptotic reactions investigated; in nuclei exposed to early apoptotic extracts where no caspase activity was present, and in nuclei exposed to intermediate apoptotic extracts in which caspase processing could be detected. These observations imply that the decrease of hsp 70 might occur independently of caspase activity.

The role of hsp 70 in apoptosis is controversially discussed. Some studies showed that overexpression of hsp 70 reduces CD95-induced apoptosis (Schett et al., 1999) and that downregulation of hsp 70 is sufficient to kill tumour cells or to facilitate the induction of apoptosis (Nylandsted et al., 2000). Contrarily, there are reports demonstrating that vector-mediated hsp 70 overexpression failed to protect Jurkat T-cells from apoptosis (Mosser et al., 1997), and that CD95-mediated apoptosis in Jurkat T-cells is enhanced upon hsp 70 overexpression (Liossis et al., 1997).

Moreover, apoptosis induction via CD95-L leads to inhibition of HSF-1, the transcription factor for stress-induced hsp 70 activation and hsp 70 synthesis (Schett et al., 1999). However, more and more evidence for a protective role of hsp 70 in cell death is being gathered at the molecular level. Hsp 70 binds to Apaf-1 and thereby blocks the assembly of a functional apoptosome (Saleh et al., 2000) and it can interact with AIF thereby inhibiting its nuclear import (Gurbuxani et al., 2003; Ravagnan et al., 2001), indicating an involvement in caspase-dependent and -independent pathways.

A possible explanation for the permanently reduced hsp 70 protein levels in the performed proteomic experiments is that hsp 70 might be released during the cell-free apoptotic reaction from the nucleus. If this translocation depends on the experimental setup - the cell-free apoptosis reaction - or whether it has a specific role in apoptotic signalling is still unclear.
Protein phosphatase 1 alpha isoform

A further protein identified in the proteomic experiments was the catalytic subunit of the alpha isoform of the protein phosphatase 1 (PP1α). All catalytic subunits of PP1 (α, β, γ₁, γ₂) can be converted into many different forms which have distinct substrate specificities, restricted subcellular locations and diverse regulations. Regulation of PP1α occurs by its interaction with a broad variety of regulatory protein subunits. These subunits target PP1 to distinct subcellular locations, and they can be classified depending on the location of their target. It was shown, for example, that targeting to the mitochondria occurs by interacting with members of the Bcl-2 family of proteins. In addition to the regulatory proteins, there are modulators of PP1 function such as inhibitors, activators or proteins with chaperone activity (Cohen, 2002).

In apoptosis, the serine/threonine protein phosphatase family including the protein phosphatase 1 (PP1) and 2A (PP2A) were described as being involved in signalling processes (reviewed in (Garcia et al., 2003)).

PP1α is involved in the regulation of the Bcl-2 family of proteins. It was shown that Bcl-2, Bcl-w and Bcl-xL, which are all anti-apoptotic members, target PP1α to the pro-apoptotic member Bad, which is then dephosphorylated and subsequently promotes apoptosis (Ayllon et al., 2002; Ayllon et al., 2001).

This involvement in apoptotic signalling does not explain the observed decrease in PP1α-level in apoptotic nuclei, but it allows for different hypotheses. First, PP1α itself could be post-translationally modified through one of these protein-protein interactions with regulatory or modulatory proteins. Furthermore, through complex formation with distinct proteins, it could be converted into a form which has different substrate specificities and different target locations, resulting probably in a translocation out of the nucleus.

Chromatin organisation and architecture

One group of proteins identified by the proteome analysis to be involved in nuclear apoptosis are chromatin organisational and chromatin architectural proteins. Among them were three different histones, two proteins of the high mobility group (HMG) protein family, the hepatocellular carcinoma protein 1 (hcc-1) and the proto-oncogene DEK. All these proteins have direct or indirect connections to cell death.

Biochemical and immunochemical analysis gave more precise information on the fate of the HMGBs and the proto-oncogene DEK during apoptosis. The role of these proteins in apoptosis will be discussed in detail later.
DISCUSSION

Histones

In all mammalian cells, DNA is organised into nucleosomes consisting of histone octamers containing two copies of each of the core histones H2A, H2B, H3 and H4. Histone H1 is located outside the nucleosomes on the internucleosomal DNA (Hengartner, 2001). Histones are highly post-translationally modified proteins, mainly at the N-terminus by acetylation, methylation, phosphorylation, poly(ADP-ribosyl)ation and ubiquitylation (Fu et al., 2003). Each change of the modification status influences the chromatin structure, to facilitate gene transcription, DNA replication and DNA repair.

Recently, a conserved histone code has been proposed, indicating that, dependent on the modification of histones, different functions of the proteins are possible (Wang et al., 2004). Likewise current studies reported on apoptosis-specific post-translational modifications, such as phosphorylation of histone H2B at Ser14 (Cheung et al., 2003). Others showed that poly(ADP-ribosyl)ation of histone H1.0 facilitates the internucleosomal DNA fragmentation by increasing the susceptibility of chromatin to endonuclease activity (Yoon et al., 1996). In addition, underacetylation of histones in apoptosis triggers a tight binding of the high mobility group protein B1 (HMGB1) (Scaffidi et al., 2002).

In the proteome screen three different histones, histone H1.0, H1.2 and H4, were identified to be altered in apoptosis. The three histone variants showed protein alterations in intermediate stage as well as in early stage apoptotic nuclei. Histone H4 was always identified in one single spot and permanently increased in signal intensity during apoptosis, whereas histone H1.0 and H1.2 were identified in more than one spot and showed increased or decreased protein levels depending on the localisation of the spot. All spots corresponding to one protein migrated at the same molecular weight, but with different isoelectric points. Taking into account the known post-translational modifications of histones in apoptosis, it is most likely that different apoptotic variants of the proteins were detected.

Hcc-1

Hcc-1 is a nuclear protein which was first described in 2001. It contains a SAP-box which mediates its DNA-binding activity (see 5.4 for more information). Hcc-1 cDNA levels were found increased in pancreatic adenocarcinoma and in well-differentiated hepatocellular carcinoma, but decreased as the carcinoma progressed to a poorly differentiated stage (Choong et al., 2001). The function of hcc-1 is not clear yet, but it might function as an architectural chromosomal protein through its SAP-box (Choong et al., 2001). Additionally, it was postulated to function as a putative splicing factor since it co-localises with snRNPs
In the 2D gel-analysis, two separate hcc-1 spots were identified, one increased and the other decreased in intensity, migrating at the same molecular weight, but with different isoelectric points, indicating that the protein might be post-translationally modified, probably phosphorylated, during apoptosis.

**RNA transport and metabolism**

The last group of proteins the level of which were altered already in the early stage of apoptosis comprises factors involved in RNA transport and metabolism. From the moment pre-mRNA emerges from the transcriptional complex and throughout its lifetime in the nucleus, pre-mRNA/mRNA never exists as free RNA, but rather is associated with proteins (Pinol-Roma and Dreyfuss, 1992). These proteins comprise the abundant hnRNP proteins (heterogeneous nuclear RNA-binding proteins) and further proteins involved in transcription and pre-mRNA processing, such as the small nuclear ribonucleoprotein particles (snRNPs). Altogether they are a group of about 20 major proteins (Nakielny et al., 1997).

In the proteomic experiments, decreased protein levels of the proteins of the 40S-ribonucleoprotein core particle, the heterogeneous ribonucleoproteins A2/B1 and C1/C2 (hnRNP A2/B1 and C1/C2) and a member of the small nuclear ribonucleoprotein particle (snRNP A’) were always observed.

### 7.3.2 Heterogeneous ribonucleoproteins and their role in apoptosis

The best characterised complex in mammalian cells is the 40S-ribonucleoprotein core particle associated to pre-mRNA. The hnRNPs A, B and C represent the major proteins present in the core particle (Pino et al., 2003).

The main structural feature of all hnRNPs is that they contain RNA-binding motives, which can be the RNA-binding domain (RBD), the RNA-recognition motif (RGM) or the arginine/glycine-rich box. In addition, another characteristic feature of hnRNPs is the number of isoforms generated by alternative pre-mRNA splicing and post-translational modifications (Krecic and Swanson, 1999).

Some hnRNPs, such as hnRNP A and hnRNP K shuttle continuously between the nucleus and the cytoplasm, although they remain predominantly nuclear (Nakielny et al., 1997). More recently this list has increased and includes now the hnRNP E, I, D, and possibly L, indicating that the shuttling hnRNPs have probably not only nuclear functions, such as RNA
transcription and pre-mRNA splicing, but also non-nuclear functions such as transport of mature mRNA to the cytoplasm, translation and regulation of mRNA stability (Kim et al., 2000).

Amongst hnRNPs, the hnRNP C proteins are the proteins for which most post-translational modifications affecting the affinity to mRNA have been reported. SUMO modification at the single lysine residue K237, for example, decreases the binding to nucleic acids (Vassileva and Matunis, 2004). A cycle of phosphorylation changes of hnRNP C was postulated to regulate mRNA binding: in a basally phosphorylated state the protein binds to pre-mRNA, additional phosphorylation releases such RNA-molecules, followed by dephosphorylation (Stone and Collins, 2002). Phosphorylation additionally occurs in different hnRNPs, such as hnRNP A2 and A1. In this case it was possible to show phosphorylation via CK2 \textit{in vitro} and \textit{in vivo} (Pancetti et al., 1999).

Poly(ADP-ribosyl)ation of hnRNP A1 and A2/B1 has been shown previously (Prasad et al., 1994) and a recent report demonstrates that many of the hnRNPs, the hnRNP A2/B1, C1/C2, E1, G, H, K and M can establish a strong non-covalent link to poly(ADP-ribose) upon DNA-damage (Gagne et al., 2003).

\subsection*{7.3.2.1 hnRNP A1 and A2/B1}

On the one hand, hnRNP A variants are reported to be cleaved by caspases in the course of apoptosis. The first hnRNP protein described to be cleaved by caspase-3 into three distinct fragments of the apparent sizes of 32, 29 and 16 kDa was the hnRNP A1 (Brockstedt et al., 1998). Later on, putative caspase cleavage sites were reported for hnRNP A2/B1 (Thiede et al., 2001). On the other hand, the constitutive shuttling of hnRNP A1 has been discussed in detail (Michael, 2000) and was demonstrated also for the hnRNP A2/B1 protein (Nichols et al., 2000).

First observations on the extrusion of RNA from the nucleus of apoptotic cells (Biggiogera et al., 1998) have been later interpreted at the molecular level. Although in control cells hnRNP A2 and B1 are localised primarily in the nucleus, excluding the nucleolus, inhibition of RNA polymerase II transcription by actimocyn D caused transcription dependent translocation from the nucleus to the cytoplasm (Kamma et al., 1999). This finding goes along with observations from proteomic studies, indicating a translocation of hnRNP A2/B1 from the nucleus to the cytoplasm and mitochondria (Thiede et al., 2002).
DISCUSSION

The data presented here also support the hypothesis that hnRNP A2/B1 is released from apoptotic nuclei. First of all the level of many different modified isoforms was decreased in the proteomic analysis (Fig. 14). This was supported by immunocytochemical experiments, where granular cytoplasmic staining could be detected upon apoptotic stimulation with an antibody recognising all hnRNP A1/A2 and B1. This staining pattern could not be repeated using a monoclonal antibody directed only at hnRNP A1, indicating that the granular cytoplasmic staining might result from hnRNP A2/B1-translocation. Western blot analyses of cytosolic extracts from Jurkat T-cells demonstrated the degradation of hnRNP A1 in apoptosis.

Interestingly, using WGA as an inhibitor of nuclear transport, the release of hnRNP A1 from the nucleus was blocked in late apoptotic reactions. Additionally, in nuclei pre-incubated with WGA and exposed to apoptotic extracts an additional band appeared in the nuclear fraction, migrating at a molecular weight smaller than all described hnRNP A/B isoforms, most likely representing an apoptotic cleavage product of one isoform. These observations suggest that cleavage of one hnRNP A/B protein occurs inside the nucleus in apoptosis and under normal conditions, without nuclear transport inhibition these fragments might subsequently translocate to the cytoplasm. Further, a possible explanation could be that cleaved hnRNPs do not bear the nuclear targeting sequence and are therefore not recognised by the active transport machinery, so that the fragments are excluded from the nucleus in late stage apoptosis. Further experiments will be necessary to elucidate changes in hnRNP transport in the course of apoptosis.

7.3.2.2 hnRNP C1/C2

Structurally, hnRNP C1/C2 is a heterodimer (C1_C2), in which C1 and C2 are splice variants of the same gene, differing by the presence of an additional 13 amino acids in C2 (Barnett et al., 1989; Burd et al., 1989). Several hnRNPs, including the hnRNP C1/C2, are restricted to the nucleus in interphase cells and they do not shuttle between the nucleus and the cytoplasm. It is presumed that hnRNP is released from newly formed mRNAs in the nucleus prior to their export. Where and how these nonshuttling hnRNPs are exactly released from mRNAs in the nucleus is currently not known, but it is discussed to take place at the nuclear pore complex (Iborra et al., 2000). The restriction of hnRNP C1/C2 to the nucleus is not due to the lack of a nuclear export signal (NES) but rather due to a specific retention sequence. The nuclear retention signal (NRS) comprises 78 amino acids (aa 88-165) and contains a proline-rich
region, clusters of basic residues, potential phosphorylation sites for CK2 and PKC and a potential glycosylation site. However, the probable importance of these motives for NRS function have not been investigated so far (Nakielny and Dreyfuss, 1996).

In apoptosis, the cleavage of hnRNP C1/C2 was reported upon caspase-3 activation (Waterhouse et al., 1996) with putative cleavage sites at the C-terminus (between aa 276-313). Recently, first evidence of a regulating function of hnRNP C in apoptosis was observed, as a direct interaction of both the C1 and C2 variants with the XIAP (X-chromosome-linked inhibitor of apoptosis) IRES (internal ribosome entry site) element could be demonstrated. The authors speculate that the proteolytic cleavage of the hnRNP C proteins at the onset of apoptosis may target and stop the synthesis of XIAP, an inhibitor of apoptosis (Holcik et al., 2001). Furthermore, it could be shown that after treatment with hydrogen peroxide rapid phosphorylation of hnRNP takes place, resulting most probably in a release of mRNA molecules from the protein (Stone and Collins, 2002).

In the present study hnRNP C1/C2 was strictly confined to the nucleus of untreated cells. Following different approaches evidence of a translocation from the nucleus to the cytoplasm upon apoptosis induction was gathered.

Again, similarly to hnRNP A2/B1, the intensity of all protein spots identified in proteome experiments, most likely reflecting different post-translational modifications of hnRNP C1/C2, were always decreased, in early as well as in intermediate stage apoptotic reactions (Fig. 14). This reduction was most likely due to protein release from the nucleus, as underscored by the immunocytochemical analysis, clearly detecting hnRNP C1/C2 in the cytoplasm after induction of apoptosis by TRAIL. Cytosolic granular structures were observed, which increased in intensity and size in the course of apoptosis (Fig. 22). Western blot analysis of cytosolic extracts gained from apoptotic Jurkat T-cells revealed a definite time-dependent increase of hnRNP C1/C2 in the cytosolic fraction (Fig. 21A).

Due to the fact that hnRNP C1/C2 harbours a nuclear retention signal which would not allow the protein to leave the nucleus, there must be an event overriding this signal so that the protein can translocate to the cytoplasm during apoptosis. The first possible explanation might be that caspases cleave the protein within the NRS (aa 88-185) and the protein domain is not functional any more. Although there is one putative cleavage site within the NRS (aa 131-134: DYYD), no caspase-dependent cleavage was reported at this site. So far, the only observed cleavage occurred in the C-terminus, but the cleavage site has not been identified yet (Waterhouse et al., 1996). Due to the fact that the protein is not cleaved within the NRS, the
DISCUSSION

only further explanation is a possible post-translational modification in the course of apoptosis which may lead to a non-functional domain, most probably occurring either at the potential phosphorylation sites for CK2 and PKC or at the potential glycosylation site within the NRS. Verification of this hypothesis awaits further experimentation and was beyond the scope of this work.

7.3.3 High mobility group proteins and their role in apoptosis

The high-mobility-group (HMG) proteins are a family of ubiquitous and abundant nonhistone nuclear proteins, consisting of six proteins and subdivided into three subfamilies: the HMGB (previously HMG-1/-2), the HMGA (previously HMG-I/-Y) and the HMGN (previously HMG-14/-17) subfamily. They are all chromatin binding proteins, but each subfamily harbours a specific functional sequence motif: the HMG-box (HMGB), the AT-hook (HMGA), and the nucleosome binding domain (HMGN) (Bianchi and Beltrame, 2000). All the HMG proteins are considered to function as architectural elements that modify the structure of DNA and chromatin to generate a conformation that facilitates and enhances various DNA-dependent activities.

In this work, only HMGB proteins were found to be affected by induction of apoptosis. Therefore, this subfamily and its contribution to cell death signalling will be illustrated in detail. The HMGB family comprises three proteins, HMGB1, HMGB2 and HMGB3 which are all composed of three different domains (Muller et al., 2001): the two homologous DNA binding domains, HMG boxes A and B, and the highly negatively charged C-terminal domain (reviewed in (Bustin, 1999)). HMGB1 and HMGB2 proteins bind to the minor groove of DNA, causing a local distortion of the double helix. They have no sequence preference and they are recruited to the site of action by specific DNA binding proteins. HMGB1 has been shown to interact with and increase the apparent binding affinity of several transcription factors and some viral proteins to DNA (Muller et al., 2001).

HMGB1 has been shown to have different functions depending on the mode of cell death. In surprising contrast to its intranuclear role, it can be secreted by necrotic cells and can trigger inflammation. In apoptotic cells, HMGB1 is immobilised on chromatin even when cells lose integrity of their membranes in late apoptosis, thereby preventing inflammation (Scaffidi et al., 2002).
DISCUSSION

For HMGB2 a role in the apoptotic signal cascade could also be demonstrated. It can stimulate the activity of DFF40 nuclease \textit{in vitro}, providing the first hint of the involvement of DNA-bending proteins in apoptotic DNA fragmentation (Toh et al., 1998). Finally, both HMGB1 and HMGB2 were shown to be part of a nuclear protein complex containing, hsp 70, ERp60 and GAPDH that identifies chemically modified nucleotides and thus contributes to DNA surveillance mechanisms (Krynetski et al., 2003).

In the case of HMGB1 the experimental data gained in this work cannot be interpreted unequivocally. A constant decrease of the level of HMGB1 was always detected in one single spot in all proteomic approaches. Using immunocytochemistry and Western blot analysis no change in signal intensity or distribution could be detected. In confocal images of both healthy and apoptotic cells a strictly nuclear localisation can be seen, going along with the observation that the protein is tightly bound to chromatin also during apoptosis (Scaffidi et al., 2002). The result of the proteomic screen must likely reflect a post-translational modification occurring in apoptosis, probably influencing the interaction between the protein and chromatin.

In contrast, the HMGB2 protein is not tightly restricted to the nucleus as demonstrated in confocal images and in Western blots. In addition, two variants were detected by Western blot analysis, one migrating at the lower molecular weight of 19 kDa predominantly present in murine cell nuclei and one migrating at the molecular weight of 28 kDa, mainly detected in the cytosolic extracts from Jurkat T-cells. Therefore, it was possible to follow translocations of the HMGB2 protein in apoptosis in the \textit{in vitro} apoptosis reaction.

In cytosolic extracts after late stage apoptosis reactions the bands specific to the nuclear HMGB2 form were detected. In addition, a slight increase of the cytosolic HMGB2 signal was observed both in the cytosolic and the nuclear samples after the cell-free reaction. This goes along with the permanently observed increase of the protein levels in proteomic experiments, occurring even in different spots. However, it is still unclear whether this increase of the murine HMGB2 in the cytosolic fraction is a specific apoptosis-triggered event. If this is the case, it could be controlled via post-translational modification. Further experiments have to be performed to explain the changes of HMGs in apoptosis.
7.4 The proto-oncogene DEK

The mammalian nuclear protein DEK was identified about 10 years ago as one part of the fusion protein DEK-Can in patients suffering from acute myeloid leukaemia (AML) (von Lindern et al., 1992). Since then, DEK was found to be involved in many diseases, such as in juvenile rheumatoid arthritis (JRA), systemic lupus erythematosus (SLE) and other immune disorders (Dong et al., 2000) where DEK was shown to be the target of auto-antibodies. In addition, several groups have reported that expression of DEK is increased in tumour cells derived from a variety of lineages (Grottke et al., 2000; Kondoh et al., 1999; Kroes et al., 2000; Larramendy et al., 2002).

In cells expressing endogenous hDaxx, which has transcriptional repression activity, DEK was shown to associate with hDaxx (Hollenbach et al., 2002). In contrast, DEK was also shown to interact with the transcription activator AP-2α protein (Campillos et al., 2003). Subsequently, results from other groups suggested a role for DEK in the regulation of human immunodeficiency virus type 2 (HIV-2) expression (Faulkner et al., 2001; Fu et al., 1997). However, despite a number of clinical observations which have been made, a defined cellular function of DEK is still unknown.

7.4.1 Properties of DEK

DEK is an abundant nuclear phosphoprotein expressed in all higher eukaryotes but not in yeast. DEK is a protein consisting of 375 amino acids (aa) and has a molecular weight of 43 kDa. No splicing variants of the protein are known. It has a high percentage of acidic amino acids (shown in red at 30-49, 228-236, 241-254, 300-310 aa), and harbours a nuclear localisation signal (Fig. 31).

DEK does not belong to any characterised family of proteins and its sequences do not contain any functional or enzymatic domains. The only motif to which DEK bears homology is the SAP-domain, a DNA-binding motif found in other chromatin-associated proteins (SAF/Acinus/PIAS) (Aravind and Koonin, 2000).

The calculated isoelectric point of human DEK is 8.6, but phosphorylation shifts the isoelectric point down to values ranging from 6.8 to 7.3 (Sierakowska et al., 1993).
DEK is similar to other chromatin associated proteins in eluting from nuclei only at high salt concentrations (250 mM). It is associated with oligonucleosomes and is equally distributed on transcriptionally active and inactive chromatin regions (Kappes et al., 2001). However, a subpopulation of the protein is localised within the nuclear matrix (Kappes, unpublished data).

DEK exhibits distinctive structure-specific rather than sequence-specific DNA binding properties. It has a strong preference to bind negatively supercoiled DNA compared to relaxed or linear DNA (Waldmann et al., 2003). The element recognised is a 4WJ (four-way junction) DNA (Waldmann, personal communication). Binding to this region is a common property of different architectural proteins, such as HMGs and histone H1 (Zlatanova and van Holde, 1998). In yeast two hybrid experiments only one protein was identified to bind with DEK, namely DEK itself. A second functional domain, localised between amino acid 270 and 350 (shown in blue, Fig. 31), was identified as having various functions: a second DNA binding activity, a multimerisation domain, and it harbours most of the phosphorylation sites (Kappes et al., 2004b).

DEK was shown to be phosphorylated in vitro and in vivo mainly by CK2 and with less efficiency by PKC. The phosphorylation peaks in the G1 phase of the cell cycle (Kappes et al., 2004a). Additionally it was demonstrated that the dephosphorylated form of DEK binds strongly to DNA, while phosphorylated DEK binds rather weakly. 50 mM salt did not affect the different binding affinities, but at 100 mM salt the amount of DNA bound to phosphorylated DEK was decreased compared to unphosphorylated DEK.

The main CK2-dependent phosphorylation sites were mapped in an in vitro approach by Nano-ESI-Q-Trap-MS and were localised in the C-terminal part of the protein including the second DNA-binding domain. No phosphorylation sites were mapped within the SAP-domain (Kappes et al., 2004a). First in vivo mapping attempts support the observed in vitro data, and
implicate possible further phosphorylation sites located between amino acids 66 to 78 and 124 to 143 (Kappes, unpublished data).

7.4.2 Localisation of DEK

DEK is a nuclear protein which is strictly restricted to the nucleus, where the majority of the protein is associated with oligonucleosomes, and a minor fraction associates with RNA (Kappes et al., 2001).

The localisation of DEK was analysed by staining untreated HeLa cells with DEK-specific antibodies. In these immunocytochemical studies the localisation was clearly confirmed to be strictly nuclear (Fig. 21). However, different staining patterns were observed depending on the antibody used. Staining with the DEK-specific affinity purified serum labelled cells homogeneously and the signal was distributed all over the nucleus. The monoclonal DEK-specific antibody additionally detected bright cells which showed an exclusion from the nucleolus. Both antibodies stained granular structures inside the nucleus and bright foci in the nucleoli. Experiments to establish whether the DEK-positive foci might localise to the fibrillar centres at nucleoli yielded negative results, leaving the question as to the meaning and function of these intranucleolar structures open.

7.4.3 DEK in apoptosis

The above mentioned subcellular localisation of DEK (see 7.4.2) is not changed during apoptosis. By immunocytochemistry, a decrease in the intensity of the nuclear DEK-specific signal could be detected concomitantly to alterations of chromatin morphology during apoptosis, but no translocation to other cellular compartments was detected (Fig. 23). Additionally it could be demonstrated that the DEK protein is neither transcriptionally nor translationally upregulated during apoptosis (Fig. 25 and Fig. 26).

In the proteomic screen two protein spots migrating at the same molecular weight but at distinct isoelectric points were attributed to the DEK protein (Fig. 14). The intensity of the more acidic spot was decreased, whereas the more basic spot was increased. Since DEK is a phosphoprotein with many phosphorylation sites (Kappes et al., 2004a), and such variations in
DISCUSSION

isoelectric points are most commonly related to changes in protein phosphorylation, the data indicate that the phosphorylation status of DEK might be altered during apoptosis. Additionally it was shown that the phosphorylated form of DEK migrates at a molecular weight of approximately 60 kDa in SDS-PAGE. Dephosphorylation resulted in a shift of mobility to 43 kDa.

The data shown here indicate that the binding affinity of DEK to DNA is reduced in cells undergoing apoptosis. In intermediate stage apoptosis, when morphological changes and caspase processing have already started, more soluble DEK was extracted at low ionic strength and in parallel, less DEK was observed in the insoluble protein fraction (Fig. 27). This was observed on Western blots using two DEK-specific antibodies, although the intensity change was more evident when the monoclonal antibody was employed. This was in accordance with experiments on the binding characteristics of the two antibodies (Fig. 22). In contrast to the affinity purified serum, recognizing all forms of DEK, the monoclonal antibody does not recognize the unphosphorylated form migrating at 43 kDa. Further, it is likely that the monoclonal antibody only binds to one specific phosphorylated form, with the phosphorylation site located within the epitope from amino acid 19 to 167 recognised by the antibody, whereas the serum might recognize different phosphorylated forms. This would explain the differences in the signal intensities of the phosphorylated DEK in Western blots depending on the antibody used (Fig. 22). First attempts to identify phosphorylation sites in vivo revealed two putative phosphorylation sites inside the binding epitope of the monoclonal antibody (F. Kappes, unpublished data).

The release of DEK from DNA is not dependent on PKC, but can be abolished when inhibiting CK2, which is known to be the kinase mainly responsible for DEK phosphorylation (Kappes et al., 2004a). However, the CK2 inhibitor itself has major effect on the viability of the cells. Although the cells seemed to be protected from apoptosis, as no caspases were activated and no nuclear fragmentation occurred, the morphology of the cells was altered. Both unstimulated control and apoptotic cells were shrunk and the nuclei were condensed. Since it was previously reported that the CK2 inhibitor TBB decreases cell viability (Ruzzene et al., 2002), cell-free apoptosis reactions were performed. Inhibition of PKC did not change the release of DEK from DNA, whereas CK2-inhibition again significantly impaired it, in line with the results obtained in intact cells. The same degree of inhibition was observed when caspases were blocked (Fig. 29).
DISCUSSION

The open question is how the release of DEK from DNA is triggered during apoptosis. It is known that dephosphorylated DEK binds strongly to DNA, whereas the phosphorylated form binds rather weakly. In addition, filter binding experiments demonstrated that the DEK/DNA interaction is dependent on ionic strength. Phosphorylated and unphosphorylated DEK bind equally well to DNA at 50 mM salt. At 100 mM salt the amount of DNA bound to the phosphorylated form of DEK is lower than the amount of DNA bound to the dephosphorylated form (Kappes et al., 2004a).

The results from the proteomic approach in this work indicate that there might be a phosphorylation event during apoptosis, which could lead to the observed release of DEK from DNA. Whether post-translational modification by phosphorylation occurs in apoptosis and which phosphorylation sites are affected, needs to be examined further.

From the data presented and discussed above different hypothetical mechanisms for apoptotic release of DEK from chromatin can be envisaged:

1. It is clear that CK2 has an effect on DEK release. It is therefore likely that phosphorylation via this kinase triggers the release of DEK, but it remains open how caspases are involved in this phosphorylation event.

2. Caspases themselves might have a direct influence on DEK. This seems unlikely, since proteolysis of DEK was not observed and no consensus site for caspase cleavage could be identified in DEK’s primary sequence.

3. Possibly, caspases might target an additional protein which might bridge DEK to chromatin. This hypothetical cleavage could subsequently reduce the interaction between DEK and DNA. However, no protein which binds to DEK was found in yeast two-hybrid screens apart from DEK itself (Kappes et al., 2004b).

4. It is more likely that caspases interact directly or indirectly with CK2 leading to the activation of the kinase, consequently to a higher phosphorylation of DEK and therefore to a loss of its affinity towards chromatin. Similar influences of caspases on kinases have been described previously, such as the activation of the human serine/threonine kinase, mammalian STE20-like kinase (MST) (Lee et al., 2001) and MEK kinase 1 (MEKK1) (Widmann et al., 1998) by caspase-3. In addition, it was shown that MEKK1 induces mitochondrial permeability transition upon cleavage by caspases resulting in a potentiation of apoptotic execution (Gibson et al., 2002).
Over the last years, there has been growing evidence that nuclear events are crucial steps in the process of apoptosis. Caspases, the major effector proteases in apoptosis were identified to directly or indirectly play a role in the process of chromatin fragmentation. In addition, factors independent of caspase activation were characterised to have influences on the nuclear execution of cell death, such as proteases (cathepsins and calpains), nucleases, and kinases.

In present study it was possible to apply a mass spectrometry-based proteomic approach and to identify 13 nuclear proteins to be altered as a consequence of apoptosis induction. In subsequent biochemical analyses it was possible to validate the observed nuclear alterations of individual candidates, such as hnRNPs, HMGBs and DEK.

DEK is a chromatin-binding protein whose physiological function has not been identified so far. There was no previous evidence which supported the involvement of DEK in apoptotic signalling.

In this work, changes in the binding affinity of DEK to chromatin in the course of apoptosis were observed which seem to depend on the phosphorylation status of the protein. Many questions regarding the apoptosis-specific changes of DEK are still open.

Which is the nature of the signals leading to DEK release from DNA? How are CK2 and caspases linked to each other: is CK2 downstream of caspases or vice versa? What are the molecular mechanisms involved in this process and do other proteins, apart from caspases and CK2, participate?

In order to address these questions, incorporation experiments with radioactive labelled phosphates should be performed. A molecular mapping of the phosphorylation sites affecting DEK-DNA binding affinity in apoptosis together with the analysis of site specific mutant forms of DEK might lead to a better understanding of the role of this post-translational modification in apoptosis.

Additionally, the physiological function of the DEK protein is unknown so far. Although the protein is ubiquitously expressed, it has not been investigated if the cell needs DEK for viability. Further evidences for the role of DEK in chromatin reorganisation and nuclear execution during cell death could be gained by studying the fate of cells overexpressing DEK in apoptosis as well as of cells lacking the DEK protein.
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two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. *Mol Cell Biol*, **12**, 1687-1697.


## APPENDIX

### 10.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>afc</td>
<td>aminotrifluoromethylcoumarine</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>Apaf</td>
<td>apoptotic protease activating factor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Brij 35</td>
<td>polyoxyethylene (35) lauryl ether; DAE</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>caspase-activated DNase</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cloamidopropyl)-dimethylammonio]-propanesulfate</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CID</td>
<td>collision induced dissociation</td>
</tr>
<tr>
<td>CK2</td>
<td>casein kinase 2</td>
</tr>
<tr>
<td>CPAN</td>
<td>caspase-activated nuclease</td>
</tr>
<tr>
<td>CT</td>
<td>threshold cycles</td>
</tr>
<tr>
<td>cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>2DE</td>
<td>two-dimensional electrophoresis</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
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<tr>
<td>DEVD</td>
<td>Asp-Glu-Val-Asp</td>
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<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signalling complex</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylenglycol-bis(β-aminooethyl-ether) tetraacetic acid</td>
</tr>
<tr>
<td>Endo G</td>
<td>Endonuclease G</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GUA</td>
<td>guanidin hydrochloride</td>
</tr>
<tr>
<td>HCCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>hydroxyethyl-piperazysulfonic acid</td>
</tr>
<tr>
<td>HexB</td>
<td>β-hexosaminidase</td>
</tr>
<tr>
<td>hnRNP</td>
<td>heterogenous ribonucleoprotein</td>
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<tr>
<td>HMG</td>
<td>high mobility group</td>
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<tr>
<td>hsp</td>
<td>heat shock protein</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
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<tr>
<td>ICAD</td>
<td>inhibitor of CAD</td>
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<tr>
<td>ICAT</td>
<td>isotope-coded-affinity-tag</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1β-converting enzyme</td>
</tr>
<tr>
<td>ICPL</td>
<td>isotope-coded-protein-label</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focussing</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilised pH gradient</td>
</tr>
<tr>
<td>kbp</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted Laser Desorption/Ionisation</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>m/z</td>
<td>mass/charge</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxy succinimide ester</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose)polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMF</td>
<td>peptide mass fingerprint</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethysulfonylfluoride</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>Q-VD-OPh</td>
<td>N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone disulfonate</td>
</tr>
<tr>
<td>RuBPS</td>
<td>ruthenium baphophenaltrolane disulfonate</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
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<tr>
<td>TBB</td>
<td>4,5,6,7-tetramethylbenzotriazole</td>
</tr>
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<td>TCA</td>
<td>trichloro acetic acid</td>
</tr>
<tr>
<td>TCPE</td>
<td>tris(2-carboxyethyl)phosphate</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
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<td>TFA</td>
<td>trifluoro acetic acid</td>
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<td>TMRE</td>
<td>tetramethylrhodamine ethylester</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)amino-methane</td>
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<td>WGA</td>
<td>wheat germ agglutinin</td>
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<tr>
<td>zVAD-fmk</td>
<td>z-Val-Ala-Asp-fluormethylketone</td>
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