INCREASED MULTIDRUG RESISTANCE IN ADRIAMYCIN SENSITIVE MCF-7 BREAST TUMOR CELLS OVEREXPRESSING HUMAN PLACENTAL THIOLTRANSFERASE

By

Elizabeta Borer Meyer

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ABSTRACT

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Human placental thioltransferase (hpTT) cDNA was isolated including its 5'- and 3'-untranslated regions. The nucleotide sequence of the cDNA and the deduced amino acid sequence agreed with those of cDNAs isolated simultaneously from other human tissues. The deduced amino acid sequence in general is highly conserved throughout mammalian species. Human TT differs from other mammalian TTs by lacking an internal methionine residue and the presence of a cysteine rather than serine at residue 7.

The recombinant hpTT cDNA was overexpressed in *E. coli* and purified; both the dithiol-disulfide oxidoreductase and DHA reductase activities were comparable to that of purified native erythrocyte TT. The purified recombinant hpTT also cross-reacted with antibodies generated against the purified human erythrocyte TT or the recombinant pig liver TT protein.

To determine whether elevated levels of TT would increase resistance to Adriamycin, MCF-7 WT breast tumor cells were transfected with the TT cDNA in an expression plasmid. Several stably transfected MCF-7 WT cell lines were established that constitutively overexpressed TT 9-66 fold and had 2 to 10-fold elevated resistance to Adriamycin, indicating a correlation between TT levels and drug resistance. In addition, Adriamycin resistance in both MCF-7 WT and transfected MCF-7 WT cells was independent of L-

ascorbate 2-phosphate, indicating that increased Adriamycin resistance is not related to DHA reduction. An alternate TT activity such as protein mixed disulfide:GSH exchange may contribute to the elevation in drug resistance.

Dedicated to my mother, Dr. Katarina T. Borer, who was my inspiration and role model.

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ABBREVIATIONS

AA	ascorbic acid
AAP	L-ascorbate-2-phosphate
ABC	ATP-binding cassette protein
ADF	adult T-cell derived leukemic factor
AP-1	activator protein-1
ATP	adenosine triphosphate
BCIP	bis-4-chloro-indolyl phosphate
BSO	L-buthionine-(S,R)-sulfoximine
cAMP	cyclic adenosine 5'-3'-monophosphate
cDNA	complementary DNA
CMV	cytomegalovirus
DEPC	diethylpyrocarbonate
DHA	dehydroascorbate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
FCS	fetal calf serum
FSH	follicle stimulating hormone
G_o	growth arrested phase of cell cycle
G1	growth phase 1 of cell cycle
G418	Geneticin, aminoglycoside antibiotic
G6P	glucose 6-phosphate
G6PDH	glucose 6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulfide
GST	glutathione S-transferase
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
hpTT	human placental thioltransferase
IPTG	iso-1-thio-β-D-galactoside
kDa	kilodalton
K_m	Michaelis-Menten constant
λ	lambda bacteriophage

LH	leutinizing hormone
LD_{50}	lethal dose 50
LTR	long terminal repeat
MCF-7 WT	Michigan Cancer Foundation wild-type breast tumor cell line
MCF-7 ADR^R	Michigan Cancer Foundation Adriamycin-resistant cell line
MDR	multi-drug resistant
MMTV	mouse mammary tumor virus
mwco	molecular weight cutoff
NADH	nicotinamide dinucleotide, reduced
NADPH	nicotinamide dinucleotide phosphate, reduced
NBT	nitro blue tetrazolium
NFκβ	nuclear factor kappa B
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	Protein disulfide isomerase
pfu	plaque-forming units
Pgp	P-170 glycoprotein
PKC	protein kinase C
PMSF	phenylmethylsulfonylfluoride
redox	reduction-oxidation
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Rosewell Park Memorial Institute
RT-PCR	reverse-transcriptase polymerase chain reaction
S	DNA synthesis phase of cell cycle
SDHA	semi-dehydroascorbate
SDS	sodium-dodecyl sulfate
SOD	superoxide dismutase
TBS	tris buffered saline
TDOR	thiol-disulfide oxidoreductase
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TPA	12-O-tetradecanoyl 13-acetate
Tris	Tris-hydroxymethylaminomethane
TRX	thioredoxin
TT	thioltransferase
V_{max}	maximal enzyme velocity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER ONE

LITERATURE REVIEW

To avoid redundancy, this review is presented in seven major sections: I. Cellular oxidative stress; II. Ascorbate and related reductases; III. Glutathione and related enzymes; IV. Thiol-disulfide oxidoreductases and their postulated cellular functions, including thioltransferase (TT, glutaredoxin), the enzyme of particular research interest; V. Adriamycin, the antitumor drug of interest; VI. Drug resistance; and VII. MCF-7 cells, that provide the model system used. Extensive reviews already are available for each area; the discussion here is of details germane to the research question.

The details presented here are designed to give the reader an understanding of reactive oxygen species (ROS) involved in Adriamycin damage; the roles of various enzymes that detoxify these ROS in drug resistance, especially enzymes that are structurally or mechanistically related to TT; changes in these enzyme activities in the MCF-7 WT and ADR^R cells that constitute the model system; and the roles of ascorbic acid (AA) and glutathione (GSH) in detoxification, particularly with respect to thioltransferase (TT).

I. Cellular Oxidative Stress

Cellular oxidative stress is modulated by the presence of many biological antioxidants and enzymes, many of which are altered in drug resistance. Responses to oxidative stress affect resistance to antitumor drugs that generate toxic oxygen radical species. Detailed here

for the reader are many of the mechanisms whereby ROS are generated in the cell, and an overview of cellular detoxification systems, particularly those changed in drug resistance, is presented.

In recent years, there has been an increasing interest in oxygen free radicals and their reactions, especially with respect to biological systems. Mammalian cells are continuously subjected to ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radicals ($^{\cdot}OH$). When cellular mechanisms which detoxify ROS are exceeded, cytotoxic lipid peroxidation, nitroperoxidation, protein oxidation, DNA damage and cell lysis result. ROS play a role in inflammation, rheumatoid arthritis, atherosclerosis, hepatic diseases, aging, mutagenesis, chemotherapy, and xenobiotic metabolism (reviewed in 1). Cellular reduction-oxidation balance regulating the effects of ROS is a "housekeeping" function of the cell, as changes in oxidant or reductant levels in the cell affect biosynthetic reactions, including activities of proteins regulating metabolism (Section IV); expression of cytokines (2), adhesion molecules (3), and protooncogenes (4); and the activities of several transcription factors (Section IV).

Free radicals are extremely reactive molecules containing an unpaired electron in molecular orbitals. These radicals are produced in cells through a wide variety of reactions and external influences. Homolytic fission of covalent bonds results in two radical products. In biological systems, ROS result most commonly from reduction-oxidation (redox) reactions catalyzed by transition metals or enzymes and aerobic metabolism.

H_2O_2 is generated in cells through several mechanisms. Dismutation of $O_2^{\cdot-}$ generates H_2O_2 [1] (5).

[1] $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$

Hydrogen peroxide is also generated through mixed-function oxidase enzymic reactions such as that of D-amino acid oxidase and urate oxidase (6). Activated neutrophils undergo a respiratory burst releasing $O_2^{\cdot-}$ and other noxious substances to destroy invading organisms (7). Electrons passing down electron transport chains can react with O_2 rather than cytochrome *c* oxidase, producing $O_2^{\cdot-}$ (8). Enzymatic reactions can generate $O_2^{\cdot-}$. Superoxide is generated during the metabolism of arachidonate to eicosanoids by lipoxygenases and cyclooxygenases (9-12). Xanthine oxidase (E.C. 1.1.3.22), produces $O_2^{\cdot-}$ during the oxidation of xanthine to uric acid (13). $O_2^{\cdot-}$ can also be generated through redox cycling of quinones which are structural components of many chemotherapeutic agents (see Section V). At low pH, the superoxide radical is protonated ($HO_2^{\cdot}$).

Superoxide and hydrogen peroxide generate the hydroxyl radical ($^{\cdot}OH$) through a metal-catalyzed Haber-Weiss reaction [2] (14).

[2] $O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe^{2+}} O_2 + {}^{\cdot}OH + OH^-$

Free radical Fenton reactions (15) can also be initiated if Fe^{2+} comes into contact with hydrogen peroxide [3].

[3] $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$

Species such as H_2O_2 and $O_2^{\cdot-}$ are not highly reactive, and may diffuse from generation

sites. H_2O_2 can cross membranes, thereby affecting more than one cellular compartment, where $O_2^{\cdot-}$ cannot. H_2O_2 is a weak physiological oxidant and inactivates some enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose 1,6-bisphosphatase by oxidation of essential thiol groups (16). $O_2^{\cdot-}$ exerts toxic effects when converted to more reactive species such as $^{\cdot}OH$ and $HO_2^{\cdot}$. $HO_2^{\cdot}$ is a stronger oxidant than $O_2^{\cdot-}$ and will directly attack polyunsaturated fatty acids, oxidizing membranes (17). Hydroxyl radicals are highly reactive, immediately interacting with biomolecules in the vicinity (18), often resulting in damage to cellular components. Hydroxyl radical attack on deoxyribose and nucleotide ring structures damages DNA, resulting in DNA strand breakage (19). If excessive, this strand breakage can lead to NAD^+ depletion (20), since the adenosine diphosphate moiety in NAD^+ is removed and placed on the damaged DNA by poly(ADP-ribose) as a repair signal. Membrane damage results from $^{\cdot}OH$ abstraction of a H atom from bilayer lipids leaving lipid radicals. These radical lipids react with molecular oxygen to form peroxyradicals, and continue chain oxidation reactions (21), resulting in significant membrane damage (22).

Amino acid oxidation, decarboxylation, deamination, and inappropriate cleavage or cross-linking are results of radical-induced damage in proteins. Cysteine thiols can be oxidized to stable disulfides or sulfenic (SOH), sulfinic (SO_2), and sulfonic (SO_{3-}) groups. Free radical damage to proteins is often irreversible, and can lead to inactivation and unfolding (reviewed in 23). Evidence strongly suggests that proteins damaged under oxidative conditions are insufficiently catabolized, and accumulate in the cell (24-27), leading to aging-related disease, such as cataracts in eye tissue (28).

Biological antioxidants such as vitamins C (ascorbic acid, AA, Section II) and E (α-Tocopherol), glutathione (GSH, Section III), carotenoids, α-lipoic acid, and uric acid protect

cells from ROS toxicity as do cellular enzyme systems including superoxide dismutase (SOD) (29), catalase (30), glutathione peroxidase (GSH-Px) (31,32), peroxyredoxins (previously known as thiol-specific antioxidant) (33), GSH-S-transferases (GSTs) (34), and ascorbate peroxidases (35), as well as thiol-disulfide oxidoreductases (Section IV).

α-Tocopherol is membrane-bound, and protects against lipid peroxidation. The one-electron oxidation of α-Tocopherol results in the α-chromanoxyl radical. Cytoplasmic AA can reduce the α-chromanoxyl radical, recycling α-Tocopherol at the surface of biological membranes (36-38).

AA is probably the most effective and least toxic antioxidant identified in mammalian cells (39,40). AA reduces semi-quinones, H_2O_2, $O_2^{\cdot-}$, HQ , $\cdot OH$, ^{1} Q , thiyl radicals and hypochlorous acid produced at sites of inflammation or during other cellular processes. Comparisons of cellular antioxidant ·OH radical scavenging abilities revealed that AA is superior to GSH and uric acid in scavenging ability (41). AA also proved to be the significant scavenger of membrane fatty acid nitroxide radicals when compared with other cellular detoxification systems such as catalase, GSH, GSH-Px, SOD, and α-tocopherol (42). GSH at high concentrations was able to enhance the AA scavenging ability, and AA was an insignificant radical scavenger when cellular levels fell below 0.1 mM. This suggests that nitroxide radical reduction may be impaired in cells that have low levels of AA, or insufficient ability to recycle oxidized AA. AA radical scavenging activity results in the oxidation products dehydroascorbate (DHA) or semi-dehydroascorbate (SDHA) (Fig.1), which are regenerated by cytoplasmic DHA reductases (Section II A) and membrane-bound NADH-dependent SDHA reductase (Section II B), respectively (Fig.2).

GSH (Fig.1) functions as an antioxidant directly, and as a cofactor or substrate in

many reactions. Reactive species reduced by glutathione include free radicals, H_2O_2, organic hydroperoxides, epoxides, alkenes, quinones, and aldehydes. One-electron reduction of radicals with GSH yields thiyl radicals; two-electron reductions using two moles of GSH results in glutathione disulfide (GSSG).

GSH is a cofactor in several detoxification reactions, either through conjugation with reactive compounds, or as a substrate to reduce oxidized thiols. Many proteins form mixed protein disulfides with GSH under oxidizing conditions. GSH is involved in maintaining cysteine and coenzyme A as well as cellular proteins in their reduced state. Intracellular GSH levels are high (1-10 mM), but oxidative stress can shift the thiol:disulfide equilibrium elevating GSSG (43,44).

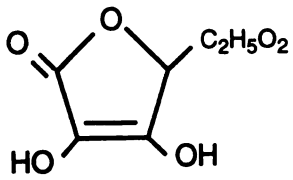
A series of enzymes participate in the removal of ROS from cells. These enzymes are found in diverse cellular locations, as well as in varying levels, depending on the oxidative demands of the particular tissue.

Superoxide dismutase (E.C. 1.15.1.6) catalyzes the dismutation of $O_2^{\cdot-}$ [1], which decreases cellular $^{\cdot}OH$. Three types of superoxide dismutases (SOD) are known (reviewed in 45). Two are found in humans; a Cu/Zn-containing enzyme, originally termed hepatocuprein or erythrocuprein, and a Mn-containing SOD. The Cu/Zn SOD is found in cell cytosol, lysosomes, between inner and outer mitochondrial membranes, and in the nucleus, whereas the Mn-containing SOD enzyme is found in the mitochondrial matrix. A third Fe-containing SOD is found in bacteria and plants, but not mammals.

Glutathione reductase (46) catalyzes the regeneration of GSH from glutathione disulfide (GSSG) [4],

Fig. 1. Structures of antioxidants.
A. Ascorbic acid (AA) B. Dehydroascorbic acid (DHA), the two-electron oxidation product of ascorbic acid C. Semi-dehydroascorbic acid (SDHA), the one-electron oxidation product of ascorbic acid D. Glutathione (GSH)

A.



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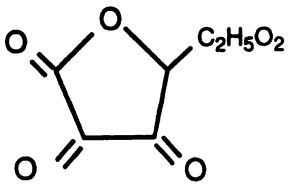

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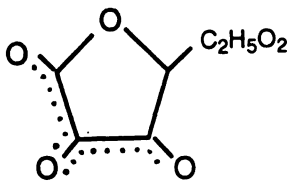

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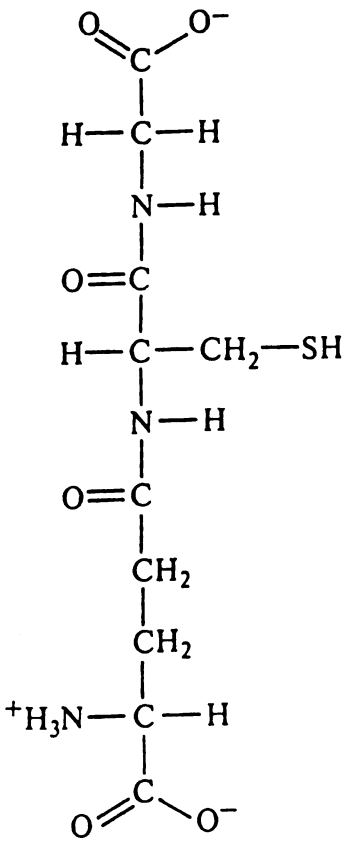


Fig. 1. Structures of antioxidants.

Fig. 2. Oxidation-reduction relationships of AA, DHA, and SDHA in a model cell. Intracellular AA (AAH_2) reacts with free radicals ($R^{\cdot}$), superoxide free radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and other oxidants. Single-electron oxidation results in SDHA ($^{\cdot}AAH$), which may disproportionate into DHA and SDHA. ($^{\cdot}AAH$) also may be reduced back to AA using NADH-dependent SDHA reductase. Intracellular DHA may be recycled by DHA reductases such as TT, utilizing GSH generated by GSSG reductase. AA is transported into the cell through unknown mechanisms, and may diffuse out of the cell. Extracellular AA in plasma or extracellular space is oxidized to DHA and transported into the cell through two types of transport mechanisms. Taken from Wells, W.W. and Xu, D.-P. (1995) Dehydroascorbate reduction. J. Bioenerget. Biomemb. 26, 369-377, with permission.

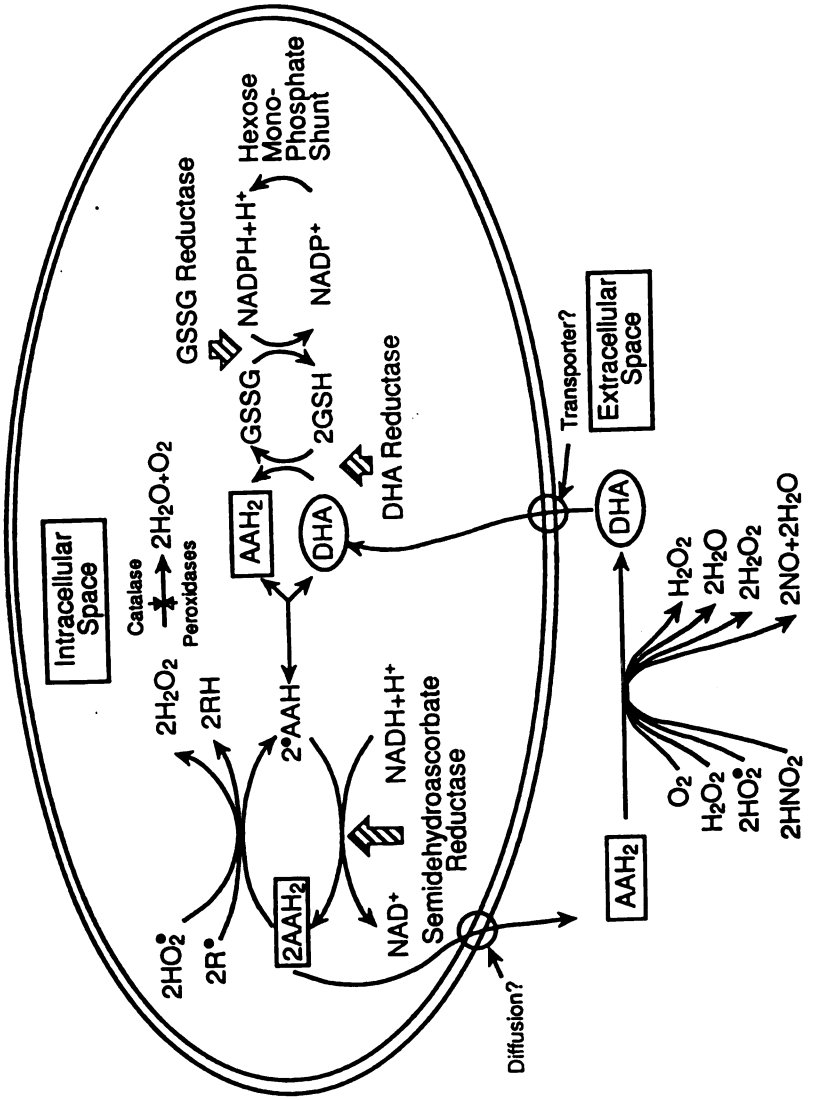


Fig. 2. Oxidation-reduction relationships of AA, DHA, and SDHA in a model cell.

[4] $GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$

where the NADPH utilized is generated in the oxidative portion of the pentose phosphate pathway [5,6].

G-6-P dehydrogenase

[5] $G\text{-}6\text{-}P + NADP^+ \rightarrow 6\text{-phosphogluconate (6-PG)} + NADPH + H^+$

6-PG dehydrogenase

[6] $6\text{-}PG + NADP^+ \rightarrow CO_2 + NADPH + H^+ + \text{ribulose-5-P}$

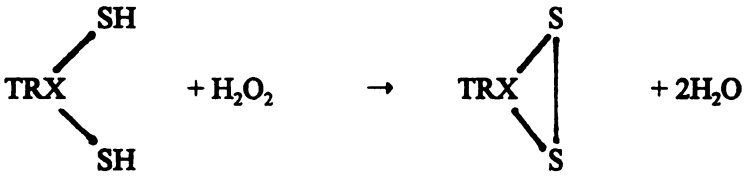
In addition to the inducible dehydrogenases (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), the rate of the pentose phosphate pathway is controlled by cellular NADP+ levels; and therefore is dependent on the energy and oxidative state of the cell (47).

Hydrogen peroxide is removed by a variety of enzymes; catalase (E.C. 1.11.1.6, H_2O_2:H_2O_2 oxidoreductase) [7] and peroxidases [8] detoxify H_2O_2 generated in peroxisomes or mitochondria.

[7] $H_2O_2 \rightarrow 2\ H_2O + O_2$

[8] $RH_2 + H_2O_2 \rightarrow R + 2\ H_2O$

Catalase is located in all body organs, and is predominant in erythrocytes and liver. Although catalase is located primarily in peroxisomes, catalase has also been found in the cytoplasm (48). Glutathione peroxidase (E.C.1.11.1.9, GSH:H_2O_2 oxidoreductase) uses GSH as a reductant (RH_2) and will use peroxides other than H_2O_2 [8] as substrates (31,32). Activity of GSH-Px is high in the liver, and moderate in heart, lung and brain. GSH-Px is distributed primarily in the cytoplasm, although GSH-Px has been found in mitochondria (49) and plasma (50). Peroxyredoxins (33) utilize thioredoxin (TRX) as a reductant in the removal of hydrogen peroxide [9].

[9] 

Ascorbate peroxidase is found in chloroplasts of higher plants and green algae [10].

[10] $AA + H_2O_2 \rightarrow 2\,H_2O + DHA$

Many compounds are removed from cells through consecutive detoxification reactions (Fig.3); sequential reactions involving hydroxylation or oxidation (Phase I) followed by conjugation to soluble compounds such as carbohydrates (Phase II), facilitating degradation or elimination. GSTs transfer GSH onto a variety of oxidized substrates (51). Studies on human glutathione transferase have described three distinct groups of enzymes which are commonly referred to as basic (α-E), near neutral (μ), and acidic (Pi, rho) transferases according to their isoelectric points (52). The acidic transferases are the dominant forms

found in human placenta, lung, brain, erythrocytes, and the lens of the eye, and are present in low but variable amounts in human liver. The Pi transferase is found in most fetal tissues including liver. Within each group of transferases differences in physical, catalytic, and immunological characteristics suggests the presence of more than one enzyme in each group.

Proteins postulated to have an antioxidant role are metallothioneins and heat shock proteins. Metallothioneins are 6.5 kDa cytoplasmic proteins composed predominantly of cysteine residues (23-33%), that store heavy metals such as Zn^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+}. Cys-S^- residues tetrahedrally ligand metals decreasing free metals available to catalyze $\cdot OH$ formation. Heat shock induces SOD activity (53),as well as heat shock proteins. Exposure to H_2O_2 also induces heat shock proteins, leading to the possibility that heat-shock may induce oxidant stress within cells.

II. Ascorbate

AA acts as a reducing agent (electron donor) in chemical reactions. AA is a demonstrated cellular antioxidant and can reduce a variety of radical and reactive species (Section I). One-electron oxidation of AA yields the semi-dehydroascorbate radical (SDHA, Fig.1); further-one-electron oxidation gives dehydroascorbate (DHA, Fig.1). SDHA is not very reactive due to a highly delocalized unpaired electron structure, and can spontaneously disproportionate [11] at a rate of $10^5\ M^{-1}\ s^{-1}$ at pH 7.0, yielding AA and DHA (93), (Fig.2).

[11] $\quad 2SDHA \rightarrow AA + DHA$

SDHA may function as both a pro- and anti-oxidant. SDHA inhibits several enzymes (54,55), and can attack cellular components. SDHA also exhibits protective capacity in

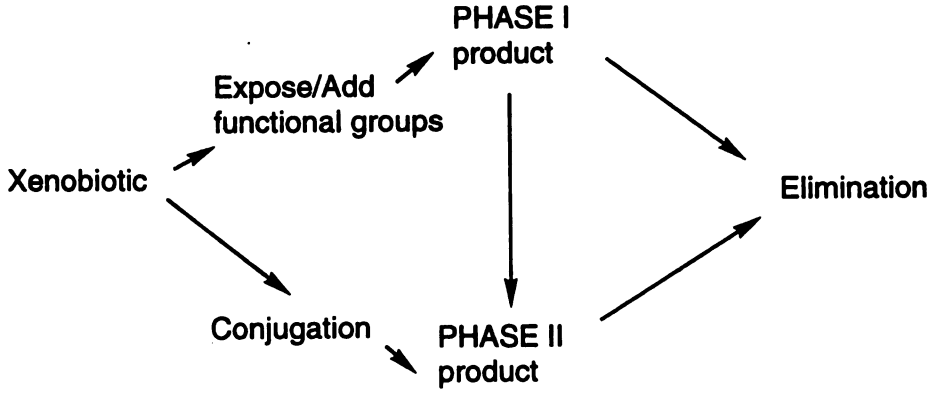


Fig. 3. Phase I and Phase II detoxification reactions.
Xenobiotics and compounds that are lipid soluble readily enter cells. Methods of excretion for these compounds include two-phase reactions. First, compounds are made more water-soluble through hydroxylation and oxidation reactions (Phase I). Some compounds are biologically more active as a result of these Phase I reactions, including antitumor drugs. Secondly, these metabolites with increased solubility are conjugated in glycosylation, sulfonation, glutathionylation and other reactions that facilitate their elimination (Phase II).

radical quenching of reactive and toxic free radical intermediates (56-57). In plants, SDHA has been implicated in cell elongation (58) and electron transport in the transplasma membrane redox system (59). Pulse radiolysis studies of the SDHA free radical determined that typical reactions involve one-electron oxidation and reduction, and that the radical rarely serves as a biological reductant (60).

At netural pH, DHA is very unstable and rapidly degrades (61). Aqueous solutions of AA are stable unless transition metals are available. Transition metals together with superoxide catalyze the oxidation of AA at neutral or slightly alkaline pH. In the presence of H_2O_2, AA can function as a prooxidant, stimulating $^{\cdot}OH$ formation by the Fenton reaction [2].

Plants and most animals synthesize AA from glucose (Fig.4). Humans and other primates (62,63), as well as guinea pigs (64) and fruit bats (65) cannot due to the lack of an enzyme, L-gulono-lactone oxidase (66), which catalyzes the conversion of 2-keto-L-gulonolactone to 1-gulonolactone (67), which is spontaneously converted to AA (Fig. 4). Insects, invertebrates and fishes are also incapable of AA synthesis (68). Since AA is essential for normal physiological processes, organisms that cannot synthesize AA rely on recycling oxidized AA and ingestion of exogenous AA for survival. Extreme AA deficiency causes scurvy, and if prolonged, results in death. Scurvy in humans is characterized by poor wound healing, weakness, hemorrhagic phenomena, hyperkeratosis, and abnormal bone growth (69). Scurvy in humans can be prevented by ingestion of as little as 10 mg AA daily.

The adult recommended dietary allowance (RDA) for AA in the United States is currently 60 mg daily. A recent pharmacokinetic study (70) determined that 60 mg is too low and recommended that the RDA be changed to 200 mg, the amount that one could eat in a healthy diet of vegetables and fruit. There appeared to be no value in taking daily doses

beyond 400 mg.

Ascorbic acid regeneration from dehydroascorbic acid (DHA) and semidehydroascorbate free radical (SDHA) depends on both the DHA reductase activity [12] (Section IIA) and the SDHA free radical reductase activity (71)[13] (Section IIB) as well as the availability of GSH and NADH, respectively.

[12] DHA + 2 GSH → GSSG + AA

[13] SDHA + 2 NADH → AA + 2 $NADP^+$

AA is accumulated in cells by a Na^+-dependent, incompletely characterized transporter that shows saturable kinetics. The K_m of this transporter appears to be 70 uM, the same concentration that plasma levels attain when daily AA ingestion is 200 mg (72,73). AA can also enter cells through the Na^+-independent active transport of DHA via GLUT-1 glucose transporters (74-77), and the subsequent rapid reduction to AA by cellular GSH-dependent DHA reductases (76,77) (Fig.2, Section IIA).

AA is absorbed in the gut as the oxidized form, DHA. When multiple pig tissues were analyzed, stomach contained the highest levels of DHA reductase activity (78), and very high levels of TT (79), a known DHA reductase (80). In most biological tissues, the level of DHA is low and the level of AA is high (81). Gastric ascorbic acid concentrations are several times higher than in plasma (82). AA is also secreted in the reduced form into the digestive tract, where AA facilitates iron absorption by reducing Fe(III) to Fe(II).

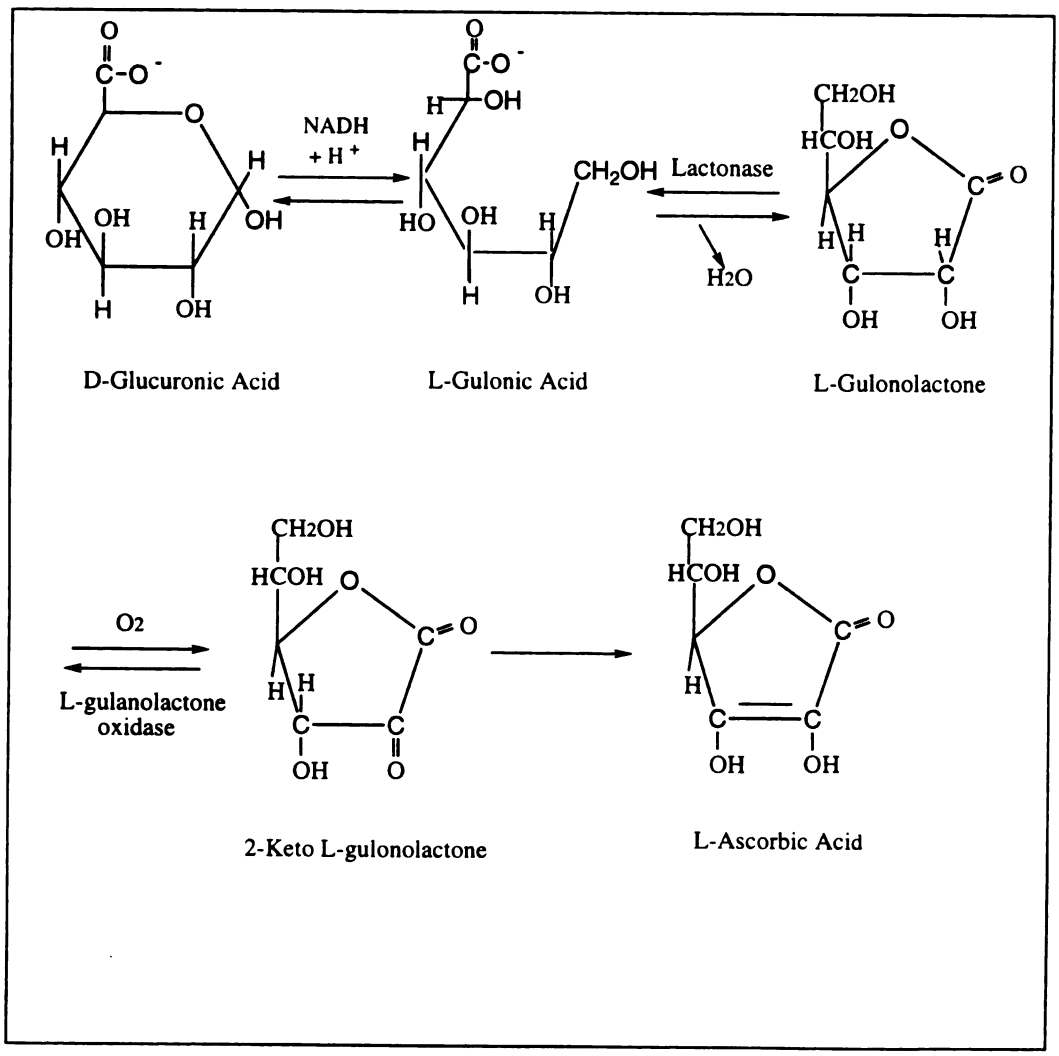


Fig. 4. AA synthesis.
AA is synthesized from glucose. L-gulonolactone oxidase catalyzes the formation of 1-gulonolactone from 2-keto-L-gulonolactone, which then is spontaneously converted to AA. Humans, other primates, guinea pigs, fruit bats, insects, invertebrates and fishes lack L-gulonolactone oxidase and cannot synthesize AA.

Several enzymes have an AA requirement for effective catalysis. AA is required as a cofactor for proline hydroxylase (83,84) and lysine hydroxylase (84), enzymes that participate in collagen biosynthesis. AA is presumably required by the hydroxylases to keep the metal centers in the reduced state, and to protect the enzyme. If AA is deficient then individual collagen fibers are insufficiently hydroxylated, resulting in improperly formed fibers, which ultimately leads to poor wound-healing and fragile vessels. Other enzymes requiring AA as a cofactor are γ-butyryl betaine hydroxylase (71), which converts γ-butyryl betaine into carnitine, and dopamine-B-hydroxylase (84), which converts dopamine into norepinephrine.

Interrelationships between AA and GSH synthetic processes have been demonstrated. Administration of L-buthionine-(SR)-sulfoximine (BSO), a specific γ-glutamylcysteine synthetase inhibitor (85), results in glutathione deficiency in animals or cells in culture. When BSO-treated animals are supplemented with 2 mM AA/kg/day, mortality decreases and levels of GSH rise. When 2 mM DHA/kg/day is added instead of AA, GSH levels do not change, and mortality increases to 100% (reviewed in 86), indicating that AA effects GSH metabolism.

Studies also indicate that one antioxidant level (GSH or AA) increases when levels of the other antioxidant decrease under conditions of oxidative stress (87). When adult mice are GSH-depleted by BSO administration, AA synthesis is elevated, and sufficient to protect the mice from toxic ROS. The AA level first doubles in 4 hours; then AA levels decrease and DHA levels rise. This finding is consistent with the inability to use GSH to recycle DHA [12]. In a related experiment, Vitamin C-depleted (scorbutic) guinea pigs were fed a GSH analog, glutathione monoethyl ester. The untreated scorbutic guinea pigs live 21-24 days, whereas the GSH-analog supplemented guinea pigs survive longer, suggesting that GSH may alter AA

metabolism (88). GSH depletion will affect AA recycling ability [12] and DHA levels will rise, however, AA and DHA levels were not measured under these conditions.

GSH is required, at least in part, for AA recycling, *in vivo*. Newborn rats are less capable of AA synthesis than adult rats. GSH-depleted newborn rats had tissue damage and mitochondrial swelling in lung (89), liver (90), brain (89), and lens (91), presumably due to the buildup of H_2O_2 produced as a metabolic byproduct of aerobic respiration. In addition, in tissues of BSO-treated animals, AA was depleted and the DHA percentage of total AA was higher than in untreated controls. This demonstrated both the *in vivo* need for DHA recycling to AA, and indicated that non-GSH mechanisms of AA recycling are insufficient to regenerate cellular AA.

A. DHA Reductases

Cellular sources of DHA include cytoplasmic radical scavenging by AA (92); SDHA free radical disproportionation (92) [11], and extracellular DHA transported into the cell (76,77, Fig.2). DHA is rapidly reduced to AA in the cell. DHA may be reduced by GSH chemically (61), or by glutathione:dehydroascorbate oxidoreductases (E.C. 1.8.5.1)[12]. The extent of catalyzed and uncatalyzed DHA reduction in mammalian systems is unknown.

Observations support a strong role for the enzymatic recycling of DHA. *In vivo* AA recycling was demonstrated in γ-gulono lactone oxidase-deficient species where DHA supplementation prevented scurvy (90). In human lymphocytes and neutrophils, the rate of DHA uptake is proportional to that of cytosolic DHA reductase activities (93). Neutrophils have increases in cytoplasmic AA (up to 14 mM) after activation (75), although external AA stays at physiological levels (50-150 μM). This indicates that extracellular DHA is a source of AA.

Several mammalian DHA reductases have been partially or completely characterized since 1990. Two GSH-dependent DHA reductases, PDI and TT (80), also have dithiol-disulfide exchange activity (Section IV). PDI has TRX-like domains responsible for the dithiol-disulfide transfer activity, although TRX does not have DHA reductase activity. Three other GSH-dependent DHA reductases without dithiol-disulfide oxidoreductase activity have been isolated; an incompletely characterized 31 kDa rat liver protein (94), a 32-kDa human DHA (95) and a 23 kDa spinach chloroplast protein (96). Lipoamide dehydrogenase catalyzes the NADH-lipoic acid dependent reduction of DHA to AA (97), and 3α-hydroxy steroid dehydrogenase catalyzes the NADPH-dependent reduction of DHA (98). TT is the most efficient DHA reductase characterized to date (Kcat/Km) (Table 1).

The mechanism of DHA reduction and whether a free radical intermediate is required still need to be resolved. Site-directed mutagenesis studies (99,100) of the pig liver TT active site residues characterized the dithiol-disulfide exchange mechanism, and led to a postulated mechanism of DHA reduction (Fig.5). The proposed scheme for DHA reduction by TT is a nucleophilic attack followed by successive exchange with 2 molecules of GSH. First, the nucleophilic Cys^{22} attacks the DHA C2 carbonyl carbon, resulting in a thiohemiketal intermediate. Then GSH displaces reduced AA, leaving an enzyme mixed disulfide, which is reduced with a second molecule of GSH.

B. SDHA Reductases

The primary reaction known to generate SDHA is the one-electron reduction of the α-chromanoxyl radical to α-Tocopherol by AA in membranes. SDHA has not typically been detected in the cytosol. Several groups have reported the presence of a NAD(P)H-dependent ascorbate free radical reductase (monodehydroascorbate reductase, E.C. 1.6.5.4) (71)[13]

activity in cellular membranes such as chloroplasts (96,101-103), and mammalian mitochondria (104-106), microsomes (107,108), and Golgi (109).

Several SDHA reductases have been purified to homogeneity; 47 kDa protein from cucumber fruit (110), 39 kDa protein from soybean root nodules (111), and a 66 kDa protein from *Neurospora* (112). A 47 kDa SDHA reductase cloned from pea (113) has a deduced amino acid sequence highly homologous to soybean and cucumber fruit SDHARs as well as microbial flavin oxidoreductases, containing the GXGXXG/A NAD(P)H- and FAD-binding domains (114) of oxidoreductases. A SKL motif in the carboxyl terminus presumably targets this pea SDHA reductase to the peroxisomes.

Attempts at purification of mammalian SDHA free radical reductases have resulted in complete loss of activity, indicating that there is probably more than one protein involved in the electron flow from NADH to the ascorbyl radical (115). P-chloromercuribenzoate and 5,5'-dithiobisnitrobenzoic acid inhibit purified plant SDHARs, indicating that a thiol group is involved in the active site.

III. Glutathione

Explained here for the reader are the various functions for glutathione (GSH), as an antioxidant, and substrate for many cellular detoxification reactions; as well as the synthesis and transport of GSH. This is germane to the research question, as oxidative stress changes cellular GSH levels, and changes in GSH-containing enzymes are noted in drug resistance. In addition, an enzyme structurally and mechanistically related to TT may modulate the rate of L-cysteine transport into cells, effecting levels of GSH synthesis. Finally, GSH is a substrate for the dithiol-disulfide reductase activity of TT.

Table 1
Relative DHA Reductase activity

Parameter	Pig TT[a]	Bovine PDI[a]	Rat DHAR[b]	Rat 3αHSDH[c]	Rat LA/LDH[d]	Human DHAR[e]
M_r (kDa)	11.7	57	31	37.5		32
$K_{m(app)}$ (mM)	0.26	2.8	0.245	4.6	1.4	0.21
k_{cat}/K_m (M^{-1}/sec^{-1})	2.4×10^4	1×10^2	3.9×10^3	2.1×10^2	1.2×10^4	1.5×10^3

[a]Taken from (454). [b]Taken from (91). [c]Taken from (85). [d]Taken from (88). [e]Taken from (86). TT = thioltransferase, PDI = protein disulfide isomerase, DHAR = dehydroascorbate reductasae, 3αHSDH = 3α hydroxy-steroid dehydrogenase, LA/LDH = lipoic acid/lipoamide dehydrogenase.

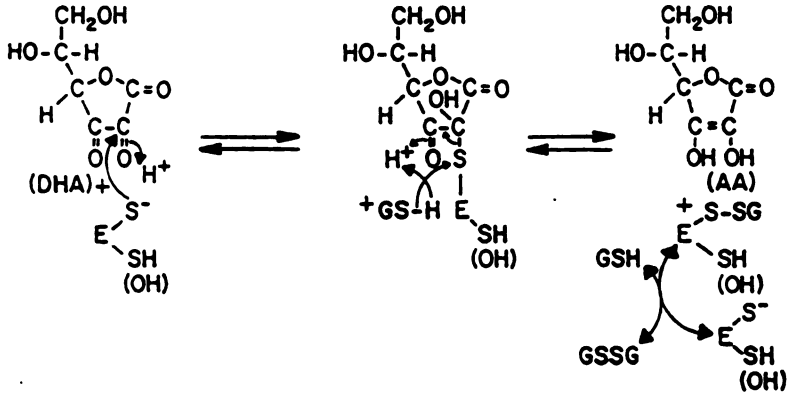


Fig. 5. Proposed mechanism of DHA reduction.
DHA reduction by TT is proposed to involve a nucleophilic attack by C^{22} on the C2 carbonyl carbon of DHA. GSH then displaces AA from the thiohemiketal intermediate, leaving an enzyme mixed disulfide, which is reduced with a second molecule of GSH through a dithiol-disulfide transfer mechanism. From (100), with permission.

Glutathione (GSH), or L-γ-glutamyl-L-cysteinyl-glycine (116), is a tripeptide at physiological pH with two negatively charged carboxyl groups and one positively charged amino group (Fig.1). The most reactive group of glutathione is the sulfhydryl of the cysteinyl residue, which can serve as an electron donor, therefore participating in reactions as a nucleophile, reductant and free radical scavenger.

Evolutionary comparisons indicate that GSH emerged when the atmosphere changed from anaerobic to oxygen-containing (116), suggesting that part of the function of GSH is to detoxify the reactive products of oxygen metabolism (117) (section I). Glutathione is present in high intracellular concentrations (0.1-10 mM) in higher eukaryotes (43,44). GSH or analogous thiols appear to be ubiquitous in all species examined, indicating an important universal function.

In addition to one- and two-electron reductions of ROS, GSH stores cysteine, and is a cofactor or substrate for many enzyme reactions. Tissue concentration of free cysteine is low, maintained in the range of 10-100 μM (118), as high tissue levels of cysteine may result in toxicity. Spontaneous oxidation of cysteine leads to H_2O_2; cysteine may interfere with the transport or function of some metal ions; and cysteine can also form mixed disulfides with essential protein sulfhydryls.

GSH is a cofactor for a large number of enzymes that perform a wide variety of metabolic functions, including glyoxalase, formaldehyde dehydrogenase, maleylacetoacetate isomerase, prostaglandin endoperoxide isomerase, and DDT dechlorinase (reviewed in 86). GSH is also a substrate for many enzymes that maintain cellular homeostasis or metabolize xenobiotics in plants and animals, including TT (Section IV C), GSTs and GSH-Pxs (Section I), and DHA reductases (Section II A).

The γ glutamyl cycle first described by Alton Meister (119) is involved in the synthesis, degradation, and recycling of glutathione (Fig.6). Glutathione is synthesized by two enzymatic reactions [14,15].

$$[14]\quad \text{L-glu} + \text{L-cys} + \text{ATP} \xrightarrow{\gamma\text{ glutamylcysteine synthetase}} \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + P_i$$

$$[15]\quad \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{gly} + \text{ATP} \xrightarrow{\text{glutathione synthetase}} \text{GSH} + \text{ADP} + P_i$$

GSH can be efficiently transported out of cells, and is postulated to function to protect cell membranes, and to provide a reducing environment in the immediate environment of the cell membrane in the extracellular space (reviewed in 86). GSH is not transported directly into cells, and only cells containing membrane-bound γ-glutamyl transpeptidase (120) such as the liver and kidney import γ-glutamyl amino acids.

Most normal cells have excess GSH. Studies selectively inhibiting γ-glutamyl-cysteine synthetase [14] using BSO found that cellular GSH export continues even when there is insufficient GSH synthesis, resulting in decreasing GSH levels in BSO-treated cells (119). Cells that are slower to export GSH have slower decreases in GSH when administered BSO. GSH depletion to about 5% of normal results in oxidative damage and cell sensitivity to radiation or chemical agents (122).

When erythrocytes are subjected to high levels of oxidative stress, intracellular GSSG levels initially increase, then excess GSSG is transported out of the cell into the plasma,

possibly to prevent protein mixed disulfide formation (123,124). GSSG can be transported out of the cell in an ATP-dependent manner through two incompletely characterized transport systems; one a low K_d, high V_{max} transporter, the other a high K_d, low V_{max} transporter (125). There is some debate in the literature as to whether GSSG transport occurs through the same mechanisms used to transport GSH adducts out of the cell. The high affinity, low V_{max} transporter for GSH adducts is competitively inhibited by GSSG, whereas the low affinity, high V_{max} transporter is not (126).

IV. Thiol-disulfide Oxidoreductases

Thiol-disulfide oxidoreductases (TDOR) are heat-stable enzymes postulated to be involved in cellular sulfhydryl homeostasis. TDOR enzymes include protein disulfide isomerase (PDI), thioredoxin (TRX), and TT. There has been a virtual explosion of information available about TDOR enzymes over the past five years; however, TT is least well characterized physiologically. Details about the related enzymes PDI and TRX are presented here as many activities and functions attributed to these other TDORs are postulated to apply to TT.

A common structural feature of TDOR enzymes is an active-site pair of cysteine residues in a 14 atom loop (CXXC) that can exist in the reduced (dithiol) and oxidized (intramolecular disulfide) forms. These TDOR enzymes, together with their electron donors, reductases, and NADPH have been shown to catalyze the reduction of disulfide bonds in a wide variety of substrates.

Three-dimensional models predict similar structures between TRX, TT, and PDI (127-129). The 2.2 Å crystal structure of pig liver TT (129) shows that the protein folds into an almost spherical α/β structure with a four-stranded β-sheet in the core, flanked on either side

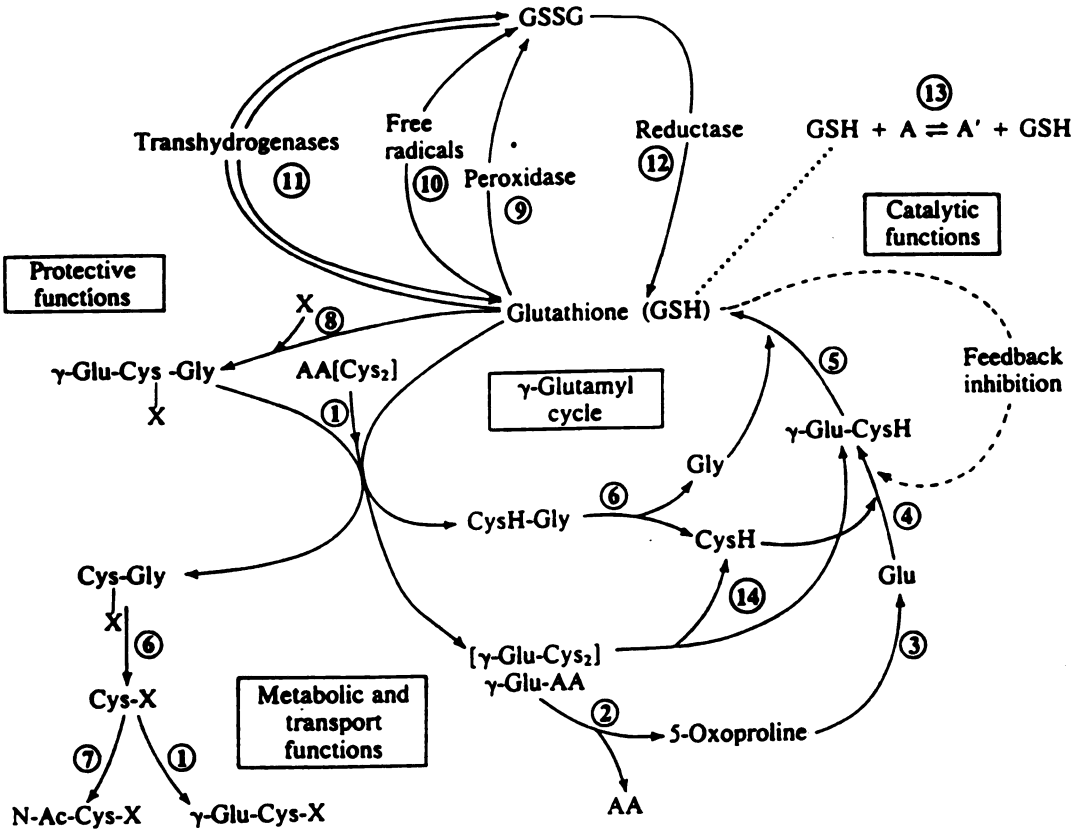


Fig. 6. Glutathione synthesis, functions, and the γ-glutamyl cycle.
Overview of the metabolism and function of GSH. GSH is synthesized by γ-glutamyl cysteine synthetase (4) and glutathione synthetase (5). GSH is exported by γ-glutamyl transpeptidase (1). Precursors for GSH synthesis are recycled GSH degradation producs as well as L-cysteien, which is transported into the cell by γ-glutamyl transpeptidase (1). GSH participates in many cellular defense mechanisms; as a substrate for GSTs (8), and GHS-Pxs (9), and functions directly as an antioxidant (10). GSH also is required as a cofactor in other reactions (13) such as those catalyzed by formaldehyde dehydrogenase, glyoxylase, maleylacetoacetate isomerase, DDT-dechlorinase, and prostaglandin endoperoxide isomerase. (AA= amino acids, X= compounds forming GSH-conjugates)
(Taken, with permission, from Holmgren, A., Branden, C.-I., Jornvall, H., and Sjoberg, B.-M., eds, *Thioredoxin and Glutaredoxin Systems: Structure and Function*, Raven Press, New York, 1986, p.340)

by helices. The active site disulfide bridge protrudes from the protein surface flanked by a hydrophobic area on one side, and by a cluster of charged amino acids on the other (Fig.7). The folding pattern is similar in TRX (130) and the TRX-like domains in PDI (128) (Fig.8), although the only sequence similarity is within the active sites. Interestingly, glutathione peroxidase (131) also has a similar folding pattern and a similar active site disulfide placement (132), even though it does not have dithiol-disulfide reductase activity. Even with similar tertiary structures, antibodies to TT, TRX and PDI do not cross-react (133).

PDI is predominantly located in the lumen of the rough and smooth endoplasmic reticulum (ER) and is loosely associated with the ER surface (134,135). TT and TRX are found primarily as soluble enzymes in the cytosol (136-145), although localization of TRX to the cytoplasmic membrane has been reported (145). The endoplasmic reticulum has a redox environment more oxidative than cytoplasm (44). Differences in cellular location, substrate specificity and redox potential of these TDOR enzymes, in addition to the redox states of the cellular compartments in which they are located, suggest separate physiological functions (147-149) (Table 2). Little is known about the regulation of these TDOR enzymes.

A. Protein Disulfide Isomerase

Protein disulfide isomerase (EC 5.3.4.1, PDI), also known as glutathione-insulin transhydrogenase (EC 1.8.4.2, GIT) (150), is a multi-functional protein first independently identified by Anfinsen (151), Straub (152) and Tomizawa (153). PDI has been purified to homogeneity from a variety of tissues and species (154-158). The enzyme is a homodimer with subunits of approximately 57 kDa, and is extremely acidic with a pI between 4.2 and 5.0 (155). Amino acid sequences from rat, bovine, chicken and human PDIs, and are identical in 406 of the 493 residues that comprise the mature subunits (reviewed in 159), indicating a

protein highly conserved throughout evolution. The PDI cDNA has been cloned from yeast (160), sea urchin eggs (161), rat pancreas (162) and human liver (163-166). Each monomer is comprised of several domains (reviewed in 159, Fig.9). At the N-terminal of the protein is a classical signal peptide sequence, followed by an internal domain highly homologous to TRX. A second internal domain following has high homology to mammalian estrogen receptors, which is followed by 200 residues of internal repeat sequences. Towards the C-terminal end of the protein another domain with high TRX homology is observed, adjacent to a very acidic region. Like other ER proteins, PDI possesses a carboxy-terminal KDEL sequence (167) which targets PDI to the lumen.

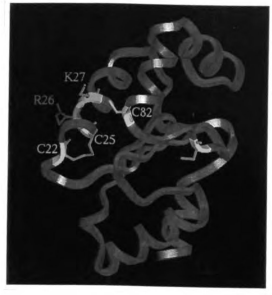
PDI is widely distributed throughout animal and plant tissues. Highest levels of PDI activity and protein are in tissues where synthesis of disulfide-containing proteins is predominant, such as wheat endosperm, chick embryo, as well as mammalian liver, pancreas, and lymphoid tissues (168). PDI has been reported to be induced during lymphocyte maturation when a strong immunogenic stimulus, bacterial lipopolysaccharide is administered (169), indicating that PDI may be induced under conditions of oxidative stress.

PDI catalyzes thiol-disulfide exchange reactions (153) *in vitro* in a broad range of protein substrates, leading to the isomerization of intramolecular disulfide bonds and the associated folding of nascent secretory proteins (168,170,171). PDI also functions *in vitro* as a chaperone-antichaperone (172,173), a glycosylation site binding protein (174), a thyroid hormone-binding protein (175), a Ca^{2+}-binding protein (176), and a DHA reductase (80, Section IIA).

The mechanism of PDI oxidoreduction (Fig.10) uses TRX as the electron donor, and a second molecule of TRX to regenerate PDI (177). Oxidized TRX is regenerated by TRX

Fig. 7. Predicted human TT structure.
A. Three-dimensional structure of pig liver TT. The active site cysteine residues (C22,C25) are shown in yellow (with a disulfide bridge), as are the two other conserved mammalian cysteine residues (C78,C82). P24 and F26 side chains are not shown; R26 and K27 are shown in red and orange, respectively.
B. Three-dimensional structure of human TT using the X-ray coordinates for pig liver TT and replacing non-conserved amino acids. The overall structure is the same as that of other thiol-disulfide oxidoreductases in the general folding pattern; an almost spherical α/β structure, with a four-stranded β sheet in the core, flanked by helices on either side. The active site cysteine residues, shown in yellow, form an intramolecular disulfide bridge. The three other cysteine residues are shown in yellow; the conserved C78, C82, and the N-terminal C7 near the active site. The other active site residues are shown in red (P23), green (Y24), and orange (R26,R27).

A.



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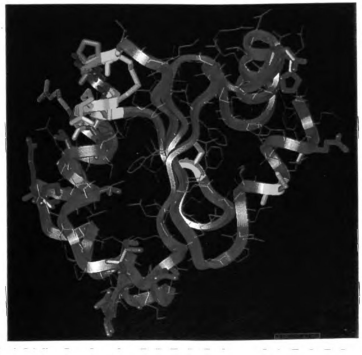

Fig. 7. Predicted human TT structure.

Fig. 8. TDOR enzyme folding similarities.
Schematic representations comparing polypeptide chain folds of (A.) Thioltransferase, (B.) T4 bacteriophage thioredoxin, and (C.) *E. coli* thioredoxin. The folding patterns and placement of active site residues are similar, despite different primary structures. β-strands are depicted as thick arrows, and α-helices are rectangular boxes. Striped helices are forward of the β-sheet, and clear helices are behind. The active site is represented by a circle. (From Katti, S.K., Robbins, A.H., Yang, Y.Y., and Wells, W.W. (1995) Protein Science, 4, 1998-2005, with permission.)

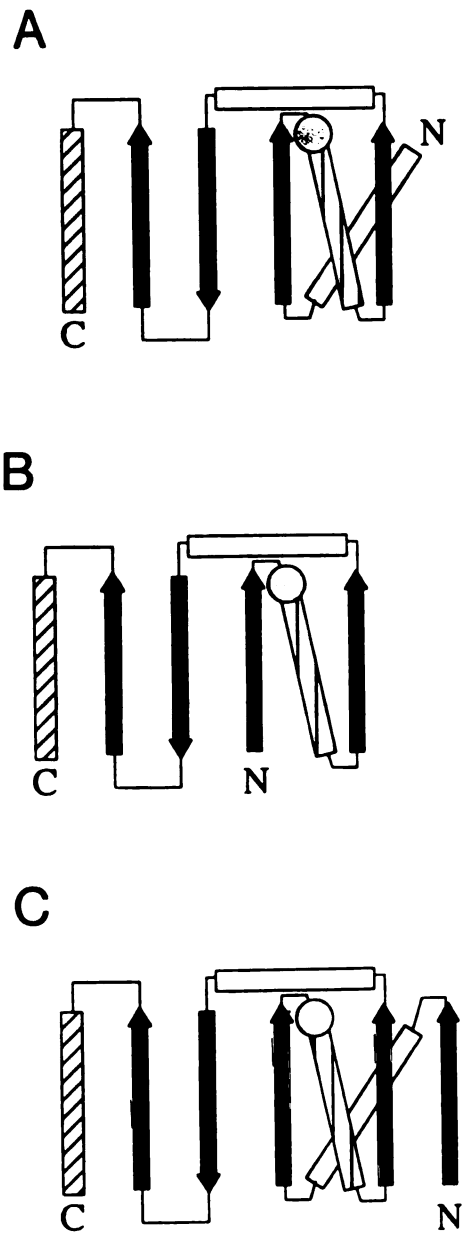

Fig. 8. TDOR enzyme folding similarities.

Table 2
Redox Potentials of TDOR Enzymes

Enzyme	Reduction potential
Pig liver thioltransferase	-0.159 $\pm$ 0.004 V
E. coli glutaredoxin	-0.260 V
Protein disulfide isomerase	-0.11 to -0.19 V

The redox potentials of the three TDOR enzymes indicate that for dithiol-disulfide oxidoreduction, TRX and TT are more effective in the cytoplasm, which is a more reducing environment than the ER; whereas PDI is more effective in the ER, which has a more oxidizing environment. (From Jung, C.-H., and Thomas, J.A. (1996) Arch. Biochem. Biophys. 335, with permission.)

reductase, an NADPH-dependent enzyme. Four WCGHCK sequences (178) homologous to the WCGPCK found in TRX (179) exist in native PDI. Mutagenesis studies have demonstrated that the cysteine residues in these motifs are the redox-active groups, that only one redox-active cysteine residue is required for efficient protein folding (180), and that the N- and C-terminal TRX-like domains are functionally non-equivalent (181), although each domain functions independently in catalysis (182,183). Whether or not there is cooperativity between these redox-active domains is not yet known.

B. Thioredoxin

TRX (TRX), an enzyme of approximately 12 kDa, has been purified, cloned and sequenced from a variety of species and tissues (reviewed in 137). Amino acid sequences have been determined for purified TRX from *Anabena sp 7119* (184), *Rhodobacter sphaeroides Y* (185), Rat liver (186), calf liver (187), human leukemic cells (ADF, 188), T4 phage (189,190), and a joint TRX-TRX-reductase in *Mycobacterium leprae* (191). TRX has been cloned from bacteria, yeast, plants, and mammals, including *E. coli* (192), *Bacillus brevis* (193), cyanobacterium *Anacystis ridulans* (194), yeast (195,196), spinach (197), and several human tissues (198,199).

TRX amino acid sequences from archaebacteria to yeast and humans are between 27-69% identical to *E. coli* TRX (127). The active site, highly conserved between prokaryotes and eukaryotes, is comprised of the amino acid sequence VDFXAXWCGPC(K/M/I)XP and conserved residues distant in primary structure. Mammalian TRXs also contain two additional C-terminal cysteine residues which easily undergo oxidation to disulfides with subsequent aggregation and inactivation of the protein (200). TRX is an acidic protein, with a redox active Cys containing an acidic pK_a (around 6.7 in human TRX). TRX uses NADPH

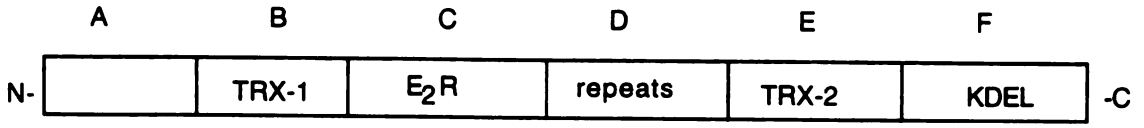

Fig. 9. PDI primary structure.
PDI is a homodimer with subunits of approximately 57 kDa. Each monomer is composed of several domains. A. N-terminal signal sequence. B. TRX-like domain 1. C. Estrogen-receptor-like domain. D. Internal repeat sequences. E. TRX-like domain 2. F. Acidic region with KDEL that targets PDI to the ER.

as an electron donor, and oxidized TRX is directly regenerated in a NADPH-dependent manner by TRX reductase (Fig.11).

TRX is found in multiple systems involved in the redox regulation of cellular functions. Physiological roles have been postulated for TRX including dithiol reducing activity, protein posttranslational modification for secretion, growth stimulation, facilitation of interactions between transcription factors and their target DNA sequences, signal transduction, cytokine activity, and a role in cellular protection from cytotoxic agents.

TRX functions as an endogenous reducing agent (201). Sulfate reduction in *E. coli* relies upon either TT or TRX (202). Deletion of both TRX genes in yeast results in methionine auxotrophy indicating that in yeast, TT cannot assimilate sulfate (195). Several mammalian species such as mouse contain one functional TRX gene, and a processed pseudogene (203), but the biological significance is not yet understood.

Plants contain multiple TRXs essential for photosynthetic growth (204). TRX regulates photosynthetic chloroplast enzymes such as fructose 1,6 bisphosphatase, phosphofructokinase, glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and NADP+-malate dehydrogenase by dithiol-disulfide exchange (205).

E. coli TRX reduces exposed S-S bridges in a variety of proteins *in vitro* (137), including oxidized ribonuclease (152), choriogonadotropins (137), proteolytic enzymes (206), factor VIII and other coagulation factors (207,208), glucocorticoid receptor (209), Vitamin Ko and Vitamin K reductase (210), and insulin (211,212). Mammalian TRX has broader substrate specificity than the *E. coli* enzyme and will react with non-thiol compounds such as alloxan, menadione (213), and selenite (214). TRX also functions as an electron donor to plasma GSH-Px (215), involved in detoxification of peroxides (Section I). TRX reductase

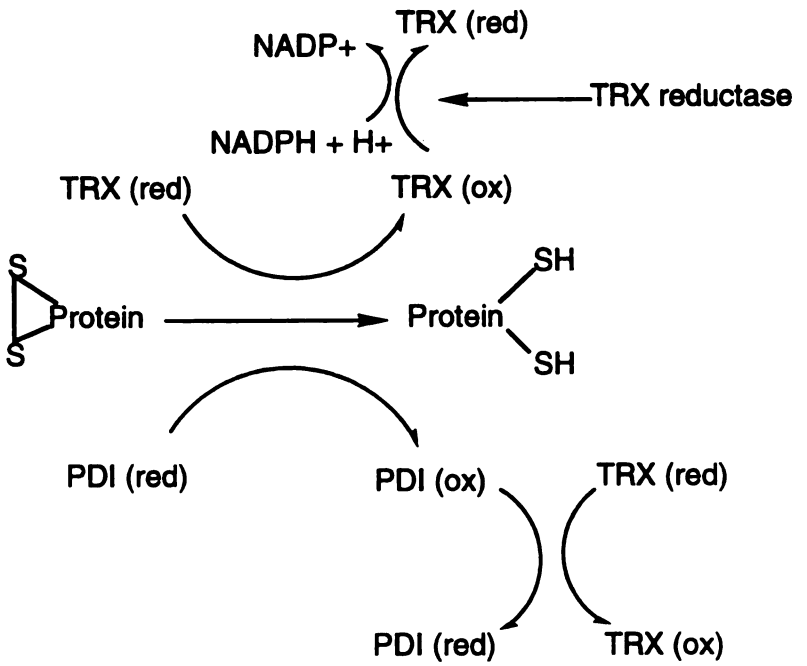

Fig. 10. PDI oxidoreduction.
TRX donates electrons to PDI, which catalyzes the reduction of protein disulfides. Oxidized PDI is regenerated by a second molecule of TRX. TRX is reduced by NADPH-dependent TRX reductase.

will directly reduce lipid hydroperoxides by NADPH, a new pathway for detoxification which is strongly stimulated by selenols (216). These selenols are then reduced by TRX. TRX preferentially reduces oxidized protein monothiols that have been converted to sulfenic or sulfinic residues over thiols oxidized to disulfides (217).

TRX is found in high levels in nerve cells and axons corresponding to areas of neurotransmittor synthesis and secretion (138), indicating that TRX may be involved in the posttranslational modification of proteins prior to secretion. Immunolocalization of TRX in secretory cells revealed that TRX is associated with intracellular membranes (145). TRX has been found localized to plasma membranes of nucleated cells (218). TRX-like domains are found in PDI (Section IV A), a multi-functional ER protein involved in disulfide formation, protein secretion, and protein folding.

There is growing evidence that TRX is involved in eukaryotic growth control. Exogenous TRX functions as a growth factor in many cell types. ^{125}I TRX added to media is taken up by cultured cells (219). Recombinant TRX stimulates proliferation in murine 3T3 fibroblasts and human cancer cell lines when applied at 100 nM levels (reviewed in 220). Exogenous TRX added to serum-free lymphoblastoid and Burkitt's cell lines stimulates DNA synthesis (221,222) and redox-dependent proliferation (223,224). In contrast, elevated TRX secretion results in growth arrest and morphological changes in human HepG2 hepatoma cells (225).

TRX levels are increased in tumors (226-229) when compared with those in normal tissues, suggesting that TRX may function as a growth factor for human cancers. Redox activity of TRX is essential in lymphocyte immortalization by human T-lymphocyte virus-1 and Epstein-Barr virus (230). TRX is expressed at high levels in rapidly dividing cells, such

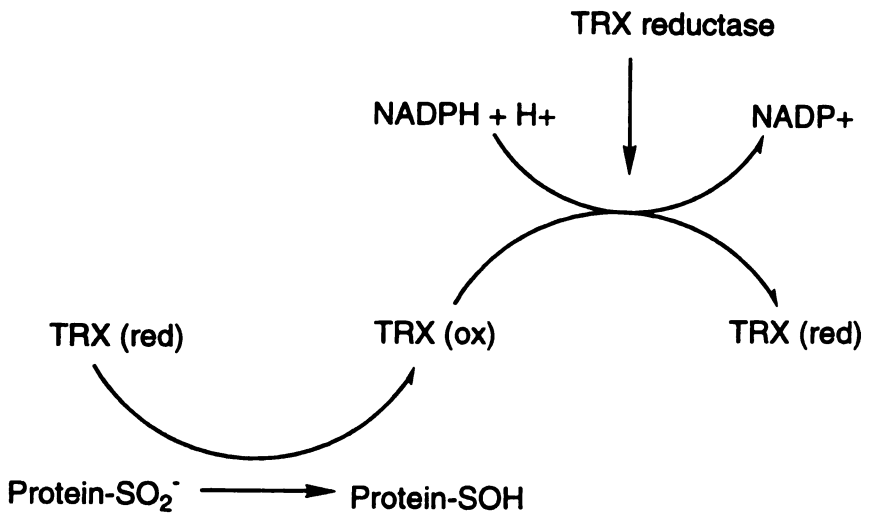

Fig. 11. TRX oxidoreduction.
TRX reduces oxidized protein sulfhydryl groups, especially those in sulfenic and sulfonic oxidation states. The electron donor for TRX is NADPH. Oxidized TRX is reduced by NADPH-dependent TRX reductase.

as human lymphoid and hematopoietic cells, and at low levels in resting cell types, such as lymphocytes or monocytes (231).

TRX can be excreted by normal and tumor cells in culture (232) using a leaderless secretory pathway (233). For example, CD4+ T-cells secrete TRX, promoting growth in normal and leukemic B cells (234). ADF, an autocrine growth factor identical to TRX, is produced in human T-lymphocyte virus I-infected cells. ADF synergizes with interleukin-1 and interleukin-2, cytokines involved in proliferation (223,235-236), induces interleukin 2-receptor α chain production and stimulates cell growth (237-238). Interestingly, interleukin 2-receptor α chain itself is under strong control of NFκB, a redox-sensitive transcription factor.

Several mechanisms are proposed for growth control by TRX. First, reduced TRX may activate protein kinase C (PKC), which subsequently activates phophoinositol-specific phospholipase C activity (221). PKC has several thiol-rich regions in each of the catalytic and regulatory subunits containing critical cysteine residues that are extremely sensitive to oxidative modification (239-241). PKC activity has been shown to be activated and inactivated through oxidative changes (242,243). The zinc-thiolate structure of PKC is required to bind phorbol ester and DNA (244). TRX increases B-cell proliferation through a PKC-dependent mechanism, as the PKC inhibitors staurosporin and calphostin C both block TRX-induced proliferation.

A second potential mechanism is based on the observation that TRX promotes L-cysteine transport into cells, increasing intracellular GSH content. Thiol compounds such as L-cysteine and GSH are involved in the activation and cell cycle progression of stimulated lymphocytes (224,245). There is a close association between the TRX and GSH systems, and

redox regulation by these systems plays an important role in regulating cell proliferation and activation. Oxidation of cellular thiols including TRX with diamide induces apoptosis in Jurkat T cells and human lymphoblasts (246).

Observations in yeast support TRX involvement in cell cycle regulation. There are two TRX genes in yeast, which are 74% identical. Loss of either of these genes affects neither cell growth nor morphology, however, deletions in both alter cell cycle and morphology. G1 phase is absent, and the S phase is 3-fold longer, suggesting slow DNA replication. The overall generation time increases by 33%, and there is a significant increase in cell size, and a greater proportion of budded cells (195).

These observations in yeast also support the theory that TRX is involved in DNA synthesis and hence regulates the rate of proliferation. TRX catalyzes the reduction of ribonucleotide reductase in bacteria and mammals (247), and is therefore involved in providing deoxynucleotides critical for the DNA synthesis that occurs in the S phase of the cell cycle.

TRX has other effects related to development and growth in varied systems. A *Drosophila* TRX homolog, "deadhead", also indicates a role for TRX in cell cycle and development. "Deadhead" is not essential for viability, but is essential for female meiosis and early embryonic development *in vivo* (248). *Drosophila* "deadhead" eggs are fertilized, but cannot complete mitosis. TRX is involved in the regulation of eosinophil migration (249), and is expressed in the ovary throughout the menstrual cycle (250). TRX also serves a role in bacteriophage replication and assembly. T7 DNA polymerase contains p5 protein and TRX in a 1:1 relationship, where TRX confers processivity to the oligomeric complex (251). The redox activity of TRX is separate from the role in T7 replication. TRX may help keep the p5

protein in the DNA binding cleft, similar to the function for the 'thumb-like" domain in *E. coli* DNA polymerase I Klenow fragment (reviewed in 251). Filamentous phage assembly also requires host TRX.

TRX-like domains are found in a phosphoinositide-specific phospholipase C (252) and in two gonadotropin hormones (253), introducing the possibility that TRX is involved in signal transduction. Insulin-like growth factors and their binding proteins contain vicinal cysteine residues similar to those found in PDI and TRX, and have dithiol-disulfide exchange activity (254). Thiol redox reactions play a role in regulating conformational changes in both the insulin receptor and possibly in the insulin-like growth factor receptor, indicating that intrinsic dithiol-disulfide exchange may be important in activating these receptor classes (254). Redox changes in thiols are involved in lymphocyte activation (236). T-cell F_c receptor signal transduction occurs via tyrosine kinase and TRX redox regulation (255). Redox regulation of $p56^{lck}$, a member of the src family of tyrosine kinases also occurs in T cells (256).

TRX may have a role in transcription. *In vitro*, TRX can modulate transcription factor IIIC DNA binding (257), and activate the human immunodeficiency virus-1 enhancer binding protein via thiol-redox mechanisms (258) as well as modulate glucocorticoid receptor steroid binding (209). Glucocortocoid receptor DNA binding requires reduced -SH groups (259).

TRX is postulated to have effects on both the activation and the DNA-binding of NFκB and activator protein-1 (AP-1), transcription factors involved in the inducible expression of genes responsive to oxidative stress as well as cellular defense mechanisms. Both AP-1 and NFκB are easily inhibited by the oxidation of sulfhydryl groups, and reactivated by thiols (260). Redox effects regulate the amount of these transcription factors, their DNA-binding affinities, and their nuclear or cytoplasmic distributions.

The NFκB/Rel/dorsal oncoprotein/transcription factor family binds target DNA sequences through a conserved basic region with a single cysteine flanked by basic residues (RXXRXRXXC) (261). NFκB is composed of two subunits, p65 and p50. Transcriptional activation of NFκB-dependent genes is usually induced by the dissociation of the inhibitory IκB protein from the cytoplasmic heterodimer, and the subsequent translocation of active NFκB into the nucleus.

DNA damaging agents such as oxidative stress and UV irradiation induce cellular immediate early genes including *c-fos* and *c-jun*. Fos and Jun are individual DNA-binding subunits that interact with each other through a leucine-zipper to form AP-1. DNA binding by Fos and Jun (AP-1) is regulated posttranslationally by phosphorylation of the C-terminal of Fos as well as redox changes (260). A lysine-cysteine-arginine sequence in the C-terminal DNA-binding domain is conserved in all the members of the family except the constitutively active viral protooncogene, *v-jun*.

The cationic environment surrounding the critical cysteine residue in both transcription factors renders the thiol highly reactive and particularly susceptible to oxidation. This cysteine binds DNA when reduced; oxidized cysteine is not permissive for DNA binding.

Oxidative conditions potentiate the activation of NFκB and AP-1 in intact cells, and have mixed effects on DNA binding activity *in vitro*. In tissue culture, NFκB and AP-1 activities (both DNA binding and transcriptional transactivation) are modulated by exogenous application or transient expression of TRX in a dose-dependent manner (262). GSSG also modulates activation and binding in intact cells and *in vitro* (263). Experiments suggest that the two transcription factors differ in the redox potentials of their critical cysteine residues, their sensitivity to oxidative inactivation, and responses to antioxidants and exogenous TRX

(231,263-269).

Activation by TRX involves PKC-independent *de novo* transcription of *c-jun* and *c-fos* (262). TRX affects *in vitro* AP-1 DNA binding (264) through the ubiquitous nuclear redox factor Ref-1. Ref-1 is subject to redox control, and stimulates Fos-Jun and NFκB interactions as well as apurinic/apyrimidinic endonuclease DNA repair activity. Ref-1 activity is augmented by TRX.

Another series of TRX functions are cytoprotective. TRX protects human macrophages from human immunodeficiency virus expression (270), prevents TNFα-induced cytotoxicity (271), and inactivates some toxic venoms (272). All venom neurotoxins inactivated by TRX (snake, scorpion, and bee) are disulfide-containing proteins. *In vitro* reduction of these toxins increases their susceptibility to tryptic proteolysis, and decreases toxin activity, whether via phospholipase-A_2 (B-bungarotoxins in bee and snake venoms) or acetylcholine receptor (α-bungarotoxins) mechanisms.

TRX attenuates ischemia-reperfusion injury in culture (273). TRX expression is elevated in gerbil astroglial cells after transient global ischemia (274). Neuroprotection from ischemia and reperfusion injury in culture by central nervous system glial cells has been correlated to secreted TRX, increased cellular GSH, and requires reducing conditions (275). GSH and TT are incapable of protecting neurons subjected to injury without TRX. Exogenously added B-mercaptoethanol, a reducing agent, increased cellular survival, whereas added BSO, which reduces GSH levels, decreased survival.

TRX also increases resistance to cis-diamine dichloroplatinum (II)-induced cytotoxicity (276). Antisense stable transfection of TRX into drug-resistant kidney cells found decreased TRX expression correlates with increased sensitivity to drugs that generate ROS,

such as cisplatin, mitomycin C, doxorubicin, and etoposide (277). One mechanism for this resistance is that TRX functions as an endogenous radical scavenger (278), and protects endothelial cells from injury by H_2O_2 and other ROS released by activated neutrophils (279).

TRX is induced by a number of stimuli *in vivo* and *in vitro*. Oxidative stresses such as H_2O_2 and ischemia result in TRX induction in rat retina (280), keratinocytes, lymphoid cells (281), and retinal pigment epithelial cells (282). TRX induction was localized to the mitochondria in the retinal pigment epithelial cells (284). Retinol stimulated TRX expression 8-10 fold after 4 hours in monkey conducting airway epithelial cells (283) without concurrent protein synthesis. In yeast, the YAP1 transcription factor responds to oxidative stress by elevating expression levels of TRX2, resulting in resistance to hydroperoxides and thioloxidants (216). Yeast cells deficient in the YAP1 were hypersensitive to thioloxidants and hydroperoxides.

Site-directed mutagenesis and NMR studies have determined protein conformations and functions for several active site residues (284-290). Replacement of the TRX active site proline with histidine, mimicking the PDI active site, increased the disulfide isomerase activity 10-fold (291). The redox-active cysteine 31 in human TRX has been determined to be responsible for both the ROS-reducing (H_2O_2) and the protein refolding activities, indicating a redox requirement for radical and ROS reduction (284). Replacement of either active site cysteines in human TRX competitively inhibited TRX reductase and the mitogenic activity (290). Replacement of lysine 36 in human TRX affected growth rates and reduction rates but did not change the redox activity (289).

As most of these experiments have been carried out *in vitro*, or in tissue culture conditions, the biological functions of TRX are continuing to be investigated by many

research groups. It remains to be seen how significant these TRX activities are *in vivo*.

C. Thioltransferase

Transhydrogenases, thiol-disulfide oxidoreductases or thiol-disulfide exchange enzymes were first documented by Racker in 1955. Racker found an enzyme of approximately 12 kDa molecular weight in beef liver cytosol that catalyzed the conversion of homocystine to homocysteine in the presence of GSH, GSSG reductase, and NADPH which he termed glutathione cystine transhydrogenase (EC 1.8.4.1) (292). Since then it has been determined that the GSH-dependent reduction of low-molecular weight thiol substrates was actually not a transhydrogenase reaction [16],

[16] $RSSR + 2\ GSH \rightarrow 2\ RSH + GSSG$

but actually two consecutive ionic S_N2 displacement reactions [17,18],

[17] $RSSR + GSH \rightarrow RSSG + RSH$

[18] $RSSG + GSH \rightarrow GSSG + RSH$

and the resulting GSSG is reduced by GSSG reductase using NADPH as the electron donor (293) [19], regenerating the reduced GSH.

[19] $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

Elucidation of this mechanism resulted in a enzyme title change from "transhydrogenase" to "thioltransferase" (E.C. 1.8.4.1).

Glutaredoxin, another cytosolic protein of approximately 12 kDa that also catalyzes the GSH-dependent reduction of mixed protein-disulfides and uses GSSG reductase and NADPH to regenerate GSH, was discovered in *E. coli* TRX deletion mutants that still could reduce ribonucleotide reductase (294). Comparison of amino acid sequences (295), catalytic activities, size, immunoreactivities, pIs, and substrate preferences determined that TT and glutaredoxin were two names for the same enzyme.

Currently, TT has been cloned from many varied species, including three different genes in *E. coli* (296,297), as well as from *Haemophilus influenzae* (298), *Pyrococcus furiosus* (299), T4 bacteriophage (300,301), vaccinia virus (302), *Trypanosoma cruzii* (303), yeast (304), rice (305), castor bean (306), pig liver (307), and several human tissues (308-312) with 48.6 to 90.7% similarity and 25.3 to 82.2% identity to human placental TT. TT is found in all GSH-containing organisms, as well as in one GSH-negative species, *methanobacterium thermautrophum* (313). TT amino acid sequences are 80-91% conserved among mammalian species (Fig.12).

The active site amino acid consensus sequence (314) CP(Y/F)C is conserved in bacterial, mammalian and plant TTs (Fig.12). Mutagenesis studies on recombinant pig liver TT revealed that these cysteine residues (C^{22} and C^{25}) are the redox active residues, and that C^{22} is essential for catalytic activity (100). Pig liver TT C^{22} has an unusually acidic pK_a of 3.8 (314), facilitated by the proximal R^{26}. Two other C-terminal half-cystine residues exist in all mammalian TT, but site-directed mutagenesis of pig TT, replacing these residues with alanine determined that they were not redox active. Human TT contains a third half-cystine residue

at residue 7, possibly increasing the protein susceptibility to oxidation (315), and decreasing the heat stability of the protein (316).

In the presence of GSH, TT catalyzes the formation of GSSG from disulfide substrates such as cystine, S-SO_3-cysteine, hydroxyethyldisulfide, cysteinyl-bovine serum albumin mixed disulfide, oxidized ribonucleotide reductase and various GSH-containing mixed disulfides (317-319). Cysteamine is an alternate reductant for TT (320). TT displays selectivity for glutathionyl substrates and catalyzes their reduction more efficiently than TRX (321).

Site-directed mutagenesis and isotope studies revealed a potential mechanism for dithiol-disulfide transfer (99,100) (Fig.13). Reduced TT first reacts with a dithiol substrate, such as a protein mixed disulfide, then with a thiol substrate such as GSH. Oxidized TT is regenerated by a second molecule of GSH, producing GSSG, which is then reduced by GSSG reductase.

Structural studies have supported this model. Two-dimensional NMR results show few changes between oxidized and reduced TRX, as major differences are associated only with active site and neighboring residues (323). TT-mixed disulfides formed with GSH using recombinant *E. coli* TT are visible by NMR (324,325). Solution structures show the GSH-binding site is conserved between human and *E. coli* TT (310). Studies investigating TT-catalyzed GSH and protein-GSH mixed disulfides determined that the γ-L-glutamyl-L-cysteinyl moiety of GSSG and GSH-mixed disulfides is an essential determinant for recognition by TT (326).

TT inhibitors include diamide and other sulfhydryl-oxidizing agents. Anti-inflammatory and anti-histaminic drugs also inhibit purified TT non-competitively (327), as

Fig. 12. TT amino acid sequence comparisons.
TT amino acids sequences are highly conserved, ranging from 48.6 to 90.7% similarity to human TT in species as diverse as GSH-negative bacteria, viruses, yeast, plants, and mammals. The active site CPY/FC is conserved in all species, and all mammalian TTs sequenced to date contain an additional conserved C-terminal CIGGC motif. Amino acid sequences and deduced amino acid sequences are aligned for human (deduced, 308), pig (deduced, 307), bovine (295), rabbit (322), yeast (deduced, 304), vaccinia virus (deduced, 302), castor bean (deduced, 306), rice (deduced, 305), *H. influenzae* (deduced, 298), *E. coli* (deduced, 297,298), *Pyrococcus furiousus* (deduced, 299), and *Methanobacterium thermautrophum* (deduced, 313) using the Genetics Computer Group (GCG, Wisconsin) programs.

```
          1                                                                  60
   human  .......... .......... .......... .......... .......... ..........
     pig  .......... .......... .......... .......... .......... ..........
  bovine  .......... .......... .......... .......... .......... ..........
  rabbit  .......... .......... .......... .......... .......... ..........
   yeast  .......... .......... .......... .......... .......... ..........
vaccinia  .......... .......... .......... .......... .......... ..........
  castor  .......... .......... .......... .......... .......... ..........
    rice  .......... .......... .......... .......... .......... ..........
 haemoph  .......... .......... .......... .......... .......... ..........
   ecoli  .......... .......... .......... .......... .......... ..........
   pyrof  MGLISDADKK VIKEEFFSKM VNPVKLIVFV RKDHCQYCDQ LKQLVQELSE LTDKLSYEIV
 methano  .......... .......... .......... .......... .......... ..........

          61                                                                 120
   human  .......... .......... .......... .......... .......... ..........
     pig  .......... .......... .......... .......... .......... ..........
  bovine  .......... .......... .......... .......... .......... ..........
  rabbit  .......... .......... .......... .......... .......... ..........
   yeast  .......... .......... .......... .......... .......... ..........
vaccinia  .......... .......... .......... .......... .......... ..........
  castor  .......... .......... .......... .......... .......... ..........
    rice  .......... .......... .......... .......... .......... ..........
 haemoph  .......... .......... .......... .......... .......... ..........
   ecoli  .......... .......... .......... .......... .......... ..........
   pyrof  DFDTPEGKEL AKRYRIDRAP ATTITQDGKD FGVRYFGLPA GHEFAAFLED IVDVSREETN
 methano  .......... .......... .......... .......... .......... ..........

          121                                                                180
   human  ...MAQEFVN CKIQPGKVVV FIKPTCPYCR .RAQEILSQL PIKQGLLE.. FVDITATNHT
     pig  ...MAQAFVN SKIQPGKVVV FIKPTCPFCR .KTQELLSQL PFKEGLLE.. FVDITATSDT
  bovine  ....AQAFVN SKIQPGKVVV FIKPTCPYCR .KTQELLSQL PFKQGLLE.. FVDITAAGNI
  rabbit  ....AQEFVN SKIQPGKVVV FIKPTCPYCR .KTQEILSEL PFKQGLLE.. FVDITATSDM
   yeast  VSQETVAHVK DLIGQKEVFV AAKTYCPYCK ATLSTLFQEL NVPKSKAL.. VLELDEMSNG
vaccinia  ...MAEEFVQ QRLANNKVTI FVKYTCPFCR .NALDILNKF SFKRGAYE.. IVDIKEFKPE
  castor  ...MAMTKTK ELVSSNAVVV FSKTYCPYC. TSVKKLLDQL G...AKYK.. VVELDTESDG
    rice  ...MALAKAK ETVASAPVVV YSKSYCPFC. VRVKKLFGQL G...ATFK.. AIELDGESDG
 haemoph  .......... .....MFVVI FGRPGCPYC. VRAKNLAEKL KGEVADFDYR YVDIHAEGIT
   ecoli  .......... .....MQTVI FGRSGCPYC. VRAKDLAEKL SNERDDFQYQ YVDIRAEGIT
   pyrof  LMDETKQAIR NIDQDVRILV FVTPTCPYCP .....LAVRM AHKFAIENTK AGKGKILGDM
 methano  .......... ....VVKIEV FTSPTCPYCP .....MAIEV VD....EAKK EFGDKIDVEK

          181                                                                240
   human  NEIQDYLQQL TGAR..TVPR VFIGKDCIGG CSDLVSLQQS GELLTRLKQI GALQ......
     pig  NEIQDYLQQL TGAR..TVPR VFIGKECIGG CTDLESMHKR GELLTRLQQI GALK......
  bovine  SEIQDYLQQL TGAR..TVPR VFIGQECIGG CTDLVNMHER GELLTRLKQM GALQ......
  rabbit  SEIQDYLQQL TGAR..TVPR VFLGKDCIGG CSDLIAMQEK GELLARLKEM GALRQ.....
   yeast  SEIQDALEEI SGQK..TVPN VYINGKHIGG NSDLETLKKN GKLAEILKPV FQ........
vaccinia  NELRDYFEQI TGGR..TVPR IFFGKTSIGG YSDLLEIDNM DALGDILSSI GVLRTC....
  castor  SEIQTALAEW TGQR..TVPN VFIGGKHIGG CDSTTAKHSQ GQLVPLLTEA GAV.......
    rice  SELQSALAEW TGQR..TVPN VFINGKHIGG CDDTLALNNE GKLVPLLTEA GAIASSAKTT
 haemoph  KE...DLSKS VGKPVETVPQ IFIDEKPIGG CTDFEALMKE QFGIVA.... ..........
   ecoli  KE...DLQQK AGKPVETVPQ IFVDQQHIGG YTDFAAWVKE NLDA...... ..........
   pyrof  VEAIEYPEWA DQYNVMAVPK IVIQVNGEDR VEFEGAYPEK MFLEKLLSAL S.........
 methano  IDIMVDREKA IDYGLMAVPA IAI....DGV VRFVGAPGRE ELFEAISDEI ..........

          241
   human  ...
     pig  ...
  bovine  ...
  rabbit  ...
   yeast  ...
vaccinia  ...
  castor  ...
    rice  ITA
 haemoph  ...
   ecoli  ...
   pyrof  ...
 methano  ...
```

Fig. 12. TT amino acid sequence comparisons.

Fig. 13. TT dithiol-disulfide transfer mechansim.
E = TT, S^- = thiolate anion of C^{22} (TT), SH = sulfhydryl of C^{25} and OH = the hydroxyl group of a C25S TT mutant. RSSR = disulfide substrate, RSH = reduced product, GSH = glutathione, GSSG = glutathione disulfide, IAM = iodoacetamide, and HI = hydriodic acid. Studies in pig liver TT resulted in this proposed mechanism of action. (1,5): Reduced TT preferentially reacts with glutathionyl-containing dithiol substrates, such as protein mixed disulfides. (4): The enzyme mixed disulfide then reacts with a molecule of GSH to produce reduced protein/product, and a TT mixed disulfide with GSH. (5) A second molecule of GSH reduces TT, producing GSSG, which can be regenerated by GSSG reductase (not shown). From (100) with permission.

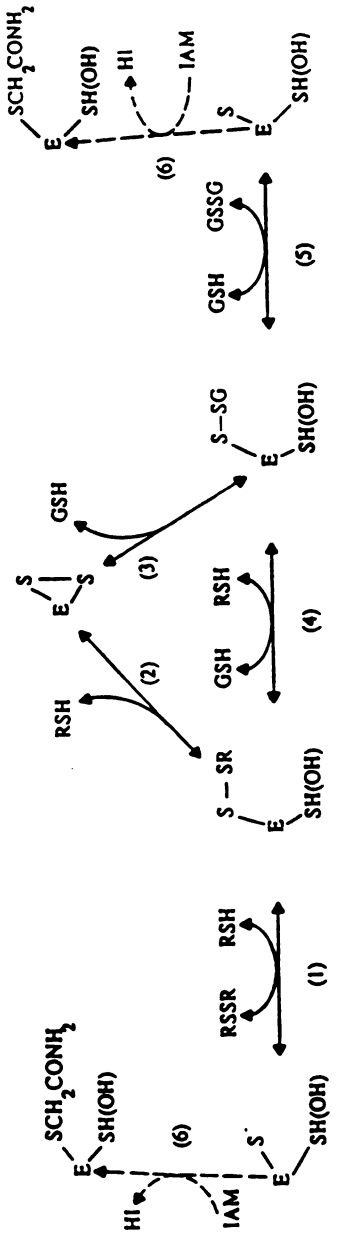

Fig. 13. TT dithiol-disulfide transfer mechanism.

well as chloramphenicol, an antibiotic (328), and cisplatin, an antitumor drug (329). No specific TT inhibitor has been discovered to date.

D. Postulated physiological roles for TT:

As this doctoral research commenced, no *in vivo* function for TT was established. Postulated physiological functions include a role in protein sulfhydryl maintenance, protection of cytoplasmic proteins from oxidative stress, regulation through posttranslational modification of critical cysteine residues, signal transduction, and cancer through growth regulation and resistance to anti-cancer drugs. A structurally and mechanistically related protein, TRX, has been examined in more detail to date; many of these functions postulated for TT are thus extensions of functions attributed to TRX.

1. Protein processing:

TT may be involved in degradation and posttranslational modification of proteins. Protein degradation may involve reduction of disulfides which maintain the native structure of proteins by TT (330). TT may complete posttranslational processing of cytosolic protein domains produced in the ER lumen. Since the ER is a more oxidizing environment (44) than the cytosol, nascent protein sulfhydryl groups could have GSH adducts (protein-SSG). As the protein enters the cytoplasm, TT may catalyze the GSH-dependent reduction of protein-SSG derivatives (146).

2. Protection against cellular oxidative stress:

Proteins form mixed disulfides as an early response to oxidative stress (reviewed in 331). This has been termed S-thiolation, and occurs primarily with GSH through a mechanism other than dithiol-disulfide transfer, possibly as an oxidation-reduction reaction with protein thiyl radicals. TT-catalyzed reduction or deglutathionylation of protein-SSG disulfides formed

under oxidative stress could return proteins to their native state. TT is an efficient deglutathionylase under physiological conditions (146,331-335), removing GSH adducts from carbonic anhydrase II, actin, creatine kinase, GAPDH, phosphorylase b and glutathione S-transferase. For example, TT can effectively catalyze the reduction of hemoglobin-glutathione disulfide adduct, a byproduct of oxidative stress in red blood cells (336). TT has a deglutathionylation rate more than 10-fold that of TRX and PDI. The specificity of deglutathionylation depends on the type of thiol modification; TT is more effective with protein-GSH mixed disulfides, whereas TRX and PDI are more effective with sulfhydryls in varied oxidation states, such as RSOH, $RSO_2^{\cdot}$, and $RSO_3^{\cdot}$ (146,321).

A second role in protection from oxidative stress is related to the DHA reductase activity (Section II) of TT (80). TT regenerates AA, a cellular antioxidant, from DHA. TT is responsible for much of the DHA reduction in normal human neutrophils (312).

TT, GSSG reductase and GST are inducible in liver by a variety of agents including phenobarbital and the dietary antioxidant BHA (2,3-t-butyl-hydroxyanisole) (337). TT is highly inducible by ultraviolet B radiation in rat keratinocytes (338), characteristic of immediate early genes, and genes induced by DNA damage.

Finally, immune complexes have been shown to stimulate TT release from rabbit polymorphonuclear leukocytes (328), under conditions of oxidative stress. TT release was not dependent on cytolysis, indicating an extracellular function for TT in response to oxidative stress.

3. Redox regulation of enzyme function:

Thiol-disulfide exchange reactions may regulate enzymatic activities in a manner analogous to phosphorylation-dephosphorylation mechanisms of control. Studies investigating

enzyme regulation by thiol oxidation/reduction support this theory. Bulk transfer of GSH-equivalents to and from total cellular protein was demonstrated (339). Disulfide exchange catalyzed by TT has reactivated many oxidized (inactive) proteins *in vitro*, such as ribonucleotide reductase (340), pyruvate kinase (316), phosphofructokinase (217,316), GAPDH (217,341), ornithine carboxylase (342), and glutathione S-transferase (341,343). Phosphofructokinase is inhibited by thiol oxidation, whereas the opposing regulatory enzyme, fructose 1,6-bisphosphatase is activated (344,345). TT and TRX both reactivate phosphofructokinase and ribonucleotide reductase *in vitro*, however TT is more efficient than TRX with respect to both substrates (149). TT may play a role in the oxidative activation/deactivation of metabolic and regulatory enzymes.

4. Role in signal transduction:

TT could affect signal transduction pathways by reducing cytosolic enzyme, receptor or transcription factor disulfides to modulate function in response to intracellular redox changes.

Several observations support dithiol-disulfide exchange and oxidation-reduction reactions in hormone-induced receptor activation. First, TT enhances L-triiodothyronine binding to its receptor *in vitro*, supporting a role in growth and differentiation pathways (346).

Secondly, two gonadotropic hormones, leutropin B subunit (LH) and follicotropin (FSH), have tetrapeptides homologous to that of the TDOR CXXC motif; (CGPC) and (CGKC), respectively (347). Structural and functional similarities also exist; the LH and FSH tetrapeptide CGXC motif is predicted to be located in a β-turn similar to that of the TDOR enzyme active sites; and LH and FSH were 60 and 300-fold more active than TRX,

respectively, when dithiol-disulfide exchange activities are measured with the standard ribonuclease reactivation assay (347). Receptors for these gonadotropins are composed of disulfide-containing subunits (348), suggesting that LH and FSH may utilize thiol-disulfide exchange to modify and subsequently activate their receptor. The gonadotropin CGXC motif is also immediately adjacent to the "determinant loop" (253) proposed as responsible for biological specificity.

Thirdly, TT has a marked similarity to conserved regions between the TGF-β factors (349), a family of proteins involved in the control of cell growth and differentiation, and implicated in inflammatory processes (Fig.14). Specific regions of similarity are around the TT redox active CXXC residues, and the second C-terminal dithiol site, CXXXC. TGF-β signals through contacting two distantly related transmembrane receptors that are serine/threonine kinases (350). TGF-β binds receptor II, a constitutively active kinase, which then starts the signal transduction cascade. The predicted receptor II structure includes a single hydrophobic transmembrane domain, a cytoplasmic serine/threonine kinase domain, and a cysteine-rich extracellular domain (351). Activation of the TGF-β receptor may involve dithiol-disulfide interchange.

Finally, several functions of TRX (Section IV B), another TDOR enzyme with similar dithiol-disulfide exchange activity may also be functions of TT. TRX has been shown to restore the ligand-binding capability of oxidized glucocorticoid receptor, *in vitro* (209), and modulate F_c receptor signal transduction (255). TRX modulates *in vitro* DNA binding abilities of transcription factors Fos/Jun (AP-1), and NFκB (263,352).

The GSSG/GSH ratio also effects transcription factor DNA binding (263); DNA binding was activated by low levels of GSSG, and inactivated by high levels of GSSG,

indicating these transcription factors may form mixed disulfides under oxidizing conditions. Since TT has greater glutathionyl substrate specificity than TRX (321), future experiments may show TT to be the more significant enzyme with respect to NFκB and AP-1 activation.

5. Role in cancer:

Based on the involvement of TT in maintaining cellular redox homeostasis, TT may function both positively and negatively in cancer by regulating growth and differentiation as well as modulating drug resistance. TT has not directly been shown to function as a growth factor, however several related proteins have a demonstrated role in growth and differentiation.

TRX (ADF) is detected in many tumor tissues, and is a growth-promoting factor (227). TGF-β has structural similarities to TT, and regulates the action of cyclin-dependent kinase inhibitors, affecting cell cycle progression (353). Finally, thiols other than GSH are indicated in redox stimulation of apoptosis in T-lymphocytes (246).

Chemotherapy involves treatment of cells with alkylating agents (such as BCNU, bis-cloroethylnitrosourea) or quinones such as Adriamycin (doxorubicin) that generate ROS including electrophiles, radicals, oxidants, and peroxides (354), which result in DNA, lipid and protein damage (Section I and V). Systems to detoxify ROS have already been mentioned (Section I); typically GSH and various enzymes associated with sulfhydryl biochemistry that serve to conjugate, degrade or excrete the toxic species.

Anti-neoplastic-dependent inhibition and induction of GSH-utilizing enzymes has also been studied, although seldom including the thiol-disulfide oxidoreductase enzymes. Sulfhydryl enzymes important in proliferating cells are inactivated via oxidation or alkylation. Many cancer cells have depressed levels of repair and detoxification enzymes (356), resulting

Fig. 14. TT and TGF-β amino acid sequence similarities.
Several TTs (II) are aligned with the C-terminal domain of the TGF-β family members (I). Residues around the TT active site consensus CPY/FC and around the C-terminal CIGGC motif are conserved between TTs and TGFβs. Residues at the bottom of the figure are the residues in the TGF-β consensus pattern for the first similar region in that domain that are also conserved in TTs. With the exception of a large gap, almost all the residues in the TGF-β amino acid consensus sequence are conserved in TTs. Residues marked with ** are residues absolutely conserved between both TTs and TGF-βs. Residues marked * are equally or more conserved between TGF-βs and TTs, than within TGF-βs. Taken from (349) with permission.

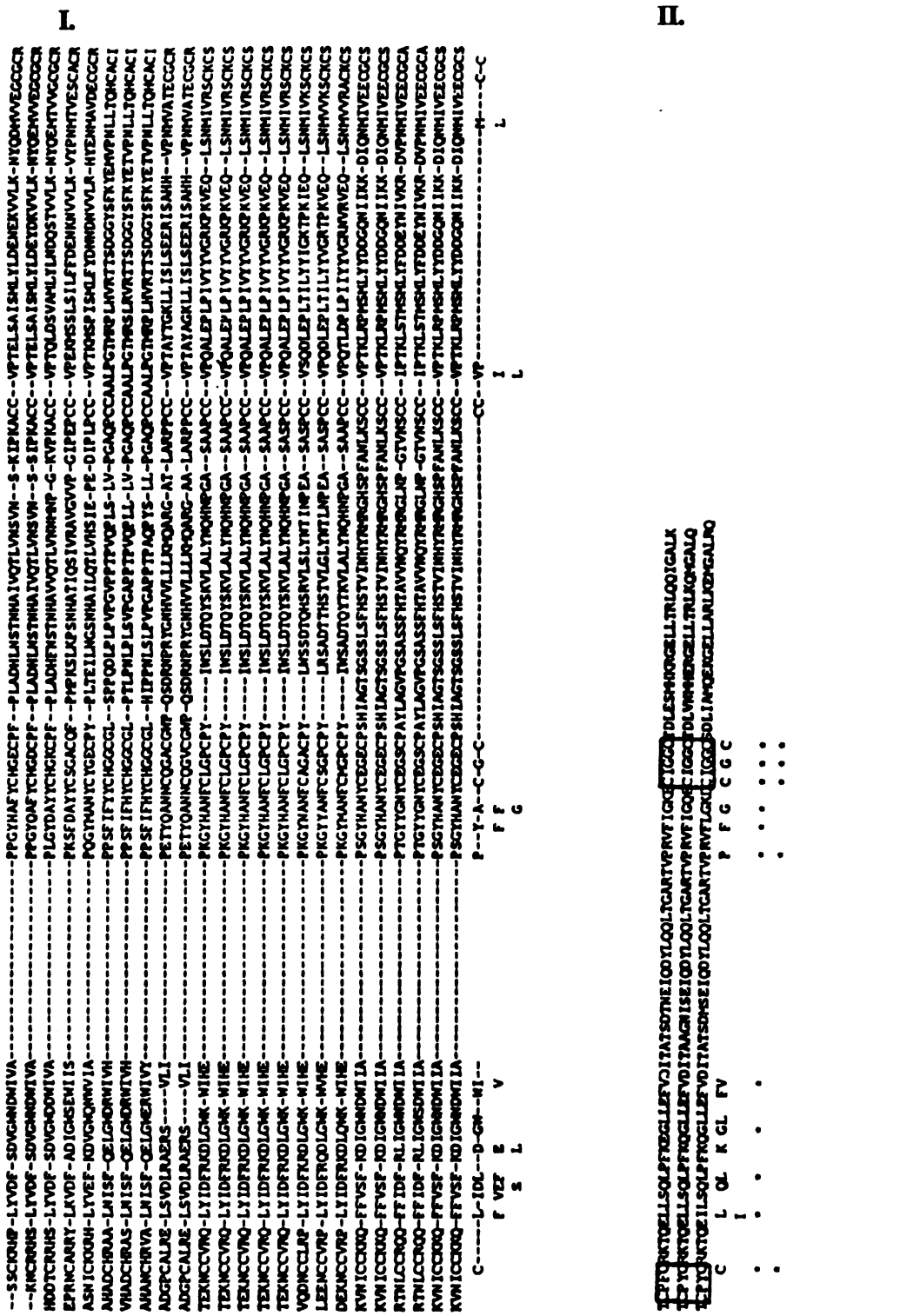

Fig. 14. TT and TGF-β amino acid sequence similarities.

in decreased protection from ROS. Many studies have shown that changes associated with elevation in the levels of these enzymes, substrates and cofactors in cancer cell populations contribute to drug resistance (357-361, Section VI). By protecting cells from alkylating or oxidative processes, GSH and TDOR enzymes may decrease the cytotoxic effects of chemotherapeutic agents.

Generation of ROS such as $O_2^{\cdot -}$ and $^{\cdot}OH$ may be involved in tumor promotion (reviewed in 362). Antioxidants may inhibit tumor promotion; ROS generation is an immediate early event involved in the stimulation of cell growth; and H_2O_2 and $O_2^{\cdot -}$ induce *c-fos* mRNA expression and DNA synthesis. As previously described, TT reactivates oxidized enzymes, including GST (341), an important electrophile scavenger (355), and functions as an electron donor to GSH-Px (215), as well as reduces oxidized AA (80).

V. Adriamycin

Adriamycin (doxorubicin) is an anthracycline antitumor antibiotic most commonly used for the treatment of malignancies in acute leukemias, non-Hodgkin's lymphomas, Hodgkin's disease, and sarcomas (363), and is considered to be the most effective single agent in the treatment of advanced breast cancer (364). Adriamycin was originally isolated from *Streptomyces peucetuis caesins* (365) and contains an aminosugar, daunosamine, linked through a glycosidic bond to a napthacenequinone, adriamycinone (Fig.15). The mechanism of action of anthracyclines is unknown, although effects on cells include: 1) intercalation between DNA bases; 2) DNA strand scission; 3) altered plasma membrane structure; 4) disruption of electron transport; and 5) redox cycling between quinone and semiquinone, generating toxic ROS such as $O_2^{\cdot -}$ in the process.

Adriamycin forms complexes with nucleotides, proteins, and a broad range of

biologically active compounds such as NAD^+ and caffeine (366) in aqueous solution at 37 molecular O_2 to $O_2^{\cdot-}$ in the presence of physiological thiol levels (386-388). Thiols, such as GSH, are believed to reduce the complexed Fe^{3+} to the Fe^{2+} state, which reacts with H_2O_2 produced by $O_2^{\cdot-}$ dismutation forming $^{\cdot}OH$ via Fenton and Haber-Weiss reactions (Section I). Adriamycin-copper complexes analogously cause ROS-dependent damage (389). In addition, the Adriamycin quinone moiety is enzymatically reduced to a semiquinone free radical form by NADH dehydrogenase (390,391), xanthine oxidase and cytochrome P450 reductase (392). The semiquinone free radical rapidly reacts with O_2 to produce $O_2^{\cdot-}$ (393-397). Both the C. Adriamycin intercalates into DNA readily; the tetracyclic rings insert between and parallel to adjacent DNA base pairs, and the amino sugar ionically interacts with the minor groove sugar-phosphate backbone (367). Interestingly, intercalation requires a reducing agent (368). Adriamycin-DNA interactions such as intercalation and adducts disrupt transcription and DNA replication (369,370).

Adriamycin has been shown to cause toxicity by action at the cell surface at or below the concentration causing other biochemical effects in the cell *in vitro* (371,372). Adriamycin inserts into the membrane bilayer by intercalation between anionic phospholipids (373), and modulates many membrane characteristics including lectin interaction (374), glycoprotein synthesis (375), phospholipid structure and organization (376), fluidity (377), fusion properties (378), transport of small molecules and ions (379), expression of hormones and receptors (377), spectrin and cardiolipin binding (380), and Na^+ permeability (381).

ROS are involved in most proposed mechanisms of Adriamycin action. Adriamycin is an effective chelator of iron (382), abstracting Fe^{3+} from ferritin (383,384) and myoglobin (385). Adriamycin-iron complexes are redox-catalysts capable of non- enzymatically reducing

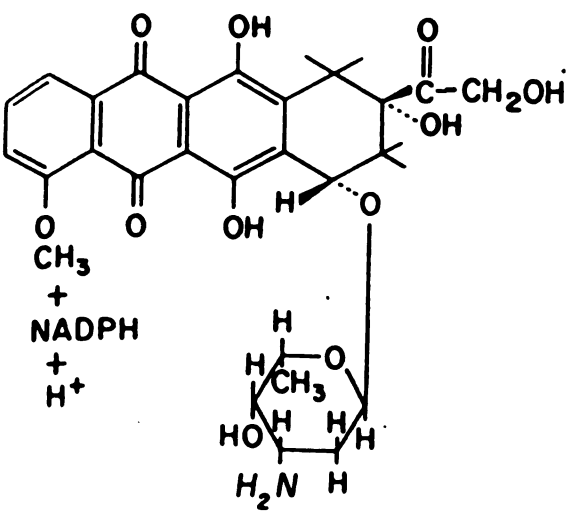

Fig. 15. Adriamycin structure.
Adriamycin contains an amino sugar, daunosamine, linked through a glycosidic bond to a tetracyclic napthacequinone moiety, adriamycinone.

enzymatic and non-enzymatic reactions can redox cycle, producing large amounts of ROS from one molecule of Adriamycin (383,398,399).

Hydroxyl radicals damage and cleave DNA, and cause lipid peroxidation and protein oxidation (Section I). Further DNA damage results from Adriamycin-stabilized topoisomerase II-DNA complexes (400), which cleave DNA. Quinones react with sulfhydryl groups on GSH and proteins; therefore Adriamycin may interfere with cellular defense and repair systems by inactivation of GSH-dependent enzymes and various TDOR enzyme system components.

Other cellular effects of Adriamycin include calcium release from the sarcoplasmic reticulum and impaired mitochondrial calcium sequestration. $O_2^{\cdot-}$-damaged cell membranes result in increased membrane permeability to Ca^{++} and interference with mitochondrial electron transport. Adriamycin-mediated $O_2^{\cdot-}$ production occurs in the initial steps of electron transport (397), leading to the possible formation of a large number of free radicals. Increased cytoplasmic and nuclear calcium has been linked with Adriamycin-induced apoptosis (401-403).

High cardiotoxicity is a side effect of Adriamycin chemotherapy, the etiology of which has not been clearly determined. A single dose of Adriamycin rarely causes clinical heart failure, instead cardiac dysfunction generally develops slowly over a period of several weeks (reviewed in 404). Murine models indicate cardiac ROS defense mechanisms differ from other tissues (405,406), depending primarily on SOD and GSH peroxidase, as cardiac catalase activity is low. Adriamycin treatment results in a rapid drop in cardiac GSH peroxidase. Cardiac toxicity is suggested to be due to a combination of decreased ROS detoxification and abundant cardiac mitochondria, which contain unusually active electron transport chains, generating large amounts of $O_2^{\cdot-}$.

Cell culture studies adding antioxidants, or free radical scavengers (Section I) support a mechanism of action involving ROS. Addition of SOD and catalase to cell culture medium reduced Adriamycin toxicity, supporting a mechanism for oxygen radical toxicity outside cells (407). AA addition significantly decreases Adriamycin-elevated lipid peroxide levels (408). Interestingly, AA administration to mice and guinea pigs prevented Adriamycin-induced cardiomyopathy (409).

VI. Drug resistance

Tumor cells are highly susceptible to anticancer drugs and radiation when compared with normal cells (356). The mechanism for this differential drug sensitivity is undetermined, although two popular theories exist: rapidly dividing cells are more susceptible to antitumor drugs which target DNA replication and cell cytoskeleton; and these drugs are more effective against tumors as they accumulate to higher concentrations inside tumor cells than in normal cells.

Chemotherapy improves long-term survival in metastatic breast cancer where there is a possibility of tumor reoccurrence (reviewed in 410). However, most metastatic cancers are either intrinsically resistant to chemotherapy or respond to chemotherapy with a cell subpopulation resistant to the chemotherapeutic agent.

Multidrug resistance (MDR) is a broad term for tumor cell mechanisms evading cytotoxic drug effects. MDR tumor cells have decreased sensitivity to a broad spectrum of drugs with no similar intracellular targets nor obvious structural homology. Drugs commonly involved in the MDR phenotype are generally natural products or their derivatives and include anthracyclines, vinca alkaloids, epidophyllotoxins, and actinomycin D (411). MDR cells are typically more resistant to the treatment drug than others (412,413). While the MDR

mechanism is unknown, many cellular changes correlate with the MDR phenotype.

Common changes observed in cells that develop MDR are a decreased accumulation of cytotoxic drugs; changes in activity and expression of certain proteins; and changes in cellular physiology (reviewed in 414). In any MDR cell any or all of these changes may be noticed, suggesting that there may be more than one mechanism giving the MDR phenotype. The most common change in cells acquiring MDR is a decreased cellular accumulation of cytotoxic drugs; via either increased drug efflux (415-420), decreased drug influx (415,416,421-426), or decreased cytosolic (427-429) or subcellular (429,430) drug retention.

Proposed mechanisms of MDR fall into several categories. Drug efflux models include ATP-dependent drug transport or increased exocytosis rate which removes drugs from the cell; drug distribution models including reduced drug accumulation through an alkaline shift of cytoplasmic pH or changes in nuclear or plasma membrane permeability, and compartmentation in cellular organelles. Theories include altered cellular functions changing drug sensitivity, such as changes in drug-DNA interactions; altered DNA repair; and changes in expression and function of detoxification enzymes (reviewed in 410,414). None of the proposed mechanisms can account for all of the MDR phenotypes observed.

The predominant hypothesis is that chemotherapeutic agents diffuse into the cell down a concentration gradient and that the drugs are removed from membranes or cytoplasm by ATP-driven "pumps" or "flippases" (reviewed in 410). Several potential drug transport proteins with strong homology to ATP-binding cassette (ABC) protein membrane transporters found in yeast, *E. coli* (431-433), the human major histocompatibility antigen peptide transporter (434), and the human cystic fibrosis membrane regulator (435-439) are overexpressed in MDR. P-glycoprotein (Pgp), the product of the human *MDR1* gene, is a

170-Kda membrane glycoprotein associated with both the plasma membrane and internal organelles (440). MDR-associated protein, a 180 Kda protein, is found on intracellular organelles (441-443), and a 110 Kda protein is found primarily in lysosomes (444). A vacuolar H^+-ATPase subunit is also overexpressed in MDR (445). ABC proteins are involved in translocating ligands, especially proteins, across membranes. Overexpression of ABC-type proteins also is associated with increased ion channels (435-439). The cystic fibrosis membrane regulator is a cAMP-controlled Cl^- channel which regulates secretory activity in epithelia.

Most of the research on MDR phenotypes has focused on these drug "transport" proteins, especially the Pgp, as Pgp is most commonly associated with MDR. Studies demonstrated that Pgp can transport drugs (446,447); antibodies and ATP-pump inhibitors stop drug efflux (448-450); site-directed mutagenesis of Pgp residues results in drug specificity changes and alters efflux (451-453); and both drugs and MDR reversal agents (chemosensitizers) can bind the Pgp (448-450,454,455). Pgp is highly overexpressed in normal tissues such as adrenal gland, kidney, and pancreas, suggesting a function of normal secretion of metabolites into the bile, urine, and lumen of the gastrointestinal tract (456,457). Pgp overexpression is not associated with all MDR phenotypes. The broad Pgp substrate specificity is unusual for a membrane pump, as most membrane pumps show high substrate specificity. MRP expression also confers MDR to NIH-3T3 cells (458).

Antitumor drugs are weak bases with pK_as between 7.4 and 8.2 (459-461) that readily traverse membranes when neutral in charge. Once protonated in the cell, antitumor drugs are retained and biologically active. Cellular pH affects both retention and exocytosis of drugs: binding of cytosolic targets such as DNA (462-467), RNA (466,468) and tubulin (469,470),

has an acidic optimum pH. The nucleus is the primary drug target although major drug accumulation sites are the trans-Golgi and the lysosomes (471,472), which are acidic cellular compartments involved in cellular exocytosis and endocytosis (429,430,473,474). Microscopic observations comparing cellular drug fluorescence in drug-sensitive and MDR lines find a shift in drug distribution in MDR cells from nuclear to peripheral locations (429,475-480), with the drugs distributed primarily in the Golgi and lysosomes (429,471,472,474,475,481-487). Drug translocation across the nuclear membrane may facilitate these changes.

Drug-sensitive tumor cells have a pH more acidic (6.85) than that of normal cells (7.6) (487) whereas the pH in MDR cells (7.30-7.6) (481,488-490) resembles normal pH. Increased cellular pH correlates with elevated drug resistance in tumor cells (481), and raising the cytosolic pH of drug-sensitive cells using ammonium chloride or CO_2 without MDR protein overexpression resulted in increased drug resistance (429). In addition, the MDR-like pH quantitatively accounted for the level of drug efflux seen in MDR cells. However, there are MDR cells with acidic cellular pH similar to the drug-sensitive lines (482). It is possible that in MDR, subcellular compartment pH changes occur undetectable when measuring total cellular pH, or that altered pH only can explain a subpopulation of MDR phenotypes.

An alternate hypothesis is that these ABC-like proteins alter drug accumulation by influencing transmembrane ionic equilibria or secretory mechanisms. Transfection of cells with *MDR1* results in both the MDR phenotype (550,551) and increased Cl^- channel activity (432). Pgp overexpression may facilitate elevated cellular pH by functioning as a Cl^- channel (414), decreasing proton entry into cells. Supporting this theory, drug-sensitive cells transfected with PgP have alkaline pH shifts (488) and increased Cl^- conductance (435) in addition to the

display of MDR phenotypes. Changes in secretion have also been noted: Pgp overexpression in vinblastine-resistant human lymphoblastic leukemia cells correlates with enhanced exocytosis-mediated secretion of lysosomal enzymes (491).

Decreased drug influx is often observed in MDR cell lines (415-419,421-426). Since most antitumor drugs are lipophilic and cross lipid membranes freely, hypotheses are that the plasma membrane structure has changed, limiting drug influx, or altering drug binding, affecting the transmembrane signaling leading to cytotoxicity. Studies on membrane changes in resistant cell lines found that plasma membrane composition and binding characteristics change in the MDR phenotype (493,494). Anthracyclines such as Adriamycin and Daunomycin bind to the plasma membrane of cells and exert a cytotoxic effect without entering the cell (372,404,492-497). Treatment of cells with different detergents stimulates Pgp ATPase activity (498) and modifies the accumulation, binding (497), and cytotoxicity of antitumor drugs (426,499,500). MDR cells with enhanced membrane recycling (501,502) remove plasma membrane components to endosomes, vacuolar compartments and intracellular organelles (485,503), resulting in more drug "sinks" in the cytoplasm that separate drug from target. The membrane recycling is inhibitable by Ca^{++} channel blockers and MDR reversal agents.

MDR reversal agents or chemosensitizers cause physiological changes which alter drug accumulation in MDR cells. Chloroquine causes an alkaline shift of organelle pH, decreasing drug trapping in organelles (reviewed in 414). Amiloride (481) and verapamil (483) acidify the cytoplasm, decreasing drug efflux, and accumulate in the lysosomes (484,485), altering lysosomal structure and function (430,486). This is accompanied by increased cytoplasmic and nuclear fluorescence intensity (429,475-479), indicating changes

in drug distribution and accumulation.

In addition to previous mechanisms described, elevations in metabolic enzymes, detoxification enzymes, PKC activity (357-361), topoisomerase II activity, and altered GSH metabolism relate to the MDR phenotype without changes in transport processes that extrude drugs or drug-adducts. As anticancer drugs tend to be either strong alkylating agents, or quinones that participate in redox cycling (Sections I and V), MDR cells exhibit altered repair and protective processes. Decreases in drug metabolic activation resulting from downregulation of monooxygenase and related enzyme activities (504) also increase resistance in murine leukemia cells.

Anticancer drugs such as acridines, actinomycins and anthracyclines inactivate replication and transcription by inducing topoisomerase II-mediated single-strand breaks in the DNA (505,507) (Section VI). Decreased topoisomerase II activity (505-509) reduces drug effects in some MDR phenotypes.

Correlations have been observed between enhanced Pgp expression, thymidylate synthase, and metallothionein (Section I) (reviewed in 414). Enzymes commonly found overexpressed in MDR phenotypes include GSH-Px, GSH S-transferases, glucose 6-phosphate dehydrogenase (G6PDH, Section I), TRX (Section IV B), and TT (Section IV C). GSH-Px detoxifies radicals generated by redox active anticancer agents and GSH S-transferase protects cells by forming GSH adducts with alkylating agents. TT and TRX would presumably reverse the damage from oxidizing agents and free radicals. Glutathione disulfide reductase (Section I) provides for adequate GSH by reducing GSSG using NADPH generated by the oxidative portion of the pentose phosphate pathway [5,6], which includes G6PDH (Fig.16).

GSH levels have a significant role in cellular responses to antitumor agents. Cells exposed to oxidative anticancer agents or xenobiotics decrease the GSSG/GSH ratio during oxygen stress, probably due to the induction of the two synthetases involved in GSH production (Section III) [14,15]. Many cancer cells in both tumors and tissue culture express high levels of GSH-dependent enzymes, especially the class Pi GSTs and GSH-Px (510). Tumors that have acquired elevated resistance against cytotoxic drugs often display further increases in GSH and GSH-dependent enzymes (511-516).

The demand for reduced GSH and NADPH is the ultimate support for the GSH-dependent enzyme systems. Numerous studies support MDR mechanisms involving GSH- and NADPH-based detoxification. GSH is elevated in many MDR cell lines (517). Mouse leukemia cells resistant to Adriamycin had G-6-P levels and 6-PG levels that were nearly doubled (518,504) in addition to 4-fold elevations in glucuronyltransferase activity (504); and a human MDR line exposed to oxidizing agents had activated levels of pentose phosphate pathway enzymes (519), increased GSH, and large increases in GSH-Px activity.

Many studies correlate changes in various GSH-dependent enzymes with MDR (407,520-523). An MDR human lung cancer line with no elevation in PgP and a 6-fold lower GSH concentration than the drug-sensitive line had elevations in GST and GSSG reductase activities (521). An unusual GST with elevated organic peroxidase activity was increased along with Adriamycin resistance (522) in human breast cancer cells. A study that mechanically disrupted human MCF-7 cell membranes transiently to introduce GST and GSH-Px without phenotype or genotype changes found increased resistance to Adriamycin and quinones (407). In a vincristine-resistant human MCF-7 cell line, GST Pi was overexpressed, and increased activity of GST, GSH-Px, and PKC was noted (523).

GSH depletion increases tumor sensitivity to the effects of sulfhydryl-reactive chemotherapeutic drugs (524). MDR breast tumor cells preincubated with BSO showed increased drug sensitivity, where MDR cells given BSO and etoposide together had no changes in drug sensitivity (525). Neither drug efflux nor drug retention changed significantly, and the effects were identical, although quantitatively smaller with drug sensitive breast tumor cells. Chemosensitization was therefore believed to be due to increased intracellular protein binding by sulfhydryl-reactive drugs.

Overexpression of enzymes such as TRX and TT, capable of regenerating oxidized proteins via dithiol-disulfide exchange activity, also correlates with MDR phenotypes. A human MDR kidney cell line was transfected with TRX antisense DNA. MDR kidney cells that underexpressed TRX had decreased drug resistance (277). MCF-7 ADR^R breast tumor cells have 4-fold elevations in TT activity over the MCF-7 drug-sensitive cells. Supplementation with an AA derivative, L-ascorbate 2-phosphate, resulted in further increases in resistance in the MDR line (526), indicating that perhaps both dithiol-disulfide transfer and DHA reduction play a role in resistance (Section VII).

One common denominator in these studies is that individual enzyme activity levels and the changes in drug resistance are not quantitatively correlated, indicating that there are additional or synergistic mechanisms responsible for the MDR phenotype.

VII. MCF-7 breast tumor cells:

MCF-7 breast adenocarcinoma cells are a well-characterized line of cells preserving many of the biochemical and endocrinological characteristics of breast tissue. A sub-population of MCF-7 breast adenocarcinoma cells (MCF-7 ADR^R) have developed a pleiotropic resistance to antitumor drugs after being cultured in the presence of increasing

doses of Adriamycin (527), and demonstrate up to a 1000-fold increased viability in the presence of Adriamycin as compared to the parental sensitive MCF-7 strain. Growth of these MCF-7 ADR^R cells in culture is hormone-independent; when transplanted into nude mice, MCF-7 ADR^R cells promote estrogen-independent tumor growth. Membrane characteristics changed in MDR include elevated epidermal growth factor receptor levels, increased Pgp levels, and decreased estrogen receptor levels.

Previous studies on the mechanism of Adriamycin cytotoxicity in MCF-7 WT cells have implicated the generation of toxic ROS (407,517,528-542). As greater doses of Adriamycin are administered to MCF-7 cells, increasing levels of ROS are apparent by electron spin resonance (393).

Pleiotropic mechanisms producing increased Adriamycin resistance in MCF-7 ADR^R cells include decreased cellular drug accumulation, especially in the nucleus; increased drug detoxification, and increased DNA repair activity. In addition, MCF-7 ADR^R cells generate several-fold less $^{\cdot}OH$ from Adriamycin redox cycling (528), are less sensitive to extracellular and intracellular ROS (529), and are sensitized to Adriamycin cytotoxicity after GSH depletion with BSO (436) (Section III). Postulated mechanisms for these changes in the MCF-7 ADR^R cells include the increased expression of both seleno-cysteine containing GSH-Px (543), GST with intrinsic peroxidase activity (522), increased expression of PgP drug transporter protein (544), decreased estrogen dependence, diminished drug-activating enzyme cytochrome P-450 Ia1 activity (545), moderately increased SOD activity (543), and elevated cytosolic pH (429) (Table 3). Studies transfecting the GST Pi (546), μ and α (547) demonstrated that overexpression of these isozymes alone is not sufficient to confer resistance to Adriamycin or other anticancer agents that generate ROS.

Fig. 16. GSSG reductase, GSH, and G6PDH interrelationships .
In aerobic cells, NADPH is generated primarily in the pentose phosphate pathway via (1) G6PDH and (2) 6-phosphogluconate dehydrogenase. The NADPH generated is required for biosynthesis; reduction of GSSG to GSH via (3) GSSG reductase; metabolism of Adriamycin to Adriamycinol via (4) daunorubicin reductase, and activation of Adriamycin to a semi-quinone radical by (5) various NADPH-dependent flavoprotein reductases such as cytochrome P450 reductase. Reduced GSH is used to (*) either reduce glutathionylated protein -SH groups or disulfide bonds by (6) TDOR enzymes such as TT, and PDI, or to detoxify H_2O_2 by (7) GSH peroxidases. (8) SOD dismutation of $O_2^{\cdot-}$ (generated from Adriamycin redox cycling between quinone and semiquinone in the presence of O_2) produces H_2O_2. NADPH and GSH production are essential for these processes removing ROS. Abbreviations: G6P=glucose 6 phosphate, G6PDH= glucose 6-phosphate dehydrogenase, 6PG= 6-phosphogluconate, R5P= ribulose 5-phosphate, GSH= glutathione, GSSG= glutathione disulfide, SOD= superoxide dismutase, NADPH= nicotinamide dinucleotide phosphate, reduced, TDOR= thiol-disulfide oxidoreductase.

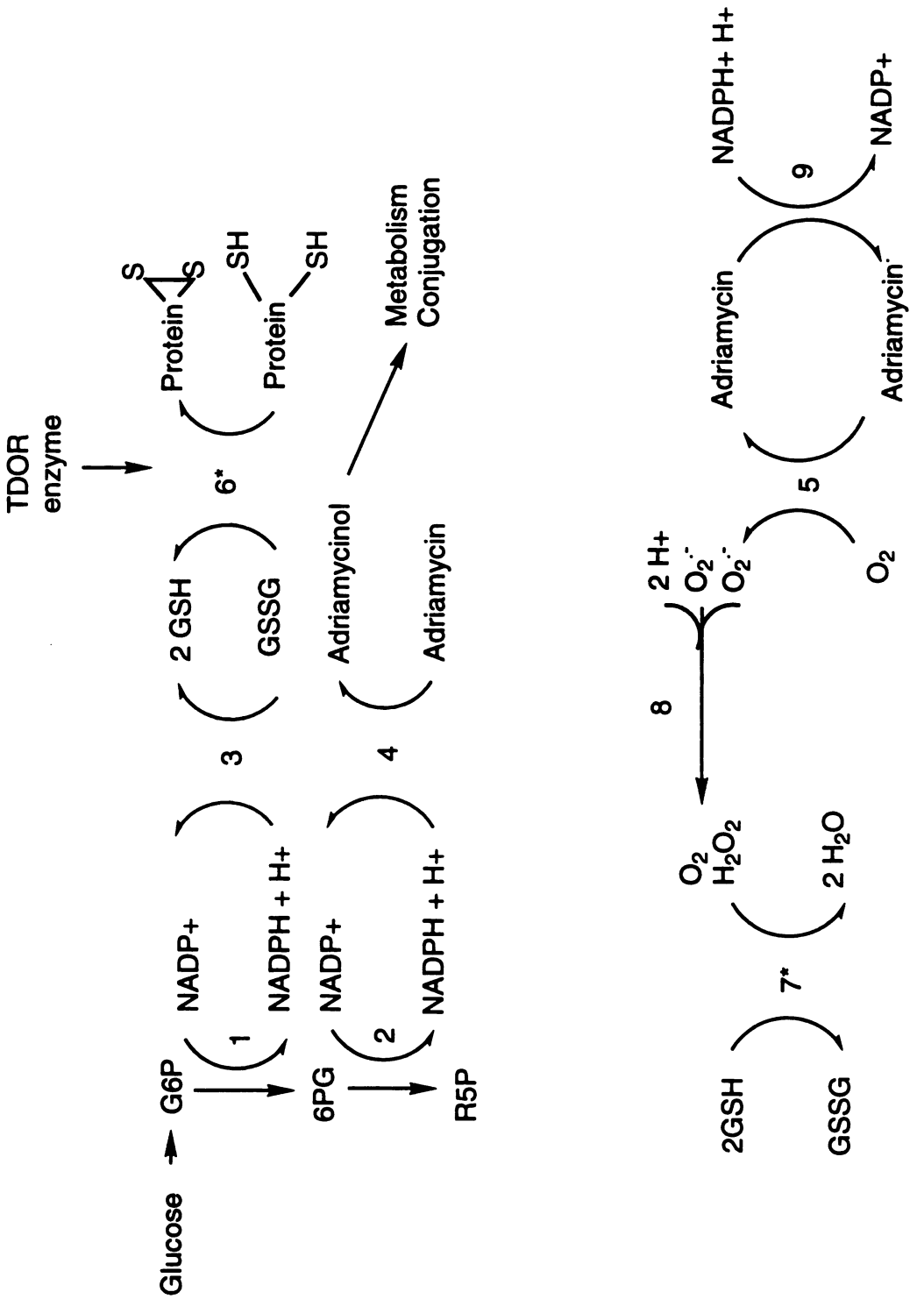

Fig. 16. GSSG reductase, GSH, and G6PDH interrelationships.

Maintenance of GSH (Section III) and the vitamins α-Tocopherol (Section I) and AA (Section II) is essential in providing resistance to oxyradical-generating drugs, since both vitamins scavenge ROS and are closely integrated with one another and the GSH redox system (Section III). AA is not a component of tissue culture media, due to its short half-life of 0.9 hours in tissue culture media (548) and its potential prooxidant activity in the presence of transition metal ions (549). Human cells cannot synthesize AA, leading to the study of "scorbutic" human tissue culture cells. Cultured cellular AA levels are dependent on the relative DHA and SDHA reductase activities (Section II). ROS generated in scorbutic cells will rapidly result in oxidative stress and subsequent cytotoxicity.

We recently showed that MCF-7 WT and MCF-7 ADR^R cells cultured in media supplemented with L-ascorbic acid 2-phosphate (AAP) have intracellular AA and DHA at levels indicative of a cell-dependent dephosphorylation of the supplemented AA ester (526). The supplementation of 2 or 10 mM AAP had no effect on Adriamycin resistance in MCF-7 WT cells, but 2 mM AAP resulted in enhanced resistance (up to 3.4 μM Adriamycin) over a 5-day incubation in MCF-7 ADR^R cells. Levels of SDHA reductase and PDI were extremely low in both cell types, indicating that an alternate mechanism was responsible for the reduction of oxidized AA in MCF-7 cells.

Interestingly, MCF-7 ADR^R cells show 4-fold constitutively elevated levels of TT protein relative to the MCF-7 WT strain (526). TT is a known DHA reductase, and therefore elevations in TT correlate with increases in drug resistance in the MCF-7 ADR^R cells. In order to determine if elevated TT increases Adriamycin resistance in the MCF-7 WT cells, TT cDNA was cloned, overexpressed in bacteria to ensure the cDNA generated a functional protein with characteristics of the wild-type protein, and then stably transfected into MCF-7

WT cells. Cells constitutively overexpressing TT were examined for changes in sensitivity to Adriamycin.

Table 3.
MCF-7 WT and MCF-7 ADR^R Differences.

Activity (nmol/min/ug DNA)	MCF-7 ADR^R	MCF-7 WT	ADR^R/WT
GSSG reductase	0.43 ± 0.27	0.56 ± 0.31	0.8
Thioltransferase	0.16 ± 0.12	0.04 ± 0.04	4.0
GSH-Px (Cu-ROOH)	1.31 ± 0.35	0.11 ± 0.009	11.6
GSH-Px (tBu-ROOH)	0.83 ± 0.18	0.02 ± 0.09	34.7
Thioredoxin	160.0 ± 50.0	70.0 ± 20.0	2.3
SDHAR (mitochondria)	18.5 ± 16.0	15.4 ± 11.5	1.2
SDHAR (microsomes)	8.8 ± 12.4	8.8 ± 4.4	1.0
a-Tocopherol (membrane)	0.162 ± 0.067	0.057 ± 0.024	2.84
a-Tocopherol (cytoplasm)	1.181 ± 0.512	0.400 ± 0.126	2.95

Taken from (454), with permission.

CHAPTER TWO

HUMAN TT SEQUENCE AND DISTRIBUTION

Introduction

At the initiation of this thesis, very little was known about human thioltransferase (TT) sequence or structure, although a high degree of homology (>80%) across mammalian species (rabbit, cow, pig) was noted for TT (307). Predominant characteristics for TTs are the highly conserved active site residues CPF/YC, which have been shown to be essential for function by site-directed mutagenesis studies (100); two additional half-cystines (CXXXC) found in the C terminal which are not essential for function (100); and high amino acid conservation throughout with a less conserved C-terminal. The recently determined crystal structure of pig liver TT determined at 2.2 A (129) showed the N-terminal to be near the active site, both of which are located on the protein surface.

To explore human TT structure and distribution, the cDNA was cloned from a human placental cDNA library, and sequenced. The tissue distribution of TT mRNA expression was examined. Human erythrocyte TT was purified, and used to generate polyclonal antibodies. These polyclonal antibodies were then used to screen pooled protein samples from various human tissues.

Materials

Restriction endonucleases were purchased from New England Biolabs (NEB), Boehrringer-Mannheim Biochemical (BMB), and Gibco-BRL. DNA fragments were purified through Spin-X 22 μm cellulose acetate membranes (Costar). The 1 kb DNA ladder was purchased from Gibco-BRL. Competent DH5α *E.Coli* were purchased from Clontech. Standard reagents were purchased from Sigma, Difco Laboratories, BMB, Gibco-BRL and NEB. Glutathione disulfide (GSSG) reductase, glutathione (GSH), and reduced nicotinamide dinucleotide phosphate (NADPH) were purchased from BMB, and L-ascorbate (AA) was purchased from Sigma Chemical Co. Dehydroascorbate (DHA) was generated by the bromine oxidation of 20 mM Ascorbic acid (AA), following the procedure of Bode et al (552). LE392 *E. coli* host strain was a gift from Brian Smith-White.

Methods

Pig liver TT probe generation:

The expression vector pKK233-2 containing the pig liver TT cDNA insert (553) was amplified in JM105 *E. coli* and isolated using standard alkaline lysis followed by ion-exchange chromatography (554, Qiagen, Promega). Purified plasmid DNA was then digested with *NcoI* and *PvuII* restriction endonucleases to release a 423 bp cDNA fragment containing 318 bp of coding sequence, and 105 bp of 3' untranslated sequence (Fig.17). This 423 bp cDNA fragment was isolated following electrophoresis and purification through Spin-X membranes (Costar), then radiolabelled (by random priming) with α-^{32}P dCTP (3000 Ci/mmol, DuPont New England Nuclear, NEN) as in Feinberg and Vogelstein (554).

Southern analysis of human genomic DNA:

Human genomic DNA (Novagen, cat# 69237-1, lot #1) was individually digested with restriction endonucleases *EcoRI*, *HindIII*, *NcoI* and *XhoI* overnight, denatured in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA at 65°C for 10 min. Digested DNA (20 µg of each digest), together with Ficoll loading buffer (0.25% bromophenol blue, Sigma, 0.25% xylene cyanol FF , Sigma, 15% Ficoll, type IV, Pharmacia) was electrophoresed on a 0.8% agarose gel in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA) with 0.125 mg/ml ethidium bromide for 16 h at 20 mA. The DNA in the gel was then depurinated 15 min. using 0.25 N HCl, and transferred to Hybond N+ (Amersham) positively charged nylon membranes for 90 min using 0.5 N NaOH as the vehicle using a Hoeffer TransBlot vacuum transfer apparatus. Transferred blots were rinsed with 0.1X SSC (150 mM NaCl, 15 mM sodium citrate, pH 8.0) to remove excess salt, and baked at 80°C for 20 min under vacuum. Once dry, the membranes were prehybridized for 2 h with 5x Denhardt's (0.01% Ficoll, Type 400, Pharmacia, 0.01% polyvinylpyrrolidone, 0.01% BSA, Fraction V, Sigma), 0.5% SDS, 6X SSC (0.9 M NaCl, 90 mM sodium citrate, pH 8.0) at 65°C. Hybridization was performed in the same buffer containing 5 x 10^6 dpm/ml ^{32}P radiolabeled probe at 60°C for 20 h. Successive washes at 65°C in 2X SSC, 0.1% SDS, 1X SSC, 0.5% SDS, and 0.5X SSC, 0.5% SDS were followed by a room temperature rinse in 0.1 X SSC. Membranes were exposed to film at -70°C for 24 h.

Screening the human placental cDNA library:

A human placental λgt11 cDNA library with a high proportion of low molecular weight cDNAs (Clontech) was screened with the 423 bp pig liver TT cDNA fragment probe. LE 392 *E. coli* host cells were grown overnight in LB media in the presence of 0.1% maltose.

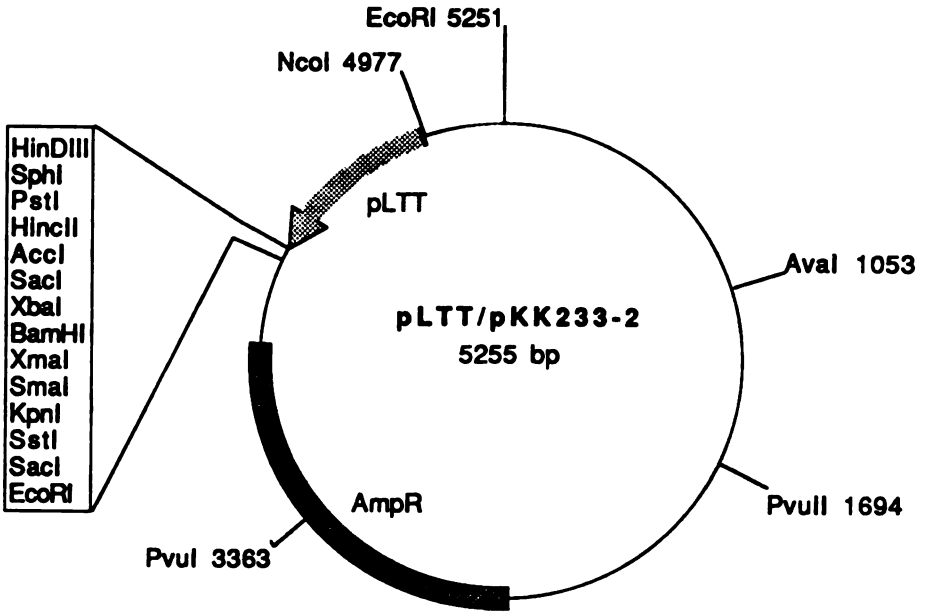

Fig. 17. Generation of 423 bp pig liver TT probe.
Plasmid pKK233-2 DNA containing the pig liver TT cDNA insert and M13 polylinker sequences (552) was subjected to restriction digestion with *NcoI* and *PvuII*. A 423 bp cDNA fragment containing the entire 318 bp coding sequence (from +1 to +318 relative to the translational start site) and 105 bp of untranslated sequence 3' of the coding sequence was isolated as described in Methods.

Approximately 10^4 pfu of λ phage were then added to 200 µl bacteria, and incubated 15 min at 37°C. The infected host cells were then plated in 0.7% LB agarose on 1.5% LB agar plates, and incubated 6-8 h at 37°C until plaques were evident. Colony-Plaque membranes (NEN) were used in duplicate for immobilization of phage DNA. These membranes were then treated with 0.1 N NaOH, 1.5 M NaCl for 2 min to lyse the bacteria, neutralized twice with 1.0 M Tris-HCl, pH 7.5 for 3 min, then rinsed in 2X SSC (0.3 M NaCl, 30 mM sodium citrate, pH 8.0) and air dried to fix the DNA to the membrane. Once dry, the membranes were prehybridized 2 h with 5x Denhardt's (0.1% Ficoll, Type 400, Pharmacia, 0.1% polyvinylpyrrolidone, 0.1% BSA, Fraction V, Sigma), 0.5% SDS, 6X SSC at 65°C, then hybridized in the same buffer containing 5 x 10^6 dpm/ml ^{32}P radiolabeled 423 bp probe at 65°C for 16-24 h. Positive signals on duplicate filters were used to locate the corresponding positive plaques. Plaques were extracted with a sterile pipet base into SM (100 mM NaCl, 8.1 mM $MgSO_4$, 50 mM Tris-Cl, pH 7.5, 0.01% gelatin), and left at 4°C overnight. Four positive plaques were found in 10,000 independent plaques. These were purified to homogeneity by four rounds of re-screening and plaque isolation, as described above.

Subcloning and sequencing the TT cDNA:

Liquid culture lysate method (556) was used to generate large amounts of phage DNA. LE 392 *E. coli* were grown to stationary phase in LB with 0.1% maltose, then 200 ul bacterial growth were infected with 10^6 plaque-forming units (pfu) of phage for 15 min at 37°C to allow phage to adsorb. Four ml NZCY media was added, and the samples were agitated at 37°C for 6-12 h to completely lyse the bacteria. Lysed bacteria were treated with 2 drops of chloroform for 15 min at 37°C, and bacterial debris was removed after centrifugation at 4000 x g at 4°C for 10 min. The supernatant solution containing the phage was treated with 1 drop

of chloroform, and placed at 4°C until phage DNA was harvested. Lambda DNA was purified using the Magic™ DNA purification system (Promega) according to maufacturer's instructions. Human TT inserts were PCR amplified from λ clone DNA using a Perkin-Elmer 9600 Thermocycler with 25 rounds of thermocycling at 95°C for 30 secs, followed by 55°C for 30 secs, and then 72°C for 1 min using Taq DNA polymerase (BMB) and λgt11 β-galactosidase sequencing primers (5'-dGGTGGCGACGACTCCTGGAGCCCG-3' and 5'-dTTGACACCAGACCAACTGGTAATG-3', NEB) which flank the TT cDNA insert on either side (Fig.18). PCR-generated fragments of approximately 1 kb were isolated and subcloned into the PCRII plasmid (Invitrogen) as follows. Linear PCRII DNA and 38-40 ng of the PCR fragments were ligated in a 1:3 molar ratio at 16°C overnight. Eight or nine ng of the resultant constructs designated pCR1, pCR2, pCR3, and pCR4 (Fig.19) were then used to transform INVaF' supercompetent *E. coli* (Invitrogen) and plated on LB plates containing 100 μg/ml ampicillin and 75 μg/ml X-gal. Four separate recombinant plasmids were isolated, amplified in *E. coli*, and purified using the Wizard™ (Promega) purification system according to manufacturer's recommendations. Complete sequencing in both orientations of the TT inserts from the double-stranded templates was performed using a combination of ^{35}S dATP, Sequenase 2.0 enzyme and reagents and automated fluorescent sequencing by the MSU-DOE-PRL Plant Biochemistry Facility using the ABI Catalyst 800 for Taq cycle sequencing and the ABI 373 A Sequencer for the analysis of products. Primers used for this sequencing were M13 primers and internal human TT specific nt sequence primers corresponding to A^{29}QEILSQ^{35} and T^{96}RLKQIGAL^{104} (Macromolecular Structure Facility, MSU, Fig.20). All four approximately 1 kb inserts were sequenced completely.

Fig. 18. PCR amplification of cDNA inserts.

λ gt11 contains two phage arms of approximately 24,100 and 19,600 bp, and insert cDNA, cloned in at *EcoRI* sites. The *E. coli* β-galactosidase gene is interrupted by the cDNA insert. hpTT cDNA inserts were PCR amplified as described in Methods using the β-galactosidase sequencing primers for λgt11 (NEB).

PCR-generated fragments of approximately 1 kb were isolated and subcloned into pCRII to generate pCR1, pCR2, pCR3, and pCR4, as described in Methods. Lane 1: 1 kb DNA ladder, lane 2-5 Purified DNA from pCR1, pCR2, pCR3, and pCR4, respectively, was digested with *EcoRI* to release the cDNA insert. pCRII is a cloning vector for PCR products that contains an*EcoRI* site on either side of the A-overhangs. Digestion with *EcoRI* will result in the release of any insert. All the hpTT cDNA clones are the same size, of approximately 1 kb.

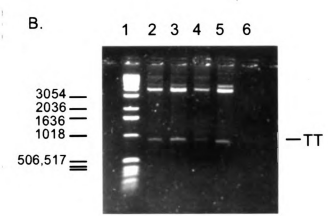

Fig. 18. PCR amplification of cDNA inserts.

Fig. 19. Construction of pCR4.
PCR-generated fragments of approximately 1 kb from four separate λgt11 clones were subcloned into pCRII as described in Methods. PCRII contains 5' T-overhangs to facilitate ligation to Taq polymerase-generated amplicons with 3'A-overhangs. The resultant constructs were designated pCR1, pCR2, pCR3, and pCR4.

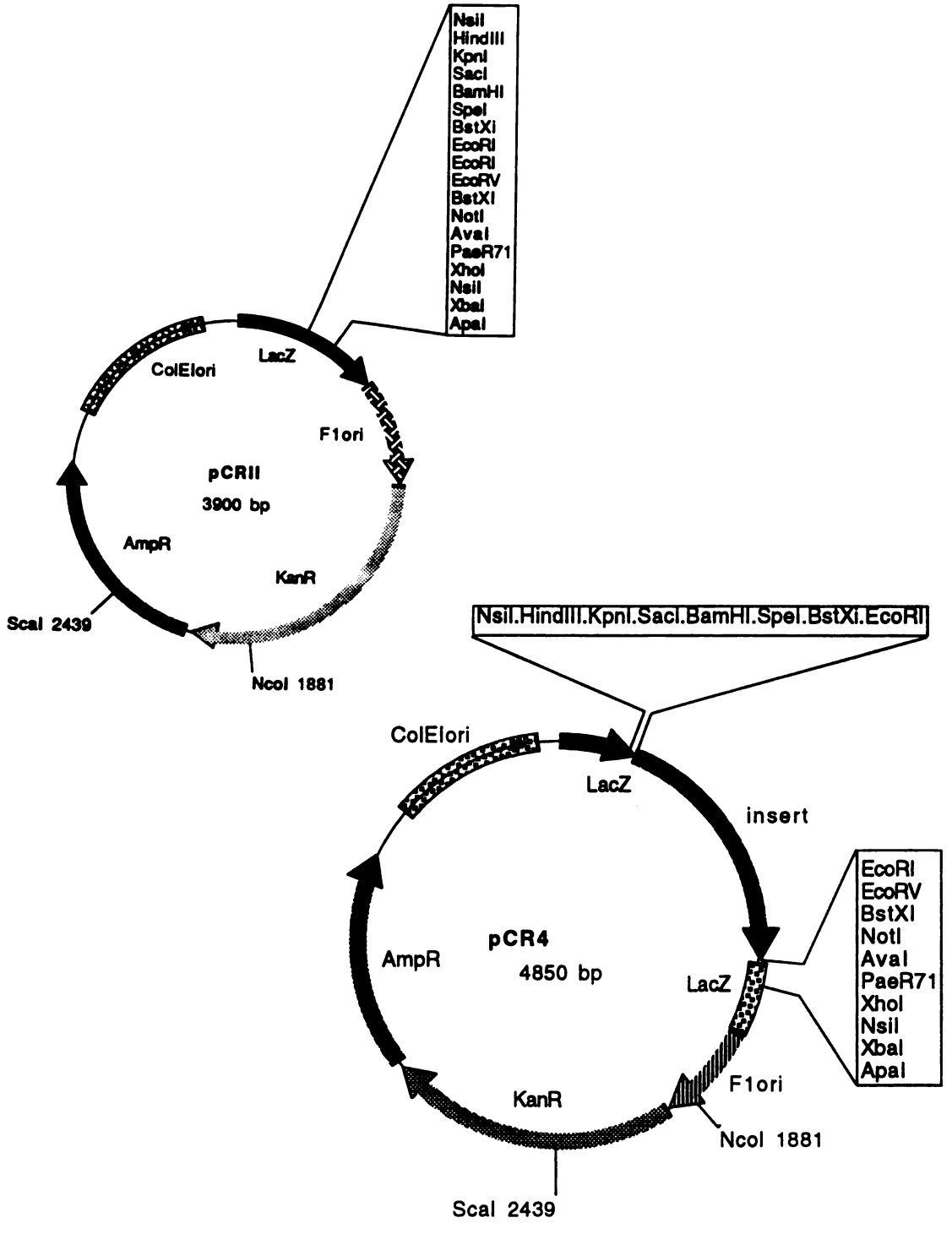

Fig. 19 Construction of pCR4.

Fig. 20. HpTT cDNA sequence and deduced amino acid sequence.
The entire 318 bp coding region of the hpTT cDNA is shown (uppercase) and the corresponding deduced amino acid sequence (below). In addition, 106 bp of 5'-UTR, and 438 bp of 3' UTR were also sequenced (lowercase). The translational start and stop codons are highlighted in hatched boxes. All numbers shown are relative to the putative ATG translational start site. The 3'-UTR is 438 nucleotides long with a putative polyadenylation tract (uppercase) 19 nucleotides after. The conserved active site $\mathbf{C^{22}PYC^{22}}$ is shown in bold type. Major differences between human TT and that of other mammalian TTs include the lack of an internal methionine residue and an additional cysteine at position 7. Primers used for sequencing are denoted by lines.

```
                                                5'-gacaccaga        -101
ccaactggta atggtagcga ccggcgctca gctggttaaa tcccctagca        -51
atacctgcaa ctgaggattc ttcccgggga gaccgcagcc catcggc ATG          3
                                                            M

GCT CAA GAG TTT GTG AAC TGC AAA ATC CAG CCT GGG AAG GTG         45
 A   Q   E   F   V   N   C   K   I   Q   P   G   K   V        [14]

GTT GTT TTC ATC AAG CCC ACC TGC CCG TAC TGC AGG AGG GCC         87
 V   V   F   I   K   P   T   C   P   Y   C   R   R   A        [28]

CAA GAG ATC CTC AGT CAA TTG CCC ATC AAA CAA GGG CTT CTG        129
 Q   E   I   L   S   Q   L   P   I   K   Q   G   L   L        [42]

GAA TTT GTC GAT ATC ACA GCC ACC AAC CAC ACT AAC GAG ATT        171
 E   F   V   D   I   T   A   T   N   H   T   N   E   I        [56]

CAA GAT TAT TTG CAA CAG CTC ACG GGA GCA AGA ACG GTG CCT        213
 Q   D   Y   L   Q   Q   L   T   G   A   R   T   V   P        [70]

CGA GTC TTT ATT GGT AAA GAT TGT ATA GGC GGG TGC AGT GAT        255
 R   V   F   I   G   K   D   C   I   G   G   C   S   D        [84]

CTA GTC TCT TTG CAA CAG AGT GGG GAA CTT CTT ACG CGG CTA        297
 L   V   S   L   Q   Q   S   G   E   L   L   T   R   L        [98]

AAG CAG ATT GGA GCT CTT CAG TAA  ccaccacaga tctcatagga        347
 K   Q   I   G   A   L   Q   *                              [105]

aatgttcaac aattctgtga aaggtcacag gacccaattg gagaaatcat        397
atgaaaagca tagttggtct tggtgtcata tggatcagag gcacaagtgc        447
agaggctgtg gtcatgcgga acactctgtt atttaagatg gctatccaga        497
taatcctgaa cactgtgtat ttattttatt tagactacca gcaaagatta        547
aagcatgaaa tgtaaaacat ctgataaaac ttacagcccc ctacaccaag        597
agtgtatctg tgaaagagct cctacacttt gaaaacttaa gaatccctta        647
tcatgaagtt tgcctgttct agaattgtaa gttgttaatt tccttcaatc        697
tctagtgaca acacttaatt tctttctAAT AAAAAAAAcc tatagatgat        747
tcagtgAAAA AAAAAAAA                                          765
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Fig. 20. HpTT cDNA sequence and deduced amino acid sequence.

Human erythrocyte TT Purification:

The human TT purification procedure followed a modified procedure of Terada *et al.* (316). Six units of outdated packed red cells were received from the Red Cross, placed on ice, and washed twice with 0.9% NaCl. Each wash was followed by centrifugation at 4080 x g in a Sorvall RC2B centrifuge with a GSA rotor for 15 min at 4°C, and the supernatants from each wash were discarded. Erythrocytes were hemolyzed with 2 volumes of 10 mM NaH_2PO_4, pH 6.0 (Buffer A). The hemolysate pH was subsequently adjusted to 6.0 with 7% acetic acid, then heat treated at 65°C for 5 min with subsequent cooling at 4°C overnight. Two volumes of Buffer A were added to the heat-treated hemolysate prior to centrifugation at 10,800 x g for 15 min at 4°C. The 840 ml supernatant solution was treated with 105 g pre-swelled CM-cellulose or CM-Sepharose to remove hemoglobin. 4 l of hemolysate supernatant were precipitated with 85% ammonium sulfate and pelleted at 16,000 x g for 15 min at 4°C. The precipitated pellets were resuspended in Buffer A and dialyzed using Spectrapor 1 (mwco 6000-8000) tubing against four 4-l Buffer A changes. After centrifugation at 10,000 x g for 15 min at 4°C to remove particulate matter, the dialyzed sample was applied in 20 consecutive 100-160 ml aliquots to a Sephadex G-75 column (85 cm x 6 cm). TT was fractionated from larger proteins using a running buffer of 50 mM NaH_2PO_4 (pH 6.0). Fractions were assayed for TT activity as described below and active fractions were collected, pooled and concentrated under 50 psi N_2 using a Diaflo PM 10,000 mwco membrane and an Amicon concentrator. The partially purified fractions were then reduced with 5 mM DTT at 30°C for 30 min, dialyzed against two 2 l buffer changes of N_2-saturated 100 mM Tris-Cl, pH 7.5, 5 mM EDTA, 5 M NaCl (Buffer B) and applied at 10 ml/h to a thiopropyl sepharose 6B column (2 ml bed volume, Pharmacia) equilibrated with 50 ml of Buffer B. After a 100

ml wash with N_2-saturated Buffer B, 100 ml 100 mM NaOAc, pH 4.6, with 5 mM β-mercaptoethanol was applied to the column. No TT activity was noted in either of these washes. TT was eluted with a 160-ml 20 mM-to-50 mM DTT gradient in a 100 mM Tris-Cl, pH 8.0 buffer. Fractions were assayed for TT activity as described below.

Protein Assay:

Protein samples and bovine serum albumin (BSA) standards were precipitated in ice-cold acetone at -20 C for 30 min, and then pelleted to remove the DTT. After acetone evaporation, the samples and BSA controls were resuspended in 0.01 N NaOH, and protein concentrations were determined by the bicinchinoninic acid (BCA) protein assay protocol according to the maunfacturer's directions (Pierce Chemical Co.).

TT Activity Assay:

The enzyme activity was assayed as described by Gan and Wells (314). TT activity was measured as the GSH-dependent reduction of the prototype substrate S-sulfocysteine (Cys-SO_3^-). The standard TT assay mixture contained enzyme, 0.5 mM GSH, 1.4 U of glutathione reductase, 2.5 mM S-sulfocysteine (Cys-SO_3^-), 0.35 mM NADPH, 0.137 M sodium phosphate buffer, pH 7.5. The reaction was initiated by the addition of Cys-SO_3^-. Formation of GSSG was coupled to NADPH oxidation by glutathione reductase and measured spectrophotometrically by a decrease in A_{340} at 30°C relative to a blank reaction without enzyme simultaneously monitored. One unit of TT activity is defined as that amount of enzyme catalyzing the formation of 1 μmole of GSSG per min under standard conditions (314). Samples were measured in triplicate.

DHA Reductase activity assay:

DHA reductase (DHAR) assays followed the direct spectrophotometric assay of Stahl *et al.* (557) following the relative change in absorbance at 265.5 nm as DHA is reduced to AA. Standard assay conditions were enzyme, 0.137 M sodium phosphate buffer, pH 6.8, 1 mM EDTA, 2 mM GSH, and 1 mM DHA incubated at 30°C. Blanks were run without the enzyme. The reaction was initiated by the addition of DHA, and was linear up to 2 min at 30°C. One unit of DHA reductase activity is defined as that amount of enzyme catalyzing the formation of one μmole of AA per min under standard conditions (557).

Rabbit anti-TT antibody generation:

Polyclonal antibodies were raised by subcutaneous immunization of two female New Zealand rabbits with 240-360 μg of human erythrocyte TT together with 0.5 ml Freund's complete adjuvant, followed four weeks later with 225 μg TT in 0.5 ml Freunds' incomplete adjuvant. Blood was collected approximately every two to three weeks for seven months and antisera retained. Antisera were titered using dot blots against 0.01-1.00 μg recombinant pig liver TT, purified human erythrocyte TT and purified recombinant human placental TT. Rabbits were boosted with approximately 250 μg TT for increased immune response each time the titer decreased substantially.

SDS-PAGE and Western analysis of purified erythrocyte TT:

Laemmli SDS-PAGE analysis (558), using a 6% stacking gel and 15% separating gel was used. Samples were loaded in 2X SDS loading buffer, and electrophoresed for 1 h at 200V using a Tris-glycine-SDS buffer. One gel was stained with Coomassie Brilliant Blue R-250, while proteins from a duplicate gel were electrophoretically transferred to a nitrocellulose filter for 1 h at 100V using a 25 mM Tris-HCl (pH), 92 mM glycine, 20% methanol buffer.

Non-specific binding was blocked by incubating the membrane overnight at 4 C in 0.1% Tween-20-TBS (Buffer A) with 5% non-fat dry milk. After blocking, a 4 min wash in buffer A was performed followed by incubation with the primary rabbit anti-human TT antibody (1:20,000) in Buffer A for 60 min at room temperature. The blot was then rinsed twice in Buffer A for 5 min at room temperature, and incubated with the secondary alkaline phosphatase-conjugated goat anti-rabbit antibody (1:3000) in Buffer A for 60 min at room temperature. After the blot was rinsed two times for 5 min, the blot was incubated with BCIP and NBT (BioRad) to visualize immobilized TT bands.

Western blots of pooled human tissues:

A commercially prepared Western blot (Clontech) containing 75 μg total protein from each tissue (skeletal muscle, liver, heart, lung, brain, kidney) per lane and stained with Ponceau S was photographed (Fig.26), rehydrated in 100% MeOH, then equilibrated with water. The membrane was then rinsed with 1X phosphate-buffered saline (PBS) for 10 min and blocked for 2 h in PBS containing 5% nonfat dry milk at room temperature. After blocking, a 5 min wash in 0.2% Tween-20, TBS (Buffer A) was performed followed by incubation with the primary rabbit anti-human TT antibody (1:20,000) in Buffer A containing 1% milk (Buffer B) for 60 min at room temperature. The blot was then rinsed twice in Buffer A at room temperature for 15 min, and incubated with secondary HRP-conjugated goat anti-rabbit antibody (1:2000, Amersham) in Buffer B for 60 min at room temperature. After briefly rinsing in Buffer A, the blot was washed once for 15 min, then 3 times for 5 min each in Buffer A. The ECL chemiluminescent detection system (Amersham) was used to visualize the signals for one min, and the blot was exposed to ECL-Hyperfilm (Amersham) for 90 sec.

HpTT probe generation:

The plasmid PCR4 containing the human TT cDNA insert was digested with *EcoRI* and *SacI* to release a 304 bp cDNA fragment containing the entire coding region except for the nucleotides corresponding to the five terminal amino acids. This fragment was subcloned into pT7T318U (Pharmacia) at the *EcoRI* and *SacI* sites (Fig.21), and was purified and random-primed to generate a probe as previously described for the pig cDNA fragment.

Northern blots of pooled human tissues:

Commercially prepared Northern blots (Clontech) containing poly A+ mRNA from pooled human tissues (pancreas, testis, ovary, brain, colon, small intestine, skeletal muscle, prostate, liver, thymus, lung, peripheral blood leukocyte, spleen, placenta, kidney, and heart) were probed using a radiolabeled 304 nt human placental TT cDNA probe. Prehybridization of the membranes at 42°C for 6 h in 10X Denhardt's (0.02% Ficoll, Type 400, Pharmacia, 0.02% polyvinylpyrrolidone, 0.02% BSA, Fraction V, Sigma), 5X SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 5 mM EDTA, pH 7.4), 50% formamide, 2% SDS and 100 µg/ml freshly denatured salmon sperm DNA was followed by hybridization for 18 h with 5 x 10^5 dpm/ml ^{32}P radiolabeled 304 nt human placental TT cDNA fragment, then washed twice with 2X SSC (300 mM NaCl, 30 mM sodium citrate) containing 0.05% SDS at room temperature. After exposure to X-OMAT film (Kodak) for 48 h at -70°C, the membrane was stripped in 0.01% SDS at 60°C for 30 min, and reprobed with 5 x 10^5 dpm/ml ^{32}P radiolabeled $-actin cDNA probe (Clontech) in hybridization buffer, washed as previously described, and exposed to film for 24 h at -70°C. Densitometry using the BioImage Visage 110 system (Milligen, Ann Arbor, MI) was used to quantitate the signal intensity in order to determine relative amounts of TT expression. TT signal intensities were normalized to mRNA concentration, as poly

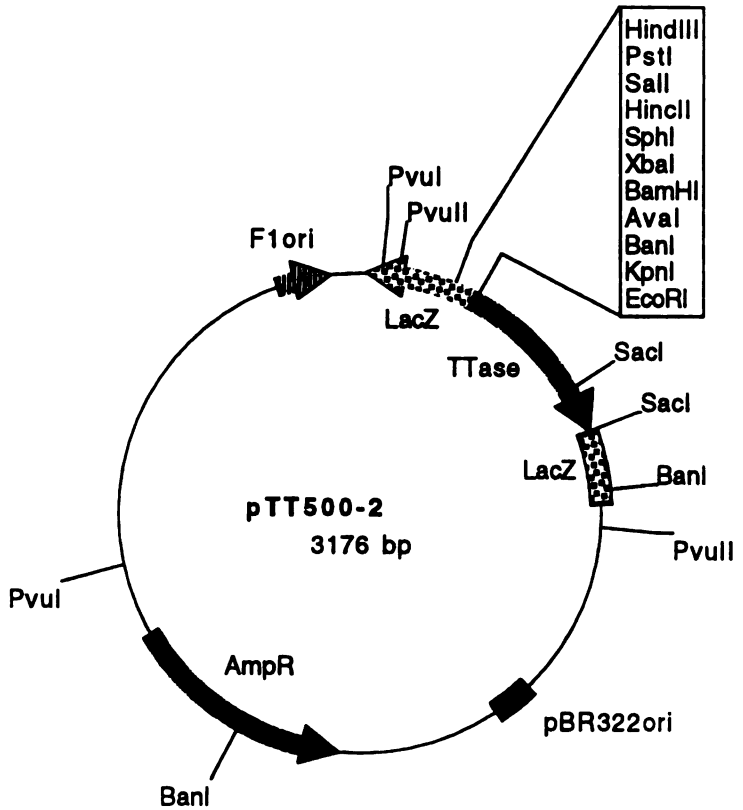


Fig. 21. Generation of the 425 bp hpTT probe.
Purified pCR4 DNA was subjected to digestion by *EcoRI* and *SacI*. Digestion of pCR4 DNA with *EcoRI* and *SacI* generates four fragments; the *EcoRI-EcoRI* plasmid fragment (3977 nt); a central *SacI-SacI* fragment (299 nt); an *EcoRI-SacI* fragment (153 nt) corresponding to 3'-UTR sequences and 5 nucleotides from the pCR4 vector that flank the cDNA insert to the 3' *EcoRI* site; and the *EcoRI-SacI* probe fragment (425 nt) corresponding to the entire 5'-UTR (106 nucleotides) and coding sequence (+1 nt to + 311 nt relative to the translational start site) of hpTT, as well as 3 nt 5' from the pCR4 vector that flank the cDNA insert to the *EcoRI* site. This 425 bp fragment was then subcloned into purified *EcoRI* and *SacI* -digested pT7T318U, as described in methods, and designated pTT500-2. The probe was isolated by *EcoRI-SacI* digestion from pT7T318U and purified as described in Methods.

A+ mRNA amounts were adjusted by the manufacturer to yield a detectable β-actin signal in every lane.

Results

Cloning the hpTT cDNA:

Four λgt11 clones containing human placental TT were purified to homogeneity through repeated rounds of screening. Upon phage DNA isolation and purification, *EcoRI* digestion of the cloned phage DNA yielded unexpected results. Because *EcoRI* restriction sites flank the cloned cDNA insert in the λ phage arms, it is expected that *EcoRI* digestion would result in the release of the cDNA insert from the two arms of the λ phage, generating two phage arms of approximately 24,100 and 19,600 bp in length, and the insert cDNA (Fig.18). However, *EcoRI* digests of the four human TT clones apparantly partially digested the phage DNA, producing fragments appearing to be greater than 23000 bp with no smaller bands observable (Fig.22). Because each of the four isolated human TT λ clones displayed similar refractory properties to *EcoRI* digestion, a possible problem with the library construction, and not an isolated problem with an individual λ clone is hypothesized. In order to circumvent this digestion problem, primers flanking the cDNA insert were used to PCR amplify the inserts in these four human TT cDNA clones. PCR amplified fragments were then subcloned into pCRII and sequenced. The sequences of all four clones were identical, indicating that there were no PCR-introduced errors in this amplification and isolation of the human placental TT cDNA inserts (Fig.20).

Human genomic Southern analysis:

Southern analysis showed that genomic fragments encoding hpTT. *HindIII* and *NcoI* digestion of human genomic DNA resulted in approximately 4.5 kb fragments that putatively

encoded the entire human TT gene. *XhoI* digestion resulted in a approximately 6 kb fragment hybridizing to the full-length cDNA, where *EcoRI* digestion resulted in several bands (10, 8, 7.5, 6, and 3.1 kb, approximately). The Southern did not indicate the presence of any pseudogenes, but did indicate that there may be *EcoRI* sites in the promoter region or in introns (Fig. 23). There are no *EcoRI* sites in the hpTT cDNA.

Human erythrocyte TT purification:

TT was purified from 6 units of packed human erythrocytes donated by the American Red Cross (Table 4, Fig.24). The purified enzyme produced a single band upon both Coomassie -stained SDS-PAGE (Fig.25) and western analysis using rabbit anti-recombinant pig liver TT antibodies (Fig.26). The apparent molecular weight of 11,300, as determined by gel electrophoresis agrees with that previously determined for human TT (317). The purified protein had an associated thiol-disulfide exchange activity of 118.7 U/mg comparable to that previously reported (321) and DHA reductase activity of 22.4 comparable to that reported by (310).

TTs are in general quite stable proteins; resistant to heat treatment, and stable upon storage. Purification of TTs are therefore generally straightforward. Human TT is an exception, being very susceptible to oxidation during the purification, resulting in denaturation and aggregation. Reducing human TT with DTT during the purification decreased the oxidation problem, but resulted in difficulty interpreting protein concentrations and DHA reductase activity by interfering with assay measurements.

Anti-human erythrocyte TT antibody generation:

Polyclonal antibodies were produced against purified human erythrocyte TT. All antisera exhibited cross-reactivity towards pig liver TT and human TT. Antisera collected at different

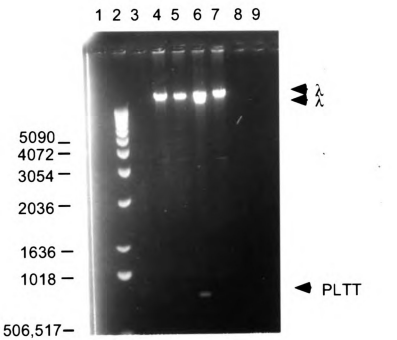


Fig. 22. *EcoRI* digestion of λgt11 hpTT cDNA clones.
Shown here are *EcoRI* digests of purified lambda DNA from three hpTT λgt11clones, compared to *EcoRI* digestion of purified lambda DNA from the pig liver TT λgt11 clone. Lane 1-blank, lane 2- 1 kb molecular weight marker, lane 3- blank, lanes 4-7: *EcoRI*-digested λgt11 DNA. lane 4- hpTT clone 1, lane 5- hpTT clone 2, lane 6- pig liver TT, lane 7- hpTT clone 4. Not shown: λgt11 hpTT clone 3, result is the same as the other three human clones. None of the λgt11 human cDNA clones had inserts that were released by *EcoRI* digestion, whereas the pig λgt11 cDNA was released from lambda DNA. The hpTT λg t11 clones are partially digested by *EcoRI*, as two lambda arms of high molecular weight are evident. A problem with the library construction is hypothesized, as the same problem existed for all the hpTT lambda clones.

Fig. 23. Southern analysis of human genomic DNA.
A. Ethiduim bromide stained 0.8% agarose gel with human genomic DNA digests. Lane 1-*EcoRI*, lane 2-*HindIII*, lane 3- *NcoI*, lane 4- *XhoI*, lane 5- blank, lane 6-1 kb DNA ladder. Genomic smears were readily produced by digestion with *EcoRI, HindIII, and NcoI*. *XhoI* digestion did not produce a strong visible smear.

B. Southern blot. The gel in A. was transferred to a Hybond N+ membrane (Amersham), and probed with a 423 pig liver TT probe as described in Methods. *NcoI* and *HindIII* digests indicate that the entire gene for human TT is encoded in approximately 4.5 kb. *XhoI* digestion resulted in a single, larger genomic fragment of about 6 kb. *EcoRI* digestion gave multiple bands, ranging from 10, 8, 7,5, 6, and 3.1 kb, approximately. There may be an *EcoRI* site in the promoter region or in the intron structure of the TT gene. There is no indication that multiple genes or pseudogenes for TT exist.

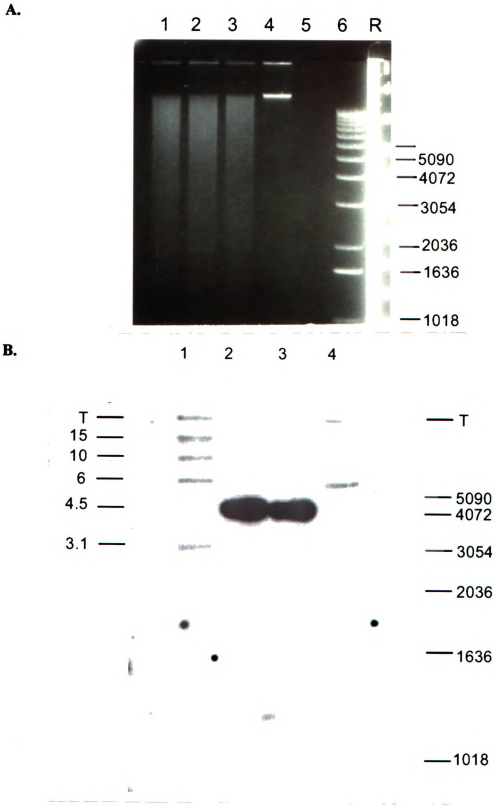


Fig. 23. Southern analysis of human genomic DNA.

Fig. 24. Thiopropyl sepharose 6B purification of human erythrocyte TT.
Reduced human erythrocyte TT was eluted from a thiol-disulfide affinity exchange column using a 20-50 mM DTT gradient as described in Methods. TT activity and elevated protein levels correspond to the same eluted fractions. Protein concentration is on the left (blue) and TT activity is on the right (red).

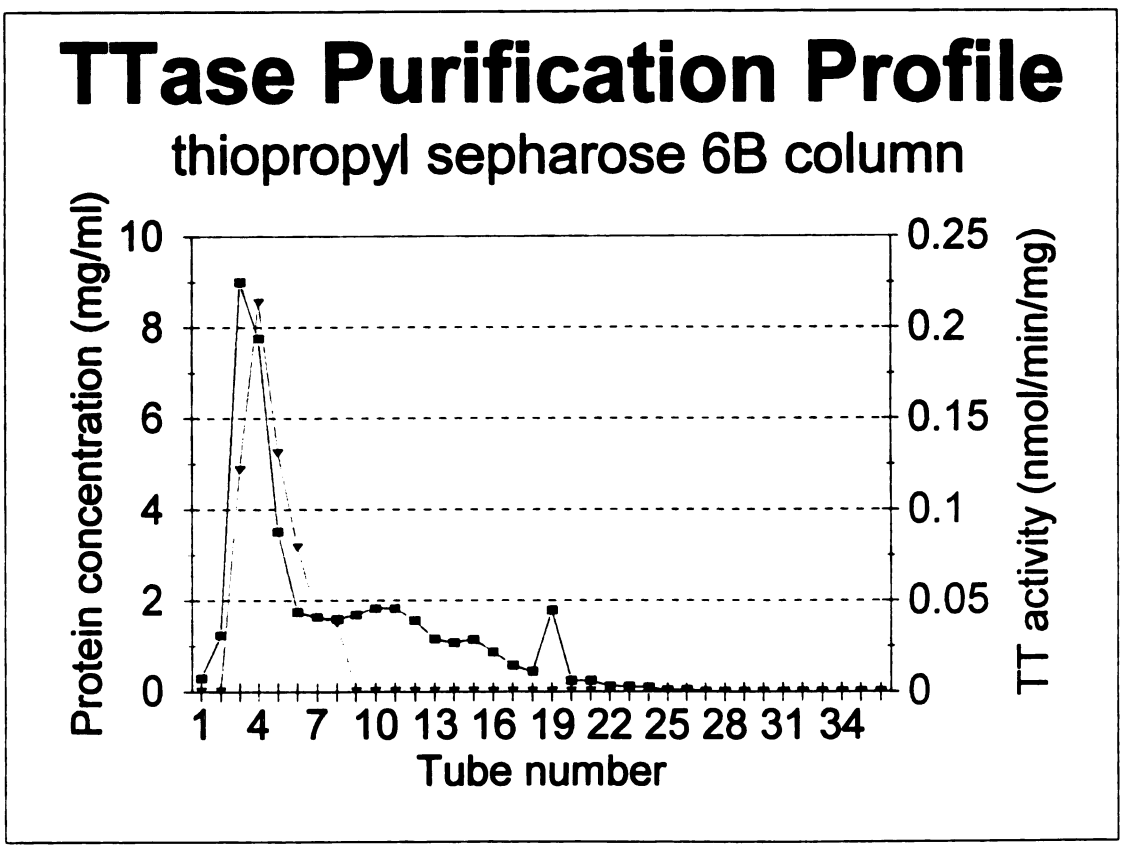


Fig. 24 Thiopropyl sepharose 6B purification of human erythrocyte TT.

Table 4.
Human erythrocyte TT purification.

Steps	Specific Activity[a] (U/mg)	Total Protein[b] (mg)	Fold Purification	% Yield
CM-cellulose	0.006	5100	1	100
40-85% ammonium sulfate	0.011	3900	2	76
Sephadex G-75 and Diaflo (10,000 mwco)	0.559	57	93	1.5
Thiopropyl sepharose 6B	34.9	1.9	5800	0.04

TT activity and protein levels were assayed as described in Methods. [a] Specific Activity is measured in units of thioltransferase per mg of protein where a unit of thioltransferase is defined as catalyzing the formation of one micromole of GSSG per min at 30°C. [b] Total protein is relative to milligrams of BSA.

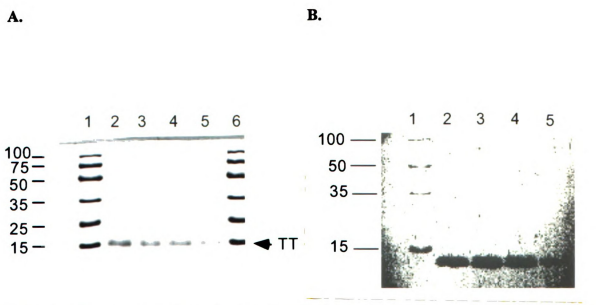


Fig. 25. SDS-PAGE and Western blot analysis of purified human erythrocyte TT.
A. SDS-PAGE. Four different Thiopropyl-Sepharose 6B fractions of human erythrocyte TT were analyzed for purity. Approximately 10 μg protein was loaded per lane. Lane 1- molecular weight markers, lane 2- fraction 3, lane 3- fraction 4, lane 4- fraction 5, lane 6-fraction 6, lane 7- molecular weight markers. The gel was Coomassie stained to visualize protein bands as described in Methods.
B. Western blot analysis of four thiopropyl sepharose 6B human erythrocyte TT fractions was performed as described in Methods. Lane 1- molecular weight markers, prestained, lane 2- fraction 3, lane 3- fraction 4, lane 4- fraction 5, lane 5-fraction 6. The only immunodetectable band is at approximately 11 kDa, indicating high purity for these TT fractions.

Fig. 26. Human tissue Northern analysis for TT content.
Northern blots were performed as described in Methods. (26 a) A. TT mRNA tissue distribution. B. B-actin tissue distribution. (26 b) A. TT mRNA tissue distribution. B. B-actin tissue distribution. C. Multiple Tissue Northern relative TT mRNA density. Lanes are labeled as follows: H=heart, B= brain, Pl= placenta, Lu= lung, Li=liver, SM=skeletal muscle, K=kidney, Pa= pancreas, S=spleen, Ty=thyroid, Pr=prostate, T=testis, O=ovary, SI= small intestine, C=colon, PBL= peripheral blood leukocyte. Samples were normalized to the amount of mRNA loaded, as mRNA was loaded unequally by the maunfacturer to produce a detectable B-actin signal in each lane. mRNA levels were as follows (in μg): (26 a) Lanes 1-8: 6.7, 1, 4, 2, 1, 1.5, 1.5, 1, and 5, respectively; (26 b) Lanes 1-8: 2, 2, 2.25, 2, 4, 2, 1.75, and 1.75, respectively.

Fig. 26a

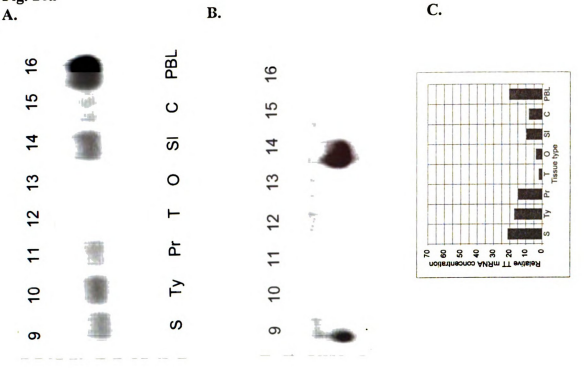


Fig. 26b

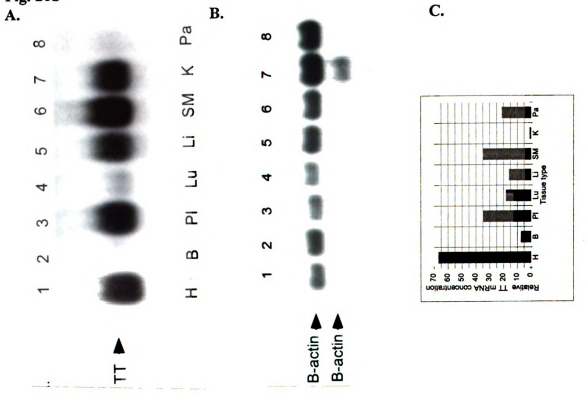
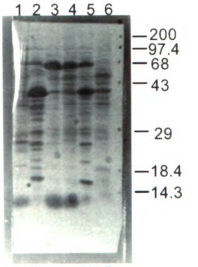


Fig. 26. Human tissue Northern analysis for TT content.

Fig. 27. Human tissue Western analysis for TT content.
A. Ponceau S stained nitrocellulose membrane. B. Western blot. Molecular weight markers are indicated to the right. Nitrocellulose preparation and western analysis was performed as indicated in Methods. Lanes are identified as 1:Li=liver, 2:SM=skeletal muscle, 3: Lu=lung, 4: K=kidney, 5:H=heart, and 6:B=brain. TT protein is distributed differently in the tissues sampled.

A.



B.

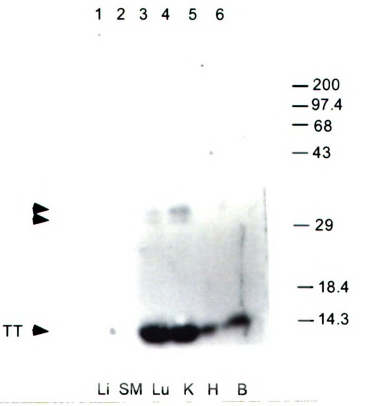


Fig. 27. Human tissue Western analysis for TT content.

times varied in recognition of 0.1 μg dot blotted purified protein. The highest titer polyclonal antisera was used for subsequent immunoblotting analysis.

Tissue distribution of TT:

Human TT mRNA was found to be expressed in every tissue tested. TT mRNA was low in endocrine glands such as pancreas, testis, and ovary; slightly higher levels in brain, colon and small intestine; intermediate levels in skeletal muscle, prostate, liver, thymus, and lung; high levels in peripheral blood leukocytes, spleen, placenta, and kidney, and at extremely high levels in the heart (Fig.27).

Several pooled tissues were immunoblotted for TT protein content. Skeletal muscle had almost non-detectable levels; liver, heart, lung, and brain had 12-20 fold higher detectable TT; kidney had the highest level of TT (Fig.28). Interestingly, two tissues, liver and lung, had another 31-32 kDa band that cross-reacted with the polyclonal antibodies.

Discussion

The amino acid sequence deduced from the human placental TT cDNA sequence is in almost complete agreement with the sequence, reported concurrently by Fernando, *et al.*, deduced from a human brain cortex cDNA clone (309), except for a single nucleotide difference at position 285, which would translate the corresponding amino acid as leucine, and not as valine, as reported by Fernando, *et al.* The sequence in this report is in exact agreement with the amino acid sequence reported for human erythrocyte TT by Papov *et al.* (559) as well as the deduced sequences from human spleen (311), brain (310), and neutrophil (312). Papov *et al.* reported the human erythrocyte TT possessed either asparagine or alternatively aspartic acid at position 51, and suggested there were either two protein isoforms or that a partial chemical degradation of Asn to Asp occurred during the isolation of peptide fragments.

The nucleotide sequence reported here clearly supports the latter explanation, since in all four independent clones, only asparagine codons were observed at this position (Fig.20). A high degree of sequence similarity, allowing for conservative amino acid changes, is observed between all reported mammalian TTs (Fig.12), including 99-100% to other reported human sequences (309-312), 90.7% to pig liver (307), 90.5% to calf thymus (295), and 90.6% to rabbit bone marrow (322). Amino acid similarity with non-mammalian TTs is 67.3% to vaccinia virus (302), 53.6% to Haemophilous influenzae (298), 48.6% to *Pyrococcus furiosus* (299), 49.4% to *Methanobacterium thermautrophum* (313), 35.7% to T4 bacteriophage (300), and 54.9% to the three *Escherichia coli* enzymes (296,297) , as well as 52.9% to rice endosperm (305), 48.1% to yeast (304), and 48.5% to castor bean (306). The high degree of conservation between species indicates a potentially important biological function for TT.

Human TT differs from other mammalian TTs in that there is no internal methionine and a unique cysteine is present at position 7. No significant function has been attributed to the lack of methionine. The unique Cys^7 of human TT is predicted to be on the surface near the conserved active site, $C^{22}PF/YC^{25}$ by analogy with the pig liver TT crystal structure, and may be involved in the decreased stability of the human enzyme relative to other TTs by increasing susceptibility to oxidation reactions. Supporting this hypothesis, Cys^7 was converted to Ser^7 by site-directed mutagenesis. The C7S mutant protein retained full enzymatic activity and had a significantly reduced tendency to form protein dimers under non-reducing conditions (315).

Although much is known about TT *in vitro* activity, little is known about the *in vivo* function, regulation or distribution of human TT. TT is believed to function in modulation of cellular redox status through the two possible mechanisms of thiol-disulfide transfer and DHA

reduction (100,526). Supporting this theory about the physiological function of TT is the finding that tissues such as heart, kidney, spleen, placenta, leukocytes, liver, and lung which would be habitually exposed to high levels of ROS are also those with high TT mRNA and protein levels.

TT mRNA and TT protein levels were similar from most pooled human tissue samples examined, indicating that TT transcription and expression may be similarly regulated. Previous studies examining TT activity, and TT protein distribution in tissues from calf, pig, and rat (136-144) found similar TT levels to those in human tissues, with a few notable differences. TT protein levels and activity were low in most endocrine and secretory tissues in the pig (144), and elevated in spleen in both the calf (141) and pig (144); both in agreement with human mRNA levels. In the calf (141) and pig (144), TT immunodetectable protein and activity levels were high in skeletal muscle and moderate in kidney, whereas the opposite was determined with rat TT activity levels (142) and immunodetectable human TT levels. Human TT mRNA is highest in heart, while moderate TT protein levels are found in human and pig heart muscle, and extremely low heart TT was detected in calf.

The relatively high levels of TT mRNA and protein in human heart leads to an interesting issue related to the role of TT in Adriamycin resistance (Section V.). Since elevated TT has been shown to correlate with increased Adriamycin resistance, it has been postulated that TT may participate in the development of drug resistance. From the high TT levels found in human heart tissue, one might expect the heart to be a particularly resistant organ to Adriamycin. In fact, the opposite is true: Adriamycin is selectively toxic to cardiac muscle through an as yet undetermined mechanism (reviewed in 410). This suggests that increasing levels of TT alone are not a complete solution to Adriamycin toxicity.

CHAPTER THREE

EXPRESSION AND PURIFICATION OF RECOMBINANT hpTT

Introduction

Prior to using the hpTT cDNA in experiments to determine cellular functions, the cDNA was first overexpressed, purified, and characterized to ensure that it contained native dithiol-disulfide transfer activity and DHA reductase activity. The 865 bp hpTT cDNA was amplified by PCR to generate a unique *NcoI* restriction site 5' of the coding region, and a unique *BamHI* restriction site 3' of the coding region. The altered hpTT cDNA was cloned into the expression vector, pET23d+ (Novagen) between the unique *NcoI* and *BamHI* restriction sites, and expressed in *E. coli* BL21(DE3) (Novagen) at 3-10% of the total soluble protein. The soluble and unfused product was purified using two different strategies and activity was measured by dithiol-disulfide exchange assay, DHA reductase assay, and purity measured by immunoblot analysis.

Materials

Restriction endonucleases were purchased from New England Biolabs (NEB), Boehrringher-Mannheim Biochemical (BMB), and Gibco-BRL. Taq polymerase, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from BMB. Standard reagents were purchased from Sigma, Difco Laboratories, BMB, Gibco-BRL and NEB. PCR primers were

synthesized in the MSU Macromolecular Structure Facility. Glutathione disulfide reductase, glutathione (GSH), reduced NADPH and IPTG were purchased from BMB. Broad and low range molecular weight protein standards, Tween-20, nitrocellulose membranes, and goat anti-rabbit alkaline phosphatase conjugate were purchased from BioRad. BCIP, NBT, Coomassie Brilliant Blue R-250, and L-ascorbate (AA) were purchased from Sigma Chemical Co. Polyclonal antibodies to human erythrocyte TT were generated by immunization of rabbits as described in Chapter 2.

Construction of the recombinant hpTT expression vector:

HpTT cDNA was amplified using two primers: the 5' primer-(5'-ATCGCCATGGCTCAAGAGTTT-3'), corresponding to nt -6 to nt +15 relative to the translational start site, contained the artificially introduced *NcoI* restriction site (CCATGG) at the translation start codon ATG; the 3' primer (5'-CGGGATCCCGTTACTGAAGAGCTCCAATC-3'), corresponding to nt +315 to nt + 345 relative to the translational start site, contained the artificially introduced *BamHI* restriction site (GGATCC) 17 nucleotides 3' to the translation stop codon TAA. PCR amplification of the pCR4 plasmid (Fig.19, Ch.2) template using these primers generated a 411 bp amplicon, which was subsequently ligated into pCRII (Invitrogen), designated pCRTT, and 7µl was used to transform DH5α *E. coli*. pCRTT plasmid DNA was extracted from a 25 ml culture following alkaline lysis and ion exchange purification using the Promega MAGIC™ purification system following manufacturer's reccomended procedures (Promega). Purified double-stranded pCRTT DNA was sequenced completely in both orientations using M13 unversal and reverse primers (BMB), ^{35}S dATP, Sequenase 2.0 enzyme and reagents. Purified pCRTT plasmid DNA was subjected to restriction digestion with restriction endonucleases

NcoI and *BamHI* to release the insert DNA corresponding to the hpTT cDNA. Following electrophoresis, the 395 bp hpTT fragment was purified by excision from a 1.2% agarose gel, and centrifugation through a Costar Spin-X membrane, directionally ligated to the *NcoI-BamHI* digested pET23d+ plasmid (Novagen) (Fig.28) immediately 3' of the T7 polymerase promoter, and was designated pEThTT. *E. coli* BL21(DE3) were transformed with 9 µl of pEThTT, and transformed bactera were cultured and plasmid DNA harvested as described above. BL21(DE3) bacteria contain T7 polymerase gene under *lac* repression. Upon IPTG induction of the cell lines, T7 polymerase is induced, and TT is transcribed at high levels.

Overexpression and purification of hpTT in *E. coli*:

BL21(DE3) cells transformed with pEThTT were cultured overnight at 37°C, then diluted 1 to 100 in 2 ml fresh LB medium with 50 µg per ml ampicillin and aerated at 37°C until an A_{600} of 0.4-0.7 was attained. IPTG was then added to 0.4 mM, and the culture was continuously aerated for 3 h at 37°C. Bacteria sampled at various times after induction were adjusted to the same OD_{600}, diluted with equal volumes of 2X SDS loading buffer, heated at 70°C for 5 min, and electrophoresed for 1 h at 200V in Tris-gly-SDS buffer.The gel was then stained with Coomassie Brilliant Blue R-250. Two different recombinant purification strategies were utilized.

Recombinant purification 1:

A 1 l growth of BL21(DE3) *E. coli* containing the pEThTT vector were harvested 3 h after IPTG induction by centrifugation at 5,500 x g for 10 min at 4°C using a GSA rotor in a Sorvall RC2B centrifuge. The bacterial pellet was resuspended in 100 ml of 50 mM Tris-Cl, pH 7.5, 10 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 400 µg per ml egg white lysozyme, and 10% sucrose. The suspension was sonicated 3 times for 30 sec at 70% power on ice, and the

Fig. 28. Construction of pEThTT.
pCR4 (Fig. 19, Ch.2) was amplified with two primers, introducing an *NcoI* site at the translation start codon (nt -6 to nt +15 relative to the translational start ATG) and a *BamHI* site 17 nucleotides 3' to the translation stop codon (nt +315 to +345 relative to the translational stop TAA), generating a 411 bp amplicon which was ligated into pCRII and designated pCRTT as described in Methods. PCRII contains 5'-T overhangs to facilitate cloning of Taq polymerase-generated amplicons containing 3'-A overhangs. pCRTT (not shown) was restriction digested with *NcoI* and *BamHI* to release the insert DNA corresponding to the hpTT cDNA, and ligated into *NcoI* and *BamHI* -digested pET23d+ immediately 3' of the T7 promoter, and designated pEThTT, as described in Methods.

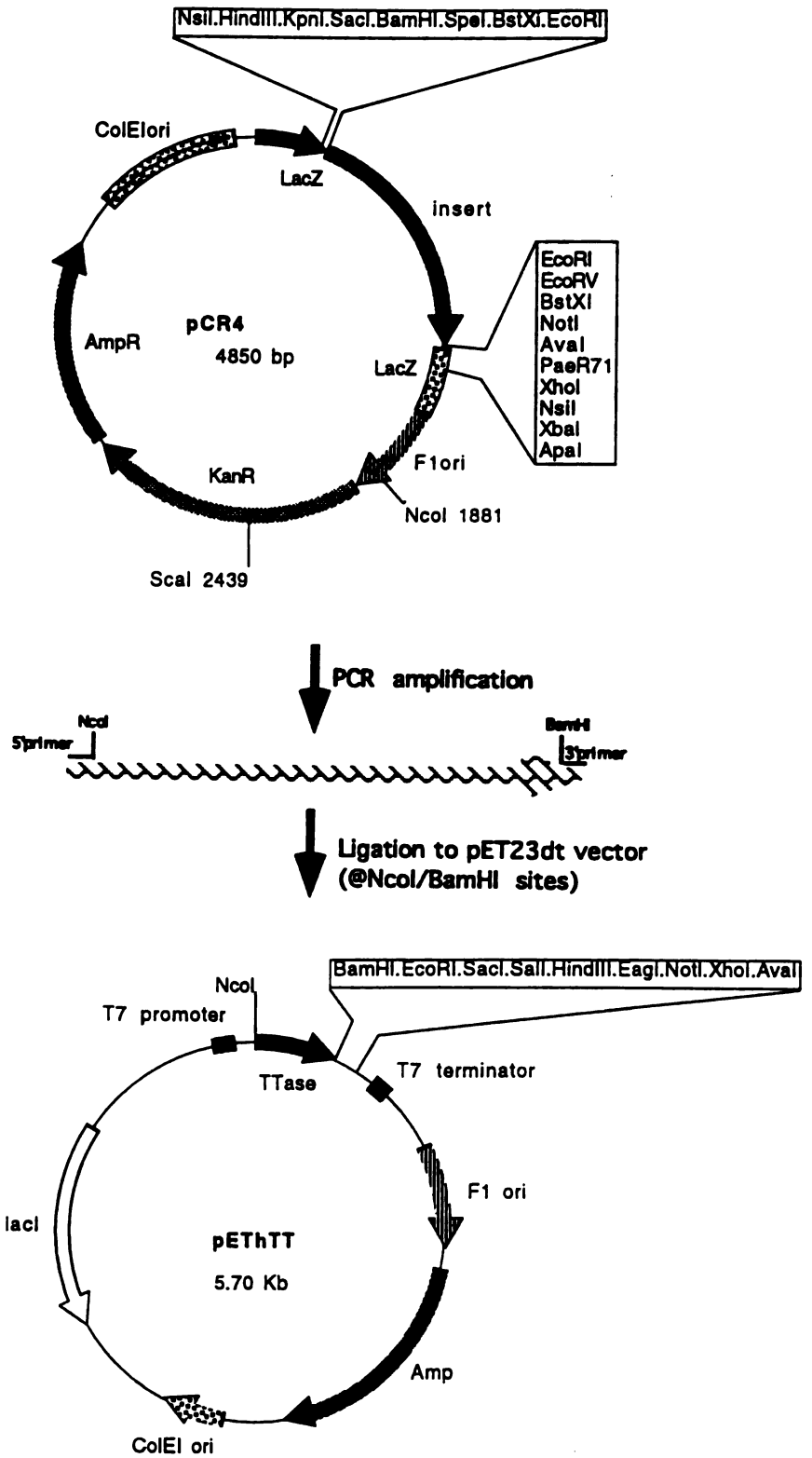


Fig. 28. Construction of pEThTT.

supernatant was retained after centrifugation at 5,500 x g for 10 min at 4°C. After heat treatment for 5 min at 65°C, a high-speed supernatant was collected (20,000 x g for 30 min at 4°C), and fractionated by 40-85% ammonium sulfate. The pellet was resuspended in 60 ml 10 mM NaH_2PO_4, pH 6.0, 2 mM EDTA, 2 mM DTT, applied to a Sephadex G-75 column (Pharmacia, 85 cm x 6 cm), and eluted with the same buffer. Active fractions were pooled into two groups, then concentrated with Centricon concentrators (Amicon, mwco 3000), and stored at -70°C with 25% glycerol added until used.

Recombinant purification 2:

A 3 l culture of Bl21(DE3) bacteria containing the pEThTT plasmid were induced and harvested as described above. The bacterial pellet was resuspended in 300 mls of 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 2 mM DTT and 1 mM PMSF, sonicated 3 times for 30 sec at 70% power on ice, and supernatant was retained through two successive centrifugation steps at 5,500 x g for 10 min at 4°C, and 18,000 x g for 30 min at 4°C, respectively. The high-speed supernatant was fractionated with ammonium sulfate, and the 40-85% ammonium sulfate pellet was resuspended in 30 ml of 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 mM DTT (Buffer A) and dialyzed against 4 l of Buffer A. The dialyzed crude enzyme fraction was then loaded onto a DEAE-Sepharose column (13.5 cm x 2.5 cm) equilibrated with Buffer A. The 28 5-ml flow-through fractions containing TT activity were collected, concentrated to 15 mls using Centriprep 3 fractionators (Amicon, mwco 3000), and then reduced with 5 mM DTT for 30 min at 37°C, and finally dialyzed against N_2-saturated 100 mM Tris-Cl (pH 7.5), 5 mM EDTA, 5 M NaCl (Buffer B). The dialysate was loaded onto a thiopropyl sepharose 6B (Pharmacia) column (10 ml bed volume), and washed with 200 ml Buffer B, followed by 300 mls of 0.1 M NaOAc (pH 4.6) plus 5 mM β-mercaptoethanol. No TT activity came

through the column on either wash. Bound hpTT was eluted with a 20-50 mM DTT gradient in 100 mM Tris-Cl, pH 8.0. Fractions containing TT activity were concentrated in a Centricon 3 microconcentrator (Amicon, mwco 3000) to less than 1 ml and frozen with 15% (v/v) glycerol at -80°C until use.

SDS-PAGE and Immunoblotting analysis:

SDS-PAGE (558) using a 6% stacking gel and 15% separating gel was performed. Two pooled samples from the G-75 fractionation of Purification 1 were electrophoresed (20 ug each), and stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid for 20 minutes at 50% power in a conventional microwave oven. The gel was then destained 3 times for 20 minutes at 50% power in a conventional microwave. Proteins from a duplicate gel were electrophoretically transferred to a nitrocellulose filter for 1 hour at 100V using a 25 mM Tris-Cl (pH 8.3), 92 mM glycine, 20% methanol buffer. Non-specific binding was blocked by incubating the membrane overnight at 4°C in 0.1% Tween-TBS (Buffer A) with 5% non-fat dry milk. After blocking, a 5 min wash in Buffer A was performed followed by incubation with the primary rabbit anti-human TT antibody (1:20,000) in Buffer A for 60 min at room temperature. The blot was then rinsed twice in Buffer A at room temperature for 15 min, and incubated with the secondary alkaline phosphatase-conjugated goat anti-rabiit antibody (1:3000) in Buffer A for 60 min at room temperature. After the blot was rinsed 2 times for 5 min, the blot was incubated with BCIP and NBT (BioRad) to visualize immobilized TT bands. Purified protein eluted from the thiopropyl sepharose 6B column in Purification 2 was treated in the same manner.

TT Activity Assays:

TT enzyme activity was assayed as described for pig liver TT (314). Briefly, the reaction mixture contained 0.5 mM GSH, 1.4 units of glutathione reductase, 2.5 mM S-sulfocysteine (Cys-SO_3^-) prepared by the method of Segle and Johnson (560), 0.35 mM NADPH, 0.137 mM sodium phosphate buffer, pH 7.5.TT activity was measured as the GSH-dependent reduction of the prototype substrate Cys-SO_3^- in a coupled spectrophotometric assay. The reaction was initiated by the addition of Cys-SO_3^-. Formation of glutathione disulfide (GSSG) was coupled to NADPH oxidation by GSSG reductase and measured spectrophotometrically by a decrease in A_{340} at 30°C. A decrease in A_{340} due to the conversion of NADPH to NADP+ corresponds to the amount of Cys-SO_3^- reduced and GSH oxidized by TT. A blank reaction without enzyme was monitored simultaneously and subtracted from enzyme-catalyzed reaction rates. One unit of TT was defined as that amount of enzyme catalyzing the formation of 1 µmol of GSSG/min under standard conditions (314).

DHA Reductase Assays:

DHA reductase assays followed the direct spectrophotometric assay of Stahl *et al.* (557) based on the change in absorbance at 265.5 nm and 30°C as DHA was reduced to AA. The standard assay was 0.137 M sodium phosphate buffer, pH 6.8, 1 mM EDTA, 2 mM GSH, 1 mM DHA, and various amounts of enzyme in a total volume of 500 µl. Blanks run simultaneously without the addition of the enzyme were subtracted from enzyme-catalyzed reaction rates. The reaction was initiated by the addition of DHA, and was linear up to 2 min at 30°C. DHA was generated by the bromine oxidation of 20 mM ascorbic acid, following the procedure of Bode et al (552). One unit of TT was defined as that amount of enzyme catalyzing the reduction of one µmol of AA per min under standard conditions.

Protein Assays:

Protein samples and bovine serum albumin (BSA) standards were precipitated in ice-cold acetone at -20°C for 30 min, and then pelleted to remove the DTT. After acetone evaporation, the samples and BSA controls were resuspended in 0.01 N NaOH, and protein concentrations were determined by the bicinchinoninic acid (BCA) protein assay protocol according to the manufacturer's direction (Pierce Chemical Co) .

Results

Human placental TT in pEThTT was expressed at about 3-10% of soluble protein 3 h after IPTG induction (Fig. 29). The soluble TT was purified using two protocols, and activity was assayed to determine if the cDNA encoded a functional protein with native TT activity.

The first purification strategy, (Purification 1), i.e., sonication followed by heat treatment for 5 min at 65°C, resulted in almost 50% losses in enzyme activity. Ammonium sulfate fractionation resulted in a small purification, and gel filtration using a G-75 resin resulted in 3-fold increased purity. TT co-eluted from the G-75 column together with two major protein peaks, and was not resolved from either peak (Fig.30). The recombinant enzyme extract possessed both dithiol-disulfide oxidoreductase and DHA reductase activities, indicating a functional protein.

The second purification strategy, (Purification 2) was applied to determine whether using DEAE Sephacel under conditions where TT did not bind would result in a greater purification from *E. coli* proteins than the standard protocol of CM Sepharose cation exchange resin. The DEAE Sephacel treatment resulted in only a 4.2-fold purification, and

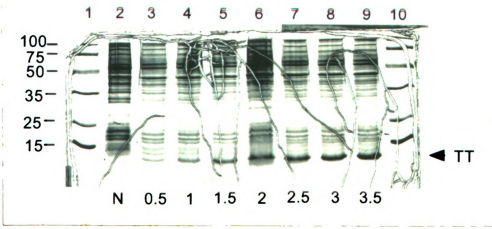


Fig. 29. Timecourse of hpTT expression in *E. coli*.
Bacteria were sampled at various times after induction, adjusted to the same OD_{600}, and electrophoresed as described in Methods. Lanes 1 and 10 are molecular weight markers; corresponding molecular weights are on the left. Lane 2: non-induced, lanes 3-9 induction of (h) 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5, respectively. The induced band that corresponds to recombinant hpTT is indicated on the right.

Fig. 30. G-75 purification of recombinant hpTT.
G-75 gel filtration was performed as described in Methods. Recombinant TT elutes between two peaks. Protein concentration (as measured by OD_{260}) is indicated on the left Y axis, and as a line. TT activity is indicated on the right Y axis, and is indicated in bars. Fraction numbers are indicated on the X axis. Active fractions were separated into two pools, corresponding to the peaks they co-eluted with, and assayed for purity by SDS-PAGE and immunoblotting.

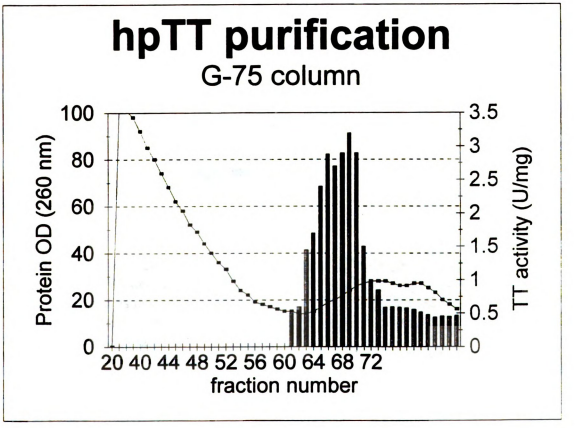


Fig. 30 G-75 purification of recombinant hpTT.

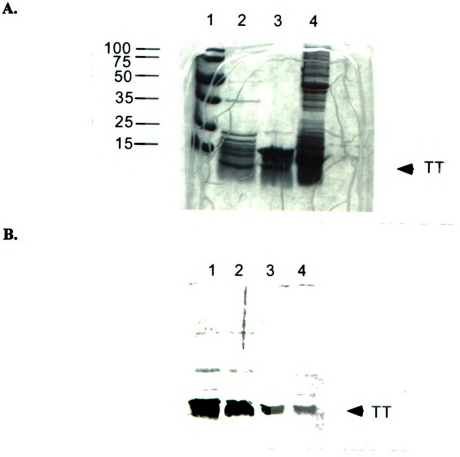


Fig. 31. SDS-PAGE and Western analysis of purified recombinant hpTT.
SDS-PAGE and Western analysis of G-75 fractions containing TT activity from Purification 1 was performed as described in Methods. A. SDS-PAGE. Marker sizes are shown on the left. Bands corresponding to TT are marked with an arrow at the right. Lane 1: molecular weight markers (Novagen), lane 2: pooled G-75 peak 2, lane 3: pooled G-75 peak 3, lane 4: bacterial suspension induced for TT expression. The TT activity elutes with two peaks. B. Western analysis. The markers are the same as in A. Lanes 1-4: Pooled G-75 fractions containing TT activity. Lane 1: Peak 2 pooled fractions for the first half of the peak eluting; lane 2: peak 2 pooled fractions for the second half of the peak to elute; lane 3: peak 3 pooled fractions for the first half of the peak to elute; lane 4: peak 3 pooled fractions for the second half of the peak to elute. The G-75 fractions are partially pure, with only one predominant protein band detectable using the polyclonal anti-TT antibody. No differences in immunuodetectable protein were observed in the different pooled fractions.

substantial losses in protein activity followed during the time involved in concentration of the extremely dilute sample. SDS-polyacrylamide (SDS-PAGE) gel electrophoresis and Western blot analysis indicated a significant band at approximately 11000 kDa that cross-reacted with polyclonal rabbit anti-human TT (Fig. 31). The purified recombinant protein had an associated thiol-disulfide exchange activity of 121.4 U/mg comparable to that previously reported (317) and DHA reductase activity of 19.7 comparable to that reported by (310).

Discussion

TTs in general are quite stable, in both storage conditions and heat treatment, however the human enzyme is more susceptible to loss of activity over time both in purification and storage, and loses activity during heat treatment, potentially due to oxidation. One explanation for this instability is an extra cysteine at position 7 unique to the human enzyme, which may lead to thiol oxidation products that irreversibly denature the enzyme. Site-directed mutagenesis of Cys^7 to Ser demonstrated no effect on either thiol-disulfide transfer activity nor the DHA reductase activity, and significantly enhanced the stability of the enzyme during purification and storage (315). The adaptive significance of this extra cysteine residue remains a mystery.

As large amounts of enzyme activity are lost during various gravity-fed chromatography steps, a DEAE Sephacel "reverse" purification step was examined to accelerate purification. Under the conditions utilized, recombinant hpTT does not bind DEAE Sephacel, and elutes in the void volume, leaving many *E. coli* proteins bound to the resin. The total volume recovered from the DEAE Sephacel step was larger than estimated, however, as the TT eluted in 108 5-ml fractions. Large losses in activity were noted for hpTT while

dilute, leading to the conclusion that this is a less desireable purification method.

Activity comparisons with those of other reported recombinant human TT purifications were not performed. Activity comparisons to the native human enzyme are similar to that reported by Mieyal, *et al* (317). Mieyal reported dithiol-disulfide activity of 121 U/mg for the purified erythrocyte enzyme using Cys-SO_3^- as the substrate, and we find the recombinant enzyme to have the same activity, 121.4 U/mg, after activity losses. Padilla *et al* (310) reported DHA reductase activity of 29 U/mg, where we find DHA reductase activity of 19.7 U/mg. The hpTT cDNA therefore encodes a functional protein with native activities, and is suitable for further overexpression studies on physiological function in tissue culture cells.

CHAPTER FOUR

INCREASED RESISTANCE TO ADRIAMYCIN IN MCF-7 BREAST TUMOR CELLS CONSTITUTIVELY OVEREXPRESSING hpTT.

Introduction

Thioltransferase (TT) has two known *in vitro* activities: thiol-disulfide oxidoreduction and dehydroascorbate (DHA) reduction, and is proposed to be involved in regulating cellular redox homeostasis. Two lines of evidence indicate a correlation between increased cellular levels of TT and enhanced resistance to Adriamycin in MCF-7 mammary adenocarcinoma cells. First, an Adriamycin-resistant MCF-7 cell subpopulation (MCF-7 ADR^R) had four-fold higher levels of TT than the drug-sensitive cell line (MCF-7 WT), and showed further increases in drug resistance when an ascorbate (AA) precursor, L-ascorbic acid 2-phosphate (AAP), was added (526). This suggested that the DHA reductase activity of TT contributed to increased Adriamycin resistance in the MCF-7 ADR^R cells. Secondly, MCF-7 ADR^R "revertant" cells that had been grown for 20 passages without Adriamycin in the media had both significantly reduced resistance and no longer had immunodetectable TT. Similarly passaged MCF-7 ADR^R cells grown in the presence of Adriamycin retain both their drug resistance and immunodetectable TT levels.

To further test the hypothesis that the increases in Adriamycin resistance were due to an increased ability to recycle AA, MCF-7 WT cell lines were stably transfected with the TT cDNA in a constitutive overexpression plasmid. In addition, in both the MCF-7 WT and

ADR^R cell lines, stable cell lines that overexpress TT in response to glucocorticoid induction were also established. These cell lines may prove useful for future studies.

Methods

Culture of MCF-7 cells:

MCF7 ADR^R and MCF-7 WT cells were grown in 75 cm^2 plastic tissue culture flasks in HEPES-buffered RPMI 1640 (Gibco-BRL) with 2 mM glutamine, 100 U/ml penicillin, 10 U/ml streptomycin (Gibco), 50 µg/ml gentamycin (Gibco) and 10% fetal calf serum (Gibco) in a humid atmosphere at 37°C in 5% CO_2. MCF-7 WT transfected cells were maintained under the same conditions with the addition of 400 µg/ml active Geneticin to the media.

Partial fractionation of cell extracts:

Medium was removed from 20 to 30 tissue culture flasks (75 mm^2) of cells grown to 100% confluency. Cells were rinsed twice with 5 ml PBS, manually scraped into 1 ml PBS, and centrifuged at 2,500 x g for 5 min at 4°C. The cell pellet was resuspended in 1 ml PBS with 0.05 mM PMSF, homogenized 15 strokes in a 1.5 ml Microfuge tube using a micropestle (Eppendorf). The soluble fraction containing cytoplasmic TT was obtainined by successively retaining the supernatants of a low-speed spin at 5,500 x g, 5 min at 4°C in a Sorvall SS-34 followed by a-high-speed spin at 105,000 x g, 60 min at 4°C in a Beckman L-2 Spinco ultracentrifuge. The supernatant was then fractionated through Centricon-30 (mwco 30,000) microconcentrators to remove high-molecular weight proteins. The flow-through containing TT was subsequently concentrated through a Centricon-3 (mwco 3000) microconcentrator to less than 500 µl.

Protein assays:

Protein samples and bovine serum albumin (BSA) standards were precipitated in ice-cold acetone at -20°C for 30 min, and then pelleted to remove the DTT (566). After acetone evaporation, the samples and BSA controls were resuspended in 0.01 N NaOH, and protein concentrations were determined by the bicinchinoninic acid (BCA) protein assay protocol (567) according to the manufacturer's direction (Pierce Chemical Co). Protein assays were performed on both the crude homogenate and the partially fractionated samples.

SDS-PAGE and Western Blot Analysis:

Each lane of a Laemmli SDS-PAGE 15% separating and 6% stacking gel (558) was loaded with 15 μg protein, which was separated using a25 mM Tris, 192 mM glycine, 0.1% SDS running buffer (pH 8.3) for 1 h at 200 V. The proteins on the gel were then electrophoretically transferred to an ECL-nitrocellulose membrane (Amersham) in 25 mM TrisCl, pH 8.3, 192 mM glycine, 20% methanol for 1 h at 100 V. Non-specific binding was blocked by incubating the membrane overnight at 4°C in 0.1% Tween-20-PBS (Buffer A) containing 5% non-fat dry milk. After blocking, a 5 min wash in Buffer A was performed followed by incubation with the primary rabbit anti-human TT antibody (1:10,000) in Buffer A, 1% milk (Buffer B) for 60 min at room temperature. The blot was then rinsed twice in Buffer A at room temperature for 15 min, and incubated with the secondary HRP-conjugated goat anti-rabbit antibody (1:2000, Amersham) in Buffer B for 60 min at room temperature. After briefly rinsing in Buffer A, the blot was washed once for 15 min, then 3 times for 5 min each in Buffer A. The ECl chemiluminescent detection system (Amersham) was used to visualize TT signals for 1 min, and the blot was exposed to ECL-Hyperfilm for 5 min.

PCR amplification of the hpTT cDNA:

Two primers were synthesized for the PCR amplification of a hpTT coding region insert (Michigan State University Macromolecular Structure Facility). The 5' primer (5'-TGGTTAAATCCGCTAGCAATA-3'), corresponding to nt -65 to -45 (relative to the translational start site), and contained the artificially introduced *NheI* restriction site (GCTAGC); the 3' primer (5'-GCTCTAGAGCTTACTGAAGAGCTCCAATC-3'), corresponding to nt +302 to +330 (relative to the translational start site), contained the artificially introduced *XbaI* restriction site (TCTAGA) 3 nt 3' to the translational termination codon TAA. PCR amplification of the coding region of hpTT cDNA from the pCR4 plasmid (Fig.19, Ch.2) template using these primers catalyzed by Taq polymerase (Perkin Elmer) for 25 cycles (30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C) in a Perkin Elmer 9600 thermocycler resulted in a 392 bp amplicon, which was subsequently ligated into pCRII (Invitrogen) (Fig.32). The resultant plasmid, designated pCRTTeu, was used (8 µl) to transform INVaF' supercompetent *E. coli* (Invitrogen). Plasmid DNA was extracted from 500 ml bacterial cultures following alkaline lysis and ion exchange purification using the Promega MAGIC™ purification system according to maufacturer's protocols (Promega). Purified pCRTTeu plasmid DNA was subjected to restriction digestion with *NheI* and *XbaI* to release the insert DNA corresponding to the human TT coding region. The 377 bp DNA fragment was purified following electrophoresis by extracting the band from a 1.2% agarose gel, and centrifugation through a Costar Spin-X membrane (Costar, 561).

Construction of the constitutive hpTT expression vector:

pCDNA3 (Invitrogen) is a constitutive mammalian expression vector with transcription of the cDNA insert under the control of the cytomegalovirus (CMV) immediate

Fig. 32. Construction of pTTeu.
hpTT cDNA was PCR amplified from pCR4 using two primers, a 5' primer corresponding to nt -65 to nt -45 (relative to the translational start site), which artificially introduced an *NheI* restriction site, and a 3' primer corresponding to nt +302 to nt +330 (relative to the translational start site), which artificially introduced a *XbaI* site, as described in Methods. The hpTT encoding amplicon was ligated into pCRII as described in Methods, and designated pTTeu.

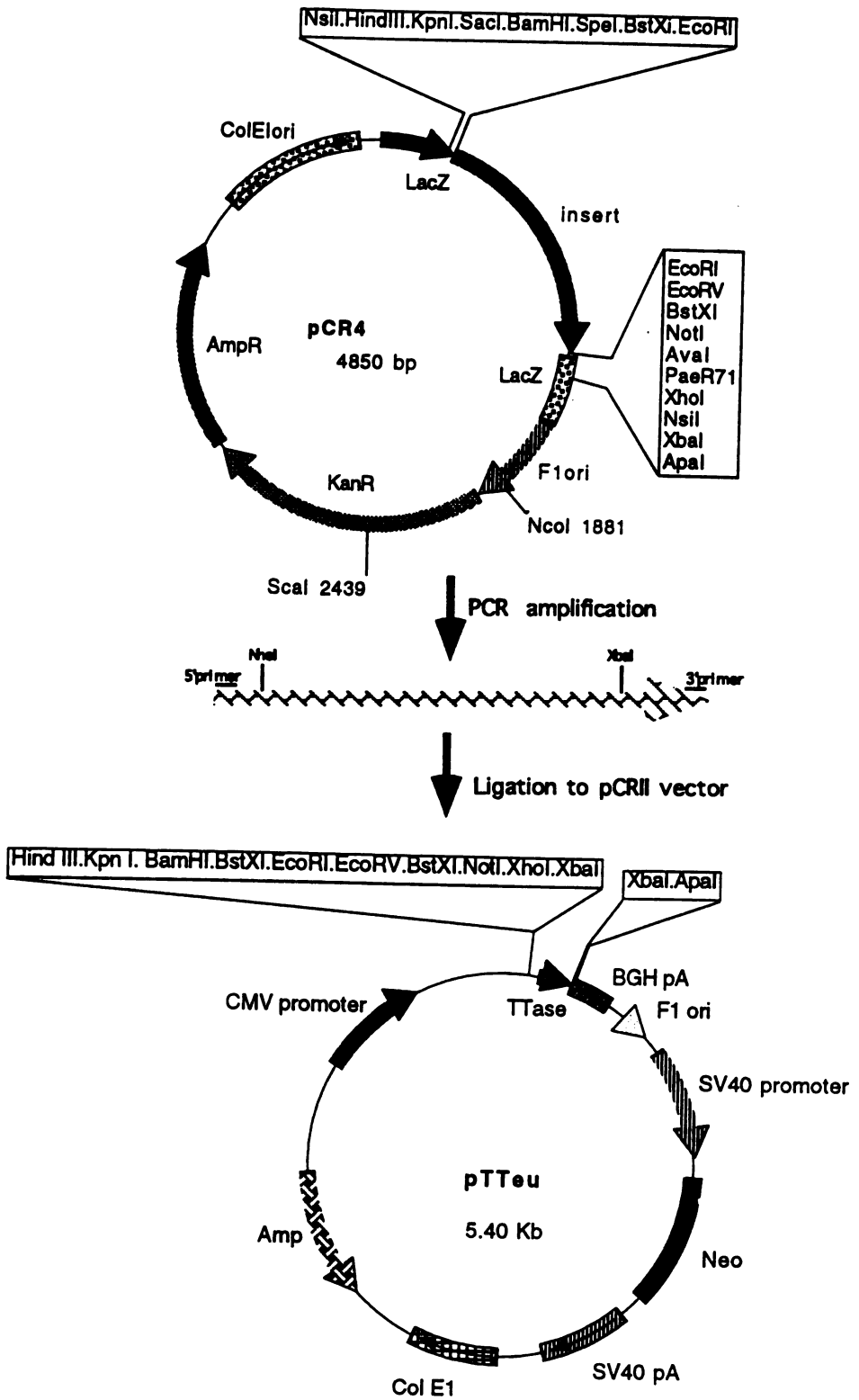


Fig. 32. Construction of pTTeu.

early promoter (563). The *NheI-XbaI* PCRTTeu hpTT coding region corresponding to -53 to +327, relative to the translational start site, was subcloned into the *XbaI* site of pCDNA3. Orientation of the hpTT cDNA insert in the pCDNA3 vector with respect to the CMV promoter was confirmed using a single *XhoI* restriction digest of the recombinant plasmid. Constructs in which the CMV promoter directed hpTT synthesis in the sense direction were designated pTTcmv (Fig.33).

G-418 Dose-Response in MCF-7 Cells:

G-418 sulfate (Geneticin, Gibco-BRL) viability dose-response in both MCF-7 WT and MCF-7 ADR^R cells were determined using selective media containing variable concentrations of added G418 with no other antibiotics or antimycotics, changed weekly. Levels of G-418 sulfate tested were 50, 100, 200, 300, 400, and 500 µg active Geneticin per ml media. After four weeks, cell were counted and cytotoxicity rates calculated for both MCF-7 WT and MCF-7 ADR^R cells.

Transient transfection of MCF-7 cells with a luciferase expression plasmid:

In order to estimate optimal transfection protocols for the MCF-7 WT and MCF-7 ADR^R cell types, a trial transfection using the pGL (Promega) plasmid was performed. pGL contains the firefly luciferase gene under the control of the SV40 promoter. Confluent MCF-7 WT and MCF-7 ADR^R cells were trypsinized and plated at 1-2 x 10^5 cells/ 100 cm^2 plate, grown to 40-60% confluence, and transfected with pGL. Dose-response curves were established to determine optimal DNA:lipofectin ratios for transfection. For each plate transfected, 0, 1, 2, or 4 µg DNA were used, together with either 2 or 10 µl lipofectin (BMB,565) according to manufacturer's transfection protocols. After 48 h, transfected cells were washed twice with 0.9% NaCl at room temperature, lysed *in situ* with 100 µl of

Fig. 33. Construction of pTTcmv.
pTTeu plasmid DNA was digested with *NheI* and *XbaI* to release the 377 bp insert cDNA, which was purified as described in Methods, then ligated into *XbaI*-linearized pCDNA3 (Invitrogen), and designated pTTcmv. Orientation of the hpTT insert in the sense direction with respect to the CMV promoter was confirmed with a single *XhoI* restriction digest of the recombinant pTTcmv plasmid.

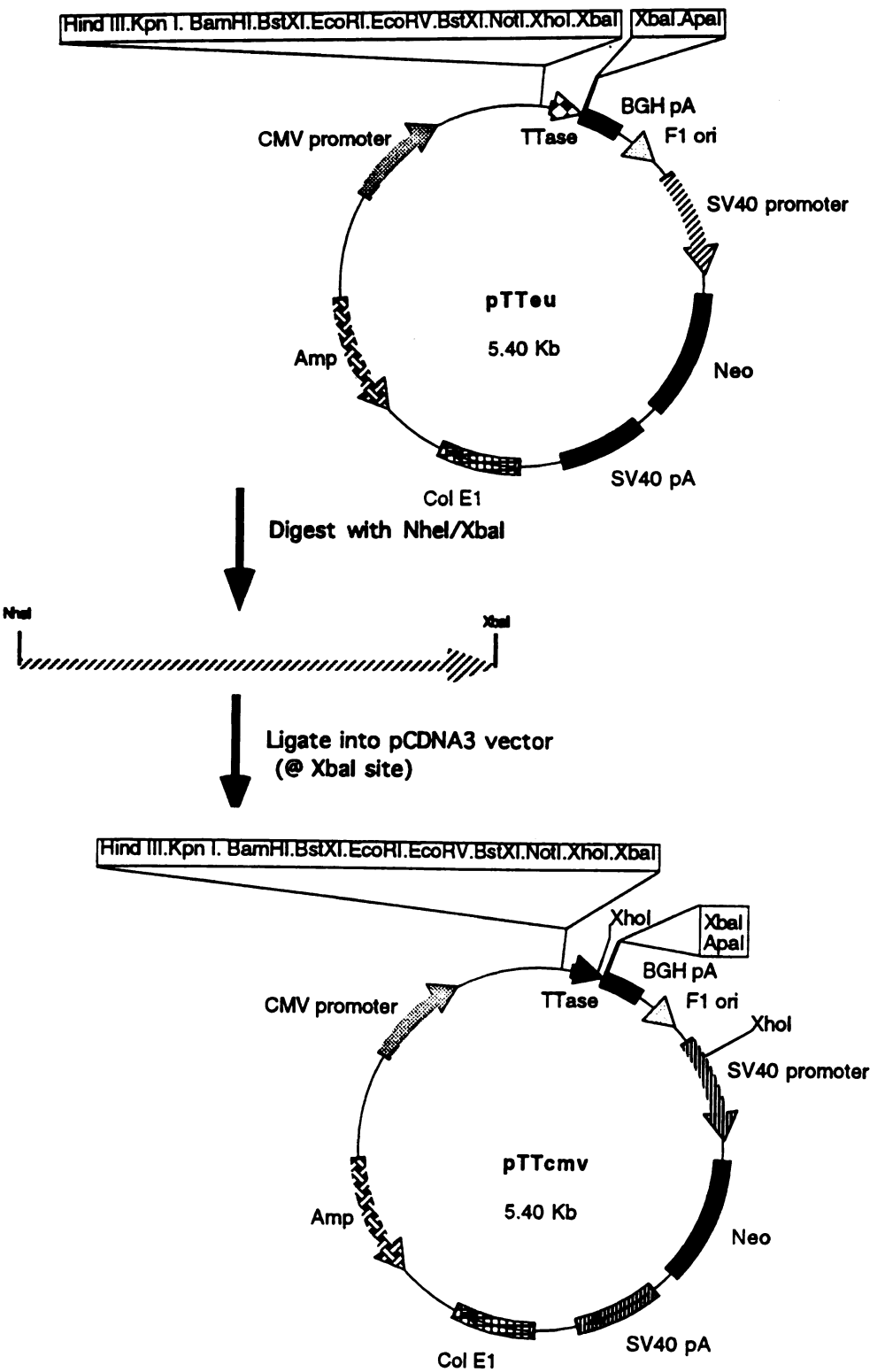


Fig. 33. Construction of pTTcmv.

Reporter Lysis Buffer (Promega,568) for 15 min, then scraped into a 1.5 ml microfuge tube. Cell debris was pelleted at 14,000 x g for 5 min, and the supernatants were transferred to a new microfuge tube. Luciferase activity was determined using 20 µl of cell lysate in 100 µl Reporter Assay Buffer (Promega) using a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA). A control containing no cell lysate was used as a blank, and subtracted from sample luminescence. Optimal conditions were determined to be 3 µg DNA and 10 µl lipofectin per plate.

Stable transfection of MCF-7 cells with a TT overexpression plasmid:

Confluent cells were trypsinized and split 1:10 from a 150 cm^2 tissue culture flask into 100 cm^2 tissue culture dishes, grown to 30-50% of confluency, and transfected with the hpTT expression plasmid pTTcmv or pCNDA3 (without insert), as follows: For each plate transfected, 3 µg plasmid DNA and 10 µl lipofectin were each diluted into a final volume of 100 µl serum-free media. The aliquots were mixed and cells transfected according to maufacturer's transfection procedures (BMB). Forty-eight h after transfection, each 100 cm^2 plate of cells were subcultured 1:5 by trypsinizing and replating into 5 new plates together with media containing 400 µg/ml active G-418 sulfate (Gibco-BRL) to select for plasmid incorporation. The pCDNA3 parent plasmid from which the hpTT expression plasmid was constructed contains the bacterial neomycin aminoglycoside phosphotransferase gene conferring G-418 resistance under the control of the constitutive SV40 promoter. Cells were refed selective (400 µg/ml active G-418) media every week and examined for the formation of colonies. Once isolated colonies were 100-1000 cells in size, they were trypsinized using cloning rings or 3 MM Whatman discs and subcultured individually. Approximately 6 x 10^7 MCF-7 ADR^R and 6 x 10^7 MCF-7 WT cells were transfected with 3 µg of the pTTcmv vector

and 10 µl lipofectin per ml of media.

Characterization of integration cell lines that constitutively overexpress hpTT:

To verify that the hpTT coding region together with the CMV promoter was integrated into cellular chromosomes in the intact form, RT-PCR (569) was performed on total cellular RNA isolated from each of the pTTcmv-transfected MCF-7 WT stable cell lines.

RNA isolation:

Confluent cells in 150 cm^2 tissue culture flasks were trypsinized and split (1:5) onto 100 cm^2 tissue culture dishes. Medium was removed from the dishes and cells were lysed *in situ* to determine RNA, DNA and protein content following the method of Chomszynski *et al* (564). Briefly, 1 ml of Tri-Reagent (Molecular Research Corp.) was added to each 100 cm^2 dish, and the cells were detatched by gentle pipetting, then transfered to an 1.5 ml microfuge tube. Nucleoprotein complexes were dissociated by incubation at room temperature for 5 min. Total cellular RNA was extracted using 200 µl chloroform for 10 min. After centrifugation at 12,000g for 15 min at 4°C, the aqueous phase containing the mRNA was removed, retained, and precipitated using 500 µl isopropanol for 10 min at room temperature. Following centrifugation at 12,000g for 12 min at 4°C, the RNA pellet was washed once with 2 ml of 95% ethanol, pelleted at 8,500 x g for 5 min at 4°C, then stored at -20°C in 95% ethanol until use. Two separate RNA extractions were performed for each of the 38 pTTcmv-transfected MCF-7 WT cell lines.

RT-PCR:

Reverse transcription of total RNA extracted MCF-7 WT, MCF-7 WT transfected, and MCF-7 ADR^R cells was performed following manufacturer's directions. Prior to use, RNA was treated with 10 units RNAse-free DNAse for 30 min at 37°C. Coupled cDNA

synthesis and PCR amplification of extracted RNAs was carried out in two steps. First, cDNA synthesis was performed in 250 mM Tris-HCl, (pH 8.3), 375 mM Kcl, 15 mM $MgCl_2$, 100 mM DTT, and 10 mM each dATP, dCTP, dGTP, and dTTP. The first strand synthesis mixture alsoncontained 0.5 U RNAsin (Promega), 200 U Superscript Reverse Transcriptase (Gibco-BRL), 1 μm of an SP6 promoter primer (5'-ATTTAGGTGACACTATAG-3', corresponding to plasmid sequences at +350 nt to +332 nt, relative to the translational start site), and 15 μg total cellular RNA. Reactions were incubated at 42°C for 60 min, followed by enzyme inactivation for 15 min at 70°C. The cDNA synthesized was used as a template for subsequent PCR reaction. PCR amplification of 10 % of the RT-generated cDNA mixture was performed using a 5' primer (5'-TGGTTAAATCCGCTAGCAATA-3', Michigan State Macromolecular Structure Facility) corresponding to nt -65 to nt-45 (relative to the translational start site in the pTTcmv vector), and containing the altered nucleotides for the artificially induced *NheI* site, and a 3' SP6 promoter primer (5'-ATTTAGGTGACACTATAG-3', Gibco-BRL), corresponding to plasmid pTTcmv sequences at nt +350 to nt +332 (relative to the hpTT translational start site) (Fig. 33). PCR amplification using Taq polymerase (Perkin Elmer) for 25 cycles (30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C) in a Perkin Elmer thermocycler results in a 415 bp amplicon in the plasmid-containing cell lines only.

TT Activity Assays:

Cells were harvested and partially fractionated to enrich TT concentration as described previously (p.134). TT enzyme activity was assayed as previously described (314). Briefly, the reaction mixture contained 0.5 mM GSH, 1.4 units of glutathione reductase, 2.5 mM S-

sulfocysteine (Cys-SO_3^-) prepared by the method of Segle and Johnson (560), 0.35 mM NADPH, and 0.137 M sodium phosphate buffer (pH 7.5). The reaction was catalyzed by the addition of Cys-SO_3^-. Formation of GSSG was coupled to NADPH oxidation by glutathione reductase and measured spectrophotometrically by a decrease in A_{340} at 30°C. A simultaneously monitored blank reaction not containing cell homogenate or enzyme was subtracted from sample reaction rates. One unit of TT was defined as that amount of enzyme catalyzing the formation of 1 μmole of GSSG/min under standard conditions (314).

Adriamycin Cytotoxicity Assays:

Fluorescent 96-well assays were used to determine viability of the different cell lines after exposure to varied levels of Adriamycin and AAP. Fluorimetric viability assays were chosen since they are extremely sensitive and minimize interference by Adriamycin or AAP. The viability assay chosen measures not only cells with intact membranes, but metabolically active cells, which distinguishes it from trypan dye exclusion. The 96-well plate assay allows large numbers of samples to be processed using fewer numbers of cells. Other types of 96-well plate assays such as tetrazolium colorimetric assays were incompatible with either the Adriamycin or the AAP. Adriamycin does not interfere with the assay until levels of Adriamycin exceed 400 μg/well. At the higher levels of Adrimaycin, the drug coats the plates, and interferes with detection.

Each well in a 96-well opaque tissue culture dish (Costar) contained 3 x 10^5 cells in 200 μl media, which were allowed to attach overnight. The media were removed from the wells and replaced with media ± Adriamycin, ± AAP treatments for 72 h. Treatments consisted of AAP (Wako Pure Chemical Industries, Ltd., Richmond, VA) at 0, 2 or 5 mM, and Adriamycin (Adria Labs, Columbus, OH) ranging from 0.034 μM to 856 μM. After 72

h, the cells were refed with the same treatment media for another 72 hours. To determine viability, media were aspirated, the cells were rinsed twice with 200 µl PBS, then 100 µl 3 µM Calcein-AM (Molecular BioProbes) in dH_2O was added to each of the wells. Calcein-AM is a non-fluorescent, membrane-permeant dye that is cleaved by active cellular esterases into a fluorescent polyanionic form, Calcein. Calcein is retained in the cytoplasm, and fluoresces bright green at 530 nm upon excitation at 485 nm. Fluorescence was determined using a 96-well fluorescence microplate reader (Model 7620, Cambridge Technologies). Control wells containing cells not exposed to Adriamycin for each treatment level of AAP were used as the standard for total viability. Control wells treated with 95% ethanol for five minutes were used as a comparative measure of complete morbidity. Viability comparisons were made using LD_{50} levels relative to those of MCF-7 WT cells. Calculations were perfomed using Graph Pad PRISM™ statistical analysis program.

Results:

G-418 Dose-response:

Both the MCF-7 WT and MCF-7 ADR^R cell lines exhibited the same dose-response to G-418. Cells were viable under conditions of 100 µg active Geneticin per ml, and morbidity was 100% when 400 µg per ml active Geneticin was administered (Fig 34).

Western Blot Analysis:

Chemiluminescent western blots were performed on non-transfected MCF-7 WT and MCF-7 ADR^R cell lines as well as an MCF-7 ADR^R cell line that had not been maintained on Adriamycin for 20 passages (MCF-7 $ADR^R_{revertant}$), and had lost its resistance. TT was detectable in the MCF-7 ADR^R cell line, but not in either the MCF-7 $ADR^R_{revertant}$ or the

MCF-7 WT cell lines (Fig. 35), indicating that there is both an upregulation of TT in the MCF-7 ADR^R, which have previously been demonstrated to be significantly more resistant to Adriamycin, and a downregulation of TT protein in cells with less drug resistance, such as the MCF-7 WT and MCF-7 $ADR^R_{revertant}$.

Transient transfection:

Transient transfection of both MCF-7 WT and MCF-7 ADR^R cells with pGL resulted in luciferase activity. The quantity of purified pGL DNA used in the transfection was linear with luciferase response from 1 μg to 2 μg, and the luciferase response declined when 4 μg was used (Fig.36). 2-3 μg DNA was determined to give reasonable transfection efficiency with 10 μl lipofectin in both MCF-7 cell lines.

Constitutive stable cell lines:

Thirty-eight cell lines were isolated containing the pTTcmv vector stably integrated into the MCF-7 WT cell line. No clonal lines were isolated after transfection of the MCF-7 ADR^R cells.

RT-PCR:

Amplified bands corresponding to the hpTT cDNA insert were detected in each pTTcmv-transfected MCF-7 WT cell line, and not in the MCF-7 WT and MCF-7 ADR^R un-transfected cell lines.

TT Activity Assays:

TT activity was examined in the untransfected MCF-7 WT, MCF-7 ADR^R, and MCF-7 $ADR^R_{revertant}$ cell lines in addition to the ten pTTcmv-transfected MCF-7 WT cell lines. Untransfected MCF-7 WT cells had extremely low levels of TT activity (0.26 U). By

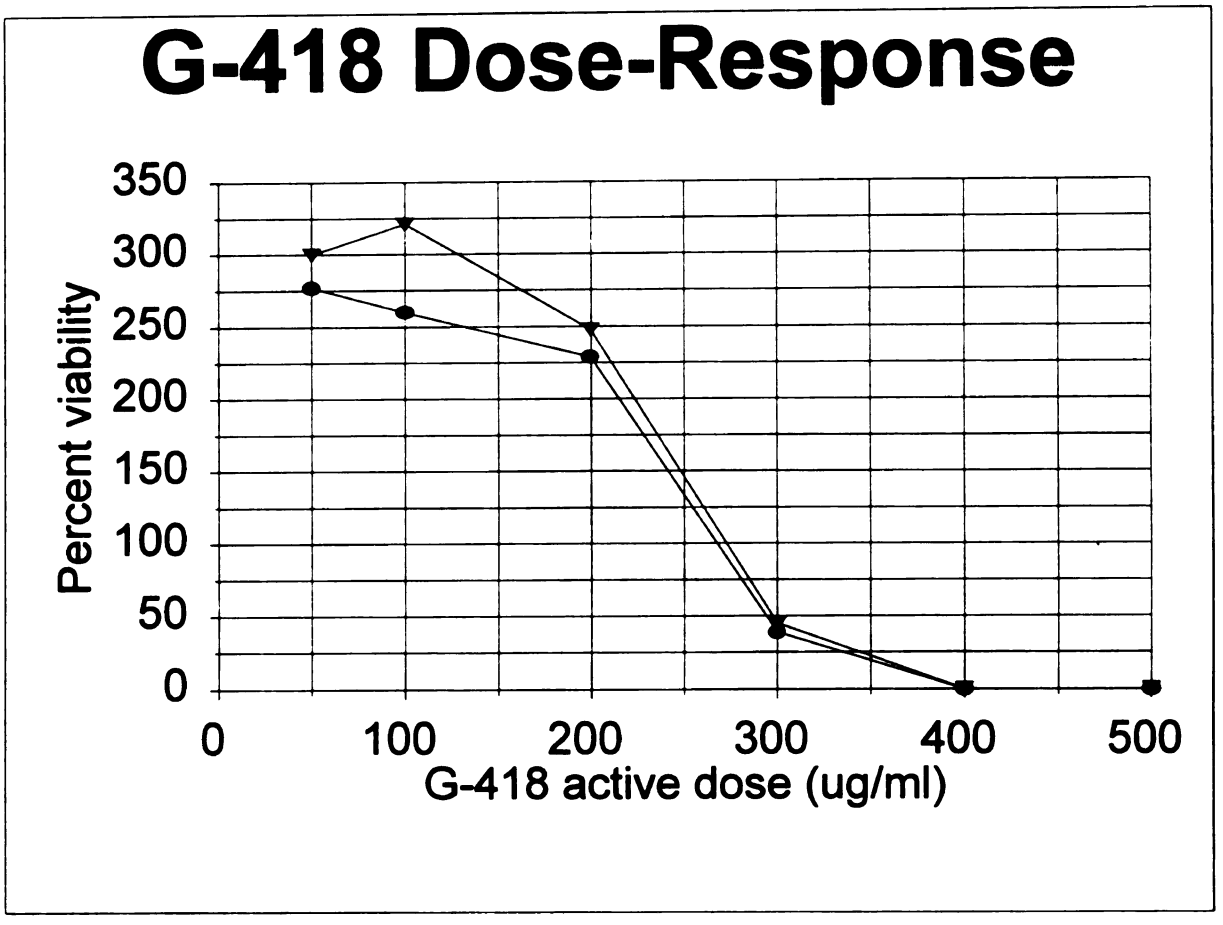


Fig. 34. G-418 dose-response.
MCF-7 WT and MCF-7 ADR^R viability in the presence of 50, 100, 200, 300, 400, and 500 μg active G-418 sulfate (Geneticin) per ml media was tested. At concentrations of active Geneticin at or below 100 μg per ml, cells had no changes in viability. At levels at or above 300 μg active Geneticin per ml, cells had markedly decreased viability, and at levels at or above 400 μg active Geneticin per ml, 100% morbidity was observed.

Fig. 35. Western analysis of TT content in MCF-7 extracts.
MCF-7 WT, MCF-7 $ADR_{revertant}$, and MCF-7 ADR^R cells were harvested and partially fractionated to enrich for TT as described in Methods. Chemiluminescent western analysis was performed as described in Methods. TT was immunodetectable in the MCF-7ADR^R cell line, but not in the MCF-7 $ADR_{revertant}$ or the MCF-7 WT cell lines. Lane 1: human placental TT , lane 2: MCF-7 WT, lane 3: MCF-7 $ADR_{revertant}$, lane 4: MCF-7 ADR^R.

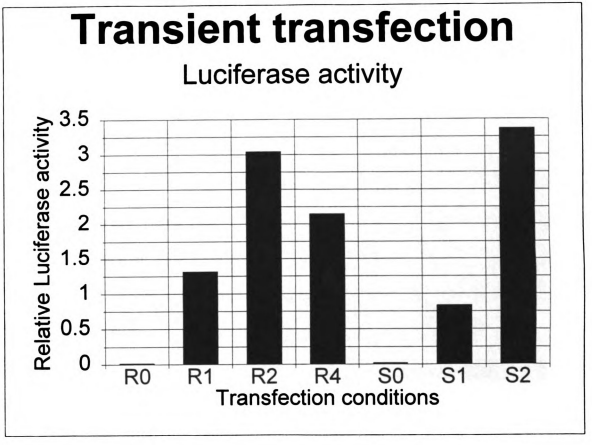


Fig. 36. Transient transfection of MCF-7 WT and ADR^R cells.
Cells were transfected with varied amounts of pGL plasmid DNA using 10 µl lipofectin (BMB) as described in Methods. Luciferase activity was monitored 48 hours post-transfection. Relative luminescence is measured on the X-axis; transfection conditions (Cell type: S= WT, R= ADR^R; 0,1,2,4 indicate µg plasmid DNA used) are on the Y-axis. Luminescence was linear with DNA concentration to 2 µg; at 4 µg the response was diminished.

comparison, MCF-7 ADR^R and $ADR^R_{revertant}$ lines were substantially higher in activity, 23 and 6.5-fold, respectively. All ten stably pTTcmv-transfected MCF-7 WT cell lines possessed dithiol-disulfide (TT) activity 9-fold to 66-fold higher than the original MCF-7 WT strain, and generally higher than the MCF-7 ADR^R cells (Fig.37).

Adriamycin Cytotoxicity Assays:

The cytotoxic effect of exposure to Adriamycin was tested in 15 stably pTTcmv-transfected MCF-7 WT cell lines. Increased resistance to Adriamycin, as measured by LD_{50} comparisons to the original MCF-7 WT cell line, was observed in all hpTT overexpression cell lines, ranging from 2-fold increases to 10-fold increases in viability (Fig. 38). The MCF-7 $ADR^R_{revertant}$ cell line showed resistance approximately 5-fold higher than that of the MCF-7 WT line, but 60-fold lower than that of the Adriamycin-resistant MCF-7 ADR^R cell line. The Adriamycin resistance of the MCF-7 $ADR^R_{revertant}$ cell line was comparable to that of most of the hpTT-overexpressing MCF-7 WT cell lines. Neither 2 mM nor 5 mM AAP increased the resistance to Adriamycin in the stably pTTcmv-transfected cells (Fig.39); instead, interestingly, resistance appeared slightly lower when AAP was added. At the extremely low levels of Adriamycin (< 1 μM), MCF-7 WT pTTcmv-transfected cells, MCF-7 $ADR^R_{revertant}$ cells, and MCF-7 ADR^R cells unexpectedly showed slightly increased viability as compared with the viability of cells without Adriamycin treatment.

Discussion

Since the physiological function of TT has not been fully characterized, it was uncertain, upon the initiation of this project, what the effect would be of high constitutive TT expression in MCF-7 cells. For instance, TT could conceivably have toxic consequences when continuously expressed. Our results indicate that there may indeed be a negative effect

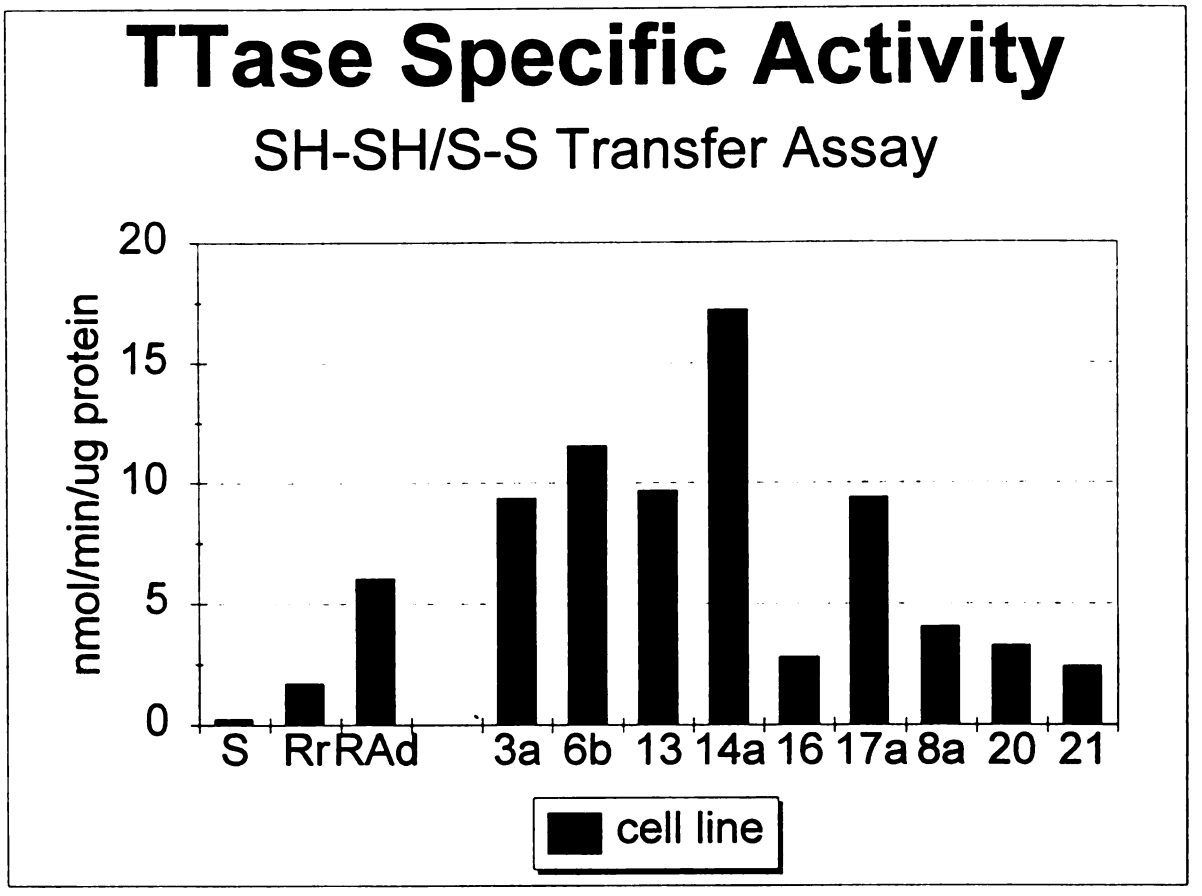


Fig. 37. TT activity in MCF-7 WT, ADR^R, and WT transfected cells.
TT activity was monitored in partially fractionated cell extracts as described in Methods. Relative TT activity is indicated on the Y-axis, and cell type on the X-axis. Legend: S=MCF-7 WT, Rr= MCF-7 ADR$_{revertant}$, RAd= MCF-7 ADR^R. Numbers indicate average results of ten MCF-7 WT transfected lines. The values represent the mean $\pm$ standard deviation of three separate experiments.

Fig. 38. Adriamycin cytotoxicity in MCF-7 WT, ADR^R and WT transfected cells. Fluorescent 96-well cytotoxicity assays were performed as described in Methods. S= MCF-7 WT, R_T = MCF-7 ADR $_{revertant}$, RAd = MCF-7 ADR^R, all others labeled are MCF-7 WT transfected cell lines. The values represent the mean $\pm$ the standard deviation of five separate experiments, run in triplicate. Cytotoxicity comparisons were performed at the LD_{50} levels relative to the MCF-7 WT cells using the statistical package PRISM™ by Graph Pad.

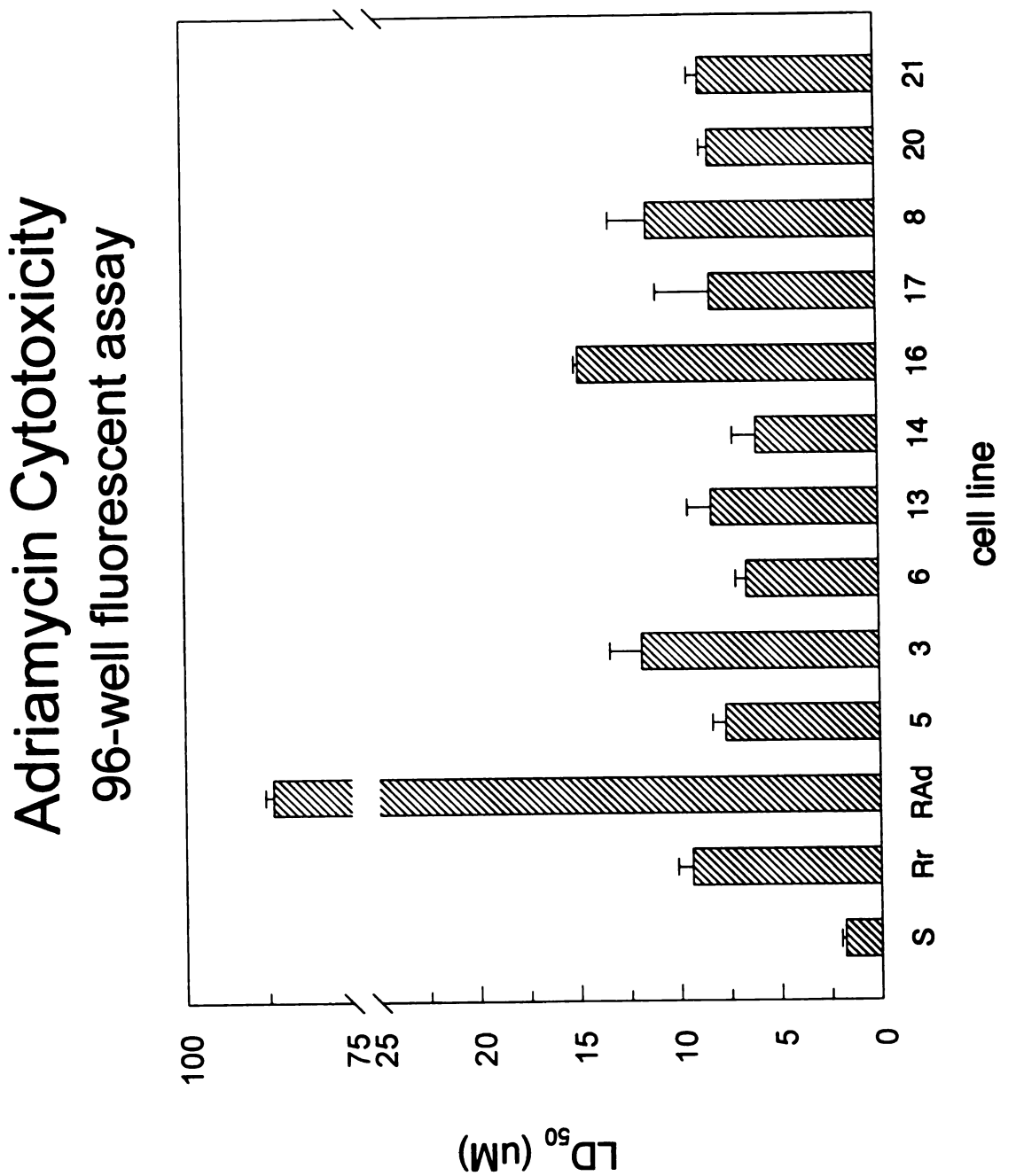


Fig. 38. Adriamycin cytotoxicity in MCF-7 WT, ADR^R and WT transfected cells.

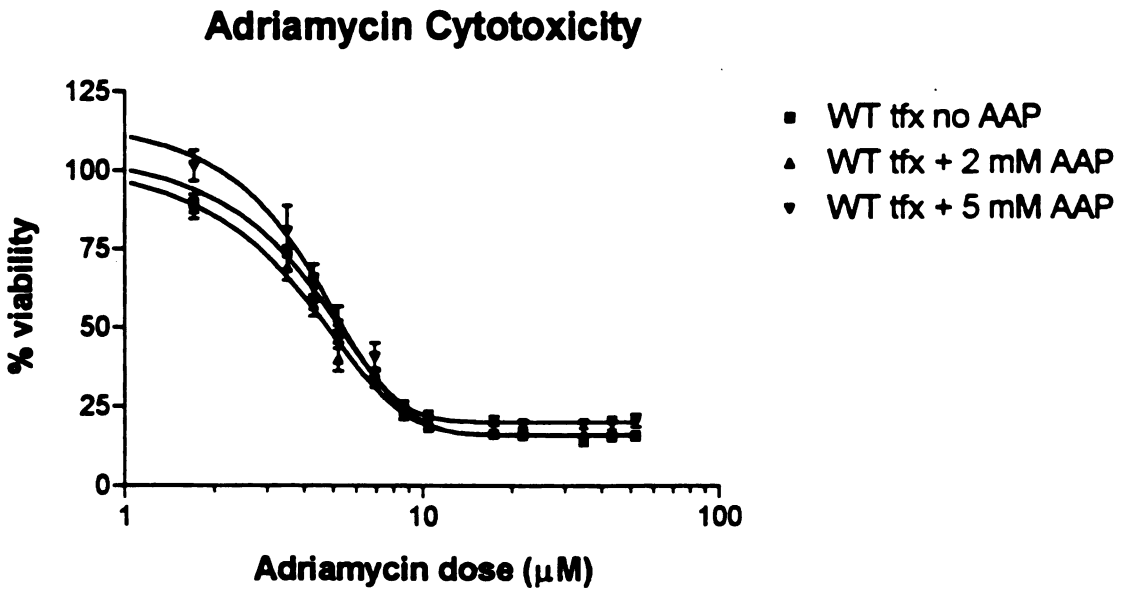


Fig. 39. Effect of AAP on Adriamycin cytotoxicity in MCF-7 WT transfected cell lines. Relative Adriamycin cytotoxicity is shown on the left. A representative WT transfected cell line is shown. Fluorescent cytotoxicty assays were performed as described in Methods. ■ represents WT transfected with no AAP added, ▲ represents WT transfected with 2 mM AAP added, ▼ represents WT transfected cells with 5 mM AAP added. The values represent the averages ± standard error of the mean of three separate experiments, run in triplicate. Cytotoxicity comparisons were performed at the LD_{50} levels using the statistical package PRISM™ by Graph Pad.

associated with hpTT overexpression in MCF-7 ADR^R cells. When matched numbers of MCF-7 WT and MCF-7 ADR^R cells were transfected, only the MCF-7 WT cells integrated the constitutive overexpression pTTcmv plasmid into genomic DNA. The TT activity levels in 10 MCF-7 WT cell lines transfected with pTTcmv ranged from 9- to 66-fold above that of the untransfected MCF-7 WT cell line. Several of these cell lines, in fact, had TTactivity significantly exceeding that of the MCF-7 ADR^R lines. Since TT activity in MCF-7 ADR^R cell lines is already 6.5- to 23-fold higher than in the MCF-7 WT line, one explanation for the inability to isolate any pTTcmv stably transfected MCF-7 ADR^R colonies is that the strong constitutive cytomegalovirus promoter may elevate TT dithiol-disulfide activity levels to where they are toxic to the MCF-7ADR^R cell. MCF-7 ADR^R cells have multiple measureable differences from the parental MCF-7 WT cell lines, including hormone independent growth (545), altered membrane receptor levels (544), decreased drug accumulation, increased drug detoxification (522, 543), increased DNA repair activity, elevated cytosolic pH (429), and alterations in various GSH-dependent enzymes that detoxify ROS (526, 543, 545). TT and a related dithiol-disulfide oxidoreductase, TRX, have been demonstrated to modulate several receptor and enzyme activities, *in vitro*, including enzymes that are altered in the MCF-7 ADR^R cell lines. Therefore, elevations in TT activity may adversely effect the MCF-7 ADR^R cell lines, and not the MCF-7 WT cell lines. An alternative explanation of decreased transfection efficiency of the MCF-7 ADR^R cells is not sufficient, since the luciferase control plasmid (pGL) was easily and similarly transfected into both MCF-7 WT and MCF-7 ADR^R cell types. Future studies beyond the scope of this dissertation may determine if high levels of TT induction are lethal to MCF-7 ADR^R cells using inducible promoters rather than constitutive promoters.

In order to test the hypothesis that increases in TT activity increase resistance to Adriamycin, Adriamycin dose-response was compared between the extremely Adriamycin-sensitive MCF-7 WT cell line and 10 MCF-7 WT pTTcmv-transfected cell lines which constitutively overexpress hpTT. All pTTcmv-transfected MCF-7 WT cell lines overexpressing TT activity were more resistant to Adriamycin than untransfected MCF-7 WT cells.

Our experimental results demonstrated a possible association of TT activity with Adriamycin resistance independent of the levels of L-ascorbate 2-phosphate administered. Unlike MCF-7 ADR^R cells, neither MCF-7 WT nor pTTcmv-transfected MCF-7 WT cell lines showed increased drug resistance when AAP was administered, and in fact, several lines even had slightly decreased resistance when 2 or 5 mM AAP was administered. This indicates the DHA reductase activity of TT is not correlated with increased drug resistance, as previously suggested (526). Adriamycin resistance apparantly involves another intrinsic activity of TT, such as thiol:disulfide oxidoreduction. Work published after these studies commenced supports a possible thiol:disulfide oxidoreduction function in drug resistance (277). TRX, another protein of the same approximate molecular weight also catalyzing dithiol-disulfide transfer was shown to be related to drug resistance in cisplatin-resistant T4 bladder cancer cells (277). TRX levels are also elevated 2-fold in the MCF-7 ADR^R cells (526). Perhaps both TT and TRX together modulate cellular oxidation-reduction status to increase cellular viability in the presence of drugs known to generate reactive oxygen species.

CONCLUSION

The cloning, sequencing and overexpression of human TT (308-312) has provided novel information about the human TT structure. Human TT is the only characterized and cloned mammalian TT to have an extra half-cystine residue, although the adaptive value of such a cysteine is as yet undetermined. This cysteine is predicted to be on the protein surface near the conserved active site $C^{22}PY(F)C^{25}$, by analogy with the pig liver TT crystal structure (129), and may be involved in the decreased stability of the human enzyme relative to other mammalian TTs. Mutagenesis studies of the recombinant protein have determined that if this cysteine residue at position 7 is replaced with a serine, the catalytic activity of TT for both DHA reduction and dithiol-disulfide oxidoreduction stays the same, however, the stability of the protein increases to that of other mammalian TTs (315).

TT protein and activity has been known to be distributed differently in varied tissues in other mammals (136-145). The examination of TT distribution in human tissues has not yet been reported. Both mRNA and protein levels indicate that TT is distributed differentially in human tissues; the tissues with the lowest expression and protein are endocrine glands; whereas tissues that are habitually exposed to highest levels of ROS are those with the highest levels of TT, such as heart, liver, lung, kidney, spleen, placenta, and peripheral blood leukocyte. Recently, it was determined that stresses such as ultraviolet light (UV) B can induce the expression of TT in rat keratinocytes (338). UVB induction mechanisms are not well

characterized; related UVC light can induce transcription of genes through NFκB, serum response elements, and src tyrosine kinases, in addition to DNA repair mechnisms (reviewed in 338). The induction of TT as an immediate-early response indicates that TT is important for repair or regeneration of damaged cellular components at an early stage. TT induction brings to light certain questions about TT regulation. How is TT regulated, both transcriptionally, and possibly post-transcriptionally? Is TT regulation tissue-specific? What role does TT regulation play in normal development and growth, as well as oxidative stress, and do aberrations result in pathophysiological conditions? What is the promoter structure of TT, and how does this relate to TT regulation? Can the UVB induction of TT in rat keratinocytes be seen in other tissues and from different species?

A recent explosion of research on the thiol-disulfide oxidoreductase enzymes has illuminated the fact that redox mechanisms are important in both maintaining cellular homoestasis as well as responding to acute stresses, such as oxidative stress. The study of TT follows the more advanced study of PDI and TRX, other members of the same class. At the same time, the recent interest in cellular antioxidants has prompted increased research on enzymes that recycle oxidized AA, such as the DHA and SDHA reductases. Here, also, much of the mechanism and function of TT remains to be elucidated. Clearly, recent research has demonstrated that TT is important in maintaining both reduced AA (79,80), as well as deglutathiolation of proteins (331-333). Other biological or physiological roles for TT remain to be determined, and may lead to many research avenues on TT function in cells.

The overexpression of human TT in cells is novel. MCF-7 breast tumor cells that typically are highly sensitive to the anti-cancer drug, Adriamycin, were found to have increased resistance when overexpressing TT. The resistance is apparently not related to the

DHA reductase ability of TT, as elevated levels of AAP were added, with no net effect on cell- viability. IF DHA reductase activity were part of the resistance mechanism, expected results would be that cells administered the AA precursor, AAP, would have greatly enhanced resistance due to the large amounts of AA available for scavenging Adriamycin-generated oxyradials. The postulate, therefore, is that the dithiol-disulfide oxidoreductase activity of TT may be a significant part of the drug resistance mechanism.

In support of a role for thiol-disulfide oxidoreduction in drug resistance, and cell growth, TRX, a related thiol-disulfide oxidoreductase of very similar molecular weight has very recently been reported to have an effect on drug resistance (277, 570). Reducing the TRX expression levels in MDR kidney cells resulted in a loss of drug resistance (277). Eliminating TRX dithiol-disulfide oxidoreduction with an active site mutant in MCF-7 WT breast tumor cells eliminated the transformed phenotype (570); and TRX overexpression in MCF-7 WT cells increased cell proliferation rate. This leaves interesting questions as to the potential mechanism for TT-increased drug resistance in MCF-7 WT cells. Does TT also function to stimulate cell proliferation? If so, does TT effect proliferation directly; or through the action of several redox-sensitive cellular signalling factors, such as receptors, or transcription factors; or through the regulation of key metabolic pathways by regulating the enzymes that modulate these pathways, or modulating the rate of transport of L-cysteine, necessary for the synthesis of GSH critical for the function of multiple detoxification enzymes? Does TT modulate the activity of the various drug detoxification enzymes, such as GSTs, and GSH-Pxs, therefore modulating drug resistance? Because of the pronounced impact that knowledge of TT function in drug resistance could have in the field of cancer chemotherapy as a potential drug target, and the interest in the scientific community about

drug resistance mechanisms and oxidative stress, it is likely that these and other related questions will be the focus of much research in this field in the future.

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