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Structure and function in c-Myc and Grx4: two key proteins involved in transcriptional activation and oxidative stress

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To my family

ABSTRACT

The proto-oncogene *c-myc* is critical for growth and development and deregulation of *c-myc* expression affects the initiation and expansion of a wide range of human cancers, many of them aggressive. c-Myc is a multidomain protein, where it's C-terminal basic helix-loop-helix leucine zipper (bHLH-Zip) domain heterodimerizes with Max to accomplish gene regulatory activity. A biophysical investigation of the biological important regions flanking the bHLH-Zip of Max found that they promote increased folding of Max and play a regulatory role in determining the affinity to DNA.

The activity of the N-terminal c-Myc transactivation domain (TAD) is regulated by binding to a range of proteins. A molecular mapping of c-Myc TAD regulatory activities has utmost relevance as a molecular drug would contribute substantially to cancer treatment. An extended c-Myc TAD, $Myc_{1–167}$, includes the unstructured c-Myc TAD (aa 1–143) together with a C-terminal segment, which were found to promote folding. Surprisingly, $Myc_{1–167}$ displays the characteristics of a helical molten globule, and binds both to Myc Modulator-1 (MM-1) and TATA box-binding protein (TBP). Although its C-terminal region ($Myc_{92–167}$) has a partly helical fold and binds both MM-1 and TBP, neither N- nor C-terminal regions of $Myc_{1–167}$ bind target proteins with as high affinity as the entire $Myc_{1–167}$, or display molten globule properties.

A screen of common interaction patches in c-Myc TAD binding proteins resulted in identification of a peptide binding motif in TBP, which interacts with c-Myc TAD and reduces cell growth in c-Myc overexpressing cells. Structural mapping of interacting residues in the TBP derived peptide by NMR and Biacore lead to the identification of a peptide segment belonging to the same binding motif in the histone acetyltransferase complex member TRRAP, which bound c-Myc TAD with even higher affinity. The location of a common c-Myc interacting patch in TBP and TRRAP proposes a mechanism for recruitment of c-Myc to the preinitiation complex and suggests novel routes for therapeutic strategies.

Glutaredoxins are ubiquitous proteins found in most living organisms and they function by performing redox regulation in the cell. Despite the wealth of information regarding glutaredoxins employing a dithiol mechanism for the reduction of their substrates, little is known about the monothiol glutaredoxins. *E. coli* Glutaredoxin 4 (Grx4), which showed no activity in the classical glutaredoxin (HED) assays, could still be oxidized by glutathione and form an internal disulfide. This internal Grx4 disulfide was a direct substrate for NADPH and *E. coli* Thioredoxin reductase, while the mixed disulfide was reduced by *E. coli* Glutaredoxin 1, suggesting extremely narrow substrate specificity with regulatory implications.

The three-dimensional structure of reduced Grx4 was determined by NMR and comprises a glutaredoxin-like α-β fold with stringently conserved structural features, likely to be present in all monothiol glutaredoxins. The absence of classical glutaredoxin activity in *E. coli* Grx4 is understood based on small but significant structural differences in the glutathione binding region, and through the lack of a conserved second GSH binding site. MALDI experiments suggest that disulfide formation on glutathionylation is accompanied by significant structural changes, and together with detailed structural evaluation suggests that the disulfide-forming region forms an active site.

LIST OF PUBLICATIONS

I. Pursglove, S. E., **Fladvad, M**., Bellanda, M., Moshref, A., Henriksson, M., Carey, J., and Sunnerhagen, M. (2004) Biophysical properties of regions flanking the bHLH-Zip motif in the p22 Max protein. *Biochem Bioph Res Co*, 323, 750-759

II. **Fladvad, M**., Zhou, K., Moshref, A., Pursglove, S., Säfsten, P., and Sunnerhagen, M. (2005) N- and C-terminal subregions in the c-Myc transactivating region and their joint role in creating versatility in folding and binding. *J Mol Biol*, 346,175-189

III. Fernandes, A. P., **Fladvad, M**., Berndt, C., Lillig, C. H., Neubauer, P., Sunnerhagen, M., Holmgren, A., and Vlamis-Gardikas, A. (2005). A novel monothiol glutaredoxin (Grx4) from Escherichia coli can serve as a substrate for thioredoxin reductase. *J Biol Chem,* 2005, 280, 24544-24552

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V. **Fladvad, M**., Andersson, K., Kanmert, D., Säfsten, P., and Sunnerhagen, M. Identification and characterization of a consensus motif in TBP and TRRAP, which binds the c-Myc transactivation domain. *Manuscript.*

TABLE OF CONTENTS

1 GLUTAREDOXINS 1

1.1 Redox regulation and oxidative stress 1
1.1.1 ROS 1
1.1.2 GSH 2

1.2 The thiol redox control system 4
1.2.1 The Thioredoxin system 5
1.2.2 The Glutaredoxin system 8

1.3 Dithiol glutaredoxins 11
1.3.1 The active site 12
1.3.2 Conserved residues 13
1.3.3 Glutathione mixed disulfide complexes 15
1.3.4 *E. coli* dithiol glutaredoxins 16
1.3.5 Yeast dithiol glutaredoxins 17
1.3.6 Mammalian dithiol glutaredoxins 17
1.3.7 Glutaredoxin like proteins 19

1.4 Monothiol glutaredoxins 20
1.4.1 The active site 20
1.4.2 Conserved residues 21
1.4.3 Bacterial monothiol glutaredoxins 22
1.4.4 Yeast monothiol glutaredoxins 22
1.4.5 Distinct differences between dithiol and monothiol glutaredoxins 23
1.4.6 Iron 23
1.4.7 Open issues in the glutaredoxin field 23

2 MYC PROTEIN FUNCTIONALITIES 25

2.1 Transcription 25
2.1.1 Transcriptional regulation 26

2.2 Proto-oncogenes 28

2.3 The Myc family 28

2.4 Myc structure 30

2.5 The Max network of transcription factors 33
2.5.1 Transcriptional activation by the Myc-Max complex 33
2.5.2 E-box 34
2.5.3 Max 34
2.5.4 Transcriptional repression by Max binding partners 35

2.6 The Biological activity of Myc - Action through target genes 36
2.6.1 Cell cycle 36
2.6.2 Protein synthesis and cell growth 37
2.6.3 Metabolism 37
2.6.4 Differentiation 38
2.6.5 Apoptosis 38
2.6.6 Transcription 39

2.7 Myc activation in human cancer 48
2.7.1 Myc as a therapeutic target in cancer 48
2.7.2 Open issues in the Myc field 49

3 PRESENT INVESTIGATION 51

3.1 Aim of the study 51

3.2 Methodology 52
3.2.1 Limited proteolysis 52
3.2.2 Circular dichroism 52
3.2.3 Fluorescence 53
3.2.4 Biacore 53
3.2.5 MALDI 54
3.2.6 NMR 55

3.3 Results and discussion 57
3.3.1 Paper I 57
3.3.2 Paper II 58
3.3.3 Paper III 59
3.3.4 Paper IV 60
3.3.5 Paper V 61

4 CONCLUSIONS 63

5 FUTURE PERSPECTIVES 65

6 ACKNOWLEDGEMENTS 68

7 REFERENCES 71

LIST OF ABBREVIATIONS

AMY-1	Associated of Myc 1
ASK-1	Apoptosis signaling kinase 1
BIN1	Bridging integrator 1
CBP	cAMP-responsive element binding protein
CD	Circular dichroism
DTT	Dithiothreitol
E-box	Enhancer box
FAD	Flavin adenine dinucleotide
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
HAT	Histone acetyltransferase
HED	B-hydroxyethyl disulfide
HSQC	Heteronuclear single quantum coherence
Krim-1	Krab box protein interacting with Myc 1
MALDI	Matrix-assisted laser desorption/ionization mass spectrometry
MAX	Myc associated protein X
MB	Myc box
MM-1	Myc modulator 1
MNT	Myc binding protein
MYC	Myelocytomatosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
P-TEFb	Positive transcription elongation factor b
RNR	Ribonucleotide reductase
SH	Thiol
SPR	Surface plasmon resonance
S-S, S_2	Disulfide bond
TAD	Transcriptional activation domain
TBP	Tata-box binding protein

TERT	Telomerase reverse transcriptase
TR	Thioredoxin reductase
TRRAP	Transactivation/transformation associated protein
Trx	Thioredoxin
Wt	Wild type

1 GLUTAREDOXINS

The focus of this overview is to provide a background on the structure/function relationships in the glutaredoxin family.

1.1 REDOX REGULATION AND OXIDATIVE STRESS

Reactions in biological systems causing a change in the oxidation number (oxidation state) of an atom are generally called redox reactions and play an important role in numerous biochemical mechanisms. A reduction is a process in which electrons are gained or a decrease in oxidation number occurs. A loss of electrons or an increase in oxidation number is consequently called an oxidation. Substances performing reducing reactions, known as reducing agents, include metals (Li, Na, Mg, Zn, Fe, and Al), hydride transfer elements ($NaBH_4$, $LiAlH_4$) and H_2 with palladium, platinum or nickel catalysts. Compounds with high oxidation numbers (H_2O_2, MnO_4^-) or highly electronegative substances (O, F, Cl, and Br) are oxidizing agents. The particular concentration of electrons in a cell, the redox state of the cell, is normally carefully regulated and the environment inside the cell is kept reduced by enzymes like thioredoxin and the tri-peptide glutathione. Pathological conditions such as oxidative stress can disturb the balance in the cell, creating an oxidative environment, which promotes protein aggregation and cell death.

1.1.1 ROS

Oxidative stress is a concept describing the process in which cells are damaged by reactive oxygen species (ROS) such as superoxide anion, H_2O_2, hypochlorite anion and hydroxyl radicals. ROS are formed from O_2 by ionization radiation, by the immune system during inflammation, by metal catalysed reactions or are by-products of cellular respiration. The high reactivity of ROS is due to the presence of unpaired valence shell electrons. Normally ROS functions as cell signalling molecules, performing regulation of important processes such as cell division, apoptosis and differentiation, but at high concentrations, ROS can be toxic and damage proteins, nucleic acids and lipids leading to cell membrane degeneration (Beckman and Ames, 1998; Ghezzi, 2005a). Cell damage by ROS can be prevented by the action of non-enzymatic antioxidants such as vitamin C, vitamin E, carotenoids, thiol antioxidants and flavonoids or enzymes like

SOD (superoxide dismutase), catalase or glutathione peroxidase (GPx) (see equations below).

$$2O_2^- + 2H^+ \overset{SOD}{\Leftrightarrow} O_2 + H_2O_2$$

$$2H_2O_2 \overset{catalase}{\rightarrow} 2H_2O + O_2$$

$$2GSH + H_2O_2 \overset{GPx}{\rightarrow} GSSG + 2H_2O$$

$$2GSH + ROOH \overset{GPx}{\rightarrow} GSSG + ROH + H_2O$$

Although antioxidants are effective in abolishing ROS, some accumulation of ROS will occur with age and may give rise to diseases such as cancer, arthritis, arteriosclerosis and neurodegenerative disorders.

1.1.2 GSH

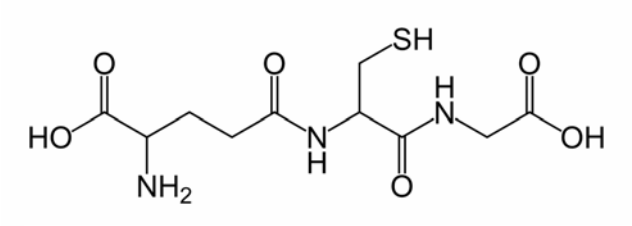


Figure 1. The structure of the glutathione tri-peptide.

The major thiol oxidant detoxifying ROS is the tripeptide glutathione (figure 1), which rescues cells by i) neutralizing ROS species ii) regenerating antioxidants and iii) working as a cofactor of several detoxifying enzymes e.g. glutathione peroxidase (GPx) and glutathione transferase (GST). Glutathione is highly abundant in the cell, 0.5-20 mM (Meister and Anderson, 1983), and the majority is found in its reduced state (GSH) in the cytosol, the primary location for GSH synthesis. GSH is synthesised from L-glutamate by γ-glutamylcysteine synthetase and glutathione synthetase and is then transferred to the other cell compartments. The nucleus and mitochondria have similar levels of GSH as the cytosol, while the extra cellular GSH concentration is much lower (100-1000 times) and proteins found outside the cell are hence often oxidized (Schafer and Buettner, 2001).

The sulphur atom of GSH is the key to the antioxidant efficiency of the molecule, since it can accommodate loss of a single electron, forming a thiyl radical ($GS^{\bullet}$). The thiyl

radical has the ability to dimerize, forming oxidized glutathione (GSSG), which can accumulate in the cell.

$$GSH + R^{\bullet} \rightarrow GS^{\bullet} + RH$$
$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG$$

The GSH/GSSG couple works as the major redox buffer in the cell together with the enzyme thioredoxin. The ratio of GSH/GSSG in the cytoplasm is usually 30-100 under normal conditions in both mammalian cells and bacteria, which means that most cysteines have free thiol groups. A decreased level of GSH/GSSG is a good indication of oxidative stress in the cell, which can lead to apoptosis and at extreme levels to necrosis (Voehringer, 1999). Other compartments in the cell have normally a highly oxidative environment, for example the endoplasmic reticulum, which has a GSH/GSSG ratio of 1, promoting disulfide formation important in protein folding.

1.1.2.1 Protein glutathionylation

In addition to its antioxidative effect, GSH can perform protein glutathionylation, ie formation of mixed disulfides between GSSG and a protein. The process of glutathionylation is reversible and deglutathionylation is catalysed by glutaredoxins and to a certain extent thioredoxins. Normal untreated cells contain certain amounts of glutathionylated proteins, e.g. approximately 1 % of GSH found in normal liver is present as mixed protein disulfides (Brigelius et al., 1982; Brigelius et al., 1983). However, oxidative cellular conditions trigger accumulation of GSSG, which react with proteins to produce protein–GSH mixed disulfides. This results in inactivation of transcription factor nuclear factor 1 and protein tyrosine phophatase-1 or restoration of function of GST (Shelton et al., 2005). Glutathionylation does not only target the proteins enzymatic activity by direct glutathionylation of active site cysteines, but can also change the isoelectric point of the protein or creating steric hindrance on the surface of the target. This can lead to regulation of mechanisms such as oligomerization (Davis et al., 2003; Wang et al., 2001; Wang et al., 2003), DNA binding (Cao et al., 2005; Klatt et al., 1999) and membrane anchoring.

The specificity of protein-glutathionylation is considered to be determined by the accessibility of the free thiol in the protein 3D structure and the nature of the

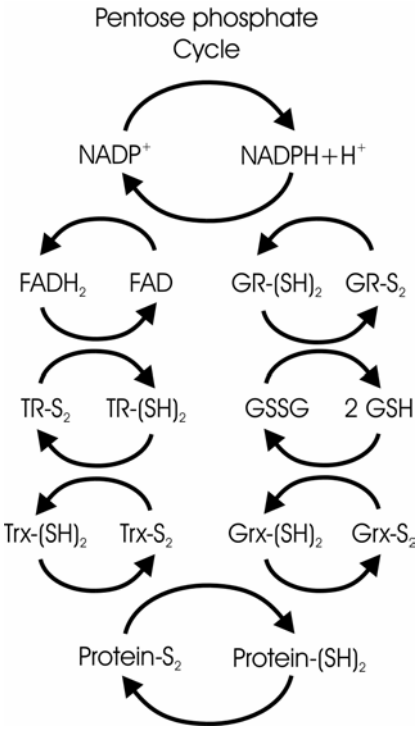
surrounding amino acids (e.g. surrounding basic amino acids favour glutathionylation by interacting with γ-glutamyl of GSH and thereby stabilizing the protein-GSH complex) (Ghezzi, 2005b). Neighbouring thiols usually prevent formation of a mixed disulfide by glutathionylation due to their reducing effect, which is observed for the CXXC active site in human thioredoxin (Casagrande et al., 2002).

1.1.2.2 Glutathionylation in redox signalling

Mixed disulfides formed between cysteine residues and GSH are unstable complexes, which are susceptible to the action of other thiols. Like glutathionylation, the deglutathionylation process can be catalyzed by a number of enzymes such as glutaredoxin (Grx), thioredoxins (Trx), thioredoxin reductase (TR) and protein disulfide isomerases (PDI) (Ghezzi, 2005b). The reversibility of the glutathionylation process have lead to the suggestion that GSH could be a suitable molecule for redox dependent signalling (similar to the role of protein phosphorylation) and this was recently supported by Fratelli *et al*. (Fratelli et al., 2005), who showed that induction of gene expression in response to H_2O_2 was GSH-dependent. A number of other biochemical processes have been implied to be redox regulated by glutathionylation, e.g. apoptosis, transcription and phosphorylation (Shelton et al., 2005).

1.2 THE THIOL REDOX CONTROL SYSTEM

Glutaredoxins (Grx) and thioredoxins (Trx) are small thiol-disulfide oxidoreductases, which are the major enzymes that control the thiol redox status in the cell. The Trx/Grx system reduce enzymes that catalyse the reduction of protein disulfides and GSH-protein mixed disulfides, but it also has more direct effects in the antioxidant defence system catalysing reduction of disulfides through self oxidation and formation of intramolecular disulfides between the two cysteines in their CXXC active site. Thioredoxin reductase and GSH/Glutathione reductase function as reducing partners for Trx and Grx by utilizing electrons form NADPH (Figure 2).



Figur 2. Protein reduction by the Trx and Grx system. Trx is reduced by thioredoxin reductase (TR) via transfer of electrons from NADPH to the redox active cysteines in the FAD domain while Grx is reduced by glutathione reductase (GR) and GSH.

The levels of Trx in *E. coli* are 100-1000-fold less than those of GSH, but at least 10-fold higher than the Grx concentration (Holmgren, 1979; Holmgren et al., 1978). Lack of Trx in *E. coli* increases the levels of Grx, and vice versa, in order to maintain the deoxyribonucleotide pool necessary for DNA synthesis (Miranda-Vizuete et al., 1996). In human cells, the compartmentalized function of Trx and GSH can however be different in the cell and/or the effects of the two systems sometimes overlap as seen for the regulation of NF-κB and AP-1 (Hirota et al., 2000) .

1.2.1 The Thioredoxin system

1.2.1.1 The Thioredoxin family and structure

Thioredoxin is found in all known organisms and is essential from archaea to man. Lower organisms such as bacteria and yeast contain several cytoplasmic Trxs, which exhibit sensitivity to H_2O_2 and therefore imply involvement in the oxidative stress defence (Carmel-Harel and Storz, 2000). Plants have a particularly well developed Trx system consisting of several different Trx isoforms localized to different compartments such as the chloroplast, mitochondria, cytosol and even the nucleus (Gelhaye et al.,

2005). The localization of Trx seems to be important also in mammals and they generally have two different Trx isoforms, the cytosolic Trx1, which is secreted from the cell or translocated to the nucleus upon oxidative stress, and Trx2 found in the mitochondria.

The sequence similarity between Trxs is relatively high even between distant organisms (30 % between *E. coli* and man) and the redox active site containing the CGPC sequence, in which the two SH-groups of the cysteines form a disulfide upon oxidation, is conserved. The considerable sequence similarity among Trx's suggests a common 3D structure, named the Trx fold, which consists of a mixed α/β-motif. Unusually little variation is seen in the fold when comparing different Trx species, with small differences in the loop length between the secondary structure elements (Eklund et al., 1991). All Trx structures have a well conserved hydrophobic core and the few variations within the Trx sequence that are present between species are preferentially located on the surface of the protein, only affecting local surface patches.

The Trx family members, which all contain Trx domains, include bacterial proteins, like *E. coli* DsbA, B and C, that promote disulfide bond formation in periplasmic proteins (Nakamoto and Bardwell, 2004), and the eukaryotic protein disulfide isomerase (PDI) family acting as chaperones and catalysing the formation and rearrangement of disulfide bonds in the endoplasmic reticulum (Puig et al., 1994). Trx-like protein 2 (Txl-2) also contain a Trx domain and binds to microtubules and peroxiredoxins (Prxs) are Trx-dependent peroxidases (Nakamura, 2005). Last but not least are the Glutaredoxins which will be discussed later in this thesis.

1.2.1.2 The biological function of Thioredoxin

Trx has an important function in direct thiol-disulfide exchange reactions required for electron transport, which supports the mechanism of essential enzymes such as ribonucleotide reductase (RNR), involved in DNA synthesis (Koc et al., 2006; Laurent et al., 1964). Peroxiredoxins, which catalyse reduction of H_2O_2, require thioredoxin for reduction (Chae et al., 1994) and aid in the defence against oxidative stress and cell death. In bacterial phage, Trx increases the processivity of T7 DNA polymerization 1000-fold (Huber et al., 1987) and participate in filamentous phage assembly and export (Feng et al., 1999). Trx also assists in assimilation of sulphur in bacteria and

yeast by functioning as a hydrogen donor for 3'-phosphoadenylsulfate (PAPS) reductase (Lillig et al., 1999; Schwenn et al., 1988). In humans, Trx1 play a role in controlling protein function by changing the redox state of SH groups involved in catalysis, DNA binding and protein folding. Major targets for this redox regulation function are transcription factors, including NF-κB, Ap-1, HIF-1, and p53, which govern cell proliferation and death (Valko et al., 2006). Trx1 can also bind to proteins such as thioredoxin- interacting protein (Txnip) and apoptosis signal-regulating kinase 1 (ASK-1) and thereby regulate their activity (Patwari et al., 2006). The mitochondrial Trx2 prime target is cytochrome c release and apoptosis (Wang et al., 2006).

1.2.1.3 Thioredoxin reductase

TRs are dimeric flavoenzymes belonging to the pyridine nucleotide-disulfide oxidoreductase family, which are present in all living organisms. The TR monomer contains both a flavin adenine dinucleotide (FAD) and NADPH-binding site in addition to its redox active disulfide. Two forms of TR have evolved through evolution; TR found in lower organisms such as bacteria, fungi, plants and lower eukaryotes and the TR found in higher eukaryotes. The bacterial TR consists of a 70 kDa TR dimer related to alkyl hydroperoxide reductase while higher eukaryotes have a larger, around 110 kDa, TR dimer, more similar to GR, with a redox active FAD-domain containing a pair of redox active cysteines and an elongated C-terminal domain (Williams et al., 2000). The C-terminal elongation domain, not present in lower organisms, includes a dimerization interface and the redox active Gly-Cys-SeCys-Gly sequence containing an essential selenocysteine (Zhong and Holmgren, 2000). The smaller prokaryotic and archea TRs have a very narrow substrate specificity and can usually only target Trx from the same or closely related organisms. This has been suggest to be related to the nature of the catalytic mechanism in which the pyridine nucleotide domain undergo a rotation of 66 ° with respect to the FAD domain (figure 3) in order for the redox active dithiol to reach its somewhat bulky Trx substrate (Lennon et al., 1999; Lennon et al., 2000). The larger TR variant found in higher eukaryotes has much broader substrate specificity and can, except for Trx, also reduce proteins like PDI (Lundstrom and Holmgren, 1990), glutaredoxin (Johansson et al., 2004) and a number of low molecular weight compounds (Arner et al., 1996; Holmgren, 1977; Holmgren and Lyckeborg, 1980; Luthman and Holmgren, 1982b) and concomitantly displays a completely different catalytic mechanism. The substrate reduction by eukaryotic TR is performed

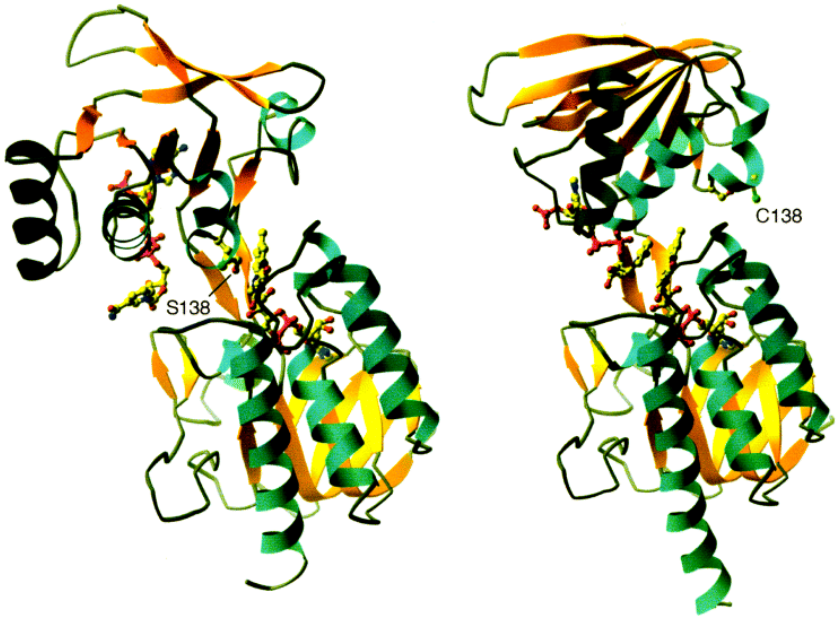


Figure 3. The two different conformations of *E. coli* TR. The left conformation promotes transfer of electrons from the FAD to the enzyme disulfide while a 66 deg rotation creates the conformation seen to the right which allows FAD reduction by NADPH and reduction of Trx.

by transfer of electrons from NADPH via the FAD to the redox active disulfide in the N-terminal domain. The electrons are then transferred from the N-terminal active site of one subunit of the TR dimer to the sulphur group of the selenocysteine in the C-terminal end of the other subunit. Both the nature of the flexible C-terminal, which allows bulky substrates, the strong nucleophilicity and low pKa of the selenocysteine contribute to the broad substrate specificity of the large eukaryotic TRs.

1.2.2 The Glutaredoxin system

Grx was discovered as a hydrogen donor of RNR in *E. coli* using GSH for reduction as a complement to the Trx system. Most organisms have Grx except for a number of gram positive bacteria, which are deficient in GSH, such as *Bacillus subtilis*, *Mycobacterium tuberculosis* and several *Streptococcus* and *Streptomyces* strains (Fahey, 2001; Newton et al., 1996). Bacteria and yeast have generally a small number of different Grxs while the plant *grxs* genes are numerous, similar to the plant *trx*, with around 30 *grx* in *A. thaliana*. All plant Grxs are expected to be localized in the nucleus but are probably exported to different compartments (Rouhier et al., 2004). Mammalian species have at least one cytosolic and one mitochondrial Grx.

1.2.2.1 Catalytic mechanism

Grxs and Trxs can partially substitute for each other in the reduction of disulfide bonds through a CXXC active site (Prinz et al., 1997) but the Grx/GR system has a 5000-fold greater catalytic efficiency for deglutathionylation of protein mixed disulfide substrates compared to the Trx/TR system (Chrestensen et al., 2000). Grx is also 50000-100000 more effective in deglutathionylation *in vitro* compared to GSH and dithiothreitol (DTT) (Shelton et al., 2005). Grx uses GSH in two different ways for catalysis; 1) dithiol reduction through a dithiol mechanism demanding a dithiol active site or 2) by a monothiol mechanism only demanding one cysteine in the active site, resulting in reduction of protein GSH-mixed disulfides.

A dithiol reduction starts with a nucleophilic attack of the N-terminal active site cysteine on one of the sulphur atoms in the protein disulfide, resulting in the formation of a mixed disulfide between Grx and the target protein. Deprotonation of the C-terminal active site cysteine generates a nucleophilic attack on the N-terminal active site, breaking the mixed disulfide and releasing reduced target protein and oxidized Grx with an intramolecular disulfide in the CXXC active site (Figure 4). The oxidized form of Grx is then reduced by two molecules of GSH.

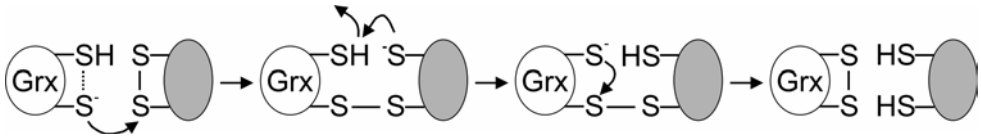


Figure 4. The dithiol Grx mechanism. Glutaredoxin mediated reduction of protein disulfides

The monothiol reduction is a ping pong mechanism where protein GSH-mixed disulfides are reduced by a nucleophilic attack on the GSH sulphur by a dithiol Grx N-terminal active site cysteine (Bushweller et al., 1992) or by a monothiol Grx cysteine. The C-terminal active site cysteine found in dithiol glutaredoxins does not effect the specificity of the reaction (Yang et al., 1998). The nucleophilic attack initiated by the N-terminal cysteine results in a release of the reduced non-GSH target protein and formation of a rate limiting Grx-GSH-mixed disulfide intermediate (Srinivasan et al., 1997). The intermediate is reduced by a second GSH molecule, creating GSSG, which is subsequently reduced by GR to GSH (Figure 5).

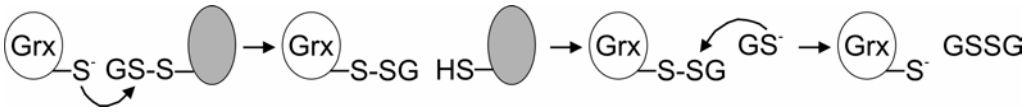


Figure 5. The monothiol Grx mechanism. Glutaredoxin mediated reduction of GSH-mixed

Factors like the pK_a value of the active site cysteines and specific molecular interactions between the Grx and the GSH moiety are hypothesised to be important for the nucleophilic and redox properties governing the catalytic mechanism of the Grx. The pK_a value of the N-terminal active site cysteine is generally low, ~3-5 (Foloppe et al., 2001; Gan and Wells, 1987; Mieyal et al., 1991; Tamarit et al., 2003), making it very reactive to GS·, but not GSSG. This displays an important feature of the reaction mechanism since protein-GSH formation using GSSG would demand intracellular GSH/GSSG ratio of approximately 100, which only occur at severe oxidative stress. Normal GSH/GSSG levels in the cell is usually closer to 1 (Fratelli et al., 2005). Low pK_a values are commonly associated with a more positive redox potential, hence a more oxidative protein (Mossner et al., 2000). The C-terminal active site cysteine has a more basic pK_a, around 8 (Foloppe et al., 2001; Tamarit et al., 2003) and is usually buried (Mossner et al., 2000).

The structure of complexes containing Grx and GSH has been determined on Grx active site mutants, which only contain the N-terminal cysteine and therefore traps GSH in the bound state. Interaction between the GSH moiety and the Grxs is overall relatively similar (Nordstrand et al., 1999; Yang et al., 1998), the GSH is localized in a cleft on the protein surface formed by helix h1, h2 and h3, the loop connecting b4 and h3 and the active site (*E. coli* Grx3 (Nordstrand et al., 1999), Figure 6).

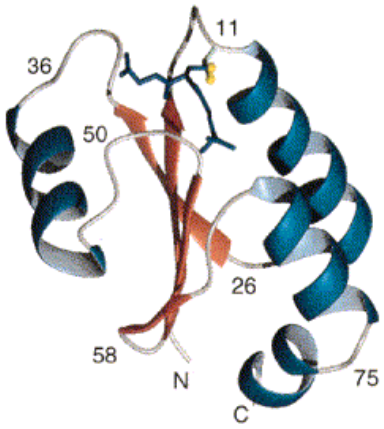


Figure 6. NMR structure of *E. coli* Grx3-SG complex. The glutathione molecule and the sidechain of cysteine 11 are displayed as sticks with the sulphurs as spheres.

The efficiency of the Grx catalysis can, based on the structure of the complex, be related to interaction of a second GSH molecule with the conserved active site tyrosine and a nearby conserved arginine in the Grx-SG complex. Movement of the tyrosine ring upon reduction, which is supported by a hydrogen bond between tyrosine and a close by asparagine in the Grx3-SG complex, can subsequently displace the formed GSSG from the active site (Nordstrand et al., 1999). A detailed comparison of different Grx-GS complexes displays small but important differences in the number and nature of the interactions between the two molecules. *E. coli* Grx1 forms less saltbridges and hydrogen bonds to the Glycine carboxylate and carbonyl oxygenes in the GSH moiety than human grx1 (Yang et al., 1998), which may reflect differences in stabilization of the GSH adduct.

1.2.2.2 Glutathione reductase

GR is a homodimeric flavoprotein, which is a major component of cellular defence against oxidative injury by catalyzing the reduction of GSSG using NADPH as a cofactor. The GR family adopts the Rossman fold where each monomer of GR contains a FAD and an active site disulfide, which create separate binding sites for GSSG and NADPH (Dym and Eisenberg, 2001). Electrons are transferred from NADPH to the FAD and then to the two active site cysteines, followed by reduction of GSSG. Two human isoforms of GR exists, one cytosolic and one mitochondrial, and since the mitochondria is the main location of intracellular ROS, it is an important location for GR mediated antioxidant protection (Kelner and Montoya, 2000). The GR activity is determined indirectly by the measurement of the consumption of NADPH, which follows on GSSG reduction, as demonstrated by a decrease in absorbance at 340 nm as a function of time (Carlberg and Mannervik, 1985).

1.3 DITHIOL GLUTAREDOXINS

The glutaredoxins belong to the thioredoxin structural family of mixed αβ-fold even though the sequence similarity between Grx and Trx is low. The Grx structures differ from the Trxs by having generally shorter secondary structure motifs and by lacking the fifth β-strand and additional N-terminal residues. Bigger variation in chain and loop length is also commonly seen for the Grxs compared to the Trxs (Eklund et al., 1991)

and the network of charged residues in the vicinity of the exposed surface loop forming the active site is completely different (Carvalho et al., 2006).

The knowledge of the Grx structure originates to a very large extent from structures of dithiol Grxs in different species, including; *E. coli* (Sodano et al., 1991; Xia et al., 2001; Åslund et al., 1996), bacteriophage T4 (Wang et al., 2004), pig (Katti et al., 1995), and human (Johansson et al., 2006; Sun et al., 1998). The monothiol Grx family is only represented by a single PDB entry showing a subdomain of the mouse protein PICOT, for which no publication is available and the structure of *E. coli* Grx4, which will be presented in the results section of this thesis. Commonly, the Grx fold comprises a 3-layer αβα-sandwich with a four-stranded β-sheet surrounded by a number of α-helices ranging from 3 in bacteriophage T4 glutaredoxin (Eklund et al., 1992) up to 5 in human Grx2 and poplar GrxC1 (Feng et al., 2006; Johansson et al., 2006). Two of the helices, one of which containing the active site, are usually positioned parallel to the β-strands on one side of the sandwich and at least one antiparallel on the other side. The strands in the β-sheet run in a parallel/antiparallel fashion forming a β-bulge between strand 3 and 4, which is conserved through most of the Trx/Grx superfamily. The β-sheet has normally a right-handed twist and contains a large number of hydrophobic residues, forming a hydrophobic core, which is prone to stabilize the fold, especially the interaction between the β-sheet and the active site containing helices. Most dithiol Grxs are monomeric in solution, but recent findings indicate that the poplar GrxC4 self-associates with a low millimolar K_d (Noguera et al., 2005). The dimer interface involves the active site and the GSH-binding pocket and intracellular concentrations of GSH is likely to stabilize GrxC4 in the monomeric state, possibly postulating a mechanism for regulation of the monomer- dimer equilibrium.

1.3.1 The active site

Dithiol Grxs has a highly conserved active site sequence, CPYC, which is usually positioned in a loop preceding a helix or in the first turn of a helix, making the N-terminal active site cysteine accessible to substrate reaction. Stabilization of the active site is likely performed by hydrogen bonds from the N-terminal cysteine to the active site C-terminal cysteine and tyrosine (Foloppe et al., 2001). Further stabilization of the active site is presumably performed by N-terminal capping of the helix containing the active site and by the conserved active site proline residue. Human Grx1 active site

mutants in which the proline was exchanged for a serine displayed significantly reduced specific activity against mixed disulfides of RNase (RNase-SG) and B-hydroxyethyl disulfide (HED) (Johansson et al., 2004). The conserved tyrosine is stabilized in the oxidized form of Grx by packing against the protein surface, which reduces the solvent accessible surface of the tyrosine ring. Replacement of the tyrosine residue by phenylalanine in pig, vaccinia virus and some plants induce little change in the conformation of the active site (Nordstrand et al., 2000), while a proline or alanine mutation in T4Grx1 results in a marked reduction in the activity of GSH-mixed disulfides (Nikkola et al., 1991), highlighting the importance of the hydrophobic contribution to the aromatic ring. The recently discovered mammalian Grx2 has an active site composed of CSYC that differs from the other dithiol Grxs. In an attempt to mimic the more common CPYC active site found in e.g. human Grx1, an exchange of the active site serine to a proline in human Grx2 resulted in an increased specific activity towards HED substrates (Johansson et al., 2004), similar to human Grx1.

1.3.2 Conserved residues

A number of residues outside the active site display a large degree of conservation within the dithiol Grx family (figure 7). Most well-known is the *cis*-proline in the conserved TVP motif preceding the third β-strand close to the active site. The conserved *cis*-proline is found through all the Trx/Grx super family and has been proposed to stabilize the active site area and to participate in GSH-binding. An alanine replacement of the *cis*-proline in T4 Grx and *E. coli* Trx1 decreases the efficiency of reduction by TR (Gleason, 1992; Nikkola et al., 1993). The *E. coli* Trx1P76A mutant also displays decreased biological activity and stability of the protein, proposing an at least partly unfolded structure *in vivo* (Kelley and Richards, 1987). Many of the conserved residues in Grxs participate in binding of the GSH moiety, including the threonine and valine in the TVP sequence, which forms part of the GSH-binding groove and hydrogen bonds to the GSH moiety (Bushweller et al., 1994; Nordstrand et al., 1999; Yang et al., 1998). A conserved lysine C-terminal to the first β-strand and an asparagine positioned C-terminal to the last β-strand provides complementary charges for binding of the GSH moiety. Two consecutive conserved glycines following the fourth β-strand are present in both Grx and Trx and they confer flexibility to the positioning of α-helices in the GHS binding cleft (Berardi et al., 1998; Nordstrand et al., 1999). Single point mutations of this GG motif in *S. cerevisiae* Grx5 cause

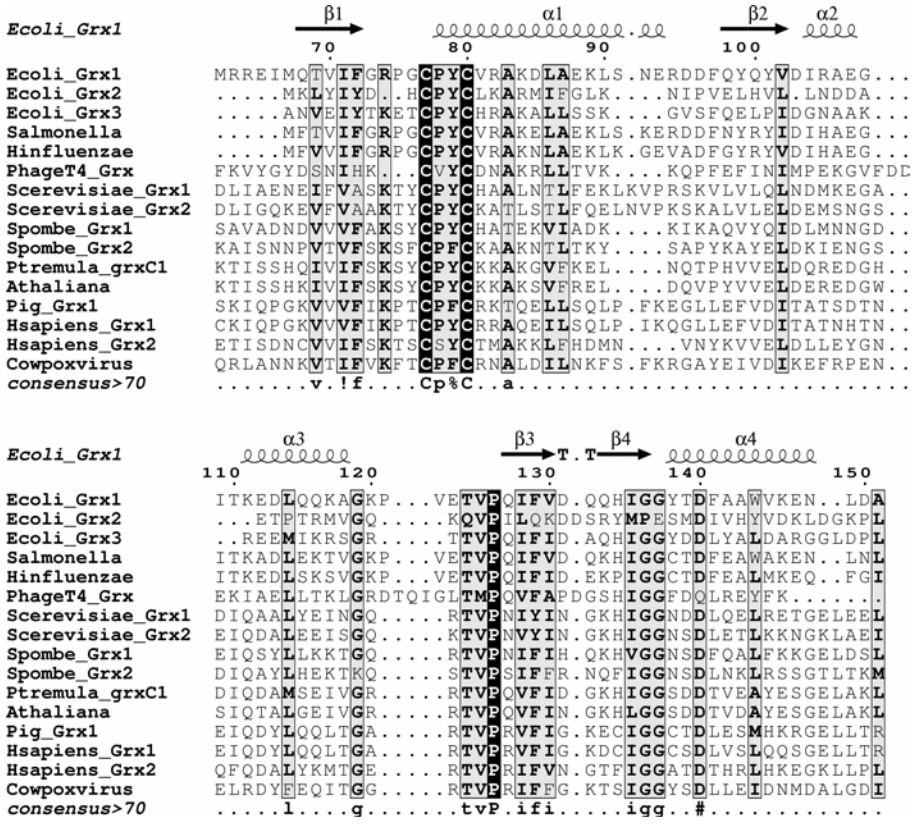


Figure 7. Multiple alignment of dithiol glutaredoxins. The secondary structure of *E. coli* Grx1 is seen at the top. Black boxes indicates strict identity, bold characters shows similarity within a group of amino acids and black frames around a grey box displays similarity across different group of amino acids.

increased sensitivity to oxidative stress and elevated iron levels (Belli et al., 2002) indicating the importance of the motif. The remaining conserved residues are located in either α-helices or β-strands, where their conserved properties are important for the formation of the secondary structure. The mammalian dithiol Grxs have all been shown to have cysteines additional to those in the active site, often located in the most C-terminal β-strand or the following helix. Replacement of two C-terminal cysteines did not affect the ability of pig or human Grx1 to perform reduction of GSH–mixed disulfides or disulfide substrates, respectively (Yang et al., 1998; Yang and Wells, 1991) but the human Grx1 C82S mutant was shown to be involved in hydrogen binding to the GSH moiety in the GSH –Grx complex. Human Grx1 has a fifth cysteine residue located in the N-terminal helix, which is involved in Grx1 dimerization by disulfide formation during oxidative conditions (Padilla et al., 1996).

1.3.3 Glutathione mixed disulfide complexes

The structure of several complexes containing Grx and GSH has been determined (Bushweller et al., 1994; Nordstrand et al., 1999; Yang et al., 1998). Glutathionylation of both Grx1 and Grx3 induce a shift in the position of α-helix 1 (only Grx3) and 2, which is accompanied by a number of side chain conformation changes, particularly by the active site tyrosine. Hydrogen bond formation between the tyrosine and a nearby conserved asparagine in Grx3 allows packing of the active site tyrosine against the protein surface (Nordstrand et al., 2000). A general conformational change model for Grx binding to GSH may explain the low affinity of Grxs to GSH-analogues (Hoog et al., 1982; Srinivasan et al., 1997). Nevertheless, the GSH binding site of Grx1 and Grx3 differs both in charge distribution and chemical properties of the residue involved, hence altering the GSH interaction network (Bushweller et al., 1994; Nordstrand et al., 1999). The structure of Grx3 close to the GSH binding pocket is much more rigid due to shorter connecting loops between the secondary structure elements and an additional C-terminal helix. Such decreased plasticity in Grx3 makes the formation of the GSH binding transition state entropically more favourable and may account for the twofold increase in turnover rate of GSH-mixed disulfides compared to Grx1 (Berardi and Bushweller, 1999). An inspection of the GSH binding patch in Human and *E. coli* Grxs show differences in the saltbridge and hydrogen binding pattern, *E. coli* having the smaller number of interactions (Yang et al., 1998). A RNR peptide was shown to form a stable mixed disulfide with *E. coli* Grx1 in a similar orientation as the GSH moiety but with a different interaction network in order to achieve binding specificity and affinity (Berardi and Bushweller, 1999). The RNR peptide binding groove in Grx1 is lined with charged residues, which together with some hydrophobic sidechains form the bases for the interaction. Electrostatic interactions between R16 and K23 in helix 1 ,which takes place upon RNR binding, may also contribute to a conformational change of the active site loop and neighbouring helices, and adapt the structure of Grx1 to its current substrate. Interestingly, the restricted structural plasticity and presence of a negatively charged residue in the potential RNR binding cleft of *E. coli* Grx3 disfavours conformational change adaptation and catalysis of RNR, and may therefore explain why *E. coli* Grx3 is such a poor reductant of RNR (Berardi and Bushweller, 1999).

1.3.4 *E. coli* dithiol glutaredoxins

The *E. coli* dithiol Grxs, glutaredoxin 1, 2 and 3 (Grx1, 2 and 3) have been thoroughly investigated and they display both common features like substrate preference and some major differences regarding for example structure (Fernandes and Holmgren, 2004). All three *E. coli* Grxs catalyse reduction of mixed disulfides of Arsenate (ArsC) and PAPS reductase, but to different extent (Lillig et al., 2003; Shi et al., 1999). Grx 1 and 3 can also function as electron donors for RNR even though the activity of Grx3 is very week (Holmgren, 1976; Åslund et al., 1994), something that can be explained by the differences in charge distribution around the active site (Åslund et al., 1996). All three *E. coli* Grxs display activity in the reduction of HED with Grx2 being the most efficient catalyst (Vlamis-Gardikas et al., 1997). In addition, both Grx1 and 2 are involved in the response to H_2O_2. Grx2 levels decrease after H_2O_2 treatment (Potamitou et al., 2002) and Grx2 null mutants are sensitive to H_2O_2 (Vlamis-Gardikas et al., 2002). Grx1 was found to be regulated by the transcription factor OxyR, which activates expression of several oxidative stress defensive genes in response to elevated H_2O_2 levels (Carmel-Harel and Storz, 2000). Grx2 can also contribute to the protection of cerebellar granule neurons from dopamine-induced apoptosis by activating the Ras/phosphoinositide 3-kinase/Akt/NF-kB and the JNK1/2/AP-1 cascade causing for example translocation of NF-kB from the cytoplasm to the nucleus and enhancing its DNA binding activity (Daily et al., 2001a; Daily et al., 2001b).

The structures of Grx1 and Grx3 are nearly identical, both displaying a four-stranded β-sheet surrounded by 3 α-helices (Nordstrand et al., 2000; Xia et al., 1992). Small differences between the oxidized forms of Grx1 and 3 can be seen in the length of helix 1, which is truncated in Grx3 and hence contains one helical turn less, and also in the charge distribution around the active site sequence, most likely influencing substrate binding (Åslund et al., 1996). On the contrary, Grx2 is more then double the size of Grx1 and 3, containing an N-terminal Grx domain connected through a linker to a C-terminal helical domain (Xia et al., 2001). The two domains in Grx2 make close contact, partly burying the active site and making it inaccessible to some bulky substrates such as RNR. The overall Grx2 structure is similar to the one of the GST but Grx2 displays no activity against common GST substrates such as 1-chloro-2,4-dinitrobenzene or 1,3,5-trinitrobenzene (Chen et al., 1988; Graminski et al., 1989).

1.3.5 Yeast dithiol glutaredoxins

Saccharomyces cerevisiae contains two dithiol Grxs, glutaredoxin 1 and 2 (Grx1 and Grx2), which display large similarities to its homologues in *E. coli* and plants. Null mutants for Grx1 or 2 are sensitive to generation of oxidative stress by superoxide anion or H_2O_2, respectively (Luikenhuis et al., 1998) displaying their different tasks in the oxidative stress defence. Grx2 mutants accounts for most of the Grx activity during exponential growth (Luikenhuis et al., 1998) and null mutants cause protein oxidative damage (Rodriguez-Manzaneque et al., 1999). No structure of the yeast Grxs are available but the crystallization of Grx2 have been published (Discola et al., 2005).

1.3.6 Mammalian dithiol glutaredoxins

The two dithiol Grxs found in mammalian species are the well characterized Grx1 and the recently identified Grx2. Grx1 is mainly localized in the cytosol but it has also been shown to be translocated to the nucleus (Padilla et al., 1992), where it can regulate the activity of several transcription factors, and through membranes by an unknown mechanism, ending up in the plasma (Nakamura et al., 1998) or inside neural cells (Daily et al., 2001a). The general functions performed by Grx1 are; donation of hydrogen for RNR (Luthman and Holmgren, 1982a), reduction of dehydroascorbate to ascorbate (Yang and Wells, 1991), and protein activation/deactivation by deglutathionylation (Srinivasan et al., 1997; Yang et al., 1998). Protein deglutathionylation by Grx1 regulates many important processes involved in disease, such as synthesis of dopamine, affected in Parkinson's (Borges et al., 2002) and maturation of HIV (Davis et al., 1997). The structure of human Grx1 consists of a four-stranded β-sheet surrounded by five α-helices, one additional helix in the N- and C-terminus, respectively, in comparison with the *E. coli* Grxs (Sun et al., 1998). Residues in the β2-α3 loop, which in *E. coli* Grx1 is involved in binding to RNR (Berardi et al., 1998), display signs of conformational change implicating possible substrate adaptation. A comparison with *E. coli* Grxs shows displacement and elongation of helix 3, resulting in a narrowing of the RNR binding groove, a possible adaptation to the human RNR, which has residues with much smaller side chains compared to *E. coli* RNR (Sun et al., 1998).

Mammalian Grx2 has two isoforms, Grx2a, which is mainly mitochondrial and Grx2b, localized in the cytosol (personal communication C.H. Lillig, Lundberg et al., 2001).

The levels of human Grx2 (hGrx2) is also generally 20-fold lower than those of hGrx1 (Lundberg et al., 2004). hGrx2 is active in the HED-assay and serves as a substrate for mammalian TR in the reduction of RNase-SG (Johansson et al., 2004). A monothiol active site mutant of hGrx2 displayed even higher reactivity for TR demonstrating that hGrx2 is well suited to control the level of s-glutathionylation during oxidative stress in the cell, especially in the mitochondria. Silencing of hGrx2 expression using siRNA increased the cells sensitivity to cell death inducing agents (Lillig et al., 2004) and overexpression of hGrx2 was shown to attenuate apoptosis (Enoksson et al., 2005), proposing a crucial role for hGrx2 in the regulation of the mitochondrial redox status and regulation of cell death. Recently, hGrx2 have been implicated in the iron metabolism of the cell. Iron is an essential nutrient used in various ways by the cell, for example in Fe-S clusters, hemes and diiron-oxo metal centres in enzymes. hGrx2 forms a [Fe_2S_2] cluster by homodimerization, involving binding to two GSH molecules (Berndt et al., 2006) (figure 8), and the dimeric holo-enzyme was found to be enzymatically inactive (Lillig et al., 2005). Oxidation of the GSH pool in the cell caused by oxidative stress is proposed to dissociate the Fe-S cluster dimers and activate the protein, suggesting a function of the Fe-S cluster as a redox sensor for activation of hGrx2. A Fe-S cluster similar to hGrx2 has been seen in poplar Grx (Feng et al., 2006). Remarkably, when the hGrx1 active site proline was changed for the corresponding serine in hGrx2, hGrx1 was also able to form Fe-S clusters (Berndt et al., 2006). Potential Grx2 proteins have also been found in other vertebrates such as dog and chicken.

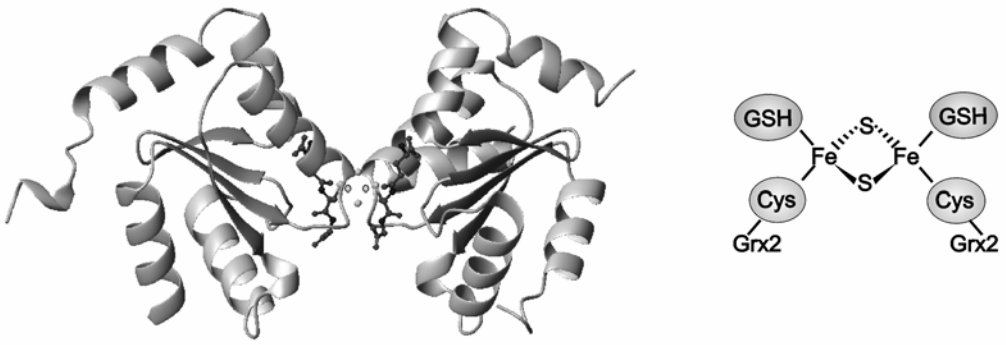


Figure 8. The structure of the human Grx2 dimer. The crystal structure containing the Fe-S cluster is seen to the left (PDB 2HT9) and a schematics of the Fe-S cluster is seen to the right.

1.3.7 Glutaredoxin like proteins

The NrdH proteins belongs structurally to the Grx family although their active site CXXC sequence differ from Grx by having CVQC or CMQC and the protein can form domain–swapped dimers, which has not been seen otherwise in the Grx family (Stehr and Lindqvist, 2004). A wide conserved hydrophobic pocket on the surface of NrdH, similar to Trx, may explain the Trx activity of NrdH and its dependence on reduction by TR (Stehr et al., 2001).

Some larger proteins also contain a Grx fold motif, for example the GSTs, which catalyse the conjugation of non-polar compounds that contain an electrophilic carbon, nitrogen or sulphur atom to reduced glutathione. In this way, GSTs contribute to the metabolism of cancer drugs, environmental xenobiotics and products of oxidative stress. The GST family is subdivided into three classes; the cytosolic (cGST), mitochondrial (mGST) and membrane bound microsomal GSTs (MAPEGs), of which the cytosolic and mitochondrial GSTs are both dimeric and structurally related to the Trx/Grx family (figure 9).

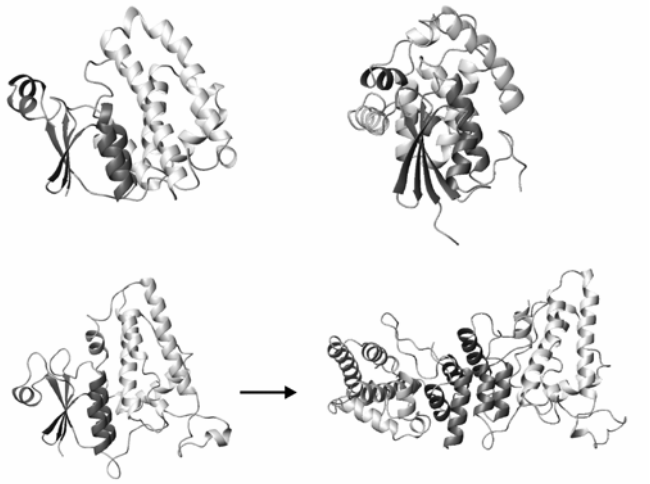


Figure 9. Crystal structures of GST proteins. A cytosolic human class pi cGST (PDB 1ZGN, upper left), the mitochondrial rat class kappa GST (1RW4, upper right), the chloride ion channel CLIC1 in its monomeric reduced form (PDB 1K0M, lower left) and oxidized dimeric form (PDB 1RK4, lower right). The Grx domain is displayed in dark grey.

The cGSTs contain an N-terminal Grx domain, which binds GSH and an α-helical C-terminal domain containing a hydrophobic pocket (H-site) to, which hydrophobic co-substrates bind (Oakley, 2005). Several cGSTs use a catalytic serine or tyrosine hydroxyl to activate GSH during catalysis. The omega class of cGSTs is somehow uniqe since it forms mixed disulfides involving GSH and a cysteine residue in the N-terminal Grx domain, hence performing the classical glutaredoxin activity (Garcera et al., 2006), linking the GST family to the Grx system. An interesting member of the

cGST family is the intracellular chloride ion channel (CLIC), which exists both in soluble monomeric and membrane bond form, has a Grx-like active site and binds GSH. Oxidation of CLIC1 results in dimerization by formation of an intramolecular disulfide bond and concomitant exposure of a hydrophobic dimerization interface, possibly facilitating membrane integration (Littler et al., 2004). The fold of the mitochondrial GSTs was recently determined and is similar to the cGSTs except that the α-helical domain is inserted in the middle of the Grx domain (Ladner et al., 2004). Future studies of the GST family provides hope for the development of isozyme-specific GST inhibitor, which can be used in chemotherapy.

1.4 MONOTHIOL GLUTAREDOXINS

Monothiol glutaredoxins are found in a wide range of species, including prokaryotes, plants and eukaryotes, are evolutionary conserved and many of them can substitute for each other (Molina-Navarro et al., 2006). Many monothiol Grxs consist of a single monothiol Grx motif, including all bacterial monothiol Grxs together with *S. cerevisiae* Grx5 and *H. sapiens* Grx5. In contrast, *S. cerevisiae* Grx3 and 4 have large N-terminal extensions, which are normally seen in most multicellular eukaryotic members of the monothiol family. The extension can sometimes be a highly conserved duplicate of the more C-terminal region e.g. in the case of human and rat PICOT proteins.

1.4.1 The active site

The potential active site found in monothiol Grxs generally contains the CGFS sequence but little is still known about the activity of this family and the role of the active site. A G61V active site mutant in *S. cerevisiae* Grx5 was found to be sensitive to oxidative stress and to have increased iron levels. Replacement of the entire active site with the GST omega CPWA sequence resulted in a low but significant activity against HED (Garcera et al., 2006), providing evidence for the important role of the residues adjacent to the active site cysteine.

1.4.2 Conserved residues

The residue conservation within the monothiol motif share some features with the dithiol family, for example a conserved *cis*-proline and GG motif followed by a D, but the rest of the sequence conservation is much more distinct compared to the dithiol Grx family (Figure 10).

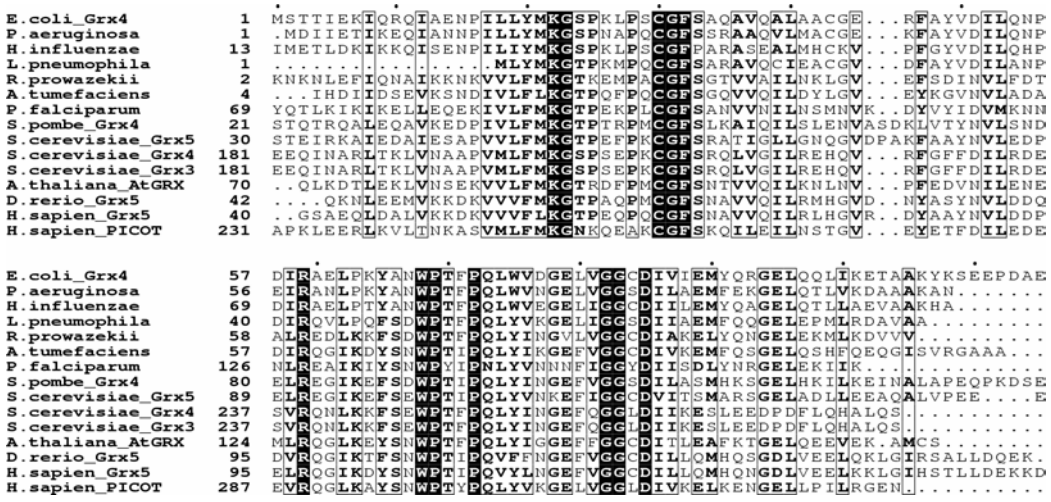


Figure 10. Multiple sequence alignment of monothiol glutaredoxins from different species. The black boxes indicate strict identity, bold characters similarity within a group of amino acids and black frames surrounding a grey box displays similarity across different group of amino acids.

A stretch of hydrophobic residues N-terminal to the potential monothiol active site ends with a conserved KG motif, and a highly conserved WP motif is located two residues before the conserved *cis*-proline. Additionally, there is a number of semi-conserved hydrophobic stretches, which may be implicated in formation of buried secondary structure and a highly conserved I/L/VR motif initiating the second half of the monothiol sequence. The number of cysteines present except for in the potential active site, which may be involved in substrate binding or disulfide formation, varies substantially within the monothiol family. *E. coli* and *H. influenza* have two additional cysteines and *S. pombe* Grx4 and *S. cerevisiae* Grx3 and Grx4 have none. The role of these cysteine residues are not deeply investigated but the C-terminal C117S mutant in *S. cerevisiae* Grx5 was discovered to be unable to perform deglutathionylation (Belli et al., 2002), suggesting proximity to the active site residue.

1.4.3 Bacterial monothiol glutaredoxins

A monothiol Grx protein in *Plasmodium falciparum*, the *P. falciparum* 1-Cys-glutaredoxin-like protein-1 (PfGLP-1) has a mitochondrial targeting signal and can perform thioloxidoreductase activity through protein deglutathionylation and reduction of insulin. No activity could on the other hand be seen with a low molecular weight substrate such as HED and PfGLP-1 could not function as a substrate for GR/GSH or TR (Rahlfs et al., 2001). A bacterial monothiol Grx from *E. coli* will be presented later as a part of this thesis.

1.4.4 Yeast monothiol glutaredoxins

The *S. cerevisiae* monothiol glutaredoxins (Grx3, 4 and 5) have a protective role against oxidative stress (Rodriguez-Manzaneque et al., 1999) and a null mutant for all the three yeast monothiol glutaredoxins was not viable (Rodriguez-Manzaneque et al., 1999). The *grx5* null mutant displayed severe growth reduction in low nutrient media and inability to grow at 37 º (Rodriguez-Manzaneque et al., 1999) and absence of *grx5* prevents respiratory growth, causes accumulation of free iron in the cell and constitutive oxidation of proteins (Rodriguez-Manzaneque et al., 2002). Interaction of Grx5 with mitochondrial proteins involved in iron biogenesis resulted in the discovered a mitochondrial localization of the protein and involvement in the formation of iron-sulphur clusters (Muhlenhoff et al., 2003; Rodriguez-Manzaneque et al., 2002). Deficiency in Grx5 leads to iron accumulation and concomitant inactivation of manganese superoxide dismutase (SOD2), which plays an important role in the defence against oxidative stress (Yang et al., 2006). The biological activity of Grx5 has so far been restricted to deglutathionylation of carbonic anhydrase since neither glutathione peroxidase, dehydroascorbate reductase or HED assay activity could be detected (Tamarit et al., 2003). Grx5 can be glutathionylated by GSSG alone but the concomitant reduction by GSH was very inefficient, about 20 times slower than for *E. coli* Grx1 (Tamarit et al., 2003). Similar to *S. cerevisiae, S. pombe* was recently shown to have three monothiol Grx homologues, Grx3, Grx4 and Grx5. Both *S. pombe* monothiol Grx4 and Grx5 are induced by nitrosative and osmotic stress while ROS inhibited Grx5 expression and induced Grx4 expression (Kim et al., 2005a; Kim et al., 2005b). Opposite to the *S. cerevisiae* Grxs, the nuclear Grx4 was the only monothiol Grx in *S. pombe* essential for growth while overexpression of nuclear Grx3 and mitochondrial Grx5 strongly retarded growth (Chung et al., 2005).

1.4.5 Distinct differences between dithiol and monothiol glutaredoxins

The characterized monothiol Grxs differ significantely from the dithiol Grxs by lacking activity towards classical Grx substrates such as HED and insulin. A summary of the characterized monothiol Grxs are displayed in Table 1.

Table 1.

Monothiol glutaredoxin	**HED assay activity**	**Insulin assay activity**	**Substitute for yeast Grx5**	**Localization**	**Impact on iron levels**
E. coli Grx4 (Fernandes et al., 2005; Molina-Navarro et al., 2006)	no	no	yes	-	Expression increased upon depletion
S. cerevisiae Grx5 (Tamarit et al., 2003)	no	no	-	Mitochondria	Reduced levels
A. thaliana AtGrxcp (Liu et al., 2006)	no	no	yes	Chloroplasts	Reduced levels
P. falciparum PfGLP-1 (Rahlfs et al., 2001)	no	low	nd	Mitochondria (predicted)	nd
Zebrafish (Wingert et al., 2005)	nd	nd	yes	Mitochondria	none

nd- not determined

1.4.6 Iron

Similarly to the dithiol Grxs, some monothiol Grxs have recently been implicated in iron metabolism. In yeast, Grx5 from *S. cerevisiae* is essential for mitochondrial biogenesis of Fe-S cluster (Muhlenhoff et al., 2003; Rodriguez-Manzaneque et al., 2002) while Grx3 and Grx4 interact with the iron responsive transcriptional activator Aft1, partly inhibiting it's function (Ojeda et al., 2006). However, the Aft1 interaction does not seem to be directly linked to the Fe-S cluster biogenesis. Deficiency of Zebra fish Grx5 causes loss of Fe-S cluster assembly, which blocks haem biosynthesis leading to hypochromic anemia (Wingert et al., 2005).

1.4.7 Open issues in the glutaredoxin field

A careful investigation of the dithiol Grxs has leed to a good understanding of structure and function relationships, especially concerning the classical CPYC containing subfamily. The more recently discovered mammalian Grx2-like proteins belong to an emerging field of Fe-S cluster containing Grxs which are proposed to function as redox sensors during conditions of oxidative stress and intensive research in currently going

on in this field in order to identify the molecular targets of these proteins *in vivo*. The monothiol Grxs are also suggested to be associated with Fe by regulating the activity of Fe-S containing enzymes Fe, but the mechanism is not fully investigated and none of the monothiol Grxs has so far been shown to host a Fe-S cluster. The actual targets of the essential monothiol Grxs are currently unknown but future studies may hopefully shed light on this mysterios member of the Grx family.

2 MYC PROTEIN FUNCTIONALITIES

In this summary, the focus is on the field of oncogenesis and in particular c-Myc, providing an introduction to c-Myc transcriptional activation and protein interactions.

2.1 TRANSCRIPTION

Transcription is the process through which the DNA in the cell is enzymatically copied by RNA polymerases in order to transfer the information to RNA. A number of different RNAs perform a wide range of separate tasks connected to transcription; rRNA form the core of the ribosome, mRNA carries the copied information from the DNA, snRNA direct the splicing of pre-mRNA to mRNA and tRNA selects amino acids and hold them in place onto the ribosome during the building of a protein. Eukaryotic species have three nuclear RNA polymerases, which transcribe different types of genes; RNA polymerase I (Pol I) transcribes ribosomal RNA (rRNA), RNA polymerase II (Pol II) transcribes messenger RNA (mRNA) and small nuclear RNA (snRNA) and RNA polymerase III (Pol III) transcribes transfer RNA (tRNA) and other small RNAs such as 5S rRNA. A number of additional proteins called transcription factors are required by Pol II and they form the transcription preinitiation complex. The TATA box binding protein (TBP) recognises and binds to the TATA box sequence located slightly upstream of the transcription initiation site followed by association of a number of other factors such as TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH (figure 11).

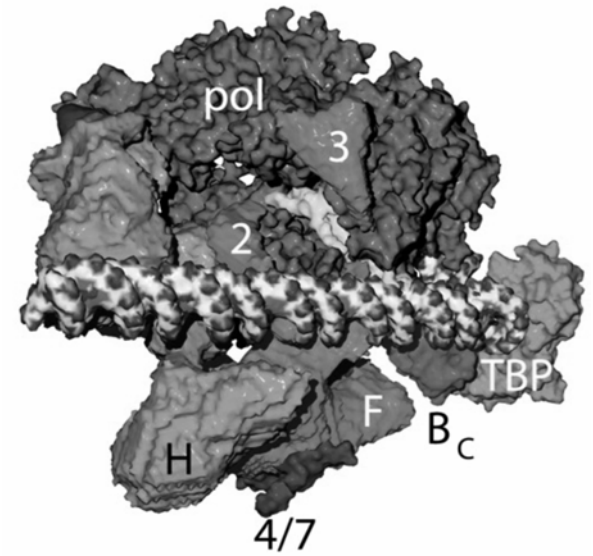


Figure 11. A model of the RNA polymerase II transcription initiation complex, adapted from (Bushnell et al., 2004).

Additionally, binding of the Pol II complex to DNA is facilitated by chromatin remodelling enzymes, which loosen up the binding of the DNA to the histones, histone acetylases, which acetylate the histone tails and thereby destabilize the chromatin structure, making it more accessible for target proteins.

2.1.1 Transcriptional regulation

Gene expression is commonly regulated by transcriptional activators and repressor proteins that bind to the DNA regulatory sequences and interact with the Pol II complex bound to the promoter region, allowing individual genes to be turned on and off specifically. Most transcriptional activator proteins are multidomain proteins consisting of several different domains separated by flexible linker regions. Two main domains mediate transcription; a DNA binding domain recognizing a specific DNA regulatory sequence and a transcriptional activation domain (TAD), which recruits a wide variety of coactivators, suppressors and components of the basal transcriptional machinery to gene promoters and thereby control the activity of the activator protein, hence also the rate of transcription. The focus of this introduction is the transcriptional activation domain of c-Myc which will be described below but excellent overviews of DNA binding domains can be found in (Luscombe et al., 2000).

2.1.1.1 Transcriptional activation domains

Protein structures are often pictured by tightly folded single domain proteins, which display only small local disordered regions close to e.g. their active site. However, the structure can also be modular, i.e. contain independently folded domains separated by flexible linker regions, or be intrinsically disordered. Intrinsically disordered protein regions have a high proportion of polar and charged residues and a low content of bulky hydrophobic residues, which normally forms the core of a folded globular protein (Dyson and Wright, 2005). True random coil proteins are most likely very rare and intrinsic disordered proteins therefore often have either local regions, which form isolated secondary structure elements or consist of a compact, but disordered molten globule state (Bochkareva et al., 2005; Demarest et al., 2004; Mark et al., 2005; Wahlberg et al., 2003). Molten globule was originally defined as being a part of the folding pathway from denatured to native state but later studies have shown that molten globule can also be the native state of a protein (Pande and Rokhsar, 1998). The characteristics of the molten globule are a native like secondary structure with little or

disordered tertiary structure, which is not cooperatively folded. Biophysical methods such as CD (structure content investigation and thermodynamic stability) and ANS fluorescence are commonly used to investigate the molten globule state.

Many intrinsically disordered proteins gain secondary or tertiary structure upon binding to their target molecules (Dames et al., 2002) and the functional advantage of a coupled folding process includes strict control over the thermodynamics of the binding event and the ability to gain different conformation upon binding to a variety of targets (Dyson and Wright, 2002). Induced structure by binding can occur through different mechanisms, including wrapping of a target onto the surface of the binding partner or through a folding-on-binding process (Dames et al., 2002; Juo et al., 2003). Both these routes allows burial of extremely large surface areas even if the interacting partners are relatively small, which would not be possible for well folded proteins. Binding by intrinsically unstructured proteins is characterized by high specificity linked to relatively low affinity, which is important in order to achieve binding to different partners in response to a specific signaling process (Dyson and Wright, 2005). A flexible structure will also create a simple mechanism for binding to both physiological- and post-translational modified targets and many intrinsically disordered domains contain a relatively high degree of serine and threonine residues, which are potential targets of phosphorylation. Phosphorylation changes the net charge of the molecule, making it highly hydrophilic and can disrupt protein-protein interactions (Campbell and Lumb, 2002) and several studies show a correlation between phosphorylation and transcriptional activation activity (Bode and Dong, 2004).

Despite long-standing biological characterization of transactivation, little is still know about the molecular mechanism behind the interaction between TADs and the basal transcriptional machinery. Each class of TADs have an outstanding capability in binding to multiple targets but no clear sequence homology between different TADs have be identified, not even among a particular class. The first examples of TADs were highly charged or Q/P rich domains, thought to function mainly by charge interactions (Erkine, 2004) but more recent results suggested that short stretches of hydrophobic amino acids (Choi et al., 2000; Lin et al., 1995; Razeto et al., 2004) are likely to be involved in interaction with target proteins. Several studies have shown a large degree of intrinsically disordered regions, especially in human TADs (Minezaki et al., 2006),

and an emerging class of TADs appear to interact with their targets through sequence-specific folding-on-binding mechanisms. Several of these interactions are believed to be mediated by formation of short α-helices (Choi et al., 2000), including VP16 (Uesugi et al., 1997), the aryl hydrocarbon receptor (Watt et al., 2005), p53 (Kaustov et al., 2006) as well as c-Myc and AF1 (Hermann et al., 2001; Kumar et al., 2004).

2.2 PROTO-ONCOGENES

The Nobel price in 1989 was awarded to J. M. Bishop and H. E. Varmus for the discovery of "the cellular origin of retroviral oncogenes". An oncogene is a gene that has sustained some genetic damage and, therefore, produces a protein capable of cellular transformation. The cellular counterpart to oncogenes, proto-oncogenes, is originally normal genes, which by overproduction or mutation become oncogenes and hence contribute to the transformation of a normal cell into a tumor cell (Bishop, 1983). Functionally, proto-oncogenes are regulators of cell growth control and consists of 1) growth factors, 2) growth factor receptors, 3) signal transducers, 4) transcription factors, and 5) others, including programmed cell death proteins (Holland and Frei, 2006). A number of different proto-oncogenes have been identified, including *EGFR*, *RAS*, *SRC*, v-*fos*, *Bcl2*, *MDM2* and finally Myc, the main subject of the second part of this thesis.

2.3 THE MYC FAMILY

Deregulated expression of the nuclear transcription factor Myc affects the expression of a large set of target genes involved in cell proliferation, cell growth, differentiation and apoptosis and is a well known hallmark in many human cancers. Extensive sequence conservation can be seen within the Myc family, consisting of c-, N-, L-, S-, and B-Myc, especially in the Myc homology box regions I and II (MBI and MBII) in the N-terminal transactivation domain (TAD), and the C-terminal DNA binding domain (figure 12).

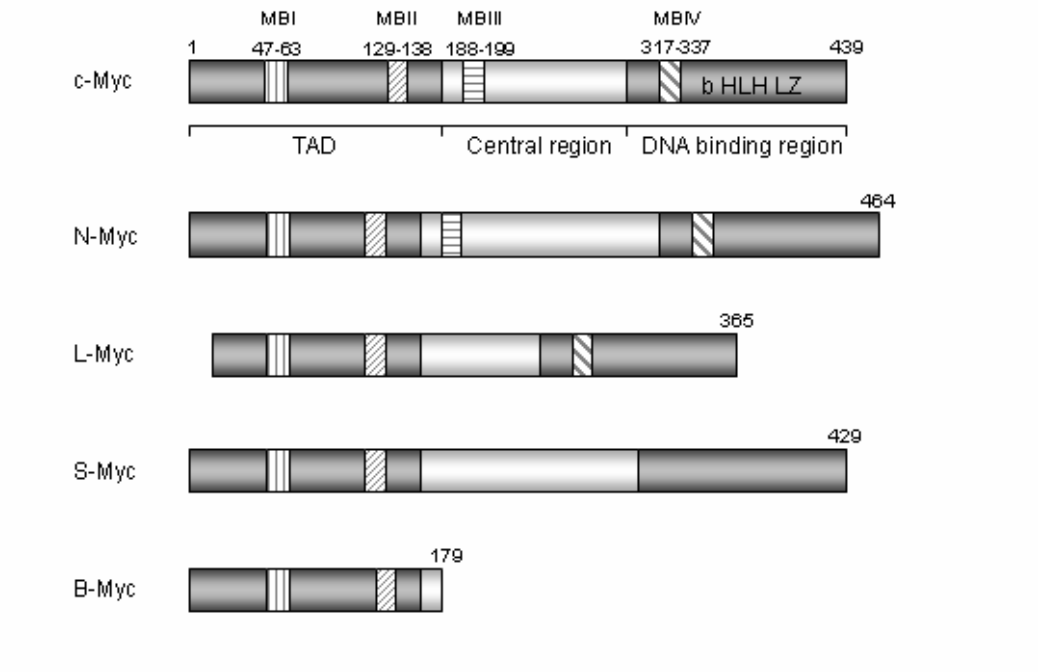


Figure 12. Myc family members. Adapted from (Oster et al., 2002).

The remains of the N-terminal region and the central region show very low degree of homology except for Myc Box III (MBIII), which is conserved between c- and N-Myc and Myc box IV (MBIV) only found in c-, N-, and L-Myc. c-, N-, and L-Myc are oncogenes and have transforming abilities while S-, and B-Myc mainly have inhibitory effects on Myc transactivation (Resar et al., 1993). B-Myc lacks the C-terminal DNA binding and dimerization motif and is largely unstructured in solution (Burton et al., 2006).

The levels of c-Myc in normal healthy cells are tightly regulated by a wide range of signalling cascades guiding proliferation, and both c-Myc mRNA and protein have very short half-life (20-30 min) (Salghetti et al., 1999). Resting cells generally lack Myc (Lacy et al., 1986) but exposure to various growth stimuli cause rapid elevation of c-Myc expression, giving Myc the role of an early growth response gene. In contrast to c-Myc, expression of both N- and L-Myc is absent in normal cells. N-Myc is highly expressed in pre-B cells, kidney, forebrain, hindbrain and intestine, and high L-Myc levels can be found in developing kidney, lungs from newborns and in proliferative and differentiative parts of the brain and neural tube (Nesbit et al., 1999). We will hereon

mainly refer to data describing c-Myc, which is the most common deregulated Myc family member in cancer although similar findings have sometimes been made for the other Myc oncoproteins, N- and L-Myc.

2.4 MYC STRUCTURE

c-Myc structurally consists of the N-terminal TAD linked to the C-terminal basic-helix-loop-helix-leucine zipper (bHLH-Zip) motif by a central low homology region (Kato et al., 1990; Landschulz et al., 1988; Murre et al., 1989). Investigation of a c-Myc TAD construct containing residue 1-143 showed little or no evidence of secondary structure (McEwan et al., 1996) but secondary structure prediction methods estimate the total structural content of c-Myc TAD to around 30 % α-helix and 10 % β-sheet with strong helical preference around the MBI and MBII. The Myc Box regions were originally merely defined by conservation but they display important features concerning the activity of the proteins. MBI and MBII are necessary for induction of apoptosis and blockage of differentiation and contain several mutational hotspots (Evan et al., 1992; Freytag et al., 1990; Stone et al., 1987). Several stretches of c-Myc TAD are necessary for *c-myc/H-ras* cotransformation by primary rat embryo fibroblasts, including MBII and a more N-terminal region (McMahon et al., 1998). Two of the hotspots in MBI are also important phosphorylation sites, T58 and S62, while MBII does not house any residues targeted for phosphorylation (Henriksson et al., 1993). The more recently discovered MBIII, located in the central region, and MBIV in the DNA binding region are also necessary for transformation but have opposite roles in apoptosis; MBIII inhibits apoptosis while MBIV is necessary for apoptosis induction (Cowling et al., 2006; Herbst et al., 2005). The transactivation function of Myc is dramatically reduced by MycΔMBII (Cowling et al., 2006) but the gene activation potential, as measured by a *lacZ* reporter gene fused to the Pho4-responsive *PHO5* promoter, lies in regions outside the MBII, namely aa 1-41 and 66-127 (Flinn et al., 2002). Point mutations of Thr58 and Ser62 results in a 50-60 % decrease in transcriptional activation of the LacZ gene driven by a glucocorticoid response element (McEwan et al., 1996). MycΔMBI causes no change in transactivation activity while deletion of MBIII and IV slightly reduces transactivation (Cowling et al., 2006).

The DNA binding domain of Myc consists of a basic motif (aa 354-367), which is involved in binding to DNA, a HLH (aa 368-407) and leucine zipper (aa 413-434) that specify heterodimerization (Nair and Burley, 2003). The inability of Myc to form homodimers has been linked to a region N-terminal to the bHLH-Zip since a minimal bHLH-Zip Myc protein lacking this region form homodimers that bind to the E-box sequence (Kato et al., 1992). Several different cellular factors have been implicated to bind to c-Myc bHLH-Zip region, e.g. Nmi, AP2, Brca1 and Miz1 (reviewed in Oster et al., 2002) and thereby regulate gene expression.

2.4.1.1 Phosphorylation

c-Myc is subjected to several types of post-translational modifications, including phosphorylation, ubiquitination, glycosylation and acetylaytion but the effect on biological activity is only well studied for phosphorylation. Five residues in c-Myc TAD have been identified as targets for phosphorylation *in vivo*; T58, S62, S71, S82 and S164 (Henriksson et al., 1993; Lutterbach and Hann, 1994a; Lutterbach and Hann, 1997). but potential kinases catalysing the phosphorylation has only been identified for T58, S62 and S71, which all have adjacent proline residues, making them potential targets for proline-directed kinases. Evidence shows that Glycogen synthase kinase 3 (GSK3) handles T58 phosphorylation but require a prior phosphorylation of S62 (Gregory et al., 2003; Lutterbach and Hann, 1994a). The identification of S62 and S71 kinases has been complicated but a number of different signal pathways have recently been recognized; mitogenic and oxidative stress stimulation leading to phosphorylation by the extracellular-signal regulated kinase (ERK), stress-activated checkpoint controlled phosphorylation by the c-Jun N-terminal kinase (JNK) and cell cycle regulation and mitosis targeting phosphorylation by the cell cycle regulated kinase (Cdk1) (reviewed in Hann, 2006). Phosphorylation by a specific pathway can be initiated by different cellular conditions and may vary among the Myc family members.

2.4.1.2 Ubiquitination and protein turnover

The low stability of the c-Myc protein, typical half-life of 20-30 min, (Hann and Eisenman, 1984; Salghetti et al., 1999) suggests that proteolytic breakdown of c-Myc could be an important issue for tumorogenesis, since several oncogenic cell lines have prolonged c-Myc half-life (Malempati et al., 2006). Ubiquitination is a major pathway for proteolysis, which uses Ubiquitin (Ub) ligases for polyubiquitination of lysine

residues in target proteins, resulting in ubiquitin-mediated proteolysis and protein breakdown. c-Myc TAD contains several lysine residues, which are ubiquitinated by the S-phase kinase associated protein 2 (Skp2) and signal Myc turnover (Kim et al., 2003). Skp2 is primarily expressed in G1/S-phase and displays transcriptional coactivator activity similar to several other Ub-ligases. Recognition by Skp2 of a region including MBII in c-Myc TAD as well as residue 379-418 in the Myc DNA binding region, activates Myc dependent transcription of target genes, linking Myc activation to destruction.

Another ubiquitin ligase containing the F-box substrate recognition protein (Fbw7) has also been suggested to ubiquitinate c-Myc TAD. c-Myc binding and ubiquitination by Fbw7 is dependent on T58 phosphorylation, hence the GSK3 activity (Welcker et al., 2004; Yada et al., 2004). In contrast to Skp2, Fbw7 is expressed throughout the cell cycle, is mutated in many cancers and transcriptionally represses c-Myc, suggesting a role as a tumor suppressor (Minella and Clurman, 2005). Finally, Jun N-terminal kinase (JNK) can also mediate c-Myc ubiquitination and degradation through a region in the c-Myc TAD (aa 127-189), which shares homology with the delta domain of c-Jun required for JNK association (Alarcon-Vargas and Ronai, 2004).

The role of phosphorylation in c-Myc proteolysis has been the subject of many investigations, most of them showing a stabilizing effect in c-Myc protein lacking T58 phosphorylation (T58 mutation) (Gregory and Hann, 2000; Salghetti et al., 1999). Similar results have also been seen for many Burkitt's lymphoma cell lines, which have mutations clustered around T58 and c-MycT58A clearly shows a general enhanced transforming ability (Conzen et al., 2000). Defective T58 phosphorylation has a dramatic effect on tumorogenesis, enhancing both the penetrance and latency of pre-B cell lymphomas, which is suggested to be linked to an impaired apoptotic mechanism in these mutants (Hemann et al., 2005). Mutations of S62 on the other hand display a decreased transforming ability (Pulverer et al., 1994), suggesting that phosphorylation of S62 is necessary for oncogenic activity and cell cycle progression. Normal degradation of c-Myc generally follows a pathway where S62 phosphorylation precedes T58 phosphorylation (Lutterbach and Hann, 1994b) but that final degradation of c-Myc is triggered by S62 dephosphorylation, possibly induced by phosphorylated T58 (Sears et al., 2000). Further evidence in favour of this theory was recently

supported by (Malempati et al., 2006) who found abnormally high phosphorylation of S62 compared to T58 in paediatric acute lymphoblastic leukemia patients, leading to a disturbance in the proteolytic degradation pathway and thus increased high c-Myc levels. Therefore, both T58 phosphorylation and subsequent dephosphorylation at S62 are important for polyubiquitination and timely degradation of c-Myc.

2.5 THE MAX NETWORK OF TRANSCRIPTION FACTORS

2.5.1 Transcriptional activation by the Myc-Max complex

Transcriptional activation by Myc is dependent on heterodimerization with the small bHLH-Zip protein Max in order to bind to the E-box DNA motif and regulate target genes (Blackwood and Eisenman, 1991a; Blackwood et al., 1992). *In vitro* experiments showed that the Max-v-Myc heterodimer was thermodynamically more stable than the Max-Max homodimer and the preference for the Max-Myc complex was also verified *in vivo* (Grinberg et al., 2004). A structural investigation of the Max-Myc complex (figure 13) indicate that complementary polar residues in the leucine zipper, not found

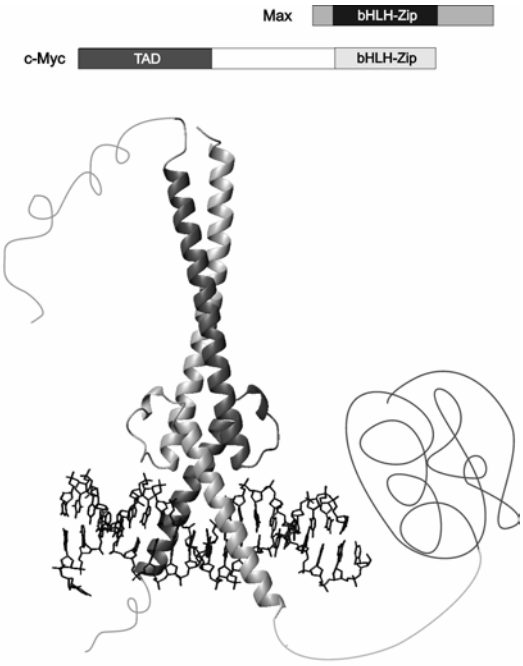


Figure 13. The structure of Myc-Max heterodimer bound to DNA. Myc bHLH-Zip is shown in grey and Max bHLH-Zip in black (PDB id 1npk). The structurally unknown parts of Myc and Max is drawn by hand, showing the c-Myc TAD in dark grey and the flanking regions of Max in light grey.

in the Max-Max complex, result in hydrogen bond pairing and hence stabilization of the heterodimer interface (Nair and Burley, 2003). The Max-Max homodimer is however a better DNA binder (Fieber et al., 2001). Phosphorylation of one of the N-

terminal serine residues inhibits Max-Max interaction, thus favoring Myc-Max heterodimerization (Berberich and Cole, 1992; Koskinen et al., 1994).

2.5.2 E-box

The consensus binding site for Myc-Max heterodimers consists of the CACGTG E-box sequence, which is a binding target for all Myc family members *in vitro* (Nesbit et al., 1999). *In vivo* studies show that target genes appear to need only this core sequence (Fernandez et al., 2003) while *in vitro* binding assays indicate that flanking C/G residues are required for maximum affinity (Boyd and Farnham, 1997). A favored position for the E-box sequence is 50-60 bases downstream of the RNA initiation site, something that may facilitate interactions between a transcriptional mediator and the RNA pol II complex (Hulf et al., 2005).

2.5.3 Max

The ubiquitously expressed Max protein contains a bHLH-Zip motif, which easily forms homodimers that bind to the E-box sequence (Blackwood and Eisenman, 1991b). Two Max isoforms, p21 and p22, are commonly expressed and the difference between the two forms consists of a nine amino acid insertion close to the N-terminus in p21, preceding the basic region (Blackwood et al., 1992). The crystal structure of the Max-Max dimer bound to DNA consist of an N-terminal helix, comprising the basic region and the adjacent helix (h1), followed by a loop and a C-terminal helix (h2) and the leucine zipper element (Brownlie et al., 1997; Ferre-D'Amare et al., 1993). Residues in the basic region mediate specific recognition of the DNA and the interaction is further stabilized by binding to the Max loop and h2 region. The NMR solution structure of a Max dimer free of DNA shows that most of the basic region is unfolded and that the loop in the HLH-motif is flexible (Sauve et al., 2004), indicating that these domains are prone to undergo conformational change upon binding to DNA, as previosly suggested (Ferre-D'Amare et al., 1994).The remaining C-terminal half of Max, residue 83-160, is not shown in the crystal structure due to disorder (Brownlie et al., 1997) and biophysical experiments conducted in solution indicate that both the N- and C-terminus of p21 Max is unstructured in the absence of DNA and that the leucine zipper is partly unfolded (Naud et al., 2005).

2.5.4 Transcriptional repression by Max binding partners

In addition to transcriptional activation by the Max-Myc complex, Max can also mediate transcriptional repression and block Myc-dependent cell transformation by forming heterodimers with the bHLH-Zip proteins Mxd1-4, Mnt and Mga (figure 14) (Ayer et al., 1993; Hurlin et al., 1995; Hurlin et al., 1999; Meroni et al., 1997; Zervos et al., 1993). None of these proteins can form homodimers or bind to DNA alone, but heterodimerization with Max makes them capable of binding the E-box sequence. The transcriptional repressor activity of the Max interacting partners Mnt and Mxd1-4 is related to binding of the corepressor Sin3 to an N-terminal region of Mnt or Mxd1-4. Sin3 mediates histone deacetylase activity, which makes the chromatin less accessible for transcription, by recruiting HDAC1 and HDAC2 (reviewed in Ayer, 1999). The mechanism behind the repressor activity of Mga is currently not known but a second DNA binding site in Mga, similar to a T-domain, has been identified, which binds to T-box associated DNA and represses T-box target gene activity (Hurlin et al., 1999).

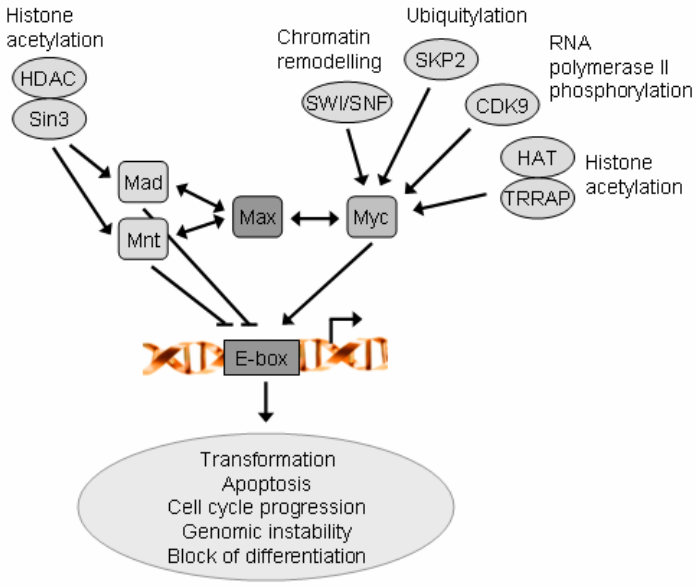


Figure 14. The Max network. Myc-Max heterodimers recruit cofactor complexes to the E-box DNA sequence near the transcriptional start site of target genes which facilitates transcription. On the contrary, dimerization of Max with Mad or Mnt mediates repression of target genes.

In contrast to the nature of the Myc protein, transcription and protein synthesis of Max is relatively constant and generate only low levels of Max in the cell. The half-life of Max is however longer compared to Myc, nearly 24 h (Hurlin and Huang, 2006).

Similar constitutive expression has been seen for the Max partner Mnt, proposing that the Max-Mnt complex represents the ground state in the cell and that heterodimerization with Myc or Mxd occurs upon mitogenic or cell cycle related stimulation, respectively. Both Mnt and the Mxd family of proteins have tumor suppressor functionalities, thereby suppressing Myc induced cell transformation. Mice lacking the Mxd-family mainly have proliferative, differentiative or apoptotic defects and all are not predisposed to tumor formation while lack of Mnt causes early death (Hooker and Hurlin, 2006; Prochownik et al., 1998). A possible explanation for these differences may be that the Mxd-family could have a specialised role in Myc antagonizing and/or only regulate Myc to a limited extent since they can also interact with other bHLH-Zip proteins besides Max (Meroni et al., 2000).

2.6 THE BIOLOGICAL ACTIVITY OF MYC - ACTION THROUGH TARGET GENES

Myc is a multifunctional protein that regulates a large number of target genes involved in cell cycle regulation, metabolism, protein synthesis, cell adhesion, cytoskeleton, angiogenesis and apoptosis in response to signals from the cellular environment. The regulation is thought to be achieved at the transcriptional level and targets a specific subset of genes leading to signalling cascades, which affect separate biological activities. Identification of targets genes has escalated during the past 5 years through a number of high-throughput screens using microarray gene expression profiling, SAGE, ChIP and Myc-methylase chimeric proteins. The number of human Myc target genes is currently approaching a total of 4000 but the result varies significantly between different studies and only a fraction of the genes seem to be universally regulated independently of cell type and specie (Zeller et al., 2003).

2.6.1 Cell cycle

The transition from the G_0 resting phase to the S phase normally requires specific growth and survival factors but is also be dependant on Myc (de Alboran et al., 2001), which can substitute for the mitogen PDGF (Jones and Kazlauskas, 2001). Myc expression is maintained throughout the whole cell cycle and may also affect later stages as was suggested by (Mateyak et al., 1997), who showed that lack of *c-myc* caused a prolonged G_2 phase. Common Myc targets include the cell cycle regulatory

genes *cyclin D1*, *D2*, *E* and *A* and *CDK4*, some cell cycle check-point genes such as *gadd45* and *gadd153* and several cyclin kinase inhibitors, causing growth arrest (Dang et al., 2006). Additionally, Myc works in an independent pathway by inducing the E2F transcription factors, which promotes G_1 to S phase progression (Leone et al., 2001).

2.6.2 Protein synthesis and cell growth

The accelerated cell entry into S-phase by Myc creates the need for increased protein synthesis, which is achieved by Myc overexpression (Fernandez et al., 2003). Induction of ribosomal genes by Myc promotes ribosome biogenesis and protein synthesis (Mateyak et al., 1997) and a number of RNA polymerase I and III associated genes for rRNA, tRNA and TFIIIB have recently been identified as direct Myc-binding targets (Gomez-Roman et al., 2003; Grandori et al., 2005). Interestingly, Myc overexpression is also associated with larger cell size both in drosophila and vertebrates (Dang et al., 2006) and although the mechanism of growth regulation is not clear, a number of potential Myc target genes regulating cell growth have been suggested, including the mRNA cap binding proteins eIF4E and 2α, RNA helicases, and the iron regulatory protein-2, IRP2, reviewed in (Oster et al., 2002).

The neoplastic transformation of cells by Myc may be promoted by down-regulation of genes encoding cell adhesion and cytoskeleton, resulting in changes in the cellular morphology and growth in an anchorage independent manner. Cell lines of human small cell lung cancer show Myc-related repression of α3β1 resulting in anchorage independent growth (Barr et al., 1998) and cytoskeletal proteins such as actin have been identified as Myc target genes, suggesting a role in fibroblast mobility.

2.6.3 Metabolism

A wide range of genes involved in cell metabolism have been identified as Myc targets, including several key enzymes in glucose and iron metabolism. Increased c-Myc expression results in enhanced glucose uptake and glycolysis by targeting e.g. enolase A, hexokinase II, lactate dehydrogenase and phosphofructokinase. Another effects of Myc upregulation is increased intracellular iron content through the iron transporter proteins TFRC1 and ferritin and iron regulator IRP1 and IRP2 (Dang et al., 2006). Nucleotide synthesis and DNA repair occurs also to be affected by c-Myc through a

number of different enzymes (Bello-Fernandez et al., 1993; Miltenberger et al., 1995; Shiio et al., 2002).

2.6.4 Differentiation

Cell differentiation, the process by which cells turn into a specific "type", is largely affected by the Myc family members. Suppression of Myc expression is essential for exit of the cell cycle and full differentiation (Chang et al., 2000a). This can be mediated by the "master regulon" of the specific cell type, e.g. C/EBPα can form a complex with e2f/pRb and thereby repress *c-myc* through the E2F binding site in the *c-myc* proximal promoter region (Johansen et al., 2001). Ectopic expression of *c-myc* blocks differentiation in a wide variety of cell types (Brewer, 2000; Facchini and Penn, 1998) leading to growth arrest and apoptosis in both normal and leukemic myeloid cells (Hoffman et al., 2002).

2.6.5 Apoptosis

The well known hallmarks of Myc as a main actor in cell cycle regulation and proliferation are in sharp contrast to the more recent finding that Myc also induces apoptosis (Askew et al., 1991). Even more bewildering is the fact that the apoptosis mechanism of Myc has common features with transformation in requiring MBII and DNA binding through dimerization with Max (Evan et al., 1992). A possible distinction between the two mechanisms is that the apoptosis function of Myc seems to be regulated mainly through repression of target genes while cell growth and proliferation are usually targeted through transactivation activity (Conzen et al., 2000; Oster et al., 2003). One important target for Myc-mediated apoptosis by repression is the transcription factor Miz-1, which is essential for cell cycle arrest by activating the cell cycle inhibitor p21 (Seoane et al., 2002). Repression of Miz-1 also inactivates transcription of the anti-apoptotic Bcl2 protein, which is suggested to be essential for apoptosis induction (Patel and McMahon, 2006).

The apoptosis mechanism of Myc can be both p53-dependant and –independant. The p53 tumor suppressor acts on cellular damage and steers the cells towards growth arrest allowing cellular repair or apoptosis. p53-dependant Myc-induced apoptosis is well characterized and it involves activation of the tumor suppressor ARF by Myc, leading to inactivation of the p53 regulator MDM2 and activation of p53, (reviewed in Meyer

et al., 2006). A substantial number of p53 mutations have been found in Burkitt lymphoma cell lines, indicating that the inhibition of the ARF-Mdm2-p53 pathway may be necessary for tumorogenesis.

A key player in p53-independent Myc-induced apoptosis is the Bcl2 family, which ultimately control the decision on mitochondrial cell death. Myc affects the balance between the pro- and anti-apoptotic members of the Bcl2 family by e.g. indirect suppression of the anti-apoptotic Bcl2 and Bcl-X_L (Eischen et al., 2001) or by regulating oligomerization of the pro-apoptotic BAX (Annis et al., 2005), which is required for cytochrome mediated apoptosis.

2.6.6 Transcription

The transactivation activity of Myc is modest with recent results showing around 2-fold activation of a large number of target genes (Patel et al., 2004). Addition of the transcription factor TFIID related TATA-box binding protein can however increase the transactivation potential significantly, up to 35-fold (Barrett et al., 2005). The mechanism for Myc transactivation is a complex biochemical event but seems to be relatively conserved among different species and cell types. Transcription is driven by recruitment of nuclear co-factors to target gene promoters by the Myc N-terminal transactivation domain, originally mapped to residue 1-143 (Kato et al., 1990), while the C-terminal of Myc forms a complex with Max and binds to the E-box DNA sequence . Alternatively, some proteins interact directly with the C-terminal domain, often resulting in transcriptional repression. Identification of c-Myc interacting proteins has mainly been done by yeast two-hybrid screens and the number of proteins interacting with c-Myc TAD is constantly rising. This thesis will only present a selection of these proteins, focusing on the more carefully characterized targets interacting with c-Myc TAD (figure 15).

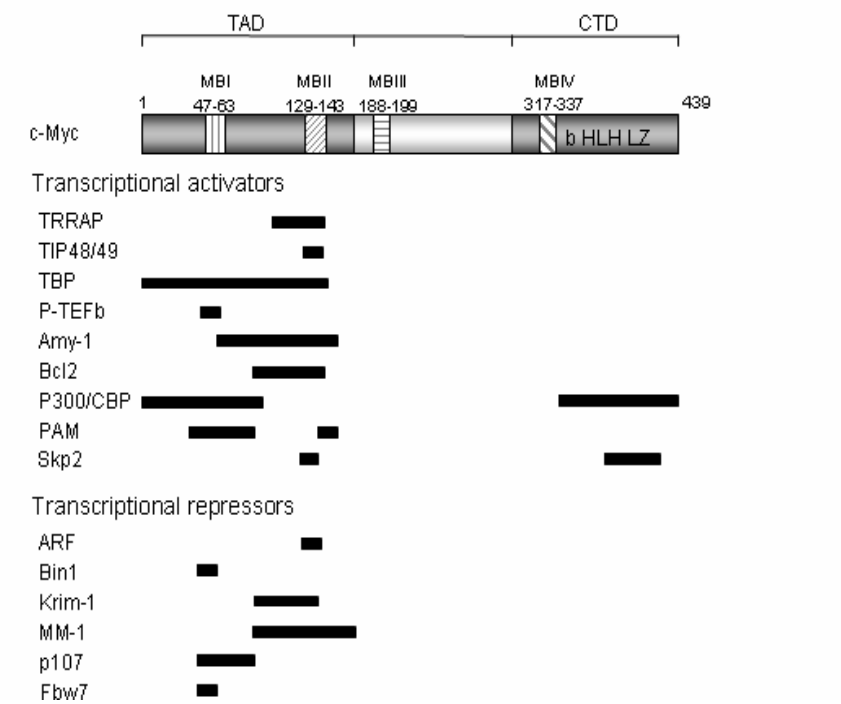


Figure 15. Proteins interacting with Myc and their located binding sites.

2.6.6.1 Transcriptional activation

Activation of many Myc target genes occurs through increased histone acetylation by histone acetyltransferase (HAT) complexes. This is thought to induce an open chromatin structure that increases the accessibility of the DNA for the transcriptional machinery. Myc not only controls histone modification at certain target genes but impact the genome wide levels of histone H3 and H4 modifications (Knoepfler et al., 2006). Such global chromatin changes may explain the large range of biological processes affected by Myc. However, activation of several Myc target genes occurs independently of HAT activity (Eberhardy et al., 2000), which may be mediated by e.g. HAT-independent TRRAP complexes. Another mechanism for HAT-independent activation by Myc is through stimulation of RNA polymerase II promoter clearance and elongation, involving recruitment of mediators such as P-TEFb, TFIIH or RNA polymerase II kinases (Eberhardy and Farnham, 2001; Eberhardy and Farnham, 2002).

2.6.6.1.1 TRRAP

The TRansactivation/tRansformation Associated Protein (TRRAP; also called PAF400) is a highly conserved, 3859 residues, nuclear protein belonging to the

phosphatidylinositol 3-kinase-related protein kinase family (PIKK) (Jiang et al., 2006). The C-terminal of PIKK proteins contains a conserved FAT/kinase domain/FATC region, which in TRRAP is found at residue 2704-3275, 3528-3826 and 3827-3859. The exact function of the FAT and FATC domains of PIKK proteins is unknown but FATC seems to mediate the interaction between ATM and TIP60 (Jiang et al., 2006). Although TRRAP have a C-terminal 300 residue kinase-like domain, which displays large similarities to the P13 kinase/ATM family, critical active site residues required for kinase activity are not conserved and TRRAP has no kinase activity. TRRAP was first identified by copurification with c-Myc TAD and three regions in TRRAP have been implicated in c-Myc interaction, aa 1261-1579, 1899-2026 and 3402-3828 (McMahon et al., 1998). An extended c-Myc region, including MBII and a more N-terminal stretch, is required for binding to TRRAP (McMahon et al., 1998; Park et al., 2001).

Regulation of transcription by TRRAP is performed by recruitment of HAT activity to an activator bound promoter. The C-terminal region of TRRAP containing the ATM and FATC region together with a well conserved part of the ATM region (3692-3713) are both necessary for this recruitment. TRRAP functions as a subunit of two diverse histone acetyltransferase (HAT) complexes, the GCN5-type (TBP- free TAF containing complex (TFTC), STAGA or GCN5/PCAF) or TIP60/Esa1 type (reviewed in Carrozza et al., 2003). Additionally, TRRAP can also form complexes with several NuA4 subunits such as p400, TIP48 and TIP49. Interaction with the GCN5 complex Ada requires MBII but SAGA, SWI/SNF and NuA4 all need larger c-Myc fragment for interaction. The function of the HAT complexes is not limited to transcriptional activation but can also mediate repression of gene expression or contribute to chromatin modification involved in DNA repair (Peterson and Cote, 2004).

Functionally, TRRAP is essential for early development and cell cycle progression (Herceg et al., 2001) but has no effect on Myc mediated apoptosis (Dugan et al., 2002). Recruitment of TRRAP is necessary for expression of some genes, like the telomerase reverse transcriptase gene (TERT), but is dispensable for several other basally expressed genes in exponentially growing fibroblasts (Nikiforov et al., 2002). The levels of TRRAP in the cell are rate limiting for Myc-dependent transformation (McMahon et al., 1998) implicating a key role in tumor formation.

2.6.6.1.2 TIP48 and TIP49

TIP48 and TIP49 are ATPase/helicase like proteins, which contain the Walker A and B motif found in proteins that bind and hydrolyse ATP (Wood et al., 2000). Both TIP48 and TIP49 can be found in TRRAP complexes but they can also interact independently with c-Myc TAD and MBII and W135 are both essential for binding. *c-myc* knockout cells display a 3-4 fold reduction in TIP48 and TIP49 mRNAs, leading to the hypothesis that TIP48 and TIP49 are dependent on Myc. TIP49 alone was also shown to be required for Myc dependent transformation even though non-functional TIP49 did not inhibit normal cell growth (Wood et al., 2000). Both TIP48 and TIP49 are highly conserved in evolution and their ability to interact with Myc is conserved in drosophila (dMyc) where they seem to be important for growth and proliferation (Bellosta et al., 2005). Recruitment of TIP48 and TIP48 via the HAT complex TIP60 to chromatin is also mediated by Myc, which contributes to histone acetylation in response to mitogenic signals (Frank et al., 2003).

2.6.6.1.3 General transcription factors

General transcription factors (GTF) are involved in the formation of the transcriptional initiation complex and recruit the RNA polymerases. The GST TATA-box binding protein (TBP) is a key factor in the recruitment of all three RNA polymerases because of its ability to recognize the eukaryotic core promoter motif, the TATA-box sequence. Structurally, TBP contains a highly conserved pseudosymmetric core consisting of mixed αβ-motif with an N-terminal unconserved extension that differs significantly, both in sequence and in length, between human and yeast and is truncated in A. thaliana. The core of TBP forms a saddle- like motif where a number of conserved amino acids in the concave surface primarily interacts with the TATA-element by bending the DNA (Juo et al., 1996; Nikolov and Burley, 1994) while the convex surface and the two adjacent stirrup loops are targets for interaction with other GTSs (Juo et al., 2003; Nikolov et al., 1995; Tan et al., 1996) such as TFIIF (McEwan et al., 1996) and TFIIIB related Brf1 (Gomez-Roman et al., 2003). The convex surface of TBP is also involved in interaction with a number of trans-activation/repression domains such as Vp16 (Dion and Coulombe, 2003), the Mediator head module (Lariviere et al., 2006), p53 (Crighton et al., 2003), and NC2 (Kamada et al., 2001). TBP binds c-Myc TAD both *in vivo* and *in vitro* (Hateboer et al., 1993; Maheswaran et

al., 1994) and increases transcription in a dose-dependent manner (McEwan et al., 1996).

2.6.6.1.4 P-TEFb

P-TEFb, the positive transcription elongation factor b, is a cyclin-dependent kinase complex consisting of a cyclin (cyclin T1, cyclin T2a, cyclin T2b or cyclin K), cdk9 and 7SK small nuclear RNA. Myc recruits P-TEFb to target genes with stalled RNA polymerase II in order to activate transcriptional elongation by promoting phosphorylation of the RNA polymerase II C-terminal domain (Eberhardy and Farnham, 2001). Cyclin T1 interacts with c-Myc TAD near or at MBI and the N-terminal 70 residues of c-Myc TAD is sufficient to activate transcription (Eberhardy and Farnham, 2002).

2.6.6.1.5 Amy-1

The 11 kDa Amy-1 protein (associated of Myc-1) is normally localized in the cell cytoplasm but it is translocated to the nucleus in response to elevated c-Myc levels during early S-phase (Taira et al., 1998). Translocation of Amy-1 is dependent on c-Myc TAD interaction since mutants lacking the C-terminal interacting region in Amy-1 were not subjected to nuclear translocation upon elevated c-Myc levels. c-Myc/Amy-1 interaction required MBII and its N-proximal flanking residues (aa 58-148) in c-Myc TAD and co-transfection of this region with Amy-1 resulted in E-box dependant transcriptional activation. Nevertheless, Amy-1 appears also to display c-Myc independent activities through protein-protein interaction in the mitochondria and *trans*-Goli network (Furusawa et al., 2001; Ishizaki et al., 2006).

2.6.6.1.6 Bcl2

The Bcl2 protein is deeply involved in oncogenesis by functioning as an anti-apoptotic agent, extending cell survival, and promoting tumor development through enhancement of DNA damage and attenuation of DNA repair. Recent results show that Bcl2 colocalizes with c-Myc in the nucleus, enhances c-Myc transcriptional activity and inhibits DNA repair. Cells deficient in c-Myc have accelerated DNA repair although Bcl2 is overexpressed, suggesting c-Myc to be essential for Bcl2 mediated DNA repair inhibition. Interaction between the BH4 domain of Bcl2 and c-Myc TAD (aa 106-143) is necessary for these effects (Jin et al., 2006).

2.6.6.1.7 CBP/p300

The cAMP- responsive element binding protein (CBP) and p300 are two highly homologous proteins, which function as mediators of transcription for many transcription factors due to their intrinsic HAT activity. Acetylation of transcription factors by CBP and p300, targeting e.g. p53 and E2F, controls transcriptional activation, tumor suppression and DNA binding properties (Rajabi et al., 2005). Mutations in CBP or p300 has been found in several cancers (Goodman and Smolik, 2000) and loss of either protein leads to embryonic death due to developmental abnormalities, suggesting a central role in proliferation and development (Yao et al., 1998). Despite their extensive similarity, binding of CBP/p300 to c-Myc appears to occur to different regions, CBP targeting the C-terminal DNA binding domain of c-Myc while p300 binds to c-Myc TAD residue 1-110 (Faiola et al., 2005; Vervoorts et al., 2003). Indeed, CBP and p300 are both essential for preventing cells from entering S-phase by repressing *c-myc* (Rajabi et al., 2005) and independently activating transcription and acetylating c-Myc at specific lysine residues, resulting in reduced c-Myc ubiquitination and protein turnover (Faiola et al., 2005; Vervoorts et al., 2003).

Noteworthy, mapping of residues in the cAMP response element binding protein (CREB) targeted by CBP binding suggested sequence similarities between the binding region in CREB and c-Myc TAD, possibly revealing a common mechanism of interaction (figure 16).

Figure 16. Alignment of the CBP interacting region of CREB and c-Myc TAD. Black boxes indicate strict identity, bold characters show similarity within a group of amino acids and a grey box displays similarity across different group of amino acids. Stars indicate residues in CREB involved in interaction with CPB.

2.6.6.1.8 PAM

PAM, protein associated with Myc, was first identified in a GST-Myc pulldown assay as a 510 kDa multidomain protein that contains several different domains; a ring-zinc finger motif, two zinc-finger motif, a NLS, two leucine zipper regions and a regulator of chromatin condensation RCC1-like motif. The RCC1-like motif consists of a 7-fold

repeat of a 50-60 amino acid motif, which is divided into two regions by a 134 aa insertion and suggests a function in DNA and/or chromatin binding (Guo et al., 1998). PAM is expressed in many tissues with the highest levels found in brain and thymus and can interact with c-Myc *in vivo*. The region of interaction in c-Myc was located between residue 44-107, containing MBI, and residue 140-154.

2.6.6.2 Transcriptional repression

In addition to transcriptional activation of target genes, Myc has been associated with down regulation of gene activity. Transcriptional repression affects a wide variety of target genes, including C/EBP-α, which is required for adipogenesis by Myc, p21Cip1, which arrest cells in the G1 phase and several other genes affecting cell adhesion and angiogenesis. The mechanism for Myc-dependant transcriptional repression is not so well characterized but has been suggested to occur either through recruitment of Myc to DNA by target proteins or through direct interaction of the Myc/Max complex with core promoters. A more precise mechanism was recently suggested by (Brenner et al., 2005) in which Myc recruits the DNA metyltransferase Dnmt3a to Miz-1 bound target sites, resulting in local histone deacetylation and concurrent repression of transcription.

2.6.6.2.1 ARF

The tumor suppressor ARF (p19ARF in mouse and p14ARF in humans) is stimulated by oncogenes resulting in inhibition of cell proliferation (Sherr, 1998). Association and colocalization of ARF and Myc in the nucleoplasm has been detected *in vivo* and the binding to c-Myc TAD requires MBII (Amente et al., 2006). Myc-ARF binding inhibits Myc-dependent transactivation, leading to reduced mRNA levels of several Myc target genes (Datta et al., 2004). Induction of ARF by Myc upregulates p53, leading to inhibition of Myc-dependent oncogenesis by cell cycle arrest or induction of apoptosis. ARF expression was also shown to block c-Myc-induced S-phase progression independently of p53 (Datta et al., 2004).

2.6.6.2.2 Bin1

The nucleocytoplastic bridging intergrator 1 protein (Bin1) is a tumor suppressor that has two ubiquitously expressed splice variants in normal cells while other splice forms such as 12A are expressed in transforming cells only. Transforming cells are often devoid of Bin1 or have a dysfunctional Bin1 splice variant but reintroduction of wt

Bin1 can reduce cell growth, induce differentiation and trigger tumor cells to undergo apoptosis (Ge et al., 1999; Ge et al., 2000). Structurally, Bin1 consists of an N-terminal BAR domain, a central domain and a C-terminal SRC homology 3 domain (SH3). The central domain of Bin1 was earlier proposed as the region for interaction with c-Myc MBI primarily while MBII was needed for full binding and inhibition of cell transformation (Sakamuro et al., 1996). However, recent structural data indicate that c-Myc TAD does not interact with the central domain of Bin1, but with the C-terminal SH3 domain and a SH3-binding motif was identified in c-Myc MBI (Pineda-Lucena et al., 2005). Intramolecular interactions between the SH3 domain and the tumor specific Bin1 exon 12A hinders Bin1 binding to c-Myc and may function as a regulatory mechanism for abolishing the suppressor function of Bin1 in tumor cells.

2.6.6.2.3 Krim-1

Another protein that represses c-Myc mediated transcription is the Krim-1 protein (Krab box proteins interacting with Myc 1), which contains an N-terminal KRAB box domain, known for having transcriptional repressor activity, and 12 C-terminal zinc-fingers (Hennemann et al., 2003). The KRAB box in Krim-1 has a general repression effect on E-box dependant transcription, which is Myc independent but Krim-1 could also repress the transactivation function and transforming ability of c-Myc. Protein-protein interaction with c-Myc TAD requires the second N-terminal zinc finger in Krim-1 and concomitantly, MBII with an adjacent N-terminal region in c-Myc TAD (aa 104-143) is essential for the interaction. Furthermore, the core repressor Tif-1β interacts and stabilizes Krim-1, suggesting a possible repression mechanism involving recruitment of histone methyltransferases and histone deacetyltransferases to c-Myc occupied chromatin.

2.6.6.2.4 MM-1

The Myc modulator -1 (MM-1) was discovered through a yeast two-hybrid screen of a human HeLa cell cDNA library and was found to be a 167 residue nuclear protein that interacts *in vitro* and *in vivo* with a c-Myc region (104-166) containing MBII (Mori et al., 1998). Most of MM-1 is required for interaction with c-Myc TAD but structural characterization by NMR suggests a mainly random coil conformation although the N-terminus of MM-1 contains a putative leucine zipper and displays helical properties by CD (Prof G Otting, personal communication). Coexpression of MM-1 and c-Myc

suggests that MM-1 transcriptionally represses c-Myc, most likely throughout the entire cell cycle except for early G1 phase (Fujioka et al., 2001; Mori et al., 1998; Satou et al., 2001). The repression mechanism by MM-1 seems to be mediated by interaction with TIF1β/KAP1, which activates a transcriptional repressor complex composed of e.g. HDAC and mSin3A (Satou et al., 2001), associated with histone deacetylase activity. The finding of an point mutation in MM-1 (Ala157Arg) that is frequent in lymphoma, leukemia and tongue cancer cells and abrogates the inhibitory effect of MM-1 on c-Myc, suggested that MM-1 function as a tumor suppressor (Fujioka et al., 2001).

2.6.6.2.5 p107

The retinoblastoma family of tumor suppressor proteins mediates E2F transactivation and can inhibit the proliferation of certain cell types by arresting the cells in G1. The pRB protein was early on suggested to interact with c-Myc TAD (Rustgi et al., 1991) but later studies confirmed that it was the pRB- related p107 protein that mediated binding (Beijersbergen et al., 1994; Gu et al., 1994). The conserved pocket region in p107, which mediates interaction with viral oncoproteins and E2F transcription factors, interacts with an intermediate TAD region (41-103) and suppresses transactivation. Additionally, p107 mediates *c-myc* repression by TGFβ through binding to the Smad3-E2F4/5 complex (Chen et al., 2002).

2.6.6.3 Phosphorylation and transcriptional activation

The stimulating effect of c-Myc TAD phosphorylation on transactivation is widely studied and S62 and T58 mutants display modest reduction in transactivation in several different studies (reviewed in Hann, 2006). Phosphorylation of S62 is required for regulation of several Myc target genes involved in cell cycle progression and tumorogenesis such as regulation of hTERT and γ-GCS promoter activity while T58 phosphorylation regulates the c-Myc target gene *Bim,* a pro-apoptotic Bcl2 family member (Hemann et al., 2005). Phosphorylation has also been shown to prevent interaction between c-Myc TAD and target proteins as seen for Bin1, where binding to c-Myc TAD was prevented by S62 phosphorylation. The effect of the phosphoryaltion on binding can be explained by electrostatic repulsion between the phosphor group and the acidic binding pocket of the SH3 domain of Bin1 (Pineda-Lucena et al., 2005). Furthermore, the c-Myc TAD *in vivo* interaction with α-tubulin was disrupted by hyperphosphorylation (Niklinski et al., 2000).

2.7 MYC ACTIVATION IN HUMAN CANCER

Deregulated expression of any of the three Myc oncogenes leads to uncontrolled proliferation upon alterations in the apoptotic pathway and an increased risk of acquiring mutations leading to tumorogenesis. Myc overexpression has been found in up to 50 % of all human cancers (Alitalo and Schwab, 1986) and is the result of point mutations, chromosome translocation, gene amplification, enhanced translation or increased protein stability. The most common alteration in solid tumors is amplification and it has been found in a wide range of tumor types such as lung cancer, breast cancer, cervix cancer, hepatocellular carcinoma, melanoma and prostate cancer (Vita and Henriksson, 2006). Burkitt's lymphoma is probably the most well known Myc related tumor disease and it has a 100 % incidence of chromosomal translocation that places c-Myc in the proximity of an immunoglobulin enhancer that promotes constitutive high c-Myc mRNA and protein levels (Boxer and Dang, 2001). However, several other tumor forms are also subjected to rearrangements such as multiple myeloma and B-acute lymphocytic leukaemia (Vita and Henriksson, 2006). Mutations within the c-Myc TAD is found in a large number of cancer forms, especially in Burkitt's lymphoma, and they frequently occur at sites for phosphorylation (Bhatia et al., 1993). Common features for these Myc mutants are increased protein stability as well as enhanced transforming ability. Three cancer associated mutants (A21, A44V and T58A) were found to have similar ubiquitination patterns and the same extent of activation by UB-ligase Skp2 as wt despite their increase stability, resulting in an active stabilized form of Myc, which can perform extended target gene stimulation (Kim et al., 2003).

2.7.1 Myc as a therapeutic target in cancer

Several studies of transgenic models using either conditional regulation of Myc transcription via a tetracycline regulatory system or post-transcriptional regulation of Myc by the Tamoxifen system show that inactivation of Myc commonly results in tumor regression by inducing proliferative arrest, differentiation and apoptosis, making Myc induced tumorogenesis reversible (reviewed in Arvanitis and Felsher, 2006). The final outcome of Myc inactivation depends largely on tumor type and the genetic context of the tumor, some tumors experience sustained tumor regression (Jain et al., 2002) while others differentiate into normal cells (Shachaf et al., 2004). A drawback of differentiation into normal cells is that some of these cells still are dormant cancer stem cells, which regain their neoplastic properties upon Myc reactivation. A few tumors

have also been found to be able to escape from Myc dependence by e.g. new chromosomal translocations (Karlsson et al., 2003).

Even though Myc seems to be an ideal target for tumor treatment, no drugs are yet approved for treatment of human cancers. Several different strategies for targeting Myc are currently investigated and a few potential drugs have been tested in animal models and one has reached phase I clinical trials (Ponzielli et al., 2005). Inhibition of Myc/Max interaction has been targeted by a mutant protein that interferes with Myc binding to the E-box or peptides/small molecules disrupting Myc heterdimerization. Another approach aims at downregulating Myc expression by using antisense oligonucleotides that binds to mRNA, RNA interference, antioxidant treatment or oligonucleotides, which hinders transcription by blocking the promoter region, alternatively G quadruplex formation. The most unique region of Myc is the transactivation domain, harbouring the well conserved MBI and MBII segment, which makes it a potential drug target for anti-cancer therapy but few strategies aiming at Myc transcriptional activation has so far been investigated. Targeting the c-Myc TAD includes dealing with the frequent mutations found in the TAD region. Myc mutants have however been shown to primarily affect oncogenic and apoptotic activities, not regulation of target genes, suggesting that the interactions required for transcription is not effected by such small sequence variations (Chang et al., 2000b; Kuttler et al., 2001). Especially, the essentiality of MBII for transformation but not proliferation makes it a suitable target for creating a tumor specific drug that would not affect normal healthy cells (Nikiforov et al., 2002; Oster et al., 2003). Another possibility is to disrupt interactions between Myc and transcriptional activators such as TRRAP. Reviewed in (Ponzielli et al., 2005)

2.7.2 Open issues in the Myc field

Myc is involved in a vast number of different protein-protein interactions, both structurally ordered systems like DNA binding to Max by the bHLH-Zip motif, but also by binding of transcriptional regulators to the partly disordered c-Myc TAD. Very little is known about this kind of interactions in Myc TAD and so far only one structure of a Myc TAD complex is known, namely between the SH3 domain of the tumor suppressor Bin1 and c-Myc MBI (Pineda-Lucena et al., 2005). Another crucial issue is the formation of Myc associated multiprotein complexes such as the TIP48/TIP49/HAT

complex since little is know about the assembly and specific action of these large molecules. It is also surprising that strictly conserved regions appear in the TAD outside the proposed regions targeted by hotspot mutations, such as the linker region between MBI and MBII.

The problem with studying multidomain proteins such as Myc is a well known issue in structural biology. Many high resolution structures involve prefolded regions while in Myc, it is evident that folding-on-binding must be the case.

Additionally, the mechanism for transactivation is still a mystery. Simple charge interactions are far too limited to explain the diversity of transactivation in real systems, in terms of structure and specificity. c-Myc is a particularly complicated target since it regulates a wide variety of target genes. The complexity is further empathised by various regulatory proteins and the lack of available three-dimensional structure.

3 PRESENT INVESTIGATION

3.1 AIM OF THE STUDY

The current study aims at characterizing proteins using biophysical and structural methods. The accumulated knowledge from several different biophysical techniques can aid in shedding light on protein functionalities even when full structural determination is prevented by practical problems concerning protein production, solubility or concentration.

According to their active sites, the glutaredoxins can be divided into two subfamilies: the well characterized dithiols and the less known monothiols. Despite the wealth of information regarding glutaredoxins employing a dithiol mechanism for the reduction of their substrates, little is known about monothiol glutaredoxins. No structural information is available and biochemical analysis of the protein family has so far not been able to reveal any specific substrate. A molecular in-depth analysis of the essential Grx4 in *E. coli* will facilitate functional understanding of monothiol Grx homologues in a wide range of both pro- and eukaryotic species, including humans.

Numerous studies concerning the Myc/Max network have been published since the discovery of the cellular homologue to v-Myc, c-Myc in 1982 (Vennstrom et al., 1982) but the relatively few structural investigations pursued have mostly concerned the well conserved DNA binding regions in these proteins. The function of the transactivation domain in c-Myc and the N- and C-terminal flanking regions of the DNA binding motif in Max have so far not been able to be interpreted on the basis of their static 3D structures. The analysis of such flexible regions, protein intermediates or molten globule is however relevant for a number of biophysical and biological aspects of these proteins and, in particular, for re-assessing the protein structure–function paradigm in the current quest for drugs against Myc induced cancer.

3.2 METHODOLOGY

3.2.1 Limited proteolysis

Limited proteolysis utilizes proteases that bind to protein regions, which are complementary to their active site and induce cleavage of the peptide chain. Native globular proteins are often resistant to proteolysis due to their rigid conformation and the techniques of limited proteolysis take advantage of this fact in order to probe conformational features of proteins. The cleavage sites of limited proteolysis along the polypeptide chain of a protein are characterized by enhanced backbone flexibility and limited proteolysis is therefore useful for identifying protein fragments that can fold autonomously and thus behave as protein domains. Protein fragments identified by limited proteolysis are often native-like and are thus likely able to functionally interact in protein complex. Furthermore, limited proteolysis can be used to probe the structural and dynamic differences between holo and apo forms of a protein, investigate ligand binding sites or compare wt/mutant proteins. Thermolysin, subtilisin, proteinase K, and pepsin display broad substrate binding, and are therefore useful for studying well folded proteins while proteases with more narrow specificity such as trypsin, can in some cases be more suitable for molecules with intrinsic flexibility or molten globules, which show large conformational flexibility. (Carey, 2000)

3.2.2 Circular dichroism

Circular dichroism (CD) spectroscopy uses the differences in the absorption of left/ right-handed polarized light to estimate the amount of structure in a peptide or protein. The absorption differences arise due to chirality of a chromophore that is caused by 1) a chiral atomic structure (e.g. the dihedral angel of a C-S-S-C bond, 2) a covalent link to a chiral centre or 3) structural asymmetric surroundings due to the 3-dimensional structure of the protein. The chromophores of most interest in a protein are peptide bonds, which are used to study the secondary structure (absorption below 240 nm), and aromatic amino acid side chains, which provide a fingerprint of the tertiary structure (absorption between 260-320 nm). Ligand binding and affinity can also be investigated by CD and is especially useful for studying the interaction that induces structural changes in a protein. CD is a quantitative but low resolution structural technique that can not give information at atomic level of resolution, but the sample requirements and time consumption is very low compared to more advanced techniques such as NMR and X-ray crystallography. CD has in this study been used for measurements of thermal

stability, investigation of secondary and tertiary structure, assessment of conformational alterations upon ligand binding and estimation of ligand binding affinities. (Greenfield, 2004; Kelly et al., 2005)

3.2.3 Fluorescence

Fluorescence is a phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light of longer wavelengths. Protein molecules contain three aromatic amino acids, which contribute to their intrinsic fluorescence; tryptophane, tyrosine and phenylalanine. All three residues have distinct absorption and emission wavelengths but the fluorescence spectrum of a protein still usually resembles that of tryptophane due to the high fluorescence and quantum yield of tryptophanes and resonance energy transfer from phenylalanine to tyrosine and from tyrosine to tryptophane. The wavelength of maximum fluorescence emission of tryptophane is very solvent dependent, leading to a shift in the fluorescence spectrum to shorter wavelength and increased intensity of the fluorescence as the polarity of the solvent surrounding the tryptophane residue decreases. Intrinsic fluorescence is commonly used to study protein structure, folding dynamics and molecular interactions. Technically, intrinsic fluorescence is non-invasive and highly sensitive (less than 10^{-8} mole can be detected) but specific attention should be paid to achieve a stable temperature since small changes can cause big alterations in the measurements. c-Myc TAD is a suitable target for measurements of intrinsic fluorescence since it contains two tryptophanes, one in the N- and C-terminal half of the protein, respectively.

The molten globule characteristics of c-Myc TAD were investigated by ANS (8-anilino-1-naphthalenesulfonate)-fluorescence, which is particularly strong for ANS-binding to hydrophobic exposed cores in partially folded intermediate such as a molten globule. The ANS-binding event itself may induce protein conformational changes, making it necessary to correlate ANS fluorescence measurements with data obtained using other biophysical techniques.

3.2.4 Biacore

Surface plasmon resonance (SPR) occurs when light is reflected off thin metal films. A fraction of the light energy incident at a sharply defined angle and can interact with the

delocalised electrons in the metal film (plasmon) thus reducing the reflected light intensity. In the equipment developed by Biacore, target molecules are immobilised on a gold film and binding to the immobilised target induce changes in the local refractive index on the backside of the film, leading to a change in SPR angle, thereby producing a sensograms. The SPR signal can be analysed and fitted to mathematical models to yield apparent rate constants and affinities for protein or a DNA binding to small molecules, peptides or other proteins. Further experimental setups are e.g. screening for binding partners, concentration determination and monitoring multimolecular complex formation. Advantages with the technique is that only small quantities of material (sub-μg) are needed to yield detectable signals, both strong and weak interactions can be studied and a wide range of methods are available for immobilization. The amine coupling immobilization technique used for c-Myc TAD was carefully evaluated and a number of control experiments using histidine antibody for immobilization was used to verify that the coupling of c-Myc TAD to the CM5 chip had minimum impact on binding. Addition of the p20 detergent (Biacore AB) to the running buffer was shown to minimize unspecific binding but had no effect on the affinity to c-Myc target proteins.

3.2.5 MALDI

Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry, MALDI-TOF, operate on the principle that when a temporally and specially well defined group of ions of differing mass/charge (m/z) ratios are subjected to the same applied electric field and allowed to drift in a region of constant electric field, they will traverse this region in a time, which depends upon their m/z ratios. Improved mass resolution in MALDI can be obtained by utilizing a single- or dual-stage reflectron, which compensate for the difference in flight times of the same m/z ions of slightly different kinetic energies by focusing the ion packets in space and time at the detector. MALDI is also a "soft" ionisation method and results predominantly in the generation of singly charged molecular-related ions regardless of the molecular mass, therefore the spectra are relatively easy to interpret. Fragmentation of the sample ions does not usually occur. A MALDI sample is embedded in a chemical matrix, which consist of crystallised molecules that greatly facilitate the production of intact gas-phase ions from compounds such as proteins, oligonucleotides, and synthetic polymers. The matrix plays a key role in this technique by absorbing the laser light energy and causing

a small part of the target substrate to vaporize. Important considerations when using MADLI are to have a sample free of contaminants such as strong ionic detergent or high salt and to use a suitable matrix material at the right concentration ratio relatively to the analyte. The use of MALDI (often in conjunction with limited proteolysis) can provide information about purity of a sample, blocked amino termini, post-translational modifications, mutation and binding sites in known proteins of high molecular mass (most instruments can measure masses to within 0.01% of the molecular mass of a sample up to around 40,000 Da). Glutathionylation and oligomerization properties of intact Grx4 was investigated using MALDI but more precise investigation of the glutathionylation of specific residues by analysis of samples subjected to limited proteolysis could not be performed.

3.2.6 NMR

When the nuclei of atoms with unpaired nuclear spin are immersed in a static magnetic field and exposed to a second oscillating magnetic field a phenomenon named Nuclear magnetic resonance, NMR, occurs. The use of pulses of different shapes, frequencies and durations allows extraction of many different types of information about the molecule such as determining the structure of small molecules and proteins, investigating molecular dynamics, diffusion coefficients and monitoring ligand binding. The most commonly used atoms in protein NMR are ^{1}H, ^{13}C and ^{15}N and these are incorporated in the protein by either addition of deuterated solvent or isotopical labelling during recombinant protein production. Common problems with sample preparation are protein solubility (usually at least 1 mM samples are necessary for structure determination) and the need for extensive conformational stability due to long data recording times. A typical first experiment when investigating a protein is the ^{15}N-2D heteronuclear single quantum correlation spectrum, ^{15}N-HSQC, that in theory has one peak for each amino acid along with some for N-containing side chains. The spectrum occurs like a fingerprint of the protein and aids in investigating the number of conformations, the degree of folding and possible sample heterogeneity. The basic process of structure determination by NMR was developed in the late 1980s, and includes distinct steps of data collection, data processing, peak picking and editing, resonance assignment, structural parameter assignment and calibration, structure calculation and, finally, structure validation (Clore and Gronenborn, 1991; Wüthrich, 1986). Recent methodological advances, such as TROSY, specific magnetic labelling

schemes, and the use of residual dipolar couplings, has made it possible to study increasingly larger proteins (Tugarinov et al., 2004). To achieve higher efficiency and throughput in structure determination, a range of automated and semi-automated strategies have been developed, both in terms of assignment and structure calculations (Altieri and Byrd, 2004). Together, this has put NMR into the position as a successful and competitive technique for structure determination of proteins in current Structural Genomics efforts (Yee et al., 2006). The current investigation uses NMR for structural determination of Grx4 and investigation of peptide binding to c-Myc TAD. Additionally, HSQC spectra of Max were used to study the structural content of different Max proteins.

3.3 RESULTS AND DISCUSSION

3.3.1 Paper I

Biophysical properties of regions flanking the bHLH-Zip motif in the p22 Max protein.

The functional relevance of the N- and C-terminal regions flanking the bHLH-Zip of Max has been pointed out in several different studies concerning DNA binding, transcriptional activation and transformation. The structural information regarding these regions is however minor since apparent crystal structures mainly include the bHLH-Zip motif of Max or display no electron density for the flanking regions (Brownlie et al., 1997; Ferre-D'Amare et al., 1993; Nair and Burley, 2003). Identification of structured entities affected by DNA binding were performed by limited proteolysis experiments, showing that deletion of a 3 kDa fragment from the C-terminus of Max was not affected by DNA binding and formed a stable fragment, Max_{1-132}. Additional cleavage of Max_{1-132}, containing the b-HLH-Zip region and 50 additional flanking residues, in the presence of DNA gave rise to two stable fragments, Max_{18-106} (corresponding to the structured region in the p21 crystal structure (Brownlie et al., 1997)) and Max_{1-106}. All these fragments, including Max_{18-132}, were subcloned and expressed as individual proteins for further studies of the effect on the N and C-terminal flanking regions on DNA binding. Investigation of secondary structure content by CD showed that the flanking regions increase the helical content of Max compared to the crystal structure, both in presence and absence of DNA, and NMR experiments supported these findings. Thermal stability measurements also showed that Max_{1-132} is the most stable protein in absence of DNA. Deletion of the Max flanking ends increases the affinity for DNA and produces the most thermally stable DNA bound Max construct. The presence of folded regions flanking the bHLH-Zip of Max agrees with previous findings that caspases cleave of these biologically significant regions during apoptosis, maybe in order to increase the DNA affinity.

3.3.2 Paper II

N- and C-terminal subregions in the c-Myc transactivating region and their role in creating versatility in folding and binding.

A large part of the human proteins of high medical interest are multidomain proteins, which are normally present at low concentrations in the cell. Recombinant production of the intact form of these proteins is often complicated by low expression, proteolytic instability and poor solubility, making the identification of domain borders highly important for production and studies of the separate domains alone. The c-Myc multidomain protein contains an N-terminal transactivation domain (TAD) that is regulated by binding to a wide range of proteins. However, little is known about the structural or biophysical properties of c-Myc TAD related to its ability to transactivate.

The classical definition of the c-Myc TAD mapped the domain borders to residue 1-143 (Kato et al., 1990) but bioinformatical investigation disagreed somewhat, suggesting an extended domain, including a conserved C-terminal α-helix. An extended construct including this helix (Myc_{1-167}) showed a higher structural content and affinity to target molecules. The chosen target molecules used for activity assays, TATA box-binding protein (TBP) and Myc Modulator-1 (MM-1), display different functional properties, both concerning transcriptional activation/repression and structure (mixed α/β-structure versus mainly predicted α-helical content). Further studies showed that the C-terminal of c-Myc TAD (Myc_{92-167}) is α-helical and contains the main determinants for protein interactions while the N-terminus (Myc_{1-88}), containing MBI, is mainly random and contribute to target protein binding, although it does not bind to target proteins alone. Myc_{1-167} had higher binding affinity to target molecules compared to the N- and C-terminal halves of c-Myc TAD and also displayed molten globule features, as investigated by ANS-binding and CD. The flexible nature of a molten globule suggests a possible mechanism for how the relatively small c-Myc TAD mediates binding to a large number of different target molecules. A recent study proposes similar molten globule characteristics in B-Myc, especially for the MBI and MBII regions (Burton et al., 2006). Furthermore, the requirement for both MBI and MBII in c-Myc TAD binding to TBP and MM-1 was supported in this article.

3.3.3 Paper III

A novel monothiol glutaredoxin (Grx4) from Escherichia coli can serve as a substrate for thioredoxin reductase.

A careful investigation of the *E. coli* genome resulted in the identification of a gene, *ydhD*, which codes for a 12.7 kDa protein containing a CGFS active site. A bioinformatical analysis showed high homology to the monothiol glutaredoxin family, especially to *S. cerevisiae* Grx5, and the protein was therefore named glutaredoxin 4 (Grx4). Construction of null mutants for *ydhD* using several different methods did not result in any viable cells, suggesting that Grx4 is essential. An earlier gene footprinting study showing that *ydhD* is necessary for robust aerobic growth supported this finding (Gerdes et al., 2003). Accordingly, the levels of Grx4 were found to be elevated in rich media and during robust aerobic growth, especially in strains deficient in thioredoxins. Iron depletion also resulted in elevated Grx4, suggesting a possible compensatory mechanism upon iron depletion involving Grx4.

Grx4 was assayed for classical glutaredoxin activity by using the HED–assay but similarly to *S. cerevisiae* Grx5, no activity could be seen. A number of active site mutants with no cysteine (Grx4-SGFS), an additional C-terminal cysteine (Grx4-CGFC) and a classical dithiol active site (Grx4-CPYC) were also tested in the HED-assay but no activity could be detected, implicating that the determinants for reduction of HED substrates are most likely located outside the active site sequence. A structural investigation of Grx4 by CD confirmed a well structured protein with a thermal stability similar to *E. coli* Grx1 (T_m of 67 ºC) excluding possible misfolding as the reason for the lack of HED-activity. Most characterized glutaredoxins, including *S. cerevisiae* Grx5, interact with GSH and are subsequently reduced but Grx4 was instead oxidized by GSSG, resulting in the formation of a disulfide and a monoglutathionylated residue. Treatment with *E. coli* TR and NADPH reduced the disulfide in Grx4, suggesting that Grx4 can function as a substrate for TR. The remaining mixed disulfide not subjected to TR reduction could further be reduced by *E. coli* Grx1. Taken together; this suggests extremely narrow substrate specificity for Grx4 with possible regulatory implications.

3.3.4 Paper IV

Molecular mapping of functionalities in the solution structure of reduced Grx4, a monothiol glutaredoxin from Escherichia coli.

The structural characterization of reduced Grx4 was started in parallel to the biochemical analysis in order to find out more detailed information about folding properties and substrate specificity. Previous experiments by CD demonstrated that Grx4 was well folded and had high temperature stability, suggesting it as a suitable target for structure determination by NMR. A 0.8 mM sample of Grx4 in 125 mM KCl resulted in narrow line-width and well spread signals in the ^{15}N-HSQC spectrum (figure 17).

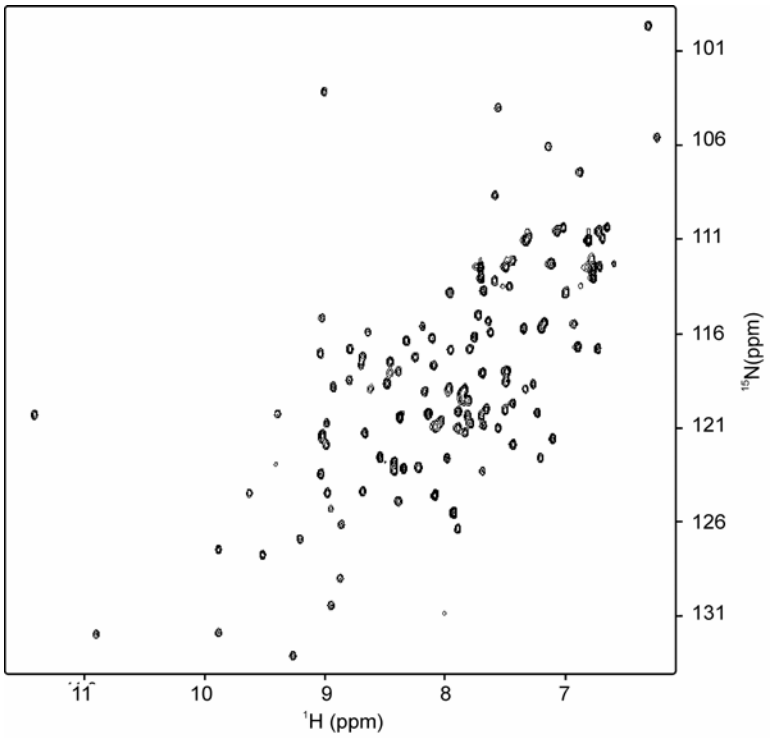


Figure 17. ^{15}N-HSQC spectra of Grx4.

The solution structure was obtained by use of heteronuclear assignment, and NOE cross-peaks from 2D NOESY/3D NOESY spectra and chemical shift assignments were used as input to the structure calculation program ARIA (ref). Additionally, backbone torsion angle restraints derived from chemical shifts using TALOS (ref) were added to improve the structure. The calculated structure of Grx4 consists of a mixed α-β fold with well defined hydrophobic core and resemblance to the structures of the dithiol

glutaredoxins. Several residues in and close to the active site gave only a limited set of restraints and showed signs of conformational exchange but P72, which is well conserved within the Grx family, shows a distinct *cis*-peptide conformation and adds rigidity to the active site loop. The surface of Grx4 displays two hydrophobic patches, which may be involve in oligomerization as suggested by initial spectral recordings showing broadening of line-widths for protein concentration above 1 mM and salt concentrations below 100 mM. Furthermore, several well conserved residues within the monothiol Grx family cluster on the surface on the side of the hydrophobic patches, close to the active site, implying a conserved potential substrate binding pocket. Careful investigation of residues in Grx4 potentionally involved in glutathione binding suggests similarities to *E. coli* Grx3. Small but notable differences are nonetheless present in the second GSH-binding site of Grx4, concerning residues involved in displacement of oxidized glutathione from the active site, thus suggesting an explanation for the lack of reactivity in the HED assay. MALDI experiments suggest that disulfide formation on glutathionylation is accompanied by significant structural changes, and together with detailed structural evaluation indicates that the disulfide-forming region forms an active site. These results contrast in a major way to thioredoxins and dithiol glutaredoxins, where differences between oxidized and reduced forms are subtle and local.

3.3.5 Paper V

Identification and characterization of a consensus motif in TBP and TRRAP, which binds the c-Myc transactivation domain.

The nature of the c-Myc TAD as a relatively small region that binds to a large number of different target proteins proposes that some of the target molecules may have common interaction patches, since the number of ways binding can be achieved is limited. A search for conserved interaction patches among c-Myc TAD interacting proteins resulted in identification of a peptide binding motif presented both in AMY-1 and in the transcription factor TATA-box binding protein (TBP), which is central for c-Myc transactivation. A TBP derived peptide identified as the best binder protected c-Myc TAD from proteolysis, induced secondary structure changes in c-Myc TAD and reduced cell growth in c-Myc overexpressing cells when introduced in-vivo linked to a TAT-NLS peptide. Structural evaluation by NMR showed that the HSQC spectrum of $Myc_{92\text{-}167}$ displays severe overlaps indicating < 50% secondary structure (figure 18),

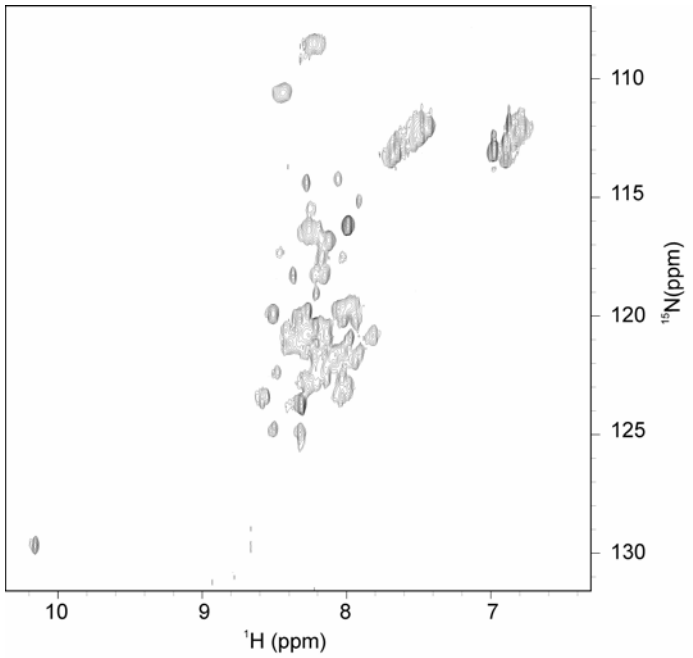


Figure 18. ^{15}N-HSQC spectra of Myc$_{92-167}$.

which is in agreement with the previous findings presented in paper 2. Addition of T1 to Myc$_{92-167}$ causes only small changes in the HSQC for selected NH resonances and the dominant feature is that the line widths are significantly sharper in the presence of the peptide, indicating less unspecific internal Myc$_{92-167}$ protein interactions, or less dynamics of the protein in the bound form. A combination of the results from the Biacore binding assay and structural data from trNOE experiments led to the identification of c-Myc-interacting residues in the TBP derived peptide. The consensus binding pattern thus identified was used in a refined search among c-Myc TAD binding partners, where a new binding motif was found within the histone acetyltransferase complex member TRRAP. The TRRAP motif displayed even stronger affinity to c-Myc TAD, providing a proof-of- principle that a true c-Myc binding motif has been identified. The location of a common c-Myc interacting patch in TBP and TRRAP increases the knowledge about how c-Myc is recruited to the preinitiation complex, supports the current model for transcriptional initiation by the TBP-free TAF complex, and suggests novel routes for therapeutic strategies.

4 CONCLUSIONS

4.1 PAPER I

A study of the biologically significant regions flanking the bHLH-Zip of Max show that these regions have a helical content significantly larger then predicted from present crystal structures and are resistant to proteolysis both in the absence and presence of DNA. Deletion of these regions is required for maximum DNA affinity and thermal stability in presence of DNA.

4.2 PAPER II

A new construct of c-Myc TAD, $Myc_{1–167}$, were found promotes folding, displays the characteristics of a helical molten globule, and binds both to MM-1 and TBP. Although its C-terminal region ($Myc_{92–167}$) has a partly helical fold and binds both MM-1 and TBP, neither N- nor C-terminal regions of $Myc_{1–167}$ bind target proteins with as high affinity as the entire $Myc_{1–167}$, or display molten globule properties.

4.3 PAPER III

A molecular in-depth analysis of the essential Grx4 in *E. coli* showed that Grx4 was highly abundant in the cell, regulated by 3', 5'-tetraphosphate and elevated upon iron depletion, suggesting an iron related function for the protein. Grx4, which showed no activity in the classical glutaredoxin (HED) assays, could still be oxidized by glutathione, with a completely unexpected concomitant formation of an internal disulfide. This internal Grx4 disulfide was a direct substrate for NADPH and *E. coli* TR, while the mixed disulfide was reduced by *E. coli* Grx1.

4.4 PAPER IV

The three-dimensional structure of reduced Grx4 was determined by NMR and comprises a glutaredoxin-like α-β fold. Several stringently conserved structural features were identified, which allowed functional predictions for the entire monothiol glutaredoxins family. The absence of classical glutaredoxin activity in *E. coli* Grx4 is understood based on small but significant structural differences in the glutathione-binding region, and through the lack of a conserved second GSH binding site. Investigation of cysteines targeted by glutathionylation implies structural changes upon disulfide formation and proposes a potential active site in the disulfide-forming region.

4.5 PAPER V

A bioinformatic screen for conserved sequence motifs in c-Myc TAD binding proteins resulted in identification of a motif in TBP, which binds c-Myc TAD with micromolar affinities and decreased c-Myc induced cell growth. Mapping of the TBP derived peptide interaction surface by trNOE-NMR and concomitant Biacore/SPR analysis of a constructed peptide library was employed to refine the binding determinants. This resulted in identification of a peptide segment belonging to the same binding motif in TRRAP, and which bound c-Myc TAD with even higher affinity.

5 FUTURE PERSPECTIVES

In paper I we found that the flanking regions of the bHLH-Zip of Max are important for folding of the intact protein and regulates binding to DNA. The DNA affinity measurements were performed by EMSA but a more careful investigation using Biacore would give a more detailed comparison of the affinities of the different Max constructs to diverse DNA sequences. Initial trials using biotinylated double stranded E-box DNA bound to a streptavidin chip gave good binding curves for Max AA and AB and a curve evaluation showed that the binding follows a two-state model, possibly by binding of a Max monomer to DNA followed by dimerization on the surface of the DNA as suggested by (Kohler et al., 1999). The shorter Max construct could not be evaluated due to extensive binding to a reference surface with non-E-box DNA. A possible explanation for this behavior could be that the flanking regions of bHLH-Zip of Max are necessary for achieving binding specificity or changes the binding pathway, but the length and sequence of the used DNA could also be critical. Future work would include further experiments including the MaxBA and MaxBB, control experiments verifying the assembly pathway of Max and construction and application of a suitable binding model. Other Max partners such as Myc and Mad could also be included in the binding analysis, leading to studies of multidomain complexes involving Max and Myc together with proteins targeting the DNA binding region of Myc for transcriptional regulation such as Miz-1, Ini1, Nmi and YY-1.

The results from paper II and V show interaction between c-Myc TAD and target proteins and peptides. The binding specificity of these Myc targets are in these studies only evaluated in context of the unmodified wt c-Myc TAD and an expansion involving common c-Myc TAD mutants found in e.g. Burkitt's lymphoma and phosphorylated c-Myc TAD would contribute to the understanding of the biology behind the binding mechanistic involved in tumor formation. Further experimental work should also be performed *in vivo* in order to verify the c-Myc TAD-peptide interaction.

Structure determination of c-Myc TAD has so far been hindered by the flexible nature of the domain hindering crystallization and poor solubility and stability properties making structural investigation NMR unfeasible. The C-terminal of c-Myc TAD,

Myc_{92-167}, is however stable in solution but the HSQC spectra show low amide protein dispersion and possible conformation averaging making assignment complicated. A successful assignment of c-Myc TAD would make mapping of target protein interactions onto c-Myc TAD at a residue level possible, giving a detailed picture of how the numerous Myc targets interact in relation to their biological activity. Addition of target proteins to c-Myc TAD may also induce folding and hence stability in the TAD, making a proper structure determination of a Myc complex possible. A partial assignment of the main chain of b-Myc, a Myc family member lacking most of the linker region and the whole the DNA binding domain, was recently published (Burton et al., 2006) and the HSQC spectra of b-Myc show similar amide proton dispersion but narrower peaks compared to c-Myc TAD, suggesting different structural properties in the two family members.

Another interesting target is c-Myc TAD from chicken, which lacks a low complexity region spanning residue 72-93 in human c-Myc. This region has no predicted secondary structure but is conserved in all higher eukaryotic species and is suggested to function as a linker in-between MBI and MBII. Linker regions often display propensity to be highly extended due to their specific amino acid composition, especially in higher eukaryotic species (Dyson and Wright, 2005), and deletion of this region may improve the properties of c-myc TAD, making chicken c-Myc TAD a suitable target for structure determination.

The results from the structure determination of reduced Grx4 by NMR (paper IV) and the MALDI investigation of the glutathionylated sample (paper III) proposed possible conformational change in Grx4 upon treatment with GSSG and concomitant disulfide bridge formation. More careful MALDI investigation of proteolytically cleaved Grx4 samples was however hindered by the inefficient cleavage of Grx4, even after addition of acetonitrile, which has been shown to facilitate cleavage of proteins with the rigid structure. Further structural studies of the fully oxidized and the TR reduced Grx4 by NMR would therefore be necessary to allow studies of these events in molecular detail. Investigation of oxidized Grx4 may be beneficial from using Grx4 cysteine mutants in order to produce homogenous and stable protein samples but the stability of these mutants is currently not known.

The function of the essential Grx4 is still not revealed. Since many biological processes are dependant on specific protein-protein interactions we initiated search for protein partners by ligand fishing in *E. coli* lysates by using Grx4 affinity columns, which resulted in the identification of several possible targets. Refinement of the method by using 2D-gels for analysis should improve the resolution and generate more possible partners. In parallel, a bacterial-two-hybrid system in *E. coli* that uses the cAMP signaling cascade to identify proteins-protein interactions (Karimova et al., 1998), could be used to identify proteins binding to Grx4.

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