MECHANISMS OF APOPTOSIS INDUCED BY A PROTEIN COMPLEX ISOLATED FROM HUMAN MILK -with focus on the role of mitochondria

by

Camilla Köhler
To my family
ABSTRACT

Human milk contains a protein complex comprising of a folding variant of human α-lactalbumin and oleic acid (C18:1). Both, a milk fraction containing this complex (MAL, for multimeric α-lactalbumin), as well as native α-lactalbumin converted in vitro into the folding variant (HAMLET, for human α-lactalbumin made lethal to tumour cells), kills various transformed cells via apoptosis—a highly regulated and conserved form of cell suicide.

Upon treatment of transformed cells, MAL/HAMLET binds to the cell surface, enters the cell and exhibits a granular pattern in the cytoplasm. Subsequently, the complex translocates to and accumulates in the nucleus.

MAL/HAMLET-treated Jurkat or A549 cells showed typical biochemical characteristics of apoptotic cells, such as caspase activation and cleavage of specific caspase substrates. The caspase activation did not depend on death receptor ligation. However, mitochondrial cytochrome c was released, an event required for activation of pro-caspase-9 in the presence of Apaf-1 and dATP. Active caspase-9, in turn, cleaves and activates executioner caspases.

Mitochondria play a critical role in apoptosis by releasing apoptogenic proteins, including cytochrome c to the cytosol. At the time of investigation, cytochrome c and AIF (apoptosis-inducing factor) were the only proteins known to be released from mitochondria during apoptosis. We showed that another intermembrane space protein, adenylate kinase (AK)-2, was released together with cytochrome c, while AK-3—a matrix protein—remained inside mitochondria during apoptosis induced by different stimuli, including MAL. This suggested a general release of intermembrane space proteins, rather than a specific release of cytochrome c and AIF during apoptotic cell death.

Co-localization of MAL/HAMLET with the mitochondrial dye MitoTracker Red indicated that MAL/HAMLET may cause the release of intermembrane space proteins in treated cells by direct targeting of mitochondria. Addition of MAL/HAMLET to Ca²⁺-loaded isolated mitochondria induced loss of ∆Ψₘ, swelling and release of proteins from the intermembrane space, including cytochrome c and AK-2. These effects were prevented by cyclosporin A, an inhibitor of mitochondrial permeability transition (MPT), indicating that MAL/HAMLET induced cytochrome c release via opening of MPT pores. Moreover, in the absence of Ca²⁺-loading MAL/HAMLET increased the rate of state 4 respiration and induced a sub-maximal drop of ∆Ψₘ in isolated mitochondria, suggesting that this complex possesses uncoupling properties. This uncoupling effect of MAL/HAMLET was due to the presence of fatty acids in the complex since it was completely abolished by bovine serum albumin (BSA). In contrast, BSA failed to prevent MPT, but slightly delayed it, indicating that the fatty acids in MAL/HAMLET rather facilitated than were responsible for MPT induction.

Treatment of cells with HAMLET caused a rapid decrease in ∆Ψₘ that was accompanied by cytochrome c release and a decreased ability of mitochondria to accumulate Ca²⁺. MPT inhibitors did not block the loss in ∆Ψₘ in HAMLET-treated cells. However, when the inhibitors were added in combination with BSA, they markedly prevented the drop in ∆Ψₘ and restored the ability of mitochondria to accumulate Ca²⁺, suggesting again that the mitochondrial changes in HAMLET-treated cells were a consequence of both MPT induction and uncoupling.

Thus, our data demonstrate that a protein complex containing a folding variant of human α-lactalbumin is directly toxic to mitochondria resulting in MPT pore opening, release of cytochrome c, and activation of the caspase cascade; taken together, these events leads to apoptotic death of transformed cells.
LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts, which will be referred to in the text by their Roman numerals:


The published papers are reprinted with permission from the respective copyright holders.

Additional paper:

# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>List of publications</td>
<td>5</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>8</td>
</tr>
<tr>
<td><strong>A. Introduction</strong></td>
<td>9</td>
</tr>
<tr>
<td>1. Background</td>
<td>9</td>
</tr>
<tr>
<td>Properties of milk</td>
<td>9</td>
</tr>
<tr>
<td>A fraction of human milk kills tumour cells via apoptosis</td>
<td>9</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>10</td>
</tr>
<tr>
<td>Characterisation of the killing factor in milk</td>
<td>11</td>
</tr>
<tr>
<td>HAMLET (human α-lactalbumin made lethal to tumour cells)</td>
<td>12</td>
</tr>
<tr>
<td>Cellular uptake and localisation of MAL/HAMLET</td>
<td>13</td>
</tr>
<tr>
<td><strong>2. Apoptosis</strong></td>
<td>15</td>
</tr>
<tr>
<td>2.1. Characteristics of apoptotic cell death</td>
<td>15</td>
</tr>
<tr>
<td>Historical remarks</td>
<td>15</td>
</tr>
<tr>
<td>Apoptosis versus necrosis</td>
<td>16</td>
</tr>
<tr>
<td>Physiological and pathological significance of apoptosis</td>
<td>17</td>
</tr>
<tr>
<td>2.2. Molecular mechanisms of apoptosis</td>
<td>18</td>
</tr>
<tr>
<td>The caspase family</td>
<td>18</td>
</tr>
<tr>
<td>Activation of the caspase cascade</td>
<td>19</td>
</tr>
<tr>
<td>Death receptor-mediated apoptosis</td>
<td>20</td>
</tr>
<tr>
<td>Role of mitochondria in apoptosis</td>
<td>21</td>
</tr>
<tr>
<td>Mechanisms for cytochrome c release</td>
<td>24</td>
</tr>
<tr>
<td>Other pathways for activation of caspases and other proteases</td>
<td>26</td>
</tr>
<tr>
<td>Cleavage of caspase substrates</td>
<td>28</td>
</tr>
<tr>
<td>Caspase-independent pathways of apoptosis</td>
<td>29</td>
</tr>
<tr>
<td>Regulation of apoptosis</td>
<td>29</td>
</tr>
<tr>
<td>The Bcl-2 family</td>
<td>30</td>
</tr>
<tr>
<td>The IAP family</td>
<td>31</td>
</tr>
<tr>
<td>The HSP family</td>
<td>32</td>
</tr>
<tr>
<td>Other mechanisms of regulation</td>
<td>32</td>
</tr>
<tr>
<td><strong>B. The present study</strong></td>
<td>34</td>
</tr>
<tr>
<td>Aims</td>
<td>34</td>
</tr>
<tr>
<td>Methodology</td>
<td>35</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>40</td>
</tr>
<tr>
<td>Summary</td>
<td>54</td>
</tr>
</tbody>
</table>
Table of contents

Future directions.............................................................. 55
Concluding remarks.......................................................... 56
Swedish summary-svensk sammanfattning............................... 58
Acknowledgements........................................................... 61
References........................................................................ 63

Papers I-IV
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocator</td>
</tr>
<tr>
<td>APAF</td>
<td>apoptotic protease-activating factor</td>
</tr>
<tr>
<td>BA</td>
<td>bongkrekic acid</td>
</tr>
<tr>
<td>BAR</td>
<td>bifunctional apoptosis regulator</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma gene-2</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology domain</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CD95</td>
<td>Fas/Apo-1</td>
</tr>
<tr>
<td>CED</td>
<td>cell death abnormal</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>mitochondrial transmembrane potential</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signalling complex</td>
</tr>
<tr>
<td>endo G</td>
<td>endonuclease G</td>
</tr>
<tr>
<td>Egl-1</td>
<td>egg laying defective</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-inhibitory protein</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain-containing protein</td>
</tr>
<tr>
<td>FLICE</td>
<td>FADD-like ICE</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-inhibitory protein</td>
</tr>
<tr>
<td>HAMLET</td>
<td>human α-lactalbumin made lethal to tumour cells</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>HSP</td>
<td>heat-shock protein</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
</tr>
<tr>
<td>MAL</td>
<td>multimeric α-lactalbumin</td>
</tr>
<tr>
<td>MPT</td>
<td>mitochondrial permeability transition</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose)polymerase</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TMRE</td>
<td>tetramethylrhodamine ethylester</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TPP</td>
<td>tetraphenylphosphonium</td>
</tr>
<tr>
<td>zVAD-fmk</td>
<td>z-Val-Ala-Asp-fluoromethylketone</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. BACKGROUND

While investigating the anti-bacterial effects of milk, Håkansson et al. (1995) determined that human milk killed tumour cells. A human lung carcinoma cell line was initially used as experimental model and the influence of milk on the bacterial adherence to these cells were studied. Although human milk inhibited bacterial cell surface binding in these experiments it, surprisingly, killed the lung cancer cells.

Properties of milk
In addition to essential nutrients, milk contains many components that protect the newborn in different ways. For instance, anti-viral and anti-bacterial factors protect the infant against infections at a time when its own defence mechanisms are poorly developed. Moreover, epidemiological studies on infant nutrition indicate that breast-feeding protects against diseases that develop later in life. Among other effects, breast-feeding has been reported to protect against cancer. In particular, breastfed children have a lower incidence of lymphomas than bottle-fed children, and the frequency decreases with the length of breast-feeding [Davis et al., 1988; Mathur et al., 1993; Hardell & Dreifaldt, 2001].

The composition of milk varies both quantitatively and qualitatively between and within species. The main components of human milk are water (88%), lipids (3.3%), lactose (6.8%) and proteins (1.3%), mainly $\alpha$-lactalbumin and $\beta$- and $\kappa$-casein [Findlay, 1974].

A fraction of human milk kills tumour cells via apoptosis
The component in human milk linked to cell death has been isolated and characterised. Human milk was fractionated, and the death-inducing activity was found in a fraction obtained after subjecting the casein fraction of milk to anion exchange chromatography [Svensson et al., 1999]. The active fraction was shown to markedly reduce cell viability of transformed cells. When separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the milk fraction was shown to contain several bands ranging from 14 to 100 kDa. N-terminal amino acid sequencing showed complete sequence homology of all bands to human $\alpha$-lactalbumin. Hence, the isolated milk fraction was shown to contain monomers, oligomers and multimers of $\alpha$-lactalbumin.
The milk fraction killed transformed cells and immature cells while differentiated non-transformed cells were resistant [Håkansson et al., 1995]. The dying cells exhibited morphological changes similar to those that occur in cells dying by apoptosis (i.e. cell shrinkage, plasma membrane blebbing, nuclear condensation and appearance of apoptotic bodies), which is described in detail in Section 2, p. 15. Tumour cells of different origin varied in their sensitivity to apoptosis induction by the milk fraction; leukaemia cells were more sensitive while sarcoma and carcinoma cells were less sensitive. Interestingly, some strains of bacteria were also killed by this milk fraction, although the mechanism of this effect is unclear [Håkansson et al., 2000].

**α-Lactalbumin**

Human α-lactalbumin (14.2 kDa) is a globular, acidic (pI 4-5) protein present in the milk of most mammals [Permyakov & Berliner, 2000]. The crystal structure of human α-lactalbumin has been resolved (at 1.7 Å resolution) [Acharya et al., 1991]. α-Lactalbumin contains one large α-helical domain and one small β-sheet domain (Fig. 1).

*Figure 1. The structure of α-lactalbumin.* The protein consists of two domains: one α-helical domain (red) and one β-sheet domain (blue). The four disulfide bridges are shown in yellow and the Ca²⁺-binding site in green. The structure was taken from Brookhaven Protein Data Bank (# ALC, Baboon).
The two domains are divided by a deep cleft and are connected by a Ca\textsuperscript{2+}-binding loop. Four disulfide bonds stabilize the molecule; two bonds are formed in the \(\alpha\)-helical region, one in the \(\beta\)-sheet region, and the last one connects the two domains. \(\alpha\)-Lactalbumin also contains a high affinity Ca\textsuperscript{2+}-binding site and binding of the Ca\textsuperscript{2+} ion is crucial for the proper folding of the native protein [Ewbank & Creighton, 1993; Anderson et al., 1997].

\(\alpha\)-Lactalbumin is the major whey protein and constitutes 28% of the total protein content in milk [Heine et al., 1991]. It is expressed in secretory epithelial cells in the lactating mammary gland and the \(\alpha\)-lac gene expression begins in late pregnancy [Vilotte & Soulier, 1992]. \(\alpha\)-Lactalbumin is synthesized into the lumen of the endoplasmic reticulum (ER). It is transported to the Golgi apparatus where it forms a transient complex (the lactose synthetase binary complex) with \(\beta\)-1,4-galactosyltransferase (UDP-galactosyltransferase) [Qasba & Kumar, 1997]. The galactosyltransferase, which is an enzyme bound to the \textit{trans} face of the Golgi apparatus, normally transfers uridine diphospho-galactose (UDP-galactose) onto the N-acetylglucoseamine of oligosaccharide chains in glycoproteins. \(\alpha\)-Lactalbumin binding changes the substrate specificity of the enzyme. Specifically, UDP-galactose will be transferred to glucose—not to N-acetylglucoseamine—resulting in lactose production. A continuous synthesis of \(\alpha\)-lactalbumin is required during the entire period of lactation since both \(\alpha\)-lactalbumin and lactose are secreted into the milk. Lactose, which is an important nutrient for the neonate, together with other sugars and ions are necessary for the osmotic pressure in milk. It was shown that \(\alpha\)-lactalbumin knock-out mice could not feed their offspring since the milk they produced was highly viscous and rich in fat and protein but devoid of \(\alpha\)-lactalbumin and lactose [Stinnakre et al., 1994].

Characterisation of the killing factor in milk
Native monomeric \(\alpha\)-lactalbumin purified from human milk whey did not have any apoptosis-inducing ability, even at very high concentrations. As mentioned, oligomers and multimers of \(\alpha\)-lactalbumin were also present in the isolated milk fraction; consequently, the active component in milk was suggested to be a multimeric form of \(\alpha\)-lactalbumin and the milk fraction was named MAL (\textit{multimeric \(\alpha\)-lactalbumin}) [Håkansson et al., 1995].

The \(\alpha\)-lactalbumin in MAL was not altered by post-translational modifications such as glycosylation or phosphorylation as determined by mass spectrometry. However, spectroscopic data showed that the protein in MAL was preserved in a partially unfolded state [Svensson et al., 1999]. The \(\alpha\)-
lactalbumin, which has been studied extensively for its protein folding, had undergone a conformational change to a molten globule-like state. The molten globule state of α-lactalbumin is characterised as an intermediate state between the native and the denatured state. It has a native-like secondary structure but a fluctuating tertiary fold [Kuwajima, 1996; Chang et al., 2000]. The molten globule-like states of α-lactalbumin are normally only found at altered conditions, e.g. at low pH, at elevated temperatures or in a partially unfolded state produced by removal of the bound Ca²⁺ [Kuwajima, 1996]. Interestingly, the protein in MAL was stable in this conformation even at physiological conditions.

In addition to α-lactalbumin, MAL also contains fatty acids. Lipid extraction from MAL yielded 5-10% (w/w) lipids. The major part was oleic acid (C18:1), which is the most abundant fatty acid in human milk [Jensen, 1996].

**HAMLET (human α-lactalbumin made lethal to tumour cells)**

If the apoptosis-inducing component in human milk is the folding variant of α-lactalbumin, then it should be possible to convert native α-lactalbumin into the active form. Indeed, native α-lactalbumin purified from human milk whey was converted into the active molten globule-like form [Svensson et al, 2000]. The protein was first partially unfolded by removing the Ca²⁺ ion with EDTA and then subjected to anion exchange chromatography. A cofactor, oleic acid was needed for the conversion and the apoptosis-inducing fraction contained mostly monomeric α-lactalbumin, but higher molecular weight forms were also present. The converted protein is referred to as HAMLET (human α-lactalbumin made lethal to tumour cells). HAMLET contains oleic acid, which is believed to stabilise the molten globule-like state of α-lactalbumin. Oleic acid was added to cells at a concentration corresponding to the amount found in HAMLET. The fatty acid did not influence cell viability, which indicated that the lipid component itself was not responsible for the apoptosis-inducing effect. Moreover, nuclear magnetic resonance (NMR) spectra of HAMLET suggested that the fatty acid had become an integral part of HAMLET [Svensson et al., 2000]. It has been reported that fatty acids can interact with α-lactalbumin and this may occur via a specific fatty acid-binding site [Cawthern et al., 1997]. It was then concluded that the apoptosis-inducing component in milk is a folding variant of human α-lactalbumin stabilised by oleic acid.
Cellular uptake and localisation of MAL/HAMLET
When biotinylated MAL was incubated with cells, it first bound to the plasma membrane and was then taken up by the cells [Håkansson et al., 1999]. Internalised MAL initially exhibited granular staining in the cytoplasm after which it subsequently translocated to the nucleus (Fig. 2). The binding of MAL to the plasma membrane did not vary between cells with different sensitivity.

![Figure 2. Cellular localisation of MAL.](image)

**Figure 2. Cellular localisation of MAL.** Jurkat (T cell-derived leukaemia), A549 (lung carcinoma) and HRTEC (primary culture of renal epithelial) cells incubated with biotinylated MAL for different lengths of time. The cells were fixed, permeabilised, and stained with FITC-conjugated streptavidin. At 30 min, MAL was bound to the plasma membrane of all cell types. At 3 h, MAL was found in the cytoplasm of sensitive cells and later MAL accumulated in the nucleus. This figure was kindly provided by A. Håkansson.
to apoptosis induction by MAL. However, the total cellular uptake varied; cell types sensitive to MAL internalised the complex more rapidly and to a larger extent [Håkansson et al., 1999]. MAL binds to the plasma membrane of resistant (differentiated non-transformed) cells, but is not internalised. Hence, the cellular uptake of MAL in different cells was proportional to their sensitivity to MAL-induced apoptosis.

Native $\alpha$-lactalbumin was internalised to a lesser extent compared to MAL and it was never detected in the nuclei of treated cells [Håkansson et al, 1999]. $\alpha$-Lactalbumin is a water-soluble protein known to associate with membranes upon conformational change toward the molten globule state [Lala et al., 1995, Banuelos & Muga, 1996]. The protein in this state exposes hydrophobic residues and interacts more strongly with lipid bilayers [Mulqueen & Kronman, 1982]. Moreover, the translocation of proteins across membranes may involve the molten globule state [Bychkova et al., 1988]. This could explain the difference in cellular uptake between MAL and native $\alpha$-lactalbumin as MAL is molten globule-like and thus more likely to penetrate cell membranes.
2. APOPTOSIS

2.1. Characteristics of apoptotic cell death
Apoptosis, sometimes referred to as programmed cell death (PCD), is a highly regulated and conserved form of cell suicide that is an essential part of life for all multi-cellular organisms. PCD is the term often used when referring to the physiological cell death that occurs during embryogenesis, morphogenesis and regulation of tissue homeostasis. This process is controlled by different physiological factors, such as hormones, growth factors etc.

Historical remarks
Physiological cell death in tissues was first described more than 100 years ago [for details see the reviews by Majno & Joris, 1995, Clarke & Clarke, 1996 and Lockshin & Zakeri et al., 2001]. As early as 1842, Carl Vogt reported cell death in the notochord and adjacent cartilage of metamorphic toads. In 1885, Walther Flemming found that cell death involved in the regression of ovarian follicles was associated with the generation of cells containing nuclei that were breaking up, along with small cellular particles in the cavity of the follicle. Flemming named this process chromatolysis. In the beginning of 20th century, Schröder observed fragmented cells with condensed chromatin in the premenstrual uterine mucosa and Saunders described cell death in the chick limb bud during embryogenesis. These are all examples of a phenomenon that later became known as PCD. In 1972, Kerr, Wyllie and Currie reported that cell death with distinct morphology (contrasted from necrosis) occurred in many pathological conditions and in normal tissues during development and in adult life. They introduced the term apoptosis to describe these specific morphological changes. Apoptosis is a Greek word meaning “falling off” as the falling of leaves from a tree (apó, means from and ptósis, means a fall). Nowadays, these terms (apoptosis and PCD) are used (sometimes mistakenly) as synonyms to describe both the biochemical and morphological changes that occurs in dying cells. Evidence for the genetic regulation of PCD was first reported by Horvitz and his colleagues [Ellis & Horvitz, 1986]. During the normal development of the nematode Caenorhabditis elegans, 131 of the 1090 somatic cells are predetermined to die by apoptosis. Four genes in the nematode were found to be required for execution of the apoptotic process. These are: egl-1, ced-3, ced-4 and ced-9 (egl and ced for egg laying defective and cell death abnormal, respectively) [Ellis & Horvitz, 1986; Ellis et al., 1991; Conradt & Horvitz, 1998]. EGL-1, CED-3, and CED-4 are pro-apoptotic proteins since loss-of-function mutations in egl-1, ced-3, or ced-4 resulted in
survival of the 131 cells doomed to die. In contrast, worms lacking a functional CED-9 died early during development, whereas gain-of-function mutations in \textit{ced-9} prevented the 131 naturally occurring deaths, demonstrating CED-9 to be a suppressor of cell death. Normally, the membrane-associated CED-9 binds CED-4 and thereby prevents the activation of pro-CED-3, which is inactive unless its pro-domain is removed by cleavage. When receiving a death signal, EGL-1 interacts with CED-9 and displaces CED-4. Free CED-4 binds to pro-CED-3, which facilitates pro-CED-3 processing and activation. CED-3 mediates downstream events of apoptosis by proteolytic cleavage of specific target proteins. Although the apoptotic process in mammalian cells is more complex than in the worm, the studies in \textit{C. elegans} have provided a great deal of information about cell death in mammalian cells since the apoptotic process is highly conserved in the animal kingdom. Thus, the \textit{ced-3} gene encodes a cysteine protease that is highly homologous to a family of mammalian cysteine proteases, the caspases [Yuan et al., 1993]. The mammalian counterparts of the anti-apoptotic CED-9 protein are the anti-apoptotic proteins of the Bcl-2 family, and the homologues of CED-4 are the adapter molecule Apaf-1 (apoptotic protease-activating factor-1) and other caspase recruitment domain (CARD)-containing proteins [Hengartner & Horvitz, 1994; Zou et al., 1997]. The molecular mechanisms of apoptosis in mammalian cells are discussed in section 2.2., p. 18.

\textbf{Apoptosis versus necrosis}

Currently, several types of cell deaths are recognised; the best described are apoptosis and necrosis. Necrosis is a passive form of cell death that occurs when the damage is so severe that the cell cannot maintain membrane integrity. The cell and its organelles swell and burst as a result of water influx. Thus, the cell content is spilled into the extracellular space. Enzymes (including lipases, proteases, and nucleases) and by-products of metabolism (e.g. reactive oxygen species, ROS) will cause severe damage to the surrounding tissue. In addition, neutrophils and macrophages will be attracted causing an inflammatory response and additional damage to the tissue. Apoptosis, on the other hand, is an energy-dependent process defined and characterized by morphological changes. These changes are remarkably similar across cell types and species and include cell shrinkage, plasma and nuclear membrane blebbing, and chromatin condensation. At a later stage, the cells are fragmented into membrane-enclosed vesicles (apoptotic or pyknotic bodies) containing cell organelles and nuclear fragments. \textit{In vivo} these vesicles are quickly phagocytosed by macrophages and neighbouring cells, thereby
avoiding an inflammatory response. In apoptotic cells, genomic DNA is fragmented by specific nucleases into high and low molecular weight fragments (HMW and LMW fragments, respectively) [Wyllie, 1980; Tomei et al., 1993; Oberhammer et al., 1993]. HMW fragments are believed to be a result of cleavage between rosettes and loops, which are chromatin subdomains of 300 and 50 kbp, respectively. In most cell types, the DNA is further fragmented into LMW fragments. Cleavage at internucleosomal sites generates DNA fragments with lengths corresponding to multiple integers of approximately 180 bp. The LMW fragments are often referred to as ‘DNA ladder’ due to the migration in a ladder pattern during conventional agarose gel electrophoresis. This pattern is not seen in necrotic cells where nucleases cleave the chromatin randomly as shown by a “smear” appearance on the gel.

**Physiological and pathological significance of apoptosis**

Apoptosis is important for both normal development and tissue homeostasis, which is maintained through a balance between cell proliferation and cell death. During embryogenesis, apoptosis helps to sculpture the body, shape the organs, and carve out fingers and toes. It is also involved in eliminating unwanted (abnormal, misplaced or harmful) and unneeded cells. The general strategy during tissue development is to produce cells in excess followed by removal of those not needed for proper organ function [Jacobson et al., 1997]. In the mammalian nervous system, up to 50% or more of the neurons die before the embryonic development is complete [Barde, 1989; Oppenheim, 1991]. Only those neurons receiving enough neurotrophic support from their target cells will survive, and the rest are eliminated by apoptosis. Another example is the Müllerian duct that during embryogenesis gives rise to the uterus and oviduct in females. This structure is removed in males [Roberts et al., 1999].

Dysregulation of the death process resulting in too much or too little apoptosis is implicated in developmental defects as well as in various disorders, such as neurodegenerative diseases, autoimmunity and cancer [Thompson, 1995; Fadeel et al., 1999]. For example, increased neuronal apoptosis is likely involved in Alzheimer’s disease (AD), a common form of adult-onset dementia. It has been reported that mutations in the genes encoding presenilin-1 and -2 result in accumulation of β-amyloid peptide [Blacker & Tanzi, 1998]. This peptide is secreted and forms extracellular β-amyloid plaques in the brain that are a characteristic pathological hallmark in AD. Moreover, the β-amyloid peptide induces apoptosis of neurons *in vitro* and may be the causative agent for the neuropathological lesions observed in
patients with AD [Loo et al., 1993]. In contrast to AD, too little apoptosis is the underlying cause for autoimmunity. Developing T cells that produce self-reactive receptors are normally eliminated via apoptosis. However, these potentially harmful cells are not removed in patients suffering from autoimmune disorders. In rheumatoid arthritis (RA) T cells recognise and react to antigens present in the joints and thereby mediate autoimmune organ damage [Marrack et al., 2001].

Tumour formation was originally believed to be a result mainly of enhanced cell proliferation. However, recent research shows that a decreased level of apoptosis also contributes to tumour development [Zornig et al., 2001]. In many cancers, it is an impairment of the apoptotic machinery that leads to unrestrained cell proliferation and insensitivity to chemotherapeutic agents. The resistance to apoptosis induction can be caused by mutations in certain tumour-suppressor genes and oncogenes, which may lead to up-regulated levels of anti-apoptotic proteins or decreased levels of pro-apoptotic proteins.

2.2. Molecular mechanisms of apoptosis

Various signals (drug treatment, withdrawal of trophic factors, virus infection, ionizing radiation, etc.) can induce apoptosis. The initiation of the apoptotic process differs among stimuli, but the signals appear to converge on a common execution pathway. When a cell receives an apoptotic stimulus, a cascade of proteolytic cleavages that mediate biochemical and morphological changes is initiated. Since the ‘death gene’ ced-3 in C. elegans was found to encode a protein homologous to the mammalian protease interleukin-1β-converting enzyme (ICE, today also referred to as caspase-1) [Yuan et al., 1993], a family of related proteases has been described. This family, termed the caspases (for cysteine aspartate-specific proteases), has been shown to play a central role in initiation and execution of apoptosis [Alnemri et al., 1996; Samali et al., 1999b].

The caspase family

The importance of caspases for the apoptotic process was documented by several findings: a) overexpression of caspases efficiently kills cells; b) synthetic or natural inhibitors of caspases effectively inhibit apoptosis induced by diverse stimuli; and c) knock-out animals lacking certain caspases show profound defects in apoptosis [Ekert et al., 1999; Chang and Yang, 2000; Zheng and Flavell, 2000].
The caspase family of proteases consists of 14 known mammalian members that are constitutively expressed in most cell types as inactive pro-enzymes (zymogens) that become processed and activated in response to a variety of pro-apoptotic stimuli. The pro-caspases (32-56 kDa) contain four domains: an N-terminal pro-domain, a large subunit (17-21 kDa), a small subunit (10-13 kDa), and a short linker region between the large and the small subunit [Earnshaw et al., 1999]. Caspase activation involves proteolytic processing of the pro-enzyme at specific aspartate residues between the domains, resulting in removal of the pro-domain as well as the linker region and formation of a heterodimer containing one large and one small subunit [Liang and Fesik, 1997]. The active caspase is a tetramer composed of two such heterodimers.

All caspases share two important characteristics. First, the caspases are cysteine proteases containing a conserved QACXG pentapeptide containing the active-site cysteine [Cohen, 1997]. Second, these enzymes have a unique strong preference for cleavage of the peptide bond C-terminal to aspartate residues. Despite the requirement of aspartate at the substrate P1 site, the caspases differ in their substrate specificity and can be divided into three subgroups based on the preferred tetrapeptide sequence (P4 to P1) after which the active enzymes cleave their respective substrates. The P4 site (four amino acids N-terminal to the cleavage site) is the most critical determinant of the substrate specificity [Talanian et al., 1997; Thornberry et al., 1997]. Caspases in group I (caspase-1, -4 and -5) cleave preferentially after the WEHD motif, group II (caspase-3 and -7) after DEXD, whereas the optimal cleavage sequences for caspases belonging to group III (caspase-6, -8, -9 and -10) are (I/L/V)EXD [Thornberry et al., 1997]. Caspase-2 was shown to preferentially cleave after the pentapeptide sequence VDVAD [Talanian et al., 1997]. The cleavage sites for caspase-11, -12, -13, and -14 are not yet clear.

Even though caspase-independent apoptosis exists (see p. 29), most experimental models of apoptosis involve the activation of caspases. Until now, no activation of caspases has been found during necrotic cell death [Armstrong et al., 1997; Dong et al., 1997]. However, inactivation of caspases might switch the death process from apoptosis to necrosis.

**Activation of the caspase cascade**

Evidence for the sequential activation of caspases has led to the concept of a caspase cascade [Grossmann et al., 1998; Hirata et al., 1998]. This cascade begins with autocatalytic activation of initiator caspases that, in turn, transmit signals by cleaving and thereby activating the downstream effector caspases
Mechanisms of apoptosis induced by a protein complex isolated from human milk

[Salvesen & Dixit, 1999]. The initiator caspases (pro-caspase-2, -8, -9, -10 and 12?), as well as the so-called inflammatory caspases involved in the activation of cytokines (pro-caspase-1, -4, -5, -11 and -13) (which are, with some exceptions, not involved in apoptosis) contain long pro-domains of up to over 100 amino acids, while the pro-domains of effector caspases (pro-caspase-3, -6 and -7) are short and usually less than 20 amino acids [Earnshaw et al., 1999]. The long pro-domains contain distinct motifs, including death effector domains (DEDs) present in pro-caspase-8 and -10 and CARDs found in pro-caspase-1, -2, -4, -5 and -9, which are important for the activation of these enzymes. There are two main intracellular pathways by which the caspase cascade can be activated; one depends on ligation of death receptors, while the other depends on the release of apoptogenic factors from mitochondria (Fig. 3).

Death receptor-mediated apoptosis

Death receptors (e.g. TNF-R1 and 2, CD95/Fas/Apo-1, NGF-R, and TRAIL-R1, 2, 3 and 4) belong to the tumour necrosis factor (TNF) receptor superfamily and are expressed on the cell surface of many cell types. These receptors all contain a cytoplasmic sequence termed the ‘death domain’ (DD). Upon ligation of specific ligands, these receptors transmit a signal that results in apoptotic cell death. The most intensively studied and best-characterised death receptor is the CD95/Fas/Apo-1 (CD95) receptor. Upon binding of the CD95/Fas/Apo-1 ligand (CD95L), CD95 receptors aggregate resulting in the assembly of the so-called death-inducing signalling complex (DISC) (Fig. 3). The adapter molecule FADD (Fas-associating death domain-containing protein) recruits pro-caspase-8, via interaction through DEDs, to the intracellular DD of the CD95 receptor. Several molecules of pro-caspase-8 are recruited to the DISC resulting in a high local concentration of the zymogen. The induced proximity model postulates that the low intrinsic protease activity is sufficient to allow the pro-enzyme molecules to mutually cleave and activate each other [Salvesen & Dixit, 1999]. Active caspase-8 then cleaves and thereby activates pro-caspase-3, giving rise to a proteolytic cascade. In addition, caspase-8 cleaves the cytosolic pro-apoptotic Bcl-2 family protein Bid, and its truncated form (tBid) translocates to mitochondria and induces release of cytochrome c from this organelle [Li et al., 1998; Lou et al., 1998; Gross et al., 1999a]. The Bcl-2 family of proteins are further discussed on p. 30.
Figure 3. The two major pathways for caspase activation. The caspase cascade can be activated via ligation of the death receptors TNF-R1 or CD-95 and via mitochondrial release of cytochrome c resulting in formation of the apoptosome complex.

Role of mitochondria in apoptosis
The role of mitochondria in apoptosis was originally suggested by Newmeyer et al. [Newmeyer et al., 1994]. Using a cell-free system of Xenopus egg extracts the authors provided evidence for the requirement of mitochondria for nuclear condensation and DNA fragmentation. During the last years, a number of publications have shown that several apoptogenic proteins, which normally reside within the mitochondrial intermembrane space, are released during the apoptotic process. These proteins include cytochrome c, AIF (apoptosis-inducing factor), adenylate kinase-2 (AK-2), HSPs (heat shock proteins), DIABLO/Smac (direct IAP binding protein with low pI/Second mitochondria-
Mechanisms of apoptosis induced by a protein complex isolated from human milk

derived activator of caspase), sulfite oxidase, HtrA2 (high temperature requirement A2)/Omi, endo G (endonuclease G) and several pro-caspases [Mancini et al., 1998; Köhler et al. 1999; Samali et al., 1999a; Susin et al., 1999b; Zhivotovsky et al., 1999; Kluck et al., 1999; Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001; Li et al., 2001]. The best characterised of these proteins is cytochrome c, the release of which today is regarded to be an almost universal feature of apoptotic cell death. The normal function of cytochrome c is to transport electrons between the complexes III and IV in the mitochondrial respiratory chain, thus participating in oxidative phosphorylation. In the cytosol, cytochrome c binds to the apoptotic protease-activating factor-1 (Apaf-1). Apaf-1 is a cytosolic protein containing a CARD, a CED-4-like domain, and a WD-40 repeat domain. Binding of cytochrome c and hydrolysis of dATP (or ATP) induces a conformational change that allows Apaf-1 to oligomerise via its CED-4-like domain into a multimeric complex, the so-called apoptosome (Fig. 4) [Li et al., 1997; Zou et al., 1999]. Simultaneously, pro-caspase-9 is recruited to the exposed CARD of Apaf-1. This subsequently leads to autocatalytic activation of pro-caspase-9. Caspase-3 is consequently recruited to the apoptosome through an interaction with caspase-9 [Bratton et al., 2001]. In this scenario, again the activated initiator caspase (caspase-9), in a process similar to the one initiated by caspase-8, starts the proteolytic cascade by cleaving the downstream located effector caspases. The activation of caspases by the apoptosome complex is also regulated by HSPs and IAPs (inhibitor of apoptosis proteins) [Beere et al., 2000; Bratton et al., 2001].

In addition to the involvement of cytochrome c in activation of the caspase cascade, the proteins released from mitochondria fulfil different functions during the execution of apoptosis. Smac/DIABLO was found to promote caspase activation by binding and inhibiting the activity of IAPs [Du et al., 2000; Verhagen et al., 2000]. More recently, the serine protease HtrA2/Omi was also reported to promote apoptosis, in part, by inhibiting IAPs in a Smac-DIABLO-like manner [Suzuki et al., 2001]. The function of the mitochondrial pro-caspases-2, -3 and -9 are not understood in detail, but they may be processed and activated within the mitochondria [Samali et al., 1999a]. HSP60 and HSP10 form a complex with pro-caspase-3 inside the mitochondria and upon apoptosis induction mitochondrial caspase-3 was activated and dissociated from these HSP proteins. Moreover, recombinant HSP60 and HSP10 accelerated the activation of pro-caspase-3 in vitro, suggesting that the mitochondrial HSPs may accelerate caspase activation in the cytoplasm of intact cells. AIF and endo G represent caspase-independent pathways of
apoptosis initiated from the mitochondria. Upon their release, both AIF and endo G translocate to the nucleus. AIF causes chromatin condensation and HMW DNA fragmentation, while endo G cleaves the DNA into LMW fragments [Zamzami et al., 1996a; Daugas et al., 2000; Li et al., 2001].

Although the function of many intermembrane space proteins released during apoptosis is well described, the role of others (i.e. AK-2 and sulfite oxidase) is less clear and requires additional investigation.

**Figure 4. Caspase activation by the apoptosome complex.** Released cytochrom c mediates, in the presence of dATP, a conformational change of Apaf-1 and formation of the apoptosome complex. This leads to activation of pro-caspase-9 and pro-caspase-3.
Mechanisms for cytochrome c release

The mechanism by which cytochrome c manages to cross the mitochondrial outer membrane during apoptosis is not fully understood, but two main hypotheses have been proposed (Fig. 5). First, it may be released via specific channels in the outer mitochondrial membrane that are regulated or formed by pro-apoptotic Bcl-2 family members (such as Bid and Bax) (p. 30). Alternatively, cytochrome c may be released as a consequence of outer membrane rupture caused by swelling subsequent to opening of the so-called mitochondrial permeability transition (MPT) pore [Skulachev, 1996; Scarlett & Murphy, 1997]. This pore is a polyprotein complex formed at the contact sites between the inner and the outer mitochondrial membranes. Opening of the MPT pore renders the mitochondrial inner membrane non-selectively permeable to small molecules (<1.5 kDa). As a consequence, water and solutes enter the mitochondrial matrix via osmosis resulting in loss of the mitochondrial transmembrane potential (ΔΨm). Since the inner membrane with its cristae is considerably larger than the surrounding outer membrane, this swelling of mitochondria ultimately results in rupture of the outer membrane and an unspecific release of intermembrane space proteins. This process is normally defined as MPT.

Hunter & Haworth were the first to describe MPT as a “Ca²⁺-induced membrane transition” in a model of isolated mitochondria [Hunter & Haworth, 1979]. They reported that Ca²⁺ could trigger a sudden increase in permeability of the inner mitochondrial membrane. Although, the Ca²⁺-dependent MPT pore remains poorly characterized it appears to consist of several proteins located both in the inner and outer mitochondrial membranes. These include: the adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC), and cyclophilin D. Other proteins suggested to be included in the pore complex are hexokinase, peripheral benzodiazepine receptor, and creatine kinase. VDAC forms large pores in the outer mitochondrial membrane through which low molecular weight solutes freely can pass. ANT is an ADP-ATP antiporter located in the inner membrane; it translocates ADP into the mitochondrial matrix and exports ATP to the cytosol. Both VDAC and ANT are the predominant proteins at mitochondrial contact sites between the inner and outer membrane [Brdiczka, 1991]. Cyclophilin D is a matrix protein that interacts with ANT and has the ability to catalyse cis-trans-isomerisation of specific peptide bonds thereby mediating protein conformational changes. During apoptosis ANT, VDAC, and cyclophilin D are believed to physically interact to form the MPT pore.
The molecular mechanisms of MPT pore opening remains elusive, but it may involve a conformational change of ANT into an open-pore state, the so-called c-state ("cytosolic-state"). Binding of cyclophilin D or oxidation of vicinal dithiols may cause such change in conformation of ANT [Crompton, 2000a]. The well-characterized MPT inhibitors, cyclosporin A (CsA) and Bongkrekic acid (BA), prevent MPT by inhibiting the interaction of cyclophilin D with ANT and stabilisation of ANT in the m-state ("mitochondrial"-state), respectively [Woodfield et al., 1998; Vieira et al., 2000]. Moreover, atracyloside, an agent inducing MPT via interaction with ANT, favours the c-state of the protein. Although the Ca$^{2+}$ ion itself is an inducer of MPT, several other factors facilitate pore opening, including oxidative stress, $P_i$, decreased $\Delta\Psi_m$, depletion of adenine nucleotide, and oxidized pyridine nucleotides [Gogvadze et al., 1996; Halestrap et al., 1997]. In addition, agents that interact with ANT (e.g. the HIV-1 protein Vpr, Bax) or VDAC (e.g. Bax) have been reported to stimulate MPT [Ferri et al., 2000; Brenner et al., 2000; Shimizu et al., 2000].

Figure 5. Cytochrome c release mechanisms.
MPT was first proposed to play a major role in necrosis and later also in apoptosis [Crompton & Costi, 1988; Vayssière et al., 1994; Zamzami et al., 1996b; Crompton, 1999]. In many models of apoptosis, CsA and BA, prevent the dissipation of ∆Ψm, release of cytochrome c, and concomitantly inhibit/delay apoptosis [Marzo et al., 1998a; Pritchard et al., 2000; Feldmann et al., 2000]. Other studies have shown that cytochrome c release may occur in a CsA-insensitive manner and in the absence of decreased ∆Ψm, suggesting that the intermembrane space protein was not released as a consequence of MPT in these apoptosis models [Bossy-Wetzel et al., 1998; Goldstein et al., 2000]. Moreover, cytochrome c release and caspase activation have been reported to occur prior to CsA-sensitive drop of ∆Ψm, implying that opening of MPT pores also can be a downstream event to caspase activation. The caspases themselves can induce MPT pore opening thereby creating self-amplifying feedback loops [Susin et al., 1997; Marzo et al., 1998b].

Other pathways for activation of caspases and other proteases

The pro-caspases are found in multiple intracellular compartments, although the majority of them are localised in the cytosol. Pro-caspase-2 is present in the nucleus and in the Golgi complex, caspase-12 in the endoplasmatic reticulum (ER), and a low amount of pro-caspase-2, -3 and -9 are present in mitochondria [Mancini et al., 1998; Samali et al., 1998; Colussi et al., 1998; Zhivotovsky et al., 1999; Mancini et al., 2000; Yoneda et al., 2001]. These differences in pro-caspase localisation may reflect organelle-specific caspase activation. An example is caspase-12 that specifically is activated in apoptosis induced by ER stress (e.g. disruption of ER Ca2+ homeostasis, accumulation of excess or malfolded proteins in ER) [Nakagawa et al., 2000; Bitko & Barik, 2001]. It was shown by Yoneda et al. that the TNF receptor-associated factor-2 (TRAF-2) was involved in activation of pro-caspase-12 [Yoneda et al., 2001]. The ER stress sensor, IRE1α, may initiate apoptosis by forming a complex with TRAF-2, which in non-apoptotic cells is bound to pro-caspase-12. This results in dissociation of pro-caspase-12 from TRAF-2 and the processing and activation of the caspase-12 is promoted. The authors speculate that TRAF-2 may act as an adaptor molecule in the ER, recruiting the pro-caspase and thereby facilitating activation of the pro-enzyme. Since pro-caspase-12 is a mouse protein and absent in human cells, it is still unclear how general this particular pathway is.

There is no doubt about the importance of intracellular proteases for apoptosis; however, caspases are not the only proteases activated during the execution phase of apoptosis. Non-caspase protease activities implicated in apoptosis include that of the calpains, granzymes, and the cathepsins. The
calpains belong to a family of neutral cysteine proteases. These proteases, including the isoforms µ-calpain and m-calpain, are commonly activated during apoptosis. The main regulators of calpains are Ca²⁺ and calpastatin. The intracellular Ca²⁺ concentration is elevated in many models of apoptosis and this may lead to increased activity of the calpains since binding of Ca²⁺ is required for maximal enzymatic activity [Saido et al., 1994; Kass & Orrenius, 1999]. In contrast, the endogenous calpain inhibitor, calpastatin, blocks the proteolytic activity of the calpains. However, it has been reported that this inhibitor is cleaved during apoptosis by activated caspases thereby resulting in increased calpain activity [Pörn-Ares et al., 1998; Wang et al., 1998]. The substrates cleaved by calpains during apoptosis include actin, fodrin and gelsolin [Johnson, 2000]. Moreover, it has been shown that activated calpains also cleave pro-caspase-3 and -9 indicating that the calpains may play a role in the activation and/or amplification of the caspase cascade [Wolf et al., 1999b]. In addition, it was recently shown that calpain can cleave Bid, which results in activation of the mitochondrial-mediated pathway [Chen et al., 2001].

A process called the ‘granule exocytosis’ is involved in the host defence mechanisms when cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells kill bacterial or viral infected cells. CTLs and NK cells contain cytoplasmic granules, which among other factors retain granzyme B and perforin. These granules are exocytosed upon target cell apoptosis. The released perforin oligomerizes and forms pores, making the plasma membrane of the target cell permeable to granzyme B [Trapani & Smyth, 1993]. This serine protease shares with caspases the ability to cleave proteins after aspartic acid residues. During granzyme B-induced apoptosis the caspases are activated. Since granzyme B cleaves caspases in vitro, it most likely induces apoptosis by cleaving and activating the caspases in the target cell [Johnson, 2000]. Granzyme B may also activate the mitochondrial pathway, since it can cleave Bid [Sutton et al., 2000; Heibein et al., 2000].

Cathepsin B (a cysteine protease like the caspases and calpains) and cathepsin D (an aspartate protease) are processed and activated during apoptosis. These cathepsins are normally localised in lysosomes and endosomes, but during apoptosis they are likely translocated to the cytoplasm. During TNF-α-mediated apoptosis in hepatocytes, active cathepsin B was reported to accumulate in the cytosol and enhance the mitochondrial release of cytochrome c and the activation of caspases [Guicciardi et al., 2000]. Cathepsin B might affect mitochondria by proteolysis of Bid since it was recently reported that Bid was cleaved in the presence of lysosomal extracts [Stoka et
al., 2001]. It is still unclear under what circumstances the release of cathepsins is important for the initiation/execution the apoptotic process.

**Cleavage of caspase substrates**

The subcellular localization of the pro-enzyme is often different from the site of action of the activated caspase [Zhivotovsky et al., 1999; Susin et al., 1999a; Porter, 1999]. Upon activation, several of the caspases are translocated to other cellular compartments, such as the ER and nucleus, where they cleave specific target proteins. Currently, more than a hundred caspase substrates have been identified and the list continues to grow [Stennicke & Salvesen, 2000; Fadeel et al., 2000]. The cleavage can result in inactivation (in most cases) or activation of the protein substrate. Among the proteins that are inactivated by cleavage are cytoskeletal proteins, including lamins, α-fodrin, and actin, and proteins involved in DNA repair and cell cycle regulation, such as polyADP-ribose polymerase (PARP) and retinoblastoma protein (Rb), respectively [for details see Chang & Yang, 2000]. Proteins can be activated by caspase-mediated cleavage either directly by removing a negative regulatory domain or indirectly by inactivating a regulatory subunit. In addition to the pro-caspases themselves, caspase substrates that are activated upon cleavage of an inhibitory domain include protein kinase Cδ (PKCδ) and the transcription factors sterol regulatory element binding proteins (SREBPs) [Emoto et al., 1995; Wang et al., 1996]. The caspase-activated DNase (CAD) is indirectly activated by cleavage of an inhibitory subunit, ICAD (inhibitor of CAD) [Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998]. This caspase-mediated cleavage results in release and activation of the catalytic subunit of CAD.

Caspase-3 is activated during most apoptotic processes and is believed to be the main executioner caspase. Its activation has been shown to be essential not only for DNA fragmentation by ICAD cleavage but also for condensation of the chromatin and plasma membrane blebbing [Sahara et al., 1999; Wolf et al., 1999a; Sebbagh et al., 2001]. Chromatin condensation is induced by cleavage of the nuclear protein acinus whereas the cleavage of the Rho-associated kinase, ROCK-1, mediates plasma membrane blebbing. The truncated ROCK-1 possesses a constitutive kinase activity and phosphorylates myosin light chains (MLCs), which in turn leads to the blebbing of the plasma membrane [Sebbagh et al., 2001; Mills et al., 1998]. The loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as actin, fodrin and gelsolin. Caspase-6 activity has also been linked to the typical changes of morphology in apoptotic cells. Cleavage of the nuclear lamins by caspase-6 is required for nuclear shrinkage and budding.
Caspase-independent pathways of apoptosis
The decision between life and death may lie upstream or be independent of caspase activation, since inhibition of caspases does not always prevent cell death [Xiang et al., 1996; De Maria et al., 1997; Adachi et al., 1998; Benson et al., 1998; Luschen et al., 2000]. Mitochondrial changes, such as dissipation of $\Delta \Psi_m$ and release of apoptogenic factors, lie upstream of caspase activation in the apoptotic process induced by many stimuli. Damage to mitochondria may cause ROS production and decreased ATP formation. If the mitochondrial damage is too severe or if the apoptotic program cannot be fulfilled (i.e., when caspase inhibitors are present) the cell may die by necrosis.

Apoptosis has been suggested to be defined as a process that requires the activation of caspases [Samali et al., 1999b]. However, during the last years, caspase-independent apoptosis has been described [Lavoie et al., 1998; Mathiasen et al., 1999; Nylandsted et al., 2000]. In the absence of activated caspases, other factors may mediate the cell death. As mentioned above, several proteins released from mitochondria in dying cells have caspase-independent functions. When released, AIF and endo G are translocated to the nucleus and cause chromatin condensation and DNA fragmentation [Zamzami et al., 1996a; Daugas et al., 2000; Li et al., 2001]. Moreover, the caspases are not the only proteases activated during the execution phase of apoptosis when specific substrates are proteolytically cleaved. Among others are the Ca$^{2+}$ activated calpains, cathepsins, the nuclear scaffold protease, and serine proteases [Zhivotovsky et al., 1997a; Zhivotovsky et al., 1997b; Wright et al., 1997].

Caspase-dependent apoptosis usually includes all morphological hallmarks of apoptosis (e.g. cell shrinkage, plasma membrane blebbing, chromatin condensation and formation of apoptotic bodies), while caspase-independent apoptosis often display less compact condensed chromatin and varying degree/comination of the other apoptotic features [Leist & Jäättelä, 2001]. Thus, it has been debated whether or not caspase-independent apoptosis is a correct term or if this type of cell death should be renamed since not all apoptotic characteristics are present.

Regulation of apoptosis
The apoptotic pathways require a high degree of regulation since errors may lead to extensive killing of healthy cells or an accumulation of damaged or aged cells in the tissue. Several families of proteins are involved in the regulation of apoptosis, such as inhibitors of IAPs, HSPs and the Bcl-2 family
of proteins [Deveraux & Reed, 1999, Garrido et al., 2001; Antonsson & Martinou, 2000].

The Bcl-2 family
One of the first genes shown to regulate apoptosis was Bcl-2. It was identified as a proto-oncogene involved in follicular B-cell lymphoma [Tsujimoto et al., 1985]. The Bcl-2 family consists of more than 25 mammalian proteins, where 10 have anti-apoptotic effects and the rest have pro-apoptotic effects [Gross et al., 1999b]. The Bcl-2 family proteins can be divided into three groups. Group I (e.g. Bcl-2 and Bcl-Xl) entails anti-apoptotic proteins that are characterized by four short conserved Bcl-2 homology (BH) domains (BH1-BH4) and a C-terminal transmembrane segment anchoring the protein into membranes [Nguyen et al., 1993]. In addition to the mitochondrial membrane, these proteins are present in the endoplasmic reticulum (ER) and in the nuclear envelope [Krajewski et al., 1993; Akao et al., 1994]. Group II (e.g. Bax, Bak) contains the hydrophobic tail and the BH1-BH3 domains, but not the BH-4 domain, and Group III (e.g. Bid, Bad) consists of proteins that show homology only with the BH-3 domain (the “BH3-only” proteins). The members of group II and III are pro-apoptotic.

The relative expression level of the anti- and pro-apoptotic Bcl-2 family members is an important death-survival determinant in cells exposed to a variety of apoptosis-inducing stimuli [Vander Heiden & Thompson, 1999; Tsujimoto & Shimizu, 2000a]. The pro- and anti-apoptotic Bcl-2 family members can form heterodimers via the BH3 domain. For example, Bcl-Xl forms heterodimers with the Bcl-2 family members Bax and Bak; it also inhibits the pro-apoptotic activities of these proteins by a mechanism that is yet unclear [Chittenden et al., 1995; Sedlak et al., 1995]. However, other studies suggest that the heterodimerization of Bcl-Xl with Bax or Bak per se is not required for the anti-apoptotic activity of Bcl-Xl [Cheng et al., 1996; Knudson & Korsmeyer, 1997].

The key function of Bcl-2 family members seems to be to regulate the release of pro-apoptotic factors (in particular cytochrome c) from the mitochondrial intermembrane compartment. Bid and Bax, which are normally cytosolic, localise to mitochondria in cells undergoing apoptosis. As mentioned earlier, Bid is cleaved into tBid by caspase-8 in CD95-mediated apoptosis [Li et al., 1998; Lou et al., 1998; Gross et al., 1999a]. tBid translocates to mitochondria and induces, alone or in combination with Bax, cytochrome c release [Zamzami et al., 2000; Eskes et al., 2000; Ruffolo et al., 2000; Crompton, 2000b]. In other models of apoptosis, Bax has been shown to translocate to
mitochondria and cause the release of cytochrome c [Wolter et al., 1997]. In contrast, this release is inhibited by Bcl-2 and Bcl-Xi [Yang et al., 1997; Jurgensmeier et al., 1998; Finucane et al., 1999; Gross et al., 1999a; Gross et al., 1999b]. When overexpressed in cells, Bcl-2 and Bcl-Xi inhibits cytochrome c release and thereby prevent caspase activation and cell death induced by agents acting via mitochondria [Hockenbery et al., 1990; Kharbanda et al., 1997; Kluck et al., 1997; Yang et al., 1997; Vander Heiden et al., 1997].

The mechanism of cytochrome c release induced by the pro-apoptotic Bcl-2 members is a subject of debate. Bcl-Xi was reported to have a 3D-structure similar to that of pore-forming domains of certain bacterial toxins, in particular the diphereria toxin, suggesting that Bcl-2 family proteins might form pores in membranes [Muchmore et al., 1996; Minn et al., 1997]. Indeed, Bcl-Xi, Bcl-2 as well as Bax can make channels in liposomes/planar lipid bilayers in vitro, but it is not known if these proteins can form pores inside the cell or whether cytochrome c could be released via such a pore in vivo [Schendel et al., 1998; Saito et al., 2000].

Rather than forming new pores, the Bcl-2 family members might instead stabilize or perturb a pre-existing channel. Bax, Bcl-2 and Bcl-Xi have been suggested to associate with the components of the MPT pore (discussed on p. 24-26), such as the voltage-dependent anion channel (VDAC) or the adenine nucleotide translocator (ANT) and thereby promote or inhibit MPT pore opening [Tsujimoto & Shimizu, 2000b; Vieira et al., 2000]. Moreover, Bax-induced release of cytochrome c from isolated mitochondria was prevented by the MPT pore inhibitor CsA [Narita et al., 1998; Jurgensmeier et al., 1998].

In addition, Bcl-2 and Bcl-Xi may inhibit apoptosis downstream of mitochondria in the cell death pathway although the precise mechanism of this phenomenon is unclear [Zhivotovsky et al, 1998; Rosse et al., 1998].

The IAP family

The inhibitor of apoptosis proteins (IAPs) are a family consisting of several known mammalian members, including neuronal IAP (NIAP), X-linked IAP (XIAP), cellular IAP-1 (c-IAP-1), cellular IAP-2 (c-IAP-2), and survivin [Deveraux & Reed, 1999]. Human XIAP and c-IAP-1 and -2 were found to inhibit the activity of caspase-3 and -7 [Deveraux et al, 1997; Roy et al., 1997]. The exact mechanism of apoptosis inhibition by IAPs remains to be determined. However, the level and activity of IAPs can be down-regulated during the apoptotic process either by proteasomes or by interaction with Smac/DIABLO or HtrA2/Omi, proteins released from mitochondria [Yang et al., 2000; Du et al, 2000; Verhagen et al., 2000; Suzuki et al., 2001].
Recruitment of pro-caspase-9 into the apoptosome complex and its activation are also controlled by IAPs although the precise mechanism of this observation is unclear [Bratton et al., 2001].

*The HSP family*

Several publications have suggested that heat-shock proteins (HSPs) play an important role in regulating apoptosis [Xanthoudakis & Nicholson, 2000; Creagh et al., 2000]. Stress, such as heat-shock, induces the expression of the majority of the HSP family members. In addition, many tumour cells have elevated levels of HSPs that may contribute to the increased resistance to apoptosis induction of these cells. The HSP proteins seem to exert both pro- and anti-apoptotic functions. For example, binding of HSP60/HSP10 to pro-caspase-3 stimulates activation of the pro-enzyme, while HSP70 binding to Apaf-1 inhibits pro-caspase-9 recruitment to the apoptosome and thereby its activation [Samali et al., 1999a; Xanthoudakis et al., 1999; Beere et al., 2000; Saleh et al., 2000]. HSP27 might also inhibit the apoptotic process by binding to cytochrome c upon its release from mitochondria, thus preventing its interaction with Apaf-1 [Bruey et al., 2000].

*Other mechanisms of regulation*

The activation of caspases can also be regulated via anti-apoptotic DED-domain containing proteins such as FLIP (FLICE-inhibitory protein), BAR (bifunctional apoptosis regulator), and Bap31. FLIP inhibits death receptor-mediated apoptosis by competing with the adaptor protein FADD for binding to pro-caspase-8 [Irmler et al., 1997]. Thus, the recruitment of pro-caspase-8 to the CD95 receptor and its subsequent activation are prevented, and apoptosis is inhibited. BAR and Bap31 also bind to the DED-containing pro-caspases-8 and inhibits apoptosis in a similar manner as FLIP [Zhang et al., 2000; Ng et al., 1997]. In addition, BAR and Bap31 can interact with Bcl-2, although the function of this binding is unclear.

The protein Aven regulates caspase activation during apoptosis induced via the mitochondrial pathway. It binds to Apaf-1 and interferes with the apoptosome formation by preventing oligorimerization of Apaf-1. This results in an impairment of apoptosome-mediated activation of pro-caspase-9 [Chau et al., 2000]. Aven also interacts with Bcl-2/Bcl-Xl, and it has been suggested that Aven in part may mediate the anti-apoptotic function of Bcl-2/Bcl-Xl.

Thus, apoptosis is a complex multistage process of cell suicide and although tremendous progress in understanding the mechanisms has been
achieved, it will require intense research efforts before the details are fully elucidated.
THE PRESENT STUDY

AIMS

The main goal of the present study was to characterize the mechanism(s) of apoptosis induction by a folding variant of human \( \alpha \)-lactalbumin.

More specifically, the aims were:

- to investigate the involvement of caspases in apoptosis induced by the protein complex isolated from human milk.

- to elucidate the pathway of caspase activation (death receptor versus mitochondria) upon MAL/HAMLET treatment.

- to investigate the release of mitochondrial proteins during apoptosis.

- to analyse, in detail, the involvement of mitochondria in apoptosis induced by HAMLET.

- to elucidate the role of Bcl-2 protein in HAMLET-induced apoptosis.
The present study: Aims and Methodology

**METHODOLOGY**

Most of the techniques used in the present study have been described in detail in papers I-IV and in the references therein. Here, the methods are listed, briefly described and commented on.

**Preparation of MAL/HAMLET**

MAL and HAMLET both contain the folding variant of human α-lactalbumin; MAL is a fraction of human milk, while HAMLET is native α-lactalbumin converted into the molten globule-like state. MAL was used in the experiments described in papers I, II, and III and HAMLET in papers III and IV. No differences between MAL and HAMLET in their capacity to induce apoptosis was observed.

For the preparation of MAL and HAMLET, human milk was thawed, centrifuged to remove fat, and separated into casein and whey by acid precipitation [Mellander, 1947].

**MAL**

The casein fraction was lyophilised, dissolved in 10 mM Tris-HCl, pH 8.5, and subjected to ion-exchange chromatography over a column packed with DEAE-Trisacryl as described [Håkansson et al., 1995]. The fraction with apoptosis-inducing activity eluted after 1 M NaCl and was desalted by dialysis (membrane cutoff 3.5 kDa) and lyophilised. Prior to experiment, it was dissolved in RPMI medium or PBS at 10 mg/ml.

**HAMLET**

Native α-lactalbumin was purified from human milk whey by ammonium sulphate precipitation followed by phenyl Sepharose chromatography and size exclusion chromatography on a Sephadex G-50 column [Lindahl & Vogel, 1984]. The purity of the protein was controlled by SDS-PAGE and by spectroscopic techniques [Svensson et al., 2000]. To convert the native α-lactalbumin to HAMLET, oleic acid (C18:1) was dissolved in ethanol by sonication, added to 10 mM Tris-HCl, pH 8.5, and applied to a column with packed DEAE-Trisacryl matrix. Apo α-lactalbumin (generated by adding 3.5 mM EDTA to remove bound Ca²⁺) was then subjected to ion exchange chromatography on the preconditioned column as described [Svensson et al., 1999; Svensson et al., 2000]. The HAMLET-eluate (after 1 M NaCl) was then handled as described above for MAL.
Cell culture
The studies presented in this thesis, papers I-IV, are based on the utilisation of the following cell lines: Jurkat (human T cell-derived leukaemia), A549 (human lung carcinoma), and HRTEC (primary culture of human renal tubular epithelial cells). These cell types displayed differences in sensitivity to MAL/HAMLET-induced apoptosis; Jurkat cells were the most sensitive, A549 less sensitive and HRTEC cells were resistant to treatment. Jurkat cells stably transfected with Bcl-2 and a vector control were kindly provided by S. Korsmeyer (Howard Hughes Medical Institute, Harvard Medical School, Boston, USA). The cell lines were maintained in culture according to standard protocols.

Procedure for apoptosis induction and viability assay
Prior to experiment, cells were washed in PBS and resuspended in serum-free medium because the presence of serum inhibits apoptosis induced by MAL/HAMLET. Serum was added back to the cells 30 min (Jurkat cells) or 90 min (A549 cells) after the addition of MAL/HAMLET.

The percentage of viable cells was determined by the trypan blue exclusion assay.

Cellular fractionation
To investigate cytochrome c release from mitochondria in Jurkat cells, papers I and II, cytosolic extracts were prepared using the hypotonic S-100 buffer for cell lysis. To study the release of cytochrome c at early time points when the cells were kept in serum-free media, paper IV, the cytosolic extracts were prepared from digitonin-lysed cells. In both cases, the lysed cells were centrifuged and the supernatant (cytosol) was subjected to Western blot analysis.

Moreover, mitochondrial fractions were isolated from Jurkat cells in order to investigate whether cytochrome c was released when MAL was added directly to isolated mitochondria (paper I), and to study the kinetics of mitochondrial targeting of HAMLET in these cells (paper IV). In paper III, where isolated rat liver mitochondria were used as the experimental model, livers were excised from male Wistar rats (150-200 g) and mitochondria were isolated by differential centrifugation in MSH (mannitol-sucrose-HEPES) buffer.

Western blot analysis
The Western blot technique was used to determine the processing of caspases
and their substrates (i.e. PARP, lamin B, and α-fodrin), to study the release of mitochondrial proteins and the mitochondrial localisation of HAMLET.

Cells or cellular fractions were mixed with conventional Laemmli’s loading buffer and loaded onto SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes. To reduce the non-specific binding, the membranes were blocked with dry milk or bovine serum albumin (BSA) dissolved in a high salt buffer. After washing, the membranes were probed with primary antibodies against proteins of interest. The binding to primary antibodies was detected by using secondary horseradish peroxidase (HRP)-conjugated antibodies, which, in turn, were visualized by enhanced chemoluminescence (ECL).

Caspase activity assay
The enzymatic activity of caspases was determined using the synthetic caspase substrates Ac-DEVD-amc and Ac-IETD-amc, which contain the tetrapeptide sequences mimicking the preferred cleavage sites for the group II and group III caspases, respectively. Upon cleavage of the substrate, the conjugated fluorochrome amc (7-amino-4-methylcoumarin) is liberated and emits green fluorescence at 450 nm (excitation, 360 nm). Briefly, lysed cells were diluted in caspase substrate buffer (containing 33.3 µM Ac-DEVD-amc or 13.6 µM Ac-IETD-amc) and the increased fluorescence intensity was analysed during 30 min at 37°C using a fluorometer. The intensity of the signal is proportional to the amount of cleaved substrate and is an indirect measure of caspase activity. The actual enzyme activity was calculated, using a standard curve, and expressed as pmoles/min per 10^6 cells.

Co-localisation studies using confocal fluorescence microscopy
In paper I, colocalisation of biotin-labelled MAL with MitoTracker Red was studied using a confocal fluorescence microscope. Briefly, the cells were incubated with biotinylated MAL for 3 h at 37°C. After washing in PBS, the cells were fixed in paraformaldehyde and permeabilised with 0.1% Triton X-100. The localisation of MAL was visualised by incubation with FITC-conjugated streptavidin. Finally, mitochondria were stained using 25 nM MitoTracker Red. After mounting on glass slides, the localisation of biotinylated MAL was compared with the localisation of mitochondria using confocal fluorescence microscopy.

DNA fragmentation
LMW DNA fragments were detected in paper I by agarose gel electrophoresis.
The cells were lysed and the supernatant obtained after centrifugation was extracted with phenol/chloroform. Precipitated oligonucleosome length DNA fragments were loaded on 1.8% agarose gels and electrophoresed at 60 mA. The DNA was stained with ethidium bromide and visualized under 305 nm illumination.

**Flow cytometry**

The change in intracellular Ca\(^{2+}\) concentration and in mitochondrial transmembrane potential were analysed by using Fluo-3-acetoxymethyl ester (Fluo-3-AM)- and tetramethylrhodamine ethyl ester (TMRE)-loaded cells, respectively. Fluo-3-AM and TMRE are both cell permeable fluorophores. Fluo-3 is a Ca\(^{2+}\)-sensitive probe that is non-fluorescent unless bound to Ca\(^{2+}\), while TMRE is a cationic dye accumulating in mitochondria as a function of \(\Delta \Psi_m\). Cells were incubated at 37°C with 25 nM TMRE for 20 min. Alternatively, the cells were co-stained with Fluo-3-AM and TMRE to simultaneously investigate the changes in Ca\(^{2+}\) and \(\Delta \Psi_m\); the cell were incubated with 1 \(\mu\)M Fluo-3-AM for 60 min at 37°C in the presence of 25 nM TMRE during the last 20 min of incubation. The cells were subjected to FACScan flow cytometry and the fluorescence intensity of the dyes was estimated. The green-fluorescent emission of Ca\(^{2+}\)-bound Fluo-3 was detected by FL-1 (excited at 525 nm) and the red-fluorescent emission of TMRE was detected by FL-3 (excited at 568 nm).

**Mitochondrial accumulation of Ca\(^{2+}\) and loss of \(\Delta \Psi_m\) in digitonin-permeabilised cells**

In addition to FACS analysis, changes in the \(\Delta \Psi_m\) were also investigated using the lipophilic cation tetraphenylphosphonium (TPP\(^+\)) that accumulates in mitochondria due to \(\Delta \Psi_m\). Treated cells were washed in PBS and resuspended in a KCl-based buffer (containing 0.2 \(\mu\)M TPP\(^+\) and succinate as a respiratory substrate). The cells were permeabilised with digitonin and the concentration of TPP\(^+\) in the incubation buffer (extra-mitochondrial) was determined using a TPP\(^+\)-sensitive electrode.

The amount of Ca\(^{2+}\) that mitochondria can accumulate before MPT is induced (the so-called Ca\(^{2+}\) capacity) reflects the stability of mitochondria. The cells were handled similarly as for the ‘TPP\(^+\)-method’ described above, but the cells were resuspended in buffer without TPP\(^+\). The ability of mitochondria in digitonin-permeabilised cells to accumulate Ca\(^{2+}\) was determined by sequential additions of this cation until the accumulated Ca\(^{2+}\) was released as a
consequence of MPT induction. Changes in the level of Ca\textsuperscript{2+} in the incubation buffer were monitored using a Ca\textsuperscript{2+}-sensitive electrode.

**Function of isolated mitochondria**
The mechanisms of MAL/HAMLET-induced release of intermembrane space proteins were analysed using mitochondria isolated from rat liver as a model system.

Mitochondrial swelling and loss of $\Delta\Psi_m$ were analysed in Ca\textsuperscript{2+}-loaded mitochondria. Swelling was estimated spectrophotometrically as a decrease in absorbance measured at 540 nm, while the TPP\textsuperscript{+}-sensitive electrode described above was used for determination of $\Delta\Psi_m$. The presence of released mitochondrial proteins (e.g. cytochrome $c$ and AK-2) in the incubation medium was determined by pelleting the mitochondria and subjecting the supernatant to Western blot analysis.

Mitochondrial respiration in isolated rat liver mitochondria was determined by measuring the rate of oxygen consumption using a Clarke-type oxygen electrode. The consumption of oxygen was initiated with 5 mM succinate as the respiratory substrate, and state 3 respiration was initiated by addition of ADP. These experiments were performed in the absence of Ca\textsuperscript{2+} loading to avoid MPT induction.

**Enucleation of A549 cells**
The adherent A549 cells ($0.4 \times 10^6$ cells) were grown overnight on coverslips to semiconfluence (about 80% confluence) in full growth medium. The cells were incubated in the presence of 10 $\mu$g/ml cytochalasin B, an inhibitor of actin polymerisation (Sigma, St. Louis, MO, USA) for 90 min at 37°C. The coverslips were then placed with the cells facing down in centrifuge tubes containing 10 ml of growth medium and 10 $\mu$g/ml cytochalasin B. After centrifugation in a pre-warmed SW28 rotor in a Beckman L8-60M ultracentrifuge at 9,000 rpm for 30 min at 37°C, the coverslips were removed gently and placed in cell culture dishes. The coverslips were rinsed 2-3 times with PBS and incubated in full growth medium at 37°C. After 2 h of incubation, when the enucleated cells had returned to the flattened shape of normal cells, they were treated with HAMLET. The percentage of enucleated cells was 75-80%. The nuclei were stained with Giemsa (Sigma, Saint Louis, MO, USA) according to the manufacturer's instructions. The cytoplasts were investigated under a Nikon Eclipse E800 upright microscope with epi-fluorescence equipment (Nikon, Japan) and images were collected using a SPOT II camera (Diagnostic Instruments Inc., Michigan, MI, USA).
RESULTS AND DISCUSSION

Activation of caspases
The caspases are believed to be the central executioners of apoptosis. The work in paper I was designed to investigate the involvement of this family of proteases in MAL-induced apoptosis. The activation of group II (caspase-3 and -7) and to a lesser extent group III (caspase-6, -8, -9 and -10) caspases were detected in MAL-exposed Jurkat and A549 cells using synthetic substrates (Ac-DEVD-amc and Ac-IETD-amc, respectively). Caspase-3 is suggested to be the major executioner caspase known to cleave many of the target proteins in the apoptotic process. Indeed, caspase-3 was processed in MAL-treated cells as detected by Western blot using an antibody against the large subunit of active caspase-3. Caspase activation resulted in cleavage of specific caspase substrates, including PARP, and the cytoskeletal proteins lamin B and α-fodrin. Pro-caspase processing, caspase activity, and cleavage of substrates were inhibited by z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a pan-caspase inhibitor, showing that these events indeed were specifically mediated by caspases.

Alternative pathways for caspase activation in MAL/HAMLET-treated cells
Since MAL/HAMLET binds to the plasma membrane, is internalised, displays a punctate localization in the cytoplasm, and, subsequently, translocates and accumulates in the nucleus (see p. 13), it could potentially induce apoptosis via several mechanisms. MAL/HAMLET may (1) interfere with a receptor, such as CD95, in the plasma membrane and thereby cause activation of the caspase cascade. While being in the cytoplasm it may either (2) activate the caspases directly in the cytosol or (3) activate them indirectly by acting via any organelle in the cytoplasm, possibly the mitochondria. Finally, (4) MAL/HAMLET may be translocated to the nucleus and induce apoptosis via a signal from the nucleus to the cytoplasm. This has been shown in other models of apoptosis. For example, the nuclear transcription factors p53 and TR3/Nur77 can target mitochondria and act directly on this organelle to induce apoptosis [Marchenco et al., 2000; Li et al., 2000]. The following work was performed in order to elucidate the mechanism used by MAL/HAMLET to activate the caspase cascade.

MAL does not activate the caspases directly or via the CD95 receptor
It has been shown that apoptosis triggered by cytotoxic drugs, such as doxorubicin, etoposide, and cisplatin is associated with induction of CD95L
The present study: Results and Discussion

[Friesen et al., 1996; Fulda et al. 1998; Friesen et al., 1999]. The apoptotic process might then be mediated by cross-linking the CD95 receptor in an autocrine/paracrine manner. Inhibition of the ligand-receptor interaction by antagonistic antibodies to the receptor has been found to prevent drug-mediated apoptosis in cell systems [Fulda et al., 2000]. ZB4, an antagonistic anti-CD95 receptor antibody, prevented the CD95-mediated activation of caspase-3-like activity as well as the proteolysis of pro-caspase-3, PARP, lamin B, and α-fodrin in Jurkat cells (paper I). However, ZB4 did not block these events in MAL-treated Jurkat cells. The A549 cells, which are killed by MAL, do not express CD95 receptors on the cell surface due to the lack of translocation of expressed protein from the cytoplasm [Nambu et al., 1998]. Moreover, caspase-8 was not activated during MAL-induced apoptosis in either the A549 or in the Jurkat cells (data not shown). Thus, it is very likely that the CD95 receptor-mediated pathway is not involved in MAL-induced apoptosis.

Mechanisms of caspase activation can be studied in vitro. Incubation of protein extracts from non-apoptotic cells with cytochrome c and dATP/ATP leads to activation of pro-caspase-9, which, in turn, activates downstream caspases [Liu et al., 1996]. This approach was used to investigate whether MAL could activate pro-caspases directly in the cytosol. The experiments described in paper I showed that MAL was not able to activate the caspase cascade when incubated with cytosolic extract from non-apoptotic cells in vitro.

Involvement of mitochondria
MAL/HAMLET displays a granular staining in the cytoplasm that could imply that this protein complex co-localises with mitochondria. In order to investigate the involvement of mitochondria, cells were incubated with MAL conjugated with biotin, and the MAL localisation was visualised by FITC-conjugated streptavidin. Upon co-staining with MitoTracker Red, a co-localisation of biotinylated MAL and the mitochondrial-specific dye was observed suggesting that MAL was associated with mitochondria in treated cells (paper I).

The involvement of mitochondria in MAL/HAMLET-induced killing was confirmed by the release of cytochrome c. This release occurred rapidly in cells treated with MAL/HAMLET and was not blocked by zVAD-fmk, suggesting that this release occurs independently of caspase activation (papers I and IV).
Mechanisms of release of mitochondrial proteins

Release of AK-2
To investigate protein release from mitochondria during apoptosis, adenylate kinases (AKs) were used as marker proteins. Three isoforms of AK have been characterised; AK-1 is mainly present in the cytosol, while AK-2 and AK-3 are localised to the mitochondrial intermembrane space and matrix, respectively [Nobutomo et al., 1998]. Two questions were addressed in paper II: a) is cytochrome c the only protein released from mitochondria during apoptosis or is it part of a general release, and b) from which mitochondrial compartment(s) are the proteins released? Apoptosis was induced by several stimuli (i.e. agonistic anti-CD95 receptor antibodies, staurosporine, etoposide and MAL). We showed that AK-2, but not AK-3, translocated from mitochondria to cytosol during apoptosis induced by these triggers. AK-2 was released concomitantly with cytochrome c reflecting a general protein release, rather than a cytochrome c-specific release. Moreover, our data also suggest that only intermembrane space proteins were released. At the outset of the study included in paper II, cytochrome c and AIF were the only known factors to be released from mitochondria to the cytosol during apoptosis [Kroemer, 1997]. Since then, a number of reports have confirmed the release of mitochondrial constituents specifically from the intermembrane space during apoptosis (p. 21). The finding that the total AK activity in the cytosol increases during apoptosis further supported our observation [Single et al., 1998].

The AKs are ubiquitous enzymes involved in the metabolism of the adenine and guanine nucleotides. The phosphorylation of AMP to ADP is promoted by AKs, and the formed ADP is then further phosphorylated to ATP by glycolysis or through oxidative phosphorylation. The role of AK during apoptosis is not known, but the levels of ADP and ATP (or dATP) may influence apoptosis. Apoptosis is an active process that requires a sufficient amount of ATP; if the ATP level is too low the cells die via necrosis [Leist et al., 1997]. ATP can be converted to dATP via the enzyme ribonucleotide reductase. dATP is hydrolysed after binding to Apaf-1 during the formation of the apoptosome complex and thereby involved in the activation of the caspase cascade [Zou et al., 1999].

Cytochrome c release induced by MAL/HAMLET
The results in paper II suggested a general release of intermembrane space proteins during apoptosis, which may be a consequence of MPT. Moreover, the fact that MAL co-localised with mitochondria in treated cells suggested that MAL itself might act on mitochondria to cause the release of apoptogenic
factors. In **paper I**, we observed release of cytochrome $c$ when MAL was incubated with mitochondria isolated from Jurkat cells. In order to investigate the mechanism of cytochrome $c$ release, MAL/HAMLET was added directly to mitochondria isolated from rat liver. The data presented in **paper III** show that addition of MAL or HAMLET to Ca$^{2+}$-loaded mitochondria resulted in mitochondrial swelling, loss of $\Delta\Psi_m$, and release of intermembrane space proteins. The swelling and release of cytochrome $c$ were Ca$^{2+}$-dependent and completely blocked by MPT pore inhibitors, such as CsA, indicating that MAL/HAMLET-induced release of cytochrome $c$ was associated with MPT induction.

Interestingly, MAL/HAMLET caused a biphasic decrease of $\Delta\Psi_m$. The first rapid phase was due to mild uncoupling of mitochondria by the fatty acids in MAL/HAMLET and was prevented by BSA, which is known to sequester fatty acids. The second phase, involving a more pronounced drop of $\Delta\Psi_m$, was due to MPT induction. BSA failed to prevent MPT, but slightly delayed it, indicating that the fatty acids in MAL/HAMLET rather facilitated than were responsible for MPT induction (**paper III**). Moreover, in the absence of Ca$^{2+}$ loading, which is necessary for MPT induction, MAL/HAMLET was only able to induce a sub-maximal shift of $\Delta\Psi_m$ in isolated mitochondria. This uncoupling effect of MAL/HAMLET was confirmed by estimation of mitochondrial respiration. MAL/HAMLET increased the rate of state 4 respiration that was completely abolished by BSA.

It is important to note that the fatty acids at the concentrations in our experiments were not responsible for MPT induction but facilitated the event. This conclusion is in agreement with other studies showing that a partial uncoupling of mitochondria favours MPT induction, which may be due to a voltage-dependent nature of the MPT pore [Bernardi, 1992; Scorrano et al., 1997]. In addition, compared to classical protonophores such as CCCP or FCCP, fatty acids may further sensitise mitochondria towards MPT pore opening due to a direct interaction with ANT [Schonfeld & Bohnensack, 1997].

**Mitochondrial changes in Jurkat cells during apoptosis induced by MAL/HAMLET is a consequence of both MPT induction and an uncoupling effect**

In **paper IV**, we further investigated the involvement of mitochondria in HAMLET-induced apoptosis. Addition of HAMLET to Jurkat cells caused a rapid drop of the $\Delta\Psi_m$, assessed by flow cytometry of TMRE-loaded cells. This drop of $\Delta\Psi_m$ was not blocked with the MPT pore inhibitors CsA or BA. According to the results obtained in **paper III**, mitochondria in the presence of CsA or BA, might still be uncoupled by the fatty acid in HAMLET. In order to
elucidate the effect of HAMLET on $\Delta \Psi_m$ in Jurkat cells, the following experiment was performed. Cells were treated with HAMLET and washed in order to remove extracellular HAMLET before permeabilisation with digitonin. The $\Delta \Psi_m$ was then estimated using the TPP+-sensitive electrode. The HAMLET-induced drop of $\Delta \Psi_m$ was not prevented by CsA, BA or BSA alone. However, the MPT pore inhibitors, especially BA, in combination with BSA significantly restored $\Delta \Psi_m$. This is in line with the results of paper III where we show that MAL/HAMLET causes both MPT induction and uncoupling. In addition, the Ca$^{2+}$ accumulation capacity of mitochondria was rapidly abolished upon incubation with HAMLET. Since the accumulation of Ca$^{2+}$ is driven by the $\Delta \Psi_m$, the restoration of $\Delta \Psi_m$ by MPT inhibitors in combination with BSA markedly restored the mitochondrial capacity to accumulate Ca$^{2+}$.

As mentioned previously, the presence of Ca$^{2+}$ is both necessary and, at concentrations above a threshold, sufficient to induce MPT. Since the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) is elevated in MAL-treated cells [Håkansson et al., 1995], it might mediate the mitochondrial changes observed during MAL/HAMLET-induced apoptosis. A slight rise in [Ca$^{2+}$] occurs within minutes after MAL/HAMLET exposure of Jurkat cells as detected using Fluo-3-AM (Fig. 6). Although the increase of [Ca$^{2+}$] was prevented by the cell-permeable Ca$^{2+}$ chelators BAPTA-AM and EGTA-AM, no effect could be seen on either loss of $\Delta \Psi_m$, cytochrome c release, caspase activity, or viability (Fig. 6 and data not shown). This suggests that the increased [Ca$^{2+}$] does not contribute to the MPT induction in MAL/HAMLET-treated cells. Thus, the slight increase in Ca$^{2+}$ may reflect its release from mitochondria as a consequence of MPT.

Although all cells rapidly lost their $\Delta \Psi_m$ after treatment with HAMLET, a subset of the cells had regained a high $\Delta \Psi_m$ 2-3 h later. Since MPT is believed to be an irreversible event, the cells with mitochondria that managed to restore the $\Delta \Psi_m$ were most likely uncoupled by oleic acid. Indeed, when oleic acid was added to the cells, the initial loss of $\Delta \Psi_m$ was fully restored with time and the cells survived (data not shown). This time-dependent recovery was also true for the Ca$^{2+}$ accumulating capacity in both HAMLET and oleic acid-treated cells. In order to explain this transient effect of oleic acid on $\Delta \Psi_m$, one should take into consideration that fatty acids could be metabolised by mitochondrial $\beta$-oxidation or used for phospholipid synthesis. Therefore, the concentration of oleic acid can decrease with time and mitochondria can regain $\Delta \Psi_m$ and hence restore their Ca$^{2+}$ accumulating capacity. Thus, in our model oleic acid did not induce MPT; nevertheless, as was the case with isolated mitochondria, the fatty acid may facilitate MPT in
HAMLET-treated cells. It has been reported that CD95-induced apoptosis is enhanced by CCCP, which similarly as fatty acids can uncouple mitochondria [Linsinger et al., 1999].

![Figure 6](image)

**Figure 6.** The loss of $\Delta \Psi_m$ in MAL-treated cells was not mediated by $Ca^{2+}$. The $[Ca^{2+}]_i$ and $\Delta \Psi_m$ were determined by flow cytometry of Jurkat cells co-loaded with 1 $\mu$M Fluo-3-AM and 25 nM TMRE.

The release of cytochrome $c$ induced by MAL/HAMLET was blocked by BA, but not by CsA. This failure of CsA to block the release may be due to the presence of fatty acids. It has been reported that accumulation of free fatty acids in mitochondria is a factor that limits the inhibition of MPT by CsA [Broekemeier & Pfeiffer, 1995]. Phospholipase A2, the main mitochondrial phospholipase, is activated in some models of apoptosis [Cummings et al., 2000]. This activation results in hydrolysis of phospholipids and in the release of free fatty acids. In such models, CsA and a phospholipase A2 inhibitor need to be used in combination to block MPT [Imberti et al., 1992; Broekemeier & Pfeiffer, 1995]. It was reported that oleic acid interacts with ANT and inhibits its activity [Bell, 1980]. Since CsA and BA inhibit MPT by interacting with different mitochondrial targets (p. 25), one could suggest that despite binding to cyclophilin D, CsA cannot prevent interaction of the fatty acid with ANT, while BA that binds to ANT directly, might prevent this interaction and thereby prevent or delay MPT induced by HAMLET.
The presence of HAMLET in the mitochondrial fraction at the time when mitochondrial changes were observed further implies that HAMLET was the MPT-inducing agent in treated cells (paper IV).

**Role of Bcl-2**
Transformation of cells often involves a decreased vulnerability to undergo apoptotic cell death that, in part, may be due to upregulation of anti-apoptotic proteins. About 50% of all tumours have increased levels of the anti-apoptotic Bcl-2 family members, which are believed to fulfil their functions at the level of mitochondria [Adams & Cory, 1998]. Since MAL/HAMLET kills transformed cells via activation of the mitochondrial pathway, anti-apoptotic Bcl-2 family members might influence this process. The role of Bcl-2 in HAMLET-induced apoptosis was examined using Jurkat cells overexpressing Bcl-2. This overexpression was detected by Western blot showing that the Bcl-2 transfectants (Jurkat-Bcl-2) expressed a significantly higher amount of Bcl-2 as compared with control (Jurkat-Neo) cells (Fig. 7A).

**Apoptosis induced by HAMLET in Bcl-2 transfected Jurkat cells**
Etoposide, a topoisomerase II inhibitor, induces apoptosis, which can be inhibited by Bcl-2 [Kamesaki, 1993; Yang, 1997]. Therefore, we used this agent as a positive control in order to verify that the Bcl-2 protein was functional in preventing apoptosis. Figure 7D shows that overexpressed Bcl-2 markedly inhibited apoptosis induced by etoposide. No significant increase in caspase-3-like activity was detected in the etoposide-treated Jurkat-Bcl-2 cells, while a substantial increase in the activity was observed in the treated Jurkat-Neo cells (Fig. 7E). In contrast, HAMLET killed both Jurkat-Neo and Jurkat-Bcl-2 cells, although the Bcl-2 overexpressing cells were slightly less sensitive to HAMLET (Fig. 7B). Both the Jurkat-Neo and the Jurkat-Bcl-2 cells showed typical apoptotic morphology upon HAMLET exposure (i.e. cell shrinkage, plasma membrane blebbing, and cellular fragmentation) (data not shown). Caspases were activated in both Jurkat-Neo and Jurkat-Bcl-2 cells, although to a lesser extent in the Bcl-2 overexpressing cells (Fig. 7C). Thus, overexpression of Bcl-2 in Jurkat cells inhibited apoptosis induced by etoposide, while it had only a minor effect on HAMLET-induced cell death.

**MPT induction by HAMLET was not blocked by Bcl-2**
To evaluate the role of Bcl-2 at the mitochondrial level the \( \Delta \Psi_m \) was estimated in HAMLET-treated cells permeabilised with digitonin using a TPP⁺-sensitive electrode. After permeabilisation of untreated Jurkat-Neo cells, the level of
Figure 7. Apoptosis induced by HAMLET and etoposide in Bcl-2 transfected Jurkat cells.

A. Expression of Bcl-2 in Jurkat-Neo and Jurkat-Bcl-2 cells. Cellular extracts were prepared and the protein level of Bcl-2 was investigated by Western blot as described in paper I.

B. Cell viability of HAMLET-treated Jurkat-Neo (filled symbols) and Jurkat-Bcl-2 (open symbols) cells. Cells were exposed to HAMLET for 6 h and the viability was assessed by the trypan blue exclusion assay.

C. Caspase-3-like activity in Jurkat-Neo and Jurkat-Bcl-2 cells exposed to HAMLET. The cells were incubated with different concentrations of HAMLET for 2 h and assayed for the cleavage of the fluorogenic substrate Ac-DEVD-amc as described in paper I. The caspase activity is expressed in pmoles per min per 10^6 cells.

D. Cell viability of etoposide-treated Jurkat-Neo and Jurkat-Bcl-2 cells. Cells were incubated with etoposide for 24 h and viability was assessed by the trypan blue exclusion assay.

E. Caspase-3-like activity in Jurkat-Neo and Jurkat-Bcl-2 cells exposed to etoposide. The cells were incubated with etoposide for 6 h and assayed for the cleavage of Ac-DEVD-amc as in panel C.
Mechanisms of apoptosis induced by a protein complex isolated from human milk

A

Neo cells

Control

B

Neo cells

Etoposide 50 μM

C

Bcl-2 cells

HAMLET 0.1 mg/ml

D

Bcl-2 cells

HAMLET 0.3 mg/ml

TPP⁺ (μM)

0.1

0.2

0.3

0.4

3 min
Figure 8. HAMLET induces loss of $\Delta \Psi_m$ in Jurkat cells overexpressing Bcl-2. Jurkat-Neo or Jurkat-Bcl-2 cells (10^7 cells) were either untreated (A) or treated with 50 µM Etoposide (B) or 0.1 or 0.3 mg/ml HAMLET (C and D, respectively) for 5 min. Cells were washed in PBS and resuspended in a buffer containing 150 mM KCl, 5 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, 5 mM succinate, 0.2 µM tetrathenylphosphonium (TPP$^+$) and 5 mM Tris-HCl, pH 7.4. Cells were permeabilised with 0.005% digitonin (dig) and the $\Delta \Psi_m$ was estimated using the TPP$^+$-sensitive electrode (p. 37). After stabilisation of $\Delta \Psi_m$, 0.5% BSA was added in order to bind fatty acids present in HAMLET. Finally, 20 nmoles of Ca$^{2+}$ were added to investigate mitochondrial function.

TPP$^+$ in the incubation buffer decreases due to its accumulation by energised mitochondria. Addition of Ca$^{2+}$ to permeabilised cells induces a transient decrease in $\Delta \Psi_m$, due to an accumulation of Ca$^{2+}$. The $\Delta \Psi_m$ is restored when the accumulation is complete (Fig. 8A). Similar results were obtained with untreated Jurkat-Bcl-2 cells, although the Bcl-2 overexpressing cells restored the initial level of $\Delta \Psi_m$ more rapidly after addition of Ca$^{2+}$. When Jurkat-Neo and Jurkat-Bcl-2 cells were exposed to 50 µM etoposide, the ‘Neo’ but not the ‘Bcl-2’ cells showed decreased $\Delta \Psi_m$ (Fig. 8B). Addition of Ca$^{2+}$ to the etoposide-treated Jurkat-Neo cells resulted in a decrease in $\Delta \Psi_m$ typical for Ca$^{2+}$ accumulation; however, they were unable to accomplish the second phase of restoration. In contrast, the Jurkat-Bcl-2 cells were still able to restore $\Delta \Psi_m$ after Ca$^{2+}$ addition, showing clear protection against mitochondrial changes induced by etoposide. Incubation of Jurkat-Neo cells with 0.1 mg/ml HAMLET causes a decrease in $\Delta \Psi_m$ (Fig. 8C). This decrease was partially restored by BSA indicating that the $\Delta \Psi_m$ drop was, in part, due to uncoupling by fatty acids. The Bcl-2-overexpressing cells demonstrate a higher initial potential, and the addition of BSA restores $\Delta \Psi_m$ completely. At this concentration of HAMLET (that do not significantly kill the cells, see Fig. 7B), Bcl-2 has some protective effect on the drop in $\Delta \Psi_m$. However, at higher concentration of HAMLET (0.3 mg/ml) the mitochondrial damage is too severe and overexpressed Bcl-2 cannot overcome the effect of HAMLET (Fig. 8D).

The reduced mitochondrial function in HAMLET-treated cells was further studied by determining the Ca$^{2+}$ accumulating capacity. Normally, mitochondria can accumulate large amounts of Ca$^{2+}$ before MPT is induced. The maximal amount of Ca$^{2+}$ that mitochondria could accumulate before induction of MPT (the Ca$^{2+}$ capacity) was determined by serial additions of Ca$^{2+}$ (20 nmoles per addition). The concentration of Ca$^{2+}$ in the buffer was monitored using a Ca$^{2+}$-sensitive electrode. The digitonin-permeabilised untreated Jurkat-Neo cells could accumulate 3 additions of Ca$^{2+}$ (60 nmoles)
Mechanisms of apoptosis induced by a protein complex isolated from human milk

A
Control

B
HAMLET 0.3 mg/ml
dig
Cells Ca^{2+}

C
HAMLET 0.3 mg/ml
BSA
dig
Cells Ca^{2+} Ca^{2+}

D
HAMLET 0.3 mg/ml
+ CsA
dig
Cells Ca^{2+}

E
HAMLET 0.3 mg/ml
+ CsA
BSA
dig
Cells Ca^{2+} Ca^{2+}

3 min
20 nmoles
Figure 9. Ca\(^{2+}\)-accumulation in HAMLET-treated Jurkat cells overexpressing Bcl-2. Jurkat-Neo and Jurkat-Bcl-2 cells (3 x 10^6) were incubated with 0.3 mg/ml HAMLET for 5 minutes in the absence (A, B and C) or presence of 10 µM cyclosporin A (CsA) (D and E). Cells were washed in PBS, resuspended in buffer containing 150 mM KCl, 5 mM KH\(_2\)PO\(_4\), 1 mM MgCl\(_2\), 5 mM succinate and 5 mM Tris-HCl, pH 7.4. Cells were permeabilised with 0.005% digitonin (dig) and 5 µM rotenone and 1 µM oligomycin were added. MPT was induced by sequential additions of Ca\(^{2+}\) (20 nmoles) and the Ca\(^{2+}\) concentration in the buffer was monitored using a Ca\(^{2+}\)-sensitive electrode. In panel C and E, 0.5% BSA was added in order to bind fatty acids.

and MPT was induced after the fourth addition (Fig 9A). The mitochondria of untreated Jurkat-Bcl-2 cells had a higher Ca\(^{2+}\) capacity than the Jurkat-Neo cells and could accumulate almost 6 additions of Ca\(^{2+}\) (120 nmoles). This suggests that the overexpressed Bcl-2 make the mitochondria in the Jurkat-Bcl-2 cells less vulnerable to MPT induced by Ca\(^{2+}\) overload. The mitochondrial ability to accumulate Ca\(^{2+}\) was completely suppressed by HAMLET in both Jurkat-Neo and Jurkat-Bcl-2 cells as a consequence of the lost ΔΨ\(_m\) (Fig 9B). When BSA was added to the permeabilised HAMLET-treated cells, the Ca\(^{2+}\) capacity was partially restored in both Jurkat-Neo and Jurkat-Bcl-2 cells (Fig 9C). In the presence of BSA, the HAMLET-treated cells accumulated about 30% of the Ca\(^{2+}\) capacity observed in untreated Neo and Bcl-2 cells, respectively. In line with our previous results, pre-incubation of Jurkat-Neo cells with CsA did not have any effect on the reduced Ca\(^{2+}\) capacity in HAMLET-treated cells, while it was completely restored when CsA was added in combination with BSA (Fig 9D and 9E). Interestingly, the same results were obtained with the Jurkat-Bcl-2 cells showing that the dissipated ΔΨ\(_m\) upon HAMLET exposure—a consequence of both MPT and uncoupling—occurred even in the Bcl-2 overexpressing cells. This suggests that HAMLET interacts with mitochondria and induces MPT independently of the Bcl-2 status.

The inefficiency of Bcl-2 to block HAMLET-induced apoptosis indicates that HAMLET may use apoptosis pathways that are not regulated by Bcl-2 family proteins or that the apoptotic signal raised in the cells can circumvent or overcome the inhibitory activity of the anti-apoptotic Bcl-2.

In conclusion, these results suggest that HAMLET, even in the presence of Bcl-2, triggers apoptosis via MPT induction, followed by the release of cytochrome c and activation of the caspase cascade.
The effect of HAMLET on cytoplasts

MAL/HAMLET is accumulated in the nuclei of sensitive cells, and it has been reported that MAL incubated with isolated nuclei from L1210 (mouse lymphoblastoid leukaemia), A549, and HRTEC cells induces cleavage of DNA, with formation of HMW and LMW fragments [Håkansson et al., 1999]. The nuclear uptake is a late event compared to the localisation of MAL/HAMLET to mitochondria; however, a role for nuclear accumulation of the milk complex in the apoptotic process cannot be excluded. It has been shown that certain stimuli can induce apoptosis in cytoplasts (i.e. cells lacking the nucleus) [Schulze-Osthoff et al., 1994; Jacobson et al., 1994; Nakajima et al., 1995]. We used this experimental approach in order to investigate if apoptosis can be

**Figure 10. HAMLET treatment of cytoplasts.** A549 cells were enucleated as described (p. 39). The figure shows cells treated with cytochalasin B, with (**C** and **D**) or without (**A** and **B**) centrifugation. The cells were incubated in the absence (**A** and **C**) or presence of HAMLET (**B** and **D**) for 2 h and were finally fixed and stained with Giemsa.
triggered by HAMLET in the absence of the cell nucleus. The adherent A549 cells were enucleated by centrifugation of cytochalasin B-treated cells as described (p. 39). The percentage of enucleation was 75-80%. The cytoplasts were then treated with HAMLET for 2h, and morphological changes were used as criteria for apoptosis. As shown in Figure 10, the cytoplasts shrunk and detached from the surface upon incubation with HAMLET, indicating that apoptosis was induced in A549 cytoplasts. These results further support our hypothesis that the apoptotic process is initiated by a direct activation of the mitochondrial pathway. The accumulation of HAMLET in the nucleus may be a consequence of the disrupted nuclear-cytoplasmic barrier during apoptosis [Faleiro & Lazebnik, 2000]. It has been reported that the nuclear pore protein Nup153 is cleaved by caspases and this cleavage may facilitate the diffusion of molecules between the cytoplasm and nucleus [Buendia et al., 1999]. Therefore, the nuclear accumulation of MAL/HAMLET may, in fact, be a consequence of apoptosis induction. However, our findings do not exclude co-existence of a nuclear pathway for HAMLET-induced apoptosis, similarly to what we have recently reported for etoposide-treated Jurkat cells, where a low concentration of the drug induces apoptosis via the nucleus and a higher dose have, in addition to nuclear, a direct mitochondrial effect [Robertson et al., 2000].
SUMMARY

Upon treatment, MAL/HAMLET crosses the plasma membrane and enters the cytoplasm of sensitive cells. The data presented in this thesis suggest that MAL/HAMLET interacts directly with mitochondria followed by a rapid release of intermembrane space proteins, including cytochrome c (Fig. 11). This release is a consequence of MPT induction—an effect that is facilitated by the presence of fatty acids in the protein complex. The release of cytochrome c leads to activation of the caspase cascade and apoptotic cell death. However, other pathways for apoptosis induction by MAL/HAMLET may also be involved.

Anti-apoptotic members of the Bcl-2 family are oncogene proteins. Upregulation of these proteins contributes both to the genesis of cancer and to difficulties in treating the disease. Since HAMLET-induced apoptosis is not inhibited by Bcl-2 overexpression, this may explain why most tumour cell lines are susceptible to HAMLET. However, the reason why most non-transformed cells are resistant to HAMLET-induced apoptosis is still unclear.

Figure 11. The proposed model for apoptosis induction by MAL/HAMLET.
FUTURE DIRECTIONS

The data obtained in this study are mainly based on experiments in which Jurkat and A549 cells have been used. These cell lines are widely used experimental models in apoptosis research. MAL/HAMLET induces apoptosis via a direct interaction with mitochondria in these cells. Nevertheless, it is necessary to investigate the induction pathways of apoptosis triggered by MAL/HAMLET in other cell types before a general conclusion can be drawn. Moreover, since MAL/HAMLET kills most transformed cell lines by apoptosis it would be very interesting to verify the mechanisms of apoptosis in primary cultures of tumour cells.

Many tumour cells are resistant to agents that induce apoptosis in non-transformed cells. In contrast, MAL/HAMLET induces apoptosis in tumour cells, but leave most other cells intact. The mechanism behind this is unknown, but the total cellular uptake of MAL/HAMLET is more pronounced in sensitive cells compared to non-transformed, resistant cells. It would be interesting to study the effect of MAL/HAMLET in cells that are non-sensitive to this treatment in order to understand if the resistance lies upstream or downstream of mitochondrial changes such as the release of cytochrome c. If the cellular uptake per se is the decisive event, then microinjected MAL/HAMLET into the cytoplasm of non-sensitive cells (i.e. a primary culture of differentiated non-transformed cells) should overcome the resistance to apoptotic cell death.

Future investigations in the ‘milk project’ should also include experiments that elucidate the molecular mechanism(s) by which HAMLET induces MPT and circumvents the antagonistic effect of Bcl-2 on mitochondrial membrane integrity. A possible physical interaction of MAL/HAMLET with any protein in mitochondria may be found by several approaches, including immunoprecipitation and affinity chromatography. Possible binding partner(s) of MAL/HAMLET could be the proteins in, or associated with, the MPT pore.
CONCLUDING REMARKS

It is interesting to speculate whether the presence of the folding variant of α-lactalbumin in human milk has any relevance for the nursing mother or the breast-fed newborn child. Lactation is dependent on regular suckling of the mammary gland. Without this stimulus, the secretion of milk ends and mammary involution is induced. Post-lactational involution of the mammary gland is mediated by apoptosis of mammary epithelial cells [Li et al., 1996; Marti et al., 2001]. It was reported that sealing of a rat mammary gland during lactation was sufficient to induce epithelial cell apoptosis, suggesting that apoptosis may be triggered by accumulation of apoptosis-inducing factors in the milk [Marti et al., 1997]. One of these factors could be the folding variant of α-lactalbumin.

In the newborn infant, the gastro-intestinal mucosa is immature with rapidly proliferating epithelial cells. Regulatory factors, such as MAL/HAMLET, may support the proper maturation of the mucosa and avoid the development of tumour cells in the tissue. Moreover, it may have effects on the development of diseases, such as cancer, later in life. The low pH in the stomach would favour a conformational change of α-lactalbumin resulting in dissociation of the strongly bound Ca\(^{2+}\) ion. In addition, lipids (triglycerides etc.) are broken down to free fatty acids by lipases in the gut. Thus, in the gastro-intestinal tract of the newborn child native α-lactalbumin may change its fold into a molten-globule-like state and thereby enhance the apoptosis-inducing activity of milk. However, further studies are required to elucidate the possible physiological role for MAL/HAMLET-induced apoptosis in the newborn child.

Many tumour cells have a defect in the ‘decision’ machinery for apoptosis but an intact execution system. Such tumour cells should die, if they are provided with an effective apoptotic signal. MAL/HAMLET is a strong MPT-inducing agent that rapidly translocates to mitochondria and thereby triggers apoptosis via the mitochondrial pathway. The mitochondria have been suggested as a target for chemotherapeutic agents [Costantini et al., 2000]. In fact, several potential anti-cancer drugs, including lonidamine, arsenite and betulinic acid, induce apoptosis by acting directly on the MPT pore [Ravagnan et al., 1999; Constantini et al., 1996; Larochette et al., 1999; Fulda & Debatin, 2000]. Traditional chemotherapeutic drugs, such as doxorubicin, etoposide and cisplatin, are antiproliferative but are not specific for tumour cells; rapidly dividing normal cells are also affected. This may
result in side effects such as decreased resistance to infections, hair loss, sterility and teratogenic effects. MAL/HAMLET preferentially kills transformed cells and is far less effective against non-transformed, differentiated cells. Other advantages would be that MAL/HAMLET seems to induce apoptotic death independently of the Bcl-2 status in the cells. As a high expression of the proto-oncogene Bcl-2 often is associated with resistance to various apoptosis-inducing chemotherapeutic agents, MAL/HAMLET may have an increased potential to become a cytotoxic drug. In addition, MAL/HAMLET is a naturally occurring factor in human milk that most likely would not cause toxic side effects upon treatment. Therefore, the knowledge provided in this thesis about the mechanisms of apoptosis in transformed cells induced by the folding variant of human α-lactalbumin may be beneficial for the design of new anti-cancer therapies.
SWEDISH SUMMARY-
SVENSK SAMMANFATTNING

Vid studier av anti-bakteriella egenskaper hos modersmjölk, där mjölkens effekt på bakteriers förmåga att binda till odlade cancerceller granskades, gjordes en oväntad upptäckt; mjölen dödade cellerna. För att undersöka vad i mjölen som orsakade celldöden fraktionerades mjölen. En fraktion som dödade tumörceller via apoptos (cellulärt självmord) isolerades [Håkansson et al., 1995]. Denna mjölkfraktion (MAL, för multimeric α-lactalbumin) visade sig till stor del innehålla en veckningsvariant av proteinet α-laktalbumin [Svensson et al., 1999]. För att studera om denna veckningsvariant var den faktor i mjölen som orsakade apoptos framställdes HAMLET (human α-lactalbumin made lethal to tumour cells). HAMLET är α-laktalbumin som på konstgjord väg konverterats till den veckningsvariant man funnit i MAL [Svensson et al., 2000]. Konverteringen krävde närvaro av en viss fettsyra, oleinsyra (C18:1), som antas vara viktig för stabilisering av proteinstrukturen. HAMLET visade sig döda tumörceller via apoptos med samma kinetik som MAL. Arbetet som presenteras i denna avhandling syftar till att karakterisera den mekanism genom vilken MAL/HAMLET framkallar apoptos i tumörceller.

Apoptos är en form av celldöd som är lika viktig för den embryonala utvecklingen som för vävnadshomeostasen i den vuxna organismen. Fel i regleringen av apoptos bidrar till uppkomsten av olika sjukdomar, såsom autoimmuna och neurodegenerativa sjukdomar och cancer. Apoptos karakteriseras av att cellen genomgår specifika morfologiska förändringar; cellen krymper, kromatinet kondenserar, DNA:t fragmenteras och cellen styckas upp i mindre membranomslutna partiklar (s. k. apoptotiska kroppar). Dessa apoptotiska kroppar känns igen av makrofager, som fagocyterar och bryter ner de döda cellerna och därmed undviks inflammation i vävnaden.

Studierna i denna avhandling baseras på celler som odlats i kultur, främst lungcancer- och leukemi- cellinjer. Resultaten visar att då dessa celler exponeras, binder MAL/HAMLET till tumörcellers yttermembran och tas därefter in i cytoplasmans, där MAL/HAMLET uppvistar en kornig lokalisation innan proteinkomplexet transporterades till cellkärnan. Denna kornighet i cytoplasmans kan förklaras av att MAL/HAMLET är associerat till mitokondrier. Denna cellorganell har under de senaste åren visat sig spela en viktig roll vid initiering av den apoptotiska processen genom att frisätta proteiner, bl. a. cytokrom c. Trots att frisättning av cytokrom c har observerats


Förhoppningen är att vår forskning bidrar med kunskap som kan bli värdefull för utveckling av kompletterande former av tumörbehandling. Potentiellt finns flera fördelar med HAMLET jämfört med andra terapiformer. För det första skadas inte friska celler, vilket ofta är ett stort problem vid behandling av cancer. För det andra tycks HAMLET inducera apoptos oberoende av cellens Bcl-2 status. För det tredje är HAMLET en naturlig produkt som finns i modersmjölk och därför troligtvis inte ger toxiska biverkningar.

Vår forskning kan också leda till ökad kunskap om effekterna av modersmjök vid ammning. Veckningsvarianten av α-laktalbumin i mjölk kan tänkas ha en reglerande effekt på cellerna i mag-tarmkanalens slemhinna, som
hos spädbarn är omogna och delar sig med hög hastighet. Möjligen fyller MAL/HAMLET en funktion vid mognaden av denna slemhinna genom att döda pre-maligna celler, som kan utvecklas till tumörceller. Därtill har epidemiologiska studier visat att ammning kan skydda mot sjukdomar som utvecklas senare i livet, t. ex. insulin-beroende diabetes, arterioskleros och även vissa typer av cancer hos barn.
ACKNOWLEDGEMENTS

This work was performed at the Division of Toxicology, Institute of Environmental Medicine at Karolinska Institutet. I would like to express my gratitude to all colleagues, co-authors and friends for your help and support. In particular, I would like to thank the following persons who in different ways have contributed to this thesis.

Docent Boris Zhivotovsky, for being my main supervisor during these years. Thank you for all support and discussions, for guiding me and letting me develop as a PhD student. For always having time (even when you don’t), for caring about me and, finally, for helping us to get excellent tickets to the ‘Swan Lake’ at the Bolshoi Theatre in Moscow.

Professor Sten Orrenius, my co-supervisor, for sharing your scientific knowledge and for creating a scientifically stimulating atmosphere in the ‘blue group’.

The ‘man at ASTRA’, Dr. Dan-Anders Lidbom, who convinced me to fulfil my PhD education.

Professor Catharina Svanborg, head of the ‘milk group’ in Lund, for nice collaboration.

Dr. Vladimir Gogvadze, for enthusiastically introducing me to the mitochondria and helping out with techniques and long (!) experiments. (Will I ever know what ‘päronska’ is?)

Dr. Anders Håkansson, who used to work in the ‘milk group’ in Lund, for supporting me both scientifically and personally during this time. Without you my work would have been much more complicated.

Dr. John Robertson, for linguistic revision of this thesis and for always being helpful.

My present room-mate Mari, for being a cheerful person that makes sure I have enough sweets; and all room-mates that passed over the years: Helene, Bettina, Carina, Bengt, Helena, Isabella, Malin and Kari, for making room C3:412 a nice working place full of laughter, friendship and discussions about
everything from experimental problems to daily reports about life in general. Thank you all!

The present members of the ‘blue group’: Margareta, Ravindra, Jessica, Andrej, Jan, Sandra, Roshan, Elisabetta, Ping and Eric; for your contribution both scientifically and socially. I would also like to thank the former ‘blue group’ members: especially, Eva, for lots of fun during the years at IMM; Bertrand, for all cups of coffee and technical help; Martin, for nice climbing; Annie and Lisa, for being nice to have around; and Birgitta for always being helpful and having a smile on your face.

Other members in the ‘milk group’ in Lund; Caroline, Malin, Oskar and, in particular, Anki, for continuously sending me batches of MAL and HAMLET.

My students, Laura and Sandra; it has been a pleasure to work with you in the lab.

From IMM, especially Lina, for being a close friend; Mattias, Martin, Ulla, Niclas, Mikael, Karin, Patrik, Michael, Anna and Lotta, for fun IMM partys and chats in the corridors.

My closest friends, especially those of you that also are (or have been) PhD students; for many nice memories, friendship and shared ups and downs during these years.

Finally, I would like to thank my family, including my mother & Janne, my father & Ewa, Jonathan, Diana, Tami, and Amanda for always being there. André, my love, thank you for trying to make me less stressed, for your endless patience and support during the last year, especially the last months. You are fantastic and very special to me.

This work was funded by the Swedish Cancer Foundation, the American Cancer Society, the Swedish Medical Research Council, the Swedish Cancer Society (Cancerfonden), and Robert Lundbergs minnesstiftelse.
REFERENCES


Bell, FP, 1980. Inhibition of adenine nucleotide translocase by oleoylcarnitine, oleoylcoa and oleate in isolated arterial mitochondria. Atherosclerosis 37, 21-32.


Bychkova, VE, Pain, RH and Ptitsyn, OB, 1988. The 'molten globule' state is


Friesen, C, Herr, I, Krammer, PH and Debatin, KM, 1996. Involvement of the CD95 (APO-I/FAS) receptor/ligand system in drug-induced apoptosis in
Halestrap, AP, Woodfield, KY and Connern, CP, 1997. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial
permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J. Biol. Chem. 272, 3346-3354.


Irmler, M, Thome, M, Hahne, M, Schneider, P, Hofmann, K, Steiner, V,


residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res. 53, 4701-4714.


Mechanisms of apoptosis induced by a protein complex isolated from human milk


Mathiasen, IS, Lademann, U and Jaattela, M, 1999. Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res. 59, 4848-4856.


Mulqueen, PM and Kronman, MJ, 1982. Binding of naphthalene dyes to the N
Porter, AG, 1999. Protein translocation in apoptosis. Trends Cell Biol. 9, 394-
Mechanisms of apoptosis induced by a protein complex isolated from human milk


Mechanisms of apoptosis induced by a protein complex isolated from human milk

Biol. Chem. 274, 6388-6396.


Vayssère, J-L, Petit, PX, Risler, Y and Mignotte, B, 1994. Commitment to apoptosis is associated with changes in mitochondrial biogenesis and


Mechanisms of apoptosis induced by a protein complex isolated from human milk


Zhang, H, Xu, Q, Krajewski, S, Krajewska, M, Xie, Z, Fuess, S, Kitada, S,