Modulation of Cytochrome c release by Mitochondrial Redox Status and Caspase-2

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Stockholm 2005
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ABSTRACT

The release of cytochrome c is an important event during apoptosis, induced by diverse stimuli. Our laboratory has previously proposed that cytochrome c release occurs via a two-step process, involving the detachment of the hemoprotein from its binding to the inner mitochondrial membrane, followed by its release into the cytosol through pores in the outer mitochondrial membrane - an event that is usually triggered by pro-apoptotic Bcl-2 family proteins, such as Bid, Bax and Bak. Cytochrome c specifically and stoichiometrically binds to cardiolipin, thus anchoring the hemoprotein to the inner mitochondrial membrane to participate in electron transport. Mitochondria are the main intracellular source of reactive oxygen species (ROS), and it has been shown that cardiolipin might become oxidized and lose its interaction with cytochrome c as a result of increased ROS production, or deficient ROS scavenging, within the mitochondria. This thesis investigates the mechanism of cytochrome c release from mitochondria, and how this may be modulated by caspase-2 or the mitochondrial redox status. Caspase-2 is one of the best conserved caspases among species, and it is unique in the sense that it shares features of both initiator and executioner caspases. We have demonstrated a new role for caspase-2 in apoptosis signaling, and propose a novel mechanism for cytochrome c release, mediated by caspase-2 and possibly involving pore formation in the mitochondrial membrane by this protease (papers I and II). Caspase-2 seemingly plays a role in apoptosis induction by exerting a direct effect on mitochondria, thereby releasing cytochrome c. Interestingly, this effect seems to also involve an interaction between caspase-2 and cardiolipin. On the contrary, we have shown that cardiolipin is not a prerequisite for Bax-mediated cytochrome c release (Paper III). However, cardiolipin must be affected by protein binding or oxidation in order for solubilization of cytochrome c to occur, allowing release of the hemoprotein through the Bax-pores. One typical dissociation factor for cytochrome c is oxidation of cardiolipin. The glutathione (GSH; \( \gamma \)-glu-cys-gly) system is one of the most important intracellular redox systems. This abundant tripeptide protects from ROS and has been linked to apoptosis by several observations. In paper IV, apart from describing a new method for GSH visualization in the cell, we also demonstrated the capability of mitochondria to scavenge GSH during oxidative stress. Moreover, papers V and VI indicated that mitochondrial Grx2 is a possible inhibitor of apoptosis, since knocking down the protein by siRNA (paper V) or overexpressing Grx2 (paper VI) influence cell death signaling, probably by preventing oxidation or degradation of cardiolipin (paper VI). It is clear that the mitochondrial redox environment is crucial for keeping cardiolipin reduced and preventing cytochrome c release. Lowering the level of Grx2, or other mitochondrial redox enzymes, may thus have a lethal effect on the cell.

In conclusion, it is clear that the release of cytochrome c may occur by different mechanisms, depending on the apoptotic inducer and on the type of cell. While caspase-2 is able to form pores in the mitochondrial membrane, as well as promote dissociation of cytochrome c from cardiolipin, we cannot exclude that this protease also may work in concert with other pore-forming agents, such as Bax. However, cardiolipin is not required for Bax pore-formation of the mitochondrial membrane. In addition, we have shown that mitochondria require a functional redox system for protection from apoptosis.
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<tr>
<td>AIF</td>
<td>Apoptosis Inducing Factor</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
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<td>BSO</td>
<td>Buthionine sulfoximine</td>
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<td>CARD</td>
<td>Caspase recruitment domain</td>
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<td>DED</td>
<td>Death effector domain</td>
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<td>DISC</td>
<td>Death inducing signaling complex</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>GR</td>
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<td>Grx</td>
<td>Glutaredoxin</td>
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<td>GSSG</td>
<td>Glutathione disulfide</td>
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<tr>
<td>IAPs</td>
<td>Inhibitor of apoptosis proteins</td>
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<tr>
<td>ICE</td>
<td>Interleukin-1β-converting enzyme</td>
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<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
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<tr>
<td>PHGPx</td>
<td>Phospholipid hydroperoxide glutathione peroxidase</td>
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<tr>
<td>Prx</td>
<td>Peroxiredoxin</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>Trx</td>
<td>Thioredoxin</td>
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<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
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<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
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1 GENERAL INTRODUCTION

1.1 APOPTOSIS

Apoptosis is a form of programmed cell death that is essential for the regulation of tissue homeostasis in multi-cellular organisms. The term apoptosis was coined by Kerr et al. (Kerr et al., 1972), although some manifestations of programmed cell death had also been described in earlier studies (reviewed in Clarke and Clarke, 1996). Importantly, the apoptotic machinery is highly conserved among species. Significant studies using Caenorhabditis elegans as a model provided a genetic framework for the cell death program (and also awarded Sydney Brenner, John Sulston and Robert Horvitz the Nobel Prize in Physiology or Medicine 2002). There are 959 somatic cells in the adult nematode and an additional 131 cells undergo programmed cell death during development. The nematode genes responsible for this tightly regulated process of cell death have human homologues (Robertson et al., 2002). Cell death plays an important role during embryonic development, and it is essential for successful organogenesis. Modulation of apoptosis can have fatal consequences; insufficient apoptosis can promote diseases such as cancer or autoimmunity, while accelerated cell death appears in acute and chronic degenerative diseases, immunodeficiency, and infertility (Danial and Korsmeyer, 2004).

1.2 APOPTOSIS VERSUS NECROSIS

In addition to apoptosis, several other types of cell death, such as necrosis and autophagy, occur. The morphological features of apoptosis are cell shrinkage, chromatin condensation, membrane blebbing and formation of apoptotic bodies (Wyllie et al., 1980). These bodies are disposed of in a neat, orderly fashion by macrophages (reviewed in Fadeel, 2003). In contrast, necrosis is a passive form of cell death that occurs during more severe circumstances, when cell damage is too profound to allow maintenance of the controlled and energy-dependent apoptotic machinery. Necrosis is characterized by organelle swelling, osmosis, energy depletion, and random DNA degradation (Wyllie et al., 1980). The outcome of necrosis is usually rupture of the plasma membrane and subsequent spillage of the intracellular contents into the surrounding tissue. If this occurs to a large extent, inflammation of the surrounding tissue may occur, causing further damage to the organism.

1.3 AUTOPHAGY

The eukaryotic cell can utilize several degradation systems. One is the ubiquitin-proteasome system, which selectively degrades most short-lived proteins, and whose discovery led to Aaron Ciechanover, Avram Hershko and Irwin Rose being awarded the Nobel Prize in Chemistry 2004. The other degradation system is autophagy, where cells recycle cytoplasm and discharge excess or damaged organelles, such as mitochondria (Shintani and Klionsky, 2004). It is the most ubiquitous system for intracellular bulk degradation in eukaryotes and is responsible for the degradation of most long-lived proteins, as well as some organelles. It is characterized by formation of
a double- or multi-membrane-bound vacuole, the autophagosome (Dunn, 1990). The vacuole membrane fuses with the lysosomal membrane, resulting in lysosomal degradation and eventually recycling of the macromolecular constituents. Apart from protein degradation and organelle turn-over, autophagy is involved in cellular remodelling during differentiation and metamorphosis, as well as aging, cancer, muscular disorder, neurodegeneration and pathogenic infections (Shintani and Klionsky, 2004). For instance, autophagy may be a way of protecting the cells against mitochondrial permeability transition during oxidative stress or mitochondrial Ca²⁺-overloading (Rodriguez-Enriquez et al., 2004). There is no clear discrepancy between autophagy and apoptosis; apoptosis may end with autophagy, and autophagy may end in cell death (Lockshin and Zakeri, 2004). It has been suggested that autophagy is a possible means to reduce cellular volume prior to apoptosis (Lockshin and Zakeri, 2004). In addition, it could also be a way for cells with dysfunctional apoptotic machinery to avoid necrosis.

1.4 THE INTRINSIC AND EXTRINSIC PATHWAYS

Two main pathways of apoptosis have been identified, the receptor-mediated (extrinsic) pathway, involving activation of death receptors on the plasma membrane, and the intrinsic pathway, involving mitochondrial signaling (Figure 1). The receptor-mediated pathway is initiated by binding of ligands to the tumor necrosis factor (TNF) family of plasma membrane death receptors, such as Fas, TNFR1, and the TRAIL receptors DR4 and DR5. The receptors form trimeric complexes upon binding of the death ligand and subsequently interact with an adaptor protein, called Fas Associated Death Domain (FADD) (Chinnaiyan et al., 1995), which in turn binds pro-caspase-8, resulting in

![Figure 1. The extrinsic and intrinsic pathways of apoptosis induction. Caspase-9 and -3 are designated as C-9 and C-3, respectively.](image-url)
formation of a protein complex called the Death Inducing Signaling Complex (DISC). Aggregation of pro-caspase-8 leads to the autoprocessing and activation of this initiator caspase. Activated caspase-8 may either directly cleave and activate effector caspases, typically pro-caspase-3, or cleave Bid to truncated Bid (tBid), a pro-apoptotic member of the Bcl-2 protein family. Subsequent translocation of tBid to the mitochondria initiates the intrinsic, mitochondria-mediated pathway (Li et al., 1998; Luo et al., 1998). The intrinsic pathway promotes release of mitochondrial proteins including cytochrome c, Apoptosis Inducing Factor (AIF), Smac/DIABLO, Endonuclease G and Omi/HtrA2 (van Gurp et al., 2003). Released cytochrome c interacts with Apaf-1, pro-caspase-9 and dATP to form a ~1 MDa protein complex called the apoptosome (Li et al., 1997). The apoptosome has seven-fold symmetry, and forms a wheel-like structure with seven spokes that radiate from a central hub (Acehan et al., 2002). Caspase-9 is activated upon apoptosome formation, and cleaves and activates pro-caspase-3. The mechanism of cytochrome c release will be discussed in more detail below. AIF is a 57 kDa mitochondrial flavoprotein (Susin et al., 1999). Activation of poly (ADP-ribose) polymerase-1 (PARP-1) in response to DNA damage causes translocation of AIF to the nucleus, where it triggers caspase-independent chromatin condensation and large scale (~50 kb) DNA fragmentation (Yu et al., 2002). Endonuclease G is a 30kDa mitochondrial nuclease that also may translocate to the nucleus upon apoptosis induction, where it digests nuclear DNA in the absence of caspase activity (van Gurp et al., 2003). Smac/DIABLO is a 29 kDa (mature form 23 kDa) mitochondrial precursor protein that is released from the intermembrane space after apoptosis induction. Smac/Diablo acts as a dimer and contributes to caspase activation by sequestering inhibitor of apoptosis proteins (IAPs) that prevent the activation of pro-caspases and inhibit the activity of mature caspases. Omi/HtrA2 is a 49 kDa (mature form 37 kDa) serine protease that also binds and sequesters IAPs (van Gurp et al., 2003).

1.5 THE CASPASE FAMILY

Caspases constitutes a protein family comprised of 14 mammalian members (11 in human). The first member of this family was described in 1992 as interleukin-1β-converting enzyme (ICE, later known as caspase-1) (Cerretti et al., 1992; Thornberry et al., 1992). In 1993, Yuan et al. demonstrated that expression of the pro-apoptotic C. elegans ced-3 gene, a homologue to ICE, could induce cell death in Rat-1 fibroblasts (Miura et al., 1993; Yuan et al., 1993). This finding was later proven to be the first representative of a whole protein family, later named the caspase (cysteine-aspartate protease) family (Alnemri et al., 1996). Caspases are responsible for both initiation and execution of apoptosis, and they are the main protease family involved in the apoptotic dismantling of the cell. They are synthesized as single-chain pro-caspases (32-56 kDa), catalytically inactive zymogens that generally require processing in order to become active enzymes.

1.5.1 Initiator and executioner caspases

Caspases can be divided into two major groups, the initiator (or apical) caspases (caspase-2, -8, -9, -10), and the executioner caspases (caspase-3, -6 and -7) (Thornberry and Lazebnik, 1998). The pro-inflammatory caspases (caspase-1, -4 and -5) are involved in the activation/maturation of cytokines. Caspase-14 is mainly found in the epidermis and may be involved in keratinocyte differentiation but has not been shown to have a pro-apoptotic function (Lippens et al., 2000). Caspase-2 signaling is part of the focus of this thesis (papers I and II) and will be discussed in more detail below.
Active caspase-8 participates in the extrinsic pathway since it is activated upon ligation of the death receptors, but it has dual mechanisms depending on cell type. Caspase-8 will cleave and activate pro-caspase-3 directly (type I cells), or it will initiate the intrinsic pathway by cleaving Bid to tBid (type II cells). An endogenous caspase-8 inhibitor, c-FLIP, is thought to function by competing with caspase-8 for binding to the DISC, but lacks protease activity. Release of cytochrome c promotes apoptosome formation, and thus the activation of caspase-9, that cleaves and activates pro-caspase-3. Caspase-10 is a close relative of caspase-8, and may also directly activate pro-caspase-3 and -7.

The effector (or downstream) caspases are usually activated by the initiator caspases, but they may also be cleaved and activated by other non-caspase proteases involved in apoptosis, such as cathepsins, calpains and granzymes (Johnson, 2000). Caspase-3 and -7 are closely related, possess similar cleavage specificity, and are believed to be responsible for the major part of the proteolysis during apoptosis execution. They cause the degradation of numerous substrates during apoptosis, including structural and regulatory proteins that are important for morphological changes, such as DNA degradation, chromatin condensation and membrane blebbing. Caspase-6 is considered as an effector caspase based on its short N-terminal pro-domain (see below), but it has different substrate specificity than caspase-3 and -7.

### 1.5.2 Active site and substrate recognition

The caspases have a conserved QACXG pentapeptide motif, which contains the active site cysteine (Thornberry and Lazebnik, 1998). The tetrapeptide substrate recognition motif differs significantly among caspases, but they all cleave at the peptide bond C-terminal to aspartate residues. Recognition of at least four amino acids N-terminal to the cleavage site is also a necessary requirement for efficient catalysis. In fact, caspases can be grouped according to substrate specificity, where group I (caspase-1, -4 and -5) recognize and cleave the WEHD motif, group II (caspase-3 and -7) the DEXD motif, and Group III (caspase-6, -8, -9, and -10) the (I/L/V)EXD motif (Thornberry et al., 1997). Only caspase-2 preferentially processes the pentapeptide VDVAD (Talanian et al., 1997). Importantly, initiator caspases have a precise substrate recognition ability, allowing them to distinguish their specific target effector caspases (Thornberry et al., 1997).

### 1.5.3 Caspase structure and activation

Caspases possess a N-terminal pro-domain of variable length, a large (17-21 kDa) and a small subunit (10-13 kDa), and a short linker region connecting the catalytic subunits (Figure 2). However, the linker region is missing in some caspase family members (Philchenkov, 2004). The N-terminal pro-domain can be of variable length, either short (20-30 amino acid residues) or long (more than 90 residues). Long pro-domains contain either death effector domains (DED) or a caspase recruitment domain (CARD), which direct the oligomeric interactions triggering autoactivation of the initiator pro-caspases (Fuentes-Prior and Salvesen, 2004). In addition, these modules may interact with adaptor proteins. DED-DED interactions are generally of hydrophobic character, while CARD-CARD interactions occur via electrostatic interactions. The initiator caspases (caspase-2, -8, -9 and -10) and the pro-inflammatory caspases (caspase-1, -4 and -5) contain a long pro-domain and are capable of autoprocessing, while executioner caspases (caspase-3, -6 and -7) contain a short pro-domain, lacking the DED and CARD modules.
Catalytic processing of two Asp cleavage sites triggers the large and the small subunits to associate, thus providing the active site of the enzyme. The active caspase is usually defined as a homodimer of two heterodimers. However, this definition is not completely correct for all caspases, since the initiator caspases does not require processing per se for activation. In fact, cleavage is neither required, nor sufficient, for the activation of initiator caspases-8 and –9, instead they exist as latent monomers that require dimerization to attain the active conformation (Fuentes-Prior and Salvesen, 2004). The processing of the linker region is not required, but may instigate stability to the active dimer. The effector caspases however, already reside in a dimeric latent form, and processing is necessary for their activation.

1.5.4 Caspase-2

Caspase-2 (NEDD-2, Ich-1) is one of the most conserved caspases among species (Troy and Shelanski, 2003). Two different forms of caspase-2 exist in human; a short, anti-apoptotic form, caspase-2S, and a long, pro-apoptotic form, caspase-2L. In our studies we decided to focus on the long form. In fact, it is not clear that the shorter form is generally expressed as a protein. Caspase-2 has been considered an initiator caspase as it shares sequence homology with the initiator caspases, especially caspase-1 and -9, but its cleavage specificity (VDVAD) is closer to the executioner caspases. It was the second caspase described (Kumar et al., 1994; Wang et al., 1994), but widespread interest in this caspase has emerged only recently. Pro-caspase-2 contains a long pro-domain and two subunits (p19 and p12). Pro-caspase-2 is present in Golgi, mitochondria, nuclei and cytosolic fractions; however, its mitochondrial localization has been challenged (van Loo et al., 2002). It is constitutively expressed in the nucleus (Zhivotovsky et al., 1999; Mancini et al., 2000). Pro-caspase-2 has been shown to interact with RAIDD, an adaptor molecule involved in the extrinsic apoptosis pathway.
As an initiator caspase, pro-caspase-2 is capable of autoactivating itself. A proposed caspase-2 activation complex, the p53-inducible death domain complex (PIDDosome), involving RAIDD and pro-caspase-2, was described recently (Tinel and Tschopp, 2004). In contrast to other caspases, few caspase-2 substrates have been found. Specifically, golgin-160, which is a Golgi complex protein (Mancini et al., 2000), and αII-spectrin (Rotter et al., 2004), which is a component of the membrane cytoskeleton, are the only known substrates. Mice carrying a null mutation for caspase-2 develop normally and lack an overt phenotype (Bergeron et al., 1998). The most prominent feature of caspase-2-deficient mice is an increased number of oocytes among females. Furthermore, caspase-2-deficient oocytes are nearly completely resistant to the chemotherapeutic drug doxorubicin, while caspase-2-deficient B lymphoblasts are resistant to granzyme B-induced apoptosis. The mechanism by which caspase-2 exerts its pro-apoptotic effect is part of this thesis, and will be discussed in more detail below.

1.6 MECHANISMS OF CYTOCHROME C RELEASE

As mentioned above, cytochrome c is extruded from mitochondria into the cytosol during the early phase of apoptosis. It plays an essential role in the formation of the apoptosome, thus triggering the activation of the caspase cascade, resulting in an accumulation of apoptotic cells. Cytochrome c is a water-soluble basic protein that is bound to the mitochondrial inner membrane by its association with anionic phospholipids, primarily cardiolipin, where it can reversibly interact with complexes III and IV of the respiratory chain. Several theories regarding release of cytochrome c have emerged. Two more popular models include Ca\(^{2+}\)-dependent release (MPT), involving mitochondrial swelling and rupture of the outer membrane, and Ca\(^{2+}\)-independent pore-formation, triggered by the pro-apoptotic Bcl-2 family members (Gogvadze et al., 2001). Recent studies have proposed a novel mechanism for cytochrome c release mediated by caspase-2. This is discussed in detail below (papers I and II).

1.6.1 Cardiolipin

Cardiolipin is an unsaturated anionic phospholipid found exclusively in the inner mitochondrial membrane of eukaryotic cells (Gallet et al., 1997; van Klompenburg et al., 1997; Schlame et al., 2000). Cardiolipin is comprised of four acyl groups and two phosphate moieties (Figure 3). The dominant acyl group is linoleoyl (C 18:2), but oleoyl (18:1) and linolenoyl (18:3) are also present. However, the cardiolipin

![Figure 3. Schematic structure of cardiolipin. The fatty acid chains, designated R1 or R2, can be of different compositions, however linoleoyl (C 18:2) is usually the dominant form.](image-url)
composition in cell culture is slightly different. Jurkat cells were shown to contain cardiolipin from mixtures of (16:1) (18:1) acyl chains (Matsko et al., 2001). The degree of unsaturation appears to determine the optimized cardiolipin - protein association and may thus influence the biological activity of cardiolipin (Schlame et al., 2000). Cardiolipin is relatively stable toward degradation. However, hydrolysis of peroxidized cardiolipin may be more efficient, since this form of cardiolipin does not bind to cytochrome c and thus has no steric protection (Tuominen et al., 2002). Formation of mono- and dilsyocardiolipin can be catalyzed by mitochondrial phospholipase A2, but the lysosomal pathway appears to be the major pathway for breakdown of cardiolipin (Hambrey and Mellors, 1975). The translocation of cardiolipin from mitochondria to lysosomes is not clearly understood, but recent studies have demonstrated that native recombinant and truncated Bid possess lipid transfer activity (Esposti et al., 2001).

1.6.2 Cytochrome c – cardiolipin interactions

In accordance with its selective intracellular distribution in the inner mitochondrial membrane, cardiolipin was demonstrated to be an essential component in many mitochondrial processes, such as electron transport, ADP/ATP translocation, ion permeability, membrane integrity and protein function and transport (Schlame et al., 2000; Schagger, 2002; Zhang et al., 2002). The activity of several mitochondrial proteins apart from cytochrome c, notably the terminal electron carrier cytochrome c oxidase and adenine nucleotide translocase, are dependent on the presence of cardiolipin. In addition, the molecular interaction between cardiolipin and cytochrome c has been extensively studied using multiple biochemical and analytical approaches. It is well established that cytochrome c specifically, irreversibly and stoichiometrically binds to cardiolipin (Rytömaa and Kinnunen, 1995; Tuominen et al., 2002), thus anchoring the protein to the inner mitochondrial membrane, ensuring its participation in electron transport and thereby allowing only a limited soluble pool of the protein. This binding involves at least two conformations; a loosely bound conformation provided by electrostatic interactions between positively charged lysine residues of cytochrome c and negatively charged phosphate groups of cardiolipin, and a tightly bound conformation where hydrophobic interactions anchor the protein to cardiolipin (Iverson and Orrenius, 2004). Several models of the hydrophobic interactions have been proposed. Some studies suggest that cytochrome c is partially embedded into the membrane (Cortese et al., 1998; Gorbenko, 1999), while others suggest that an expanded acyl chain of cardiolipin and a hydrophobic inlet of cytochrome c anchors the protein to the membrane (Rytömaa and Kinnunen, 1995).

1.6.3 The two-step process of cytochrome c release

There is ample evidence that a decreased level of cardiolipin, as a result of inhibited synthesis, accelerated break-down or oxidative stress, leads to impaired cytochrome c binding and increases the susceptibility of cells to undergo mitochondrially-mediated apoptosis (reviewed in Iverson and Orrenius, 2004). In 2002, Ott et al. suggested a two-step model for cytochrome c release (Figure 4), based on the fact that cytochrome c must become detached from the inner mitochondrial membrane before release through the outer membrane can occur. This has later been supported by other studies (Iverson and Orrenius, 2004). The first step involves detachment of cytochrome c into the intermembrane space by breaching its electrostatic and/or hydrophobic interactions with the inner mitochondrial membrane. Secondly, permeabilization of the outer mitochondrial membrane must occur in order for the solubilized cytochrome c to be released.
Previous studies on cytochrome c – cardiolipin interactions demonstrated that peroxidation of cardiolipin causes dissociation of cytochrome c from the phospholipid (Shidoji et al., 1999). Mitochondria are the main intracellular source of reactive oxygen species (ROS), which are predominantly generated as respiration side products (see below). As a result of increased ROS production, or deficient ROS scavenging within the mitochondria, cardiolipin might become oxidized and lose its interaction with cytochrome c (Shidoji et al., 1999; Nomura et al., 2000; Asumendi et al., 2002; Ott et al., 2002; Chang et al., 2004). On the other hand, antioxidants or antioxidant enzymes have been shown to inhibit apoptosis in several models (Keller et al., 1998; Manna et al., 1998; Gottlieb et al., 2000; Nomura et al., 2000; Chen et al., 2002; Damdimopoulos et al., 2002; Nonn et al., 2003; Chang et al., 2004), further pointing to the importance of keeping cardiolipin in a reduced state. In addition, cardiolipin was shown to be a mitochondrial target for the pro-apoptotic Bcl-2 family protein, Bid (Lutter et al., 2000), and to play a critical role in mitochondrial permeabilization/cytochrome c release by tBid/Bax (Epand et al., 2002; Kuwana et al., 2002). It has been demonstrated that changes in the levels, or chemical structure through oxidation, of cardiolipin (Iverson and Orrenius, 2004) result in the creation of a soluble pool of cytochrome c that can be released into the cytosol upon permeabilization of the outer mitochondrial membrane. On the other hand, release of other mitochondrial proteins such as AIF and Endonuclease G does not seem to require cardiolipin oxidation or degradation, and there is no known interaction between these proteins and cardiolipin. Adenylate kinase-2, which is a soluble intermembrane space protein, only requires pore formation of the outer mitochondrial membrane for its release to the cytosol (Ott et al., 2002).
1.7 REGULATION OF MITOCHONDRIAL OUTER MEMBRANE PERMEABILIZATION

1.7.1 The Bcl-2 family

The Bcl-2 protein family may regulate the release of proteins and ions by influencing the permeability of the outer mitochondrial membrane as well as the endoplasmic reticulum (Sharpe et al., 2004). The Bcl-2 protein family is characterized by the presence of conserved sequence motifs, known as Bcl-2 homology (BH) domains. Anti-apoptotic members, such as Bcl-2, Bcl-XL and Bcl-w, share all four BH domains, designated BH1-4 and prevent cytochrome c release from mitochondria, while pro-apoptotic members, such as Bax and Bak, promote its release (Jurgensmeier et al., 1998; Rosse et al., 1998). The anti-apoptotic Bcl-2 family members appear to function, at least in part, by interacting with and antagonizing pro-apoptotic family members (Rosse et al., 1998; Gross et al., 1999). A third subfamily, called the BH3-only proteins, are more distantly related and also promote apoptosis, possibly by regulating the former family members. Three dominant theories for how Bcl-2 family members control membrane permeability exist (Sharpe et al., 2004); they may either form protein channels in membranes, interact with and regulate pre-existing mitochondrial membrane pores or, perhaps, alter the membrane structure by interactions with membrane lipids, notably cardiolipin.

1.7.1.1 Bcl-2 and Bcl-XL

Bcl-2 and Bcl-XL potently inhibit apoptosis in response to many cytotoxic insults. These proteins are constitutively localized on the outer mitochondrial membrane, preventing cytochrome c release and thereby inhibiting apoptosis, presumably by maintaining outer mitochondrial membrane integrity (Sharpe et al., 2004). Bcl-2 and Bcl-XL operate, at least in part, by sequestering BH3-only proteins into stable complexes and thus preventing the activation of Bax or Bak. Increased expression of Bcl-2 and Bcl-XL has been detected in several cancers, such as lymphoma, prostate, breast, lung and colorectal cancer.

1.7.1.2 Bax and Bak

Bax has been shown to trigger cytochrome c release in cells and from isolated mitochondria, opposing Bcl-2-mediated inhibition of this event (Jurgensmeier et al., 1998; Rosse et al., 1998; Finucane et al., 1999). In healthy cells, Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane. Upon induction of apoptosis, cytosolic Bax oligomerizes (Tan et al., 1999; Antonsson, 2001), translocates to the mitochondria (Hsu et al., 1997; Wolter et al., 1997; Saikumar et al., 1998) and inserts into the outer mitochondrial membrane (Goping et al., 1998). Bax oligomerization is required for mitochondrial membrane permeabilization (Antonsson et al., 2000).

Bak, on the other hand, constitutively resides within the mitochondria (Griffiths et al., 1999). Voltage-dependent anion channel 2 (VDAC2), a mitochondrial outer membrane protein, was recently found to specifically associate with Bak to keep it in a monomeric, inactive conformation in healthy cells (Cheng et al., 2003). Bak also oligomerizes upon apoptosis induction and forms complexes on the outer mitochondrial membrane, colocalizing with Bax complexes. Knockout of either Bax or Bak in mice does not result in major abnormalities, while Bax/Bak double-knockout mice die in utero with dramatic defects in development, suggesting severe impairment in apoptosis signaling (Lindsten et al., 2000). Also, cells obtained from Bax/Bak double-knockout mice are resistant to a variety of apoptotic stimuli (Cheng et al., 2001).
1.7.1.3 Bid

In response to various death stimuli, Bid can be activated by multiple proteases, including caspase-8 after initiation of the extrinsic pathway (Li et al., 1998; Luo et al., 1998), granzyme B (Barry et al., 2000; Wang et al., 2001), lysosomal enzymes (Stoka et al., 2001; Reiners et al., 2002), and calpains (Chen et al., 2001; Mandic et al., 2002). Bid is normally localized in the cytosol in an inactive form. Bid cleavage occurs within an unstructured loop (Li et al., 1998; Luo et al., 1998), and cleaved Bid (tBid) is N-myristoylated (Zha et al., 2000) before translocating to mitochondria, where it enhances apoptotic signaling. In addition to binding anti-apoptotic members of the Bcl-2 family, recent data suggest a broader role for tBid in apoptosis signaling. For example, although oligomeric Bax alone can induce cytochrome c release from isolated mitochondria, it has been suggested that tBid can synergize with Bax to induce mitochondrial permeabilization (Wei et al., 2000; Kuwana et al., 2002), as well as induce Bax (Eskes et al., 2000) and Bak oligomerization (Wei et al., 2000), resulting in permeabilization of the outer membrane and the release of cytochrome c. However, many cellular insults induce Bax activation in vivo and it is difficult to know if (t)Bid functions by binding and interacting with Bax directly or indirectly by activating apoptosis via another mechanism. Also, it has been difficult to catch Bid-Bax complexes (Grinberg et al., 2002), resulting in a “kiss and run” hypothesis (Eskes et al., 2000; Letai et al., 2002), where Bid is proposed to bind to Bax, induce a conformational change and subsequently release activated Bax.

The Bid knockout mouse (Yin et al., 1999) has less developmental defects than the Bax knockout mouse, suggesting that other BH3-only proteins can substitute for Bid in activating the developmental cell death pathways controlled by Bax. Expression of a variety of BH3-only proteins, such as Bim, Bad and Bid, in Bax/Bak double-deficient cells were unable to induce apoptosis, suggesting that BH3-only proteins require Bax or Bak to mediate apoptosis signals (Zong et al., 2001).

1.7.2 Interactions between cardiolipin and Bcl-2 family proteins

In addition to providing the second step of cytochrome c release, i.e. pore-formation in the outer mitochondrial membrane, some studies have also suggested a direct interaction between the pro-apoptotic members of the Bcl-2 family, notably Bid and cardiolipin. An alternative to the Bid-Bax “kiss and run” hypothesis mentioned above (Eskes et al., 2000; Letai et al., 2002) suggests that Bid could alter the composition or curvature of the mitochondrial lipid bilayer to allow solubilization of cytochrome c and/or to induce Bax-mediated permeabilization of the outer mitochondrial membrane. Thus, a direct interaction between Bid and Bax would not be required. It has been demonstrated that Bid interacts specifically with cardiolipin-containing membranes (Lutter et al., 2000; Kim et al., 2004) and Bid-cardiolipin interaction has been suggested to induce mitochondrial cristae reorganization at mitochondrial contact sites (Scorrano et al., 2002; Kim et al., 2004). Moreover, it has been argued that Bid can cause membrane lipid transfer activity (Esposti et al., 2001), and Bid has also been shown to preferentially bind monolysocardiolipin (Esposti et al., 2003). In addition to Bid binding directly to cardiolipin, there are some suggestions that the Bcl-2 protein family could affect cardiolipin structure indirectly. The negative charge of cardiolipin can be neutralized by Ca\(^{2+}\), causing changes in the curvature of the membrane (Rand and Sengupta, 1972). Interestingly, Bax and Bak promote the movement of Ca\(^{2+}\) from the endoplasmic reticulum to the mitochondria (Nutt et al., 2002a; Nutt et al., 2002b). It has also been suggested that Bax is dependent on the presence of cardiolipin for its pro-apoptotic functions (Kuwana et al., 2002). This is discussed in more detail below (paper III).
1.7.3 Mitochondrial Permeability Transition

Mitochondrial permeability transition (MPT) may occur during both apoptotic and necrotic cell death, but MPT seems to be most relevant during ischemia-reperfusion injury or in response to mitochondrial Ca\(^{2+}\) overload triggered by cytotoxic stimuli (Orrenius et al., 2003). MPT results in loss of the mitochondrial inner membrane potential (\(\Psi_M\)), the driving force for ATP synthesis (Crompton, 1999). A diminished \(\Psi_M\) is usually a sign of mitochondrial dysfunction, and loss of \(\Psi_M\) can have a deleterious effect on cell viability or the ability of the cell to tolerate stress. High Ca\(^{2+}\)-fluxes or oxidative stress may cause a transformation of the mitochondrial inner membrane protein adenine nucleotide translocase from its native state, a gated pore (mediating ADP/ATP exchange), into a non-selective pore. As a result, the inner membrane becomes freely permeable to small ions and metabolites, causing osmotic swelling of the mitochondria, rupture of the outer mitochondrial membrane and, subsequently, release of mitochondrial proteins such as cytochrome c and Smac/Diablo from the intermembrane space. Transient pore opening may also occur, in which case mitochondria have open pores at a given time, causing a release of mitochondrial membrane proteins, but without an observable drop in \(\Psi_M\) in the entire mitochondrial population (Szalai et al., 1999).

1.7.4 Caspase-2-mediated release of cytochrome c

During the period of this thesis work, a novel mechanism for cytochrome c release, which is mediated by caspase-2, has been proposed. Since this is part of the scope of the thesis (papers I and II), it will be discussed in more detail below.

1.8 OXIDATIVE STRESS

Oxidative stress occurs as a consequence of intracellular imbalance between pro-oxidants and antioxidants. Many pro-oxidants are reactive oxygen species (ROS), capable of modifying biomolecules such as lipids, proteins, carbohydrates and nucleotides (Djordjevic, 2004). Oxidative stress and the oxidative modification of biomolecules are involved in a number of physiological and pathophysiological processes, such as aging, atherosclerosis, inflammation, carcinogenesis, and drug toxicity.

1.8.1 Reactive oxygen species

ROS are generated by aerobic cells at all times, mainly as by-products of complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxidoreductase) activity during mitochondrial respiration (Dalton et al., 1999). Several cytokines, growth factors, hormones, and neurotransmitters use ROS as second messengers in intracellular signal transduction. However, excess ROS may react with and modify cellular macromolecules and critical cellular targets that cause behavioral abnormalities, cytotoxicity, and mutagenic damage (Fridovich, 1978; Floyd, 1990; Sahu, 1990; Sohal and Allen, 1990; Floyd, 1991). The most common ROS are the superoxide radical anion (\(O_2^{-}\)), the non-radical hydrogen peroxide (H\(_2\)O\(_2\)) and the hydroxyl radical (HO\(^{\cdot}\)). Superoxide radicals are dismutated to hydrogen peroxide, which subsequently might be transformed into the hydroxyl radical (HO\(^{\cdot}\)) by the Fenton reaction, a reaction catalyzed by metal ions (Fe\(^{2+}\) or Cu\(^{2+}\)). The hydroxyl radical (HO\(^{\cdot}\)) is highly unstable, and is considered to be the most reactive radical (Djordjevic,
O$_2^-$ is less reactive than HO\(^\cdot\), but is potentially more damaging because of its ability to diffuse some distance before coming across the oxidative target. In fact, approximately 1-2% of the total oxygen consumed during electron transport is reduced to O$_2^-$.

Hydrogen peroxide is relatively stable and may, in lesser amounts, be involved in cell signaling cascades. It is suggested that an H$_2$O$_2$ concentration that is four times higher than normal could have a toxic effect (Djordjevic, 2004). ROS production is normally increased when cells become exposed to environmental oxidants, such as ultraviolet and ionizing radiation, heavy metals, redox active chemicals, anoxia, and hyperoxia (Becker et al., 1991; Carney et al., 1991; Floyd, 1991; Troll, 1991).

### 1.8.2 Lipid peroxidation

Lipid peroxidation is initiated by an electrophilic attack by the radical on one of the hydrogens at a carbon double bond in the fatty acyl chain (Djordjevic, 2004). Hydroxyl radicals (HO\(^\cdot\)) can initiate a chain reaction of lipid peroxidation by abstracting a hydrogen atom from unsaturated fatty acids (LH), leading to the generation of lipid radicals, which subsequently may combine with molecular oxygen to form a peroxyl radical (L\(^\cdot\)→LOO\(^\cdot\)), that may react with nearby lipids to form a lipid hydroperoxide and another carbon-centered lipid radical. The radicals formed propagate a radical chain reaction of lipid peroxidation (LOOH) until the free radical is scavenged by an antioxidant. The major membrane phospholipid components, such as cardiolipin, are rich in unsaturated fatty acids, which are particularly susceptible to oxygen radical attack (Imai and Nakagawa, 2003). Lipid hydroperoxides are also produced enzymatically by lipoxygenases, such as 15-lipoxygenase and cyclooxygenase. The different types of ROS may oxidize various substrates. However, due to their reactivity, proximity will probably determine the ROS target, and since cardiolipin is close to the main ROS production sites, i.e., the respiratory chain, it may be especially vulnerable even to minor changes in the ROS homeostasis.

### 1.8.3 Protein oxidation

Increased ROS production, or diminished ROS scavenging, can modulate the redox status of the cell. Many proteins contain amino acids with side chains that may be reduced or oxidized. Cysteine residues, in which the thiol side chain can be in the -SH state, or a -S-S- cystine disulfide, are among the most easily oxidized residues in proteins. This may result in intermolecular protein cross-linking, which may have considerable influence on the protein structure. Therefore, affecting the cysteine oxidation state is a potential way of modulating protein structure, function and activity (Coan et al., 1992; Cotgreave and Gerdes, 1998). However, formation of protein disulfides or protein-glutathione mixed disulfides may also prevent irreversible oxidative damage of the proteins and hence protect protein function and/or activity.

### 1.9 GLUTATHIONE

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is the most abundant low molecular weight thiol in the eukaryotic cell. It is present in millimolar concentrations (1 to 10 mM) in most mammalian cells (Meister, 1988), and it is also widely conserved among species throughout evolution, from microorganisms to plants and mammals. GSH provides a first line of defence against ROS, as it can protect reduced cysteine thiols on the surface of proteins, reduce disulfides to thiols, or scavenge free radicals and reduce H$_2$O$_2$. In addition, GSH-dependent enzymes are able to detoxify ROS.
products and prevent propagation of free radicals. GSH, together with its oxidized counterparts, mainly the glutathione disulfide (GSSG) and protein-GSH mixed disulfides, constitute the redox buffer of the cell (Schafer and Buettner, 2001). The GSH/GSSG ratio thus reflects the intracellular redox status. Normally, this ratio is 100 : 1 in resting cells (Schafer and Buettner, 2001). However, the equilibrium may shift down to 10 : 1 upon changes of the cellular redox potential, resulting in the formation of protein-GSH mixed disulfides or protein disulfides, possibly leading to alterations in protein structure and/or activity (Schuppe et al., 1992; Cotgreave et al., 2002). Reduction of disulfides and mixed disulfides, and reversion to the original (active) protein conformation, is typically mediated by redox enzymes such as thioredoxin, glutaredoxin, and protein-disulfide isomerases. The GSH/GSSG ratio is maintained by de novo synthesis of GSH, cellular excretion of GSSG, and reduction of GSSG back to GSH by glutathione reductase (GR), at the expense of NADPH. GR activity has been found in the cytosol as well as in mitochondria.

Apart from scavenging free radicals and maintaining the redox balance of the cell, GSH is involved in redox reactions, detoxification of xenobiotics and carcinogens, and the biosynthesis of DNA, proteins and leukotrienes (Schafer and Buettner, 2001). Further, protein glutathionylation may have a regulatory posttranslational effect on protein function or activity (Davis et al., 1997; Obin et al., 1998), and GSH has been found to work as a second messenger (Huang and Huang, 2002) and neurotransmitter (Oja et al., 2000). Also, glutathionylation or de-glutathionylation of key proteins may be a control-point for certain redox-sensitive gene expression (Cotgreave and Gerdes, 1998).

### 1.9.1 GSH metabolism

GSH is synthesized from glutamate, cysteine and glycine by the consecutive actions of the ATP-dependent enzymes $\gamma$-glutamylcysteine synthetase (glutamate-cysteine ligase) and GSH synthetase (Meister, 1988). GSH is degraded by $\gamma$-glutamyltranspeptidase and dipeptidases. The most widely used inhibitor of GSH synthesis is buthionine sulfoximine (BSO), which binds to $\gamma$-glutamylcysteine synthetase (Griffith and Meister, 1978; Griffith, 1982). Some cells may also export GSH. The liver is the major organ for synthesis and export of GSH into the plasma (Bray and Taylor, 1993). Also, when cells or tissues are subjected to oxidative stress, GSSG efflux has been observed (Sies and Akerboom, 1984). A putative reason for the export of GSSG may be to maintain the GSSG/GSH ratio and thus sustain a favourable redox environment in the cell (Schafer and Buettner, 2001). However, the GSH and GSSG levels found outside the cell are generally low, usually 100 to 1000 times less than intracellular GSH. GSH is not readily taken up by cells, but is broken down to its constituent amino acids, which can cross the plasma membrane and be used for intracellular GSH synthesis. The compartmentalization of GSH is discussed in this thesis (paper IV).

### 1.9.2 GSH and apoptosis

Depletion of intracellular GSH, and a concomitant increase in ROS, has been suggested to be an important part of apoptotic signaling (Bustamante et al., 1997; Macho et al., 1997; Tan et al., 1998). GSH decrease during apoptosis has been shown to be due to an increased rate of GSH efflux (Ghibelli et al., 1995; van den Dobbelsteen et al., 1996). Inhibition of glutathione efflux could rescue cells, suggesting that, at least in some cell types, the control of GSH homeostasis could have a central role in the regulation of cell death signaling. Most cells appear to be able to tolerate a reduction in GSH levels of up to about 90% without adverse consequences (Hall, 1999). Depletion of cytosolic GSH
by BSO may, however, induce a switch from apoptotic to necrotic cell death (Fernandes and Cotter, 1994).

1.10 THE GLUTAREDOXIN SYSTEM

Glutaredoxins (Grxs) are a family of low molecular weight thiol-disulfide oxidoreductases that catalyze GSH-dependent thiol-disulfide reactions, usually with a high specificity for protein-GSH mixed disulfides (Fernandes and Holmgren, 2004). Grx was originally identified in 1976 as a GSH-dependent dithiol hydrogen donor for ribonucleotide reductase in an *Eschericia coli* mutant lacking thioredoxin (Trx1) (Holmgren, 1976). There are two Grxs known to exist in humans: Grx1 and Grx2 (Padilla *et al*., 1995; Gladyshev *et al*., 2001; Lundberg *et al*., 2001).

1.10.1 The active site and the thioredoxin fold

Grxs are highly conserved throughout evolution, particularly in the region of their active site. They exist in various organisms, from prokaryotes and viruses to plants and mammals. They are structurally similar to thioredoxins (Trxs), and share a typical fold, the Trx fold, which consists of a central core of a four-stranded mixed β-sheet surrounded by three α-helices (Figure 5) (Martin, 1995). The Trx fold, described in 1975 by Holmgren *et al*., is shared among the Grxs, Trxs, protein disulfide isomerases, glutathione-S-transferases, glutathione peroxidases, peroxiredoxins, and the bacterial Dsb family (Martin, 1995; Kemmink *et al*., 1996; Choi *et al*., 1998). In addition, they share three highly conserved features: two redox active cysteines in a conserved CXXC active site motif; a GSH binding site; and a hydrophobic surface area involved in

Figure 5. Structural prediction of Grx2, containing the typical Thioredoxin fold. The model is based on the known structures of pig Grx2, human Grx1, and *E. coli* Grx3 using the Swiss-Model Service.
substrate binding (Xia et al., 1992; Bushweller et al., 1994; Yang et al., 1998). Grxs from most species contain a CPYC (Cys-Pro-Tyr-Cys) active site motif, located at the N-terminus of one of the α-helices (Fernandes and Holmgren, 2004). However, human Grx2 contains the active site motif CSYC, Cys-Ser-Tyr-Cys, (Gladyshev et al., 2001; Lundberg et al., 2001), reflecting different functions of the human glutaredoxins.

1.10.2 Catalytic mechanisms of Glutaredoxins

Grxs use one or both cysteines in the CXXC active site motif in order to catalyze two types of reaction (Figure 6). In the dithiol reaction mechanisms, Grx2 reduces protein disulfides at the expense of GSH (Holmgren, 1979b, a), while in the monothiol reactions, Grx2 reduces mixed disulfides between proteins and GSH (glutathionylation/de-glutathionylation) (Bushweller et al., 1992). In the dithiol reaction mechanism, the N-terminal cysteines in the active site initiate a nucleophilic attack on one of the cysteines in the target protein disulfide, resulting in the formation of a mixed disulfide between Grx and the target protein. The C-terminal cysteine in the active site becomes deprotonated and subsequently initiates a nucleophilic attack on the N-terminal active site cysteine, and as a result oxidized Grx (Grx-S2) and a reduced protein target are produced. Grx-S2 is reduced by two molecules of GSH via a protein-GSH mixed disulfide intermediate. In the monothiol reaction mechanism, reduction of protein-GSH mixed disulfides, only the N-terminal cysteine of the active site participates in the reaction mechanism (Bushweller et al., 1992; Yang et al., 1998). The active site cysteine exerts a nucleophilic attack on the GSH moiety of the protein-SG complex, thus releasing the reduced protein target. A Grx-SG mixed disulfide is generated, which in turn is reduced by GSH. The resulting GSSG is thereafter reduced to free GSH by GR. The monothiol reaction mechanism thus demonstrates a more general substrate specificity, since Grx recognizes and binds the GSH moiety of the protein-GSH mixed disulfide, not the protein target itself.

Figure 6. Catalytic mechanisms of the Glutaredoxins. In the dithiol reaction, Grx reduces protein disulfides by using both active site cysteines. In the monothiol mechanism, reduction of glutathionylated proteins by Grx requires only the N-terminal active site cysteine.
1.10.3 Glutaredoxin 2

Two different human glutaredoxins have been identified. The cytosolic 12kDa Grx1 has been well characterized and has a growing list of functions, including cellular differentiation, regeneration of transcription factor binding activity and apoptosis (Holmgren, 2000). Human Grx2 was discovered in 2001 (Gladyshev et al., 2001; Lundberg et al., 2001). Alternative splicing of the grx2 gene generates two different isoforms of Grx2 mRNA, corresponding to proteins with mitochondrial (Grx2a) and possibly nuclear (Grx2b) localizations (Lundberg et al., 2001). The species similarity among human, rat, and mouse Grx2 is approximately 70% and human Grx2 contains classical Grx features, including the CXXC active site, a hydrophobic surface area, and a GSH binding site (Lundberg et al., 2001). This thesis focuses on the mitochondrial form of Grx2 (papers V and VI).

The uncleaved form of mitochondrial Grx2 is 18kDa (16kDa in its processed form). The C-terminal 125 residue core is shared between the two variants, while the N-terminal extensions are different. The N-terminus of the mitochondrial form of Grx2 contains a mitochondrial localization signal that is cleaved and disposed of after transport into the mitochondria. The fact that Grx2 contains a different active site than Grx1 (CSYC instead of CPYC), as well as the mitochondrial, and possibly nuclear localization (Grx2), instead of cytosolic localization (Grx1), indicates different functions of the human glutaredoxins.

Grx2 expression has been found in several tissues, especially those constitutively exposed to oxidative stress (i.e. heart, skeletal muscle, kidney, lung and liver) (Lundberg et al., 2001; Lundberg et al., 2004). Although not characterized in detail, Grx2 has been shown to be active in a standard assay for glutaredoxins, using the GSH-coupled reduction of hydroxyethyl disulfide (HED) or dehydroascorbate, with 10-fold lower specific activity than Grx1 (Lundberg et al., 2001). However, Grx2 has a more specialized reactivity toward glutathionylated proteins, which could be of importance by influencing mitochondrial ROS production as well as cellular ROS scavenging (Johansson et al., 2004). In fact, a recent study by Beer et al. (2004) demonstrated that Grx2 is capable of catalyzing deglutathionylation / glutathionylation of mitochondrial proteins at a wide range of GSSG/GSH levels. Moreover, Grx2 can also receive electrons from both cytosolic and mitochondrial TrxR, emphasizing the importance of functional Grx2 during cellular stress (Johansson et al., 2004). Modulation of cell death signaling by Grx2 is discussed in this thesis (paper V and VI).

1.11 OTHER MITOCHONDRIAL REDOX ENZYMES INVOLVED IN APOPTOSIS

Several redox systems are present in mitochondria apart from Grx2, including glutathione peroxidase, mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx), Mn-superoxide dismutase (Mn-SOD), and the mitochondrial thioredoxin system, consisting of thioredoxin 2 (Trx2), thioredoxin reductase 2 (TrxR2) and peroxiredoxin III (PrxIII) (Imai and Nakagawa, 2003; Djordjevic, 2004). Overexpression and/or knockdown of several of these enzymes were shown to modulate cellular susceptibility to apoptosis, presumably by influencing ROS production and/or cardiolipin oxidation and thereby cytochrome c release. Each antioxidant enzyme has a distinct substrate specificity and mitochondrial distribution, suggesting that each antioxidant enzyme might have an independent role or that the
enzymes might cooperate with each other. Strikingly, there is not as much redundancy as one can expect, since apoptosis signaling may be affected by modulating the enzyme levels independently.

1.11.1 Mn-SOD

As mentioned above, superoxide radicals are readily dismutated by mitochondrial superoxide dismutase (Mn-SOD), leading to the production of hydrogen peroxide and oxygen (Djordjevic, 2004). In addition, SOD also exists extracellularly (EC-SOD) as well as in the cytosol (Cu,Zn-SOD). Cu,Zn-SOD has also been found in the mitochondrial intermembrane space. Both inducible and basal levels of ROS induce Mn-SOD expression, which protects mitochondria from ROS toxicity. Modulation of Mn-SOD levels have shown that increasing Mn-SOD levels prevents superoxide production and subsequent apoptosis (Manna et al., 1998; Bruce-Keller et al., 1999), while heterozygous Mn-SOD-deficient mice (Sod2 -/+) exhibited increased superoxide levels and enhanced apoptosis following excitotoxic damage (Murakami et al., 1998; Fujimura et al., 1999).

1.11.2 Catalase

Elimination of hydrogen peroxide by catalase and glutathione peroxidase is critical to the efficacy of SOD in reducing oxidative stress. Catalase is mainly localized in peroxisomes, but can also be found in heart mitochondria (Djordjevic, 2004). Overexpression of catalase, including a mitochondrial localization signal, prevented oxidant-induced toxicity and apoptosis in HepG2 cells. However, since most tissues lack mitochondrial catalase (Bai and Cederbaum, 2001), peroxisomal catalase is most likely of greater importance during oxidative stress and apoptosis. In fact, catalase is believed to protect cells by increasing p53-degradation, not by affecting mitochondrial signaling (Bai and Cederbaum, 2003).

1.11.3 Gpx and PHGPx

Glutathione peroxidases (Gpx) are selenocysteine-containing enzymes with a general specificity for hydroperoxides and a high specificity for reduced GSH. It reduces H₂O₂ and organic alkyl hydroperoxides to water and corresponding alcohols, and GSH is oxidized to the corresponding disulfide (GSSG). GSSG is subsequently reduced to GSH by glutathione reductase at the expense of NAD(P)H. Classical glutathione peroxidase, cGpx1, is present in both the cytosol and mitochondria of various mammalian tissues, while phospholipid hydroperoxide glutathione peroxidase (PHGPx) exists in the nucleus, mitochondria, and cytosol (Ursini et al., 1995; Imai and Nakagawa, 2003). It has been proposed that while cGpx is important in removing cytosolic hydroperoxides, PHGPx catalyzes the reductive inactivation of lipid hydroperoxides in membranes and lipoproteins and thus protects cellular membranes against oxidative damage (Thomas et al., 1990). PHGPx is synthesized as a long form (L-form; 23 kDa), which contains a mitochondrial localization signal, and a short form (S-form, 20 kDa) (Pushpa-Rekha et al., 1995). Several studies have shown that overexpression of PHGPx protects against apoptosis (Imai et al., 1996; Yagi et al., 1996; Arai et al., 1999; Nomura et al., 1999; Brigelius-Flohe et al., 2000; Nomura et al., 2000). Interestingly, mitochondrial PHGPx was shown to inhibit apoptosis by protecting cardiolipin from oxidation, which, in turn, prevented cytochrome c release (Arai et al., 1999; Nomura et al., 1999; Nomura et al., 2000). However, the mitochondrial form of this enzyme is mainly expressed in testis (Pushpa-Rekha et al.,
Hence, a general physiological significance of the mitochondrial-specific protection remains to be established.

### 1.11.4 Peroxiredoxin

Peroxiredoxin (Prx) can reduce hydrogen peroxide by using a redox-sensitive active site cysteine residue of each subunit of the Prx homodimer (Chang et al., 2004). The cysteine thiol is oxidized to cysteine sulfenic acid (Cys-SOH), which then reacts with a neighboring cysteine thiol of the other subunit to form an intermolecular disulfide. This disulfide is reduced specifically by Trx, which, in turn, is reduced by TrxR at the expense of NADPH. The mitochondrial localization of PrxIII (Chae et al., 1999), together with the identification of its mitochondria-specific electron suppliers, Trx2 and TrxR2 (Lee et al., 1999), suggest that these three proteins might provide a primary line of defense against H₂O₂ produced by the mitochondrial respiratory chain (Chang et al., 2004). In fact, overexpression of PrxIII protected WEHI7.2 thymoma cells from pro-oxidant-induced apoptosis, while depletion of PrxIII in HeLa cells by RNA interference caused an increase in apoptosis susceptibility (Nonn et al., 2003; Chang et al., 2004). Modulation of the mitochondrial Trx2 or TrxR2 level also affects apoptotic signaling (Chen et al., 2002; Damdimopoulos et al., 2002; Patenaude et al., 2004).
2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY

The release of cytochrome \textit{c} from mitochondria is a key event in apoptosis signaling. The main goal of this thesis was to investigate different perspectives of mitochondrial signaling during apoptosis, with a focus on DNA damage-induced, caspase-2-mediated, cytochrome \textit{c} release as well as modulation of this release by the oxidative environment of mitochondria (\textit{e.g.} the GSH redox system).

\textit{The specific aims were as follows:}

✓ To assess how etoposide-induced DNA damage mediates mitochondria-dependent apoptosis, leading to the release of cytochrome \textit{c}.

✓ To investigate how caspase-2 can mediate cytochrome \textit{c} release from mitochondria, and to study if caspase-2 can disturb the cytochrome \textit{c} - cardiolipin interaction.

✓ To assess if cardiolipin was necessary also for Bax-mediated cytochrome \textit{c} release.

✓ To demonstrate the importance of the mitochondrial GSH system during oxidative stress and apoptosis.
2.2 METHODOLOGY

The techniques used in this thesis project are described in detail in papers I-VI. Here, materials and methods will be listed and briefly commented upon.

2.2.1 Cell Culture

In paper I and II, Jurkat cells, a human T cell lymphoma cell line, was used. In paper IV - VI, HeLa cells, a human cervix epithelial adenocarcinoma cell line, was used. In addition, A549 cells, a lung epithelial carcinoma cell line, was also utilized in paper IV. All cell lines were purchased from ATCC. Jurkat cells were cultured in RPMI 1640 medium, while HeLa and A549 cells were cultured in DMEM medium. Both types of media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in humidified air at 37 °C and 5% CO₂, and kept in logarithmic growth phase at all times.

2.2.2 Yeast culture

Yeast mitochondria were used as a model system in paper III, due to the availability of a cardiolipin-deficient strain of *Saccharomyces cerevisiae*, ∆crd1 strain, which was kindly provided by Miriam L. Greenberg. Standard methods were used for culture of the homozygous diploid deletion strain ∆crd1 and the isogenic wild type strain BY4743 (Giaever et al., 2002). Yeast were grown aerobically as described in paper III.

2.2.3 Isolation of Yeast Mitochondria

Yeast spheroplasts were generated from zymolyase-treated cells as described previously (Daum et al., 1982). Isolation of mitochondria was performed essentially as described previously with minor modifications. Details are described in paper III.

2.2.4 Liposome preparation

Liposomes or large unilamellar vesicles (LUVs) were used as a model for the study of protein interactions with lipid membranes in papers II and III. The liposomes were prepared by using a lipid extruder according to previously published protocols (Buser and McLaughlin, 1998). For details, see Experimental Procedures section in papers II or III.

2.2.5 Transfection methods

In paper VI, cells overexpressing the mitochondrial form of Grx2 (mGrx2-HeLa cells) and truncated form (tGrx2-HeLa cells) were established by transfection of HeLa cells using FuGene6 transfection reagent. Selection for G418 resistance ensured survival of only successfully transfected cells, and individual clones were isolated by trypsinization using cloning cylinders. For further details, see Materials and Methods in paper VI.

2.2.6 Western blotting

A standard western blotting procedure was used for the immunodetection of proteins in papers I-III and VI. In paper I and II, cytosolic extracts from Jurkat cells were prepared.
by using S-100 hypotonic buffer. However, in paper VI the separation of HeLa cytosol was enhanced by using a slightly different protocol with digitonin as the membrane permeabilizing agent. In both cases, centrifugation was used for separation of the cytosol from the other intracellular organelles. Exclusion of Trypan blue was used for optimization of the fractionation protocol. Samples mixed with conventional Laemmli’s buffer were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with antibody against cytochrome c, caspase-2, Smac/Diablo, AIF and GAPDH. The membranes were further incubated with a horseradish peroxidase-conjugated secondary antibody and bound antibodies were detected using chemiluminescence. In papers II, III and VI, densitometry was used for quantification of the target protein content.

2.2.7 Flow cytometry

Flow cytometry was done utilizing a fluorescence-activated cell sorter analysis (FACScan, Becton Dickinson) with a 488-nm laser line. The results were analysed by Cell Quest (paper I and IV) or WinMDI 2.8 software (paper VI).

2.2.7.1 PS Exposure
During apoptosis, phosphatidylserine (PS) is translocated to the outer leaflet of the plasma membrane in most cell lines. In paper I, PS exposure was measured by a conventional Annexin V-binding assay. Annexin V is membrane impermeable and thus only stains cells with PS exposed on the outside of the plasma membrane. Necrotic cells with disrupted plasma membrane were distinguished by propidium iodide uptake.

2.2.7.2 GSH visualization
In paper IV, basal GSH levels and protein-GSH mixed disulphides were measured in Jurkat cells and rat liver mitochondria. The samples were fixed prior to binding of the anti-GSH antibody. In addition, the GSH levels during different cell cycle stages were assessed using double staining of GSH and DNA, the latter visualized by propidium iodide (PI) staining, see below.

2.2.7.3 Propidium iodide staining
Loss of PI staining was used for estimation of the level of apoptosis in cell culture, as apoptotic cells or bodies demonstrate lower DNA content. One molecule of PI binds DNA every four to five base pairs, and this dye can thus be used for quantification of cells with increased sub-G1 DNA content (paper VI) as well as for cell cycle analysis (paper IV). The probe was allowed to enter the cell by permeabilization with Triton X-100 over night. Fluorescence was detected in the FL-3 channel.

2.2.7.4 Cardiolipin measurement
Cardiolipin oxidation or degradation has previously been widely studied using different methods. One of these methods is staining with 10-N-nonyl acridine orange (NAO), which has a high affinity to cardiolipin (Petit et al., 1992). NAO has been used since it interacts with reduced cardiolipin, but not with the oxidized form of cardiolipin (Maftah et al., 1990; Nomura et al., 2000). This method has been partly questioned as unspecific and depending on the mitochondrial membrane potential (Keij et al., 2000; Jacobson et al., 2002). However, this was later opposed by others, claiming no significant correlation between loss of membrane potential and NAO staining of cardiolipin in isolated rat liver mitochondria. In paper VI, NAO was used for the estimation of loss of cardiolipin after apoptosis induction in HeLa cells according to a previously published protocol (Nomura et al., 2000).
2.2.8 ELISA

A specific and sensitive sandwich enzyme linked immunosorbent assay (ELISA) was used in paper VI for evaluation and final selection of the Grx2 overexpressing clones. The polyclonal antibodies used were raised against the truncated form of Grx2, thus recognizing both gene products overexpressed in the study (Lundberg et al., 2004). The secondary antibodies were conjugated with biotin. Alkaline phosphatase-conjugated streptavidin, which binds strongly and specifically to biotin, was subsequently added to the wells. Grx2 content in the samples was calculated by addition of a colourless substrate, which is is converted into a coloured product by alkaline phosphatase that is bound to the biotinylated antibodies. The absorbance of this product is measured and related to a standard curve.

2.2.9 Fluorescence microscopy

Visualization of Grx2 in papers V and VI was achieved by staining with anti-Grx2 antibodies and AlexaFluor 488 secondary antibody. Mitochondria were visualized by addition of 50 nM Mitotracker Red. Analysis was done by fluorescence microscope (Olympus BX60) and images were collected with a C4742-95-10SC digital camera.

2.2.10 Caspase assay

Caspase activity was determined by the cleavage of 7-amino-4-methylcoumarin (AMC), which becomes fluorescent after cleavage. The specificity is determined by the substrate tetra- or pentapeptide coupled to AMC. The DEVD-AMC substrate (papers I, II and VI) is cleaved by caspase-3 and-7 and LEHD is cleaved by caspase-9 (paper I), while only caspase-2 has a preference for VDVAD-AMC (paper I). However, some influence of the other caspases to cleave these substrates, although with significantly lower specificity, cannot be completely excluded.
3 SUMMARY OF THE PAPERS

3.1.1 Paper I

Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis.

Treatment of the cells with the chemotherapeutic agent etoposide induces DNA damage, thereby triggering the onset of apoptosis. Among the early changes observed is the release of cytochrome c from mitochondria, although the mechanism responsible for this effect is unclear. Our laboratory (Robertson et al., 2000) had previously demonstrated that etoposide-induced apoptosis was a caspase-mediated event. In this study, we demonstrated that caspase-2 could function as an upstream modulator for the mitochondrial apoptotic pathway after etoposide treatment. Pre-treatment of Jurkat cells with an irreversible caspase-2 inhibitor caused an inhibition of cytochrome c release after etoposide-treatment. In addition, transfection of Jurkat cells with a pro-caspase-2 anti-sense construct (Casp-2/AS cells) had the same effect, suggesting caspase-2 as an important factor in the DNA damage-induced apoptosis. Moreover, using cell-free system experiments, we showed that etoposide-induced cytochrome c release by way of caspase-2 occurs independently of cytosolic factors, suggesting that the nuclear pool of pro-caspase-2 is critical to this process. Also downstream events, such as pro-caspase-9 and -3 activation, phosphatidylserine exposure on the plasma membrane, and DNA fragmentation were inhibited in Casp-2/AS cells, or cells treated with caspase-2 inhibitor. Taken together, our data indicate that caspase-2 may provide an important link between etoposide-induced DNA damage and cytochrome c release.

3.1.2 Paper II

Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids.

In view of the conclusions presented in paper I, as well as another study from our laboratory (Robertson et al., 2004), demonstrating that fully processed caspase-2 can permeabilize the outer mitochondrial membrane, causing release of cytochrome c and Smac/DIABLO, we investigated this caspase-2-mediated effect further by using permeabilized cells, isolated mitochondria, and protein-free liposomes. We found that recombinant caspase-2 exerts a direct effect on mitochondria, which neither depends on the presence or cleavage of other proteins nor on a specific phospholipid composition of the liposomal membrane. Treatment of rat liver mitochondria or permeabilized Jurkat cells with caspase-2 stimulated a fast and dose-dependent release of cytochrome c. However, mutant caspase-2, unable of autoprocessing, was not capable of exerting the same effect. Experiments with protein-free liposomes revealed that caspase-2 could have a pore-forming effect on liposomes, thereby releasing entrapped dextran molecules or cytochrome c independently of the specific phospholipid composition of the membrane. Treatment of rat liver mitochondria or permeabilized Jurkat cells with caspase-2 stimulated a fast and dose-dependent release of cytochrome c. However, mutant caspase-2, unable of autoprocessing, was not capable of exerting the same effect. Experiments with protein-free liposomes revealed that caspase-2 could have a pore-forming effect on liposomes, thereby releasing entrapped dextran molecules or cytochrome c independently of the specific phospholipid composition of the membrane. Interestingly, caspase-2 was also shown to disrupt the interaction of cytochrome c with anionic phospholipids, notably cardiolipin, from liposomes with cytochrome c bound to the outside of the vesicle membrane. In addition, processed caspase-2 enhanced the release of cytochrome c caused by digitonin or oligomeric Bax.
Combined, our data suggest that caspase-2 possesses an unparalleled ability to engage the mitochondrial apoptotic pathway by permeabilizing the outer mitochondrial membrane and/or by breaching the association of cytochrome c with the inner mitochondrial membrane.

### 3.1.3 Paper III

**Cardiolipin is not required for Bax-mediated cytochrome c release.**

As mentioned earlier, our laboratory and others suggest that cardiolipin is required for the binding of cytochrome c to the inner mitochondrial membrane and thereby for the maintenance of cytochrome c within the mitochondria. It has been demonstrated that changes in the levels, or chemical structure through oxidation, of cardiolipin result in the creation of a soluble pool of cytochrome c that can be released into the cytosol upon permeabilization of the outer mitochondrial membrane (Iverson and Orrenius, 2004). A seemingly contradictory view is the hypothesis that cardiolipin is required for the release of cytochrome c and other mitochondrial proteins, supported by a previous study showing that cardiolipin is required for Bid/Bax-mediated pore formation in liposomes (Kuwana et al., 2002). We addressed this by comparing the spontaneous- and Bax-mediated cytochrome c release from mitochondria isolated from two strains of *Saccharomyces cerevisiae*, one lacking cardiolipin (ΔCRD1) and the corresponding wild type (WT). We show that the mitochondrial association of Bax and the resulting cytochrome c release is not dependent on the cardiolipin content of the yeast mitochondrial membranes, and that phosphatidylglycerol can partially substitute for cardiolipin in cardiolipin-deficient mitochondria. Furthermore, using models of synthetic liposomes and isolated yeast mitochondria, we found that cytochrome c was bound more "loosely" to the cardiolipin-deficient systems compared with when cardiolipin is present. These data support the two-step model of cytochrome c release (Ott et al., 2002), which suggests that cardiolipin is required for binding cytochrome c, thereby limiting its release in the event of pore formation in the outer mitochondrial membrane.

### 3.1.4 Paper IV

**Visualization of the compartmentalization of glutathione and protein-glutathione mixed disulfides in cultured cells.**

As mentioned above, it has been shown that cardiolipin might become oxidized and lose its interaction with cytochrome c as a result of increased ROS production, or deficient ROS scavenging within the mitochondria (Shidoji et al., 1999; Nomura et al., 2000; Asumendi et al., 2002; Ott et al., 2002; Chang et al., 2004). The GSH system is one of the most important systems in protection against oxidative stress, and several studies have shown that variation of the GSH level can modulate apoptosis (Ghibelli et al., 1995; van den Dobbelsteen et al., 1996; Bustamante et al., 1997). In this study, we demonstrate a novel, specific method for visualizing GSH, and glutathionylated proteins, by FACS or fluorescence and confocal microscopy. We have shown that the GSH levels in the cytosol and nucleus are similar, while the mitochondria contain 1.5-2-fold more GSH. In addition, we have shown that mitochondria are potent scavengers of GSH. Treatment with buthionine sulfoximine (BSO), causing inhibition of GSH synthesis and thus depletion of GSH in cytosol and nuclei, had only a minor effect on mitochondria, where GSH was preserved. We also demonstrate cell-cycle variation in GSH content of the cells, with the highest levels of GSH associated with the G2/M
mitotic phase of the cell cycle. Similar FACS analyses performed in isolated mitochondria presented a considerable variation in GSH content within mitochondria of uniform granularity from the same preparation.

3.1.5 Paper V

Short interfering RNA-mediated silencing of Glutaredoxin 2 increases the sensitivity of HeLa cells toward doxorubicin and phenylarsine oxide.

GSH is a poor reductant by itself, but utilizes a number of enzymes, including the glutaredoxins (Grx1 and Grx2), to exert its antioxidant effect. We have previously identified a novel dithiol glutaredoxin, human glutaredoxin 2 (Grx2) (Lundberg et al., 2001), which is present in the mitochondria and thereby differs from the cytosolic Glutaredoxin 1 (Grx1). Interestingly, Grx2 differs from the Grx1 by its higher affinity toward S-glutathionylated proteins and by being a substrate for thioredoxin reductase. In addition, Grx2 is also functional in enzyme assays after being exposed to oxidative conditions (increased GSSG/GSH ratio) while Grx1 loses its activity. Thus, Grx2 is less sensitive for oxidative conditions. Here, we have successfully established a method to silence the expression of Grx2 in HeLa cells by using short interfering RNA in order to study its role in the cell. Cells with levels of Grx2 <3% of the control were dramatically sensitized to cell death induced by doxorubicin/adriamycin and phenylarsine oxide. However, no differences were detected after treatment with cadmium, a known inhibitor of Grx1. These results indicate a role for Grx2 in the regulation of the mitochondrial redox status and of cell death at the mitochondrial checkpoint.

3.1.6 Paper VI

Overexpression of Glutaredoxin 2 attenuates apoptosis by preventing cytochrome c release.

To further investigate this putative mitochondrial protective effect of Grx2, we overexpressed the enzyme in HeLa cells and studied the effect of Grx2 on their sensitivity to apoptosis induction, in particular how Grx2 could influence cytochrome c release and cardiolipin oxidation. We have overexpressed Grx2 in HeLa cells in its mitochondrial form (mGrx2-HeLa) as well as a truncated cytosolic form, lacking the mitochondrial translocation signal (tGrx2-HeLa). The resulting clones were less susceptible to apoptosis induced by 2-deoxy-D-glucose (2-DG) or doxorubicin (Dox). Overexpression of Grx2 inhibited cytochrome c release and caspase activation induced by both agents. In addition, Grx2 prevented 2-DG- and Dox-induced loss of cardiolipin, thus inhibiting the first step in the two-step process of cytochrome c release. Overexpression of mGrx2 provided better protection than tGrx2 overexpression, especially after treatment with 2-DG. We propose that Grx2 facilitates the maintenance of cellular redox homeostasis upon treatment with apoptotic agents, thereby preventing cardiolipin oxidation and cytochrome c release.
4 GENERAL DISCUSSION

The importance of cytochrome c release during apoptosis signaling has been established for many years, even though the specific mechanism has been, and still is, under debate. As mentioned before, our laboratory suggested that cytochrome c release occurs via a two-step process, involving dissociation of cytochrome c from cardiolipin and pore-formation of the outer mitochondrial membrane, normally by pro-apoptotic members of the Bcl-2 family (Ott et al., 2002). Even though it has been known that cardiolipin binds cytochrome c to the inner mitochondrial membrane, cardiolipin has not been in the focus of attention within the apoptosis field until quite recently. However, the correlation between ROS increase, cardiolipin oxidation, detachment of cytochrome c from cardiolipin and subsequent cytochrome c release is presently well established (Iverson and Orrenius, 2004).

Cytochrome c release mediated by caspase-2

During the course of this study, we discovered that caspase-2 mediated cytochrome c release in etoposide-treated Jurkat cells (paper I). This was supported by several other groups, showing that caspase-2 activation induced by DNA-damaging agents (Lassus et al., 2002), or overexpression of caspase-2 (Guo et al., 2002; Paroni et al., 2002), triggered apoptosis via the mitochondrial pathway. However, the specific mechanism of caspase-2-mediated cytochrome c release was under debate. Although our laboratory claimed that the effect was dependent on caspase-2 only, others suggested that the Bcl-2 family proteins, such as Bax and tBid, were involved in the mechanism. However, our recent observation revealed that caspase-2-mediated release of cytochrome c occurs in Bax/Bak double knockout MEF mitochondria, suggesting that these proteins are not required for this mechanism. Moreover, caspase-2 exerted a direct effect on mitochondria, independently of both its proteolytic activity and the involvement of the Bcl-2 family members (Robertson et al., 2004). In paper II, we demonstrated that caspase-2 had a pore-forming effect on the lipid membranes, thus ruling out the possibility of a target protein interaction. Additional experiments indicated that caspase-2 could also disturb the cytochrome c - cardiolipin interaction, and thus enhance the release of cytochrome c from mitochondria treated with digitonin or oligomeric Bax. Taken together, we suggested that the mechanism of caspase-2-mediated cytochrome c release might involve both steps of the two-step process of cytochrome c release. However, we cannot exclude another scenario, where caspase-2 may take part in solubilizing cytochrome c into the intermembrane space, while pore formation occurs by pro-apoptotic Bcl-2 family members. Considering the weak phenotype of the caspase-2 knockout mice, there are probably compensatory mechanisms for induction of apoptosis, triggered by DNA damage. This has also been suggested by other studies (Troy et al., 2001). The specific mechanism remains to be elucidated, but available data can offer us some clues for speculations of how caspase-2 mediates cytochrome c release, and thus apoptosis, induced by DNA damage.

Why caspase-2 among all the caspases?

Caspase-2 was one of the first caspases to be discovered (Kumar et al., 1994; Wang et al., 1994), but the specific function of caspase-2 has been an unsolved question for many years, and the few established substrates for this caspase were not able to offer clarification of this issue. In fact, although the sequence homology of caspase-2 is
similar to the initiator caspases, the cleavage specificity is closer to the executioner caspases (Troy and Shelanski, 2003). Our results suggest that the protease activity is not important for its function once it has achieved its active protein conformation. Moreover, inhibition of the catalytic activity by inhibitors or alkylation did not abolish the permeabilizing effect of processed caspase-2 on mitochondria (Robertson et al., 2004). We speculate that the conformation of the active, processed protein can offer some clues as to how caspase-2 exerts its effect. The crystal structure of caspase-2 was solved by Schweitzer et al. in 2003, and it revealed several unique features of this enzyme compared to other caspases (Figure 7) (Schweizer et al., 2003). Caspase-2 comprises two active sites, and the cysteines situated at one of the active sites (Cys 390) form a stable intersubunit disulfide bridge, possibly stabilizing the dimer formation. This is in contrast with other apical pro-caspases that exist as monomers in solution and contain only one active site. Moreover, caspase-2 holds an additional hydrophobic specificity pocket, designated S5, which has only been found in caspase-2. Taken together, these unique structural features could be one possible explanation for the distinctive mechanisms of caspase-2 action. In fact, the caspase-2 structure may allow formation of oligomeric complexes, capable of pore-formation in the outer mitochondrial membrane, that other apical caspases are unable to form.

Figure 7. 3-D Structure of caspase 2. The disulfide bond, unique for caspase-2, is formed between Cys 390 and Cys 390# from each subunit. (Schweizer et al., 2003)
Lessons learned from Bid

Cytochrome c is not only bound to the inner boundary membrane, but is also found within mitochondrial cristae, presumably inaccessible to direct release through pores in the outer mitochondrial membrane. However, the fact that caspase-2 is stimulating a rapid and almost complete release of the hemoprotein indicates that some mitochondrial remodelling might occur. As aforementioned, tBid can bind cardiolipin, and thereby facilitate cytochrome c release (Lutter et al., 2000; Kim et al., 2004). Moreover, it has been suggested that tBid may be involved in the rearrangement of mitochondrial cristae during apoptosis (Scorrano et al., 2002; Kim et al., 2004). The explicit interaction between tBid and cardiolipin is currently not known, but according to Kim et al., treatment with the cardiolipin-specific dye NAO could prevent tBid-induced dissociation of cytochrome c. Experiments in our laboratory demonstrated that cytochrome c release, mediated by caspase-2, may be inhibited by NAO pre-treatment, indicating that tBid and caspase-2 disrupt the cytochrome c-cardiolipin interaction in a similar manner (data not shown). Cristae rearrangement would explain why caspase-2 is able to trigger a massive release of cytochrome c from mitochondria, i.e. how such a large pool of cytochrome c could become accessible for release through mitochondrial outer membrane pores. However, further experiments are required in order to clarify this effect.

Bax-mediated cytochrome c release – is cardiolipin a necessity?

Caspase-2-mediated pore formation in the outer mitochondrial membrane is a novel mechanism for cytochrome c release. Another well-described protein causing cytochrome c release is Bax, which is known to possess pore-forming ability upon oligomerization (Jurgensmeier et al., 1998; Rosse et al., 1998; Finucane et al., 1999). Kuwana et al. used monomeric Bax, oligomeric Bax and protease-cleaved Bid (tBid) to investigate the importance of cardiolipin in Bax-induced cytochrome c release (Kuwana et al., 2002). In their study, monomeric Bax was dependent on tBid and cardiolipin to induce dextran release from liposomes comprised of resealed mitochondrial or ER membranes. However, the authors suggested not only that cardiolipin is important for Bax function, but also that cardiolipin is a pre-requisite for Bax-mediated cytochrome c release. On the contrary, in paper III we show that oligomeric Bax is releasing cytochrome c from cardiolipin-deficient mitochondria, suggesting that cardiolipin is not essential for Bax-mediated release of solubilized cytochrome c. This has also been supported by another study, showing that neither cardiolipin nor its precursor, phosphatidylglycerol, is important for Bax-mediated cell death (Polcic et al., 2005). Although both Kuwana et al. and our group agree that cardiolipin is a potential target for tBid, as has also been shown by other groups (Lutter et al., 2000; Kim et al., 2004), we suggest that tBid is responsible for the dissociation of cytochrome c from cardiolipin, while Bax is important only for the pore-formation of the outer mitochondrial membrane.

Mitochondrial Grx2 may influence the release of cytochrome c

As mentioned earlier, it has been shown that cardiolipin might become oxidized and lose its interaction with cytochrome c as a result of increased ROS production, or deficient ROS scavenging within the mitochondria (Shidoji et al., 1999; Nomura et al., 2000; Asumendi et al., 2002; Ott et al., 2002; Chang et al., 2004). The GSH system is one of the most important defense systems against oxidative stress, and several studies have shown that GSH is involved in apoptosis regulation (Ghibelli et al., 1995; van den Dobbelsteen et al., 1996; Bustamante et al., 1997; Macho et al., 1997; Tan et al., 1998). In paper IV we describe a novel method for visualizing GSH, and glutathionylated proteins, by FACS or fluorescence and confocal microscopy. The GSH levels in the
cytosol and nucleus are similar, while the mitochondria contain 1.5-2-fold more GSH. We also show that mitochondria are potent scavengers of GSH. This finding indicates that the mitochondrial redox system is apparently less sensitive to GSH depletion or changes in total GSH/GSSG ratio and, possibly, that maintenance of mitochondrial GSH is critical for cell survival.

Modulation of Grx2 levels affects the release of cytochrome c

The mitochondrial GSH system is comprised of cGpx, PHGPx, and mitochondrial Grx2. While cGpx probably is more important in removing cytosolic hydroperoxide, modulation of the PHGPx levels has indicated a role for the enzyme in apoptosis signaling (Imai et al., 1996; Yagi et al., 1996; Arai et al., 1999; Nomura et al., 1999; Brigelius-Flohe et al., 2000; Nomura et al., 2000). Grx2 was discovered in 2001 (Gladyshev et al., 2001; Lundberg et al., 2001), and due to its localization, mitochondrial Grx2 came in focus of our attention. In view of the known effect of the mitochondrial redox status on cytochrome c release, together with the observation that mitochondria maintain their GSH level also during GSH depletion (paper IV), we investigated the effect of Grx2 on cell death and, especially, on cytochrome c release. In paper V we knocked down the levels of Grx2 to <3% of its endogenous levels, without causing a severe effect on oxidation status, as assessed by protein carbonylation and glutathionylation. However, since the glutathionylation status of particular proteins may alter their activity, we cannot exclude that knockdown of Grx2 may affect some specific proteins, even if alteration of the general glutathionylation level remains undetectable. We demonstrated also a sensitization of HeLa cells to cell death induction after knockdown of Grx2. Moreover, overexpression of mitochondrial (mGrx2-HeLa) and truncated, cytosolic (tGrx2-HeLa) Grx2 in HeLa cells showed that Grx2 could inhibit apoptosis induced by 2-deoxy-D-glucose (2-DG) or doxorubicin (Dox), by preventing cytochrome c release and cardiolipin oxidation (paper VI). We propose that Grx2 facilitates the maintenance of cellular redox homeostasis upon treatment with apoptotic agents. However, the exact mechanism of protection remains to be investigated. A recent study by Beer et al. (2004) demonstrated that Grx2 specifically catalyzes the removal of GSH from glutathionylated mitochondrial membrane proteins, even at relatively low GSH/GSSG ratio. Glutathionylation may activate or inactivate proteins, and Complex I has been suggested as a possible target for glutathionylation, thus affecting respiration and enhancing ROS leakage (Taylor et al., 2003). Grx2 may therefore have an indirect effect on ROS production as well as ROS scavenging, by affecting proteins in the respiratory chain. Also, Grx2 can also receive electrons from both cytosolic and mitochondrial TrxR2 (Johansson et al., 2004). Thus, under conditions where the abundance of free GSH is low, Grx2 may still be functional by interaction with, and reduction by, TrxR2.

Why are mitochondria so sensitive to modulation of redox enzyme levels?

Considering the fact that several different redox systems co-exist in the mitochondria, it is quite surprising that the compensatory mechanisms among them are seemingly minor. The Grx2 experiments in paper V and VI, as well as several studies involving other mitochondrial redox enzymes, such as PrxIII or Trx2, have shown that overexpression and especially knockdown of one of the mitochondrial redox enzymes significantly alters the sensitivity of the cells to apoptosis inducers. There can be several explanations to this phenomenon. Some enzymes, such as PHGPx, whose mitochondrial form is found exclusively in testis (Pushpa-Rekha et al., 1995), are probably cell- and/or tissue-specific. The different enzymes scavenge different types of ROS, from the initial radicals (dismutation of superoxide by SOD), intermediate ROS
scavenging (reduction of hydrogen peroxide by PrxIII) to more down-stream oxidative events, such as catalysis of reduction of glutathionylated proteins or increased GSH turnover by Grx2. Thus, even if the end-term effects are similar, the redox enzymes act at different levels of the “oxidative chain”. Sublocalization of the different enzymes is another factor that provides discrepancy between the different redox proteins, especially since it is known that radicals usually attack targets within a close proximity. Finally, some lessons could be learnt from mice expressing defective mitochondrial DNA polymerase (Trifunovic et al., 2004). Increase in ROS may cause not only protein and lipid oxidation, but also damage to mitochondrial DNA. Trifunovic et al. recently showed a correlation between increased amount of mutations in mitochondrial DNA and aging, and protection of mitochondrial DNA from oxidative attack may thus be more important than previously suspected. A functional mitochondrial redox defense is not only important for the efficient and finely tuned pathways of apoptosis within certain cells, but could also alter processes that affect aging of the whole organism.
5 CONCLUSIONS

Taken together, the release of cytochrome c is an intricate process that presumably involves several mechanisms depending on the apoptosis trigger. In this thesis we investigated the mechanism(s) of cytochrome c release from different perspectives. As a result, we have proposed a novel mechanism for cytochrome c release, mediated by caspase-2 and possibly involving pore-formation in the mitochondrial membrane by this protease (papers I and II). In vivo, several possible models involving caspase-2 might be proposed. Caspase-2 may act on its own, forming pores in the outer mitochondrial membrane and also dissociating cytochrome c from cardiolipin. However, it cannot be excluded that caspase-2 also may act in concert with Bax, where Bax is the pore-forming molecule and caspase-2 detaches cytochrome c, in a manner resembling the effect of tBid. We have shown that cardiolipin is not important for Bax-mediated pore formation (paper III). However, cardiolipin must be affected by protein binding or oxidation in order for solubilization of cytochrome c to occur, allowing release of the hemoprotein through the Bax-pores. One typical dissociation factor for cytochrome c is oxidation of cardiolipin. The GSH system is one of the most important intracellular redox systems, and while paper IV demonstrates a novel method of GSH staining, we also show that GSH is maintained within mitochondria during oxidative stress, regardless of the GSH depletion in other organelles. Moreover, papers V and VI indicate that mitochondrial Grx2 is a potential inhibitor of apoptosis, since knocking down the protein by siRNA (paper V), or overexpressing Grx2 (paper VI), influence cell death signaling, probably by preventing oxidation or degradation of cardiolipin (paper VI). It is clear that the mitochondrial redox environment is pivotal for keeping cardiolipin reduced and preventing cytochrome c release, and lowering the levels of Grx2, or other mitochondrial redox enzymes, may have a lethal effect on the cell.
ACKNOWLEDGEMENTS

This work was performed at the Institute of Environmental Medicine, Division of Toxicology and Neurotoxicology, and at the Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, at Karolinska Institutet. I would like to express my deepest gratitude to all the people that I have met and that I have been working with during my years at KI. In particular, I wish to thank the following people:

My two supervisors; professor Sten Orrenius, head of the Blue Group for most of my graduate studies, and professor Arne Holmgren, head of the Biochemistry group. You are both excellent researchers and it has been a pleasure to work in the creative environment that you have built up around you. I have learnt so much from you!
Sten, thank you for being an excellent role model, for keeping your door open for me, and for showing patience and encouragement. You rock! Arne, thanks for always taking the time, and for allowing me to find my own ways, pursue my own ideas and develop as a researcher. The combination of you two as supervisors has been outstanding!

Professor Boris Zhivotovsky, present head of the Blue Group. Thank you for your scientific input in the studies, for always always taking the time even though you are terribly busy, generously sharing ideas and not complaining too much when I play loud music and sing in the cell lab. Thank you for being so appreciative when I need it and for always wanting the best for me. I have learnt a lot from you, scientifically and personally. Also, thank you for your critical reading of my thesis.

Dr John Robertson; we all love caspase-2, and lot of the work is founded by you and the etoposide-studies. Thanks for helping me out in the lab, and for being an excellent friend as well as collaborator. It’s been really fun to work with you. Thank you also for correcting my English, and for proofreading parts of this thesis. I miss you, sweetie!

Dr. Vladimir Gogvadze, our mitochondrial expert, for your excellent help with experiments, for being patient and always having a critical eye when I get (too?) enthusiastic about beauuutiful data. Thanks for sharing your jazz music with me and for enduring my long and loud radio sessions. You are a very good friend and colleague, and I have really appreciated working with you.

Dr. Joya Chandra, for introducing me to apoptosis, for showing me how fun the field is, and for sharing your joy for research. You are an excellent role model, and you have really influenced the way I work and think about research.

Dr Bertrand Joseph and Professor Sandra Ceccatelli, for providing a great environment in the Blue Group, and for bringing in outstanding people to the lab.
Dr Ian Cotgreave, for taking the time when I was a rookie at IMM, always willing to explain and discuss research, and for constantly keeping up the enthusiasm and the good mood. Thank you also for the GSH collaboration.

Dr Bengt Fadeel, for being an outstanding dance partner at the IMM parties and for generously sharing your research experience as well as your dry sense of humour.

Dr Aristi Potamitou, for being an excellent collaborator and researcher, but most of all for being a very good and supportive friend.

Roshan Tofighi, for being a good friend and colleague. Thanks for all the excellent discussions in the sauna! I think of you every time I drink a cosmo...

Yu Sun, my Chinese Tian-Qin, for being an excellent friend, colleague, and a terrific chef!

My fantastic students, especially Malin Örn and Stefanie Prast; it has been a pleasure to work with you in the lab!

Dr Suzanne Iverson, for great collaborations and much laughter within and outside the lab. I miss our Friday lab bench discos, Doll!

Jan Schmidt-Mende, for being an excellent friend and travel companion, especially on our conference trips. I’ll never be able to camp in anyone’s garden without thinking of you.

Susanne Ahlberg, for being an excellent colleague and a great person. It has been a privilege to work with you.

My present lab room mates; Andrej Kropotov, our computer wiz, professional photographer and music master. Helin Vakifahmetoglu, for being so sweet, caring and funny. Yuan Xue, for not complaining when I occupy the computer ALL the time.

Therese Hemström, for all the discussions about football, sports in general, research (det är så rrroligt!), Piaf and every other topic one can think of.

Old members of the Blue group, especially Bettina Gleiss, Dr Camilla Köhler, Martin Ott, Dr Wanlaya Uthaisang, Dr Leta Nutt, Dr Ping Huang, Dr Jessica Freudenthal, Dr Mia Emgård, Dr Babette Bartling, Maria Karpova, Pavel Grudinkin, Minna Suomela, Jenny Karis, Maria Trieb, Agnieszka Sobczak-Pluta, Dr Eric Sleeper, Dr Elisabetta Darè, Dr Masato Sasamoto, Dr Alessandro Giuliani and Luigi Leanza.

Present Blue group members: Christoffer Tamm, Carolina Johansson, Natalia Onishchenko, Margareta Sandström, Gudrun Malmborg, Magnus Olsson, Ulrika Nyman, Pinelopi Vlachos and Tie-Jun Shi. You are unbelievably good friends and colleagues! A special thanks to Christoffer for all the discussion (will we ever agree on anything?!), Margareta for being invaluable in the lab, and to Magnus for being my occasional boxing partner.
Birgitta Blacker, for being an excellent secretary at IMM, but also for all the fun conversations we have had. You are a fabulous person!

The Biochemistry lab; Dr Anna-Klara Rundlöf, Dr Catrin Johansson, Karin Aneståhl, Linda Johansson, Olle Rengby, Malin Fladvad, Dr Klas Pekkari, Dr Horst Lillig, Maria Lönn, Geng Chang, Dr Johanna Ungerstedt, Dr Javier Vila-Carniõ, Carsten Berndt, Dr Liangwei Zhong, Dr Rong Zhao, Dr Thomas Nordman, Jacek Andrwzjewski and all other past and present members of the Biochemistry lab. Dr Elias Arnér, for sharing your enthusiasm for science, but also for the valuable comments at the Biochemistry Group seminars. Horst and Maria, thank you also for the Grx2 collaborations and discussions about redox biochemistry in general.

Dr Mathias Lundberg, the Grx2 founder, for helping me out in the lab and for being patient and helpful during my first year as a PhD student. Also, thank you for the input in the Grx2 studies.

Lena Ringdén, for being an excellent secretary – thank you for taking my last-minute-problems with a smile! You have been a great help!

MIS’ group, for welcoming me at all the “unplanned” visits on Fridays at 2-3pm.

Other friends and colleagues at IMM and MBB, especially Ilona, Martin, Therese, Niklas, Ulla, Mattias, Tobias, Kristian and Atchasai. Therese, thanks also for the GSH collaboration.

The Biochemical Research School in Lund, for giving me the opportunity to try out different laboratories and thus finding my way to KI. A special thanks to Cecilia Emanuelsson and Anders Malmström. SSF, for being an important sponsor during this year.

The Espanish mafia, especially Cristina, Juan, Juanluis, Salvador, Angel and Octavio. Thanks for all the laughters, for letting me into the “family” and for putting up with me when I abuse your language. Salva, for being a close friend the past year.

The sailing crew; Magnus, Ingela, Peter, Helena, Micke, Cecilia, Mariana, Mats, Cecilia, Maria, Fredrik, Anna, Inga-lill and Jesper, for all the wonderful moments in the archipelago and in Stockholm. Thanks for all the fun times and the great friendships.

Jesper, for sharing a lot of good memories with me, and for being supportive when things were not so easy.

My old friends, especially Martin B, Ola, Malin, Sofia, Anders S, Martin S, Anders O, Richard R, Patrik, Marie and Jenny K. Monika and Maria, for much laughter and shared wisdoms (Allt som flyger har faktiskt inte skal). Per, for putting up with the craziness during the writing of this thesis.
My friends in Stockholm, especially Malin, Amanda, Sofia, Hubert, Manne, Frida, Christian and Daniel.
Ylva Edling, for being a wonderful friend and a terrific housemate at Solvik. We have had lots of fun together, and you are so important to me!

Linda, for being a fabulous sambo the past year. More champagne!

Katheryn and Bree, for making my Aussie stay both fun and magic. I miss you…

Rickard, for always tagging along to every new thing I come up with, and for being a really good friend for many years. Jag tycker så mycket om dig!

Jenny, for being an encouraging and caring friend, ex-sambo, midnight-caller and an excellent travel companion. Even though we share very few genes, you are my second sister... What’s the next adventure?!

My extended family; all the aunts, uncles and cousins; all so important to me. A special thanks to Birgitta and Kristina, for letting me stay with you and thus making it possible for me to come to Stockholm in the first place.


... and thank you to all the other friends and colleagues I have not added into the list above…


