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MAMMALIAN THIOREDOXIN REDUCTASE AS A DRUG TARGET IN ANTICANCER THERAPY THROUGH DIRECT APOPTOSIS INDUCTION BY SELENIUM COMPROMISED FORMS OF THE PROTEIN

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ABSTRACT

The mammalian thioredoxin system, consisting of thioredoxin(s), thioredoxin reductase(s) and NADPH, participates in the regulation of many important systems in a mammalian cell. The mammalian thioredoxin reductases (TrxR) are selenoproteins containing a selenocysteine (Sec) residue within a carboxyl terminal –Gly –Cys –Sec –Gly –COOH motif. Sec is encoded by a UGA codon, normally functioning as a stop of translation, which necessitates an expansion of the genetic code. The Sec residue is thus inserted by an intricate translation machinery dependent on a Sec insertion sequence (SECIS) element located in the 3' untranslated region of the mRNA. A dysfunctional SECIS element would likely generate a form of TrxR with a truncated carboxyl terminal motif consisting of Gly-Cys-COOH lacking its normal enzymatic activity. Alkylating the selenocysteine with electrophilic compounds, a reaction that is highly favoured at physiological pH due to a high reactivity of Sec, can also generate an inactive enzyme.

The aim of this thesis project was to examine how different forms of TrxR affect cell viability, especially in cancer cells.

The *BioPORTER* technique, which makes it possible to introduce fully active proteins into cells, was used to deliver different preparations of pure TrxR1 protein derivatives into A549 (lung carcinoma) cells. Interestingly we found that selenium compromised TrxR1, either in the truncated form or produced by alkylating the Sec residue by electrophilic compounds, provoked a very rapid cell death in A549 cells. This effect could not be detected after introduction of fully active TrxR1 enzyme nor upon introduction of glutathione reductase. The selenium compromised TrxR1 forms initiating cell death were suggested to be collectively termed SecTRAPs (selenium compromised thioredoxin reductase-derived apoptotic proteins). The cell death provoked by SecTRAPs was demonstrated to trigger exposure of phosphatidyl-serine, a phenomenon characteristic of apoptotic cell death. It was also found that the apoptosis provoked by SecTRAPs required both caspase-2 and caspase-3/7 activation but not induction of protein synthesis. Either human or rat SecTRAPs could induce apoptosis in human A549 (lung adenocarcinoma) or HeLa (cervix carcinoma) cancer cells. HeLa cells overexpressing Bcl-2, which were resistant to staurosporine, were still susceptible to apoptosis provoked by SecTRAPs. Notably, human embryonic kidney-derived cells (HEK293) were resistant to SecTRAPs, illustrating that not all human cell types are susceptible to cell death provoked by SecTRAPs. We subsequently evaluated the importance of a redox-active disulfide/dithiol motif involving Cys59 and Cys64 within a CVNVGC sequence present in TrxR for the apoptotic effects of SecTRAPs. This was performed by generation of a mutant where the two redox active cysteins were replaced by Ser residues. The findings indicated that the two Cys residues of the CVNVGC motif, found in both TrxR1 and SecTRAPs, are required for the apoptotic features of the latter. This may suggest that SecTRAPs redox cycle with an endogenous substrate leading to oxidative stress and apoptosis or, alternatively, that local effects at the mitochondrial membrane may directly signal apoptosis through release of cytochrome *c* in a process involving caspase-2. The critical Cys residues could also be susceptible to posttranslational modifications.

In addition we found by *in vitro* analysis that mammalian TrxR may easily be targeted by all representative compounds of the major classes of clinically used anticancer alkylating agents and most platinum compounds whereas this was not the case with glutathione reductase. In conclusion, our results suggest that TrxR may be a prime target for anticancer alkylating agents, producing SecTRAPs that can directly induce apoptosis in cancer cells.

Key words: thioredoxin reductase, TrxR1, SecTRAPs, selenoproteins, apoptosis, caspase-2, glutathione reductase, cisplatin

LIST OF PUBLICATIONS

This thesis is based upon the following publications that will be referred to by their roman numerals:

I. **Anestål K**, Arnér E.S.J. (2003) Rapid induction of cell death by selenium-compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine. *Journal of Biological Chemistry, 278,15966-15972.*

II. Witte AB, **Anestål K**, Jerremalm E, Ehrsson H, Arnér E.S.J. (2005) Inhibition of thioredoxin reductase but not of glutathione reductase by the major classes of alkylating and platinum-containing anticancer compounds. *Free Radical Biological Medicine. 39, 696-703*

III. **Anestål K**, Arnér E.S.J. (2006) SecTRAPs are potent inducers of a caspase-dependent apoptosis in cancer cells that can not be prevented by overexpression of Bcl2. *Submitted*

IV. **Anestål K**, Arnér E.S.J. The apoptotic effects of SecTRAPs require an intact CVNVGC motif in the aminoterminal domain. *Manuscript*

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LIST OF ABBREVIATIONS

AIF	Apoptosis Inducing Factor
AP-1	Activator protein 1
AP-2	Activator protein 2
ASK-1	Apoptosis signaling kinase 1
C. elegans	Caenorhabditis elegans
CARD	Caspase activation and recruitment domain
DED	Death effector domain
DISC	Death Inducing Signalling Complex
DNCB	1-Chloro-2,4-dinitrobenzene
DNFB	1- Fluoro-2,4-dinitrobenzene
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
Endo G	Endonuclease G
FADD	Fas Associated Death Domain
GPx	Glutathione peroxidase
GR	Glutathione Reductase
Grx	Glutaredoxin
GRIM-12	Gene associated with retinoid-IFN-induced mortality number 12
GSH	Reduced glutathione
GSSG	Oxidized glutathione (glutathione disulfide)
IAPs	Inhibitory Apoptosis Proteins
INF-β	Interferon β
JNK	Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MGd	Motexafin gadolinium
MMP	Mitochondrial membrane permeability
NAC	N-acetylcysteine
NFκβ	Nuclear factor κβ
NO	Nitric oxide
NOS	Nitric oxide syntase
OMM	Outer Mitochondrial Membrane
PARP-1	Poly(ADP-ribose) polymerase
PMA	Phorbol 12-myristate 13-acetate

Prx	Peroxiredoxin
RA	Retinoic Acid
Ref-1	Redox factor 1
ROS	Reactive oxygen species
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
SecTRAPs	Selenium Compromised Thioredoxin Reductase-derived Apoptotic Proteins
SOD	Superoxide dismutase
TGR	Thioredoxin and Glutathione Reductase
TAMs	Trx80 activated monocytes
TNF-α	Tumor necrosis factor α
TNFR1	Tumor necrosis factor binding receptor 1
TRAIL	Tumor necrosis factor apoptosis-related ligand
Trx	Thioredoxin
TrxR	Thioredoxin reductase
VDAC	Voltage-dependent anion channel
VDUP	Vitamin D upregulated protein

1 INTRODUCTION

Cell death is a continuous process that goes on in all multicellular organisms to maintain the homeostasis of tissues. Old and damaged cells that might harm the organism are efficiently cleared from the system. In embryogenesis, cell death is essential for normal development, a phenomenon that has been extensively studied in the model organism *C. elegans* (Horvitz 2003). Defects in the regulation of this mechanism can result in either too little cell death and cause cancer or autoimmunity or too much cell death, which is the cause of chronic and acute degenerative diseases, immunodeficiency and infertility (Danial and Korsmeyer 2004).

In the introduction of this thesis I will start with a brief overview of an extensively studied cell death mechanisms that has connection to the work presented herein (the caspase-dependent intrinsic pathway of apoptosis). This will be followed by a specific introduction to the thioredoxin system and then continue with a description of the thioredoxin system in relation to cell death.

1.1 CELL DEATH – A REGULATED PROCESS

1.1.1 Different types of cell death

Homeostasis in tissues is maintained basically by three different cellular events namely; differentiation, proliferation and death. For a long time cell death was considered entirely as a pathological process, occurring as a result of exposure to a variety of toxic stimuli such as oxidative stress, hypoxia, compounds negatively affecting cell metabolism etc. Characteristic for this type of cell death, later termed necrosis, include swelling of cytoplasm and organelles, energy depletion and random DNA degradation. The final step is lysis of the cell membrane leading to spillage of the intracellular content and triggering of an inflammation in the surrounding tissue.

A more controlled way of a cell to die is apoptosis, a term coined by Kerr in the 1970´s, characterized by shrinkage of the cell, chromatin condensation, membrane blebbing and formation of apoptotic bodies (Kerr, Wyllie et al. 1972). The apoptotic bodies are efficiently cleared by macrophages, preventing the upcome of an inflammation

(Fadeel 2003; Fadeel and Orrenius 2005). Importantly, apoptosis should not be considered equal to programmed cell death. Programmed cell death is rather considered as a cell death resulting from genetic events, without harm to surrounding tissues, lacking the induction of biochemical and morphological changes characteristic of apoptotic cell death (Schwartz, Smith et al. 1993).

1.1.2 The intrinsic and extrinsic pathways

Two main apoptotic signaling pathways have been identified; the receptor mediated (extrinsic) pathway and the intrinsic pathway involving mitochondrial signaling. The extrinsic pathway is activated by binding of a ligand to the tumour necrosis factor (TNF) family of plasma membrane death receptors, such as Fas, TNFR1 and TRAIL. A trimeric receptor complex is generated upon binding of the ligand, and a subsequent interaction with the adaptor protein called Fas Associated Death Domain (FADD) (Chinnaiyan, O'Rourke et al. 1995) and procaspase-8 results in the formation of the Death Inducing Signalling Complex (DISC). Aggregation of procaspase-8 leads to autoprocessing and activation of this initiator caspase. Caspase-8 then has two options; either activation of effector caspases like caspase -3, accelerating a caspase cascade, or cleavage of the pro-apoptotic protein Bid to a truncated Bid (tBid) (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998). tBid can subsequently translocate to the mitochondria and initiate the intrinsic pathway, by release of a variety of death signalling proteins such as cytochrome *c*, apoptosis inducing factor (AIF), Smac/DIABLO, Endonuclease G and Omi/HtrA2 (van Gurp, Festjens et al. 2003). Released cytochrome *c* interacts with Apaf-1, procaspase-9 and dATP to form the apoptosome (Li, Nijhawan et al. 1997). Upon apoptosome formation caspase-9 is activated and procaspase-3 is subsequently cleaved and activated.

Smac/DIABLO, a 29kDa mitochondrial precursor protein, is released from the intermembrane space of the mitochondria after apoptosis induction. Acting as a dimer the protein contributes to caspase activation by avoiding the activation of inhibitory apoptosis proteins (IAPs), which prevent activation of procaspases and inhibit the activity of mature caspases. In a similar way, the Omi/HtrA2 serine protease inhibits activities of IAPs by binding or sequestering the proteins (van Gurp, Festjens et al. 2003).

AIF is a 57kDa flavoprotein that is translocated to the nucleus as a response of poly(ADP-ribose) polymerase 1 (PARP-1) activation provoked by DNA damage (Susin, Lorenzo et al. 1999). In the nucleus AIF triggers a caspase-independent chromatin condensation and a large-scale DNA fragmentation (Yu, Wang et al. 2002). Subsequently tBid translocates to the mitochondria and initiates the intrinsic pathway by release of a variety of death signaling proteins, such as cytochrome *c*, apoptosis inducing factor (AIF), Smac/DIABLO, Endonuclease G and Omi/HtrA2. In great similarity to AIF, the Endonuclease G, a 30kDa mitochondrial nuclease, may translocate to the nucleus and digest nuclear DNA as a process independent of caspase activity (van Gurp, Festjens et al. 2003). The different pathways of apoptosis are schematically summarized in Figure 1.

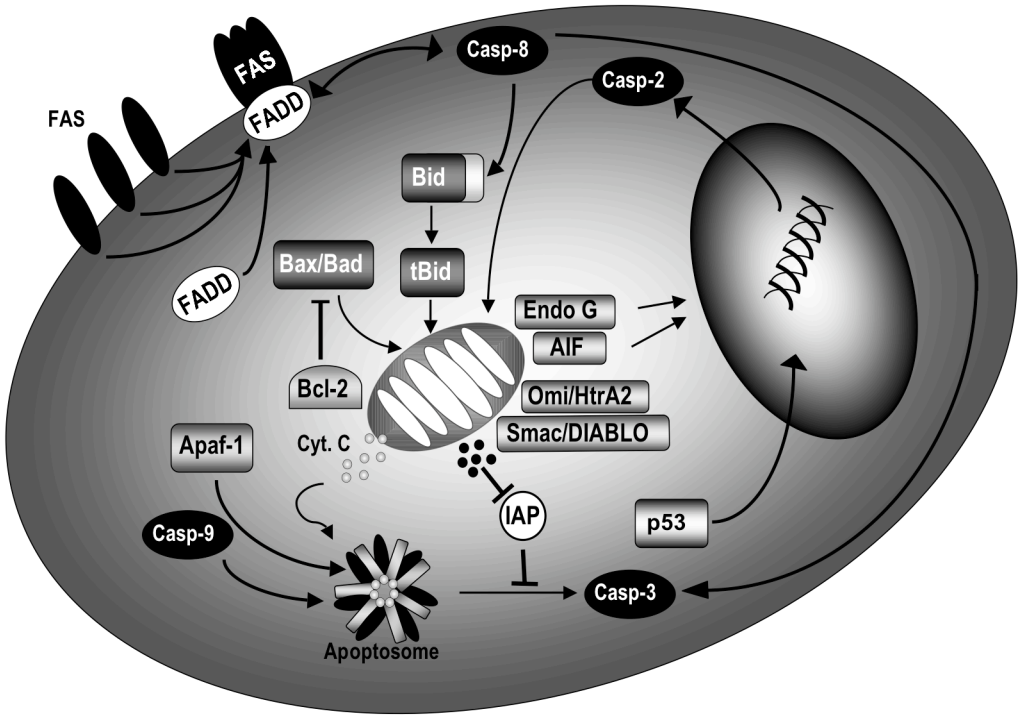

Figure 1. Intrinsic and extrinsic pathways of apoptosis. The most extensively studied apoptotic proteins in the intrinsic and the extrinsic signalling pathway are illustrated in the figure. The extrinsic receptor mediated pathway is initiated by binding of a ligand to membrane death receptors here illustrated by Fas. A trimeric receptor complex is generated upon binding of the ligand, and a subsequent interaction with the adaptor protein called Fas Associated Death Domain (FADD) and procaspase-8 results in the formation of the Death Inducing Signalling Complex (DISC). Aggregation of procaspase-8 leads to autoprocessing and activation of this initiator caspase. Subsequently caspase-8 can either activate effector caspases here illustrated by caspase -3, or cleave the pro-apoptotic BH3 protein Bid to truncated Bid (tBid).

1.1.3 Caspases

Caspases (cysteine- aspartate- proteases) are a group of well conserved cysteine proteases responsible for diverse cellular functions in inflammation and apoptosis (Cohen 1997; Fadeel, Orrenius et al. 2000; Boatright and Salvesen 2003). The caspases are named in the order they were discovered and today 12 different variants have been identified in humans. Caspases are synthesised as single chain procaspases (32-56kDa) and generally a conformational change is needed to generate the catalytically active enzyme. Common for all caspases is the conserved active site QACXG (where X is R, Q or G) located in the large subunit. Additionally, all caspases contain a small subunit and a prodomain (Figure 2).

Caspases can be divided in two different ways either based on structure or substrate preferences. Structurally caspases can be categorized depending on the size of the prodomain. Caspase-1, -2, -4, -5, -8, -9, -10, and -11 have a long prodomain that contains structural motifs of the death domain superfamily including the caspase activation and recruitment domain (CARD) or death effector domains (DEDs). These motifs enable the recruitment of the N-terminal part of monomeric caspases to other effector proteins, generating caspase-dimers in multiprotein complexes, a process essential for activation of this group of caspases.
Caspase -3, -6, -7 and -14 have a short prodomain and are present in a dimeric latent form in the cell. Proteolytic cleavage of the prodomain by other caspases activates short prodomain containing caspases.

In addition, a distinction can be made between inflammatory and apoptotic caspases. Caspase -1, -4, -5 and -11 have been found to participate in the maturation of cytokines and inflammatory responses. The remaining caspases are primarily involved in apoptosis and can be further divided into initiator (caspase -2, -8, -9, -10 and -12) and executioner caspases (caspases -3, -6, -7, -14) depending on the size of the prodomain and activation mechanism as described above.

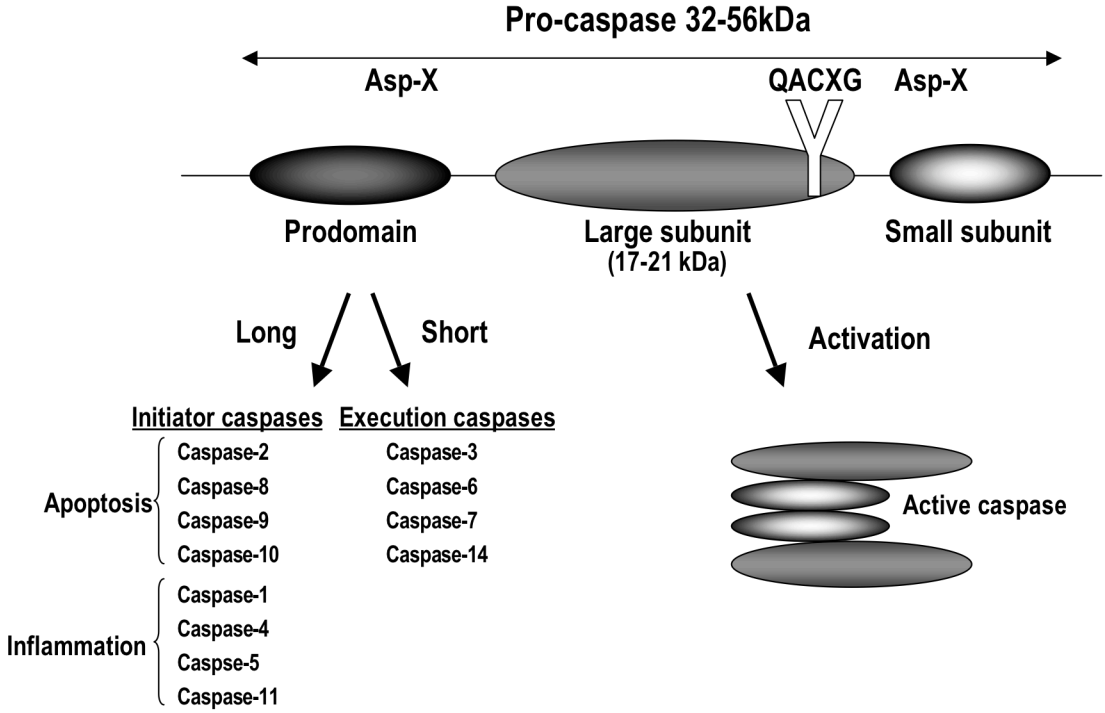

Figure 2. Division of human caspases based on structure or substrate preferences. Depending of the size of the prodomain and their function caspases can be categorized into initiator caspases and effector caspases. Initiator caspases are activated of proteins outside the caspase family, while effector caspases are activated by proteins within the caspase family. In addition initiator caspases can be divided into caspases participating in apoptotic responses alternatively inflammation responses. Essential for caspase activation is a conformational change and dimerization.

1.1.3.1 Caspase-2

The enigmatic caspase-2 has been of special interest in this study and is thus deserving a more detailed presentation.

Caspase-2 is the evolutionarily best conserved caspase, and was one of the first caspases to be identified in humans (Lamkanfi, Declercq et al. 2002) (Kumar, Kinoshita et al. 1994; Wang, Miura et al. 1994). The phenotype of caspase-2$^{-/-}$ mice is subtle, indicating that other caspases with similar function can replace its activity (Bergeron, Perez et al. 1998). Until recently very little was known about caspase-2 activation, but it has been suggested to participate in a variety of cell death pathways, and consistent with an initiator caspase it is activated rapidly in response to diverse death stimuli (Kumar, Kinoshita et al. 1994; Wang, Miura et al. 1994; Harvey, Butt et

al. 1997). Apparently caspase-2 is the only initiator caspase that can not cleave effector caspases (Van de Craen, Declercq et al. 1999).

Caspase-2 has been shown to be activated at DNA-induced apoptosis resulting in release of cytochrome *c* and Smac/DIABLO. This implies that caspase-2 acts upstream of the mitochondria. Interestingly, caspase-2 could not provoke any release of AIF (Lassus, Opitz-Araya et al. 2002; Robertson, Enoksson et al. 2002). In addition, cytotoxic stress has also been shown to cause caspase-2 activation. In the same study it was suggested that proteolytic cleavage of caspase-2 was a requirement to mediate apoptosis (Lassus, Opitz-Araya et al. 2002). However, later studies have shown that the caspase does not need to be catalytically active to perform its effect. Instead the essential step seems to be dimerization and subsequent autocleavage, resulting in stabilization and optimal enzyme interaction (Baliga, Read et al. 2004).
Interestingly, cytochrome *c* and Smac/DIABLO release after treatment with recombinant caspase-2 could not be prevented by Bcl-2 overexpression in Jurkat cells, suggesting that the mechanism for permeabilization of the outer mitochondrial membrane does not involve Bcl-2. In addition, in studies with $Bak^{-/-}$ and $Bax^{-/-}$ mouse embryonic fibroblast mitochondrial-intermembrane space proteins were found to be released after treatment with recombinant caspase-2 protein, implying that Bak and Bax proteins are not a prerequisite for outer mitochondrial membrane (OMM) permeabilization by caspase-2 (Robertson, Gogvadze et al. 2004).

In a healthy cell environment where no apoptosis signalling is going on, cytochrome *c* is attached to anionic phospholipids in the inner mitochondrial membrane and different theories have emerged about how cytochrome *c* is released at the event of apoptosis. A recently published paper indicates that caspase-2 participates in this process by permeabilizing the OMM and/or breaching the association of cytochrome *c* with the inner mitochondrial membrane (Enoksson, Robertson et al. 2004).

1.1.4 Bcl-2 family

The Bcl-2 proteins are a family of both pro- and anti-apoptotic proteins, with the major function of regulating the release of apoptotic factors from the mitochondria (Green 2000; Martinou and Green 2001). Structurally the family can be divided into three

different subfamilies (Adams and Cory 1998; Tsujimoto and Shimizu 2000). The first group, encompassing the anti-apoptotic members Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1, share sequence homology within any of the four Bcl-2 homology (BH) regions (Tsujimoto 2003). The second subfamily, encompassing the pro-apoptotic members Bax, Bak, Mtd (Bok) and Bcl-rambo, share sequence homology at region BH1, BH2 and BH3 (Tsujimoto 2003). This group is usually referred to as multi-domain apoptotic Bcl-2 proteins. The third subfamily entitled BH3 only proteins is a fast growing group of pro-apoptotic- proteins encompassing among others Bid, Bim, Bik, Bad, PUMA and NOXA. This group has only sequence homology in the BH3-region (Sharpe, Arnoult et al. 2004).

1.1.4.1 Anti-apoptotic Bcl-2 proteins

The anti-apoptotic Bcl-2 proteins are localized in the mitochondrial membrane, where they may bind to active multi-domain pro-apoptotic Bcl-2 or BH3 pro-apoptotic proteins (Tsujimoto 2003). However, this does not seem to be the main anti-apoptotic function of Bcl-2 since mutational studies have shown that Bcl-2, without anti-apoptotic activity, still binds to the multi-domain and BH3 pro-apoptotic proteins. Instead the anti-apoptotic activities of Bcl-2 have been ascribed to its ability to inhibit mitochondrial channels like VDAC, based on the finding that Bcl-2 can protect the Ca^{2+}- induced permeability transition independently of multi-domain pro-apoptotic members of the Bcl-2 family.

1.1.4.2 Pro-apoptotic Bcl-2 proteins

Bax and Bax are two extensively studied multi-domain pro-apoptotic proteins (Jurgensmeier, Xie et al. 1998; Rosse, Olivier et al. 1998; Finucane, Bossy-Wetzel et al. 1999). They are redundant in many cells but seem to have diverse functionalities in different cell types (Tsujimoto 2003). Double knockout mice of Bak/Bax are resistant to a numerous amount of apoptotic stimuli indicating that multidomain pro-apoptotic Bcl-2 proteins act as a gateway. Bax is retained in a monomeric state in the cytoplasm or in the periphery of the mitochondria until apoptosis stimulation (Hsu, Wolter et al. 1997; Wolter, Hsu et al. 1997; Goping, Gross et al. 1998; Tan, Beerheide et al. 1999), while Bak is present as an integral mitochondrial membrane protein (Griffiths, Dubrez

et al. 1999). Upon stimulation both proteins perform a conformational change and create multimer complexes in the mitochondrial membrane (Tsujimoto 2003). The BH3-only proteins are mostly localized outside the mitochondria, appearing to act as sensors of death signals and subsequently they may trigger the activity of multidomain pro-apoptotic Bcl-2 proteins (Tsujimoto 2003). Upon stimulation BH3-only proteins are modified in different ways whereupon they translocate to the mitochondria and increase the permeability of the outer mitochondrial membrane, thereby contributing to the activation of multidomain pro-apoptotic Bcl-2 proteins or to the inhibition of the anti-apoptotic activities of Bcl-2 by binding (Tsujimoto 2003).

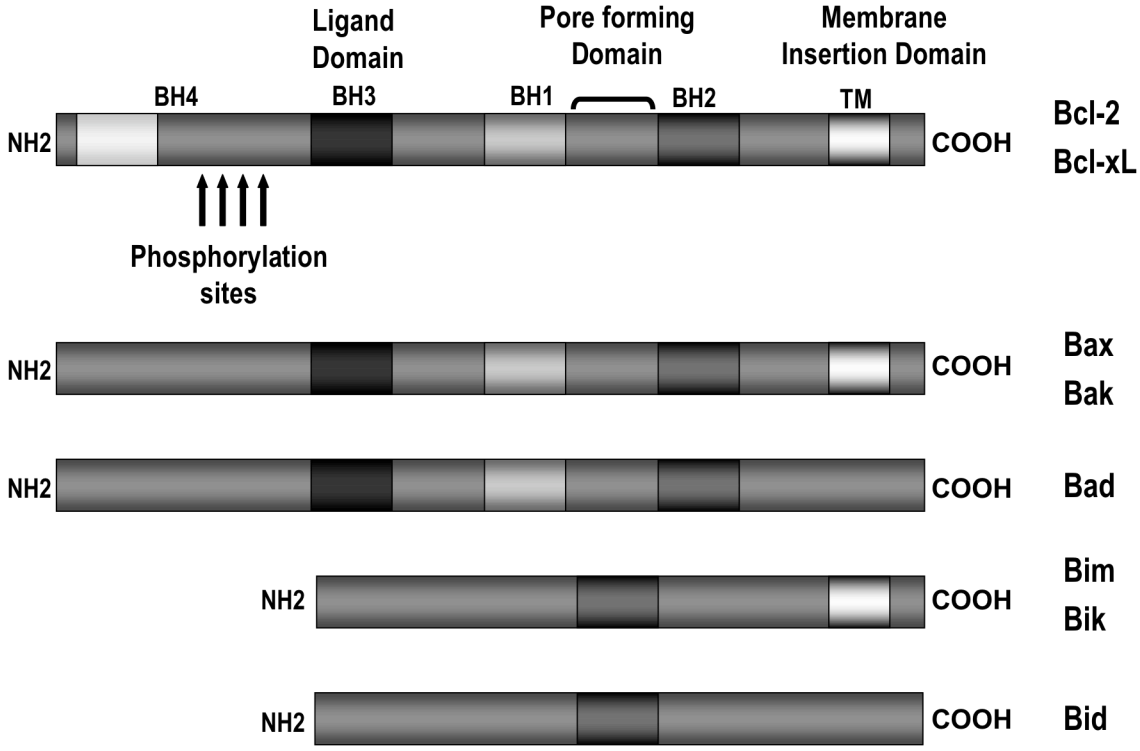

Figure 3. Bcl-2 family of proteins. The Bcl-2 family can be divided into two groups of anti- and pro-apoptotic proteins. A structural characteristic for the anti-apoptotic proteins is that they have similarities in region BH1-BH4 (Bcl-2, Bcl-xL). The pro-apoptotic group can be further divided into multidomain pro-apoptotic proteins (Bax/Bad) and BH-3 (Bim/Bid) only proteins.

1.1.4.3 Effects of Bcl-2 overexpression

Mitochondrial membrane permeabilization (MMP) is regulated by the Bcl-2 family members and can be inhibited by overexpression of Bcl-2 or Bcl-xL. Interestingly the two proteins seem to have distinct functions in cell death inhibition but MMP can be

inhibited by overexpression of both proteins. In addition, only Bcl-xL can inhibit DISC formation and Bid processing suggesting that Bcl-2 is less important for inhibition of upstream transduction pathway in TNF-induced cell death (Wang, Zhang et al. 2004). In addition overexpression of Bcl-2 could not prevent the loss of mitochondrial membrane potential upon TRAIL-induced apoptosis in several tumor cells, such as Jurkat, HeLa and MDA-MB-231 (Keogh, Walczak et al. 2000).

Several studies have demonstrated that Bcl-2 to a larger extent than Bcl-xL participate in additional signalling mechanisms not associated with apoptosis (Kim 2005). Bcl-2 overexpression has been found to be associated with G1 arrest, which promotes cellular senescence in tumor response. In addition, introduction of *Bcl-2* increases death in testicular germline tumor cells as a result of Bcl-xL downregulation. Furthermore introduction of the *Bcl-2* gene into ovarian carcinoma cells resistant to cisplatin with no consistent pattern of Bcl-2 expression increased the sensitivity to cisplatin (Beale, Rogers et al. 2000).

The findings presented above suggest that Bcl-2 may switch its anti- cell death function to a pro- cell death function, depending on the situation (Kim 2005). Subsequently the result of overexpression of Bcl-2 seems to vary a lot from one cell type to another.

1.1.5 p53

One of the first tumor suppressor genes to be identified was p53 (Wiman 1997; Asker, Wiman et al. 1999; Bykov and Wiman 2003). Activation of the p53 protein induces cell growth arrest, senescence, and cell differentiation or cell death by apoptosis. The protein is activated upon various stimuli including DNA damage, ribonucleotide depletion, oxidative stress, hypoxia and oncogene activation. The cellular level of p53 is normally very low and is negatively regulated by the p53 target gene MDM2. In the absence of stress signals the MDM2 protein exports p53 from the nucleus to the cytoplasm, where it is ubiquitinated resulting in proteasome-mediated degradation. In a stress situation p53 is stabilized by different modifications such as phosphorylation and acetylation. It is also regulated by thioredoxin system (se 1.3.4.1).

The tumor suppressive effect of p53 is performed by transcriptional regulation of several effector genes such as p21/WAF1 (leading to G1 arrest), Bax (el-Deiry, Harper et al. 1994; Wu and Deng 2002), Noxa, PUMA (Vogelstein, Lane et al. 2000) and Fas (Reinke and Lozano 1997) (leading to apoptosis). In addition, p53 can also promote apoptosis through transcriptional repression of certain genes lacking consensus p53 binding sites such as Bcl-2 (Miyashita, Harigai et al. 1994) or IGF1-R (Prisco, Hongo et al. 1997). However, the finding that p53-induced apoptosis occurred even in the presence of protein and RNA synthesis inhibitors indicated that p53 can also induce apoptosis independently of transcriptional activity, either by protein-protein interactions (Ding, McGill et al. 1998; Gottlieb and Oren 1998) or by increased transport of Fas from cytoplasmic stores to the cell surface (Bennett, Macdonald et al. 1998).

1.1.6 Oxidative stress and cell death

Reactive oxygen species (ROS) generated by side-reactions after leakage from the electron transport chain in the mitochondria, have been shown to trigger apoptosis by various stimuli including APO-1/Fas/CD95-ligands (Morel and Barouki 1999; Dröge 2002). The mitochondria are greatly affected by an increase in ROS production, illustrated by NO induced apoptosis, which is associated with a decrease in cardiolipin concentration and decreased activity of the electron transport chain resulting in cytochrome *c* release into the cytosole (Dröge 2002). In addition ROS at moderate levels are essential for the redox control of many processes in the cell, which will be described in the following part.

1.2 REDOX REGULATION IN THE CELL

Oxygen is essential for all aerobically living organisms. Immoderate levels of ROS can however interact with and harm macromolecules such as proteins, DNA (Stadtman and Levine 2000) and lipids (Girotti 1998; Mylonas and Kouretas 1999) and oxidation damage to these reactions can result in many human diseases such as cancer, neurodegenerative disorders and atherosclerosis (Dröge 2002). Furthermore ROS have been suggested to participate in the mechanisms of aging after the finding that

organisms with high metabolism have a shorter lifespan (Cadenas and Davies 2000; Finkel and Holbrook 2000; Dröge 2002).

ROS is mainly produced as by-products in the cellular metabolism, primarily in the mitochondria (Finkel and Holbrook 2000; Thannickal and Fanburg 2000; Nordberg and Arnér 2001; Dröge 2002). The major ROS derivative is the superoxide anion ($O_2^{\bullet-}$) but several other ROS molecules exist such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and nitric oxide (NO) (Nordberg and Arnér 2001). At moderate levels ROS participate in many reactions in a cell by directly affecting the conformation and/or activities of all sulfhydryl-containing molecules, including transcription factors and other important proteins, by oxidation of their thiol moieties (Finkel 1998; Rhee 1999; Allen and Tresini 2000; Hensley, Robinson et al. 2000). One specific example of how oxidants modify protein function is by glutathionylation (Cotgreave and Gerdes 1998; Klatt and Lamas 2000; Fratelli, Gianazza et al. 2004), a mechanism wherein glutathione (GSH) is covalently but reversibly added to cysteines. By binding of glutathione proteins might become inactivated but it may also facilitate access of a substrate or generate a conformational change that exposes otherwise buried specific sites. Furthermore glutathionylation can provide protection from irreversible oxidation and damage of proteins (Fratelli, Gianazza et al. 2004). Glutathionylation processes may be specifically regulated by the glutaredoxins (Fernandes and Holmgren 2004), that are unique glutathione dependent redox active proteins with a thioredoxin fold.

In order to sustain ROS at a well tolerated level several antioxidant systems have evolved. Glutathione is not just part of glutathionylation reactions, but is also the predominant thiol-based antioxidant (Anderson 1997; Anderson 1998; Dickinson and Forman 2002). The glutaredoxins are an important family of GSH-dependent antioxidants (Grx) (Holmgren 2000). One of the functions of Grx is to reduce glutathionylation reactions (Holmgren and Aslund 1995; Holmgren, Johansson et al. 2005). In addition, Grx has been found to participate in a growing list of functions such as reduction of dehydroscorbic acid, cellular differentiation, regulation of transcription factors and apoptosis (Holmgren, Johansson et al. 2005). Several other antioxidant systems have also evolved but since many of them interact with the thioredoxin system they will be covered in the following chapter.

1.3 THE THIOREDOXIN SYSTEM

The core of the thioredoxin system consists of the two antioxidant oxidoreductase enzymes thioredoxin (Trx) and thioredoxin reductase (TrxR). Thioredoxin is the main substrate of thioredoxin reductase and is reduced at the expense of NADPH. The system participates in several diverse functions in a cell such as regulation of cell growth, cell proliferation, redox control and cell death. Some functions will be described here, but for additional information see (Arnér and Holmgren 2000; Nordberg and Arnér 2001; Becker, Kanzok et al. 2003; Gromer, Urig et al. 2004; Rundlöf and Arnér 2004).

1.3.1 Thioredoxin

The thioredoxin protein (Trx1) was discovered 1964 in *Escherichia coli* as a dithiolfactor for ribonucleotide reductase in the synthesis of deoxyribonucleotides from ribonucleotides (Laurent, Moore et al. 1964). The 12kDa protein is ubiquitous from Archea to man and has a well conserved active site sequence Cys- Gly- Pro- Cys- (Holmgren 1968). Bacteria, yeast and plants contain several different cytoplasmic Trxs, but in mammals only one major cytosolic form has been found namely Trx1, although several sperm- or tissue- specific isoforms exist (Miranda-Vizuete, Sadek et al. 2004) and in virtually all cells there is also a mitochondrial Trx2 (Miranda-Vizuete, Damdimopoulos et al. 2000). Both Trx1 and Trx2 are required for normal development and life of mammals (Matsui, Oshima et al. 1996; Nonn, Williams et al. 2003). The cytosolic Trx1 is yet most studied. It plays an important role in cell viability, proliferation and activation, and is part of a general protein disulfide reducing system. Furthermore, Trx1 serves to maintain the redox environment in a cell.

The Trx promoter is rather complex and includes many possible motifs compatible with constitutive expression such as Sp1, as well as motifs associated with inducible expression such as AP-1, AP-2 and NF-κβ and Myb (Tonissen and Wells 1991; Kaghad, Dessarps et al. 1994). The Trx1 promotor contains an oxidative responsive element and at oxidative stress this motif regulates Trx1 expression (Taniguchi, Taniguchi-Ueda et al. 1996). Furthermore, an antioxidant responsive element located in

the Trx promotor responds to either hemin or PMA by different effects of either Nrf2 or Fos and Jun at the same element (Kim, Masutani et al. 2001). Retinoic acid has furthermore been demonstrated to regulate Trx in human epithelial cells (Chang, Reddy et al. 2002)

In addition, a truncated form of the TrxR1 protein, named Trx80, has been found to be secreted or located to the surface of monocytic cell lines. It functions as a mitogenic cytokine for human peripheral blood mononuclear cells independent of redox capacity (Pekkari, Gurunath et al. 2000; Pekkari, Avila-Carino et al. 2001; Pekkari, Avila-Carino et al. 2003). Recently, it was shown that Trx80 induces differentiation of human $CD14^+$ monocytes into a novel cell type, designated Trx80 activated monocytes (TAMs) (Pekkari, Goodarzi et al. 2005).

At physiological concentrations nitric oxide (NO), synthesised from L-arginin by NO-synthase (NOS), functions as an intracellular signalling molecule (Dalton, Shertzer et al. 1999; Nordberg and Arnér 2001). Furthermore, exposure of protein thiols to NO may S-nitrosylate the Cys residues resulting in modulated enzyme activity. Trx1 has been found to be regulated this way. Cysteine 69 was proposed to be S-nitrosylated under basal conditions, a posttranslatinal modification that was claimed to be a requirement for scavenging reactive oxygen species and for preserving the redox regulatory activity of Trx (Haendeler, Hoffmann et al. 2002). On the other hand, Trx1 has been found to regulate the activity of NOS by its ability to modulate thiol groups. This illustrates the complexity of the signalling between the NO- and thioredoxin systems (Dalton, Shertzer et al. 1999).

Several transcription factors such as NF-κB, AP-1 and p53 are regulated by Trx (Hirota, Matsui et al. 1997; Jayaraman, Murthy et al. 1997; Gaiddon, Moorthy et al. 1999). Upon stimulation with PMA, UV radiation or oxygen, Trx translocates from the cytoplasm to the nucleus and reduces the cysteines in NF-κB, which is crucial for its DNA binding (Matthews, Wakasugi et al. 1992; Mitomo, Nakayama et al. 1994; Hirota, Murata et al. 1999). The Trx regulation of the transcriptional activity of AP-1 is more intricate. The transcription complex consists of a heterodimer of kinase-regulated Fos and Jun, a homodimer of Jun or proteins related to these two proteins. Phosphorylation and dephosphorylation events play critical roles in the activation of

pre-excisting AP-1. Dephosphorylation of Thr-231, Ser-149 and Ser-243 in Jun, followed by phosphorylation of Ser-63 and Ser-73 is a prerequisite for the transcriptional activity of AP-1. Fos is activated by phosphorylation. However, dephosphorylation and phosphorylation is not enough to expose the DNA-binding domain of AP-1. In addition, conserved cysteines in an oxidized state cause a sterical hindrance of AP-1 binding to DNA. Reduction of the conserved cysteines is regulated by redox factor 1 (Ref-1) (Abate, Patel et al. 1990; Xanthoudakis, Miao et al. 1992; Dalton, Shertzer et al. 1999). Ref-1 is subsequently under redox control of Trx, which upon translocation to the nucleus in a reduced state enhances the activity of AP-1 about 3- to 4-fold compared to Ref-1 alone (Xanthoudakis, Miao et al. 1992; Hirota, Matsui et al. 1997).

Trx can also interact directly with proteins without catalyzing redox reactions. One example of this kind of interaction is between ASK-1 and thioredoxin (will be further discussed in the thioredoxin and cell death chapter 1.3.4. below) (Saitoh, Nishitoh et al. 1998). Reduced Trx can bind to ASK-1 and thereby inhibits its MAPKKK activity (Hodges, Smart et al. 2004).
In the interaction between Trx and Vitamin D_3 upregulated protein-1 (VDPU-1) the effect is the opposite, leading to inhibited Trx activity (Schulze, De Keulenaer et al. 2002). Interestingly, the expression of a mouse homologue to VDUP-1 is upregulated by H_2O_2 treatment and provokes apoptosis via ASK-1 and Jun as a result of inhibited Trx activity (Junn, Han et al. 2000). In human cerebellar granule cells c-Jun phosphorylation in parallel with upregulation of VDUP-1 results in apoptosis, suggesting that VDUP-1 abolishes the inhibitory effect of Trx on ASK-1 (Saitoh, Tanaka et al. 2001).

1.3.2 Thioredoxin reductase

Thioredoxin reductase (TrxR) isoenzymes are NADPH dependent homodimer oxidoreductases with one FAD per subunit that reduce the active site disulfide in oxidized Trx (Figure 4) (Holmgren 1989; Holmgren and Björnstedt 1995; Gromer, Schirmer et al. 1999; Arnér and Holmgren 2000). In plants, prokaryotes and bacteria the subunits of TrxR are about 35kDa, which should be compared to mammalian TrxR with 55kDa subunits. These properties divide TrxR into two subgroups, small TrxR (subunit size approx. 35kDa) and large TrxR (subunit size approx. 55kDa) (Gromer,

Urig et al. 2004). *E.coli.* TrxR has a very narrow substrate specificity and is in principle only reducing *E.coli* Trx. Plants and yeast TrxR have similar characteristics as the bacterial enzyme. The larger mammalian TrxR enzymes are more complex, have a wide substrate specificity and, in contrast to small TrxRs', are selenoproteins. For a schematic figure of their structure, see Figure 4.

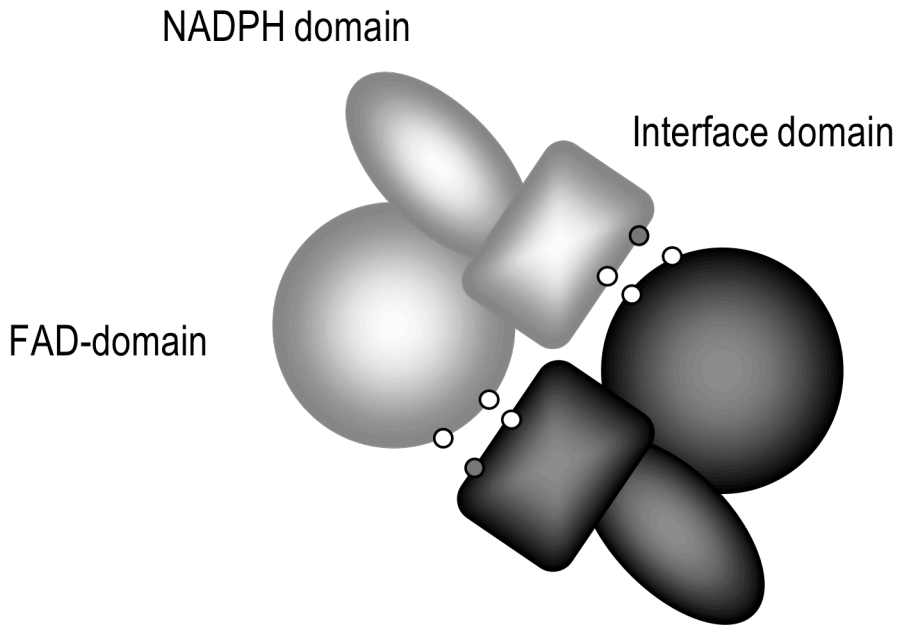

Figure 4. **Schematic structure of mammalian TrxR**. The dimer consists of two identical subunits arranged in a head to tail orientation. The NADPH, FAD and interface domain are indicated in this cartoon and the redox active cysteines are shown as white dots whereas selenocysteines are grey dots. In the proposed reaction mechanism for TrxR1, the flexible C-terminal tail with a selenolthiol motif is responsible for transferring electrons from the dithiol redox-center close to the enzyme bound flavin to any of the substrates of the enzyme (see text) For crystal structure see (Sandalova, Zhong et al. 2001).

A mammalian thioredoxin reductase 1 was first purified to homogeneity from bovine tissues in 1977 (Holmgren 1977), but it was not until 1996 the enzyme was identified as a selenoprotein (Gladyshev, Jeang et al. 1996; Tamura and Stadtman 1996).

TrxR contains selenium in the form of selenocysteine (Sec), the naturally occurring selenium analogue of cysteine being recognized as the 21st amino acid (Böck, Forchhammer et al. 1991; Stadtman 1996). The Sec residue of TrxR1 is localized to the C-terminus of TrxR and is part of the tetrapeptide motif Gly-Cys-Sec-Gly-COOH (Gladyshev, Jeang et al. 1996; Lee, Bar-Noy et al. 2000; Zhong, Arnér et al. 2000; Zhong and Holmgren 2000). Sec is encoded by a UGA codon, that normally terminates translation. However, in selenoproteins termination is recoded by the Sec insertion

element (SECIS element) located in the 3' untranslated region of the selenoprotein mRNA, thus facilitating a cotranslational insertion of the Sec residue (Böck, Forchhammer et al. 1991; Stadtman 1996; Bar-Noy, Gorlatov et al. 2001). The Sec residue is essential for the normal catalytic activity of TrxR and its activity is impaired in selenium deficient animals (Hill, McCollum et al. 1997) or human cell lines (Marcocci, Flohe et al. 1997), a fact that may explain some of the effects of selenium deficiency. Theoretically selenium deficiency may possibly generate a truncated TrxR with a carboxylterminal motif consisting of Gly-Cys-COOH. There are some implications for this in cell lines studies (Gorlatov and Stadtman 1998; Nalvarte, Damdimopoulos et al. 2004), but selenium compromised truncated TrxR protein has not yet been detected conclusively from tissues of selenium deficient animals.

Three different genes for human TrxR have been identified, one encoding cytosolic TrxR1 (Tamura and Stadtman 1996; Zhong, Arnér et al. 1998), one mitochondrial TrxR2 (Chae, Kim et al. 1999; Miranda-Vizuete, Damdimopoulos et al. 1999) and one enzyme predominantly expressed in testis (TGR) (Su, Novoselov et al. 2005). In addition it has been found that mammalian TrxR isoenzymes are subject to alternative splicing, encoding several isoforms that differ mainly at their N-terminal ends (Rundlöf, Carlsten et al. 2000; Osborne and Tonissen 2001; Rundlöf, Janard et al. 2004; Su and Gladyshev 2004). The physiological relevance of the different splice variants is today not known.

Apart from being selenoproteins, mammalian TrxRs share similarities to other flavoprotein oxidoreductases, such as glutathione reductase (GR) and lipoamide dehydrogenase, in domain arrangement (Gasdaska, Gasdaska et al. 1995; Zhong, Arnér et al. 1998), crystal structure (Sandalova, Zhong et al. 2001) as well as the mechanism of the reductive half reaction (Arscott, Gromer et al. 1997). The reductive half-reaction, which is the first part of the reduction of mammalian TrxR by NADPH, involves transfer of electrons from NADPH via the enzyme-bound FAD to an active site disulfide formed by the cysteines in positions 59 and 64 in the N-terminal active site domain sequence (numbering for rat TrxR1) being identical to the corresponding motif in GR (CVNVGC), thereby forming a dithiol with a FAD-thiolate charge transfer complex. Electrons are then transferred from the N-terminal active site of one subunit (Sub_1) to the selenenylsulfide formed by the Cys-Sec pair in the C-terminal sequence

Gly-Cys-Sec-Gly-COOH of the other subunit (Sub_2). (Zhong, Arnér et al. 2000). This transfer of electrons from one subunit to the other can be viewed as analogous to the reduction of GSSG by GR (Zhong, Arnér et al. 2000). The selenolthiol formed in the C-terminal motif can finally reduce any of the substrates of TrxR. See figure 5 for a schematic view of the catalytic mechanism of mammalian TrxR1.

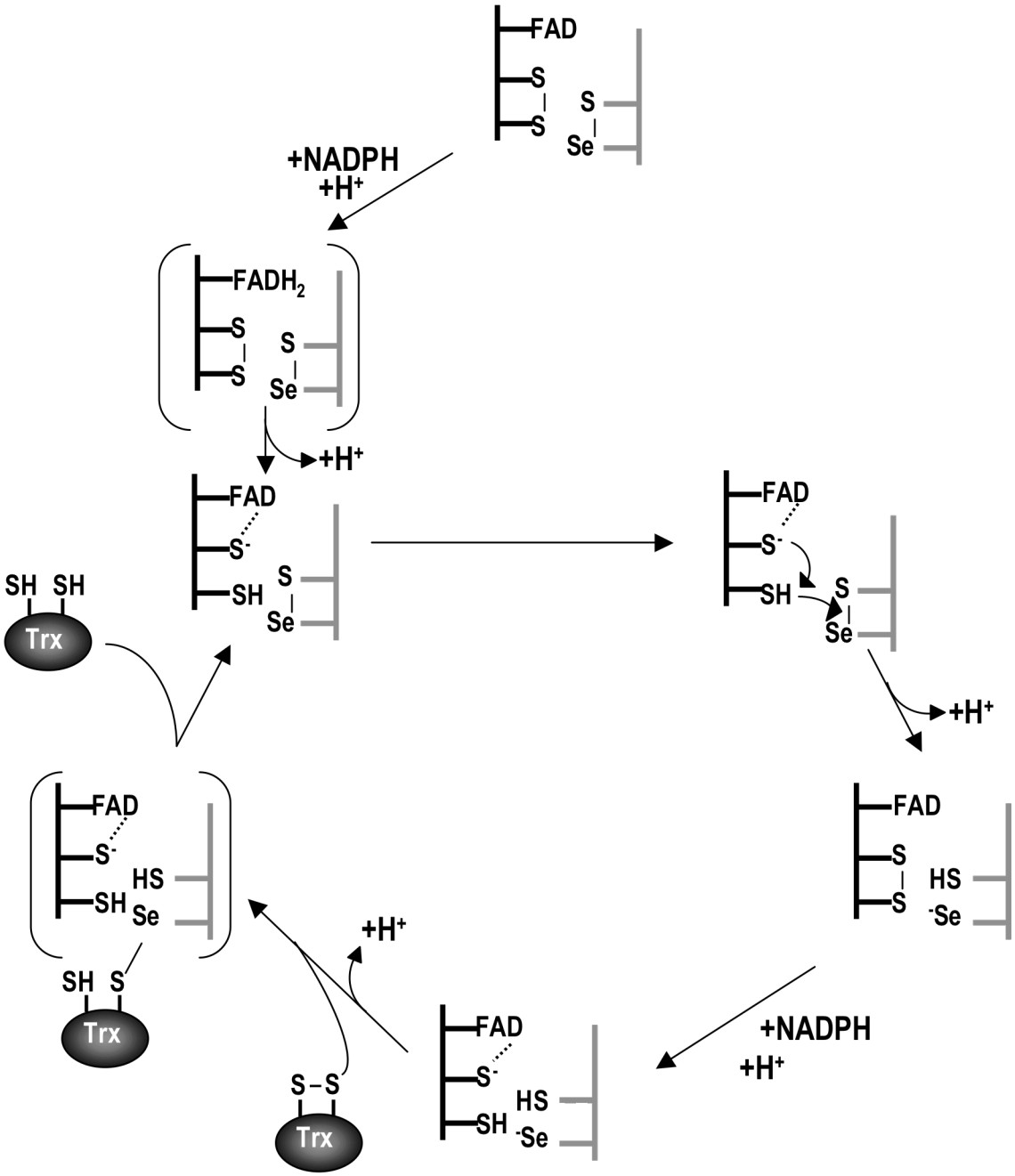

Figure 5. Simplified scheme of the catalytic mechanism of mammalian TrxR1. The enzymatic catalysis involves transfer of electrons from NADPH via the enzyme-bound FAD to an active site disulfide formed by the cysteines in position 59 and 64 in the N-terminal active site domain sequence, forming a dithiol motif (Arscott, Gromer et al. 1997; Zhong, Arnér et al. 2000). The N-terminal redox active site exchanges the electrons to the C-terminal redox active Cys-Sec pair of the opposite subunit (forming a selenenylsulfide in the oxidized enzyme). The reduced C-terminal selenolthiol can finally reduce any of the many substrates of TrxR. Note that only active site of the homodimeric enzyme is depicted in this scheme.

The main substrate for mammalian TrxR is Trx. Close to, if not all functions attributed to Trx are redox regulated, no matter if the active motif of Trx is in the disulfide or dithiol state. Thus the activity of TrxR is an integral part of the many functions of Trx. In addition, TrxR has been found to have a broad substrate specificity and the enzyme reduces not only protein disulfides but also many low molecular weight disulfide compounds such as DTNB (Holmgren 1977), lipoic acid (Arnér, Nordberg et al. 1996), and low molecular weight non-disulfide compounds such as vitamin K (Luthman and Holmgren 1982) or alloxan (Holmgren and Lyckeborg 1980). In addition TrxR can reduce ubiquinone to ubiquinol (Xia, Nordman et al. 2003) as well as other quinones (Cenas, Nivinskas et al. 2004) and it may recycle dehydroascorbate to ascorbate (May, Mendiratta et al. 1997). Several selenium containing compounds, such as selenite (Kumar, Björnstedt et al. 1992), ebselen (Zhao, Masayasu et al. 2002), selenocysteine (Björnstedt, Kumar et al. 1995), methylseleninate (Gromer and Gross 2002) and selenodiglutathione (Björnstedt, Kumar et al. 1992) are also substrate for TrxR. There are two features of mammalian TrxR that can explain the broad substrate specificity of the enzyme. The flexible C-terminal tail that transports electrons from the buried redox-center near the flavin to a more exposed position at the surface enable reduction of bulky substrates. In addition, the Sec residue in the active site has a stronger nucleophilicity and lower pK_a value compared to cysteine, resulting in a motif that is highly reactive at physiological pH.

1.3.2.1 Inhibitors of thioredoxin reductase

The properties of the active site of mammalian TrxR has made the enzyme a highly susceptible target for inhibition of different compounds. The majority of the inhibitory compounds studied until today inhibit the enzyme irreversibly, and seem to act via a reaction with one or more of the redox active residues (Cys-Sec-), as indicated by the absence of inhibitory effect on oxidized TrxR1 in the absence of NADPH.

1.3.2.1.1 Platinum compounds

The platinum containing compound *cis*-diamminedichloroplatinum (II) (Cisplatin) has been proven to be very effective in anticancer therapy. However, severe side effects such as ototoxicity and nephrotoxicity has caused an intensive search for finding new

platinum containing drugs with less severe side effects. DNA is generally considered the major target for platinum compounds. In addition, cisplatin has been found to efficiently and irreversible inhibit TrxR (Sasada, Nakamura et al. 1999; Arnér, Nakamura et al. 2001). Interestingly, the related compound *cis*-diammine-(1,1-cyclo-butanedicarboxylato)-platinum(II) Carboplatin did not inhibit TrxR (Arnér, Nakamura et al. 2001). Recently it was also reported that a series of new platinum(II) complexes efficiently inhibited mammalian TrxR, but not the closely related GR (Millet, Urig et al. 2005).

1.3.2.1.2 Nitrosoureas

Nitrosoureas are other compounds widely used as anticancer agents in the clinic especially for treatment of brain tumors and certain lymphomas. Compounds in this group, such as Carmustine, can easily react with thiols and selenols but also with other functional groups (Stahl, Krauth-Siegel et al. 1987). Different from the platinum containing compounds, nitrosoureas inhibit all members of the glutathione reductase family, including thioredoxin reductase (Schallreuter, Gleason et al. 1990; Arscott, Gromer et al. 1997; Gromer, Schirmer et al. 1997).

1.3.2.1.3 Gold compounds

Gold containing compounds are used in the treatment against rheumatoid arthritis (Gromer, Arscott et al. 1998), but has in screening trails also been proven to be as effective as 5-fluorouracil and cisplatin as antineoplastic agents (Simon, Kunishima et al. 1981). Interestingly gold compounds have been identified as most effective and selective inhibitors of TrxR, acting upon TrxR in almost stoichiometric concentrations (Hill, McCollum et al. 1997; Gromer, Arscott et al. 1998). In inhibition assays with glutathione reductase and glutathione peroxidase a micromolar range was instead required to observe an inhibition (Gromer, Arscott et al. 1998).

1.3.2.1.4 Dinitrohalobenzenes

1-Chloro-2, 4-dinitrobenzene (DNCB) is used clinically as a locally applied immuno-stimulatory agent in the treatment of malignant melanoma (Bilzer, Krauth-Siegel et al. 1984; Gromer, Urig et al. 2004). DNCB has been identified as a covalent inhibitor of TrxR (Arnér, Björnstedt et al. 1995). In addition to inhibited TrxR activity the reaction also induces a strong increase of the enzyme NADPH-oxidase activity, which results in

superoxide production (Nordberg, Zhong et al. 1998; Arnér 1999). 1-fluoro-2, 4-dinitrobenzene (DNFB), the fluoride analoge to DNCB, can also inhibit the enzyme and induce oxidase activity. In great similarity to many other TrxR inhibitors, DNCB is a poor inhibitor of glutathione reductase (Ernster and Dallner 1995).

1.3.2.1.5 Additional inhibitors of TrxR

In addition to the groups of compounds mentioned above TrxR can also be inhibited by nitroaromatic compounds such as tetryl (Cenas, Prast et al. 2005), quinone compounds (Cenas, Nivinskas et al. 2004), flavonoids (Lu, Papp et al. 2006), 4-Hydroxy-2-nonetal (HNE) (Fang and Holmgren 2006), Curcumin (Fang, Lu et al. 2005), MGd (Hashemy, Ungerstedt et al. 2006) and thiol alkylating compounds such as iodoacetamide, iodoacetic acid, 5-iodoacetamido-fluorescein (Sun, Wu et al. 1999) and 4-vinylpyridine (Nordberg and Arnér 2001; Gromer, Urig et al. 2004).

1.3.3 Thioredoxin system in antioxidant defence

The function of the thioredoxin system in antioxidant defence includes several different enzymes and low molecular compounds that will be covered below. In addition, the direct antioxidant properties of thioredoxin include removal of hydrogen peroxide and scavenging of free radicals generated by ascorbic acid and 2,6-dimethoxy-p-benzoquinone (Spector, Yan et al. 1988; Fernando, Nanri et al. 1992; Hassouni, Chambost et al. 1999; Lowther, Brot et al. 2000).

1.3.3.1 Superoxide dismutase (SOD)

Two different forms of SOD enzymes have been identified, one located in the mitochondria (Mn-SOD) and one cyotosolic variant Cu/Zn-SOD (McCord and Fridovich 1969). SOD can metabolize $O_2^{\bullet -}$ into hydrogen peroxide. Oxidative stress and Trx (Das, Lewis-Molock et al. 1997) induce expression of Mn-SOD.

1.3.3.2 Peroxiredoxins (Prx)

The peroxiredoxins are a recently found group of enzymes that directly can reduce peroxides such as hydrogen peroxide and different alkyl hydroperoxides (Chae, Kang

et al. 1999; Chae, Kim et al. 1999). In mammals at least 13 different peroxiredoxins are known (Kim, Kim et al. 1988; Chae, Kang et al. 1999; Chae, Kim et al. 1999). Oxidized peroxiredoxins generated in the catalytic cycle can be regenerated by thioredoxin (Poole, Godzik et al. 2000), and in the mammalian mitochondria the Trx system is probably a specific reductant of Prx (Miranda-Vizuete, Damdimopoulos et al. 2000).

1.3.3.3 Glutathione peroxidase (GPx)

Glutathione peroxidases are selenoproteins that catalyze the reduction of hydrogen peroxide by using glutathione as a substrate (Ursini, Maiorino et al. 1995; Arthur 2000). Furthermore they can also reduce other peroxides, such as lipid peroxides, to alcohols. The cytosolic forms of glutathione peroxidases, GPx1 and GPx4, are found in almost all tissues. However GPx2 and GPx3 are mainly expressed in the gastrointestinal tract and kidney respectively. GPx3 has been found to be a substrate to the thioredoxin system (Björnstedt, Xue et al. 1994). This property couples also this type of antioxidant enzyme to the thioredoxin system.

1.3.3.4 Glutathione (GSH/GSSG)

Glutathione is found in millimolar concentrations in the cell, mainly present in its reduced form (GSH) (Anderson 1997; Anderson 1998). The compound mainly functions as a sulfhydryl buffer but can also detoxify ROS via conjugation reactions catalyzed by glutathione S-transferases (Armstrong 1997; van Bladeren 2000; Rinaldi, Eliasson et al. 2002). Oxidized glutathione (GSSG) is reduced by glutathione reductase, but may also be reduced by the thioredoxin and glutathione reductase (TGR) (Sun, Kirnarsky et al. 2001) or in some cases Trx (Kanzok, Schirmer et al. 2000; Kanzok, Fechner et al. 2001).

1.3.3.5 Low molecular weight compounds

In addition to the enzymatic systems and glutathione mentioned above a large number of additional low molecular weight compounds can also function as antioxidants in biological systems.

Vitamin E is the major antioxidant in a biological membrane and can react with free radicals to form Vit E semiquinones that in turn can be reduced back to Vit E by ascorbic acid (Packer, Slater et al. 1979; Mukai, Nishimura et al. 1989; Babior 1997). In this reaction an ascorbyl free radical are formed, and two of these can participate in a spontaneous dismutase reaction, generating one molecule of ascorbic acid and one molecule of dehydroscorbic acid. TrxR can reduce the latter back to ascorbic acid (May, Cobb et al. 1998).

Ubiquinone functions as antioxidant by preventing lipid peroxidation (Ernster and Dallner 1995). However the main function is in the mitochondria as a part of the electron transport chain. Regeneration of ubiqionone can be catalyzed by TrxR (Xia, Björnstedt et al. 2001).

Furthermore selenium compounds and lipoic acids are part of the antioxidant defence in a cell, and as mentioned above they can be regenerated by TrxR (Nordberg and Arnér 2001).

1.3.4 Thioredoxin system and cell death

The multifaceted properties of the thioredoxin system make it not just an important regulator of cell growth and proliferation but also of cell death. The system participates in several different ways and at different levels in the regulation of cell death. The following part is an attempt to summarize these different interactions.

1.3.4.1 Thioredoxin system and p53

Trx augments the DNA binding activity of p53 and can also further potentiate Ref-1-enhanced p53 activity (Ueno, Masutani et al. 1999). Subsequently, Trx enhances p53-dependent expression of p21, which further intensifies the Ref-1-mediated activation. Cisplatin- induced p53 activation and p21 transactivation is inhibited by mutant Trx indicating that Trx-depedent redox regulation is physically involved in p53 regulation. In addition cisplatin has been found to translocate Trx to the nucleus (which may be related to TrxR inhibition - see above) (Ueno, Masutani et al. 1999).

Treatment of cells harbouring a wildtype p53 gene with electrophilic prostaglandins results in accumulation of p53 protein that is conformationally and functionally impaired (Moos, Edes et al. 2000; Mullally, Moos et al. 2001). Furthermore, such treatment did only antagonize the apoptosis mediated by p53, but not the p53-induced cell cycle arrest and, also, the same effect could not be seen if TrxR was absent. These observations have been implicated to be a result of derivatized TrxR formed by the prostaglandins (Moos, Edes et al. 2003). Interestingly, inorganic Se spares p53 from inactivation by lipid electrophiles, however if this is a result of increased TrxR expression or activity (Moos, Edes et al. 2003), or due to a larger pool of targets for the lipid electrophiles, is not yet investigated. Interestingly, auranofin, one of the most efficient inhibitors of TrxR (se above), impaired the p53 conformation in a similar manner as for electrophilic lipids (Moos, Edes et al. 2003).

However, when TrxR expression was decreased by siRNA, p53 was accumulated in the nucleus and showed an increase in DNA binding activity (Seemann and Hainaut 2005), to the same extent as observed after exposure of cells to 1ug/ml doxorubicin. At the same time oxidized Trx was sequestered from the nucleus into the cytoplasm. In addition, decreased expression of Ref-1, a protein with a thiol domain reduced by Trx (Hirota, Matsui et al. 1997), resulted in increased protein turnover and decreased p53 level and activity. However, inhibition of TrxR and Ref-1 did not prevent activation and accumulation of p53 in response to DNA damage by doxorubicin (Seemann and Hainaut 2005). These observations illustrate the truly intricate network of interactions between Trx, TrxR and p53 that are not yet fully understood.

1.3.4.2 Effects of inhibited TrxR activity

Inhibitors of mammalian TrxR are a fast growing area of interest. In the last years several new reports have been published identifying several old and new compounds as inhibitors of TrxR. In addition, *in vivo* effects as a result of inhibited TrxR enzymatic activity are studied in closer detail. Some of the recent findings will be presented here.

TrxR has been found to be a target for 15-Lipoxygenase-1 or its metabolites. Overexpression of 15-Lipoxygenase-1 in HEK293 cells inhibits TrxR activity (Yu,

Moos et al. 2004). The inhibition resulted in a corresponding inhibition of ribonucleotide reductase and cell growth arrest in G_1 as a consequence. An increase in the pro-apoptotic protein Bax was also observed (Yu, Moos et al. 2004). However this was the only way apoptosis induction was measured in that study. Since HEK293 cells contain the E1B19K protein, inhibiting essentially all apoptosis signalling pathways (Martinou, Fernandez et al. 1995; Ohi, Tokunaga et al. 1999; Hu, Zhu et al. 2004), a measurement of caspase activation would have been very interesting in light of our findings (presented below).

Furthermore, TrxR has been found *in vitro* and *in vivo* to be a target for quinones or nitroaromatic compounds such as DNCB, juglone and tetryl (Cenas, Prast et al. 2005). The compounds were found to be highly cytotoxic to HeLa (human cervical cancer) cells and induced a caspase 3/7 activation (Cenas, Prast et al. 2005). In addition, the compounds did also provoke apoptosis in Bcl-2 overexpressing HeLa cells and A549 (lung carcinoma) cells, indicating that alkylation of TrxR with nitoraromatic or quinone compounds may contribute to the apoptosis induction in exposed human cancer cells (Cenas, Prast et al. 2005). In correlation to this study growth inhibition and apoptosis in five different cancer cell lines were studied after treatment with [bis(1,2-Benzisolelenazolone-3 (2H)-ketone)]ethane (BSSKE). All cells showed a good linear correlation between TrxR activity and cell growth/proliferation inhibition and apoptosis. The apoptosis provoked was suggested to be a result of signalling via Bcl-2/Bak and caspase-3 pathways (Zhao, Yan et al. 2005).

A recently published study with 4-mercaptopyridine (4-chloro-2,2:6,2´´-terpyridine)platinum nitrate ($I_{23}2N$) and 2-mercaptopyridine (4´-chloro-2,2´:6,2´´-terpyridine ($I_{25}2N$) has demonstrated a decrease in tumor growth as a result of inhibited TrxR activity and DNA intercalation. However, the antitumor activity of $I_{23}N$ was not mediated by apoptosis but rather by cell cycle arrest (Ahmadi, Urig et al. 2006). This study correlates well with a study in A549 cells demonstrating that flavonioids inhibiting TrxR activity results in cell cycle arrest in S phase (quercetin) respectively in sub-G_1 phase (myricetin) (Lu, Papp et al. 2006). In addition, 4-Hydroxy-2-nonetal (HNE) (Fang and Holmgren 2006), Curcumin (Fang, Lu et al. 2005) and MGd (Hashemy, Ungerstedt et al. 2006) have all been found to inhibit cellular activities of TrxR. Interestingly the anticancer drugs curcumin (Fang, Lu et al. 2005) and MGd

(Hashemy, Ungerstedt et al. 2006) provoked a strong NADPH oxidase activity. However, no in-depth analysis of how the apoptotic signaling pathways in the cells were affected by the different inhibitors have yet been performed.

1.3.4.3 Apoptosis regulation by ASK-1

The mitogen-activated protein (MAP) kinase (MAPK) cascade transmits signals form the outside of the cell to the nucleus, activating transcription factors and other intracellular target proteins via phosphorylation of serine, threonine or tyrosine residues. The cascade is activated by phosphorylation of the MAP kinase kinase kinases, in turn activating MAP kinase kinases by phosporylation and finally MAP kinases are activated (Chang and Karin 2001). In mammals three different major MAP cascades have been well characterized. ERKs are activated by various cytokines whereas JNKs and p38/MAP are preferentially activated by chemical and physical stressors and therefore control stress adaptation, cell death and survival (Davis 2000; Tournier, Hess et al. 2000). One member of the MAPKKK family that activates both p38 and JNK pathways is Apoptosis Signal-regulating Kinase-1 (ASK-1) (Ichijo, Nishida et al. 1997; Minden and Karin 1997). In mammalian cells ASK-1 is activated by TNF-α and H_2O_2 exposure. The TNF-α effect can be inhibited by N-acetyl cysteine (NAC), an antioxidant interacting with reactive oxygen species (Gotoh and Cooper 1998). These findings imply that the level of reactive oxygen species is important for ASK-1 activation. Interestingly, ASK-1 activity is regulated by the thioredoxin system. In a reduced state Trx binds to the N-terminal part of ASK-1 and prevents its apoptotic signaling (Saitoh, Nishitoh et al. 1998). Decreasing intracellular levels of reduced Trx, either by antisense or inhibited TrxR activity by DNCB, has been shown to result in the activation of ASK-1.

Furthermore, the association of Trx to ASK-1 will also prevent the apoptotic activity of TNF receptor associated factor 2 (TRAF2) via JNK and p38, since this reaction also requires ASK-1 (Liu, Nishitoh et al. 2000).

1.3.4.4 GRIM-12

Although TrxR1 is generally considered a protein promoting cell growth and proliferation, redox control and defence against oxidative stress, several papers

identified the protein as an important contributor to cell death provoked after treatment with retinoic acid (RA) and interferon β (INF-β), or Tamoxifen and INF-β (Hofmann, Boyanapalli et al. 1998; Lindner, Hofmann et al. 2000; Hu, Ma et al. 2001; Ma, Karra et al. 2001; Ma, Karra et al. 2001; Lindner, Ma et al. 2002; Ma, Hu et al. 2002). Much effort was invested in identifying and understanding the mechanism behind the proposed cell death properties of TrxR1. However, one problem with those studies was that the TrxR construct used for transfecting cells was fused into a modified mammalian expression vector pCXN2-Myc, adding a Myc epitope to the carboxyl end instead of maintaining the selenocysteine-encoding UGA-codon. Consequently a truncated TrxR protein without the selenocysteine residue was expressed, so that *in essence* a form of the enzyme lacking its normal redox activity was analyze in those studies. I will in the following paragraph summarize the major results from those studies.

TrxR1 was identified in the form of GRIM-12 (gene associated with retinoid-IFN-induced mortality number 12) by the usage of an anti sense knockout approach, thus identifying TrxR1 as a gene that conferred a growth advantage in the presence of the death inducers RA and INF-β when it was expressed in an antisense orientation in HeLa and MCF-7 cells (Hofmann, Boyanapalli et al. 1998). However, when cells were exposed to RA/INF-β, the activity and protein expression of TrxR was increased, without increased mRNA expression, implying that the treatment had no transcriptional effect. Subsequently the FAD domain, the NADPH domain and the interface domain (ID) were directly expressed in MCF-7 cells in an attempt to identify the domain in the protein that potentiated the effects of RA/ INF-β treatment. The different domains had no effect on growth rate, but when cells were exposed to RA/ INF-β, the ID-construct protected cells against cell death and lowered the TrxR activity. Interestingly, however, establishing stable transfectants of GRIM-12 in MCF-7 cells slowed down the growth and increased the cell death significantly compared to control cells, an effect that was further potentiated by RA/ INF-β treatment. Further studies performed with these cells augmented an increased transcriptional activity of p53 resulting in the increased cell death. No increased protein expression was noticed in cells transfected with a construct containing a deletion of the CVNVGC motif in the myc-tagged TrxR1, which was exchanged for “GA”. Cotransfection with Trx1 and GRIM-12 further increased the p53-dependent cell death. The p53 inhibitory protein E6 to some extent decreased cell

death in GRIM-12 overexpressing cells, but not down to the same levels as seen in controls or with the "GA" mutant. Analysis of increased expression of apoptotic proteins as a result of GRIM-12 expression revealed an increased Bax expression, that was further increased by RA/ INF-β treatment followed by cytochrome *c* release. Other apoptosis promoting proteins with increased mRNA expression increased by GRIM-12 were Fas-ligand, Fas, DR3, DR4, DR5 and TRAIL. However, Western blot analysis revealed that only TRAIL and Fas were increased as proteins. In addition it was found that caspase-inhibitors prevented the effects of GRIM-12 and subsequent analysis indicated activation of caspase-8 and-9, activities that were further escalated after RA/ INF-β treatment.

Our work on SecTRAPs initiated as an analysis of "GRIM-12" experiments referred to above. Before discussing our results, we should however also consider the relation between TrxR1, selenium and cancer.

1.3.5 Thioredoxin reductase, selenium and cancer

The therapeutic window for nutritional selenium compounds is narrow, and low dose supplementation results in an increased antioxidant defence predominantly as a result of increased selenoprotein expression. However, higher concentrations of selenium are toxic and result in cell death and DNA-fragmentation (Garberg, Stahl et al. 1988; Wilson, Thompson et al. 1992). In addition, several clinical trials have shown that selenium may be effective in reducing the incidence of cancer, i.e as a cancer preventive agent (Clark, Combs et al. 1996). However, the biochemical basis for the protective effect of selenium against cancer is not well understood. Basically two different mechanisms may be considered. The first possibility is that the chemopreventive action of dietary Se is mediated by an increase of abundance of Se-containing proteins with subsequent cancerpreventive activies, and the second alternative is that low molecular weight Se-compounds are responsible for the chemoprotective effect. In the literature there are supporting evidence for both theories and they may well both be correct.

Numerous studies have found an elevated expression of TrxR in cancer cell lines, including Jurkat and A549 cells. In addition it has been demonstrated that TrxR1

protein and activity is increased in colorectal tumors compared to normal mucosa, but the importance of splice variants have not yet been considered (Rundlöf and Arnér 2004). In a study with a cancer cell line of epithelial origin it was shown that selenium in the form of selenite increased TrxR mRNA-levels 2- to 3-fold and increased the half-life of the mRNA from 10 to 21 hours (Gallegos, Berggren et al. 1997). Furthermore the study suggested that an initial increase of TrxR activity caused by selenium supplementation was due to increased TrxR1 mRNA level followed by an increase in the TrxR selenoprotein activity. However, analysis of cells of lymphoid origindid not posses any increased activity or protein expression after supplementation with selenium (Gallegos, Berggren et al. 1997). In addition, selenium supplementation to colon cancer cells increased the activity of TrxR. At the same time TrxR protein expression was increased, but to a lesser extent than activity (Berggren, Gallegos et al. 1997). In a recent study, investigating the effects of selenium-induced cytotoxicity in HEK293 cells overexpressing TrxR, it was found that overexpressing cells were more resistant to the cytotoxic effects of selenium than normal cells (Madeja, Sroka et al. 2005). Furthermore, TrxR overexpressing cells were more resistant to inhibitory effects of 50 μM selenite and demonstrated a more pronounced increase in TrxR activity after supplementation of 0.1 μM selenite (Madeja, Sroka et al. 2005). Interestingly GPx activity was decreased in TrxR overexpressing cells compared to normal cells. Based on these observations the authors conclude that TrxR is a crucial enzyme in the resistance against selenium cytotoxicity (Madeja, Sroka et al. 2005).

In studies with rats, supplementation of selenium initially increased TrxR activity in some tissues (lung, liver and kidney), but the increased activity was not sustained over time (Berggren, Mangin et al. 1999).

In TGF-α/c-Myc transgenic mice that develop liver cancer at 6 months age, Se deficiency as well as high levels of Se suppressed hepatocarcinogenesis. The subsequent analysis of selenoproteins demonstrated that GPx and Sep15 were more susceptible to selenium changes than thioredoxin reductase, indicating that thioredoxin reductase is a prioritised protein at selenium deficiency. Interesting was indeed the finding that tumor development was inhibited after both selenium deficiency as well as high-selenium supplemented diet (Novoselov, Calvisi et al. 2005). However, other studies in rats showed an unaltered GPx activity and a decrease in TrxR activity after

selenium deficient and selenium supplemented diet (Björkhem-Bergman, Torndal et al. 2005). At the same time the volume fraction of preneoplastic liver nodles were decreased after supplementation of selenite (Björkhem-Bergman, Torndal et al. 2005).

The differences between the reports described above reflect the complexity in the relations between selenium, TrxR and cancer. Conclusions that can be drawn are that effects of selenium on different organs are diverse depending on if it is a normal or a tumor tissue, and dependent on the progression state the tumor is in. One interesting phenomenon is the high priority of TrxR among other proteins at selenium deficiency. This is also reflected by the fact that the only selenoprotein found in *C.elegans* is TrxR (Buettner, Harney et al. 1999; Gladyshev, Krause et al. 1999).

2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY

The thioredoxin system at a whole is likely to be essential for the survival of a cell. In cancer cells the system is often up-regulated, helping the cell to persist an increased stress level caused by the high metabolic activity that is characteristic in cancer. This property alone makes the thioredoxin system a potential target for developing new strategies for cancer therapy. In this study, we have focused on thioredoxin reductase and its selenocysteine in the active site. The Sec residue is highly reactive at physiological pH and in TrxR it has previously been found to easily interact with electrophilic compounds, resulting in a loss of its enzymatic activity. Additionally, contradictory results have identified thioredoxin reductase as a protein contributing to the effect IFN-γ and retinoic acid treatment as GRIM-12. In the present study we therefore wanted to investigate how different enzymatic preparations of TrxR may affect cancer cells. In addition, we also wanted to examine TrxR as a target for electrophilic cancer drugs used at the clinic. The specific aims of the present study project were to:

- study how selenium compromised enzymatically inactive TrxR1 species in comparison to fully active TrxR1 may affect cancer cell viability.
- compare the inhibition capacity of electrophilic anti-cancer drugs between TrxR1 and glutathione reductase.
- identify the signalling pathways for apoptosis in cells death provoked by selenium compromised TrxR1
- investigate the necessity of an intact CVNGC motif for the apoptotic effects of selenium compromised species of TrxR1

2.2 METHODOLOGY

The methods used in this thesis project are described in detail in the material and method sections of paper I-IV. Here I will however briefly comment on some of the methods used as guidance for future users.

2.2.1 Annexin-V staining

When a cell becomes apoptotic it translocate phosphatidyl-serine to the outer side of the cell membrane as a signal to phagocytes that it should be "eaten" to prevent the upcome of an inflammation. Research has taken advantage of this phenomenon, since it is easily detected with Annexin-V antibodies. However, one important aspect to have in mind when using Annexin-V antibodies is that if a cell has a disruptured cell membrane the antibodies can give a false positive staining. Therefore an additional control should be made with propidium iodide, that will enter permeabilized cells. Thus, Annexin-V positive cells negative for PI staining have true phosphatidyl serine exposure. However, PI as a control can only be used if the antibodies coupled to Annexin-V have another colour and wavelength for detection. Annnexin-V staining can be detected by FACS analysis alternatively fluorescent microscopy. In this thesis project we used Annexin-V antibodies coupled to FITC and positive staining were visualized by fluorescence microscopy equipped with a filter for fluorescein isothiocyanate (excitation: 490 nm, emission: 525 nm).

2.2.2 BioPORTER system

Overexpression of proteins can not always be performed by transfecting cells with a plasmid containing the gene of interest. Moreover the impact of posttranslational modified proteins in cells can not easily be studied using transfection experiments. A quite new method was therefore developed based upon a reagent called *BioPORTER*. The *BioPORTER* is a lipid formulation that makes it possible to transfect proteins into cells without changing the functionality of the protein. Successful uptake can be monitored with a fluorescent microscope after labelling of the protein in question with

a fluorescent probe (or by other means). In comparison to protein transduction domains such as Tat or VP22, the *BioPORTER* was found to be a superior method for protein delivery into cells (Gupta, Levchenko et al. 2005). In our experimental setup, in order to assess delivery, we have chosen two different probes that bind to exposed thiol groups; Alexa488 and 5-IAF. After reduction with DTT both compounds successfully labelled TrxR1. However, the advantage with Alexa 488, compared to 5-AIF, is that it is more resistant to bleaching at UV-exposure, conveying easier work at the fluorescent microscope.

The cell death effect by to the *BioPORTER* system itself is around 5-10%. We have found that the *BioPORTER* system has been convenient to use for fluorescent microscopy studies. However, we also tried to use it in luminescence assays (of caspase activation), without positive results (unpublished). In addition, *BioPORTER* seems to make the cells more sticky, which may be a problem in samples used for FACS analysis. Finally, the high cost of BioPORTER limits its use to experimental set-up using a limited number of cells.

2.2.3 Caspase inhibitors

One way of evaluating if caspases participate in cell death signalling is assessing protection by caspase inhibitors. There are several aspects to have in mind when using caspase inhibitors. First of all there are very few specific caspase inhibitors; they rather preferentially inhibit one caspase in front of other caspases. In addition, the concentration of the caspase inhibitor can affect the result. In the literature a concentration of 100 μM or even higher is very common, however the trend is to use lower concentrations. While 100 μM is a good starting concentration, it is optional to go down to 25 μM or even lower to further strengthen the result in terms of specificity.

2.2.4 Fluorescent microscopy

All cell death assays in this thesis project were performed by staining non-permeabilized cells with Hoechst 3342 and propidium iodide (PI). Hoechst 3342 stains all cell nuclei and was detected by excitation at wavelength 360 nm and emission at

wavelength 460 nm (blue color). Propidium Iodide intercalate with DNA and stain cells with permeabilized nuclear membranes which was detected by excitation at 570 nm and emission at 620 nm (red color). From each sample that was evaluated, three pictures were captured showing cells at visual light, or with blue staining and red staining. Total amount of cells were then counted. In addition, morphological studies of the Hoechst stained nuclei were performed and cells having solely blue-stained normal non-condensed nuclei were considered viable, whereas blue- as well as blue- and red-stained cells (PI-staining due to collapsed membranes) having condensed nuclei were considered apoptotic and dead.

2.3 RESULTS AND DISCUSSIONS

2.3.1 Paper I

Rapid induction of cell death by selenium-compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine.

The initial purpose of this work was to study if TrxR1 protects against several different ROS producing compounds. We began by trying to establish stable transfectants overexpressing TrxR1. This was however significantly harder than we had thought. Later it was clear that also others (Nalvarte, Damdimopoulos et al. 2004), had observed this phenomenon of difficulties in establishing TrxR1 overexpressing cells and the only cell line where the transfection has been successful is in HEK293 cells. However, to circumvent the difficulties in overexpressing TrxR in cells, we took advantage of the *BioPORTER* technique, making it possible to directly introduce proteins into cells. Since we had a method of producing active recombinant TrxR1 (Arnér, Sarioglu et al. 1999; Rengby, Johansson et al. 2004; Johansson, Gafvelin et al. 2005), the *BioPORTER* method was highly suitable for us to achieve TrxR1 introduction into cells. First we used fluorescent labelled TrxR1, which was successfully delivered to A549 cells, thus verifying that this system could be used for TrxR1 delivery. The finding that TrxR was a suitable protein for delivery with the *BioPoRTER* system subsequently gave us an opportunity to analyse the studies wherein TrxR1 had been identified as a cell death factor, in the form of GRIM-12 (Hofmann, Boyanapalli et al. 1998; Lindner, Hofmann et al. 2000; Hu, Ma et al. 2001; Ma, Karra et al. 2001; Ma, Karra et al. 2001; Lindner, Ma et al. 2002; Ma, Hu et al. 2002). In those studies a modified TrxR1 protein was expressed as a result of the myc tag instead of the UGA-encoding Sec (see discussion above). An explanation to this mistake by the authors' might be that they had initiated their studies before TrxR was identified as a selenoproteins (thus implying the UGA to be a stop codon).

At the start of our *BioPORTER* studies, we observed a clear toxic effect to the cells by just using fluorescently labelled TrxR. Therefore, also in light of the GRIM-12 studies, we decided to introduce different pure preparations of TrxR1 into A549 cells. Interestingly, we found that selenium compromised TrxR1, either in a truncated form

or produced by alkylating the Sec residue by electrophilic compounds, provoked a very rapid cell death in A549 cells. The cell death effect of selenium compromised TrxR could not be detected after introduction of the fully active TrxR1 enzyme nor upon introduction of glutathione reductase, a highly homologous enzyme lacking the Sec residue. The results with GR indicated that the cell death provoked by selenium compromised TrxR was not a result of an unspecifically increased ROS production, since GR had higher endogenous NADPH oxidase activity than all the forms of TrxR that were introduced to cells. Morphological studies of the cells showed condensed DNA, indicating that the selenium compromised TrxR species provoked apoptosis, but since this was just one observation we believed that further studies needed to be performed to ascertain that a genuine apoptosis was induced. We found the result of this study to be highly intriguing, since they impaired that targeting of the Sec residue in TrxR may affect cells not only by inhibition of the Trx system, but also by a direct gain of function of the selenium compromised TrxR1 protein.

2.3.2 Paper II

Inhibition of thioredoxin reductase but not of glutathione reductase by the major classes of alkylating and platinum-containing anticancer compounds.

At the time for this study the effects of several electrophilic clinical used drugs were and still are mainly assigned to their ability to intercalate with DNA. However, nature has made the property of TrxR1 an excellent target for electrophilic compounds (Arnér, Björnstedt et al. 1995; Nordberg, Zhong et al. 1998; Nordberg and Arnér 2001; Gromer, Urig et al. 2004) and based on our findings in the previous study, we hypothesised that TrxR as a drug target could largely contributes to the anticancer effects of several electrophilic drugs. In this study we therefore wished to analyze *in vitro* the potential inhibition of mammalian thioredoxin reductase (TrxR), and of glutathione reductase (GR) as a control enzyme, by major widely clinically used anticancer alkylating agents and platinum-containing compounds. We found that nitrogen mustards (chlorambucil and melphalan) and alkyl sulfonates (busulfan) indeed inhibited TrxR (the latter less efficiently). In contrast, these compounds did not inhibit GR. Inhibitions of TrxR were concentration- and time- dependent and apparently irreversible. Anticancer anthracyclines (daunorubicin and doxorubicin) were, in

contrast to the alkylating agents, not inhibitors but poor substrates of TrxR (they had been reported as inhibitors by others). We also found that TrxR, but not GR, was efficiently inhibited by cisplatin, its monohydrated complex, and oxaliplatin. The inhibition of TrxR with cisplatin was already familiar to us (Arnér, Nakamura et al. 2001). However, in the context that the side-effects of oxaliplatin in patients are less severe compared to cisplatin treatment, the finding was at first surprising to us that oxaliplatin was as good inhibitor of TrxR as cisplatin. It implies, however, that TrxR1 may be a target in cancer cells where oxaliplatin displays therapeutic effects and other factors, such as pharmaco-dynamics, and distribution. In contrast to cisplatin and oxaliplatin the platinum containing drug carboplatin could not inhibit any of the two enzymes, further illustrating the importance of the structural design of a drug. If a drug is too bulky fewer targets exist, which necessitates a higher concentration of a drug for effects, increasing the pressure on organs participating in its clearance. These findings collectively lead us to conclude that representative compounds of the major classes of clinically used anticancer alkylating agents and most platinum compounds may easily target TrxR, but not GR. Thus, TrxR may indeed be an important target for anticancer therapy, also considering drugs already used for decades in anticancer therapy.

2.3.3 Paper III

SecTRAPs are potent inducers of a caspase-dependent apoptosis in cancer cells that can not be prevented by overexpression of Bcl2.

The extensive studies with GRIM-12 discussed above illustrated a cell death with several typical apoptotic features, such as caspase activation and positive Annexin-V staining due to phosphatidyl serine exposure (Hofmann, Boyanapalli et al. 1998; Lindner, Hofmann et al. 2000; Ma, Karra et al. 2001; Lindner, Ma et al. 2002; Ma, Hu et al. 2002). In this paper we therefore wanted to further evaluate any apoptotic signaling events provoked by delivery of selenium compromised TrxR1 species that initiate cell death in our system. To distinguish the cell death properties of TrxR1 by a gain of function from the general affects of GRIM-12 (either dependent on a functional Trx system in relation to RA/IFN-β or due to selenium compromised myc-tagged TrxR) we coined the term SecTRAPs (selenium compromised thioredoxin reductase-derived apoptotic proteins). In similarity with some of the GRIM-12 results we

demonstrated that the cell death provoked by SecTRAPs triggered exposure of phosphatidyl-serine to the outer membrane. Interestingly we found that caspase-2 inhibitors prevented apoptosis to the same extent as a general caspase inhibitor. Notably caspase-2 has been found to be part of the apoptosis signaling after cisplatin treatment (Zhivotovsky and Orrenius 2005), which agrees with the notion of formation of SecTRAPs, since SecTRAPs induce caspase-2 dependent apoptosis. The activation mechanism of caspase-2 is not recognized. Possibly there exists a close link between cisplatin treatment, generation of SecTRAPs and caspase-2 activation. The activation mechanism of caspase-2 may thus encompass SecTRAPs, either directly or together with another protein partner or low molecular compound, which should be studied further.

In this paper we also treated the cells with the protein synthesis inhibitor cycloheximide. However, the apoptotic outcome after treatment with SecTRAPs was not significantly changed, implying that no novel protein synthesis is needed for SecTRAPs to perform their effects.

One issue in our prior studies with SecTRAPs has been that we have used TrxR1 from rat as starting material in treatment of human cells, and we therefore found it important to verify that the effects were not due to some incompatibility between the two spieces. In addition we also wanted to exam the possibility that the effects observed were not just a specific event for a single cell line. Consequently we expressed recombinant human TrxR1 and introduced the truncated protein to either A549 or HeLa cells. Both cell lines were susceptible to the human truncated TrxR, as well as rat truncated TrxR implying that several cancer cell lines may be sensitive to SecTRAPs. Interestingly, however the human embryonic kidney cell line HEK293 was resistant to the effects of SecTRAPs. This result agreed with the fact that HEK293 cells but not CEM, Jurkat or HeLa cells may be used for stable transfection with overproduction of TrxR1, resulting in generation of truncated TrxR1 species (Nalvarte, Damdimopoulos et al. 2004). Unfortunately this did not give us any further information how the effects of SecTRAPs is initiated, since HEK293 cells are immortalized with adenovirus that contain E1B19K, a protein that basically turns off every apoptosis signalling pathway. However, the results further ascertained the apoptosis provocative properties of SecTRAPs.

Interestingly, HeLa cells overexpressing Bcl-2 that where not resistant to apoptosis provoked by staurosporine, were still susceptible to apoptosis provoked by SecTRAPs. This is a second result that may indicate the importance of SecTRAPs for caspase-2 activation, since release of cytochrome *c* and Smac/DIABLO could not be prevented by overexpressing Bcl-2 in Jurkat cells (Robertson, Gogvadze et al. 2004).

Based on these findings we conclude that SecTRAPs can be potent inducers of a specific apoptotic signalling in cancer cells, and we propose that the consequences of the formation of SecTRAPs during anticancer chemotherapy with electrophilic agents may contribute to the therapeutic effects of these drugs.

2.3.4 Paper IV

The apoptotic effects of SecTRAPs require an intact CVNVGC motif in the aminoterminal domain.

Previous results implied that a derivatization of the Sec residue was the only parameter that needed to be fullfilled to transform TrxR1 into a SecTRAP. However, after some preliminary *in vitro* assays demonstrating that SecTRAPs have a significant NADPH oxidase activity with the quinone compound menadione (unpublished) we felt that an investigation of the redox active disulfide/dithiol motif involving Cys59 and Cys64 within the CVNVGC sequence in thioredoxin reductase (TrxR) needed to be performed. In addition to our preliminary results, TrxR has been shown *in vitro* to be inhibited by quinone compounds and subsequently efficiently redox cycling with these (Cenas, Nivinskas et al. 2004). Further more it was found that the nitroaromatic compounds that in addition to the inhibition provoked the highest NADPH oxidase activity also induced most cell death when added to cells (Cenas, Prast et al. 2005).

We thus generated a mutant where the two redox active cysteins were replaced by Ser residues, but maintaining an intact Sec-containing motif in the C-terminal part of the enzyme. UV-spectral analyses of the mutant demonstrated that the protein sustained a NADPH electron transfer to the flavin which was bound to the mutants, but with a distorted spectrum compared to wild-type and naturally with no evidence of formation of a typical thiolate charge-transfer complex found in this class of enzymes. As

expected, the mutant also lacked classical TrxR1 activity.

The kinetic studies performed with quinone compounds illustrated none, or at least much lower, NADPH oxidation by the C69S/C64S mutant TrxR compared to recombinant Sec-containing TrxR (rTrxR) or "regular" SecTRAPs formed by derivatization of rTrxR with electrophilic compounds. Alkylation of the Sec in the C69S/C64S mutant protein with platinol did not change this activity, even though the alkylated rTrxR protein provoked a lower NADPH oxidase activity compared to rTrxR using juglone as substrate. The observation that rTrxR had a much higher NADPH oxidase activity compared to SecTRAPs was a somewhat surprising finding, since it could indicate that rTrxR1 could be more toxic than SecTRAPs (if redox cycling is a mechanism). One possible explanation may be that in the cell, where Trx is present, this competes with any redox cycling low molecular weight compound.

When the above described different TrxR1 preparations were introduced to A549 cells with the *BioPorter* system, the C59S/C64S mutant, with or without platinol-alkylated Sec, provoked no more cell death than controls, in sharp contrast to SecTRAPs (platinol-alkylated rTrxR or truncated TrxR) that provoked cell death to the same high extent as reported before. This result demonstrate that the redox active disulfide/dithiol motif involving Cys59 and Cys64 is essential for the effects of SecTRAPs, and that our assumption in the first paper that ROS have no involvement in effects by SecTRAPs may need to be reconsidered. Possibly SecTRAPs may induce apoptosis as a result of redox cycling with an endogenous substrate, through the CVNVGC/FAD motif leading to oxidative stress or, alternatively, to local effects at the mitochondrial membrane that directly signal apoptosis through a release of cytochrome *c* involving caspase-2. Another alternative may be that the Cys residues may form disulfide-linked dimers with hitherto unidentified cysteine-dependent apoptotic signaling protein partners. A third alternative could be that the Cys residues in the CVNVGC motif of SecTRAPs are subject to post-translational modification events required for SecTRAP activity.

3 CONCLUSIONS

The main conclusions from each individual paper were as follows:

Paper I: Derivatized TrxR, either as a truncated protein with a C-terminal motif consisting of Gly-Cys-COOH or with alkylation of the Sec residue with DNCB or cisplatin, provoke a rapid cell death in A549 (lung carcinoma) cells that could not be seen after treatment with fully active enzyme or with the closely related protein GR.

Paper II: Common clinically used electrophilic anticancer drugs such as cisplatin (platinol), oxaliplatin, chlorambucil, melphalan and busulfan inhibit the enzymatic activity of TrxR *in vitro*. Interestingly, these compounds did not inhibit the closely related protein glutathione reductase. In addition doxorubicin and daunorubicin could not, in contrast to previous reports, inhibit the enzymatic activity of TrxR. The results imply that several electrophilic cancer drugs, usually considered as DNA alkylators, may have TrxR as an important cellular target.

Paper III: The cell death provoked by SecTRAPs generates a translocation of phosphatidyl-serine to the outer side of the cell membrane, indicative of apoptotic cell death. In addition, the cell death is found to be prevented by caspase inhibitors, and especially efficient by a caspase-2 inhibitor. However the anti-apoptotic protein Bcl-2 could not prevent the effects by selenium compromised TrxR. The expression SecTRAPs (Selenium compromised thioredoxin reductase derived apoptotic proteins) is introduced as a collective word for the proteins having this activity.

Paper IV: The apoptotic effect of SecTRAPs is shown to require an intact CVNVGC motif in the aminoterminal domain. In addition, the effects of SecTRAPs can be prevented by the two antioxidants Vitamin E and ascorbic acid. However, a combinatory treatment with both antioxidants together with SecTRAP provokes apoptosis to the same extent as SecTRAP alone.

4 FUTURE PERSPECTIVES

The results presented here have identified TrxR1 as a protein that rapidly provokes apoptosis in two different cancer cell lines by a gain of function. A prerequisite for this property of the enzyme is a derivatization of the Sec residue generated either as a result of a dysfunctional (absent) SECIS element, or by alkylation with different electrophilic compounds. Interestingly, that redox-active disulfide/dithiol motif involving Cys59 and Cys64 within the CVNVGC sequence was found to be a requirement for the effects of SecTRAPs. The participation of ROS was further strengthened by the protective effects of the antioxidants Vitamin E and ascorbic acid. However, still there are many question marks that need to be straitened by future investigations.

In paper III we demonstrated that at least two cancer cell lines are susceptible to effects of SecTRAPs. One interesting aspect that needs to be investigated is if the cell death provoked by SecTRAPs is exclusive for cancer cells or if it can affect all types of cells. To get further information if there is a possibility that SecTRAPs are formed in tissues after treatment with electrophilic drugs, not just primary cells but also primary cancer cells should be exposed to SecTRAPs. If we hypothesize that not just cancer cells are affected, side effects of different electrophilic cancer drugs can be explained. One interesting example is the nephrotoxic side effect seen in patients after cisplatin treatment, which is a result of harm to cells in proximal tubuli that have one of the highest expressions of TrxR1 (Rundlöf, Carlsten et al. 2000). However, if cells with high TrxR expression are more sensitive to generation of SecTRAPs by electrophilic targets, many cancer cells will still be more susceptible to drugs that target TrxR. On the other hand, a dream scenario would be that only cancer cells are suspicious to SecTRAPs, which would open new possibilities for more efficient cancer treatment strategies.

In paper II we identified several electrophilic drugs as irreversible inhibitors of TrxR. Another interesting aspect for further investigations is therefore to evaluate if all electrophilic drugs that inhibit the enzymatic activity of TrxR also generate SecTRAPs. The Sec residue is the main target for many electrophilic compounds, but not an exclusive target. Our finding that the C59S/C64S mutant did not provoke cell death

indicates that drugs that bind to this part of the active site and result in ablation of the charge transfer complex, may not generate SecTRAPs. In addition, it would be interesting to investigate if the electrophilic drugs recognised to generate SecTRAPs, also create SecTRAPs when they are added as low molecular weight compounds to cells in culture media. Possibly not all compounds are easily transported into a cell resulting in a drug that seem promising in the *in vitro* assays but are useless in *in vivo* assays. Variability in the uptake of different drugs into different cells would be an easy explanation why not all cells are susceptible to a candidate drug.

In paper III we started the work of identifying the mechanism for apoptosis induction by SecTRAPs. The result with the caspase-2 inhibitor was of special interest, and we think that this certainly requires a closer analysis. *In vitro* assays with recombinant caspase-2 and SecTRAPs with or without other components of the thioredoxin system would be an interesting experiment to perform to see if SecTRAPs may directly activate caspase-2. In addition, another interesting approach would be to repress caspase-2 expression with siRNA to see if the effects of SecTRAPs are inhibited.

Another possibility is that SecTRAPs interacts with an endogenous low molecular weight compound. The results with Vit E or Vit C presented in paper IV indicated that ROS might be part of the signalling mechanism of SecTRAPs. Reactions between SecTRAPs and quinones nearby the mitochondria would possibly be a disaster to the cell, possibly provoking release of pro-apoptotic proteins from the mitochondria. Intracellular ROS production is however hard to detect. One way of doing this is by usage of the dye DCF that is cleaved and activated by ROS, however it requires a lot of optimizations to become a reliable method.

The apoptosis signalling mechanisms we have investigated so far are just the tip of the iceberg. One of the obstacles to overcome for us are the limitations in the use of the *BioPORTER* technique. The tet-ON system is an alternative technique, although in the beginning very time consuming, since it makes it possible to express the truncated TrxR in a controlled way by usage of a tetracycline-inducible promoter. This system will expand the possible analysis methods. Western blot analysis, which demands a larger amounts of cells, will not be a problem any longer and analysis of cleavage of different caspases as well as the possible release of different death inducing proteins

from the mitochondria will easily be characterized. The functional system would also facilitate FACS analysis of the effects of SecTRAPs in different growth phases of the cell cycle. In addition, several other apoptosis analysing systems can be used with a larger number of cells such as DNA degradation laddering, caspase activation assays etc etc.

One hypothesis that we have is that SecTRAPs have a protein partner. A functional tet-ON system would make it easier to find this protein partner by immunoprecipitation of SecTRAPs in combination with MS- analysis. In a similar way, detection of a possible binding of SecTRAPs to a low molecular weight compound could possibly be performed, as could analyses of covalent modifications (eg. to the Cys residues in the CVNVGC motif).

Another interesting approach to use for SecTRAPs is to introduce them to cells by microinjection. Although it is also a very time consuming technique and using even less cells than with the *BioPORTER*, it would be suitable for studies in primary cells, which seem to be more sensitive to the *BioPORTER* compound (unpublished).

Our results indicate that the effects of SecTRAPs do not necessarily encompass the endogenous Trx system. An interesting experimental setup would therefore be to analyse effects of SecTRAPs at different availabilities of selenium. Preferably this would be done by first exposing cells to selenium starvation and sort out cells that are more persistent to this treatment. Thereafter effects of SecTRAPs in addition to treatments with different selenium concentrations in both low and high dose intervals can be performed. In addition it would be interesting to see if a truncated TrxR could be detected as a result of the selenium deficiency. Possibly could part of the symptoms of selenium starvation be due to formation of SecTRAPs.

The finding in paper IV that a combinatory treatment of Vitamin E and ascorbic acid did not protect against the effects of SecTRAPs was not expected. The compounds generally act synergistically in antioxidant defence, since ascorbic acid reduces Vitamin E semiquinone, which is generated when Vitamin E reacts with free radicals at lipid membranes, thereby forming an ascorbyl radical. Two ascorbyl radicals can then be converted to one ascorbic acid and one dehydroscorbic acid by dismutation, with

TrxR1 reducing the latter back to ascorbic acid (May, Mendiratta et al. 1997). However, TrxR1 can also catalyze one-electron reactions with ascorbate (May, Cobb et al. 1998). Possibly SecTRAPs may instead redox cycle with one-electron reduction of ascorbate thus producing ascorbyl radicals, which may be propagated to the membrane by induction of Vitamin E semiquinones. Such effect would be aggravated if Vitamin E were added together with SecTRAPs and an excess of ascorbic acid. However, further studies must be conducted to ascertain this possible chain of events.

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