MECHANISMS OF NUCLEAR LOCALIZATION OF GLUTATHIONE REDUCTASE, SUBNUCLEAR COLOCALIZATION WITH THIOREDOXIN, AND GENETIC ANALYSIS OF A CHEMICALLY INDUCED GLUTATHIONE REDUCTASE KNOCKOUT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Glutathione reductase [GR; EC 1.6.4.2] is a ubiquitous, highly conserved protein that has been identified in plants, bacteria, yeast, and mammals. GR expressions and functions in cytosol and mitochondria have been studied, but little is known about mechanisms of import or functions of GR within the nucleus.

No classical nuclear localization signal has been identified in GR, but cDNAs containing either mitochondrial targeting signals (MTS) from human MnSOD or from human GR (hGR) target GR transgene products to both the mitochondria and the nucleus. In contrast, cells transfected with a construct coding for a hGR-MTS/GFP fusion protein demonstrated fluorescence only in the mitochondria. Subsequent mutational deletion analyses of the hGR MTS indicated a tight correlation between nuclear and mitochondrial levels of expression and with the numbers of amino acids in the truncated MTSs. The MTS is necessary but not sufficient for nuclear localization of GR.

Microscopy studies of cells in culture revealed GR-dependent immunofluorescence in subnuclear clusters. Subsequent investigations indicated that the GR-containing clusters do not colocalize with proteins found in subnuclear structures that exhibit morphologies similar to the anti-GR immunoreactive forms, or with p-histone 3, a protein involved in replication. In both native and transfected cells, GR was colocalized with glutaredoxin, and...
more strongly with thioredoxin, and colocalization with thioredoxin also exhibited subnuclear clustering. The functions served by the colocalization between GR and TRX protein are not known at this time.

A GR-deficient mouse (Neu) was generated by treatment with isopropyl methanesulfonate (a chemical mutagen). RT-PCR analysis indicated that the defect in the GR gene was due to a deletion. Using PCR and Southern blot strategies, the deletion was isolated to the region between intron 1 and intron 5. Sequence analysis of a PCR product that included the putative deletion identified the precise breakpoints of the deletion and indicated that the Neu mice have a 12.8 kb deletion in their genomic GR gene and are a genetic knockout. The Neu mice are surprisingly healthy despite the absence of a gene so highly conserved across such a wide range of life forms.
DEDICATION

Dedicated to my husband, Forrest, who has always been the wind beneath my wings.
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FIELDS OF STUDY

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<td>acetylated histone 3</td>
</tr>
<tr>
<td>BCNU</td>
<td>1,3-bis(2-chloroethyl)-N-nitrosoourea</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary (cells)</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>g</td>
<td>gram(s)</td>
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<td>GR</td>
<td>glutathione reductase</td>
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<td>GRX</td>
<td>glutaredoxin</td>
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<td>GSH</td>
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<td>glutathione disulfide</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>h</td>
<td>hour(s)</td>
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<td>H₂O₂</td>
<td>hydrogen peroxide</td>
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<td>kb</td>
<td>kilobases</td>
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<td>M</td>
<td>moles per liter</td>
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<tr>
<td>MTS</td>
<td>mitochondrial targeting signal</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>p-histone3</td>
<td>phosphorylated histone 3</td>
</tr>
<tr>
<td>pro</td>
<td>protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>ROOH</td>
<td>organic hydroperoxide (non-specific)</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<td>TRX</td>
<td>thioredoxin</td>
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<td>TRXR</td>
<td>thioredoxin reductase</td>
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CHAPTER 1
INTRODUCTION

The evolution of aerobic life necessitated the co-development of mechanisms to protect organisms from the deleterious effects of oxygen (Ondarza et al., 1983). Processes as fundamental as breathing and energy production generate reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and other hydroperoxides (ROOH). Additional oxidant exposures from the environment, in the form of pollutants or ingestion of foreign substances, can place additional oxidative burdens on organisms. The uncontrolled actions of ROS can cause biochemical changes in proteins, lipids, and nucleic acids, causing these biological molecules to lose essential functions or gain new functions, thereby compromising the viability of the organism. Complex organisms, such as mammals, have developed antioxidants to limit the adverse effects of these potentially harmful substances and protect essential cellular components. These antioxidants include glutathione (GSH), glutathione-dependent peroxidases (GPX) and reductase (GR), thioredoxin (TRX), thioredoxin reductase (TRXR), and glutaredoxin (GRX).

GSH is widely conserved in aerobic life forms and functions to supply reducing equivalents for use by GPX to reduce H$_2$O$_2$ and ROOHs, a reaction resulting in the generation of glutathione disulfide (GSSG). Although, in principle,
GSH availability for reduction of substrate oxidants could be supported by *de novo* synthesis, cells use a more efficient means of sustaining GSH supplies by reduction of GSSG back to GSH, in a reaction driven by NADPH and catalyzed by the enzyme GR (Figure 1.1). Alternatively, GSSG can be exported from specific cell types, such as hepatocytes and red blood cells, but the efflux rates are small compared to the quantities of GSSG recycled by NADPH/GR (Chung *et al.*, 1991). High levels of GSSG can affect cell integrity and metabolic processes by inducing the formation of disulfide bonds between the thiol groups of proteins or as mixed disulfides with GSH, thus generating inactive proteins (Olafsdottir and Reed, 1988). The large supplies of NADPH produced through the pentose phosphate pathway, make the GSH recycling system efficient in limiting GSSG levels and maintaining cell viability (Schirmer *et al.*, 1989).

The GR protein was identified in 1951 in wheat germ and pea seed and was characterized as a distinct enzyme whose primary function was the reduction of GSSG to GSH (Conn and Vennesland, 1951; Mapson and Isherwood, 1963). Later, Rall and Lehninger identified this same GSSG-reducing entity in pig and rat liver tissues (Rall and Lehninger, 1952). Subsequently, GR has been found in most aerobic life forms, including plants, bacteria, yeasts, and mammals. The only known exception to the ubiquity of GR activities is in insects, as has been characterized in *Drosophila melanogaster*. Although lacking GR, these insects are capable of reducing GSSG, apparently through the actions of an alternative form of TRX that effects the net reduction of GSSG to GSH by
thiol-disulfide exchange reactions of GSSG with TRX and reduction of the disulfide form of thioredoxin with the variant TRXR (Kanzok et al., 2001).

**Structure and mechanism of function of GR.**

In mice and humans, the GR protein is coded by a single nuclear gene that is 52 kb in length. The GR gene is comprised of 13 exons that generate a 1.5 kb transcript. The nucleotide sequence of the first exon possesses two in frame functional translational start sites (ATG) that are not separated by an intronic sequence. The nucleotides between the two translational start sites code for peptide sequences that possess the properties of a classical mitochondrial targeting signal (MTS) and are essential for mitochondrial expression of GR (Figure 1.2) (Tamura et al., 1997; Kelner and Montoya, 2000).

Little is known about transcriptional regulation of GR, but increases in GR message have been observed under specific stress conditions (Hamburg et al., 1994). Antioxidant response elements, cis elements in a DNA sequence that are binding sites for specific redox sensitive transcription factors, have been identified in the 359 bp region 5’ of the GR gene that is thought to be the promoter, and expressions of promoter/reporter constructs were induced by treatment with pro-oxidants (J. J. Gipp, 2001). In yeast, deletion of the AP-1 transcription factor binding site in the promoter region abolished stationary phase induction of GR activities (Grant et al., 1996b).

Polymorphisms in the GR protein of mice were first noted by Nichols and Ruddle (Nichols and Ruddle, 1975) and Firth et al. (Firth et al., 1979), using starch gel electrophoresis, but a much more extensive study was performed later
by Guo et al. (Guo et al., 2003). Guo analyzed the nucleotide sequences of several antioxidant genes in eight common inbred mouse strains. In their analyses of the GR coding sequences, they identified 19 single nucleotide polymorphisms. Two specific alleles, Gsr\textsuperscript{a} and Gsr\textsuperscript{b}, were identified and associated with differences in GR activities. The Gsr\textsuperscript{b} was found in the SJL/J and SWR mice strains and was associated with a substitution of an alanine for an aspartic acid residue (amino acid 39). Renal homogenates from mice expressing the Gsr\textsuperscript{b} allele exhibited higher GR activities than were observed in homogenates from NZB/N mice, which expressed the Gsr\textsuperscript{a} allele; however, the specific activities of GR enzyme isolated from the SJL/J and SWR mice were only about 65% of the activities found in the NZB/N mice. These findings indicate that the mice possessing the Gsr\textsuperscript{b} allele have up-regulated expressions of a less efficient GR protein than is expressed in the mice with the Gsr\textsuperscript{a} allele. The compensatory up-regulation of GR indicates that evolutionary conservation of GR activities might be important in natural selection.

Human genetic variations in the GR gene have also been identified but are reasonably rare in humans. The human mutations identified to date have been associated with lower GR activities and with severe clinical manifestations. The consequences of human genetic polymorphisms for GR will be discussed in greater detail later (p12).

The human GR protein is composed of 522 amino acids (numeric amino acid designations to be used in the present document include the MTS sequence, which, in humans, is 43 amino acids). The active GR protein is a
head to tail homodimer, held together primarily by the hydrophobic interactions of the interface domains in a region near the C-terminus, although a single interchain disulfide bond between the Cys-134 residues in the two chains also exists (Krauth-Siegel et al., 1982). The crystal structure of GR was first described by Schulz et al. (Schulz et al., 1978). The GR molecule was defined as containing five distinct domains, the N-terminal domain, whose function is unknown, the FAD-binding domain, which contains the two active site cysteines, the NADPH-binding domain, the central domain, which is necessary for appropriate configuration of the protein, and the interface domain, which is required for dimer formation (Figure 1.3) (Thieme et al., 1981; Krauth-Siegel et al., 1982). The GSSG binding site is buried deep within a pocket that contains two cysteine molecules, Cys-102 and Cys-107, which are linked by a disulfide bond in the native molecule. The environment of the pocket is influenced by ionic interactions of three other pairs of amino acids, His-511:Glu-516, Arg-335:Asp-375, and Lys-110:Glu-245 (Krauth-Siegel et al., 1998).

The mechanism of enzymatic reaction is defined in four steps. First, a molecule of NADPH binds to the NADPH binding site, donates reducing equivalents, and facilitates the reduction of the Cys-102 - Cys-107 disulfide bond, thus forming the reduced intermediate (EH$_2$). A GSSG molecule enters the deep pocket of the enzyme active site, and electrons are transferred from EH$_2$ to the GSSG molecule, forming a mixed disulfide between Cys-102 and one GS molecule and releasing the other GS group as a molecule of GSH. The mixed disulfide reacts with the thiolate anion of Cys-107 forming the Cys-102 - Cys-
107 disulfide bond, and the second molecule of GSH is released (Karplus et al., 1989; Karplus and Schulz, 1989).

There is currently no evidence for translational regulation of GR protein, but two models of post-translational regulation have been hypothesized. The first proposed mechanism involves the formation of a disulfide bond between Cys-46, found in the N-terminal extension, and Cys-102, found in the active site. This model would prevent binding of GSSG to the active site and render the enzyme inactive (Untucht-Grau et al., 1981). The second proposed mechanism of regulation is the reduction of the inter-subunit disulfide bond at Cys-134 (Untucht-Grau et al., 1981). This event would free the long α-helix containing the active site and could influence the arrangement of the catalytic site, thus regulating the rate of GSSG reduction. Evidence for such post-translational regulation was observed in studies of LPS administration to mice. GR mRNA and protein levels were increased in the mice given increasing doses of LPS, but disproportionate increases in GR activity levels were observed (Hamburg et al., 1994). Studies by Chung et al. (Chung et al., 1991) have suggested that GR activities are regulated at a functional level by the cellular concentrations of GSH. The general idea is that diversions from normal cell steady-state ratios of GSH:GSSG, such as caused by decreases in GSH concentrations, cause increases in GR activities and thus increased rates of reduction of cellular GSSG, but the mechanisms involved in regulation of GR activities was not addressed.
Exogenous inhibitors of GR activities have been identified and include heavy metal ions, arsenicals, and nitrosoureas (Schirmer et al., 1989). Inhibitors presently recognized are thought to affect the active site of GR in such a way as to interfere with the formation of the reduced intermediate of the enzyme. In 1977, Frischer (Frischer, 1977) described a group of people who exhibited GR activities that were lower than the control population. Analysis of the hematological parameters of patients who had been hospitalized for cancer treatments, malnutrition, or liver disease, identified a correlation between diminished GR activities and treatment with a specific anti-tumor drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), either alone or in combination with other treatments.

BCNU is the most widely studied of the nitrosoureas and is used as a therapeutic agent in the treatment of some cancers. In addition to inhibiting GR irreversibly, BCNU pretreatments potentiate the cell killing of a number of toxicants in vitro and in vivo (Smith, 1989). In fact, exacerbation by BCNU of the effects of a second agent or experimental manipulation has been offered as proof that the cell killing is mediated by oxidant mechanisms (Gerson et al., 1985; Nakae et al., 1988).

In a biological matrix, BCNU hydrolyzes to form carbamoylating and alkylating species that carbamolyate, alkylate, and cross-link proteins and DNA. Inhibition of GR activities by BCNU is attributed to carbamoylation of Cys-102 (Davis et al., 1993). Inhibition of GR activities is irreversible, and restoration of GR activities occurs with cell turnover or new protein synthesis. In contrast,
repair of DNA alterations caused by BCNU is substantial by 12 h after administration. Although the mechanisms of action are not clearly defined, the anti-tumorigenic properties of BCNU are more closely related to the alkylating species, which exhibit reactivities attributed to the 2-chloroethyl carbonium ion, formed from BCNU, and to the subsequent reactions of the alkylating species with DNA and proteins. In rapidly proliferating cells, such as tumor cells, attempts to progress through the cell cycle prior to adequate repair of DNA modifications correlate with initiation of cell death. Critical comparisons indicate that BCNU potentiation of cell killing correlates more tightly with DNA alkylation, cross-linking, and repair than with inhibition of GR activities (Tong et al., 1982; Bodell et al., 1986).

**Studies in non-mammalian species.**

GR has been studied in a wide variety of plants. Although the amino acid sequences found in plants vary from the sequences found in animals, the basic components of the protein exist and function in the same capacities. The studies of GR in plants have included a broad spectrum of topics, and much can be learned and applied by studying function and regulation of proteins in non-mammalian life forms. Foyer et al. (Foyer et al., 1995) found that increasing the GR activities in the chloroplasts of poplar trees improved the plants’ antioxidant capacities and abilities to deal with oxidant stresses. Pastori et al. (Pastori et al., 2000) reported that GR was post-transcriptionally regulated in the bundle sheath cells of maize. Although the exact nature of regulatory function was not defined, these findings might offer an additional theory for regulation of GR activities in
animals. Rudhe et al. (Rudhe et al., 2002) identified specific regions of the pea GR mitochondrial targeting signal that were responsible for targeting GR protein to either the mitochondria or the chloroplasts.

*E. coli* GR was identified in 1955 by Asnis (Asnis, 1955). The ease of genetic manipulations in *E. coli* has made this bacterium the subject of many studies concerning the structure and function of the GR enzyme. GR negative mutants were first produced by lysogeny of *E. coli* with Mu phage. The negative mutants were isolated, but no growth defects were observed in the mutants (Davis et al., 1982). Subsequent mutation studies have led to the identification of the GR enzymatic active site and the amino acids responsible for catalytic function (Berry, Scrutton et al. 1989; Deonarain, Berry et al. 1989; Scrutton, Berry et al. 1990; Deonarain, Scrutton et al. 1992; Bashir, Perham et al. 1995).

Studies involving the GSH and TRX systems in yeast have been extensive. The GR gene of *Saccharomyces cerevisiae* (*grl*) shares a 49.8% identity to the human GR gene and maintains redox active cysteines in the active site (Collinson and Dawes, 1995). Disruption of the GR gene in *S. cerevisiae* does not impair normal aerobic growth, but growth appears to depend upon compensatory functions of TRX 1 and 2. Disruption of either *trx1* (cytoplasmic and nuclear) or *trx2* (mitochondrial) and *grl* causes the yeast to grow more slowly, but the mutants are viable (Muller, 1996). Triple mutants for *grl, trx1*, and *trx2* and mutants in both *glr* and thioredoxin reductase (*trr1*) are not viable, indicating that the yeast require at least one of the thiol-disulfide reduction systems intact (Trotter and Grant, 2003).
S. cerevisiae exhibit three-fold increases in GR activities during stationary phase (Grant et al., 1996b) and are more resistant to oxidants such as H₂O₂ during this phase (Grant et al., 1996a). The stationary phase increases in GR activities are attributable to stimulation of gene expression by activation involving an AP-1 site in the promoter region of the grl gene. Blocking or inhibiting the AP-1 binding site abolishes the stationary phase increase, indicating that, in the yeast model, GR activities are regulated at least in part by a transcriptional event (Grant et al., 1996b). Yeast that are deficient in GR expressions are more sensitive to oxidants, such as H₂O₂ and diamide, and demonstrate substantial increases in cellular GSSG levels (Muller, 1996). Alternatively, disruption of the grl gene in Saccharomyces pombe, a yeast more closely related to higher eukaryotes, results in a variant that is not viable in air, indicating the S. pombe requires the presence of a functional grl gene for survival in an aerobic environment (Lee et al., 1997).

Development Ontongeny of GR.

Ontogeny of GR activities in mammals has been studied in the developing rat model (Jung and Henke, 1996; Choe et al., 2001). Gestational day (GD) 9 embryos and the visceral yolk sacs express very low GR activities. Presumably, at this early stage in development, maternal mechanisms provide adequate antioxidant protection under normal circumstances. By GD 13, the yolk sac and the head, trunk, and heart exhibit 4- to 5-fold increases in GR activities. These increases coincide temporally with the development of a cardiovascular system.
and the conversion to aerobic metabolism. Later in gestation, the GR activities found in the yolk sac decrease, and only modest increases are observed in tissues from GD 14 to birth (Choe et al., 2001). Following birth, the levels of GR activities in the lungs and livers of rats steadily increased by 20% at six months and 30% by 12 months. In the lungs, GR immunoreactivity was localized primarily to the bronchial epithelia, and in the kidneys anti-GR immunoreactivity was localized primarily to the epithelial cells (Jung and Henke, 1996; Fujii et al., 2002).

The ontogeny of GR expression has also been studied in ocular tissues of the developing rat (Fujii et al., 2001). The expression of GR protein is first detected in the ganglion cell layer late in the prenatal stage. At birth, GR is detected in the inner and outer plexyform layers and in the pigmented epithelial cells. By adulthood, GR proteins are found in virtually all of the tissues in the eye. These findings indicate that reduction of GSSG by GR is important in protection of the eye tissues against oxidant stresses.

Age-related declines in the efficiency of the glutathione system as a whole have been reported (Habif et al., 2001; Erden-Inal et al., 2002). Erythrocyte GR activities have been measured in humans from ages 0.2 to 69 years old. Steady, age-dependent declines in GR activities were reported, with the lowest activities correlating to the oldest age group tested. These decreases in GR activities also correlated with decreases in GSH levels and increases in GSSG levels in erythrocytes. Collectively, the observations in both rats and humans indicate that GR activities are relatively high at birth and during the early stages of life, but that
GR activities decline with age, and these decreases are likely to contribute to the oxidative modifications that correlate with the aging process.

Clinical Significance of GR.

Loos et al. (Loos et al., 1976; Roos et al., 1979), described a case of familial deficiencies in GR activities. Three siblings from a consanguineous marriage were found to have no measurable GR activities in their red blood cells and only 15% of control activities in isolated leukocytes. The lack of measurable GR activities in the red blood cells and the relatively low levels in leukocytes were attributed to continuous synthesis of an unstable enzyme in the nucleated cells. The decreased GR activities were not associated with deficiencies in riboflavin in the diet in the affected individuals or with malfunctions in the glucose-6-phosphate dehydrogenase enzyme responsible for the availability of NADPH. Although the siblings lived to adulthood, they experienced medical problems resulting from decreased life span of erythrocytes, acute hemolytic crises after eating fava beans, shortened respiratory bursts in activated leukocytes, progressive deafness, and development of cataracts. Fava beans contain vicine and covicine which upon digestion are metabolized to divicine and isouramyl in the intestinal tract. Although these substances are usually detoxified by glucose-6-phosphate dehydrogenase (G6PD), individuals with deficiencies in G6PD are vulnerable to hemolytic crises. The case report by Loos also links a hemolytic crisis after eating Fava beans with deficiencies in GR.

In 1981, Saha (Saha, 1981) reported three distinct phenotypes of GR in a Sudanese population, but the specific genotypes were not described. GR
deficiencies in two regions of Saudi Arabia also have been found to be associated with other genetic blood disorders, specifically sickle cell anemia and thalassaemia (el-Hazmi and Warsy, 1985). Although many diagnosed GR deficiencies found in Saudi Arabia have been associated with dietary riboflavin deficiencies (Warsy and el-Hazmi, 1999), el-Hazmi and Warsy found that as many as 24.5% of males and 20.3% of females are GR deficient due to genetic variations (el-Hazmi and Warsy, 1989a). The high frequencies of genetic defects in the GR gene appear to be localized to the Saudi Arabian populations.

Malaria is a serious world health concern, primarily in underdeveloped countries, affecting 40% of the world population. The World Health Organization estimates 300-500 million cases occur each year, and approximately one million people die from the disease (http://www.who.int/health_topics/malaria).

Plasmodium falciparum, the most common malaria parasite, has been the organism of interest in many of the studies involving manipulations of GR activities. P. falciparum multiplies in human erythrocytes, and metabolic disorders that cause deficiencies in glucose-6-phosphate dehydrogenase or glutathione reductase activities confer protection from the “severe manifestations” of falciparum malaria (Becker et al., 1996). Studies involving P. falciparum have focused on mechanistic approaches for inhibiting GR activities, both in the parasite and in the host, and have included inhibitors that bind to the inner cavity of the folded protein and inactivate the enzyme, as well as dimerization inhibitors that prevent the two monomeric subunits from joining
Thus far, manipulations of GR activities have not provided an effective treatment for *falciparum* malaria.

**Subcellular distribution of GR.**

In mammals, GR is found in multiple subcellular compartments, including the cytoplasm, nuclei, and mitochondria. The largest pool of GR is found in the cytoplasm, and, in conjunction with glutathione peroxidase and GSH, contributes significantly to redox control within the cell. Studies in which GR activities have been enhanced in the cytoplasm of cells by transient or stable transfections have indicated that increased GR activities can be cytoprotective. In CHO cells transiently or stably transfected with functional GR constructs, protection from tert-butyl hydroperoxide cytotoxicity, as measured by lactate dehydrogenase release, was observed (Tamura, 1996). In addition, cytoprotection of H441 (lung carcinoma) cells against treatment with tert-butyl hydroperoxide or exposure to hyperoxia was observed with adenoviral vector-mediated transfection of a GR cDNA transgene. (O'Donovan *et al.*, 1999; O'Donovan *et al.*, 2000).

The mitochondrial targeting signal in the 5’ region of the GR gene was first identified in mice (Tamura *et al.*, 1997) and later in humans (Kelner and Montoya, 2000). Mitochondria metabolize a large fraction of the oxygen used by most cells and are therefore logically the primary source of the chemically reactive and potentially cytotoxic byproducts of oxygen metabolism (Cai and Jones, 1999). At least some mitochondria are unable to export GSSG effectively into the cytoplasm, further supporting the need for efficient GSSG reduction systems in mitochondria (Olafsdottir and Reed, 1988; Brodie and Reed, 1992; Liu and
Kehrer, 1996). In addition, oxidant mechanisms have been implicated in the processes through which mitochondria contribute to the initiation of cellular apoptosis (Cai and Jones, 1999). Consequently, enhancing antioxidant capabilities in the mitochondria would be expected to protect cells from certain types of oxidant insults.

As mentioned previously, cytoprotection against subsequent treatment with tert-butyl hydroperoxide or hyperoxia was observed with transfection of GR constructs into both CHO and H441 cells. However, in these same studies, protection was far greater in cells transfected with constructs containing functional MTSs (Tamura, 1996; O'Donovan et al., 1999; O'Donovan et al., 2000). These findings suggested that enhancement of antioxidants, specifically in the mitochondria, have beneficial survival effects in the presence of oxidant challenges. Interestingly, the CHO cell lines used in these experiments were analyzed further for nuclear GR expressions, and those cells transfected with constructs containing a functional MTS 5' of the transgene also had higher GR activities in the nuclei than did native CHO cells (Chapter 2). The observation that the mitochondrially targeted GR constructs were also increasing the GR activities in the nuclei raises the question of the relative contributions of increases in GR activities in the mitochondria or the nucleus to the protective effects observed in cells transfected with the MTS-containing constructs.

Nuclear GR activities have been reported in several previous studies, but characterizations of the nuclear GR pools have not been described (Ochalska-Czepulis and Bitny-Szlachto, 1981; Mbemma et al., 1985; Taniguchi et al., 1986;
Rogers et al., 2002). Our interests in subnuclear pool(s) of GR were stimulated by our observation of distinct subnuclear foci of immunofluorescence in studies with a GR-deficient, stably transfected CHO cell line. This cell line had been generated by transfection of an anti-sense GR construct. These stably transfected cells exhibited GR activities that were approximately 50% of the activities found in native CHO cells. Immunofluorescence studies indicated lower overall fluorescence in the GR-deficient cells than in control cells, but the presence of subnuclear foci of immunofluorescence was striking (Figure 1.4). Subsequent studies performed with isolation by differential centrifugation of subcellular fractions of rat liver revealed GR activities associated with the nuclear and the nucleolar fractions (Rogers et al., 2002).

Current Studies.

More recently, we observed that the genomic MTS of GR placed 5’ of the GR cDNA not only caused increases in GR expression in the mitochondria but also increased GR expression in the nuclei. Little is known about the molecular mechanisms of nuclear import of GR or the possible functions GR might have in the nucleus. The studies described in Chapter 2 address the mechanisms involved in the nuclear localization of GR protein and include studies using confocal microscopy and transient transfections of deletion or mutation constructs. In Chapter 3, we describe experiments designed to identify possible functions of the subnuclear pool(s) of GR, by identifying the other proteins that colocalize with GR in the nucleus. In Chapter 4, we describe studies of a mouse strain that possesses a mutation in GR gene that causes substantially lower GR
activities in all tissues. Studies described in Chapter 4 characterize the specific mutations in the genome of the GR1\textsuperscript{a1Neu} mice and define this strain as a genetic knock-out for GR.
Figure 1.1. The glutathione recycling antioxidant system. The scheme represents the mechanisms involved in detoxification of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ by GPX. The consequence of this reaction is the formation of glutathione disulfide, which can be reduced back to GSH by GR, at the expense of NADPH.
Figure 1.2. The human GR cDNA. The entire GR cDNA consists of 1566 bp. A 129 bp MTS is located immediately 5’ to the GR gene and is responsible for mitochondrial targeting and is necessary, but not sufficient for nuclear targeting of the GR protein. Two in-frame translational start sites are present on either side of the MTS, but the mechanisms regulating translation are not known.
PROTEIN DOMAINS OF GLUTATHIONE REDUCTASE

MTS: mallpralsagagpswrraarfgflillpeaalsra

1. N-terminal segment
   macrqpnpqppampaagav

2. FAD domain:
   asydylviggsgglasaraelgaraavveshklggctvngcvkkvnmntavhsefmdadygfps
cegkfnrveikrdaysrlnaiyqnnltshieirghaftdpektevsgkktyaphiatio

3. NADPH domain:
   gmpstphespgaslgitsdggfleelpgsvigagyiavemalsgulsmirhdkvrlrfdsmistn
tceelenagvevlksqykevkktsgevsmvptlpgrplvpmipdclvwaigrvp

4. Central domain:
   ntldlsnkpiqdtgkhiiivdefqntnvkgyavgdvbgkalltvpviiagrlhrfrfeykedkldy

5. Interface domain:
   niptvshppigtvglteaihgyienvkyststfpmvhyavtrkrktckvkmcvkanekekvvgihmgq
gcdmiqgfavvakmgatkladfdntvaihptseelvtl

Figure 1.3. Functional domains of GR protein. GR protein was divided into 5 functional domains by crystal structure. The N-terminal domain has no ascribed function. The flavin adenine dinucleotide (FAD) domain contains the binding site for FAD and the two active site cysteines, Cys-102 and Cys-107. The NADPH domain contains the binding site for NADPH. The central domain plays an important role in conformation of the folded protein. The interface domain contains the sequence that is in contact with the second monomer of the dimeric protein. The amino acids highlighted in red were investigated in Chapter 2 as a putative nuclear localization sequence.
Figure 1.4. GR immunofluorescence in stably transfected GR deficient CHO cells. Stably transfected CHO cells that contain 50% of the GR activities of native CHO cells were fixed and treated with GR primary antibody and a FTIC-labeled secondary antibody. The cells were analyzed by fluorescent microscopy. Bright punctate spots were observed in the nuclear regions of the cells.
CHAPTER 2
THE MITOCHONDRIAL TARGETING SIGNAL IS NECESSARY BUT NOT SUFFICIENT FOR NUCLEAR LOCALIZATION OF HUMAN GLUTATHIONE REDUCTASE

Introduction.

Aerobic organisms are exposed to reactive oxygen species (ROS) and limit the potentially detrimental effects through diverse, interactive antioxidant functions, in which glutathione (GSH)-dependant processes are pivotal. The glutathione peroxidase-catalyzed reductions of $\text{H}_2\text{O}_2$ and organic hydroperoxides to water and the respective alcohols generate glutathione disulfide (GSSG), and the stores of GSH are sustained by reduction of GSSG to GSH in a reaction that is catalyzed by the enzyme glutathione reductase (GR; [E.C.1.6.4.2]), which couples the GSH-dependent antioxidant system to the reductive capacities of cellular NADPH. In addition, GSH-dependent mechanisms are central to modulations of redox status of proteins and other nonprotein thiols, and such modifications of thiol/disulfide status contribute to a number of cell signaling and regulatory pathways (Watson et al., 2003a; Fernandes and Holmgren, 2004).

Evidence for the toxicological importance of GR has largely been ascribed to the exacerbation of oxidant-mediated toxicities by administration of 1,3-bis(2-chloroethyl)-N-nitrosourea (BCNU, Carmustine), which inhibits GR in vivo and in
vitro. Studies of mechanisms involved indicate that the enhancements of toxicities of other drugs by BCNU are more closely associated with DNA cross-linking than with inhibition of GR activities (Tong et al., 1982; Bodell et al., 1986). However, we have used molecular methods to generate stably transfected Chinese hamster ovary (CHO) cell lines that have approximately 50% of the GR activities of native CHO cells and have found these cells to be much more susceptible to oxidant challenges (Hansen et al., 1993). In addition, we have observed enhanced resistance to oxidant challenges in stably transfected CHO cells that have greater GR activities, particularly in cells generated with human GR (hGR) constructs containing a functional mitochondrial targeting signal (MTS) and exhibiting markedly greater GR mitochondrial activities (Tamura, 1996).

Human GR (hGR) is coded by a single nuclear gene, yet GR activities and protein have been observed in cytoplasmic, mitochondrial, and nuclear compartments (Taniguchi et al., 1986; Rogers et al., 2002). Nuclear GR activities were described in 1981 by Ochalska-Czepulis (Ochalska-Czepulis and Bitny-Szlachto, 1981) in both chromatin and extra-chromatin fractions of isolated nuclei from mouse spleens. GR activities in nuclear fractions of rat liver homogenates (Taniguchi et al., 1986) and in WI-38 fibroblasts (Mbemba et al., 1985) have been reported, but the activities represented small portions of the total cell or tissue activities. In later studies, we observed substantial GR activities in purified nuclei isolated from rat livers and found that GR activities associated with nucleoli isolated by differential centrifugation (Rogers et al., 2002). Although differences were noted among these studies in the relative
proportions of nuclear GR activities to the total cellular activities, differences in cell and tissue sources or in subcellular fractionation techniques and isolation procedures are likely to contribute to the variations.

The earliest reports of mouse and human GR nucleotide sequences did not include the sequence of a functional MTS in the N-terminal sequence of the protein coded by the respective cDNAs (Tutic et al., 1990; Krauth-Siegel et al., 1996; Tamura et al., 1996). More recently, a 26 amino acid sequence in mice (Tamura et al., 1997; Iozef et al., 2000) and a 43 amino acid sequence in humans (Kelner and Montoya, 2000) that appear to function as mitochondrial targeting signals were identified. However, nuclear localization signals for GR have not been reported and the mechanisms involved in nuclear localization of GR are not understood. The primary goal of the present study was to elucidate the molecular mechanisms by which GR is targeted into the nucleus.
Methods

**Cell Culture and transfection.** Chinese Hamster Ovary (CHO) cells were maintained in McCoy’s 5A medium supplemented with 10% fetal calf serum (FCS) (Cellgro, Herndon, VA). Constructs were generated using a pUC 18 vector with the addition of a hygromycin resistance cassette and included the cDNA for hGR or the mitochondrial targeting signal (MTS) from human manganese superoxide dismutase [(MTS-hMnSOD)] placed immediately 5’ to the cDNA for hGR. To generate stable cell lines, CHO cells were transfected with either of the construct-containing vectors, and colonies were selected by antibiotic resistance, by sequential treatment with G418 and hygromycin. Resistant clones were identified, and clones that expressed GR activities greater than observed in control CHO cells were propagated. Stably transfected cells were maintained by the addition of 250 µg/ml of hygromycin to the media described above. For determinations of compartmental GR activities, cells were plated at 2 x 10^6 cells per 150 mm plate and allowed to grow for 48 h before harvesting.

Human 293T cells were used for transient transfection studies, to take advantage of the high transfection efficiencies attainable with these cells. The 293T cells were maintained in DMEM media supplemented with 10% FCS. For transient transfection experiments, 150 mm plates were treated with poly-D-lysine (Sigma Chemical Co., St Louis, MO.), washed in sterile water, and air-dried. Cells were plated at 2 x 10^6 cells per plate and allowed to grow for 24 h prior to transfection. Human 293T cells were transfected using FuGENE 6
Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN.), according to the manufacturer’s protocol. After 48 h, cells were harvested for subcellular fractionation.

**Digitonin solubilization and subcellular separation.** Subcellular fractions were prepared by the method of Bronfman et al. (Bronfman et al., 1998). At the time of harvest, the media were removed from the cells, and each plate was washed 3 times with phosphate buffered saline (PBS). The plates of cells were then individually treated with 1 ml of 1 mg/ml digitonin (Sigma Chemical Co., St Louis, MO) dissolved in 0.25 M sucrose, 3 mM imidazole, pH 7.4 (sucrose/imidazole buffer), and immediately observed under the microscope. The digitonin buffer was removed within 15-30 sec, and the plates were washed with an additional 2 ml of sucrose/imidazole buffer. The digitonin-containing buffer and the subsequent washes were combined as the cytoplasmic fractions. The remaining cellular material was scraped from the plates using 1 ml of sucrose/imidazole buffer and homogenized with a Dounce homogenizer, using 12 strokes of the tight pestle, which disrupted the cytoskeleton. The resulting homogenates were centrifuged at 10,000 x g for 3 to 5 sec. The supernatants were removed, and the pellets washed in 0.5 ml of sucrose/imidazole buffer and centrifuged again for 3-5 sec. The supernatants were discarded, and the pellets containing the nuclei were suspended in 0.3 ml of sucrose/imidazole buffer. The supernatants from the first 3-5 sec centrifugation were centrifuged again at 10,000 x g for 15 min. The supernatants were removed, and the pellets were washed in 0.5 ml of sucrose/imidazole buffer and centrifuged again for 15 min.
The supernatants were discarded, and the pellets containing the mitochondria were suspended in 0.3 ml of sucrose/imidazole buffer. Subcellular fractions used for enzyme assays and western blot analyses were treated with 0.1% Triton X-100 to solubilize the organelle membranes.

The purities of the subcellular fractions separated by the digitonin solubilization method were assessed by western blot analyses using antibodies against RAF (Transduction Laboratories, Lexington, KY.) for cytoplasm, histone 3 (Cell Signaling Technology, Beverly, MA.) for nuclei, and cytochrome C (BD Biosciences, Pharmingen, San Diego, CA.) for mitochondria. The secondary antibodies were either anti-rabbit or anti-mouse and were obtained from Sigma Chemical Co (St. Louis, MO).

**GR Constructs.** All cDNA constructs used in the transient transfections were generated in the pRC/CMV vector (Invitrogen, Carlsbad, CA), unless otherwise indicated. The MTS for hGR, [(MTS-hGR)], was amplified from human genomic DNA (H441, lung carcinoma cells) by PCR and was ligated 5’ to the hGR cDNA to generate [(MTS-hGR)•(hGR)]. As a positive control for nuclear localization, a construct containing the SV40 large T antigen NLS (PKKKRKV) was generated by annealing the appropriate complementary oligonucleotides that coded for the NLS amino acid sequence and ligating the annealed oligonucleotides 5’ to the hGR cDNA, generating the construct [(NLS-SV40)•(hGR)]. Transient transfection studies were performed with cDNA constructs coding for hGR, [(MTS-hGR)•(hGR)], or [(NLS-SV40)•(hGR)]. Transfected cells either were harvested
and fractionated as described for measurement of GR activities and proteins or were fixed for immunofluorescence studies.

To identify more specifically the portions of the GR protein that contribute to nuclear localization of GR, a series of GFP-containing cDNA constructs were generated and studied (Figure 5a). The cDNAs for the [(MTS-hGR)•, [(hGR)]•, [(MTS-hGR)• plus 1.0 kb of the 5' end of (hGR), and the [(MTS-hGR)• plus the entire 1.5 kb of (hGR) were cloned into a pWAY2 (Lo et al., 1998) GFP reporter vector, generating [(MTS-hGR)••GFP], [(hGR)•(GFP)], [(MTS-hGR)•(0 to 1.0 kb-hGR)•(GFP)], and [(MTS-hGR)•(hGR)•(GFP)], respectively. The GFP constructs were transfected into 293T cells, and the cells were analyzed subsequently by fluorescence microscopy for subcellular localizations of the GFP-containing proteins.

The contributions of the putative NLS, \(^{456}KRKTK^{460}\), to expression and subcellular localization of GR were studied by generating DNA constructs containing mutations that coded for substitutions of amino acids within this sequence, including single amino acid substitutions, substitution of the second and third amino acids together, and one construct in which all five amino acids in this sequence were mutated (Table 2.1). Constructs containing mutations in amino acids \(^{456}KRKTK^{460}\) were designed and generated using the GeneEditor In Vitro Site-Directed Mutagenesis System (Promega Corporation, Madison WI). The mutations were designed to replace the lysines (K) and the arginine (R) with alanines (A), and to replace the threonine (T) with glycine (G). The 293T cells were transfected with the mutation-containing constructs. After 48 h, the cells
were harvested, subcellular fractions were isolated, GR activities were measured on the isolated fractions, and GR concentrations assessed by western blot analyses.

The contributions to nuclear localization of GR of the two potential translational start sites were tested by mutation analyses. Constructs were prepared by PCR in which the first or second ATG was mutated to ATT, thus changing the coding for the respective methionine (M) to isoleucine (I). These constructs were thus [(MTS-(M1I)-hGR)•(hGR)] and [(MTS-(M44I)-hGR)•(hGR)]. Two additional constructs were prepared and studied. In one, the sixth nucleotide (C) of the targeting signal coding sequence was deleted, which changed the codon for the second amino acid and created a frame shift [(MTS-(FS)-hGR)•(hGR)] in the remainder of the coding sequence. Studies with the [(MTS-(FS)-hGR)•(hGR)] construct were designed to test the hypothesis that the second translational start site could function independently from a nonfunctional 5' coding sequence for the targeting signal. The fourth construct prepared in this series of studies had the nucleotides coding for the last amino acid of the MTS sequence and the first 8 amino acids of the hGR protein [(MTS-hGR)•(∆aa 43-51)-hGR)] deleted from the sequence. This construct was used to test the hypothesis that the elements within the N-terminal portion of the hGR protein itself were critical for nuclear localization of GR. These constructs were transfected into 293T cells. After 48 h, the cells were harvested, subcellular fractions isolated, and GR activities were measured.
To determine the extent to which specific portions of the hGR MTS affect mitochondrial and nuclear targeting of GR, cDNA constructs were designed and prepared in which sections of the MTS were deleted independently. In this series of studies, the coding for the first start site M was maintained, and the first series of constructs were designed to delete the first [Δaa 2-15], middle [Δaa 15-30], and last [Δaa 30-42] third of the MTS sequence, respectively. Additional constructs, deleting both larger [Δaa 2-30] and smaller [Δaa 22-30] and [Δaa 24-30] regions of the MTS, were designed, prepared, and studied, to test the necessity of specific regions of the MTS for mitochondrial or nuclear targeting. The deletion constructs were made by PCR using pfu Turbo polymerase (Stratagene, Inc., La Jolla, CA) and primers designed for the specific deletions. The cDNA constructs were transfected into 293T cells. After 48 h, the cells were harvested, subcellular fractions separated, GR activities measured, and western blot analyses performed. All mutation and deletion constructs were confirmed by restriction enzyme screening and by DNA sequencing.

**Immunofluorescence studies.** Human 293T cells were grown on coverslips and transfected as described above. After 48 h, the media were removed, and the cells were fixed with 4% paraformaldehyde for 15 min, washed, and solubilized with methanol for 5 min at −20°C. Fixed cells were blocked for a minimum of 2 h in PBS containing 10% goat serum, then exposed to primary antibodies overnight at 4°C, washed, and exposed to secondary antibodies for 2 h at room temperature (GR antibody production described on page 97). The
cells were visualized using a Zeiss 510 Confocal Microscope, and the images were acquired with the Zeiss software.

**GR activities.** Subcellular fractions were treated with Triton X-100 for 1 h at 4°C and centrifuged at 12,000 x g for 15 min. The cell supernatants were assayed for GR activities by monitoring GSSG-dependent NADPH oxidation at 340 nm, as described previously (Rogers et al., 2002).

**Protein.** Protein concentrations were measured in the Triton X-100-treated subcellular supernatants using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA.).

**Western blot.** Western blot analyses used standard protocols. SDS polyacrylamide gels (12%) were used to separate 30 µg of protein. The proteins were transferred to PVDF membranes (BioRad, Hercules, CA) which were blocked for 2 h in 5% non-fat dry milk, then incubated with the designated primary antibody. The membranes were washed thoroughly, incubated with secondary antibody for 2 h, and visualized using ECL chemiluminescence reagents (Amersham Pharmacia Biosciences, Piscataway, NJ). Quantitations of the western blots were by densitometry, using a gel documentation system.

**Statistics.** Data are expressed as means ± SEM and were assessed by one-way ANOVA, with Student-Newman-Keuls (SNK) post hoc. All analyses were preformed using SPSS, version 10.0 (Chicago, IL). Statistical significance was attributed at P≤ 0.05.
Results

The western analyses with antibodies against RAF, cytochrome c, and histone 3, as markers of cytoplasmic, mitochondrial, and nuclear proteins, respectively, indicated that the methods employed in the present studies provided fractional purities adequate for the needs of our studies. The RAF and histone 3 immunoreactivities of the subcellular fractions prepared by the method adapted from Bronfman (Bronfman et al., 1998) were 99% in the cytoplasmic and nuclear fractions, respectively, each showing 1% relative densities in the mitochondrial fractions (Figure 2.1). The immunoreactivities against cytochrome c indicated 6±4% and 3±2% relative densities in the nuclear and cytoplasmic fractions, respectively. These measurements of cytochrome c immunoreactivities in the nuclear and cytoplasmic fractions would overestimate the contamination of the respective fractions by mitochondria by any actual content of cytochrome c in the nuclei or cytoplasm, respectively, in the intact cells.

In the stably transfected CHO cell lines, the GR activities in the cytoplasmic fractions were markedly greater in cells expressing [(hGR)] and [(MTS-MnSOD)•(hGR)] than were the corresponding activities in native CHO cells, with the cytoplasmic GR activities in the [(hGR)]-transfected cells being more than twice the activities in the cells prepared with the MTS-containing construct (Figure 2.2). Mitochondrial and nuclear GR activities were higher in the CHO cells transfected with [(MTS-hMnSOD)•(hGR)] cDNA than in the native cells, but similar increases were not observed in the cells transfected with
These results indicate the ([MTS-hMnSOD]) can target hGR to the nuclei as well as to the mitochondria in CHO cells.

Human 293T cells transiently transfected with the cDNA constructs ([hGR]), ([MTS-hGR]•hGR), or ([NLS-SV40]•hGR]) resulted in greater nuclear and mitochondrial GR activities than were observed in the parent cells (Figure 2.3). The nuclear and mitochondrial GR activities of the cells transfected with ([hGR]) were twice the respective activities of the native cells, whereas the nuclear and mitochondrial GR activities of the cells transfected with ([MTS-hGR]•hGR]) were more than ten-fold greater than the respective compartmental activities of native cells. The cells transfected with ([NLS-SV40]•hGR] had GR activities that were lower than cells transfected with ([MTS-hGR]•hGR], suggesting less efficient transcription or translation of the SV40-derived construct, that the SV40 NLS was not as effective in nuclear targeting of hGR, or that the SV40 NLS peptide sequence inhibited the catalytic activity of GR.

Transfection with each of the three constructs resulted in increases in cytoplasmic GR activities. However, the activities in cytoplasmic fractions of cells transfected with ([hGR]) were twice those of cells transfected with ([MTS-hGR]•hGR]) or ([NLS-SV40]•hGR).

Immunofluorescence studies indicated low levels of hGR protein in the native 293T cells (Figures 2.4a and b), and the ([hGR])-transfected cells showed hGR protein predominantly in cytoplasmic domains (Figures 2.4c and d). The ([MTS-hGR]•(hGR]) construct targeted GR protein into mitochondria and nuclei, resulting in a distinct, spotty sub-nuclear localization (Figures 2.4e and f). The
construct containing the [(NLS-SV40)•(hGR)] cDNA increased immunoreactivity essentially exclusively in the nucleus (Figure 2.4g). Although the immunofluorescence analyses indicated that the majority of the GR protein was located within the nuclei of the cells transfected with [(NLS-SV40)•(hGR)], the GR activities of the nuclear fractions of cells transfected with this construct were not as high as in cells transfected with [(MTS-hGR)•(hGR)] (Figure 2.3), which suggests that the GR protein coded by [(NLS-SV40)•(hGR)] may be considerably less active catalytically (Figure 2.4). The confocal images presented in Figure 2.4 indicate lower fluorescence intensities in non-targeted compartments of the transfected cells than are observed in the same compartments of the cells not transfected. These visual impressions are not accompanied by lower fractional GR activities (Figure 2.3), but are consequences of adjustments in signal acquisition necessitated by the greater fluorescence intensities in targeted compartments of the transfected cells (Figures 2.4 c-h). The attenuations needed to keep the signals arising from the targeted compartments in the transfected cells within limits capable of providing interpretable images result in dimmer signals from the compartments in which GR expressions were not similarly enhanced.

Transfection of 293T cells with GFP fusion protein constructs (Figure 2.5a) indicated that cells transfected with [(MTS-hGR)•(GFP)] demonstrated GFP fluorescence predominately in mitochondria (Figure 2.5b1), whereas cells transfected with [(hGR)•(GFP)] demonstrated diffuse GFP fluorescence primarily in the cytoplasm (Figure 2.5b2). Cells transfected with a construct containing
[(MTS-hGR)] and the first 1000 bp of the [(hGR)] cDNA, [(MTS-hGR)(0 to 1.0 kb-hGR)(GFP)], demonstrated GFP fluorescence diffusely through the cells, but the majority of the fluorescence appeared to be cytoplasmic (Figure 2.5b3), while cells transfected with [(MTS-hGR)(hGR)(GFP)] exhibited GFP fluorescence in the mitochondria and nuclei (Figure 2.5b4). These findings indicate that the [(MTS-hGR)] alone is sufficient to direct GFP expression into the mitochondria of the 293T cells studied but is not sufficient for nuclear localization of the GFP model protein. The full-length [(MTS-hGR)(hGR)] appears to be necessary for both nuclear and sub-nuclear localization of GFP.

Since the [(MTS-hGR)] did not target GFP to the nuclei of 293T cells in the absence of the [(hGR)] coding sequence, other elements of the hGR protein sequence appear to be required for nuclear targeting of GFP and, as a working hypothesis, GR. The hGR protein sequence was scanned by sequence analysis software, specifically PSORT and TargetP (Swiss Protein) to identify a NLS but no consensus NLS was identified (not shown). Assessment of the hGR sequence by PSORT II gave a probability of nuclear localization of 8.7%, along with a 52.2% prediction of mitochondrial expression. Visual analysis of the peptide sequence of GR revealed a five amino acid sequence, $^{456}$KRKTK$^{460}$ (Swiss Protein), in the C-terminal region of the hGR protein that contained a cluster of basic amino acids, which is characteristic of a NLS. In transfection studies with mutant constructs of the $^{456}$KRKTK$^{460}$ sequence, in which the basic amino acids (K or R) were replaced with alanine (A) and/or the (T) replaced by glycine (G), differences between the native cells and those transfected with hGR
and all other constructs were observed in the cytoplasmic, nuclear, and mitochondrial GR activities (Table 1). Among the cells transfected with the mutant constructs, the mean nuclear GR activities ranged from 32.8 to 60.7 mU/mg pro. However, the completely modified sequence (\textsuperscript{456}KRKTK to AAAGA\textsuperscript{460}) in the MTS-containing construct gave nuclear GR activities at least as great as were observed with transfection with the unmodified construct containing the MTS. Elevations in mitochondrial GR activities required the MTS and were uniform among the variants of the \textsuperscript{456}KRKTK\textsuperscript{460} sequence studied, with a singular exception of (K456A), which gave mitochondrial GR activities that were about half of the activities obtained with the other MTS-containing constructs. The MTS-containing constructs gave cytoplasmic GR activities (range 27.9-47.9 mU/mg pro) that showed some differences, but no consistent pattern of effect was indicated. These data indicate that the sequence \textsuperscript{456}KRKTK\textsuperscript{460} is not necessary for nuclear localization of GR protein.

Cells transfected with mutant constructs in which the ATG coding for the first methionine at the N-terminal of the MTS was mutated to ATT, which codes for isoleucine [(MTS-(M1I)-hGR)•hGR] or in which a frame shift (FS) was introduced by deletion of the sixth nucleotide of the MTS coding sequence [(MTS-(FS)-hGR)•hGR], had nuclear and mitochondrial GR activities that were indistinguishable from the activities in the cells transfected with [(hGR)] (Figure 6). In cells transfected with the constructs with the (M1I) or the (FS) mutations in the coding sequence for the (MTS-hGR), the cytosolic GR activities were elevated, but mitochondrial and nuclear activities were not. In contrast, cells
transfected with constructs in which coding for the second putative translational start site was altered, by mutation (M44I) or by deletion of amino acids 43-51, gave elevations in nuclear and mitochondrial GR activities, but demonstrated no increases in cytoplasmic GR activities. These data indicate that both translational start sites are potentially functional in the [(MTS-hGR)•hGR)] construct, and that the first translational start site is essential for both nuclear and mitochondrial targeting of GR. The translational origin of cytoplasmic GR remains to be determined.

Transfection-induced increases in GR activities in nuclear fractions of 293T cells were not affected by deletion of the nucleotides that coded for [aa 2-15] of the [(MTS-hGR)] (Figure 2.7). Cells transfected with constructs containing deletions of the nucleotide sequences that coded for [aa 16-30] or [aa 30-42] of the [(MTS-hGR)] exhibited nuclear GR activities that were not different from the activities observed in cells transfected with [(hGR)] with no MTS, although all were higher than the nuclear GR activities in native 293T cells. Transfections with modified [(MTS-hGR)•hGR)] constructs [△aa 16-23], [△aa 22-30], and [△ 24-30] produced increases in nuclear GR activities that were equal to those obtained with the full-length [(MTS-hGR)] construct.

Mitochondrial and nuclear GR activities in cells transfected with the [(MTS-hGR)] deletion constructs described in Figure 2.7 were highly correlated (Figure 8a). The tightness of this correlation (P < 0.001) suggests that the MTS-dependent mechanisms that direct GR to the nucleus are not distinct from the mechanisms of mitochondrial import of hGR. Regression analyses of either the
nuclear (Figure 2.8b) or mitochondrial GR activities (data not shown, p<0.179) with the number of basic amino acids remaining in the targeting signal indicated no correlation. However, both the mitochondrial and nuclear GR activities in these cells correlated with the total lengths (numbers of amino acids) of the respective MTS constructs (Figure 2.8c and 2.8d).

Western blot analyses of subcellular fractions of cells transfected with constructs containing mutations in the $^{456}$KRKT$^{460}$ sequence (Figure 2.9a) as well as with the MTS deletion mutants (Figure 2.9b) were used to evaluate the extents to which the immunoreactive GR protein levels corresponded with the GR activities measured. No internal standard was used in the western blots, because the studies involved different subcellular fractions. At least three western blots were performed on different experiments with each construct and assessed for reproducibility. The western blots indicated that the relative protein contents of the transfected cells corresponded with the activity measurements with each experiment.

Discussion

The primary goal of the present study was to elucidate the molecular mechanisms by which GR is targeted into the nucleus. To accomplish this goal, a method of subcellular fractionation was needed that would provide reproducible isolation of nuclear, mitochondrial, and cytoplasmic fractions from cells, in purities that would allow us to study changes in the relative distributions of GR within cells transfected with cDNA constructs modified to test hypotheses related to the molecular mechanisms regulating compartmental expression of GR. The
methods employed in the present studies provided cytoplasmic, nuclear, and mitochondrial fractions with purities adequate for the needs of our studies. The immunoreactivities against cytochrome c indicated 6±4% and 3±2% relative densities in the nuclear and cytoplasmic fractions, respectively. Thus, expression of a transgene product that increases mitochondrial GR activities by 100 mU/mg protein, but does not affect nuclear GR activities in intact cells could, from these estimates, account for measured increases in GR activities in the nuclear fractions on the order of 6 mU/mg protein. The data thus indicate that contamination of nuclear fractions by mitochondrial proteins is unlikely to account for more than 10% of the enhancements of GR activities observed in the nuclear fractions of the CHO cells stably transfected with [(MTS-hGR)•(hGR)].

The absence of apparent increases in nuclear or mitochondrial GR activities in the CHO cells stably transfected with [(hGR)], despite the increases in cytoplasmic GR activities of 100 mU/mg protein (Figure 2.2), suggest that the purities of the respective subcellular fractions are at least as great as is estimated from the western analyses presented in Figure 2.1. The apparent increases in nuclear and mitochondrial GR activities of the 293T cells transiently transfected with [(hGR)], although statistically significant, are within the range of what might be attributable to contamination of these fractions with GR from cell cytoplasm (Figure 2.3). However, as with the CHO cells, the nuclear GR activities in the 293T cells transfected with [(MTS-hGR)•(hGR)] can not reasonably be attributed to contamination of the nuclear preparations by cytosolic or mitochondrial GR.
The studies with the stably transfected CHO cells indicated that a functional N-terminal MTS was necessary for nuclear, as well as mitochondrial localization of hGR. Transient transfections of 293T cells with [(hGR)], [(MTS-hGR)•(hGR)], or derived constructs offered a more readily manipulated experimental model for expression studies, and the subcellular expressions obtained were similar to the pattern of expressions in the stably transfected CHO cells. The fact that the human GR MTS construct, [(MTS-hGR)], markedly enhanced nuclear expression of [(hGR)] (Figures 2.3, 2.4e, 2.4f), but did not direct expression of GFP into the nucleus (Figure 2.5b1), indicates that the (MTS-hGR) is necessary, but not sufficient for nuclear targeting of the product protein. These results, in turn, imply that structural elements within the hGR protein are critical to nuclear targeting of GR.

The nuclear and mitochondrial expressions of hGR effected by [(MTS-hGR)•(hGR)] (Figures 2.4e and f) and the GFP-derived fluorescence observed in cells transfected with [(MTS-hGR)•(hGR)•(GFP)] (Figure 2.5b4) indicate that both the MTS and elements of hGR are vital to nuclear targeting of GR. The absence of substantive nuclear or mitochondrial localization of GFP fluorescence in cells transfected with [(hGR)•(GFP)] (Figure 2.5b2) provides additional support for the mitochondrial import function of the MTS sequence, and the absence of nuclear expression of GFP in these cells demonstrates the importance of the MTS sequence for nuclear import mechanisms. The absence of similarly striking nuclear GFP fluorescence in cells transfected with the [(MTS-hGR)•(hGR 0 to 1.0 kb)•(GFP)] construct (Figure 2.5b3), in which the last 0.5 kb of the coding
sequence for hGR was omitted, suggests that the elements of hGR that are critical for nuclear localization are located in the C-terminal part of the protein. Proteins targeted to the nucleus and the mitochondria characterized to date usually possess consensus signals for both, and often the NLS is located in the C-terminus of the protein (Lundberg et al., 2001). However, the [(MTS-hGR)(hGR 0 to 1.0 kb)(GFP)] construct also exhibited compromised mitochondrial targeting (Figure 2.5b3), despite the evidence that the intact MTS alone was sufficient for effective mitochondrial GFP expression (Figure 2.5b1).

Nuclear localization signals (NLS) identified to date have been described as either a single cluster of basic amino acids or two clusters of basic amino acids separated by 10 amino acids (Roberts et al., 1987; Makkerh et al., 1996; Christophe et al., 2000; Hodel et al., 2001). The most common method of nuclear import involves recognition of the NLS by nuclear import proteins, importins $\alpha$ or $\beta$, and subsequent binding to import receptor(s) on nuclear pore complexes. Alternatively, other mechanisms of nuclear import have been identified and involve less well-defined import proteins and receptors. One example of a protein localized to the nucleus by non-classical import mechanisms is STAT1. Nuclear translocation of STAT1 involves a tyrosine phosphorylation-dependent dimerization of two subunits to generate a nuclear targeting signal. The STAT1 NLS has unique recognition specificity and can bind to only one specific importin $\alpha$ (hSRP1/NPI-1), instead of the broad specificity found for most classical NLS sequences. Many of the RNA binding proteins and ribosomal proteins also exhibit non-classical nuclear import mechanisms (Mattaj...
Nuclear import of GR may not be a matter of simple recognition of a contiguous amino acid sequence, but appears to involve more complex mechanisms.

Sequence analysis of GR did not identify a consensus nuclear localization signal, but the sequence $^{456}$KRKT$^{460}$ contains a cluster of basic amino acid residues, which is characteristic of an NLS or typical of a component of a bipartite recognition signal. The nuclear GR activities of cells transfected with the $^{456}$KAAT$^{460}$ construct, 32.8 ± 1.3 mU/mg protein, were noticeably below the range of activities (49.4 to 60.7) observed in the cells transfected with the intact or other mutated MTS constructs (Table 1). However, the Student-Newman-Keuls post hoc statistical analyses of the data did not indicate differences in nuclear GR activities within the set of cells transfected with the MTS-containing constructs. The mitochondrial GR activities observed with cells transfected with this construct (97.8 ± 15.5 mU/mg protein) diminish concerns regarding interference with transfection efficiency with this construct or catalytic activity of the modified protein thus produced. More importantly, the nuclear GR activities of 60.7 ± 7.2 mU/mg protein in cells transfected with the fully mutated $^{456}$AAAGA$^{460}$ construct indicate that the sequence $^{456}$KRKT$^{460}$ does not function as a NLS or play a critical role in subcellular distribution of GR protein.

Several proteins that are targeted to multiple subcellular compartments have been identified, and these proteins often contain multiple targeting signals within their amino acid sequences (Lakshmipathy and Campbell, 1999; Lundberg et al., 2001; Szewczyk et al., 2001; Ikeda et al., 2002; Wang et al., 2002). Multi-
targeting of proteins usually involves one or more of four defined mechanisms of regulation: alternative transcription initiation sites, alternative splicing of mRNA, alternative translation initiation sites, or post-translational modifications (Danpure, 1995). The GR gene contains nucleotide sequences appropriate for two transcription initiation sites, one in the 5’ UTR and the second within the MTS, but studies to date have not identified two RNA transcripts for GR. Frequently, alternative splicing of the RNA eliminates one of the potential targeting signals in the translated protein, which allows the remaining targeting signal to direct the mature protein to the appropriate subcellular compartment (Otterlei et al., 1998). However, no evidence for alternative splicing of hGR mRNA has been found. In fact, with the MTS of hGR residing adjacent to the second start site and the start of the coding sequence for the mature GR protein, classical mechanisms for alternative splicing are not feasible.

GR also contains sequences for two in frame translational start sites, both containing ribosomal recognition sites necessary for translation initiation. The presence of two translational start sites, such as found are in GR, is common in proteins targeted to multiple compartments (Lakshmipathy and Campbell, 1999; Wang et al., 2002). Transient transfection studies with constructs that contained mutations in the two potential translational start sites in [(MTS-hGR)\(\times\)hGR)] revealed the necessity of the first translational start site for nuclear as well as for mitochondrial localization of GR. In cells transfected with [(hGR)] or with MTS-containing constructs in which the first start site had been mutated, we observed predominately cytoplasmic expression; however, these studies do not resolve the
question of the functionality of the second translational start site for cytosolic expression of GR in products transcribed from the endogenous gene. One hypothesis for mechanisms of regulation of subcellular expression of GR is that both translation start sites are functional, and that alternative translation or posttranslational mechanisms are responsible for regulation of subcellular distribution of hGR. The mechanisms by which the relative proportion of protein might be translated from the first as opposed to the second start site are not known but could involve conformational availability of the ribosomal recognition sites (Kozak, 1991). Whether both translational start sites are recognized on a single transcript was not addressed in the present studies.

A shared N-terminal targeting signal that contributes to mitochondrial and chloroplast targeting has been described in studies with pea GR (pGR) (Rudhe et al., 2002). The pGR presequence is described as an ambiguous targeting signal, and deletion studies indicate that the targeting signal is capable of localizing pGR protein to the chloroplasts or the mitochondria (Rudhe et al., 2002). Rudhe et al. were able to define regions of the targeting signal of pGR protein that were responsible for the relative distribution of GR. Deletion of the N-terminal 16 amino acids of the pGR presequence caused a decrease in the chloroplast levels of GR protein and increased the mitochondrial levels of pGR, but no single sequence was identified as being responsible for mitochondrial or chloroplast localization. However, Rudhe et al. did not address the nuclear expression of GR.

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Studies with hGR-containing constructs in which segments of the MTS of hGR were omitted revealed that, while the first start site was essential for nuclear and mitochondrial GR expressions, deletion of amino acids 2 through 15 of the hGR MTS did not affect the increases in nuclear or mitochondrial GR activities attained by transfection with \[(\text{MTS-hGR})\times(\text{hGR})\]. In contrast, deletion of sequences coding for residues 16-30, 30-42, or 2-30 effectively prevented increases in nuclear GR activities in transfected cells (Figure 2.7b). The failure of deletions of residues 16-23, 22-30, or 24-30 of the hGR MTS to affect nuclear or mitochondrial GR activities of transfected cells did not indicate a critical segment for hGR expression in either compartment.

Transfections with the series of deletion constructs indicated correlations between the GR activities in the nuclear and mitochondrial compartments and the numbers of amino acid residues in the modified MTS sequences, rather than with the deletion of specific amino acids or segments (Figure 2.9). In addition, the GR activities of the nuclear and mitochondrial compartments in these cells correlated very closely, suggesting that the mechanisms that direct expression of hGR to nuclear and mitochondrial compartments are not regulated differentially by elements of the MTS. In contrast to the association indicated by the tight correlation between nuclear and mitochondrial GR activities, cytoplasmic GR activities tended to be elevated less by the constructs in this series that gave greater increases in nuclear and mitochondrial GR activities (Figure 2.7). The concept that distinct and even redundant antioxidant systems are functional within cell nuclei is logical, in light of the importance of preservation and
maintenance of the genetic structure of the organism. In addition, the contributions of thiol/disulfide-dependent mechanisms to regulation of transcription and other cellular functions and responses logically could be managed more effectively with proximate mechanisms for reduction of GSSG. The results of the present studies indicate that nuclear localization of hGR is complex and is not explained readily by classical localization mechanisms. The possibility that hGR is imported into the nucleus in its catalytically active homodimeric form or as a complex with one or more other proteins merits consideration. Although further studies are needed to address the issue of nuclear import, the present findings suggest a novel mechanism of dual localization to the nucleus and mitochondria involving the same N-terminal targeting signal. Understanding the mechanisms by which hGR is targeted to cytoplasm, mitochondria, and nuclei is essential to elucidating the functions of GR in each compartment, which in turn is needed to identify possible approaches to specific enhancement of cellular resistance to oxidant challenges and perhaps to more selective therapeutic cell killing.
<table>
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<td>cyto nucl mito</td>
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</tr>
</tbody>
</table>

Figure 2.1. Western blot analyses on subcellular fractions of 293T cells. The 293T cells were plated equally and after 48 h of growth, the cells were harvested, and subcellular fractions were isolated as described in Methods. From each subcellular fraction, 30 µg of protein were loaded and separated by SDS PAGE. The proteins were transferred to PVDF membranes and probed with the indicated primary antibodies. After incubation in the appropriate secondary antibodies, the membranes were visualized by ECL, and densities of the bands were quantitated with a gel documentation system. Data are means ± SEM.
Figure 2.2. GR activities in subcellular fractions of stably transfected CHO cells.

CHO cell lines were plated equally, grown to confluence, harvested, subcellular fractions were isolated, and GR activities measured, as described in Methods. Data are expressed as means ± SEM, n=8, and were assessed by one-way ANOVA, with Student-Newman-Keuls tests post hoc. Common symbols indicate homogenous data sets within each subcellular fraction, p<0.05.
Figure 2.3. GR activities in subcellular fractions of transiently transfected 293T cells. The 293T cells were plated equally, allowed to grow for 24 h, and transfected with the designated constructs. After 48 h, the cells were harvested, subcellular fractions were isolated, and GR activities measured, as described in Methods. Data are expressed as means ± SEM, n=3-8, and were assessed by one-way ANOVA, with Student-Newman-Keuls tests post hoc. Common symbols indicate homogenous data sets within each subcellular fraction, p<0.05.
Figure 2.4. Immunofluorescence studies of transiently transfected 293T cells. Cells were plated on cover slips, allowed to grow for 24h, and transfected with the designated constructs. After 48 h, the cells were incubated in MitoTracker (Molecular Probes), washed, and fixed, as described in Methods. After blocking, the cells were exposed overnight to anti-GR antibody, washed, and exposed to fluorescently labeled anti-rabbit secondary antibody. The coverslips were mounted onto slides and visualized with a Zeiss 510 confocal microscope. The images on the left are green fluorescent only and represent GR immunoreactivities. The images on the right are merged images of green fluorescence from GR immunoreactivities, with red MitoTracker. Images a and b are native cells, images c and d are cells transfected with [(hGR)], images e and f and cells transfected with [(MTS-hGR)•hGR], and image g is a merged image of cells transfected with [(NLS-SV40)•hGR]. The images presented are selected as representative of the results from replicates of 3-5 for each construct.
Figure 2.5. Fluorescence studies of 293T cells transiently transfected with GFP constructs. a) The GFP constructs are displayed, indicating the portion of the protein coding sequence included and the relative position to the GFP fusion protein. b) 293T cells were plated on cover slips, allowed to grow for 24h, and transfected with the designated constructs. After 48 h, the cells were washed and fixed, as described in Methods. The coverslips were then mounted onto slides and visualized with a Zeiss 510 confocal microscope. The numbered images (b) correspond with cells transfected with the constructs described in (a) and numbered similarly. The images presented are selected from replicates of 3-5 for each construct.
a. \((\text{MTS-hGR})_n\text{(GFP)}\)

1. MTS-hGR \hspace{1cm} \text{GFP}

2. \hspace{1cm} \text{hGR (1.5 kb)} \hspace{1cm} \text{GFP}

\((\text{hGR})_n\text{(GFP)}\)

3. MTS-hGR \hspace{1cm} \text{hGR (1.0 kb)} \hspace{1cm} \text{GFP}

\((\text{MTS-hGR})_n\text{(0 to 1.0 kb-hGR})_n\text{(GFP)}\)

4. MTS-hGR \hspace{1cm} \text{hGR (1.5 kb)} \hspace{1cm} \text{GFP}

b.

1.

2.

3.

4.
Figure 2.6. GR activities in subcellular fractions of transiently transfected 293T cells. The 293T cells were plated, allowed to grow for 24 h, and transfected with the designated constructs. After 48 h, the cells were harvested, and GR activities were measured. Data are expressed as means \( \pm \) SEM, \( n=3-8 \), and were assessed by one-way ANOVA, with SNK post hoc. Common symbols represent homogenous subsets within each subcellular fraction, \( p<0.05 \).
GR Activities (mU/mg pro)

Cytoplasm

Nuclei

Mitochondria

GR Activities (mU/mg pro)

native hGR [MTS-hGR] [MTS-(M1I)-hGR] [MTS-(FS)-hGR] [MTS-(Δaa43-51)-hGR] [MTS-(Δaa43-51)-hGR] [MTS-(Δaa43-51)-hGR]
Figure 2.7. GR activities in subcellular fractions of 293T cells transiently transfected MTS deletion constructs. The 293T cell lines were plated, allowed to grow for 24 h, and transfected with the designated constructs. After 48 h, the cells were harvested, subcellular fractions were isolated, and GR activities measured, as described in Methods. (a) The regions of the [(MTS-hGR)] deleted in the respective cDNA constructs are indicated by the dashed lines. (b) The GR activities of cells transfected with the respective MTS deletion constructs are indicated. Data are expressed as means ± SEM, n=3-8, and were assessed by one-way ANOVA, with SNK post hoc. Common symbols represent homogenous subsets within each subcellular fraction, p<0.05.
A. MTS MALLPRALSAGAGPSWRRAARAFRGFLLLLPEPAALTRALSRA
\[ \Delta \text{aa 2-15} \]
\[ \Delta \text{aa 16-30} \]
\[ \Delta \text{aa 30-42} \]
\[ \Delta \text{aa 2-30} \]
\[ \Delta \text{aa 16-23} \]
\[ \Delta \text{aa 22-30} \]
\[ \Delta \text{aa 24-30} \]

B. GR Activities (mU/mg pro)

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<th>Mitochondria</th>
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</table>
Figure 2.8. Correlation analyses of the nuclear and mitochondrial GR activities in cells transfected with the MTS deletion constructs. (a) The mean nuclear GR activities of the cells transfected with the MTS deletion constructs are plotted against the respective mitochondrial GR activities, and the relationship was assessed statistically by linear regression analysis. (b) The mean nuclear GR activities of the cells are plotted against the number of basic amino acids in the respective MTS deletion construct and the relationship assessed statistically by linear regression analysis. (c) The mean nuclear GR activities of these cells are plotted against the numbers of amino acids in the respective MTS deletion constructs, and the relationship assessed statistically by linear regression analysis. (d) The mean mitochondrial GR activities of the cells are plotted against the numbers of amino acids in the respective deletion MTS constructs, and the relationship was assessed statistically by linear regression analysis, with P indicated.
Figure 2.9. Western blot analyses of subcellular fraction obtained from 293T cells transiently transfected with mutant (A) or deletion (B) constructs. Western blots were performed on the subcellular fractions of cells transfected with the designated constructs and probed for GR protein immunoreactivities. The density of the bands corresponded with the GR activities measured in the subcellular fractions.
Table 2-1. GR activities in 293T cells transfected with $^{456}\text{KRKTK}^{460}$ mutant constructs. The 293T cells were plated, allowed to grow for 24 h, and transfected with [(MTS-hGR)•hGR] cDNA constructs containing mutations in the $^{456}\text{KRKTK}^{460}$ sequence. After 48 h, the cells were harvested, subcellular fractions separated, and GR activities measured as described in Methods. Data are means ± SEM, n=3-8, and were assessed by one-way ANOVA, with SNK post hoc. Homogeneous subsets are depicted by common superscripted symbols, p<0.05.

<table>
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<th>transfected</th>
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<th>nuclear</th>
<th>mitochondrial</th>
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<td>60.7 ± 7.2#</td>
<td>98.9 ± 18.9†</td>
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CHAPTER 3

SUBNUCLEAR COLOCALIZATION OF GLUTATHIONE REDUCTASE WITH THIOREDOXIN AND GLUTAREDOXIN

Introduction

Glutathione (GSH) serves many biological functions both intracellularly and extracellularly, and one of the most widely studied functions of GSH is its role as an antioxidant. During normal physiological processes or during pathological challenges, GSH can serve as a cofactor for glutathione peroxidase (GPX) in reduction of $\text{H}_2\text{O}_2$, which results in the formation of glutathione disulfide (GSSG). Increased levels of GSSG can cause changes in the thiol/disulfide ratios of cells, which can have detrimental effects on cell viabilities. Although GSH levels can be restored by de novo synthesis, a more economical method for reduction of GSSG exists, through the actions of the enzyme glutathione reductase (GR, [E.C.1.6.4.2]). GR is a flavin-dependent enzyme that uses the cellular stores of NADPH to reduce GSSG to GSH. The importance of GR in normal biological functions is indicated by the ubiquity of GR in aerobic life forms (one exception is *Drosophila melanogaster*, (Kanzok *et al.*, 2001)). Both the gene and protein sequences for GR are highly conserved throughout a wide range of life forms, further suggesting that GR functions play important roles in cell viabilities.
The intracellular organization of eukaryotic cells is complex and consists of many subcellular compartments (Misteli and Spector, 1998; Matera, 1999; Dundr and Misteli, 2001; Spector, 2001). Many proteins localize in multiple subcellular compartments or structures and serve the same or even different functions within each organelle (Lakshmipathy and Campbell, 1999; Lundberg et al., 2001; Szewczyk et al., 2001; Ikeda et al., 2002; Wang et al., 2002). Considerable evidence exists for subcellular compartmentation of GSH-dependent processes. The activities of GR have been studied extensively in the cytoplasm and the mitochondria, but less is known about the function of GR in the nucleus (Ochalska-Czepulis and Bitny-Szlachto, 1981; Mbemba et al., 1985). The availability of GR in the nucleus suggests that GR might function to limit nuclear concentrations of glutathione disulfide (GSSG) and maintain a more reducing thiol-disulfide steady state environment within nuclei, thereby protecting DNA from oxidants, and provide reducing equivalents to other antioxidants such as glutaredoxin.

The nucleus is complex and dynamic and contains several well described subnuclear structures as well as many structures that are not as well understood (Misteli and Spector, 1998; Misteli, 2001a). Most nuclear activities currently recognized involve replication, transcription, or regulation of these events. Many of the crucial interactions in these processes are transitory and dynamic. However, subnuclear compartments are not membrane-limited, and interactions of proteins with other proteins, DNA, or other subnuclear elements that have been characterized to date are generally transitory and are poorly suited to
isolation and characterization by more standard biochemical methods, such as differential centrifugation. Consequently, much of what is known about subnuclear structures at the present time has been elucidated by microscopy studies (Misteli, 2001b). The present investigations were designed to use immunofluorescence confocal microscopy of cultured cells to test the hypothesis that subnuclear GR clusters localized with nucleoli. Our initial findings indicated that GR did not colocalize with nucleolin, a protein that is characteristic of nucleoli. We therefore broadened our studies to test the hypothesis that subnuclear GR immunofluorescence would colocalize with one or more of the subnuclear structures characterized to date that exhibit distributions similar to what we observed for GR. In these studies, we investigated coilin for coiled bodies, PML for PML bodies, and SC-35 for nuclear speckles (Tuteja and Tuteja, 1998; Matera, 1999).

A second working hypothesis was that the discrete nuclear clusters of GR protein were associated with other proteins that share common or complementary functions. Identification of other proteins in the clusters could provide valuable clues to understanding the functions of GR in the nucleus. In addition, the previously described functional associations between the GSH/GR pair and GRX and the possibilities for interactions with TRX led us to investigate similarly the subnuclear localization of GR, in relation to TRX and GRX.

Methods
Cell culture and transfection. Human 293T cells were maintained in DMEM media supplemented with 10% FCS. For transient transfection experiments, poly-D-lysine (Sigma Chemical Co., St Louis, MO)-treated 150 mm plates or 6-well plates with coverslips placed at the bottom of each well were used. Cells were plated at 2.5 x 10^6 cells per 150 mm plates or 2 x 10^5 cells per well in the 6-well plates and allowed to grow for 24 h prior to transfection. The cells were transfected using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN.), according to the manufacturer’s protocol. A FLAG tag hGR was generated by annealing oligos containing the FLAG sequence (DYKDDDDK) and ligating them into the hGR cDNA between the nucleotides coding for amino acids 2 and 3 of the mature protein. The cDNA constructs used in the transfection studies were generated in the pRC/CMV vector (Invitrogen, Carlsbad, CA) and contained either the complete human GR cDNA, including the mitochondrial targeting signal [(MTS-hGR)•(hGR)], or the complete human GR cDNA with a FLAG tag [(MTS-hGR)•(FLAG-hGR)]. After 48 h, cells were harvested, or the coverslips were removed and the cells fixed.

Immunofluorescence studies. Human 293T cells were grown on coverslips and transfected as described above. After 48 h, the media were removed, and the cells were fixed with 4% paraformaldehyde for 15 min, washed, and the cell membranes were solubilized with methanol for 5 min at –20°C. Fixed cells were blocked for a minimum of 2 h in PBS containing 10% goat serum or 3% BSA, then exposed to primary antibodies overnight at 4°C, washed, and exposed to
secondary antibodies for 2 h at room temperature. The anti-GR antibody used in the initial studies was a generous gift from Professors K. Becker and H. Schirmer. The latter studies were performed with an antibody generated in our lab (GR antibody production described on page 97). The GR antisera were affinity-purified with cyanogen bromide-linked Sepharose columns treated with recombinant human GR protein.

Antibodies to SC-35, PML, and coilin were obtained from BD Biosciences (Transduction Labs, Lexington KY); antibodies to nucleolin, was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA); and antibodies to thioredoxin and glutaredoxin were obtained from American Diagnostica (Stamford, CT). The fluorescently labeled secondary antibodies were obtained from Molecular Probes (Eugene, OR) and had excitation wavelengths of 488 or 546 nm.

The cells were visualized using a Zeiss 510 Confocal Microscope. A minimum of 3 slides was prepared for each antibody treatment, four to five fields were observed on each slide, and two to three representative cells were chosen for analysis. Cells that were not in direct contact with other cells were chosen for analysis, because such cells tended to lie flat along the two dimensional planes of imaging and generated more readily interpretable images down the Z-axis. Single cell images were chosen for the figures in this report to increase the resolution of the subnuclear structures. Z-stack confocal images were captured at a resolution of 2048, using a 0.2 µm depth, which generated 15-20 images per cell. The images were collected using the multi-tracking capabilities, which collect the data from each laser individually, and the filters were set at the most
narrow band-pass to prevent bleed-over of the primary fluorescent wavelengths. Images that dissected the centers of the cells were chosen for analysis.

**Western blot analyses.** Western blot analyses were performed by standard protocols. SDS polyacrylamide gels (12%) were used to separate the proteins of the subcellular fractions isolated from 293T cells. The proteins were transferred to PVDF membranes, which were blocked, then incubated with the designated primary antibody. The membranes were washed thoroughly, incubated with secondary antibody for 2 h, and visualized using ECL reagents (Amersham Pharmacia Biosciences, Piscataway, NJ).

**Overlay assays.** Subcellular fractions were isolated from 293T cells by the method of Bronfman (Bronfman *et al.*, 1998). The proteins of each of the subcellular fractions were separated by SDS-PAGE (12%) and subsequently transferred to PVDF membranes. Once the proteins were transferred, the membranes were washed briefly and incubated in human recombinant GR protein or bovine serum albumin (10 µg/ml) for 1 h at room temperature. The membranes were again washed gently and placed in a 0.05% formaldehyde solution for 30 min, washed, and blocked again in 5% milk. After blocking, the membranes were exposed to anti-GR primary antibodies for 2 h, anti-rabbit secondary antibodies for 1 h, and visualized by ECL (Leinweber *et al.*, 1999).

**Results**

Although native cells had an overall, GR-dependent green haze, the nuclei of the cells are clearly visible, and the fluorescent clusters of anti-GR antibody reactivities can be observed. No subnuclear colocalization was observed in cells
treated with anti-nucleolin and anti-GR (Figure 1, panels a-c). We similarly determined that GR protein did not colocalize appreciably with nuclear speckles, coiled bodies, or PML bodies (Figure 1, panels d-f, g-l, and j-l, respectively).

Most cellular GR protein is cytosolic, and to increase the signal-to-noise ratios in our images, we transfected 293T cells with the complete human GR cDNA, including the mitochondrial targeting signal [(MTS-hGR)•(hGR)], which directs expression of GR to nuclei as well as to mitochondria (manuscript in review). The cells thus transfected did afford greater image resolution (Figure 2) and, as with the native cells (Figure 1), subnuclear anti-GR immunofluorescence did not colocalize with antibodies against nucleolin (panels a-c), nuclear speckles (panels d-f), Cajal bodies (panels g-i), or PML bodies (panels j-l).

Cellular distribution of GRX, as observed with anti-GRX immunofluorescence, indicated focal subnuclear staining, but the subnuclear GRX-positive foci did not colocalize with anti-GR staining (Figure 3a-c). Extranuclear GRX and GR exhibited evidence of a relationship, but one that appears to be more complex than simple overlap of immunofluorescent signals. The superimposed image in Figure 3c shows distinctive green, red, and yellow foci, indicative of GR, GRX, and close overlap of GR and GRX, respectively. The extranuclear foci of staining against GR and GRX do not appear to be entirely overlapped, as would be expected for straightforward associations, such as 1:1 molecular complexes; however, the yellow foci suggest some such close association. The distinguishable green and red foci also observed indicate that the respective GR and GRX proteins each exhibit significant protein densities.
that do not appear to include the other. The extranuclear foci identified by the three colors in the superimposed images illustrated by Figure 3c do not appear to be distributed randomly, but are intimately juxtaposed. In contrast, the intranuclear patterns of immunofluorescence in these cells do not suggest similar colocalization of GR and GRX in nuclei.

Cells transfected with the Flag-GR construct, then probed with anti-Flag and anti-GRX antibodies, exhibited similar patterns of related and overlapping localizations of the two proteins, but perhaps with a greater extent of yellow overlap of Flag-GR and GRX, relative to the separate green and red foci (Figure 3d-f), than was observed with non-Flag-labeled GR (Figure 3a-c).

Similar studies of cellular distribution of GR and TRX revealed patterns of extranuclear colocalization (Figure 3g-i) that were similar to the patterns observed with GR and GRX (Figure 3a-c). In contrast with the lack of any apparent relationship between GR and GRX within the nucleus, nuclear TRX immunofluorescence showed marked colocalization with GR (Figure 3i). Cells transfected with the Flag-GR construct and probed with the Flag and TRX antibodies (Figure 3j-l) were consistent with the distributions indicated by the anti-GR and anti-TRX immunostained cells (Figure 3g-i).

Western analyses indicated that the antibodies to GR (54 kDa) used in the present studies showed no measurable cross-reactivities with TRX (12 kDa), and the anti-TRX antibody showed no cross-reactivity with GR (Figure 4). Each of the films was exposed heavily, to enhance the limits of detection of any possible
cross-reactivities, but none were detected. A second observation in this series of studies was that the mitochondrial fractions isolated by the methods used contained no detectable levels of TRX-1. The absence of detectable reactivities of proteins in the mitochondrial fractions with the anti-TRX-1 antibody in the western analyses document the minimal to absent reactivity of the anti-TRX-1 antibody with GR or with the mitochondrial form of thioredoxin (TRX-2). In addition, the western analyses document a high level of purity of the mitochondrial fractions, with no contamination by cytosolic or nuclear protein detectable by this method. In contrast, the mitochondrial form of GR is highly reactive with either of the anti-GR antibodies used.

The colocalizations of nuclear GR with TRX-1 and GRX were investigated additionally in non-transfected cells. As we had found with our initial studies of subnuclear GR, nucleolin, and the other proteins characterized in Figure 1, the immunofluorescent images of anti-GR localization obtained with the native cells (Figure 5) were less distinct than we obtained with the transfected cells. The signal-to-noise ratios in the native cells are limited in part by the lower amounts of GR protein in the nuclei than in the transfected cells. Further, the greater relative abundance of cytosolic GR in the native cells may contribute to the overall haze of anti-GR reactivity observed in the native cells, even with the high resolution confocal microscopy employed in the present studies. Nevertheless, the TRX-1 and GR colocalizations, both subnuclear and extranuclear, exhibited the same associated and overlapped patterns (Figure 5c) observed in the transfected cells (Figure 3i). The subnuclear colocalization of GR and GRX is
less visually obvious, but the subnuclear patterns of immunofluorescence observed with each antibody indicate a similar pattern of association, if not complete overlap (Figure 5d-f).

Additional colocalization experiments were conducted using antibodies to phosphorylated histone 3 and acetylated histone 3 as markers of replication and transcription. Modest colocalization of GR immunofluorescence and Ac-histone 3 was observed in the nucleus of transiently transfected cells, but intense colocalization (yellow fluorescence) was observed in the extra-nuclear regions of the cells. Further investigation of the specificity of the p-histone 3 and Ac-histone 3 antibodies indicated that the intracellular fluorescence observed with these antibodies was likely to be non-specific (data not shown).

Overlay assay analyses provided additional evidence for selective interactions between GR and TRX-1 or GRX. The readily observable immunoreactivities in the region of 12 kDa on the membranes incubated with rhGR (Figure 6A) indicate a selective interaction between GR in the incubation solution and a protein(s) of the same molecular weight as TRX-1 or GRX (12 kDa) from cytosolic and nuclear proteins that had been separated by SDS-PAGE and transferred to the membrane. In contrast, membranes incubated with BSA as a control, rather than with rhGR, exhibited no detectable anti-GR immunoreactive bands, other than those attributed to the endogenous GR at 54 kDa.

Discussion
Our earlier studies had indicated that GR immunofluorescence was localized to discrete regions of the nuclei of GR deficient (50% of control activities) CHO cells and that GR activities were present and measurable in nucleoli isolated from rat livers by differential centrifugation (Rogers et al., 2002). Subsequent studies have supported the findings that GR activities and proteins are present in the nuclei of cells grown in culture, but additional immunofluorescence microscopy indicates that GR protein is found clustered in discrete subnuclear loci. In the present studies, confocal microscopy indicated that GR immunofluorescence was not found to colocalize with anti-nucleolin immunofluorescence (Figure 1, panels a-c) in native 293T cells or in transiently transfected cells (Figure 2, panels a-c). These findings, although incorporating the best reagents presently available, do not prelude the possibility that binding of one antibody to a specific site might inhibit binding of the second antibody. Nevertheless, we regard as more likely the interpretation of the data that our isolation by differential centrifugation of GR with nucleoli from liver homogenates was an artifact of the isolation process we employed. Nucleoli are not membrane-bound entities and are isolated by incorporating non-physiological concentrations of MgCl$_2$ into the isolation buffers, a manipulation that might alter macromolecular interactions significantly. Studies that have characterized other more recently described subnuclear structures and their relative distributions in similar cell fractionation and isolation procedures are quite limited currently. We cannot preclude the possibility that one or more of the other subnuclear structures described by Dundr and Misteli (Dundr and Misteli, 2001), Spector
(Spector, 2001), and Matera (Matera, 1999) or even not identified to date would separate with the nucleolar pellet in the isolation procedures used in our earlier studies (Rogers et al., 2002).

Although the results of the present series of immunofluorescence studies with native cells indicated that GR did not colocalize with other major subnuclear structures that shared similar morphologic appearances (Figure 1), these images were difficult to interpret because the nuclear clusters of GR immunofluorescence were partially obscured by an overall background green fluorescence. Consequently, we conducted additional experiments with transiently transfected cells. In these latter studies, the subnuclear clusters of GR protein were similar in appearance to the native cells, but were more distinct. No co-immunofluorescence was observed with GR and the other subnuclear structures tested (Figure 2).

The nucleus is a dynamic organelle that is in a constant state of change and exhibits remarkable mobility of intranuclear proteins (Phair and Misteli, 2000; Misteli, 2001b). In such an environment, interactions between subnuclear structures and the proteins in rapid flux through the nucleus may be transitory and of short duration. Although we did not observe interactions between GR and the subnuclear proteins we tested, our findings do not conclusively prove that such interactions do not exist in specific circumstances.

Immunofluorescent colocalization between GR and GRX was observed in the extranuclear regions of transiently transfected cells, but similar colocalization
within the subnuclear structures was not observed. The association between GRX and the GSH/GR couple has been established in that the GSH/GR couple is responsible for the reduction of oxidized GRX (Fernandes and Holmgren, 2004). However, the dynamic state of the nucleus and transitory nature of reduction reactions could make such interactions between GRX and GR proteins difficult to observe. However, the less distinct and more diffuse patterns of GR immunofluorescence observed in the native cells do appear to co-localize with GRX immunofluorescence in all areas of the cell. These findings suggest that the associations observed between GR and GRX do not exist within the GR-rich subnuclear clusters but are distributed more uniformly throughout the cell.

Functions of GRX include reduction of ribonucleotides to provide deoxyribonucleotides for DNA repair and replication, reduction of disulfide bonds within proteins, and reduction of GS-disulfide bonds with cysteine residues of other proteins. The proximity of GR to GRX could enhance the efficiency of GRX activities.

The observation of immunofluorescent colocalization between TRX and GR raises interesting questions about the apparent relationship between these two proteins. The antibodies used for these studies are specific to GR proteins (Figure 4), and the additional immunofluorescent colocalization of the FLAG-tagged GR protein with thioredoxin argues that our observations of colocalization between GR and TRX are not artifacts of non-specific interactions with our antibodies (Figure 4, Figure 3 panels j-l). TRX-1 is primarily a cytoplasmic protein, but is transported to the nucleus upon changes in the redox state of the
cell (Powis and Montfort, 2001; Watson and Jones, 2003; Watson et al., 2003b). TRX is a substrate for thioredoxin peroxidase, provides reducing equivalents to ribonucleotide reductase, binds to several proteins in a regulatory manner, and is involved in transcriptional regulation of many redox-sensitive genes. Oxidized TRX proteins, either as a dimer or in a disulfide bond with another protein, are reduced specifically by thioredoxin reductase (TRXR) (Mustacich and Powis, 2000). Alternatively, TRX can be reduced by GSH in thiol-disulfide exchange reactions, producing GSSG, which can be reduced by GR. Under conditions that place greater demands on disulfide reduction capabilities than can be managed by the capacities of TRXR, the GSH/GR system could support TRX-mediated functions in the nucleus.

The observations of colocalizations of GR, TRX, and GRX with each other, but not with other proteins that are characteristic of presently recognized subnuclear structures suggest the possible existence of one or more structures not recognized previously. Although the reactions catalyzed by these three proteins suggest potential functional synergy, these “reductisomes” also may serve for sequestration of the proteins in catalytically inactive forms. Other subnuclear bodies serve similar purposes. For example, nuclear speckles are storage sites for RNA processing factors, and Cajal bodies are storage sites for transcriptional components (Dundr and Misteli, 2001; Spector, 2001). GR, TRX, and GRX may interact in a redox-regulated manner, possibly bound and released by formation of interprotein disulfide bonds. For example, aggregation in catalytically inactive forms would provide a means whereby cells could S-thiolate...
proteins more extensively, as might be useful in cell cycle-dependent processes or in cell differentiation. Release of the reductases in active forms would facilitate reversal of such processes or could be part of adaptive cellular responses to oxidant stress challenges. TRX is involved with transcriptional regulation by activation of several transcription factors, including NF-kB, glucocorticoid receptor, AP-1, estrogen receptor, and p53 (Hirota et al., 2000). Interactions between GR/GSH and TRX could provide a mechanism for activation or inactivation of these key transcriptional activators.

Colocalization by immunofluorescence does not provide conclusive evidence of functional interactions between two proteins. The GR antibodies used for immunofluorescence studies were affinity-purified, using recombinant human GR protein, to minimize cross-reactivity of the antibody with other proteins. TRXR cross reactivity was of greatest concern to us, but amino acid sequence analyses indicate that the human forms of GR and TRXR proteins have only 35% identity, and the more highly conserve regions of the two proteins are in the FAD and NADPH binding domains, which would not be freely accessible to react with antibodies under native conditions. The additional studies in cells transfected with a FLAG-tagged GR construct and subsequent immunofluorescent studies using anti-FLAG antibodies, rather than anti-GR, further support the specificity of the data indicating colocalization of GR with TRX.

The relative cellular distribution of a protein may be changed when the protein is expressed in greater than normal abundance, as is often the case in
transiently transfected cells. However, the similar findings in our
immunofluorescence studies with GR and TRX in both transiently transfected
cells and native cells argues against marked changes in the relative distribution
of GR at the levels of the transgene product achieved in our studies.

An additional concern was that GR and TRX were simply in the same
vicinity and were visually observed in the same plane. Crystal structures indicate
the size of the GR dimer to be approximately 119.8 x 84.5 x 63.2 angstroms, and
TRX to be 67.7 x 26.3 x 52.6 angstroms. The 0.2 \( \mu \)m confocal plane used in our
studies represents approximately 10 times the sum of a single GR-TRX pair, but
this estimate does not include the dimensions of the antibodies. The two
antigen-antibody complexes are sufficiently large, relative to the thickness of the
confocal plane employed, that the colocalization data are most reasonably
interpreted as arising from direct interactions of GR and TRX. The sizes of the
antibody complexes that are bound to these proteins in the process of
immunofluorescent visualization make the colocalizations we observe highly
unlikely to arise from non-interactive overlays of the two protein-antibody
complexes, or from random or coincidental events. Except in bound complexes,
proteins are rarely stacked upon each other, and the behaviors of other proteins
in the nucleus are better described as fluid and highly mobile, although forming
transitory interactions (Misteli, 2001b).

Proteins found to colocalize by microscopy studies often are bound to
each other in manners that allow both proteins to be isolated together, using
techniques such as co-immunoprecipitation. We have applied several co-
immunoprecipitation methods in efforts to identify a physical link between GR and TRX, but as yet have not observed evidence of such interactions (data not shown). Either the interactions between these two proteins are weak enough such that these interactions do not survive the physical manipulations, or that TRX and GR are simply in close proximity of each other, perhaps acting on a third protein, but with similarly limited strengths of association.

Immunoprecipitation techniques have proven to be useful in cases where proteins are bound together, often in large complexes. Our evidence would indicate that the interactions between GR and TRX proteins may be transitory, interacting and releasing in the dynamic nucleus as described by Misteli (Misteli, 2001b). Consequently, we do not interpret our inability to identify a GR/TRX complex by immunoprecipitation as disproving the existence of such interactions in more physiological settings. Although the overlay assay is distant from physiological conditions, the selective interaction between GR and TRX and/or GRX indicated in Figure 7 suggests a surprisingly selective interaction. The additional weakly immunoreactive bands seen on the overlay assay indicate that proteins other than TRX may be capable of interacting with GR, but the nature of these additional bands is not known at this time.

Our studies indicate that GR and TRX are associated in subnuclear clusters, while GR and GRX associations are more diffuse throughout the cell. Although the exact nature of these interactions is not yet understood, the data imply a previously unidentified role for GR in the nucleus. Future studies will be needed to investigate the potential for changes in both nuclear localization of GR
and colocalization with TRX in responses to oxidant challenges.
Figure 3.1. Immunofluorescence in native 293T cells of GR and selected proteins characteristic of selected subnuclear compartments. Human 293T cells were cultured and fixed to coverslips, as described in Methods. Each coverslip was exposed to primary antibodies against hGR and with antibodies against nucleolin (b and c), SC-35 (e and f), PML (h and i), or coilin (k and l). The slides were then exposed to the respective fluorescent secondary antibodies. Z-stack confocal images were captured at a resolution of 2048, using a 0.2 μm depth that generated 15-20 images per cell. Images that dissected the centers of the cells were chosen for analyses, which were conducted with Zeiss software.
Figure 3-2. Immunofluorescence in transiently transfected 293T cells of GR with selected proteins characteristic of other major subnuclear compartments. Human 293T cells were cultured, transfected with hGR transgene constructs that included the functional hGR mitochondrial targeting signal, and fixed to coverslips, as described in Methods. Each coverslip was exposed to primary antibodies against hGR, concomitant with exposure to antibodies against nucleolin (b and c), SC-35 (e and f), PML (h and i), or coilin (k and l), then exposed to the respective fluorescent secondary antibodies. The images were obtained as described in Methods and in Figure 3.1, and are representative of 4 to 5 fields per coverslip and of at least two individual transfection experiments.
Figure 3.3. Co-immunofluorescence of GR and GRX or TRX. Human 293T cells were cultured, transfected, and fixed to coverslips as described in Methods. Each coverslip was exposed to antibodies against GR (acgi), GRX (bcef), FLAG (efkl), or TRX (hikl), then exposed to the respective fluorescent secondary antibodies. The images were obtained as described in Methods and in Figure 1 and are representative 4 to 5 fields per coverslip and of at least two individual transfection experiments per series.
Figure 3.4. Specificity of antibodies against GR and TRX. Proteins isolated from cytosolic (C), nuclear (N), and mitochondrial (M) fractions of 293T cells were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with the anti-GR antibodies generated by Becker and Schirmer, or by us, as indicated, or with commercial anti-TRX-1 antibody, and the immunoreactivities were visualized by ECL.
Figure 3.5. Co-immunofluorescence in native 293T cells of GR and GRX, or GR and TRX. Human 293T cells were cultured and fixed to coverslips, as described in Methods. Each coverslip was exposed to antibodies against GR (acdfgi) and TRX (bc) or GRX (ef), then exposed to the respective fluorescent secondary antibodies. The images were obtained as described in Methods and in Figure 1 and are representative of 2 to 3 cells per coverslip and of at least two individual experiments.
Figure 3.6. Overlay assay with recombinant human GR (rhGR) protein. Proteins from subcellular fractions of 293T cells were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked, incubated in rhGR or bovine serum albumin (BSA), as indicated, fixed with formaldehyde, and incubated with anti-GR antibody (see Methods). The immunoreactivities were visualized by ECL. Immunoreactive bands at 54 kDa in both membranes reflect cellular GR, as expected, but the immunoreactive band at 12 kDa in the membrane incubated with rhGR protein is consistent with a selective interaction between cellular TRX in the membrane and the rhGR to which the membrane had been exposed.
CHAPTER 4

ANALYSIS OF THE GLUTATHIONE REDUCTASE HYPOMORPHIC MICE INDICATE A GENETIC KNOCK-OUT

Introduction

The only currently recognized function of glutathione reductase (GR) [E.C. 1.6.4.2] is that of catalyzing the reduction of glutathione disulfide (GSSG) to the thiol form of glutathione (GSH). This process enables cells to utilize GSH in the reduction of \( \text{H}_2\text{O}_2 \) and other substrate oxidants in a catalytic cycle coupled to NADPH, rather than requiring \textit{de novo} synthesis of GSH for each reduction, and contributes to maintaining the reduced thiol/disulfide redox status found in most cells (Chung \textit{et al.}, 1991).

Glutathione reductase (GR) is a homodimeric enzyme that is highly conserved throughout evolution and has been identified in plants, bacteria, fungi, yeast, and all mammalian species tested (Schirmer \textit{et al.}, 1989). The evolutionary conservation observed for the GR gene and the protein implies that GR is important to survival in aerobic life. The importance of GR activities is further supported by the observation that no naturally occurring knock-out for GR has been reported. Although GR activities are not necessary for viability in yeast, the abilities of GR-deficient yeast to handle oxidant stresses are severely compromised (Muller, 1996). \textit{Drosophila melanogaster} do not express a distinct GR protein, but do express a variant form of thioredoxin reductase that, in
conjunction with thioredoxin (TRX), is capable of effecting the reduction of GSSG at rates sufficient for sustaining necessary functions (Kanzok et al., 2001).

GR deficiencies in human populations have been observed in association with dietary riboflavin deficiencies (el-Hazmi and Warsy, 1989a; el-Hazmi and Warsy, 1989b) or following administration of the anticancer agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Frischer, 1977; Frischer and Ahmad, 1977). Angular stomatitis is observed in conjunction with riboflavin deficiency, but this associated manifestation may not necessarily be attributable to GR deficiencies alone (Blanck et al., 2002). In addition, genetic GR deficiencies have been characterized in Saudi Arabian populations, and such deficiencies have been associated with sickle cell disease, thalassemia, or deficiencies in glucose-6-phosphate dehydrogenase (el-Hazmi and Warsy, 1985). These studies have not directly correlated specific GR deficiencies with clinical manifestations.

Loos et al. reported a study of three siblings from a consanguineous marriage who exhibited 10-15% of normal GR activities in their polymorphonuclear leukocytes (PMLs) and monocytes and no measurable GR activities in their red blood cells (Loos et al., 1976; Roos et al., 1979). The data suggested synthesis of an unstable protein that was able to provide sufficient enzyme function for viability of the affected individuals. However, the affected siblings had severe clinical symptoms, including juvenile cataracts and progressive deafness, and one sibling presented with hemolytic crisis after eating fava beans.
In 1999, Walter Pretsch described the generation of a line of GR hypomorphic mice (Gr1a1Neu) exhibiting tissue GR activities that were substantially lower (less than 10% in liver) than the corresponding activities in control mice, yet the mice exhibited no apparent physiological consequences (Pretsch, 1999). Pretsch generated the Gr1a1Neu mice by treating (102/Ei x C3H/Ei) hybrid male mice with isopropyl methanesulfonate, breeding the treated mice, and screening the progeny for genetic deficiencies (Ehling and Neuhauser-Klaus, 1995). Out of 1707 offspring screened, one mouse exhibited erythrocyte GR activities that were 50% of the activities in control animals. The trait associated with decreased GR activities was bred to homozygosity. The mutant mice were backcrossed at least 15 generations onto a C3H background to transfer the mutation to a defined inbred genetic strain. The homozygous offspring were characterized as having substantially lower GR activities than were observed in control C3H mice in all tissues tested, specifically 2% of controls in erythrocytes, 3-8% in liver, lung, kidney, and spleen, 21% in heart, and 14% in brain. We obtained a breeding pair of the GR mutant mice on the C3H background and re-derived these mice in our animal facility for additional studies.

Pretsch mapped the genetic defect of the Gr1a1Neu mice to a region on mouse chromosome 8 flanking the Gr1 locus, but the exact nature of the defect was not characterized. Our interests in the mechanisms by which GR expressions and activities might be regulated led us to test the working hypothesis that the mutation(s) in these Gr1a1Neu mice involved a regulatory
region, such as the promoter or perhaps an enhancer region, which could explain the low but measurable GR activities in these animals. An alternative hypothesis was that the critical mutation was in a region that altered RNA splicing or translation itself. The goals of the studies presented in this chapter were to identify the region(s) of the Gr1 locus that was/were mutated or deleted.

Methods

Animals. The Gr1<sup>a1Neu</sup> (Neu) mice were received as a generous gift from Professor Walter Pretsch. After import from Germany, the Neu mice were rederived in our animal facility and were maintained in a specific pathogen free transgenic barrier. C3H mice were obtained from Harlan Sprague-Dawley to be used as control animals, and both the C3H and Neu strains have been bred and housed in identical conditions at the animal facility in Columbus Children’s Research Institute. All animals were on a 12 h light/dark cycle and were given food and water ad libitum. All protocols used in this study were approved by the institutional IACUC at Columbus Children’s Research Institute. At the time of sacrifice, the mice were anesthetized with sodium pentobarbital, and the livers were removed and freeze clamped with aluminum blocks cooled in liquid N<sub>2</sub>.

RT-PCR. RNA was isolated from frozen liver tissue using standard protocols (Sambrook, 2001). The cDNAs were generated using random hexamer primers and MultiScribe Reverse Transcriptase (Applied Biosystems). The GR gene was amplified using primers indicated in the figures. The products were separated on 1% agarose gels and visualized by UV light with a UVP gel documentation system (UVP, Inc, Upland, CA).
Genomic DNA PCR. Genomic DNA was isolated by digesting 0.05 g of liver tissue in a solution containing 10 mM Tris, pH 8.0, 75 mM NaCl, 25 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K. The digests were subsequently extracted with phenol, chloroform, and isoamyl alcohol, and the DNA precipitated with ethanol. Prior to PCR analysis, DNA samples were heated to 95 °C for 5 min, to enhance denaturation. LA taq polymerase (TaKaRa) was used for the PCR, to increase fidelity and to provide effective amplification of the longer PCR products. Addition of the GC buffer provided with the LA taq polymerase was required for the reaction to proceed through the GC-rich region at the 5’ end of the gene.

Southern Blot Analysis. Southern blot analyses were performed according to standard protocols (Sambrook, 2001). Genomic DNA isolated from frozen liver tissues was digested with either EcoRI or BamHI restriction enzymes (New England Biolabs, Beverly, MA). The fragments were separated on 0.8% agarose gels, transferred to nylon membranes, and UV crosslinked. The membranes were probed with ³²P-labeled genomic DNA probes, as defined in the individual figures. The membranes were then washed and analyzed by autoradiography.

Sequencing. DNA sequencing was performed at the Ohio State University Plant Microbe Genomics Facility, using dye-terminator systems and compared to Genbank Accession number X76341 for cDNA sequence and AC090489 for genomic sequence.

Preparation of Anti-GR Antibodies. The cDNA for the full length mGR (including the mitochondrial targeting signal) was cloned into a pET 41 vector
The cloning was verified by sequence analysis, the cDNA-containing vector was transformed into the BL21 strain of *E. coli*, and the bacteria were grown according to the specifications detailed by the supplier. Recombinant protein was isolated by affinity chromatography, using GSH-linked agarose beads. The recombinant protein was injected into New Zealand White rabbits, following the protocol described by Halliday (Halliday *et al.*, 2000). Five weeks after injection, the rabbits were bled, and anti-GR titers were measured by ELISA. For immunofluorescent studies, the anti-sera were further purified using column chromatography with CNBr-linked Sepharose 4B beads.

**Western Analysis.** Western blot analysis was performed using standard protocols. Thirty micrograms of protein, obtained from liver homogenates, were loaded in each lane and separated by SDS-PAGE. The gel was transferred to PVDF membrane and subsequently probed with anti-GR antibodies. The western blot was visualized using enhanced chemiluminescence.

**Results**

Using RT-PCR and primers that spanned the entire coding region of GR, we were able to amplify products from hepatic RNA isolated from both Neu and the C3H mice (Figure 4.1). The product generated from the RNA obtained from the C3H mice was 1.5 kb, which is the correct size for the native GR transcript, but the product generated from the RNA obtained from the Neu mice was substantially smaller, less than 1 kb. Using a forward primer within the exon 2 and a reverse primer within exon 10, RT-PCR analyses revealed an appropriate
size product with the mRNA from the C3H mice, but no product was observed with the mRNA from the Neu mice (Figure 4.1). Using primers encompassing exons 1 through 3, 1 through 5, and 7 through 13, subsequent RT-PCR analyses indicated that a deletion existed in the GR transcript(s) from the Neu mice, and the deletion involved the region including exons 2 through 5 (data not shown).

The product generated from RT-PCR of RNA isolated from the Neu mice was cut from the gel, purified, and sequenced. The results of sequencing analyses of this product indicated that the 3’ end of exon 1 was adjacent to the 5’ end of exon 6 (Figure 4.2). This sequence defines a 334 bp deletion in the Neu transcript that would generate a frame shift in exon 6 and a premature stop codon in exon 7 (Figure 4.3).

PCR analyses of genomic DNA isolated from the Neu mice, using primers that included the 5’ UTR of the GR genomic sequence and the 5’ region of intron 1, generated an appropriate size product (Figure 4.4A). Amplification was not effective with reaction buffers provided with the enzyme (lane 2), but amplification was observed only with the addition of a GC buffer, included with the LA Taq polymerase, designed to facilitate PCR of GC-rich regions (lane 3). Additional reactions that included primers in the 3’ region of intron 1 or the 3’ region of intron 2 and the 5’ region of intron 5 (Figure 4.4B) did not amplify a product in the Neu mice. Appropriate size products were generated with primers that spanned exons 6 and 7 (Figure 4.4C) with DNA isolated from both the Neu and C3H mice. The results indicate that a deletion exists in the genomic DNA of the Neu mice, and the breakpoints appear to be located within introns 1 and 5.
Southern blot analyses of BamHI and EcoRI digests of DNA from the Neu mice revealed no bands in the blots hybridized with DNA probes directed at sequences lying within the putative deletion, whereas the expected bands were observed with DNA isolated from the C3H mice. Probes A and B were designed to detect sequences in intron 1 and intron 4, respectively. Southern blots hybridized with DNA probes A and B indicated the appropriate size bands in the DNA from the C3H mice, but no bands were observed in the DNA from the Neu mice (Figure 4.5A and 5B).

In contrast, probes that were directed at sequences that included, but were not contained entirely within the putative deletion gave detectable bands, but with sizes that differed between DNA samples from the C3H and Neu mice. Probe C was designed to hybridize to the DNA sequence that lies within the central region of intron 1 (Figure 4.6). Southern blots of DNA isolated from the Neu and C3H mice, cut with BamHI and hybridized with Probe C, detected a band in Neu mouse DNA that was smaller than the band detected with the DNA from the C3H mice (Figure 4.6A). EcoRI restriction digests hybridized with probe C revealed a larger fragment with Neu DNA than with the C3H DNA. DNA cut with BamHI and hybridized with probe D, which encompassed a central region of intron 5, revealed a band in the Neu DNA that was larger than the band found with the C3H DNA (Figure 4.6B). With an EcoRI restriction digest, probe D revealed a band with the Neu DNA that was smaller than the band observed with DNA from the C3H mice.
PCR analyses and subsequent restriction enzyme digestions with \textit{Bam}HI and \textit{Hind}III localized the breakpoint of the deletion to within a 1.8 kb region of the \textit{Hind}III digest (Figure 4.7). PCR primers were designed to lie just outside of the regions identified as deleted by the previous PCR and Southern blot analyses. A PCR product from the DNA of the C3H mice would be large (16.3 kb) and would not be visible on a standard agarose gel. In contrast, the product generated from the DNA of the Neu mice was approximately 3.5 kb. The PCR product generated from the DNA isolated from Neu mice was first treated with \textit{Bam}HI, and no size difference was observed between the cut and uncut products, indicating that any \textit{Bam}HI restriction sites within the intervening sequence had been deleted. The PCR product from DNA isolated from Neu mice was again cut with \textit{Hind}III, and the resulting digest yielded the expected products of 1.25 kb on the 5’ end and 0.46 kb on the 3’ end, but the internal portion was a 1.8 kb fragment that was composed of an unknown sequence (Figure 4.7). The unknown 1.8 kb fragment was sequenced and revealed the breakpoints for the deletion. The first breakpoint was at nucleotide 10840 in intron 1, and the second breakpoint was at nucleotide 23627 in intron 5. These breakpoints predict a 12.8 kb deletion in the genomic GR gene (Figure 8).

Western blot analysis of liver homogenates obtained from Neu and C3H mice indicated no detectable anti-GR immunoreactive band in the Neu mice, while the homogenate from the C3H mice contained a readily detected band of the correct size for GR protein, 54 kDa (Figure 4.9).
Discussion

Although the original working hypothesis for these studies had been that the GR deficiency in the Neu mice was likely to involve alterations in a regulatory region, the results of our first RT-PCR analysis (Figure 4.1) indicated that the genetic deficiency in the Neu mice was more likely to be a deletion within the gene itself. A deficiency in the regulatory region would have resulted in a full length RT-PCR for the GR transcript that was the same size as observed with the mRNA from the C3H mice, but possibly of lesser intensity. Genetic alterations that produce normal transcription activities and message stabilities, but which result in translation to a less catalytically active and/or less stable protein were considered, but these working hypotheses also were not consistent with the results of our initial studies.

With mRNA from the Neu mice, the smaller size of the RT-PCR product generated using primers that spanned the full length of the transcript and the lack of observable product in the RT-PCR reaction using primers in exon 2 and exon 10 indicated that the putative mutation in the GR gene in the Neu mice was more consistent with a deletion that involved at least some portion of the regions between exons 2 and 10. Isolation and sequencing of the smaller PCR product generated from the RNA isolated from the Neu mice confirmed that exons 2 through 5 were deleted, with introduction of a frame shift in exon 6 and a premature stop codon within exon 7 (Figure 4.2). These findings indicate that the Neu mice are likely to be functional GR knockouts.
PCR analyses of genomic DNA isolated from the Neu mice support the interpretation from the RT-PCR analyses and further identify the genetic deletion as originating in intron 1 and continuing through intron 5 (Figure 4.4). The deletion apparently changes a splicing recognition site within intron 5, and the frame shift observed in the sequence of the RT-PCR product may be produced by an incorrect splicing event. The results of the Southern blots in Figures 4.5 and 4.6 further support this interpretation of the results of the PCR analyses, demonstrating no hybridization to the probes that contain DNA sequences from the deleted regions (Figure 4.5) and hybridization bands of different sizes using probes for regions that partially encompass the deleted area (Figure 4.6).

Identification of the exact breakpoints in the GR gene of the Neu mice required that the portion of the gene involving the deletion be isolated to a region that would be small enough to sequence. PCR through the breakpoint region yielded a 3.5 kb product that was further digested with HindIII to generate three products, two of predicted size and one of unknown origin. Sequencing of the 1.8 kb unknown HindIII fragment identified the breakpoint at nucleotides 10840 and 23627, indicating a deletion of 12.8 kb. The magnitude of the deletion and the frame shift in exon 6, resulting in a dramatically altered amino acid sequence, and the introduction of a premature stop codon in exon 7, indicate that the Neu mice are functional GR knock-outs.

In light of the highly conserved expression of GR in aerobic life (Ondarza et al., 1983), the suggestion of a viable mammalian genetic knock-out for GR is rather surprising. The healthy phenotype exhibited by the Neu mice offers clear
evidence that GR is not necessary for viability. However, the Neu mice did not arise from a mutation occurring in nature or animals that survived normal processes of natural selection. One working hypothesis for the viability of the Neu mice is that other disulfide reductase mechanisms provide the same functions as are normally served by GR. Kanzok et al. demonstrated that the thioredoxin/thioredoxin reductase pair isolated from *Plasmodium falciparum*, *Drosophila melanogaster*, *Escherichia coli*, or humans, was able to reduce GSSG in vitro and could support relatively high fluxes as are often found in vivo (Kanzok et al., 2000). The apparent GR activities we have measured as GSSG-dependent oxidation of NADPH (decrease in absorbance at 340 nm) in the tissues of the Neu mice (data not shown) are similar to those reported by Pretsch (Pretsch, 1999), although these activities probably are attributable primarily to thioredoxin/thioredoxin reductase, perhaps with contributions from other endogenous reductase mechanisms, rather than to GR.

If these alternative mechanisms of reduction of GSSG and other disulfides were adequate for normal viability, the high degree of conservation of GR across species would be difficult to understand. If, on the other hand, the Neu mice are viable because of compensatory upregulation of alternative mechanisms of disulfide reduction, such compensatory responses would need to be passed along with the mutated gene, through subsequent generations and back-crosses, to insure the health and survival of the genetically modified offspring. The exceedingly low rates of GSSG-dependent NADPH oxidation (3 to 8% of control in liver, for example) that are observed in the Neu mice do not suggest strong
support for the substantial upregulation of alternative mechanisms capable of compensating fully for the absent GR. A second and, in our view, a more likely hypothesis for the surprising viability of the Neu mice is that the antioxidant systems in Neu mice are capable of maintaining life in the environment of a vivarium, whereas similarly diminished disulfide reduction capacities would be insufficient to meet the demands and stresses encountered in nature, thus resulting in strong natural selection against animals with comparable deficiencies in GR activities. Although viable and healthy, studies to date indicate that the Neu mice are more susceptible than are control C3H mice to some, but not all toxicant challenges, which suggests that these mice may provide useful live animal models with which to test hypotheses regarding oxidant mechanisms of tissue injury *in vivo.*

In the studies reported by Loos et al. (Loos *et al.*, 1976; Roos *et al.*, 1979), PMLs isolated from the GR-defective subjects exhibited substantially shortened respiratory bursts that were accompanied by dramatic decreases in GSH levels and decreases, rather than increases, in hexose monophosphate shunt activities during phagocytosis of zymosan. The same results were observed in studies using isolated human granulocytes that had been treated previously with quantities of BCNU sufficient to deplete the GR activities to below 30% of the activities of non-treated cells (Cohen *et al.*, 1987). The compromised respiratory burst capacities observed in PMLs deficient in GR activities indicate that GR plays a significant role in host responses to bacterial infections, and severe selection against similar or complete GR deficiencies in nature would be
expected. The Neu mice, although not generated by traditional strategies, appear to be functional GR knockouts and offer a useful model for studies of oxidant mechanisms of cell death and tissue injury in vivo.
Figure 4.1. RT-PCR from RNAs isolated from C3H and Neu mice. RNAs isolated from C3H and Neu mice were amplified by RT-PCR. Primers that encompass the entire GR coding region revealed a major product of appropriate size in the RNA isolated from C3H mice (the minor band is an incomplete PCR product), but the product formed from the RNA isolated from the Neu mice was substantially smaller. Primers directed at sequences located within exon 2 and the 3’ end of exon 10 produced an appropriate size product from the RNA isolated from C3H mice, but no product was observed from the RNA isolated from the Neu mice.
Figure 4.2. Sequence of the RT-PCR transcript obtained with RNA from the Neu mice. The band produced by RT-PCR with RNA isolated from the Neu mice was purified and sequenced. The sequence of the PCR product from the Neu mice indicated that nucleotides observed in the 3’ end of exon 1 of the GR gene were adjacent to nucleotides found within the 5’ region of exon 6 of the product from the C3H mice. The data indicated that, in the Neu mouse, the region between exon 1 and exon 6 of the GR gene was deleted, and the deletion induced a frame shift in exon 6.
Figure 4.3. Comparison of the GR transcripts of C3H and Neu mice. The results of the sequencing in Figure 2 indicated that the GR transcripts in the Neu mice had a 334 bp deletion, which produced a frame shift in exon 6 and a stop codon in exon 7 that would prevent translation of the remainder of the protein.
Figure 4.4. PCR amplification from genomic DNA. Genomic DNA was isolated from the livers of both C3H and Neu mice, and the indicated regions of the GR gene were amplified by PCR. (A) Primers that encompassed the part of the 5’UTR and all of exon 1 yielded an appropriate size product in the DNA isolated from Neu mice. Both products are from DNA isolated from Neu mice, but amplification (second lane) was limited in the reaction without the GC buffer used (lane 3) to enhance priming through the 5’ region of the GR gene. (B) Primers that encompassed the putative region of gene deletion, including exons 2-5 and exons 3-5, yielded appropriate size products from the DNA of C3H mice, but no product was observed using primers including exons 3-5 from the DNA of Neu mice. (C) Primers that encompassed the region 3’ of the proposed deletion yielded appropriately sized products from the DNA of both the C3H and the Neu mice.
C3H Neu exon 6-7

BamHI BamHI BamHI BamHI BamHI EcoRI EcoRI EcoRI EcoRI

14.5 kb 5.8 kb

PCR fragments

exon 1

exon 2-5 exon 3-5 exon 6-7

A. 3 kb 2 kb 1 kb

M Neu Neu Neu -GC +GC exon 1

B. 3 kb 1 kb

M C3H C3H Neu exon 2-5 exon 3-5

C. 3 kb 1 kb

M C3H Neu exon 6-7
Figure 4.5. Southern blot analyses of DNA isolated from C3H and Neu mice.

Southern blots were performed as described in Methods, using probes A and B to analyze the regions between introns 1 and 4. The results suggest that the Neu mice do not possess the genomic DNA complementary to the probes indicated and/or have lost the respective restriction sites.
Figure 4.6. Southern blot analyses of DNA isolated from C3H and Neu mice.

Southern blots were performed as described in Methods, using probes C and D to analyze the regions within intron 1 and intron 5. The results suggest that the Neu mice lack a large portion of the genomic DNA sequences normally found from intron 1 through intron 5.
Figure 4.7. PCR amplification though the proposed breakpoint in the DNA isolated from Neu mice. Using primers located in intron 1 and intron 5, PCR amplification of DNA from the C3H mice did not produce detectable amounts of the predicted 16.3 kb product. However, DNA from the Neu mice produced a 3.5 kb product. Digestion of the PCR product with HindIII revealed that the breakpoint in the DNA from the Neu mice lies within a 1.8 kb HindIII fragment.
Figure 4.8. Sequence of the breakpoint region. Sequencing of the breakpoint region indicated that the DNA isolated from the Neu mice possesses a 12.8 kb deletion spanning the sequence from nucleotides 10840 to 23627.
Figure 9. Western blot analysis of liver homogenate proteins in Neu and C3H mice. Liver tissue from Neu or C3H mice was homogenized in 0.25 M sucrose, and 30 g of the homogenates were loaded onto a 12% SDS PAGE gel. The proteins were separated and transferred to a PVDF membrane. The membrane was blocked and subsequently incubated in anti-GR antibody for at least 2 h. After incubation in anti-rabbit secondary antibody, the western blot was visualized by enhanced chemiluminescence.
Glutathione reductase [GR; EC 1.6.4.2] is a ubiquitous, highly conserved protein that has been identified in plants, bacteria, yeast, and mammals. The conservation of GR throughout evolution and the importance of GR activities in the recycling of GSH suggest that GR is an important component of essential physiological functions. GR expressions and functions in cytosol and mitochondria have been studied, but little is known about mechanisms of import or functions of GR within the nucleus. The studies described in this document provide further evidence of a distinct nuclear pool of GR and address questions concerning the mechanisms and regulation of nuclear import as well as the possible functions of nuclear GR. In addition, we have identified a GR hypomorph mouse, generated by administration of a chemical mutagen, as a genetic knockout.

In the studies described in Chapter 2, we tested the hypothesis that GR localization to the nucleus was driven by a discrete amino acid sequence functioning as a NLS. Experiments with GFP fusion proteins indicated that the MTS alone was sufficient to drive GFP to the mitochondria but was not sufficient to drive GFP to the nucleus. We identified an amino acid sequence,
456KRKTK^{460}, which most closely matched the properties (sequence of positively charged amino acids within the mid-chain or C-terminal sequence) of a nuclear localization signal (NLS). A series of experiments using alanine substitution mutations of the putative NLS demonstrated that the sequence was not a functional NLS in GR. We determined that the 5' region, containing the MTS, was necessary for nuclear localization of GR. We analyzed the MTS for nuclear localization properties by a series of mutation and deletion studies. The results indicated a tight correlation between nuclear and mitochondrial localization, but no specific amino acid sequence tested was uniquely responsible for nuclear localization of GR. In addition, nuclear and mitochondrial localization of GR did not correlate with the number of basic amino acids present in the MTS deletion constructs but did correlate closely with the total number of amino acids remaining in the MTS deletion constructs studied. Our findings indicated that the 5' MTS was necessary but not sufficient for the nuclear localization of GR and that sequences within the protein (other than the MTS) also were necessary for nuclear, but not for mitochondrial import of GR.

The studies described in Chapter 3 were designed to test the hypothesis that nuclear GR has unique functions, as implicated by the formation of subnuclear aggregated structures with other nuclear proteins. To test this hypothesis, we generated an anti-GR antibody sufficiently active for confocal immunofluorescence studies and investigated subnuclear colocalization of anti-GR immunofluorescence with that arising from antibodies against other nuclear
proteins reported to exhibit similar subnuclear structural features. Using the best reagents available, we observed no colocalization of GR protein with proteins unique to the subnuclear structures we tested. We then addressed the possible functions of nuclear GR by testing for colocalization the redox-active proteins, thioredoxin (TRX) and glutaredoxin (GRX). Our results indicated the GR immunofluorescence colocalized with both TRX and GRX. The colocalization with GRX was diffuse, but colocalization with TRX was observed in foci found in the extra-nuclear regions, as well as in discrete subnuclear clusters. One possible interpretation of the data is that GR is associated with other proteins in a manner that causes the proteins involved in the cluster to be non-functional but maintains their availability to the cell. In the nucleus, the storage sites for redox proteins could have implications in the transcriptional regulation of redox-sensitive genes.

The third study described in this report (Chapter 4), characterizes the genetic mutations in a GR-deficient mouse model (Pretsch, 1999). The founder mouse was generated by treatment of the male parent with isopropyl methanesulfonate. The F1 generation was screened, and a GR-deficient mouse was identified. Our studies identified the genetic abnormality as a deletion in the genomic GR gene. The exact breakpoints of the deletion were characterized, and the Neu mouse was identified as a genetic knock-out for GR. Although these mice are viable and healthy, preliminary studies indicate that they are likely to be more susceptible to certain oxidant stresses than are their wild type counterparts. Importantly, the Neu mice are not more sensitive to all toxicant
challenges, which suggests that these mice may provide useful tools for studies of oxidant mechanisms of tissue injury in vivo.

A great deal remains to be investigated concerning the mechanism by which GR is imported into the nucleus, the specific functions of GR in the nucleus, and the relationships between GR and the other proteins with which GR interacts. Although our results indicate that amino acid sequences within GR act in conjunction with the MTS to promote nuclear localization, the identity and of these sequences and the molecular mechanisms through which the nuclear import processes are effected and regulated are yet to be defined. The additional sequence(s) responsible for nuclear localization might be folded within the mature GR (having the MTS removed) in such a manner as to be incapable of participating in the cooperative binding with the MTS and/or chaperone proteins. In this postulated mechanism, a kinetic competition between folding of GR into the configuration of the mature enzyme and associations necessary for nuclear import would regulate expressions in to the respective compartments. Alternatively, nuclear importation might be of the folded protein, in which case, distribution of GR between nuclear and cytosolic compartments might be determined by relative rates of cleavage of the MTS and binding to chaperones or import machinery.

The evidence that the hGR MTS alone is sufficient for mitochondrial import of GFP indicates that similar kinetic determinants are unlikely to function exactly the same in the mitochondria. Although we have shown that GR/GFP fusion proteins do not create a functional enzyme (the GR-GFP fusion proteins
have no measurable GR activities), fusion proteins of this nature would be one way to attempt to define the region of the GR protein that is responsible for nuclear localization. These studies could be done in a progressive manner, starting at the N-terminus and adding 150-200 amino acids to the MTS with each construct, transfecting the constructs into cells, and analyzing the cells for immunofluorescence. The addition of the key sequence to the construct should change the localization of the immunofluorescence from the mitochondrial to both mitochondria and nucleus. This approach is likely to be fraught with limitations and difficulties, if the essential sequence requires the entire protein for conformational folding or chaperone binding that dictates nuclear targeting. In addition, the GR/GFP fusion proteins are not functional enzymes, which suggests that these fusion proteins may be incapable of folding in the manner that is essential for nuclear localization.

Protein modeling techniques offer an alternative approach to identification of sequences that might contribute to regulation of nuclear import. Models of GR, with and without the MTS, in both the monomeric and dimeric states, might offer some clues as to conformationally driven interactions and amino acid sequences that might participate in conformational regulation of GR distribution. With such clues, conservative substitution mutagenesis studies could be used to elucidate the exact sequences responsible for regulation of nuclear import of GR. The mechanisms involved in nuclear import of GR and regulation of compartmental distribution are interesting as fundamental principles of cell function, and understanding these mechanisms is necessary for designing
methods for regulating independently GR expression in mitochondria and nuclei. Methods for independent regulation of nuclear, subnuclear, mitochondrial, and cytosolic GR activities are needed for studies of the relative contributions of compartmental redox regulation of cellular signal transduction mechanisms and cell death. The results of such studies are needed to determine whether compartment-specific approaches can be developed into useful therapies.

Our studies with GR colocalization have been informative but also have raised many questions as to the functions of GR in the nuclear clusters and the relationship of these GR clusters with both GRX and TRX. In Chapter 3, we speculated on the possible existence of a nuclear reductisome, as a potential explanation of the colocalization of GR and TRX, but much work is needed to determine whether the structure indicated by the immunofluorescent colocalization functions as an integrated unit. Further studies also are needed to determine how this structure might function in a cell at rest and under stress conditions. If the GR-TRX clusters that we term reductisomes are structures for storage of redox-active proteins in relatively inactive forms, the imposition of increased oxidant stresses would be expected to cause an initial decrease in the reductisome clusters, as demands for reduction of oxidized substrates increase. As the cells respond to the stress and decrease the levels of the oxidized molecules, the reductisome clusters would return to the resting levels. These studies would most easily be done in cells, because of the accessibility to our current models.
Our studies of nuclear localization and clustering of GR have been established in cell lines, but characterization of the nuclear and subnuclear pool(s) of GR in animal models will be necessary to test more critically the physiological relevance of our findings. Correlative immunofluorescence and biochemical studies in animal tissues will be needed to characterize nuclear localization and distribution of GR in vivo. Confocal immunofluorescent analysis of animal tissue sections treated with anti-GR primary antibodies and fluorescent secondary antibodies will allow us to test our working hypotheses concerning the existence of a cellular reductisome. In addition, changes in the relative distribution of GR immunofluorescence within the cells of animal tissues could be assessed in animals subjected to several different stresses, including hyperoxia, acetaminophen, furosemide, and diquat.

Transgenic expressions of GR in mouse models are currently being developed. At present, we have generated mice that contain the complete human GR transgene (including the MTS) under the control of three different promoters: Clara cell secretory protein (CCSP), surfactant protein C (SPC), and albumin, which were selected to direct transgene expression in lung Clara cells, lung type II pneumocytes, and parenchymal hepatocytes, respectively. Founder mice have been identified with all three transgene constructs, and the F1 generations are currently being characterized. We anticipate that these mice will have increased GR activities and protein concentrations in the mitochondria and nuclei of the respective cells that have been targeted by the corresponding promoters.
The transgenic animals will be subjected to selected stresses and the relative susceptibilities of the mice to the specific stresses will be determined. The transgenic animals expressing the CCSP-GR and SPC-GR transgenes are expected to be more resistant to hyperoxic lung injury, as was found in studies where *D. melanogaster* overexpressing transgenic GR were protected from hyperoxic injury (Mockett *et al.*, 1999). The animals expressing the GR transgene in the liver (albumin promoter) might be more resistant to hepatotoxins that are thought to act through thiol oxidation mechanisms, but no previous data exist to support this theory and the relative protection provided by increased GR activities in the liver remains to be determined. The importance of nuclear and/or mitochondrial GR expressions could be evaluated by making additional transgenic animals that express a GR transgene lacking the MTS. In these animals, the GR expressions would be limited to the cytoplasm of the targeted cells. Our working hypothesis is that these animals would be protected from oxidant stresses, but the changes in subcellular distribution of transgene activities in these animals may cause them react to specific toxicants in a manner quite different than would the animals expressing GR with the MTS.

In light of the findings that the Neu mice are genetic knock-outs for GR, making a knock-out mouse by recombination might be considered redundant, but we do not expect that this opinion will be universal. Not all knockout mice made using the same general strategies result in the same phenotypes, and knockout mice made from very different strategies are likely to result in different phenotypes. Therefore, we regard making GR knockout mice by the more
traditional homologous recombination methods to be worthy of the effort, if for no other reason than to limit the discussions on the concerns regarding the Neu mice as models.

We also plan to study the relative contributions of mitochondrial and nuclear GR expressions, using a knock-in strategy in which the first translational start site at the 5’ end of the MTS is mutated, to cause a loss of the initiation of translation. Mice generated with this construct should be deficient in both nuclear and mitochondrial GR, but the results of studies in cells indicate that the mice would have normal cytoplasmic GR activities (Tamura et al., 1996).

Additional studies will be performed with the Neu mice and additional knockout mice to characterize differences in susceptibilities to bacterial infections. Roos et al. (Roos et al., 1979) reported that phagocytic cells isolated from GR-deficient humans gave shorter respiratory bursts and had substantially decreased capacities to kill bacteria than did polymorphonuclear leukocytes (PMNs) from control subjects. The respiratory bursts, in addition to being necessary for the destruction of the invading bacteria, also impose increased oxidant burdens on the phagocytic cells and the surrounding tissues. The studies by Roos indicated that the PMNs isolated from the GR-deficient patients were unable to sustain a respiratory burst, presumably because the GR-deficient cells were not able to maintain a reducing environment (most likely due to the rapid depletion of GSH during the oxidant challenge imposed by the burst). The working hypothesis of studies of responses to bacterial exposures is that the
relatively normal viability of the Neu mice is dependent upon the pathogen-free environment of the vivarium. The absence of identified GR knockouts in nature suggests a strong natural selection against those organisms able to mount an appropriate response to bacterial invasion but unable to sufficiently maintain that response, due to either the vulnerability of the phagocytic cells to oxidant injury or damage to the adjacent tissues.

Studies to test the hypothesis that GR is important in survival of an organism in the face of bacterial infections should include mice deficient in GR activities, both Neu and an additional knock-out/knock-in mice generated by recombination, as well as GR-overexpressing transgenic mice. The transgenic mice should include those expressing transgenes with and without the MTS and with selected promoters. Initial studies of possible effects of differences in GR activities with bacterial resistance will employ *Pseudomonas aeruginosa*, which is a common gram negative bacteria that causes a variety of infections, primarily pneumonias, in patients with compromised immunity. Earlier studies have indicated dramatic decreases in antioxidant status in mice infected with *P. aeruginosa* (Suntres *et al.*, 2002), and similar studies in CCSP knock-out mice indicate increased susceptibilities to inflammation and decreased abilities to destroy the invading bacteria (Hayashida *et al.*, 2000). In addition, we will study *Streptococcal agalactiae* infections in the same strains of mice. Group B Streptococcal infections (*S. agalactiae*) are major concerns in pregnant women and newborns and are responsible for substantial morbidity in the general population (Center for Disease Control). Both bacterial strains are clinically
relevant, but the major purpose of the studies would be to determine the relative importance of glutathione-related antioxidant capabilities in sustaining appropriate responses to bacterial challenges \textit{in vivo}.

GR is highly conserved through evolution, but the results of the present studies indicate that GR is not essential for aerobic life. However, this seeming paradox does not take into account the protection against bacterial infections provided by phagocytic cells and the need for effective and sustained respiratory bursts in bacterial killing. Further studies investigating the role of GR in bacterial infections could identify an essential function for GR in aerobic life.
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