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ACTIVATION OF GLUTAMATE-CYSTEINE LIGASE IN LYMPHOCYTES

Cecile M. Krejsa

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

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Program Authorized to Offer Degree: Department of Environmental Health

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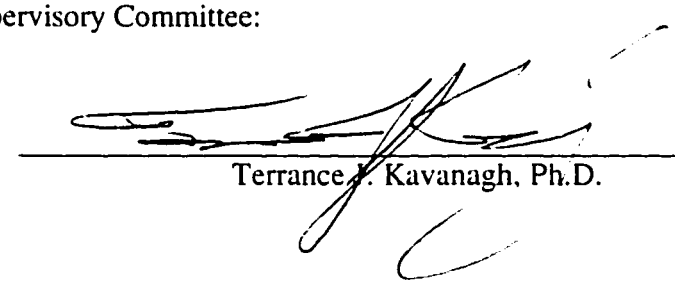

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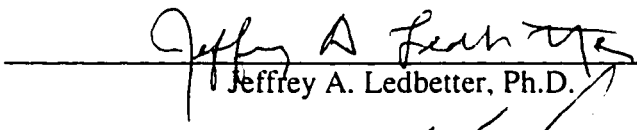

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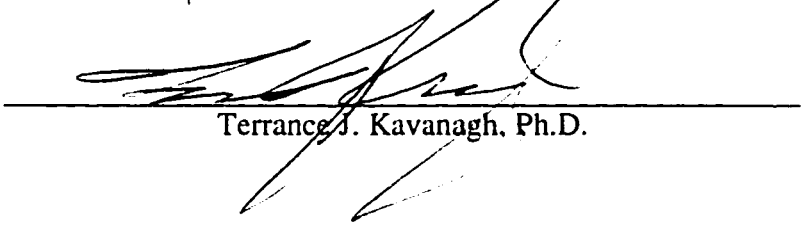

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Abstract

Activation of Glutamate-cysteine Ligase in Lymphocytes

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Lymphocytes are normally quiescent cells that react to receptor-based signals by developing into functional effector cells that orchestrate immune responses. In their resting state, lymphocytes are highly susceptible to oxidative stress, which can alter signal transduction, inhibiting antigen-specific responses. Glutathione (GSH), the major cellular thiol antioxidant, is required for robust lymphocyte responses. Glutamate-cysteine ligase (GLCL; E.C. 6.3.2.2) catalyzes the rate-limiting step in the biosynthesis of GSH. The studies presented herein characterize the induction of GLCL protein in lymphocytes following stimulation of proliferative responses by the mitogen phytohemagglutinin, and by cell surface receptors. Upregulation of GLCL protein is shown to be linked tightly with cell proliferation. In addition, the present work describes changes in GLCL activity following treatments that induce oxidative stress and deplete GSH in lymphocytes, and proposes a novel mechanism for the rapid activation of GLCL. Finally, this report provides evidence that the catalytic subunit of GLCL is cleaved in lymphocytes and other cells in a caspase-3 dependent fashion during programmed cell death. The site of cleavage is identified and results from initial investigations of the function of cleaved GLCL are presented. The studies described herein demonstrate that lymphocytes possess multiple mechanisms for the activation of GLCL and control of GSH biosynthesis.

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DEDICATION

(for Michael)

Explain to Me, Love

Your hat lifts gently, greets, floats in the wind
your bare head has captivated the clouds
your heart has business elsewhere,
your mouth is annexing new languages,
the quaking grass is taking over the country,
the summer blows star-shaped flowers alight and out,
you raise your face blind with flakes,
you laugh and cry and die of being you,
what more can happen to you--

Explain to me, Love!

The peacock in solemn amazement spreads his tail
the dove fluffs up his feather ruff,
the air stretches, glutted with cooing,
the drake screams, the whole land
eats wild honey, even in the staid park
a golden dust borders every bed.

The fish blushes, catches up the shoal
and bursts through grottoes into coral beds.
The scorpion dances shyly to the music of silver sand.
The beetle smells the Glorious One from afar;
if only I had his senses, I too would feel
that wings are gleaming under her armor,
and take the road to the distant strawberry bush!

Explain to me, Love!

Water knows how to talk,
the wave takes the wave by the hand,
in the vineyard the grape swells, bursts and falls.
So guilelessly the snail steps from its house!
One stone knows how to soften another!

Explain to me, Love, what I cannot explain:
Should I, for the short dreadful while,
be friends with thoughts only, and alone
know nothing sweet, do nothing dear?
Must someone think? Isn't he missed?

You say: Another spirit counts on him...
Explain nothing to me. I see the salamander
go through every fire.
No shudder pursues him, and nothing gives him pain.

--Ingeborg Bachmann

INTRODUCTION

I. MODULATION OF INTRACELLULAR REDOX STATUS BY GSH

Oxidative stress and antioxidants defined

An evolutionary challenge faced by all organisms is to import and utilize a chemical with sufficient reactivity to be the final electron acceptor in metabolic pathways whose primary function is to conserve electron flow in high energy molecules. The reactivity that allows molecular oxygen (O_2) to fulfill this role in aerobic organisms also makes it a potentially dangerous chemical within the cell. However the payoffs in energetic efficiency have no doubt placed enormous selective pressure on organisms which develop mechanisms to utilize O_2 with a minimum of collateral damage. Thus, many diverse and complementary safety measures have evolved in organisms that employ aerobic metabolism in the process of life. The condition of excess intracellular oxygen, especially when the oxygen exists in one or more of its damaging forms, has come to be known as *oxidative stress.* Strategies for combating intracellular oxidative stress on a molecular level have been termed *antioxidants.* Both terms have experienced a growing amount of media usage and public awareness in the past decade, fueled largely by the attention of biomedical researchers to the role of oxidative stress in aging and disease.

The intracellular reduction-oxidation (redox) balance can be shifted to a condition of oxidative stress by numerous mechanisms. The electron transport chain, mentioned above, is a major source of potentially dangerous superoxide anions ($^{\cdot}O_2$-). These are metabolized by mitochondrial superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2), which in turn can be metabolized to water by glutathione peroxidases (GPx), catalase, and peroxiredoxins. If sufficient amounts of these antioxidant enzymes are present the mitochondrial flux of $^{\cdot}O_2$- should not present a major problem to the cell (Cohen 1994). However any imbalance in the system, including mitochondrial dysfunction due to aging, metabolic defect, or toxicity, could lead to an excess of reactive oxygen species (ROS) of mitochondrial origin. Indeed, a large proportion of the cellular redox changes observed during apoptosis is likely derived from mitochondrial $^{\cdot}O_2$- flux (Garcia-Ruiz et al. 1997; Vanden Hoek et al. 1997; Wallace et al. 1997; Pierce et al. 2000). In addition, there are countless drugs and environmental toxicants that induce intracellular oxidative stress as a side effect or as a principal mode of action. For example,

many metals can shuffle between valence states, passing electrons readily to nearby molecules; this redox cycling can lead to production of ·O2-, H_2O_2, and ·OH, depletion of NAD(P)H, and in some cases, the generation of glutathionyl radicals (Stohs and Bagchi 1995).

Other sources of intracellular oxidative stress include the action of enzyme systems that are designed to produce highly reactive oxygen species. For example, NADPH oxidase produces $\cdot O_2$-; myeloperoxidase produces hypochlorous acid (HOCl); and the nitric oxide synthetases make NO· radical (el-Hag and Clark 1987; Bastian and Hibbs 1994). The activation of these enzymes may be responsible for the oxidative burst which causes ischemia/reperfusion injury and leads to tissue damage following stroke, cardiac arrest, and kidney transplantation (Schiller et al. 1991; Korthuis and Granger 1993; Walder et al. 1997; Heinzelmann et al. 1999). Other enzyme systems may produce compounds capable of redox cycling, with the generation of reactive oxygen species (ROS) as the end result. For example, metabolism of conjugated ring structures by the CYP450 system can lead to the generation of quinones, which are capable of generating H_2O_2 through redox cycling reactions. Furthermore, oxidative stress can be amplified by non-enzymatic mechanisms, such as the Fenton and Haber-Weiss reactions that produce ·OH from the less toxic $\cdot O_2^-$ and H_2O_2, and the propagation of lipid peroxides from unsaturated fatty acids (Cohen 1994; Davies 1999).

Unchecked oxidative stress may lead to the damage of all classes of cellular macromolecules, including protein, DNA, RNA, and lipid. The sites of such damage vary, depending on the site of generation of the ROS and its stability in the intracellular milieu. Damaged proteins may be targeted for proteosomal degradation, or may accumulate in the cell as insoluble aggregates (Grune et al. 1997). The presence of protein carbonyls has been used as a biomarker for oxidative injury (Berliner and Heinecke 1996; Dean et al. 1997; Stadtman and Berlett 1998). H_2O_2 and ·OH can induce strand breaks on DNA, leading to the potential for mutations during repair, or cell cycle arrest and apoptosis in cases where the damage is too great (Demple and Harrison 1994; Davies 1999). In addition to direct measurements of DNA strand breaks, 8-hydroxy-2'-deoxyguanidine adducts have been used to assess oxidative DNA damage (Bashir et al. 1993; Beckman and Ames 1997). Lipid peroxides may be repaired by some glutathione peroxidase enzymes, however if the damage caused by unchecked lipid peroxidation is severe enough,

membrane function will be compromised, resulting in receptor aggregation or failure thereof, and potential loss of membrane integrity (Comporti 1989; Pushpa-Rekha et al. 1995). Extreme damage in any cellular compartment can lead to altered cellular function or the induction of cell death through apoptotic or necrotic mechanisms.

Antioxidants

To counteract the many potentially dangerous side effects of an oxygen-based energy currency, aerobic organisms have evolved tremendous antioxidant resources to protect their cellular functions. The detoxification of ROS, in general, occurs by their conversion to water through a multi-step process enacted by both enzymatic and non-enzymatic means (Cohen 1994; Davies 1999). The first line of defense against ROS consists of small molecular weight antioxidants, often known as free-radical scavengers, which can absorb an extra electron without becoming dangerous. An example of this type of mechanism is provided by α-tocopherol, which has the effect of stopping the propagation of lipid peroxides through membranes. Thiol antioxidants may also act as free radical scavengers by donating an electron to oppose the reactive, unpaired electron in the radical or by directly reducing α-tocopherol (Sies 1993). This leads to the oxidation of GSH to GSSG. GSH may be then regenerated by enzymatic means. Both thioredoxin and glutathione (GSH) utilize this mechanism (Meister and Anderson 1983; Holmgren and Bjornstedt 1995). NADPH donates electrons to maintain thiol antioxidants, which in turn maintain both protein thiols and the reduction status of small antioxidants such as ascorbate (Meister 1994; Ishikawa et al. 1998). The enzyme glucose-6-phosphate dehydrogenase supplies NADPH, the main cellular reducing equivalent, through the pentose phosphate pathway (Tian et al. 1998). A schematic illustration of the interactions between low molecular weight antioxidants is shown in fig. 1.

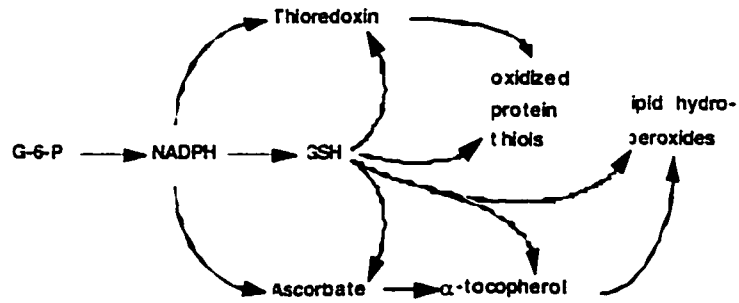

Figure 1. Interactions of antioxidant defense systems. Arrows indicate electron flow.

Enzymatic mechanisms for detoxifying ROS include SOD and CAT (mentioned above), the glutathione peroxidase/reductase (GPx/GSSGRx) system, which converts H_2O_2 to water with electrons ultimately supplied by NADPH, and the peroxiredoxins, which act in concert with thioredoxin and thioredoxin reductase to detoxify peroxides (Sies 1993; Kang et al. 1998). Glutathione-S-transferases (GSTs) act on potentially dangerous products of the CYP450 system by adding GSH to potentially reactive sites; however this may not always prevent redox cycling in quinones (Beckett and Hayes 1993). Other antioxidant enzymes work to maintain the oxidation state of proteins and lipids. Some GPx and GST enzymes will reduce lipid peroxides (Pushpa-Rekha et al. 1995). Thioredoxin reductase works to maintain protein sulfhydryls in a reduced state and prevent aggregation caused by protein disulfide crosslinks (Holmgren and Bjornstedt 1995). Overall, the cellular environment has a reducing redox tone, except in specialized compartments such as the endoplasmic reticulum where protein folding and disulfide bonding require oxidizing conditions.

Glutathione Biosynthesis

The tripeptide GSH, γ-glutamylcysteinylglycine, is the major low molecular weight cellular thiol, existing at millimolar concentrations in most cell types. In addition to the antioxidant functions described above, GSH plays an important role in amino acid transport, through the action of the γ-glutamyl transpeptidase system, and acts as a substrate for the detoxification of electrophilic compounds through the GST system (Meister and Anderson 1983). A stick model and space-filling model of GSH are shown in fig. 2.

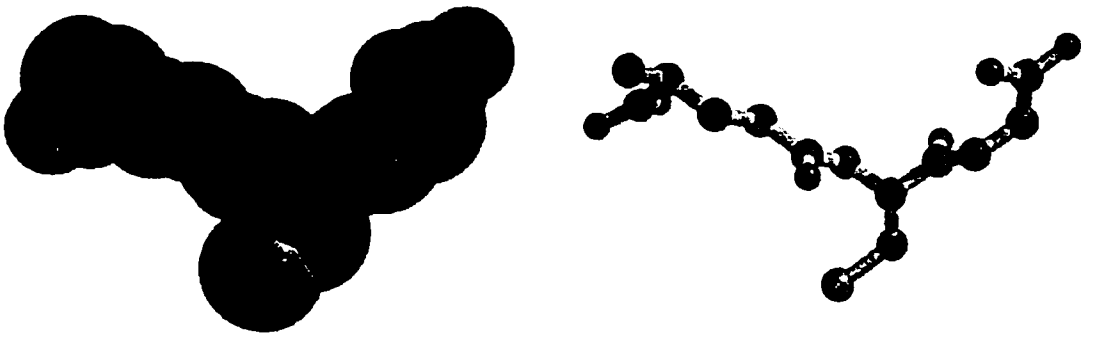

Figure 2. Two views of GSH. (Models courtesy of Dr. Elie Adman, U. Washington).

The biosynthesis of GSH occurs in two reactions; the first and rate limiting step in GSH formation is the synthesis of γ-glutamylcysteine (γ-GC), which has an unusual gamma bond that prevents the digestion of GSH by normal peptidases. This reaction is catalyzed by glutamate cysteine ligase (EC 6.3.2.2, GLCL; also known as γ-glutamyl cysteine synthetase). The second reaction, which adds a glycine to γ-GC, stabilizing the tripeptide, is catalyzed by glutathione synthetase (GS). Both reactions require ATP, and GLCL also has a requirement for Mg^{2+} as a cofactor. Whereas GLCL is rate limiting for GSH biosynthesis under saturating substrate conditions, the availability of L-cysteine may in fact be limiting in some tissues (Meister and Anderson 1983).

Mammalian GLCL, which carries out the first step in GSH biosynthesis, is composed of two separately encoded subunits, a catalytic subunit (GLCLC) of 73 kDa, and a regulatory subunit (GLCLR) of about 28 kDa (Seelig et al. 1984). The bacterial analog of this enzyme (GSH1) is monomeric and possesses a higher degree of homology to GLCLR than GLCLC (Huang et al. 1993a). The regulatory subunit, GLCLR, associates with GLCLC to form GLCL holoenzyme, a heterodimer of 100 kDa. All the catalytic activity, and the binding sites for all three substrates (cysteine, glutamate, and ATP) reside within the GLCLC subunit (Seelig et al. 1984). GLCL holoenzyme has a higher affinity for glutamate and lower affinity for GSH than GLCLC, which is subject to feedback inhibition by GSH at the glutamate binding site. In rat, the GLCLC monomer has a Km for glutamate of 18.4 mM and a Ki for GSH of 1.8 mM, whereas the GLCL holoenzyme has a Km for glutamate of 1.4 mM and a Ki for GSH of 8.2 mM (Huang et al. 1993b). Given the range of glutamate and GSH concentrations present in most tissues, this enzyme would be expected to have minimal activity except in the holoenzyme form. In the human, the differences in kinetics between GLCLC and the GLCL holoenzyme are less pronounced: the $Km_{(glu)}$ is 3.2 and 1.9 mM, respectively, for the GLCLC and GLCL holoenzyme, and the $Ki_{(GSH)}$ is 1.0 and 3.3 mM (Griffith and Mulcahy 1999). These are the only two mammalian enzymes for which the kinetics have been reported. However, despite the differences between these two species, the trend remains similar. The presence of GLCLR is thought to make GLCLC a more efficient enzyme under physiological conditions.

The function of GS has been less well studied, as it is not considered to be rate limiting for GSH production. Mammalian GS is a 118 kDa homodimer, whereas the GS from

bacteria is monomeric and has very little homology to mammalian GS (Oppenheimer et al. 1979; Gali and Board 1995). The murine GS gene has been shown to code for six alternative transcripts, derived from splice variants; these may be expressed differently in different organs, however nothing is known of the functional differences in the proteins encoded by in these variant transcripts (Shi et al. 1996a). Substrate mapping studies of rat GS suggest that it is highly specific for the cysteinyl moiety of γ-GC and less finicky about the structure of the glutamyl moiety, which differs from GLCL, whose affinity for glutamate seems to govern its kinetics (Meister and Anderson 1983).

GSH Metabolism

In some tissues, such as liver, or under certain cellular conditions, excess GSH is produced and exported in reduced form (van den Dobbelsteen et al. 1996; Lu 1999). The metabolism of GSH occurs by many routes. Its role in the secondary metabolism of xenobiotics has been well studied. GSH is consumed by reactions of the GST system, through which many electrophilic substrates and products of the CYP450 system are detoxified (Beckett and Hayes 1993). The products of these reactions are exported from the cell via anionic transport pumps, and converted to mercapturic acids in the kidneys prior to excretion. GSH also plays a major role in cellular protein import. The γ-glutamyltranspeptidase (GGT) system breaks GSH to transfer γ-glutamate to certain amino acids as a mechanism for cellular import. The remaining cysteinylglycine moiety is cleaved by dipeptidases and recycled back into GSH (Meister and Anderson 1983). The nonenzymatic donation of electrons to $^{\cdot}OH$, and the peroxidase activity of GPx, result in the formation of glutathione disulfide (GSSG). This may be exported from the cell (to protect the redox balance), or be reduced by GSSGRx, which utilizes 2 NADPH in the reduction of GSSG. GSH is also the subject of some reactions involving cellular signaling. Nitrosating species may donate $NO^{\cdot}$ to GSH, resulting in S-nitroso-glutathione, a compound which itself can spontaneously degenerate to GSSG and $NO^{\cdot}$ (Singh et al. 1996). Leukotriene C_4, an inflammatory cytokine, consists of leukotriene A_4 bound to GSH, and is synthesized by a unique, non-microsomal GST pathway (Beckett and Hayes 1993). The variety of known routes of metabolism for GSH and its tremendous diversity of known functional roles leads one to wonder what discoveries future inquiry may bring.

Regulation of GLCL in Mammals

It appears that GLCL is required for mammalian growth and development at many levels. The recent report in which the GLCLC gene was disrupted in mice showed that GLCL deficiency was lethal to embryos, which fail to gastrulate and die from distal apoptosis by g.d. 8.5 (Shi et al. 2000). There were hints that GLCLC might be important in the embryonic lethality seen in mice lacking the transcription factor MTF-1, since these animals showed diminished GLCLC mRNA levels. However, these mice died at a later time (g.d. 14) compared to the GLCLC knockouts, and showed major hepatic impairment and cell death (Gunes et al. 1998). GLCL is also important in postnatal development, as newborn mice treated with buthionine sulfoximine (BSO), an inhibitor of GLCL, invariably developed cataracts, and were subject to mitochondrial depletion, swelling of the few remaining mitochondria, and nuclear changes in the cerebral cortex (Meister 1991). Adult mice administered BSO developed skeletal muscle necrosis, with attendant mitochondrial disruption, and lung abnormalities, in particular damage to type 2 cells and swelling of mitochondria in capillary endothelial cells (Meister 1991).

Very few human cases of GLCL deficiency have been reported (a total of 8). In contrast, GS deficiency is a rare, autosomal recessive disorder which has been common enough to study and characterize (Ristoff and Larsson 1998). Severe cases of GS deficiency result in intracellular GSH levels about 15% of normal, with 5-oxoprolinuria, hemolytic anemia, metabolic acidosis and neurological defects as the major devastating symptoms of the disorder (Meister 1974; Shi et al. 1996b; Ristoff and Larsson 1998). Hemolytic anemia and neurological defects have also been reported in GLCL deficiency, but with so few cases, it is difficult to define a syndrome. Interestingly, one mutation in GLCLC which was characterized genetically predicted a substitution of His^{370} with Leu; this His residue is conserved in all species for which the GLCLC sequence is known (Beutler et al. 1999). Another coding mutation (Pro^{158} to Leu) was recently discovered in a kindred with two symptomatic (homozygous) individuals and several non-symptomatic (heterozygous) members (Ristoff et al. 2000). Several studies indicate that GLCL expression is quite variable in humans, with up to 4-fold differences observed in GLCLC and 9-fold variation in GLCLR mRNA levels from peripheral blood mononuclear cells of normal volunteers (Yao et al. 1993; Oguri et al. 1999). PBMC from cancer patients, tested prior to chemotherapy treatment, had increased variability in GLCL expression, with 24-fold differences in GLCLC and 60-fold differences in GLCLR mRNA levels (Oguri et al.

1999). There is a trinucleotide repeat of variable length in the 5' untranslated region (UTR) quite close to the transcription start site for GLCLC, and other polymorphisms have been noted in an intron and the 3' UTR (Walsh et al. 1996; Beutler et al. 1999). Studies in our laboratory suggest that the 5' trinucleotide repeat may affect GLCL transcription in response to GSH depletion (Shao, et. al., unpublished results). Less is known of variability in GLCLR among humans, however the physical mapping of GLCLR on chromosome 1p22 has located it within a region that is critically deleted in human mesothelioma (Rozet et al. 1998). The role of GLCLR in this disease has not yet been defined.

GLCL in disease

Altered levels of GLCL have been observed in several diseases. Erythrocytes from patients with non-insulin-dependent diabetes mellitis had significantly lower GLCL activity than erythrocytes from normal controls (Murakami et al. 1989; Yoshida et al. 1995). This may be due to impaired expression or protein alterations due to chronic high glucose exposure (Murakami 1991; Urata et al. 1996). Decreased plasma cysteine and cystine, and lower GLCL activity was observed in mucosa from individuals with inflammatory bowel disease, in both inflamed and non-inflamed tissue (Sido et al. 1998). Low GLCL and GS activities were also associated with subcapsular cataracts, and in normal, clear lens the level of GLCL activity was found to decrease substantially with age (Sethna et al. 1982; Rathbun et al. 1993). Individuals seropositive for HIV have decreased circulating cysteine and GSH in their plasma and lymphocytes (Buhl et al. 1989; Eck et al. 1989). This may be due to a decrease in the GLCLR mRNA levels caused by the HIV-1 Tat transactivator (Choi et al. 2000a). While the precise role of GLCL in the etiology of these diseases is not yet known, decreased GLCL levels provide evidence for disruption of GSH homeostasis as a potential causal factor or aggravating factor in each case.

Tissue levels of GLCL may also be increased as a response to oxidative stress or inflammatory cytokines in disease. A study of lung cancer patients showed that individuals with chronic obstructive pulmonary disease (COPD) had higher GLCLC mRNA levels in their alveolar epithelium than patients without COPD (Rahman et al. 2000). Subjects with hepatitis C infection also showed higher GSH synthetic activities in PBMC than did healthy controls, suggesting a peripheral response to hepatic inflammation (Boya et al. 1999). Tissue from colorectal tumors had higher GLCL activity than normal

colorectal tissue from the same patients (Ozdemirler et al. 1998). In addition, increased GLCL has been frequently noted as a factor in chemotherapy resistance in many tumors and tumor cell lines, and GLCL levels may be upregulated following chemotherapy treatment (for review, see Griffith and Mulcahy 1999). Clinical interventions based on altered GLCL in various diseases have been proposed. For example, treatment of HIV^+ individuals with N-acetylcysteine (NAC), a cysteine precursor, has been used in clinical trials to increase GSH and slow the progress of AIDS, with mixed results (Witschi et al. 1995; Akerlund et al. 1996; Herzenberg et al. 1997; Look et al. 1998). Treatment of cancer patients with buthionine sulfoximine (BSO), which inhibits GLCL activity, is under development as an adjuvant for standard chemotherapy to decrease resistance of tumors by lowering intracellular GSH (Bailey et al. 1994; O'Dwyer et al. 1996; Bailey et al. 1997).

GLCL regulation by transcriptional activation

Intensive study of GLCL mRNA levels in mammalian models of disease and oxidative stress has ensued since the original publication of the rat GLCLC sequence by Yan and Meister in 1990. Numerous reports of transcriptional regulation of GLCLC and GLCLR have examined the role of intracellular oxidative stress in the GLCL expression in a variety of cell models. Recent work on the promoters for GLCLC and GLCLR has elucidated some of the responsive elements in the two genes (Griffith and Mulcahy 1999). Whereas differential regulation of the two subunits in response to the same stress or stimulus has been reported (Cai et al. 1997), there are common elements in the upstream structures of both genes. The antioxidant response elements (ARE) appear to be especially important for transcriptional upregulation of both GLCLC and GLCLR by agents that induce oxidative stress (Moinova and Mulcahy 1999; Wild et al. 1999; Jeyapaul and Jaiswal 2000). In addition, both AP-1 and NFκB sites have been implicated in increased expression of GLCL (Iwanaga et al. 1998; Iida et al. 1999; Rahman et al. 1999). Expression of GLCLC and GLCLR varies quite dramatically with tissue type as well; however, less work has been done to characterize the factors responsible for these differences. Kang et al. (1999)(Kang et al. 1999) found that in mouse brain the expression of GLCLC and GLCLR mRNAs, which differed among brain structures, correlated well with both the GLCL activities and GSH levels in those structures. Studies in our laboratory of GLCLC and GLCLR expression in normal mouse tissues showed dramatic variation between tissue type in both the relative amounts of GLCL mRNA and protein, and the ratios between GLCLC and GLCLR (Krejsa et al., in preparation). This

was a predictable result, based on the known differences in GSH metabolism in some tissues. However the specific requirements for GSH in many tissues are unknown.

GLCL Regulation by post-transcriptional and post-translational mechanisms. Remarkably few studies have addressed the regulation of GLCL activity by post-transcriptional mechanisms. However, message stabilization of GLCLC and GLCLR has been reported in response to alkylating agents and cyclohexamide in mammalian cells, and in response to several treatments in Arabidopsis (Gomi et al. 1997; Sekhar et al. 1997; May et al. 1998). Post-translational control of GLCL has also been sparsely studied. Sun et al. (1996) reported that GLCLC, but not GLCLR, was phosphorylated in vitro by protein kinase C, protein kinase A, and Ca^{2+}/calmodulin dependent kinase II. Phosphorylation was also seen in GLCLC from cultured hepatocytes treated with pharmacological activators of these kinases. The effect of phosphorylation was to decrease GLCLC activity by about 20%. A report of autophosphorylation of GLCLC showed that in the absence of glutamate GLCLC could transfer a phosphate from ATP to an unidentified residue; this resulted in a 60% reduction in GLCL activity (Sekhar and Freeman 1999). The biological importance of this reaction is unknown.

Two reports of post-translational activation of GLCL have been published by Ochi (1995; 1996). In both cases Chinese hamster V79 cells were treated with agents that generate ROS, and the activity of GLCL was increased in cytosols very quickly (within minutes following H_2O_2 treatment; 1h. following treatment with menadione or tert-butylhydroperoxide). In all treatments, the activity increased by 50% to 200% over controls. Kinetic analysis of GLCL from menadione treated cells showed no change in the $Km_{(glu)}$. From experiments employing different antioxidants, Ochi concluded that GLCL oxidation was important for the change in activity, and that H_2O_2, not $^{\cdot}OH$, was activating the enzyme. Enzyme oxidation, by means of an intermolecular disulfide bridge between GLCLC and GLCLR, was also tentatively proposed as a mechanism for regulating GLCL activity by others (Huang et al. 1993b). This model has since been extensively cited in the literature, despite a complete lack of scientific studies designed to test it. In fact, no further work on post-translational activation of GLCL by intracellular oxidation has been reported since Ochi's groundbreaking work. The studies reported herein address the question of GLCL activation in a human lymphoblastoid cell line and

present a mechanism for the post-translational control of GLCL which differs from the model proposed by Huang et al. (1993b).

II. MODULATION OF LYMPHOCYTE FUNCTION BY GSH

Importance of intracellular redox status in lymphocyte function

The immune system is a very sensitive target of xenobiotics and oxidative stress. Peripheral blood lymphocytes (PBL) are normally quiescent cells, existing in a resting state until directed by external stimuli to differentiate into metabolically active effector cells (Crabtree and Clipstone 1994). Because resting PBL must rapidly develop appropriate responses following receptor stimulation, the maintenance of signal transduction pathways is essential for PBL function. Numerous reports have demonstrated alterations in lymphocyte signaling following changes in intracellular redox balance by environmental or pharmacological agents. Oxidative stress was shown to alter lymphocyte signal transduction pathways by activating phosphotyrosine kinases (PTKs) and inactivating phosphotyrosine phosphatases (PTPs) (Holsapple et al. 1996). The PTPs and dual-specificity phosphatases are especially sensitive to oxidative stress due to an active site cysteine which must remain in reduced form for catalysis to occur (Denu et al. 1996). As dephosphorylation is a mechanism for control of kinase activity, inhibition of PTPs usually results in the initiation of phosphotyrosine signaling cascades (Garcia-Morales et al. 1990; Hecht and Zick 1992; Krejsa et al. 1997; Krejsa and Schieven 1998). Thus, oxidation induced signal transduction results from the synergistic effects of activating kinases and inactivating phosphatases, leading to the generation of second messengers such as intracellular Ca^{2+}, inositol 1,4,5-triphosphate, and diacylglycerol (Holsapple et al. 1996). The activation of $p21^{ras}$ and the MAP kinase pathway, both important for lymphocyte proliferation and differentiation, are also triggered by intracellular oxidative stress (Stevenson et al. 1994; Lander et al. 1995).

In lymphocytes, oxidizing treatments, including H_2O_2, ionizing radiation, and UV irradiation, stimulated the activation of syk and src family tyrosine kinases, Ca^{2+} mobilization, phospholipase-Cγ1 and the activation of NFκB (Schieven and Ledbetter 1994). However, depletion of intracellular GSH by N-ethylmaleimide or 1-chloro-2,4-dinitrobenzene down-regulated the responses of lymphocytes to receptor based signals (Kanner et al. 1992; Kavanagh et al. 1993). In addition, while depletion of GSH from lymphocytes was shown to abrogate the mobilization of Ca^{2+} following CD3 stimulation, it

enhanced their responses to TNFα, an inflammatory cytokine (Staal et al. 1994). Taken together, these studies suggest that perturbing the redox balance can lead to sub-optimal antigen-specific responses while initiating or enhancing inflammatory responses.

The integration of redox-sensitive and redox-independent signals appears to be crucial for productive lymphocyte activation. For example, generation of reactive oxygen via lipoxygenase was reported to serve as a second messenger in the costimulation of T lymphocytes by CD28 (Los et al. 1995). Crosslinking CD28 with CD2, which induces long term proliferation of T cells, causes an initial depletion of cellular thiols that may be necessary for NFκB activation (Costello et al. 1993). It also appears that changes in the intracellular concentration of GSSG may play a direct role in the activation of NFκB. However, high concentrations of GSSG are inhibitory to NFκB function, as the oxidized transcription factor cannot bind DNA (Droge et al. 1994). While the ability of lymphocytes to respond to such signals may be disrupted in cases of redox imbalance, the role of intracellular GSH in signal transduction remains undefined.

GSH in lymphocyte responses

Various studies of lymphocyte function have implicated GSH as a key determinant in lymphocyte responses. The proliferative capacity of $CD4^+$ lymphocytes was positively correlated with GSH content at the time of stimulation (Kavanagh et al. 1990). However, the effects of GSH enhancement or depletion can differ markedly in early and later stages of lymphocyte activation. For example, treatment of PBLs with agents to deplete GSH markedly decreased cell proliferation, but the upregulation of IL-2 and IL-2Rα (CD25), both early response genes, was not affected (Gmunder et al. 1990; Iwata et al. 1994; Walsh et al. 1995). Furthermore, CD3-activated killer cells were found to show diminished cell killing if treated with GSH during the initial stages of their development, but enhanced function if treated with GSH later in their development (Ting et al. 1992). GSH concentration or redox balance may also shift the direction of initial signaling toward differentiation to specific phenotypes. A recent study showed that thiol antioxidants enhanced T cell proliferation but reduced the production of cells with a Th_2 phenotype (Jeannin et al. 1995). It has also been reported that GSH depletion in the antigen presenting cell inhibits Th_1 and favors Th_2 responses in stimulated T cells (Peterson et al. 1998). The incomplete stimulation of lymphocytes (receptor engagement without the proper costimulatory signals) can render them anergic or induce apoptosis; GSH depletion

and oxidative stress also caused a state of non-responsiveness to receptor stimulations (Kanner et al. 1992; Kavanagh et al. 1993; Flescher et al. 1994).

Oxidative stress in lymphocytes

As lymphocytes have lower GSH and less GSH synthetic capacity than most other tissues, their responses to oxidative stress may differ substantially from those described in other tissues. Lymphocyte trafficking can also place them in situations where they must withstand considerable oxidative stress. Sites of inflammation can often be highly oxidizing environments due to the action of NADPH oxidase, nitric oxide synthase, myeloperoxidase and polyamine oxidase (el-Hag et al. 1986; el-Hag and Clark 1987; Ward et al. 1988; Bastian and Hibbs 1994). Certain chronic diseases also lead to oxidizing environments for lymphocytes; for example, the T cells isolated from synovial fluid of patients with rheumatoid arthritis have significantly reduced GSH and impaired proliferative responses compared with donor matched T cells from peripheral blood (Maurice et al. 1997). Infection with HIV also leads to a well-documented decrease in GSH content in both peripheral blood and in PBMC (Buhl et al. 1989; Eck et al. 1989; Roederer et al. 1992; Staal et al. 1992a; Staal et al. 1992b). The functional impairments in $CD4^+$ lymphocytes from HIV infected individuals could be due to an increased production of TNFα, an inflammatory cytokine which stimulates oxidative stress in many cell types. Increased likelihood of stimulating apoptotic pathways, prolonged activation of NFκB, and alterations in tyrosine phosphatase activity due to insufficient intracellular reducing potential have also been proposed as mechanisms for $CD4^+$ lymphocyte impairment (Sandstrom et al. 1993; Sandstrom et al. 1994; Cayota et al. 1996; Ginn-Pease and Whisler 1998). Interestingly, the HIV-1 Tat transactivator was found to suppress GLCLR expression in the livers and erythrocytes of Tat over-expressing mice (Choi et al. 2000b). This suggests that inefficient GSH biosynthesis may be responsible for at least a part of the pathology observed in HIV.

Regulation of GLCL in lymphocytes

Very little is known about the control of GLCL in lymphocytes. Yao, et al. (1993) found that GLCLC mRNA expression varied over 3-fold in peripheral blood mononuclear cells (PBMC) from 20 individuals tested, and that treatment of cancer patients with BSO by intravenous injection resulted in a twofold increase in GLCLC mRNA levels in PBMC. An investigation of the antioxidant status of PBMC from individuals with chronic hepatitis

C, showed that GSH synthetic capacity was significantly higher in patients than controls (Boya et al. 1999). Lymphoblastoid cell lines from patients with Alzheimer's disease had decreased GSH levels compared to age-matched controls, however no differences in GLCL activity were noted (Cecchi et al. 1999). Walsh et al. (1995) investigated GLCLC mRNA expression in cultured human PBMC treated with BSO to inhibit GLCL activity. This study showed that GLCLC transcript levels were undetectable in primary cells but increased over the culture period (48 h.). BSO treatment substantially increased GLCLC mRNA expression in both unstimulated and concanavalin A (Con A) stimulated lymphocytes. Apart from the manipulation of GLCL activity by BSO treatment, no other oxidizing treatments have been employed to investigate the induction of GLCL subunits or activity in lymphocytes. Furthermore, the transcription factors Nrf-1 and Nrf-2, which have been reported to control antioxidant response element (ARE)-dependent GLCLC and GLCLR expression following intracellular oxidative stress, have not been described in lymphocytes (Moinova and Mulcahy 1999; Wild et al. 1999; Jeyapaul and Jaiswal 2000).

One reason for the paucity of studies of GLCL regulation in lymphocytes may be the difficulty of detecting GLCL in lymphocytes, where it is expressed at much lower levels than most other tissues. For our investigation of GLCL in human T cells, we developed high affinity antisera capable of detecting the relatively minute amounts of GLCLC and GLCLR present in lymphocytes (see Appendix A). We have directed our efforts at understanding the dynamics of GLCL regulation during lymphocyte proliferation, and in response to intracellular oxidative stress and death signals.

CHAPTER I: INDUCTION OF GLCL IN HUMAN CD4$^+$ T CELLS

ABSTRACT

The tripeptide glutathione (GSH) is a major thiol antioxidant, present at millimolar concentrations in most cell types. Glutamate cysteine ligase (GLCL), the rate-limiting enzyme in GSH production, is known to be transcriptionally controlled during cell proliferation. This study addresses the regulation of GLCL protein levels during the proliferation of primary human CD4$^+$ T lymphocytes. Stimulation by phytohemagglutinin (PHA) strongly induced the upregulation of both the catalytic (GLCLC) and regulatory (GLCLR) subunits of GLCL. The GLCLR subunit was induced to a greater extent than GLCLC (15-fold and 5-fold induction, respectively). Pre-treatment with the antioxidant α-lipoic acid increased the induction of GLCL by PHA in a dose-dependent manner. Blockade of CD28/B7 interactions during PHA stimulation did not affect the induction of GLCL in enriched CD4$^+$ T cell cultures; however, in peripheral blood mononuclear cell (PBMC) cultures stimulated with PHA, the CD28/B7 blockade inhibited the upregulation of GLCL protein levels. To investigate the receptor-based pathways leading to GLCL induction, we tested a panel of stimulations for upregulation of GLCL protein levels. We found that naive T cells differed from previously activated T cells in the induction of GLCL by receptor stimulation. Whereas CD3 ligation was necessary for the upregulation of GLCLC in naive cells, in previously activated T cells the CD28 signal was more important. These data suggest that several pathways which lead to proliferation can induce the upregulation of GLCL protein levels in lymphocytes. This redundancy in the control of GLCL underlines the importance of intracellular redox status during lymphocyte proliferation.

INTRODUCTION

Alterations in the redox poise of the intracellular environment have been reported to be important during cell proliferation. Early signaling events may induce the production of reactive oxygen species (ROS) which have been reported to act as second messengers in the propagation of certain signals and the activation of redox sensitive transcription factors. Studies in many cell types have shown that redox changes during cell stimulation are important for robust proliferation (Lander et al. 1995; Sundaresan et al. 1995). In primary T lymphocytes, Los et al. (1995) showed that part of the signaling cascade stimulated by CD28 receptor engagement induced the activation of 5-lipoxygenase, which

generated small amounts of H_2O_2. Antioxidant treatment blocked the activation of the transcription factor NFκB following CD28 stimulation, suggesting that the redox change was necessary for propagation of a complete signal.

However, once the initial message is sent, and a cell prepares for entry into the cell cycle, a reducing intracellular environment appears to be required. Lymphocytes in particular have been shown to be sensitive to intracellular redox status during cell proliferation. The major thiol antioxidant, glutathione (GSH) is present in the high micromolar to low millimolar range in lymphocytes. Kavanagh et al. (1990) showed that proliferative capacity of human PBMC and $CD4^+$ T cells was positively correlated with their intracellular GSH content prior to stimulation. In addition, depletion of GSH from human PBMC resulted in diminished early signaling responses to CD3 cross-linking (Kanner et al. 1992; Kavanagh et al. 1993). Supplementation of concanavalin A (Con A) stimulated mouse lymphocytes with GSH or cysteine was shown to increase DNA synthesis, but to inhibit the production of IL-2 mRNA. In contrast, treatment with buthionine sulfoximine (BSO), which inhibits GSH synthesis, resulted in a pronounced decrease in DNA synthesis without affecting IL-2 mRNA levels (Gmunder et al. 1990). Human T cells stimulated by crosslinking of CD2 and CD3 exhibited a dose-dependent decrease in proliferation when pre-treated with BSO; this effect was not due failure to respond to IL-2 (Suthanthiran et al. 1990). Human PBMC pre-treated with BSO also showed decreased proliferation in response to Con A, accompanied by decreased total RNA synthesis but no change in IL-2 or IL-2Rα (CD25) mRNA expression. Analysis of the timecourse of DNA synthesis and histone H3 and H4 mRNA expression in these cells suggests that they were able to enter the cell cycle up to 48 h. after stimulation, but not at later timepoints (Walsh et al. 1995).

GSH is produced in two reactions; the first, catalyzed by glutamate cysteine ligase (GLCL; also known as γ-glutamylcysteine synthetase), forms γ-glutamylcysteine; the second, catalyzed by glutathione synthetase, adds glycine to form tripeptide. GLCL activity is reported to limit the rate of GSH synthesis; however in certain cell types the availability of intracellular cysteine may in fact be rate-limiting (Meister and Anderson 1983). The enzyme is a heterodimer containing a 73 kDa catalytic subunit (GLCLC), which possesses all catalytic activity; and a 28 kDa regulatory subunit (GLCLR), which alters the kinetics of GLCLC by increasing its affinity for glutamate and reducing feedback inhibition by

GSH (Huang et al. 1993b). The activity of GLCL is regulated by transcriptional, post-transcriptional, and post-translational mechanisms (for review, see Griffith and Mulcahy 1999).

The transcriptional regulation of GLCLC and GLCLR (separately coded messages) has been intensively studied. Promoter analysis has revealed that antioxidant response elements (ARE), as well as the transcription factors Nrf2 and c-Jun, are involved in the upregulation of both subunits following oxidative stress (Moinova and Mulcahy 1999; Wild et al. 1999; Jeyapaul and Jaiswal 2000). In addition, GLCL levels have been reported to vary with cell proliferation in several model systems. Rat hepatocytes plated at low cell densities had 1-2 fold increases in GLCLC mRNA levels 24 h. later (Cai et al. 1995). Huang, et al. (1998) found that GLCLC mRNA and protein levels, and GLCL activity were increased in rats 12 h. following partial hepatectomy, but not in sham-operated animals. This increase was transient, as GLCL levels had returned to baseline by 24 h. Ray, et al. (1999) showed that the lung epithelial line A549 showed increased GLCL activity 20 h. after plating; however, it is unclear whether this effect is due to increased cell size at this stage in proliferation. Poot et al. (1995) found that de novo synthesis of GSH was a requirement for serum-stimulated NIH-3T3 cells to enter the cell cycle and proliferate. These data indicate that intracellular GSH content during cell proliferation is controlled at least in part by increased de novo biosynthesis. However, despite the documented importance of GSH in lymphocyte proliferation, the expression of GLCL in T cells following stimulation has not been characterized.

We undertook to investigate the dynamics of GLCL regulation in primary human $CD4^+$ T lymphocytes during proliferation, with particular emphasis on the role of the CD28 costimulatory pathway. CD28 costimulation is required for productive T cell proliferation following antigen receptor engagement or crosslinking of the T cell receptor (Linsley and Ledbetter 1993). The present study characterizes changes in GLCLC and GLCLR protein levels in $CD4^+$ T cells following various stimulation protocols. We found that $CD4^+$ T cells upregulate both subunits of GLCL after mitogenic stimulation and receptor engagement. However the CD28 pathway is less important than the CD3 pathway in the induction of GLCL protein in naive cells stimulated by receptor crosslinking, and is not required for the induction of GLCL by mitogenic stimulation. These results provide the first report of GLCL upregulation during lymphocyte proliferative responses.

MATERIALS AND METHODS

Lymphocyte Cultures

Peripheral blood was obtained from normal healthy volunteers according to protocols established by the Institutional Review Boards of the University of Washington and Bristol-Myers Squibb Pharmaceutical Research Institute. The mononuclear cells were isolated by gradient centrifugation over lymphocyte separation medium (Organon Teknika, Durham, NC) and depleted of adherent cells by two 30 min. incubations at 37 °C in RPMI 1640 media (Gibco-BRL, Rockville MD) containing 2% FBS in polystyrene tissue culture flasks. The remaining cells were subjected to negative selection of natural killer cells, monocytes, B cells and $CD8^+$ T cells with the mAbs Fc1.2 (anti-CD16), F13 (anti-CD14), 1F5 (anti-CD20), and G10-1 (anti-CD8) at 10 μg/ml and 25% newborn rabbit complement (Pel-Freez, Brown Deer, WI). The cells were washed and cultured in RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, sodium pyruvate and penicillin/streptomycin (Gibco-BRL). Enriched T cells were rested overnight prior to stimulations with phytohemagglutinin (PHA) or mAb. For mitogenic stimulations, 1 μg/ml PHA-L (Boehringer-Manheim, Indianapolis, IN) was added to the culture medium. In experiments employing a CD28/B7 blockade, cells were pre-treated with 10 μg/ml CTLA4Ig (a gift from Robert Peach, Bristol Myers Squibb PRI) for 30 min. prior to addition of PHA. The mouse mAbs G19-4 (anti-CD3) and 9.3 (anti-CD28) have been previously described (Hansen et al.,1984; Ledbetter et al. 1986). Solid phase mAb stimulations were performed by coating a 6 or 12 well plate overnight at 4 °C with PBS containing 10 μg/ml of the stimulating antibody, then washing extensively with PBS prior to adding cells. For solution phase mAb stimulations, 1 μg/ml mAb was added to wells containing $CD4^+$ T cells. Phorbol-12-myristate-13-acetate (PMA; Calbiochem, San Diego, CA) was used at 10 ng/ml. All cultures were performed at 37 °C in humidified air containing 5% CO_2.

Western blotting

Cells were harvested by centrifugation, and lysed with ice cold NP-40 lysis buffer (1% Nonidet P-40, 150mM NaCl, 50 mM Tris, pH 8.0) containing 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 0.5 mM PMSF, 0.5 % aprotinin (all from Sigma, St. Louis, MO), and 5 μg/ml leupeptin (Boehringer-Manheim, Indianapolis, IN). For cells stimulated with solid phase mAb, the adherent cells were lysed directly in

the incubation wells, and added to pellets from the suspended cells in those wells. Proteins were quantified using the bis-cinchroninic acid (BCA) method (Pierce, Rockford, IL). Equal amounts of protein were boiled in 4x sample buffer (240 mM Tris, pH 6.8, 40% glycerol, 20% β-ME, 0.002% bromophenol blue) and separated by SDS-PAGE, transferred to PVDF (Millipore, Bedford, MA) and stained for GLCLC and GLCLR as previously described (Thompson et al. 1999). Antibody binding was detected with goat-anti-rabbit-horseradish peroxidase (Boerhinger Manheim, Indianapolis, IN) and developed by enhanced chemoluminescence (Amersham, Arlington Heights, IL). Protein levels were quantified using a Gel Doc system equipped with Molecular Analyst software (Bio-Rad, Hercules, CA).

Statistical Analysis

Data management and descriptive statistics were performed using MS Excel software (Microsoft Corp., Redmond, WA).

RESULTS

GLCL protein is induced by mitogenic stimulation in $CD4^+$ T cells.

Human peripheral $CD4^+$ T cells were stimulated with PHA (1μg/ml) for up to 5 days as described in Materials and Methods, and samples were taken each day for GLCLC and GLCLR analysis by Western blotting. An induction timecourse for two donors is shown in fig. 3. There was clear induction of GLCL proteins over the proliferative period, but some differences between donors in the kinetics of GLCL upregulation were noted. Densitometry was performed on several similar assays; fig. 4 shows the average dynamics of GLCL protein expression in $CD4^+$ T cells stimulated with PHA. Interestingly, the GLCLC protein was induced to a lesser extent than GLCLR (5-fold induction of GLCLC vs. 15-fold induction of GLCLR). This suggests that in lymphocytes the proliferative response may require a highly efficient (holoenzyme) form of GLCL. The protein levels of both subunits peaked at 72 h., which correlates well with the peak of proliferation induced by PHA (Younkin 1972). GLCL levels were low again by day 5 after PHA stimulation, a time at which the cells had completed their proliferative response and were in a resting state. Whereas GLCLC levels returned to baseline, the level of GLCLR was approximately 4-fold higher than in unstimulated cells (fig. 4).

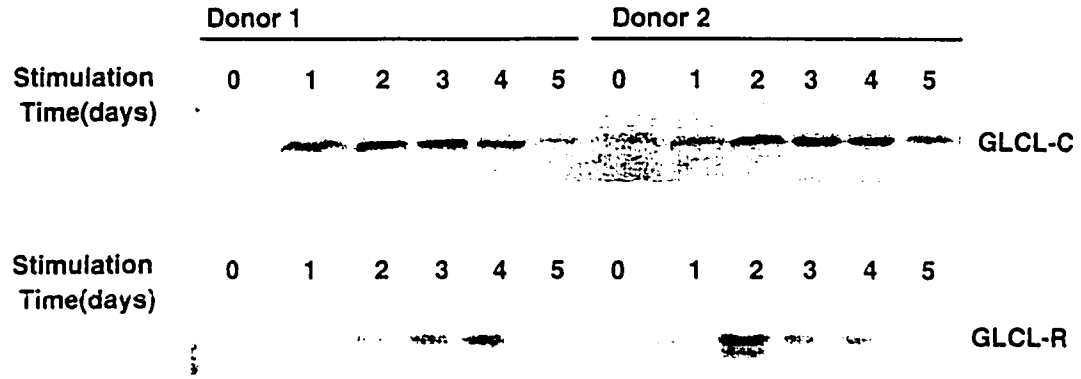

Figure 3. Induction of GLCL protein in PHA-stimulated CD4⁺ lymphocytes. Lymphocytes from two donors were stimulated with PHA and samples taken during the course of the proliferative response from day 0 to day 5. Immunoblots show levels of GLCLC (top panel) and GLCLR (bottom panel).

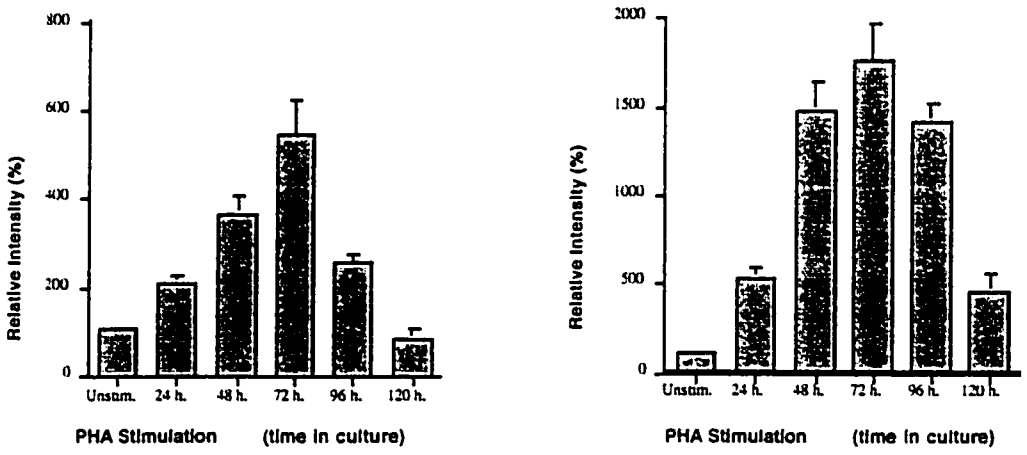

Figure 4. Average induction of GLCL protein in CD4⁺ T cells. Data from several experiments in which CD4⁺ T cells were stimulated with PHA to induce proliferation was compiled. Average relative intensity (from densitometry) of GLCLC (left) and GLCLR (right) is expressed as percent of unstimulated controls. Error bars indicate standard error of mean, with n=3 to 9 donors for each timepoint.

Effect of α-Lipoic acid on PHA stimulated induction of GLCL.

The strong induction of GLCL in CD4⁺ cells stimulated with PHA suggests that lymphocytes require an increase in GSH biosynthesis to mount a robust proliferative response. A similar requirement for GSH has been reported in other model systems, including lymphocytes treated with BSO (see above). To test whether the induction of GLCL could be inhibited by an exogenous antioxidant, we stimulated PBMC with PHA in the presence of α-lipoic acid. We reasoned that if an early oxidative signal was inducing GLCL upregulation (possibly through the activation of NFκB), the presence of α-lipoic

acid should inhibit this response (Packer et al. 1995). Interestingly, we found that GLCL protein induction by PHA was enhanced by the addition of α-lipoic acid to cultures at concentrations of 100 and 250 μM. Both GLCLC and GLCLR induction were similarly affected by α-lipoic acid, with an earlier enhancement of GLCL upregulation at the 100 μM dose than at the 250 μM dose (fig. 5). This surprising result suggests that the induction of GLCL following PHA stimulation in lymphocytes is probably not due to the generation of ROS during mitogenic signaling.

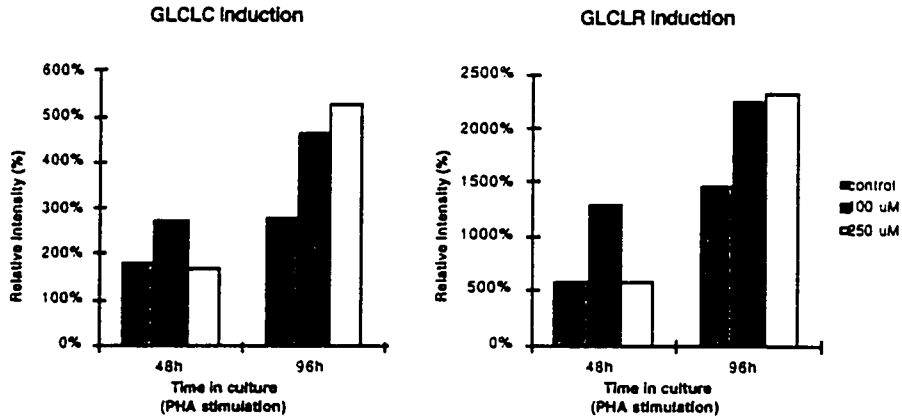

Figure 5. α-Lipoic acid enhanced GLCL induction by PHA in lymphocytes. PMNC were treated with 0, 100 or 250 μM α-lipoic acid during stimulation by 1 μg/ml PHA. Untreated cell extracts and extracts from stimulated cells were subjected to SDS-PAGE followed by Western blotting for GLCLC and GLCLR. Densitometry was performed on the autoradiographs and the relative intensities (% of untreated controls) were plotted. Data shown are averages from two donors.

Effect of CD28/B7 blockade on PHA-stimulated GLCL induction

The importance of the CD28 signal in T cell responses to antigen has been well documented. Costimulation with CD28 enhances IL-2 mRNA transcription and message stabilization, possibly through signals transmitted by the Rel family of transcription factors. T cells stimulated with CD2 plus CD28 showed long term proliferative capacity, enhanced CD25 expression, and persistent activation of NFκB. Cells stimulated in this way also had increased intracellular GSH levels (Costello et al. 1993). CD28 is also essential for the viability of T cells stimulated with CD3, as Thy 1.2^+ lymphocytes from CD28 deficient mice began to die by apoptosis within two days after stimulation (Noel et al. 1996). This may be due to upregulation of Bcl-xl, an anti-apoptotic protein, by CD28 costimulation (Boise et al. 1995). We employed CTLA4Ig, which binds to B7, preventing stimulation of the CD28 receptor, to investigate the role of CD28/B7 signaling in PHA

induced GLCL upregulation (Linsley and Ledbetter 1993). $CD4^+$ T cell preparations were treated with 10 µg/ml CTLA4Ig prior to and during stimulation with PHA, or stimulated with PHA as usual. Extracts from unstimulated cells and cells treated thus were separated by SDS-PAGE and Western blotted for GLCLC and GLCLR. The CD28/B7 blockade did not prevent GLCL induction by PHA stimulation in any of the donors tested (fig. 6, 7). However, the CTLA4Ig treatment did reduce the proliferation of these cells, compared to donor-matched cultures stimulated with PHA in the absence of CTLA4Ig (C. Krejsa, unpublished observations). This suggests that the depletion of a majority of the monocytes from the $CD4^+$ T cell preparations led to altered signaling in response to PHA. To test this hypothesis, we compared the effects of CTLA4Ig on PHA-induced GLCLC and Bcl-xl upregulation in PBMC preparations and highly purified $CD4^+$ T cells. Interestingly, the CD28/B7 blockade inhibited the induction of both proteins in the PBMC, but did not prevent induction by PHA in $CD4^+$ T cells from the same donors (fig. 7). This result suggests that mitogenic stimulation by PHA, a plant lectin, utilizes different T cell stimulatory pathways depending on the presence of antigen presenting cells in culture. Furthermore, the CD28 pathway is not required for induction of GLCL or Bcl-xl in PHA stimulated $CD4^+$ T cells.

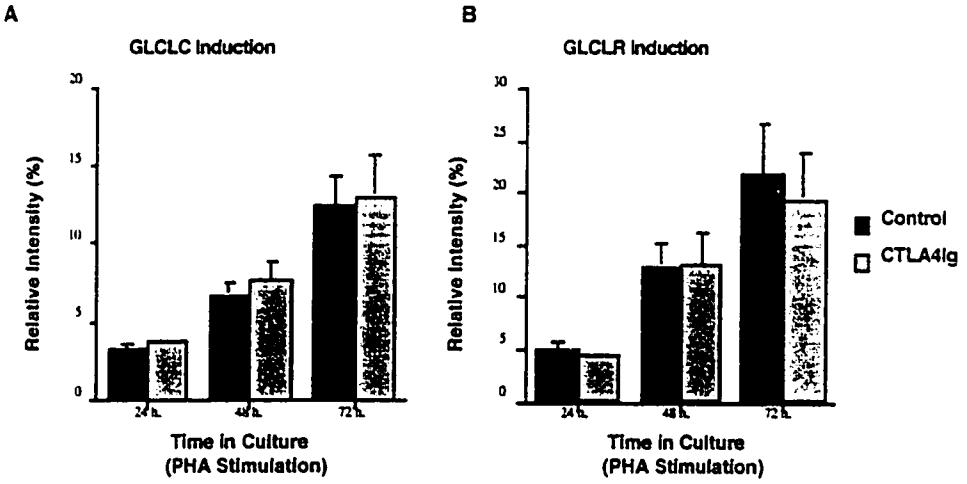

Figure 6. CD28/B7 blockade does not affect GLCLC induction by PHA. $CD4^+$ T cells were stimulated with PHA; donor-matched samples were pre-treated with CTLA4Ig, then stimulated with PHA in the presence of CTLA4Ig. Extracts were separated by SDS-PAGE and Western blotted for GLCLC (A) and GLCLR (B) protein levels. Densitometry was performed on the autoradiographs and relative intensity (% of unstimulated controls) was plotted. Error bars indicate standard error of the mean; n=4 donors.

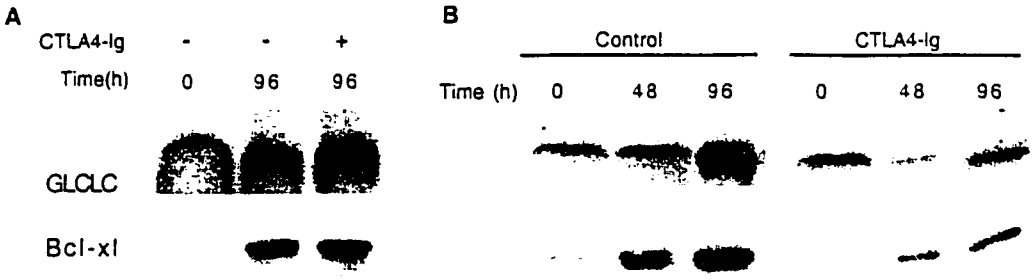

Figure 7. Effect of CD28/B7 blockade on PHA stimulation of CD4⁺ T cells (A) and PBMC (B) from the same donor. Induction of GLCLC (top panels) and Bcl-xl (bottom panels) following PHA stimulation in control cells and cells treated with CTLA4Ig prior to and during stimulation.

Induction of GLCL protein in CD4⁺ T cells by receptor stimulation.

The preceding experiments showed that GLCL protein is upregulated during mitogenic stimulation of T cells, however the use of PHA to induce proliferation in CD4⁺ T cells may not be an appropriate model for investigating the pathways responsible for GLCL induction under physiological conditions. To further investigate the regulation of GLCL protein in CD4⁺ T cells, we used a panel of stimulating mAbs to induce cell proliferation. Solid phase CD3 stimulatory mAb (G19.4) was used in coordination with either solid phase or soluble anti-CD28 (9.3), and phorbol myristate acetate (PMA). We found that different individuals varied in their responsiveness to the stimulations, however protein levels of both GLCLC (fig. 8) and GLCLR (fig. 9) were consistently induced by CD3 stimulation. The CD28 pathway appeared to be less important for GLCLC induction, as very little additional induction was seen by CD28 stimulation in the context of CD3 (fig. 8). However, the combination of CD28 and PMA, which provides a "complete" signal to T cells, also induced GLCLC.

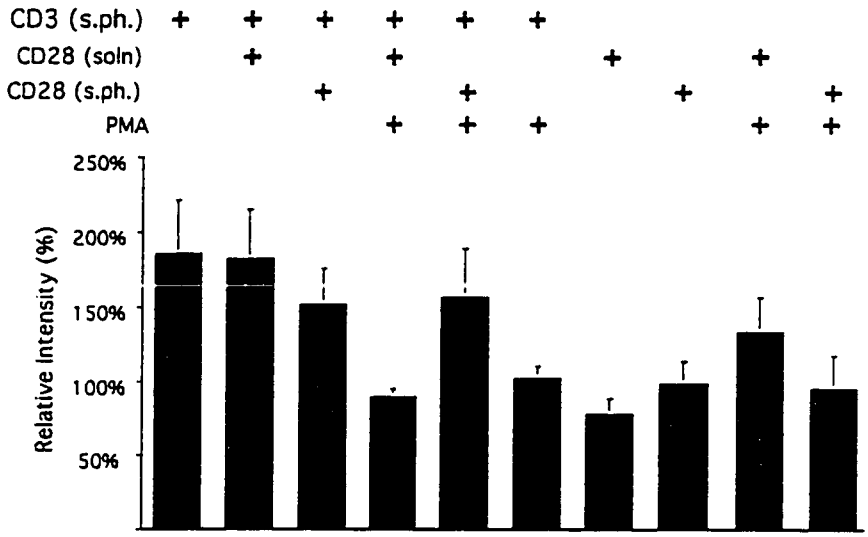

Figure 8. Induction of GLCLC following receptor stimulation. $CD4^+$ T cells were treated with solid phase anti-CD3 mAb, PMA, and anti-CD28 mAb in solution or solid phase as indicated. Cultures were harvested following stimulation at T=48 h. Average and standard deviation from four donors are shown.

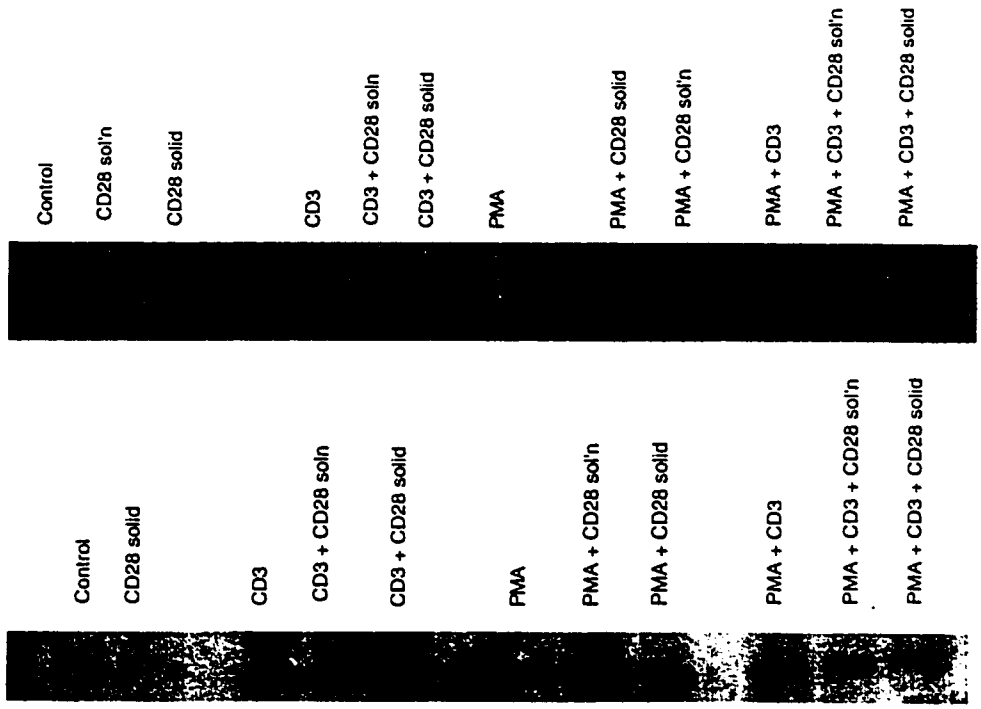

Figure 9. Induction of GLCLR following receptor stimulation. $CD4^+$ T cells were treated with solid phase anti-CD3 mAb, PMA, and anti-CD28 mAb in solution or solid phase as indicated. Cultures were harvested following stimulation at T=48 h. GLCLR Western blots from two donors are shown.

Induction of GLCL in restimulated T cells.

Following PHA stimulation, T cells enter the cell cycle and undergo a proliferative response which lasts approximately 3-4 days, after which they return to a resting state. The upregulation of Fas ligand (FasL) and APO2L then ensues, and the cells begin to undergo activation induced cell death via apoptosis (Martinez-Lorenzo et al. 1998; Martinez-Lorenzo et al. 1999; Martinez-Lorenzo et al. 2000). Human T cells previously stimulated by PHA may be restimulated by receptor crosslinking at day 5 (Younkin 1972). This provides a means of expanding the T lymphocyte population prior to performing stimulation studies. However, previously activated T cells have altered requirements for entry into the cell cycle. For example, they are highly responsive to CD28 stimulation, unlike naive cells that do not proliferate when stimulated through CD28 alone (Ledbetter and Linsley 1992). We used resting 5-day PHA blasts to test a panel of receptor stimulations for induction of GLCL protein. The level of GLCLC had returned to baseline levels at this timepoint, however there was still more GLCLR present in these cells than in naive (unstimulated) T cells (see fig. 4). Fig. 10 shows the responses of GLCLC and GLCLR to restimulation by solid phase anti-CD3 mAb alone and in combination with anti-CD28 in solution or solid phase. PMA and IL-2 were also used in the restimulation assays as indicated (Fig. 10). In contrast to naive $CD4^+$ T cells, the restimulated T cells showed a dependency on the CD28 signal for strong upregulation of GLCLC. The induction of GLCLR was also strongly enhanced by CD28 stimulation. Treatment with IL-2 and PMA induced GLCL protein upregulation in the absence of other stimuli. While donor variability allows only general conclusions to be drawn from this data, it appears that the upregulation of GLCL during proliferation of restimulated $CD4^+$ T cells may be driven by pathways other than those governing the induction of GLCL in naive cells.

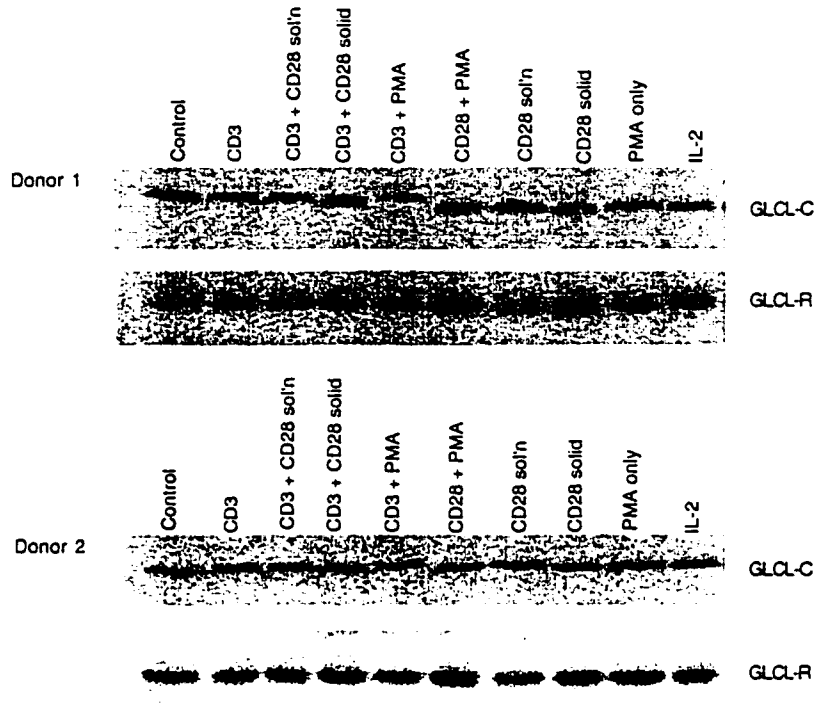

Figure 10. Induction of GLCL following restimulation of 5-day PHA blasts. $CD4^+$ T cells were treated with PHA for 5 days prior to stimulation with solid phase anti-CD3 mAb and anti-CD28 mAb in solution or solid phase, as indicated. Cells were harvested at 36 h. following stimulation and subjected to Western blotting for GLCLC and GLCLR. Western blots from two donors are shown.

DISCUSSION

We have characterized the induction of GLCL, the rate limiting enzyme in GSH synthesis, in human $CD4^+$ T cells stimulated with PHA or by receptor engagements which induce the cells to undergo proliferation. Both types of stimulation resulted in the upregulation of the GLCLC and GLCLR proteins, which exist at very low levels in resting T cells. The intracellular GSH level has been previously shown to be important for development of lymphocyte functional responses; in particular, DNA synthesis is impaired in lymphocytes with low GSH (Suthanthiran et al. 1990; Walsh et al. 1995). It appears that this requirement for GSH is met by de novo synthesis. Both subunits of GLCL were strongly induced, with GLCLR levels peaking at 15-fold, and GLCLC levels increasing to 5-fold induction over unstimulated cells. The apparent excess of GLCLR in this system may be a mechanism to predispose GLCL toward the highly efficient, holoenzyme form. We have found in studies on Jurkat cells that the heterodimerization of GLCL can be triggered by

intracellular oxidation; the role of surplus GLCLR may be to facilitate this process (see Chapter II).

It is interesting that treatment with α-lipoic acid did not inhibit the upregulation of GLCL in PHA stimulated lymphocytes. This suggests that the generation of ROS during early lymphocyte signaling is not responsible for the induction of GLCL during proliferation. Treatment with α-lipoic acid has been shown to increase the GSH content in Jurkat T cells fairly quickly (50% increase after 5 h. treatment; (Packer et al. 1995)). It could be that α-lipoic acid is acting in an unexpected way to indirectly upregulate GSH levels through increased biosynthesis. Pyrrolidine dithiocarbamate, another compound commonly utilized as an antioxidant in NFκB signaling studies, has recently been shown to induce the transcription of GLCLC and GLCLR (Wild and Mulcahy 1999). A more thorough study of the dynamics of GSH enhancement and GLCL upregulation in α-lipoic acid treated lymphocytes is needed to shed light on the mechanism of this effect.

The role of CD28 in upregulation of GLCL subunit protein levels was found to vary depending on the circumstances of T cell stimulation. The enriched $CD4^+$ T cells stimulated with PHA in the presence of CTLA4Ig (to block CD28/B7 interactions) did not show any reduction in GLCL or Bcl-xl levels compared to cells stimulated without CTLA4Ig. However PBMC from the same donors gave the opposite result, with both GLCL and Bcl-xl induction being strongly inhibited by the CD28/B7 blockade. This suggests that the $CD4^+$ T cells utilize alternative signaling pathways to upregulate these proteins in the absence of sufficient accessory cell stimulation. The relevance of this finding is not known. In naive $CD4^+$ T cells stimulated with CD28, little induction of GLCLC or GLCLR occurred. This may be due to the fact that T cells stimulated thus, with an "incomplete" signal, will not proliferate. Solid-phase CD3 stimulation appeared to be most important for the induction of GLCL in this system. This differs from the results seen in restimulated $CD4^+$ PHA blasts. Treatment of 5-day resting blasts with anti-CD28, both as solid phase stimulation and in solution, resulted in stronger induction of GLCL than did the CD3 signal. In aggregate, these results suggest that receptor based signals which produce strong proliferative responses also induce the expression of GLCLC and GLCLR proteins in $CD4^+$ T cells, and in naive cells the mechanism of induction depends on receptor engagement rather than costimulatory pathways.

Mitogenic proliferation assays provide an easily quantified endpoint for testing the effects of certain treatments or conditions on lymphocyte functional development. However, other determinants of lymphocyte function have been quantified with regard to intracellular GSH content. For example, recent reports indicate that redox alterations can affect the development of Th_0 cells into the Th_1 or Th_2 phenotype (Jeannin et al. 1995, Peterson et al. 1998). This is expected to have an effect on the course of an immune response, and may have serious implications for inflammatory diseases. Under normal circumstances, the thiol content of lymphocytes is likely to be controlled by internal mechanisms. However, some disease states cause general or localized changes in GSH status that may affect the outcome of T cell stimulation. HIV infected individuals had a decreased supply of hepatic GSH, leading to lower blood GSH levels and a decrease in circulating cysteine (Roederer et al. 1992). This may be due to interference with the expression of GLCLR mRNA by the HIV-1 Tat transactivator (Choi et al. 2000a). The GSH level in PBMC from patients with Hepatitis C and Alzheimer's disease was also decreased; in the case of Hepatitis C this corresponded with upregulation of GLCL activity (Boya et al. 1999; Cecchi et al. 1999). In rheumatoid arthritis, synovial fluid lymphocytes had lower GSH levels than cells from the periphery (Maurice et al. 1997). Sites of inflammation such as atherosclerotic lesions create localized environments with oxidizing conditions, due to the ROS production by monocytes and neutrophils (Cohen 1994). All these conditions may alter the functional development of T lymphocytes if the internal antioxidant mechanisms are not robust enough to counteract environmental oxidative stress. The induction of GLCLC and GLCLR protein by $CD4^+$ T cells as a part of the proliferative response may be one mechanism by which these cells control their intracellular redox status, and hence, proper functional outcome, as they develop into effector cells in vivo.

NOTES TO CHAPTER I

Most of the work presented in Chapter I was performed at the Bristol-Myers Squibb Pharmaceutical Research Laboratory in Seattle, WA, where Drs. Jeff Ledbetter and Gary Schieven kindly hosted me in their labs from 1994-1997. The antisera against GLCLC and GLCLR were produced there with the assistance of Dr. Jim Blake and Ms. Lorelyn Mackie (see Appendix A for details). This work was supported by NIH grants ES04696, ES07032, and by Bristol-Myers Squibb.

CHAPTER II: RAPID ACTIVATION OF GLUTAMATE CYSTEINE LIGASE BY OLIGOMERIZATION FOLLOWING OXIDATIVE STRESS

ABSTRACT

Glutamate cysteine ligase (GLCL) catalyzes the rate-limiting step in the formation of the cellular antioxidant glutathione (GSH). The enzyme consists of two seperately coded proteins, a catalytic subunit (GLCLC) which posesses all catalytic activity and a regulatory subunit (GLCLR) which is reported to influence the activity of GLCLC by increasing its affinity for glutamate and decreasing feedback inhibition by glutathione. The control of GLCLC and GLCLR transcription following cellular redox changes has been widely studied. However, little is known about the post-translational control of GLCL activity, a question this study addresses. We found that GLCL was rapidly activated by treatments that induce cellular oxidative stress. Both reactive oxygen species (ROS) generators and GSH depleting agents caused an increase in GLCL enzymatic activity in cells, without increasing GLCL protein levels. This activation of GLCL resulted in a structural change, as evidenced by the loss of native GLCLC binding to a high-affinity anti-peptide antiserum. Native gel electrophoresis of extracts from cells exposed to GLCL activating treatments revealed that both GLCLC and GLCLR shift to lower mobility forms. Size exclusion chromatography also showed that GLCLC protein eluted at a higher molecular weight following treatments that activate GLCL. While we found no evidence for an intermolecular dissulfide bridge between GLCLC and GLCLR, our data suggest that the two subunits are subject to oxidant induced changes in their interaction, and that GLCL oligomerization may lead to rapid enzyme activation. This provides the first report of a mechanism for post-translational activation of GLCL following cellular oxidative stress.

INTRODUCTION

The tripeptide glutathione (GSH) is the major cellular thiol, existing at millimolar concentrations in most cell types. It plays a role in myriad cellular functions, including amino acid transport, maintenance of reduced protein thiols, secondary metabolism of xenobiotics and endogenous substrates, enzymatic detoxification of hydrogen peroxide (H_2O_2) and lipid peroxides, and non-enzymatic scavenging of free radicals (Meister and Anderson 1983). A large body of work has focused on the anti-oxidant qualities of GSH,

which protects against cellular destruction following exposure to both chemical toxicants and receptor-mediated death signals. Resistance to chemotherapeutics is often associated with altered GSH metabolism in cancer cell lines, whereas depletion of the cellular GSH pool can make cells more vulnerable to induction of cell death by chemotherapy, radiation, and receptor based mechanisms (Meister 1991; Forman et al. 1995; Hall 1999).

The rate limiting step in GSH biosynthesis is catalyzed by glutamate-L-cysteine ligase (GLCL, also known as γ-glutamyl cysteine synthetase) which forms an unusual gamma bond between glutamate and cysteine to produce γ-GC (see Griffith and Mulcahy (1999)for an excellent review). GSH formation is completed with the addition of glycine by glutathione synthetase (Oppenheimer et al. 1979). GLCL consists of two separately expressed proteins, a 73 kDa catalytic subunit (GLCLC) and a 28 kDa regulatory subunit (GLCLR) (Seelig et al. 1984). GLCLC possesses all the catalytic activity of the enzyme, and contains binding sites for glutamate, cysteine, and ATP. However the presence of GLCLR has been shown to increase the affinity of GLCLC for glutamate, and to decrease its feedback inhibition by GSH, which competes for the glutamate binding site (Huang et al. 1993). One model for GLCL activation by oxidative stress postulates the presence of a labile disulfide bridge between the two subunits of the holoenzyme, which, when formed, would increase the specificity of the glutamate binding site, resulting in an lower K_m for glutamate and a higher K_i for GSH (Huang et al. 1993b). While this model has been commonly cited, there has been no direct evidence published in support of this hypothesis.

Indeed, most studies of GLCL upregulation have focused on the transcriptional control of GLCLC and GLCLR. There is good evidence that GLCL activity is increased by de novo expression of GLCLC, GLCLR, or both subunits following cellular oxidative stress. These increases in GLCL activity are generally measured after 6-48 hours of treatment, and they represent the action of several transcription factors which are currently being identified (Griffith and Mulcahy 1999). In contrast, very few studies have investigated mechanisms for the post-translational control of GLCL activity. Sun, et al. (1996) reported that GLCLC was phosphorylated by protein kinase C, protein kinase A, and CAM kinase in vitro; phosphorylation decreased enzyme activity by about 20%. Ochi (1995, 1996) reported that treatment of cells with H_2O_2 or menadione, which generates superoxide anion radicals, resulted in an immediate increase in GLCL activity; but the

mechanism for this activation was not indentified. The generation of ·OH radicals and increased GSSG to GSH ratios were ruled out as proximal mediators of H_2O_2- or menadione- induced GLCL activation; still, it appeared that oxidation of the enzyme was important for its activation, as reducing agents could mitigate the effects of H_2O_2. We recently reported that GLCLC is cleaved during apoptosis by a caspase-3 dependent mechanism (Siitonen et al. 1999). However, given the striking changes in intracellular redox status which also occur with apoptotic cell death, it is unlikely that this modification of GLCLC is solely responsible for the increased GLCL activity seen during apoptosis.

In the current study, we report that GLCL is rapidly activated in cells by treatments which induce intracellular oxidation. Both phorone, a GSH depleting agent, and H_2O_2, a GSH oxidizer, were effective upregulators of GLCL activity while protein levels of GLCLC and GLCLR remained unchanged. GLCL activity following cell treatment with phorone or H_2O_2 increased up to 200%. Interestingly, the structural state of GLCL appeared to be altered during activation, as immunopreciptiation by a high-affinity antibody recovered less GLCLC from cells treated to activate GLCL than from untreated controls. Furthermore, both GLCLC and GLCLR displayed decreased electrophoretic mobility on native gels following treatment of cells with phorone or H_2O_2. Treatment with phorone to activate GLCL also caused a shift of GLCLC into a higher molecular weight complex, as determined by size exclusion chromatography analysis of whole cell extracts. However, we did not observe a disulfide crosslink between GLCLC and GLCLR in control or treated cells. These results, taken together, suggest that post-translational GLCL activation is mediated by the formation of a higher order enzyme complex following intracellular oxidation.

MATERIALS AND METHODS

Chemicals and Reagents

All common laboratory reagents were purchased from Sigma (St. Louis, MO). L-glutamate and L-serine were from ICN Biomedicals (Aurora, OH). Nonidet P-40 was from Calbiochem (San Diego, CA). L-buthionine-S-R-sulfoximine (BSO) was purchased from Schweizerhall (S. Plainfield, NJ). Phorone was from Aldrich (Milwalkie, WI). Acrylamide/bis-acrylamide protein gel mix and ammonium persulfate were from Bio-Rad (Hercules, CA). A peptidase inhibitor (PI) mix was made containing 100 mg/ml

Pefablock, 10 mg/ml N-α-Tosyl-L-Lysine chloromethyl ketone, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, and 1 mg/ml leupeptin (all from Boehringer-Manheim, Indianapolis, IN) in diH_2O, pH 1.0; this PI mix was used in cell lysis and tissue homogenization buffers at a 1:1000 dilution.

Cells and Cell Treatments

Jurkat cells (clone E6.1) were obtained from American Type Culture Collection and cultured in RPMI 1640 media supplemented with L-glutamine, sodium pyruvate, pennicillin/streptomycin (Gibco-BRL, Rockville, MD) and 10% fetal bovine serum (Hyclone, Logan UT). Some experiments used the Jurkat clone BMS-2, which expresses CD28, (a gift from Dr. Robert Mittler, Bristol-Myers Squibb). Cells were cultured at a density of 0.5 x 10^6 per ml. For phorone treatment, 500μM phorone (dissolved in DMSO) or an equivalent volume of DMSO were added to treatment and control flasks, respectively. Treatment flasks were capped with non-permeable caps to prevent the escape of volatile phorone from the flask. After 90 min. incubation at 37 °C, the cells were pelleted from treatment media and resuspended in fresh RPMI supplemented as above. Cells were incubated for another 60 min., then harvested as described. For H_2O_2 treatment, cells were pelleted and resuspended in fresh RPMI, then treated with 10 mM H_2O_2 in PBS (T=0). The cells were immediately collected by centrifugation and harvested at T=10 min., as described. In other experiments, cells were treated with diethyl maelate and diamide (15 μM) for 2h. at 37 °C; exposed to ionizing radiation 1000 to 4000 rads in RPMI without FBS (^{137}Cs irradiator; J.L. Shephard, Glendale, CA, model Mark I) and harvested immediately; or pelleted and resuspended at a density of 2x10^6/ml in fresh RPMI supplemented as above and treated for 12 h. with 100 ng/ml anti-Fas antibody (CH-11, Kamiya Biochemicals, Seattle, WA).

GLCL Activity Assay

GLCL activity was assayed by a modification of the HPLC-based method of Hamel et. al. (1996; see White et al. 1999 for detailed protocol). Briefly, cells were harvested by centrifugation and sonicated on ice in TES buffer (20 mM Tris, 1mM EDTA, 250 mM Sucrose, pH 7.4) containing 20 mM serine and 1 mM boric acid and peptidase inhibitors (TES/SB/PI). The extracts were cleared by centrifugation and protein levels in the supernatants were assayed by the Bradford method (Bio-Rad, Hercules, CA). In some experiments, NP-40/PI extracts were used instead, in which case protein quantitation was

by the bis-cinchroninic acid (BCA) method (Pierce, Rockford, IL). Samples were assayed in triplicate at 37 °C in Tris/EDTA buffer containing 21.3 mM L-glutamate, 10.67 mM ATP, 10.67 mM $MgCl_2$ and between 167 and 833 μM L-cysteine (cysteine levels were adjusted to facilitate chromatography on different instruments). Reactions were started by the addition of cysteine to the pre-warmed reaction tube and stopped by the addition of ice-cold 5-sulfosalicylic acid (SSA) solution. Baseline GSH levels were determined in duplicate by the addition of samples to reaction buffer containing 50 mM SSA to immediately precipitate proteins. Precipitated proteins from GSH baseline and GLCL activity replicates were pelleted, and the supernatants were normalized with KOH and then derivatized with monobromobimane (Molecular Probes, Eugene, OR). Samples were separated by HPLC, and the γ-GC and GSH peaks were quanitfied against standard curves of MBB-derivatized GSH. All activities were calculated as total γ-GC plus GSH (adjusted for baseline), normalized to sample protein concentration, and reported as nmoles γ-GC formed per mg cytosolic protein per minute.

Polyacrylamide Gel Electrophoresis and Western Blotting

Jurkat cells were lysed in NP-40 lysis buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, pH 8.0) and the extracts were cleared by centrifugation at 4°C at 14,000 rpm. Supernatants were mixed with an equal volume of 2x native gel sample buffer (120 mM Tris, 20 % glycerol, 0.001% bromophenol blue, pH 6.8), and loaded onto 4-15% precast gradient gels (Bio-Rad, Hercules CA) for native gel electrophoresis. For denaturing SDS-PAGE, extracts were boiled 3 min. in 2x Laemmli buffer (containing 10% β-ME for reducing PAGE) and loaded onto 12.5% SDS-polyacrylamide gels. Gels were run with Tris/Gly buffer on ice for 1 hr. at 150 V, then the proteins were transferred to PVDF membrane (Millipore, Bedford, MA) and blocked with 5% milk in PBS-Tween. Blots were stained with polyclonal rabbit antisera reactive against the GLCLC sequence E^{76}TLQEKGERTNPNHPT^{91} or the GLCLR sequences E^{89}KINPDEREEMKVSAK^{104} and E^{109}SNSSSSTRSAVD^{121} followed by goat-anti-rabbit-HRP secondary antibody (Boehringer Manheim, Indianapolis, IN). Western blots were developed by enhanced chemoluminescence (Amersham, Arlington Heights, IL), and protein levels quantified by imaging through a Gel-Doc transilluminator with data processing by Molecular Analyst Software (Bio-Rad, Hercules, CA).

Immunoprecipitation of GLCLC and GLCLR

For native immunoprecipitation, Jurkat cells were pelleted and lysed with NP-40/PI buffer as described. The lysates were immunoprecipitated with a 1:200 dilution of anti-GLCLC or anti GLCLR serum, on ice, for indicated times. Immune complexes were collected on Protein-A sepharose beads (Repligen, Cambridge, MA), washed extensively with NP-40/PI buffer, and released by boiling in 2x Laemmli loading buffer containing 10% β-ME. The recovered proteins were subjected to SDS-PAGE and Western blotting as described above, except that Protein-A-HRP was used to detect antibody binding (Amersham, Arlington Heights, IL). For denaturing immunoprecipitation, cells were lysed as described above, then 50 µl of NP-40 extract was aliquotted into tubes containing 10 µl of 10% SDS. Samples were boiled for 3 minutes, then 1.2 ml of modified RIPA (MR) buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% deoxycholate, pH 8.0) was added to each tube. The samples were mixed and incubated on ice for 15 min. prior to addition of antisera as described above. For comparison, 50 µl of NP-40 extract was added to tubes containing 1.2 ml MR buffer and subjected to immunoprecipitation without the denaturing step, or 50 µl of NP-40 extract was added directly to 2x Laemmli buffer and subjected to SDS-PAGE/Western blotting.

Size Exclusion Chromatography

For preliminary studies, rat kidney cytosol was prepared by homogenization in TES/SB/PI, with or without 1mM DTT. 500 µl of clarified extract (2 mg per run) was injected into a Waters HPLC equipped with a Waters 300W size exclusion chromatography column (Waters, Milford, MA). Fractions were collected at 15 second or 20 second intervals, mixed with 2x Laemmli sample buffer containing 10% β-ME, boiled for 3 min., and separated by SDS-PAGE. The proteins were transferred to PVDF and Western blotted as described above. A 250 mm ID by 45 cm column was packed with Sepharose S-200 HR size exclusion chromatography medium (Pharmacia, Uppsula, Sweden), according to manufacturers directions. The column was fitted with an injection port and was operated at a flow rate of 1.066 ml/min using a Minipuls2 peristaltic pump fitted with 2.54 mm ID tubing (Gilson Medical Electronics, Middleton WI). The column was characterized using Bio-Rad Gel Filtration Standards Chromatography standards under established flow conditions, detected by a Gilson 112 uv/vis detector. Clarified extract from control and treated cells (total protein between .5 and 1.5 mg) was injected (T=0) and fractions were collected at 1.5 or 2.0 minute intervals starting at T=60 min. (to

include the end of the flow through peak) on a Frac-100 automated fraction collector (Pharmacia, Uppsula, Sweden). Samples were collected until T=96 min. In one experiment, samples were collected until T=120 min. to confirm that no GLCLC eluted at later timepoints. Fractions were mixed with 4x Laemmli buffer containing 20% β-ME, boiled, separated by SDS-PAGE, transferred to PVDF, and Western blotted for GLCLC. Densitometry was performed as described above to quantify GLCLC per fraction, and the sum of all fractions was used to calculate percent of total and cumulative percent of GLCLC eluted per fraction.

Statistical Analysis

Data management and descriptive statistics, and Student's T-test were performed using MS Excel (Microsoft Corp., Redmond, WA). Wilcoxon's signed rank test was used for non-parametric statistical analysis; this analysis was run using Systat v5.2 (Systat, Inc., Evanston, IL). Statistical testing was performed on raw data in all cases, whereas data are sometimes represented as percent of control in figures.

RESULTS

GLCL was rapidly activated in cells.

Jurkat cells were treated for 1.5 hr. with phorone, a glutathione-S-transferase substrate that causes depletion of intracellular GSH, and allowed to recover in fresh media for 1 hr. prior to preparation of cell extracts. Alternatively, Jurkat cells were pelleted by centrifugation and treated with 10 mM H_2O_2 in fresh media containing 10% FBS (we have found this concentration to provide a dose equivalent to 1.1 mM H_2O_2 in PBS without serum). Cells were immediately pelleted by centrifugation and cell extracts were prepared at T=10 min. Both treatments induced rapid activation of GLCL (fig 11a), and a decrease in the baseline GSH content in phorone- and H_2O_2-treated cells (fig. 11b). These changes occurred without altering the protein levels of GLCLC or GLCLR (fig. 11c). We also found that treatment with diethyl maelate, diamide, ionizing radiation, and anti-Fas stimulation activated GLCL in Jurkat cells without increasing GLCLC or GLCLR protein levels (data not shown).

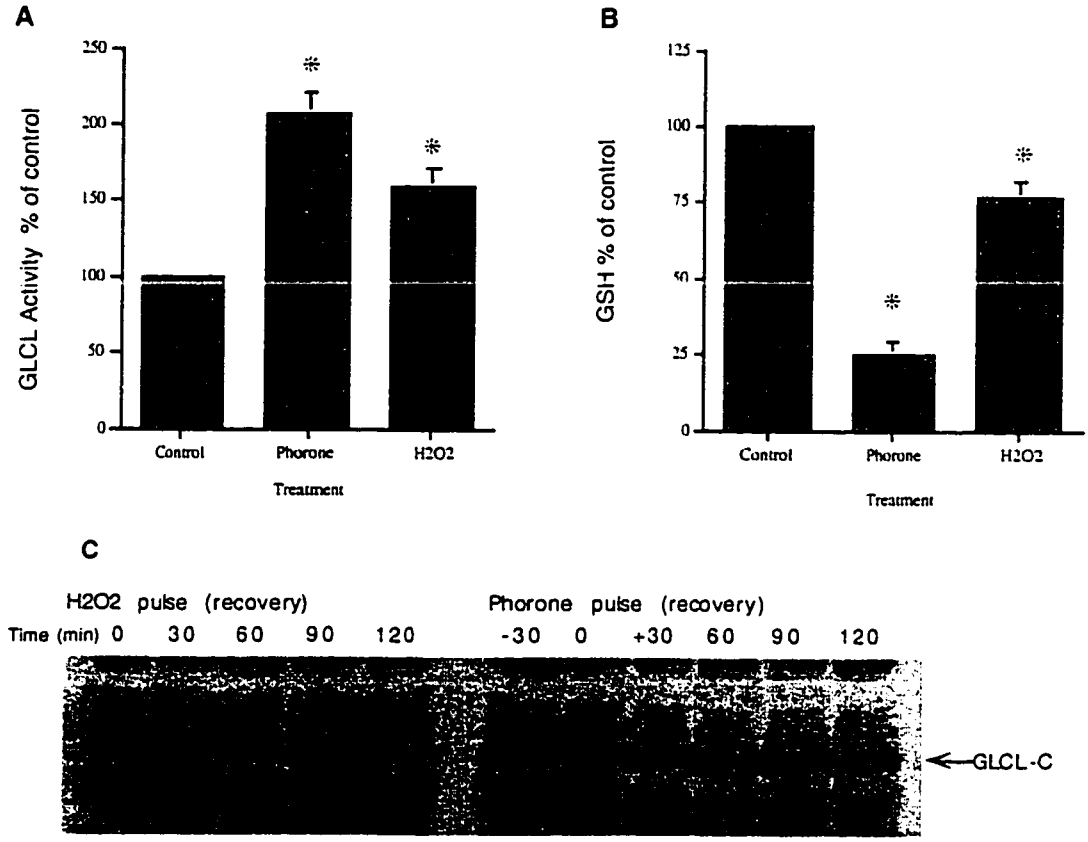

Figure 11. Activation of GLCL following oxidative stress. A. Activity of GLCL in Jurkat cells treated with phorone (90 min. pulse followed by 60 min. recovery) or H_2O_2 (10 min. pulse). B. Baseline GSH in Jurkat cells treated with phorone or H_2O_2. Average percent of control is shown, error bars indicate standard error of the mean. *Statistically significant from control by Wilcoxon signed rank test. C. Protein levels of GLCLC following treatment with H_2O_2 (left lanes) or phorone (right lanes).

Activation of GLCL was associated with a structural change.

Using native immunoprecipitation studies, we found that the recognition of an antigenic epitope on GLCLC was modulated in cells treated with phorone or H_2O_2 to upregulate GLCL enzymatic activity. Immunopreciptiation of native GLCLC by antisera directed against the N-terminal epitope E^{76}-T^{91} was decreased following enzyme activation (fig. 12a). The change in GLCLC recovery by immunoprecipitation following phorone treatment was consistent using anti-peptide sera collected in 15 separate bleeds from three different rabbits (data not shown). This indicates that decreased recovery of GLCLC was related to the reactivity of the peptide epitope, and not some peculiarity of the serum or animal in which it was produced. By using antibody directly coupled to immobilized protein A beads, we were able to greatly increase the amount of antibody used to

immunoprecipitate the GLCLC. However this strategy did not allow recovery of equal GLCLC from control and phorone treated cell extracts, suggesting that the antibody did not compete directly with whatever was blocking access to the epitope in the activated GLCL (fig 12b, c). Indeed, serial immunoprecipation demonstrated that availability of GLCLC was limited in untreated cell lysates, but with increasing time more GLCLC could be recovered (fig 12d, e). It may be that "breathing" of the enzyme occurs, allowing access to an otherwise hidden epitope. This is consistent with a model for two cellular pools of GLCLC, one in monomeric form (epitope available) and one in a protein complex (epitope masked), with dynamic flux of GLCLC between the two states. Longer periods of immunoprecipitation would essentially trap GLCLC in the monomeric pool. Alternatively, a structural change in monomeric GLCLC could hide or expose the peptide epitope; as the crystal structure of GLCLC has not been solved, it is not possible to postulate how this might occur. Interestingly, antibodies against the N-terminal peptide E^{76}-T^{91} do not support immune complex activity assays, suggesting that access to this region may be necessary for the catalytic function of the enzyme. The epitope may be relatively close to Lys^{38}, which has been identified as the Glu binding site for GLCLC (Chang 1996).

A comparison of 2 h. to overnight immunoprecipitations showed that increased native GLCLC could be recovered from the phorone treated lysates with the longer reaction time, but compared with untreated controls, there was still much less GLCLC recovery (fig. 13a). In contrast, if cell lysates were denatured by boiling with SDS, then the diluted extracts subjected to immunoprecipitation, the recovery of GLCLC by immunoprecipitation from control and treated cells was equal (fig. 13b). In aggregate, these results suggest that the form of GLCL differs between activated and resting enzyme, that the E^{76}-T^{91} epitope is masked by a structural change whose stability over time is improved by enzyme activation, and that this change occurs without direct covalent modification of the epitope peptide sequence.

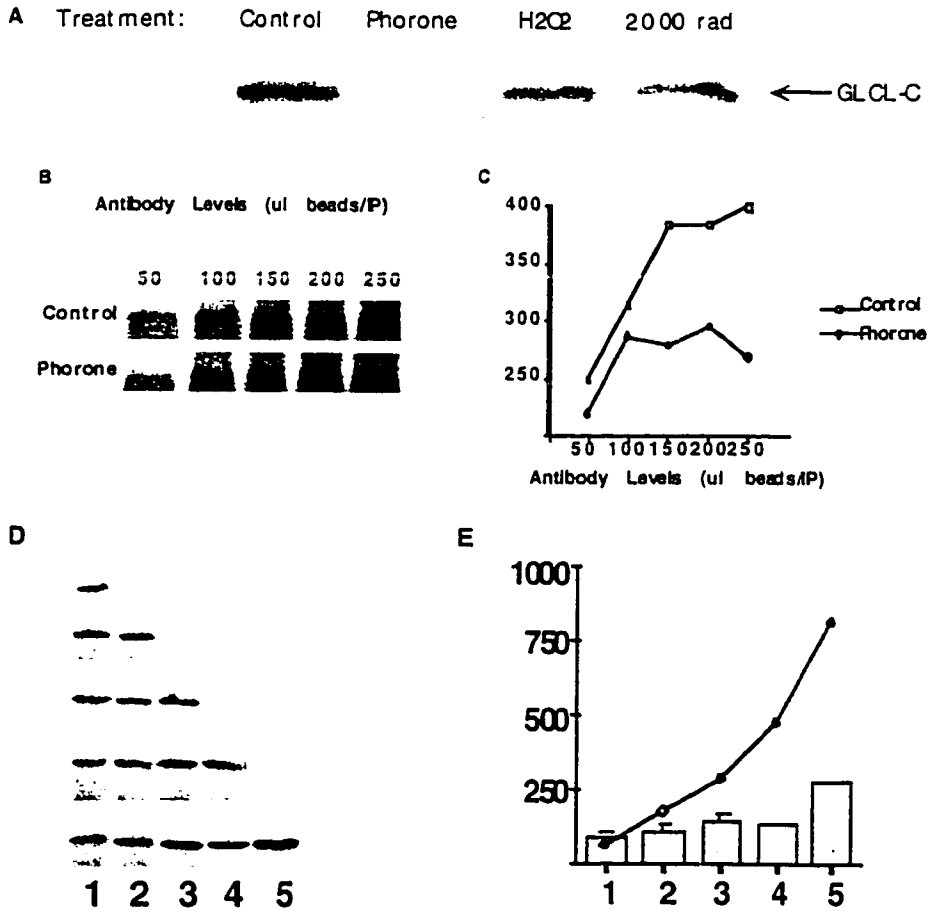

Figure 12. Epitope change in GLCLC following oxidative stress. A. GLCLC recovery by immunoprecipitation from Jurkat cells treated with 500 μM phorone (60 min. pulse followed by 60 min. recovery), H_2O_2 (10 mM pulse for 10 min.) and ionizing radiation (1000 rads). B. Recovery of GLCLC from control and phorone treated Jurkat cells using high antibody levels in the immunoprecipitation reaction. C. Densitometry from immunoprecipitates in (B) shows limited availability of GLCLC epitope in phorone treated cells. D. Serial immunoprecipitations in untreated Jurkat cells; 1-4 are 2h. reactions, 5 is overnight reaction. E. Average recovery (bars) and cumulative recovery (line) of GLCLC from (D). Error bars indicate standard deviation in recovered GLCLC.

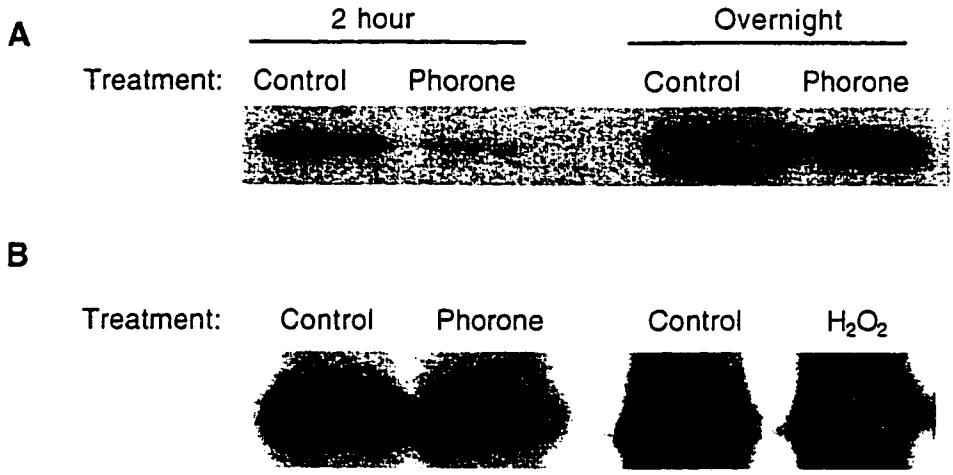

Figure 13. GLCLC epitope availability in native and denaturing immunoprecipitation. A. Lysates from control and phorone treated Jurkat cells were subjected to native immunoprecipitation for 2 h. or overnight with the same amount of GLCLC antisera. B. Lysates from control cells or cells treated with phorone or H_2O_2 were subjected to GLCLC immunoprecipitation using a denaturing/renaturing strategy.

Thiol modifying agents affect GLCLC immunoprecipitation.

To further investigate the nature of the alteration of the GLCLC epitope, we conducted native immunoprecipitation studies in the presence of thiol-modifying agents. Although there is no cysteine in the peptide epitope recognized by our antisera, oxidation of sulfhydryls at other sites could lead to alterations in the tertiary enzyme structure. Huang et al. (1993b), studying feedback inhibition of GLCL holoenzyme by GSH and its non-thiol analog ophthalmic acid, reported that the enzyme was subject to inhibition by GSH through competitive binding at the glutamate site and also by a sulfhydryl interaction at another, unidentified site. Tu and Anders (1998b) recently reported that Cys^{553} was important for the interaction of the GLCLR with GLCLC, and that mutation of Cys^{553} resulted in decreased affinity for glutamate in the GLCL holoenzyme. To investigate the effect of thiol modification on GLCL conformation, we subjected native cell extracts to immunoprecipitation in the presence of GSH, DTT, β-ME or GSSG. We reasoned that if oxidizing conditions yielded active enzyme and hid the E^{76}-T^{91} epitope, reducing conditions might return the enzyme to an inactive state and enhance immunoprecipitation. Contrary to our expectations, the thiol reducing agents GSH and DTT decreased the recovery of GLCLC by immunoprecipitation, whereas β-ME had no effect on availability of the epitope (fig. 14a). This suggests that the structural change associated with GLCL

activation may be more than simple enzyme oxidation. It is interesting that GSH and DTT should have similar effects in this assay, whereas β-ME did not alter the recovery of GLCLC by immunoprecipitation. It could be that DTT is more structurally similar to cysteine, and mimics the action of this moiety within GSH. However, the epitope availability was not affected by presence of the substrates L-cysteine, L-glutamate or the GLCL inhibitor buthionine sulfoximine (BSO), which binds the GLCLC active site as a transition state analog (fig. 14a). Nor was the recovery of GLCLR by immunopreciptation altered by presence of thiol reducing agents in the reaction (14b).

Interestingly, in the immunoprecipitations treated with GSSG, the GLCLR subunit was coprecipitated with GLCLC (fig. 14c, d). The amount of GLCLR coprecipitated in this fashion increased in immunoprecipitates from phorone treated cells (data not shown). Under no other circumstances have we observed co-precipitation of GLCLC and GLCLR using our antisera. However, in vitro treatment of the cell extracts with GSSG did not mimic enzyme activation by phorone or H_2O_2 treatment of cells, since equal amounts of GLCLC were recovered from control and GSSG treated immunoprecipitations (fig. 14c). This suggests that GLCLC epitope availability was not affected by the GSSG *per se*, and that the formation of an irrelevant disulfide bridge between GLCLC and GLCLR occurred under the non-physiological conditions employed in these experiments.

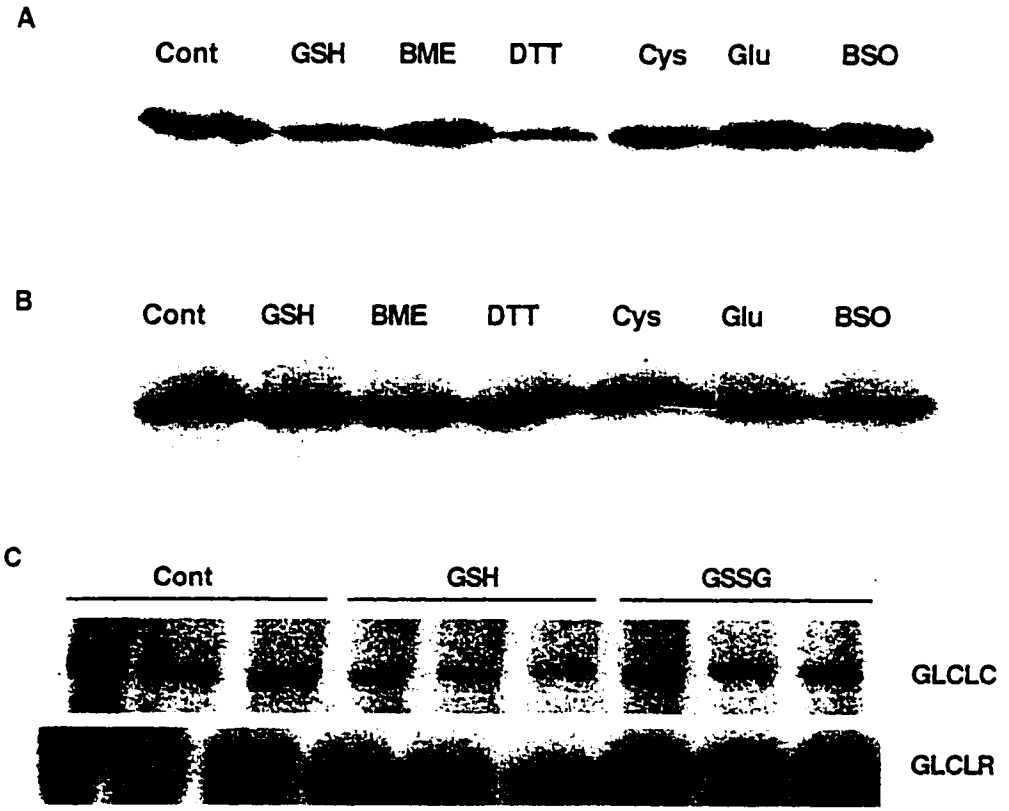

Figure 14. GLCLC epitope availability is affected by thiol modifying agents. A. Untreated Jurkat lysates were immunoprecipitated for GLCLC in the presence of 5 mM thiol, 5 mM substrates, or 1 mM BSO, and stained for GLCLC. B. Immunoprecipitation of GLCLR under the same conditions. C. Triplicate immunoprecipitates in the presence of 5 mM GSH or GSSG. The same blot was stained for GLCLC and GLCLR to detect copreciptation.

Absence of disulfide bridge between GLCLC and GLCLR.

Several investigators have reported that thiol oxidation of the GLCL holoenzyme results in a disulfide bridge between the two subunits. Indeed, GLCLR was originally identified by merit of its presence as a disulfide-linked component of GLCL preparations purified from rat kidney (Seelig et al. 1984). We also noticed higher molecular weight forms of GLCLC and GLCLR on non-reducing gels, including bands at 100, 160-170, and 200 kDa (fig 15a). However this occurred only in tissues or cell extracts which were "aged" by storage at -80 °C for extended periods. Identical banding patterns were seen in immunoblots of fresh kidney and liver homogenates prepared with or without 5 mM DTT in the homogenization buffer and separated by reducing and non-reducing SDS-PAGE (fig. 15b). This was also true for extracts from control cells and cells that had been treated

with phorone or H_2O_2 to activate GLCL (fig. 15c). In addition, if GLCL activation were mediated by disulfide formation between GLCLC and GLCLR, we would expect to see increased coprecipitation of the two subunits in following our denaturing immunoprecipitation strategy, which does not disrupt disulfide bonds. However we saw no coprecipitation of the two subunits in either control or treated cells, except when 5 mM GSSG was added to native immunoprecipitation reactions (see above). In aggregate, these data suggest that while a disulfide crosslink can be formed between GLCLC and GLCLR, the activation of GLCL by oxidative stress does not require the formation of an intermolecular disulfide bridge. Furthermore, the GLCL holoenzyme may be subject to crosslinking by non-physiological thiol oxidation but is not normally crosslinked in freshly prepared cell extracts.

Notably, phorone treatment, but not H_2O_2 treatment, caused the formation of a high molecular weight (approx 160 kDa) form of GLCL which was stable against reduction by β–ME and stained positive for both GLCLC and GLCLR (fig. 5b, c). The nature of the covalent bond responsible for this high molecular weight band and the identity of any additional members of this protein complex are unknown. Further investigation of this complex may provide insight about the proximal mediators of GLCL enzyme activation.

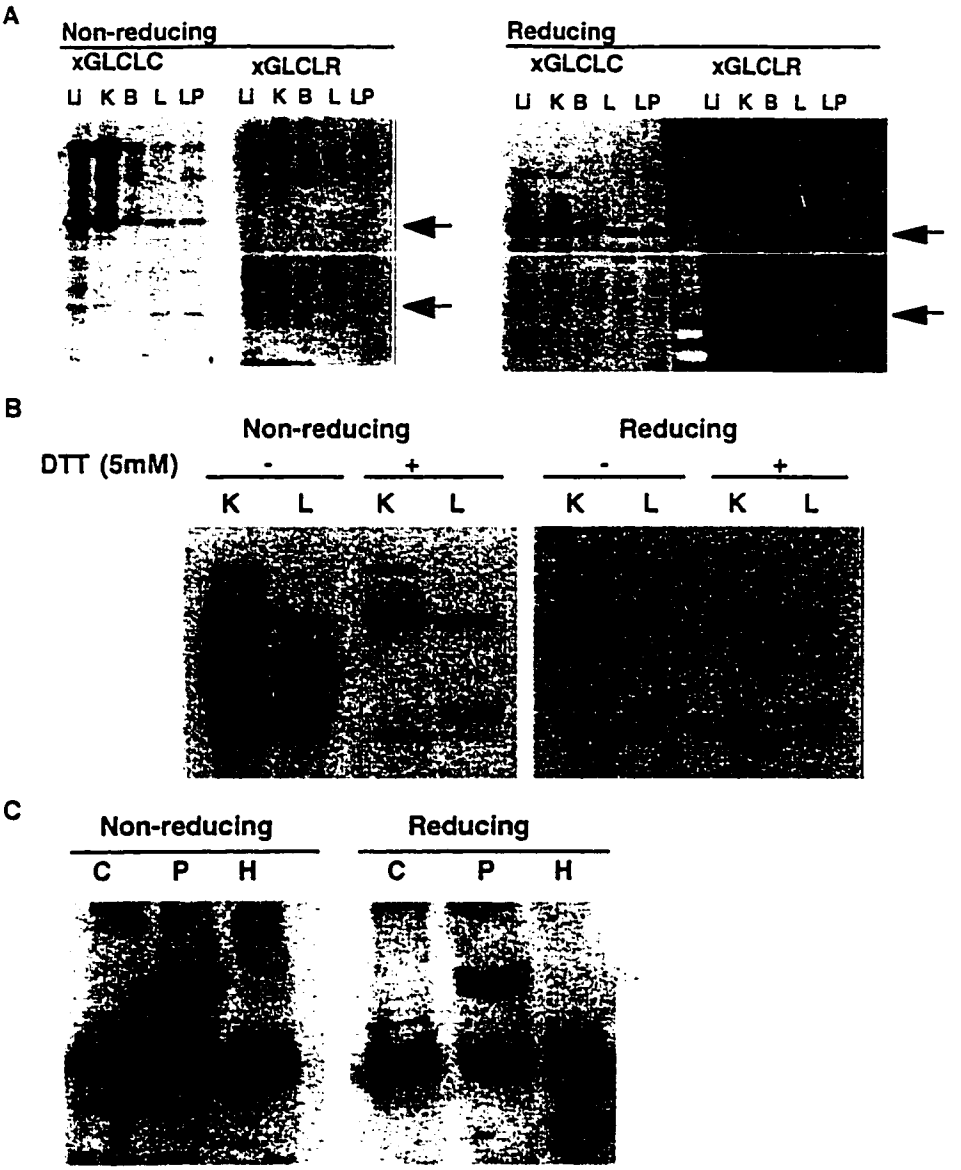

Figure 15. Non-reducing and reducing SDS-PAGE analysis of disulfide bond formation. A. Samples from mouse liver (Li), kidney (K), brain (B), and control Jurkat cells (L), and phorone-treated Jurkat cells (LP) were "aged" by freezing at -80°C prior to separation by non-reducing or reducing SDS-PAGE. Blots were stained with GLCLC and GLCLR as indicated. *Arrows* show the locations of the GLCLC and GLCLR monomers. B. Samples from fresh rat kidney (K) and liver (L) were homogenized with or without 5mM DTT and extracts separated by non-reducing or reducing SDS-PAGE and stained for both GLCLC and GLCLR. C. Extracts from control Jurkat cells (C) or cells treated with phorone (P) or H_2O_2 (H) were subjected to non-reducing and reducing SDS-PAGE, western blotted, and stained for GLCLC.

Native PAGE reveals multiple forms of GLCL

The presence of high molecular weight bands in oxidized samples and in phorone treated cell extracts, combined with the results of our immunoprecipitation studies, suggested that GLCL may exist in multiple forms within the cell. Indeed, native PAGE of rat kidney and liver homogenates showed two major bands which were partially dissociated with the addition of 5 mM DTT to the homogenization buffer (fig. 16a). We therefore employed native PAGE, followed by Western blotting for GLCLC and GLCLR, to investigate the effects of phorone and H_2O_2 on intracellular GLCL status. Fig. 16b shows that in untreated Jurkat cells, GLCLC exists mostly in two pools of protein with different mobility. The lower mobility band also stains positive for GLCLR (fig. 16c), suggesting that this band represents the 100 kDa GLCL holoenzyme while the higher mobility band is unbound catalytic subunit. In addition, other low mobility bands stain positive for GLCLC and GLCLR, which suggests that GLCL is forming a higher order enzyme complex upon activation (fig. 16b, c). Interestingly, treatment of cells with phorone or H_2O_2 results in a dramatic reduction in the intensity of the GLCLC band and an increase in the intensity of the holoenzyme band and other, higher molecular weight forms of GLCLC (fig. 16d). In both control and treated cells, there is proportionately more GLCLC as the holoenzyme form than as monomeric catalytic subunit. Phorone treatment causes a shift predominantly to the holoenzyme form, whereas the H_2O_2 treated cells have relatively more GLCLC in higher molecular weight forms. Taken together, these results provide strong evidence that the activation of GLCL in cells treated with phorone or H_2O_2 is mediated by 1) the association of monomeric GLCLC and GLCLR into an active heterodimer and 2) the further oligomerization between GLCL and additional subunits of GLCLC, GLCLR, or other as yet unidentified protein partners.

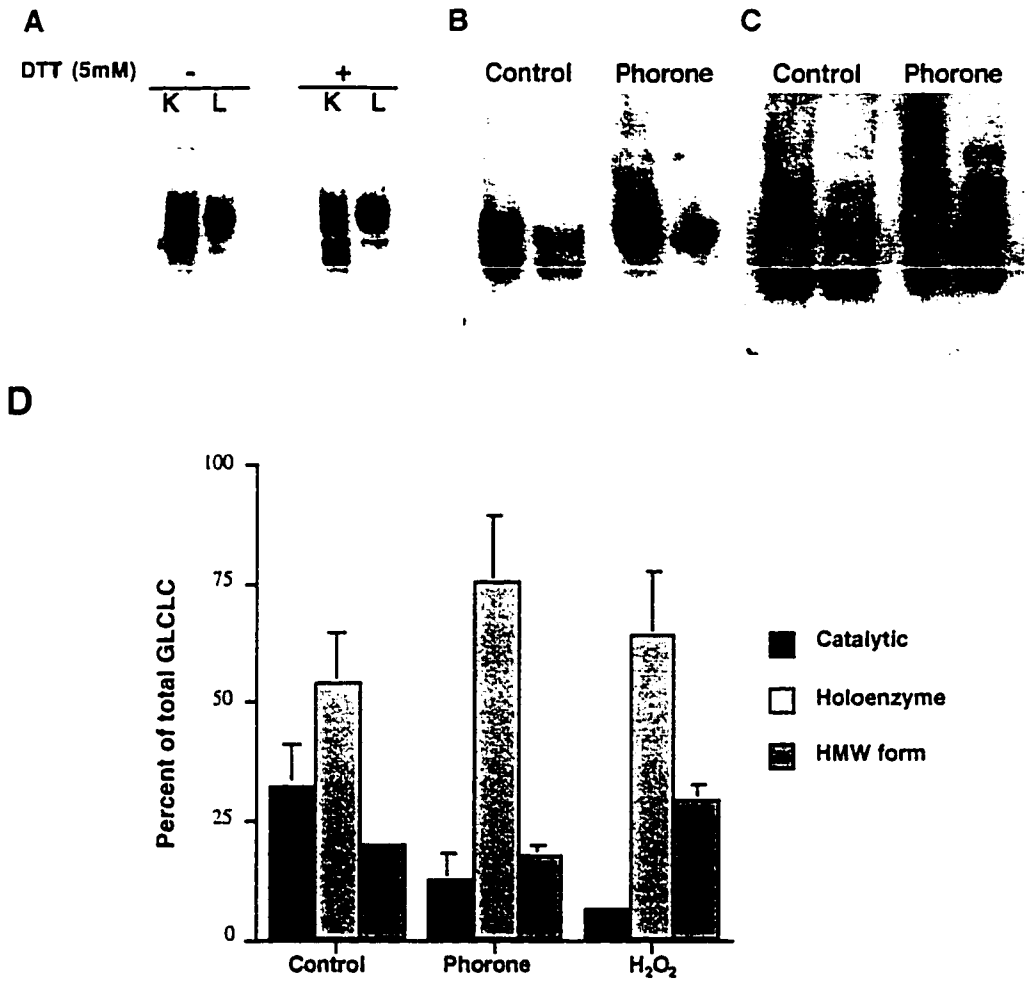

Figure 16. Native PAGE immunoblots reveal low mobility forms of GLCL. A. Native PAGE of GLCLC in freshly prepared extracts from rat liver and kidney. Homogenates were made in the presence of 1 mM DTT as indicated. B, C. Native PAGE of GLCLC (B) and GLCLR (C) from control and phorone treated Jurkat cells. Duplicate lanes were run with 100 μg and 50 μg of cytosolic protein. D. Densitometry was performed on several experiments to quantitate GLCLC monomer, holoenzyme, and higher molecular weight (hmw) forms in native PAGE Westerns from control Jurkat cells and cells treated with phorone or H_2O_2 to activate GLCL. Average percent of total GLCLC, and standard deviations, are shown.

Size Exclusion Chromatography of GLCL.

To further investigate the shifts in mobility seen with native PAGE upon GLCL activation, we employed size exclusion chromatography (SEC) on extracts from control and phorone treated Jurkat cell lysates. Preliminary studies with rat kidney extracts demonstrated that GLCLC existed in cellular fractions ranging from 60 to 200 kDa; GLCLR existed in fractions at approximately 30 kDa and from 100 to 200kDa (fig. 17). No difference in

size distribution was seen between extracts prepared with 1mM DTT in the homogenization buffer and extracts homogenized in the absence of reducing agents (data not shown).

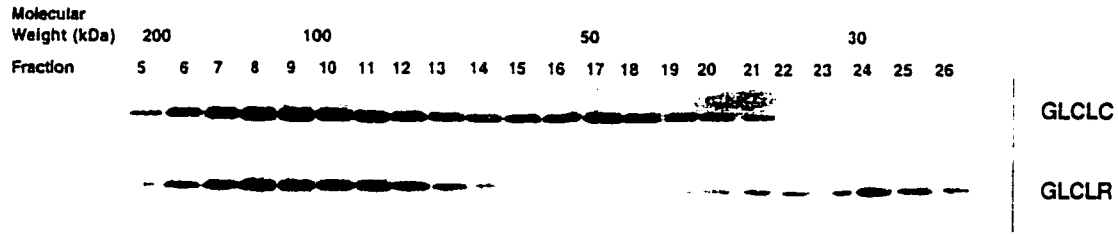

Figure 17. Size exclusion chromatography analysis of GLCLC and GLCLR from rat kidney. Clarified extract (2 mg.) was separated on a Waters 300W SEC column and the fractions run as individual lanes on SDS-PAGE, Western blotted, and stained for GLCLC (top panel) and GLCLR (bottom panel).

A large SEC column was prepared using Sephacryl S-200 HR gel filtration medium, which is recommended for separation of molecular sizes up to 250 kDa. Extracts from control and phorone treated Jurkat cells were separated on the column as described in Materials and Methods. The fractions were then run as individual lanes on SDS-PAGE gels and immunoblotted for GLCLC. Densitometry analysis of the percent of total GLCLC and cumulative percent of GLCLC per volume eluted (fig. 18) shows that GLCLC elutes in higher molecular weight fractions in extracts from phorone treated cells than untreated controls. Similar results were seen using extracts from control and anti-Fas treated Jurkat cells (data not shown). These results demonstrate that GLCL exists in several size fractions within the cell, and that the activation of GLCL by oxidative stress correlates with a shift of part of the GLCLC pool to a higher molecular weight form. Future studies will aim to identify binding partners of GLCLC in the oligomeric complexes, and address the contribution of these high molecular weight forms of GLCL to the increased cytosolic GLCL activity observed in cells following oxidative stress.

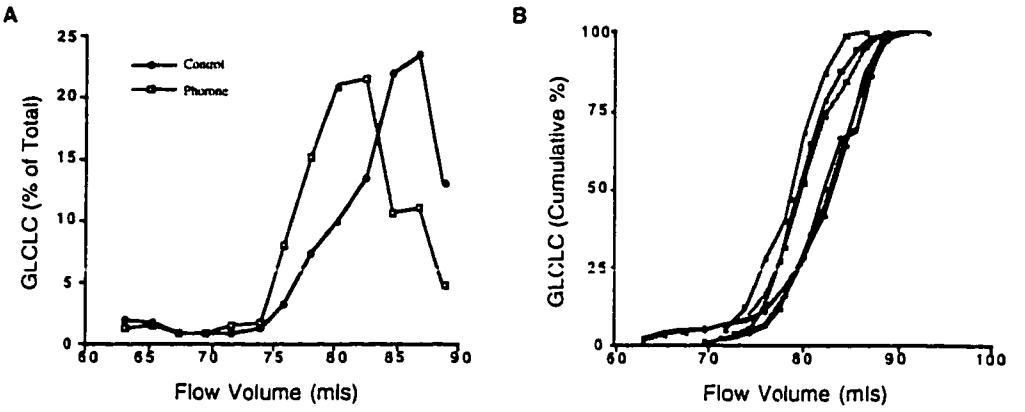

Figure 18. Size exclusion chromatography analysis of GLCLC in control and phorone treated Jurkat cells. A. Elution of GLCLC (expressed as % of total GLCLC eluted) from control cells (circles) and phorone treated cells (squares) in a representative experiment. B. Cumulative % of GLCLC eluted per flow volume from three separate experiments. Elution from control cells lags behind elution from treated cells by approximately 5 ml.

DISCUSSION

The data presented herein show that GLCL activity is rapidly stimulated by oxidizing conditions and GSH depletion in Jurkat lymphocytes. We have seen similar results in other cellular models, including mouse P19, mouse Hepa1 cells, and COS monkey kidney epithelium cells treated with phorone to deplete GSH (data not shown). Likewise, Ochi (1995,1996) observed rapid activation of GLCL in hamster V79 cells treated with H_2O_2 and menadione. These studies suggest that post-translational activation of GSH biosynthesis following intracellular oxidation is a general phenomenon, occuring in several species and diverse cell types. As a mechanism for maintaining intracellular GSH levels, GLCL activation complements the well described transcriptional up-regulation of GLCLC and GLCLR following oxidative stress. Our evidence suggests that increased GLCL activity in the extracts of treated cells is associated with both a structural change in GLCL and an increase of the molecular size of the GLCL complex. This correlates with the loss of an N-terminal peptide epitope in the native enzyme. It appears that GLCL activation occurs through the formation of GLCLC / GLCLR heterodimers, the stabilization of the heteromeric form of GLCL, and the binding of additional protein(s) to form a high molecular weight (oligomeric) complex. A model for GLCL activation is presented in fig. 19.

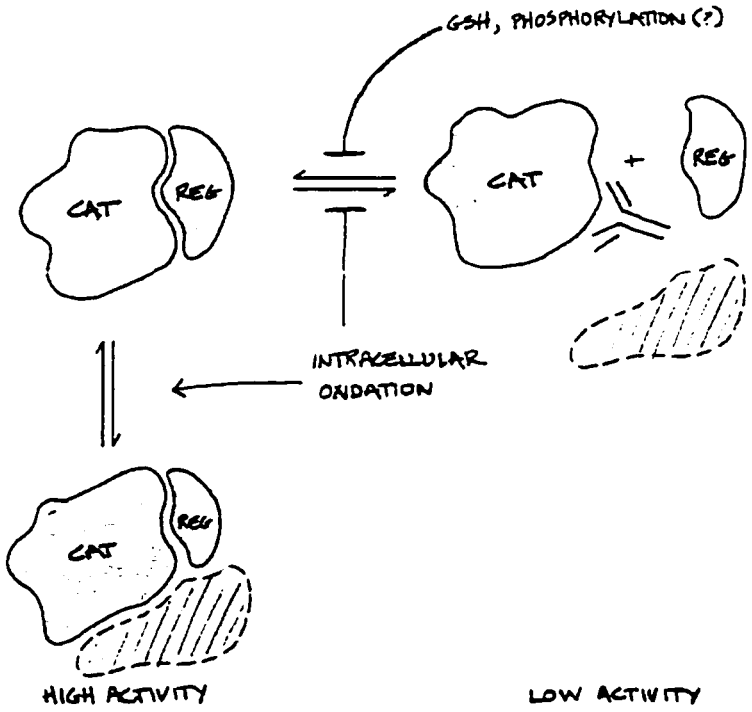

Figure 19. Model for activation of GLCL by oligomeriaztion following oxidative stress. Dynamic equilibrium between monomeric subunits and heterodimeric enzyme governs GLCL activity. Intracellular oxidation favors heterodimeric enzyme and formation of high molecular weight forms. High intracellular GSH and possibly phosphorylation favors monomeric GLCLC and GLCLR.

We found increased GLCL activity in Jurkat cell cytosols following many models of intracellular oxidation, within the range of 30-300% more activity than cytosols from untreated cells. These changes, which reflect increased Vmax, could also be a function of altered Km for glutamate, cysteine, or ATP; alternatively, they could be due to changes in turnover rate due to product release or other factors. The assay conditions employed do not allow for discrimination between these potential effects. Kinetic analysis of GLCL from control and treated cells would provide insight to the mechanism(s) of GLCL activation, at least with regard to the current paradigm of holoenzyme structure and function. In both humans and rats, for which the kinetic constants have been reported, GLCL holoenzyme has a higher affinity for glutamate, and a lower affinity for GSH, than the GLCLC subunit alone. In humans, the Vmax for GLCL holoenzyme is also considerably higher than the Vmax for GLCLC; the changes in Vmax as a function of holoenzyme formation in the rat have not been reported. An in-gel GLCL assay, developed by the authors to discriminate between the activity of GLCLC and GLCL holoenzyme, showed that recombinant mouse GLCLC had strikingly higher activity when

associated with GLCLR (C. Krejsa, unpublished results). Strategies to facilitate the kinetic analysis of GLCL from whole cell lysates, and to detect the specific activities of the different forms of GLCL, will allow for a rigorous investigation of the changes in GLCL function following intracellular oxidative stress. Future studies will address these challenging problems.

The model proposed by Huang et al. (1993b), which is widely cited in the GLCL literature, suggests that GLCL exists predominantly as an inactive heterodimer, which is oxidized to the activated state by the formation of an intermolecular disulfide bridge. While we have observed disulfide formation between GLCLC and GLCLR under non-physiological conditions and in stored cell lysates, our data do not show an intermolecular disulfide bridge in GLCL from either freshly prepared tissues or cells treated to activate GLCL. Rather, it appears that the rapid activation of GLCL involves a shift of GLCLC and GLCLR from the monomeric state to the holoenzyme form, with possible further oligomerization into highly active complexes. However, this does not rule out the involvement of cysteine groups as redox sensors on GLCL, through the formation of mixed disulfides with small molecular weight thiols or an intramolecular disulfide bond. It was determined by site directed mutagenisis of GLCLC that Cys^{553} was important for holoenzyme function in human GLCL. Substitution with Gly at position 553 resulted in an enzyme that could associate with GLCLR but did not show increased activity in the heterodimeric state (Tu and Anders 1998b). However, mutation of Cys^{553} could alter the interaction between GLCLC and GLCLR through a mechanism that does not involve an intermolecular disulfide bond. Many of these questions will be clarified when the crystal structures of GLCLC and the GLCL holoenzyme are solved.

Phosphorylation of GLCLC has been reported to down-regulate enzyme activity in rat hepatocytes (Sun et al. 1996). In preliminary studies, we found no phosphorylation of GLCLC or GLCLR in either control Jurkat cells or cells treated with phorone and H_2O_2 (data not shown). This could be due to differences between cell types (liver v. lymphocyte) or the models used (pharmacological activation of signaling pathways v. induction of intracellular oxidative stress). Until the proximal mediators of GLCL activation are identified, it will be difficult to rule out phosphorylation or dephosphorylation on the basis of negative data alone.

The observation of high molecular weight GLCL complexes in native Western blots from lymphocytes, kidney, and liver, suggests that other, as yet unidentified binding partners may participate in the activation of GLCL or act as members of a highly efficient GLCL enzyme complex. The identification of proteins that can associate with GLCLC and GLCLR, by pull-down experiments or the yeast 2-hybrid screen, will greatly increase our understanding of GLCL enzyme function. We and others have noted striking differences in GLCL expression between tissues, in both the levels of each subunit and the ratiometric expression of GLCLC and GLCLR (Krejsa et. al, in preparation; Gipp et al. 1995). If the rapid formation of heterodimeric and oligomeric complexes governs a tissue's ability to activate GLCL, the relative expression of the GLCL subunits may greatly affect the sensitivity of a tissue to oxidative injury. The activation of GSH biosynthesis is also likely to temper the activation of redox-sensitive transcription factors, and may play an important role in modulating the transcriptional regulation of GLCLC and GLCLR as well as other stress-responsive genes. In addition, the proportion of GLCL existing in higher molecular weight forms in different tissues may reflect the basal level of GLCL activity within the tissue. For example, in the liver, which continuously produces GSH for export, most of the GLCLC exists in a high molecular weight complex, whereas the kidney has higher cellular levels of GLCL but a greater proportion in the monomeric or heterodimeric forms (see fig. 5a). The significance of the size distribution of GLCL in particular tissues will be better understood when more is known about the specific activities of different size fractions within the cell.

This study presents an investigation of the physical changes in GLCL structure which are associated with increased activity following transient oxidative stress. The evidence strongly support the hypothesis that GLCLC exists in at least two forms within the cell, consisting of relatively inactive monomers and a more active pool of heterodimers and oligomers. The shift to the more active pool involves a change in GLCLC structure, such that an epitope which was available in control cells is unavailable in cells following oxidative stress. This is accompanied by a decrease in the electrophoretic mobility of GLCL in native PAGE, and by an increase in the apparent molecular weight of the GLCLC subunit in SEC studies. The proximal mediators of these changes, and signaling pathways leading to their activation, have not been identified. However it appears that there is no requirement for an intermolecular disulfide bridge between GLCLC and GLCLR, nor is the activation of GLCL a simple function of enzyme oxidation. Future studies will

address the question of relative activity in different molecular weight forms of GLCL, investigate the kinetic parameters of GLCL from control cells and cells following oxidative stress, and seek to identify other binding partners in the high molecular weight GLCL complexes.

NOTES TO CHAPTER II

We wish to thank Dr. Jim Blake of Bristol-Myers Squibb for synthesis of the GLCLC and GLCLR peptides used to develop the oligoclonal antisera and Lorelyn Mackie for competent veterinary care of the rabbits used for this purpose. The HPLC assays for GLCL activity were performed with the expert assistance of Collin White at the University of Washington. Dr. Steve Nadler of Bristol-Myers Squibb and Dennis Sloane of University of Washington assisted in the size exclusion chromatography studies. Drs. Gary Schieven, Jeff Ledbetter, and Terry Kavanagh provided guidance and helpful discussion, as well as careful readings of the manuscript. Funding was provided by NIH grants ES04696, AG01751, ES07032, and by the Bristol-Myers Squibb Pharmaceutical Research Institute.

CHAPTER III: CLEAVAGE OF GLUTAMATE-CYSTEINE LIGASE CATALYTIC SUBUNIT IN APOPTOSIS BY A CASPASE-3 DEPENDENT MECHANISM.

ABSTRACT

The intracellular level of the tripeptide antioxidant glutathione (GSH) has been shown in many studies to be an important determinant of susceptibility to the induction of apoptosis. Glutamate cysteine ligase (GLCL) catalyzes the rate limiting initial step in the biosynthesis of GSH. We report here that in both receptor-driven and chemical-induced models of apoptosis, the 73 kDa GLCL catalytic subunit (GLCLC) was cleaved by a caspase-3 dependent mechanism to a 60 kDa form. The GLCL regulatory subunit (GLCLR) was not cleaved during apoptosis. Cleavage of GLCLC was prevented in cells treated with the caspase inhibitor ZVAD-FMK and in cells lacking caspase-3. However, caspase-3 was not required for cellular depletion of GSH during apoptosis. GLCLC was also cleaved directly by recombinant caspase 3 in vitro. Mutation of aspartate at position 499 to alanine in recombinant GLCLC completely prevented cleavage to the 60 kDa form in vitro and in transfected cells. Although GLCLC cleavage occurred upstream of a cysteine residue reported to be important for association of GLCLC with GLCLR, the cleaved form of GLCLC was capable of binding GLCLR to form an active holoenzyme. GLCLC cleavage may represent a novel mechanism for the control of cellular GSH production during apoptotic cell death.

INTRODUCTION

Apoptosis is a general mechanism for the control of cell death following a wide variety of stimuli, including receptor based signals and cellular damage induced by chemicals and radiation. The apoptotic process is mediated through the activation of caspases, which cleave key protein substrates in the cell, leading to many of the changes which characterize apoptotic cells, including nuclear condensation, DNA fragmentation, and opening of the mitochondrial permeability transition pore (Nicholson 1999; Slee et al. 1999). Although the cells are committed to die, apoptosis is thought to be protective of tissues because the process prevents the inflammatory responses associated with necrotic cell death.

Intracellular redox status has been shown to be an important determinant of a cell's response to apoptotic stimuli. Numerous studies have shown that pretreatment of cells with antioxidants can confer resistance to treatments which induce apoptosis (Um et al. 1996; Deas et al. 1997; Singh et al. 1998). In addition, depletion of cellular antioxidants can make cells vulnerable to apoptosis, and in some cases can shift the cellular response from apoptotic to necrotic death (Deas et al. 1997; Nicole et al. 1998; Higuchi and Matsukawa 1999). During apoptosis, mitochondrial dysfunction results in the increased production of reactive oxygen species, which, combined with the decreased production of reducing equivalents can cause substantial oxidative stress (Garcia-Ruiz et al. 1995; Garcia-Ruiz et al. 1997; Pierce et al. 2000). Reactive oxygen species (ROS) may also play a role in the initiation phase of apoptosis, both through receptor- and mitochondria-initiated pathways (Hampton and Orrenius 1998). While there is good evidence that changes in cellular redox status occur during apoptosis, and the sources of many of the ROS have been characterized, less is known about mechanisms for control of intracellular antioxidant production during this process.

The major intracellular thiol antioxidant, GSH is maintained at millimolar concentrations in most cell types (Meister and Anderson 1983). It acts as a free-radical scavenger, and through the GSH peroxidase/reductase system, represents a first line of defense against H_2O_2 and lipid peroxides. The enzyme glutamate cysteine ligase (GLCL; also known as γ-glutamyl cysteine synthetase) catalyzes the formation of γ-glutamylcysteine, which is the rate-limiting step in GSH biosynthesis (Griffith and Mulcahy 1999). GLCL is a heterodimeric enzyme composed of a 73 kDa catalytic subunit (GLCLC), which possesses all of the catalytic activity, and a 28 kDa regulatory subunit (GLCLR), which influences the activity of GLCLC by increasing its affinity for glutamate and decreasing the feedback inhibition by GSH (Huang et al. 1993b). Several reports have shown that overexpression of GLCL confers resistance to agents which induce oxidative stress; more recently it has been shown that GLCL overexpression protects against apoptosis (Manna et al. 1999; Tipnis et al. 1999). In addition, GLCL was shown to be upregulated in some human cancer cell lines which show resistance to chemotherapy, and treatment with buthionine sulfoximine, a specific inhibitor of GLCL, overcame this resistance (Godwin et al. 1992; Mulcahy et al. 1994a; Mulcahy et al. 1994b; Mulcahy et al. 1995; Komiya et al. 1998; Siitonen et al. 1999). As many chemotherapeutic agents exert their toxicity through

apoptotic processes, these data suggest that the ability to produce GSH may be a variable in cellular sensitivity to inducers of apoptosis.

We recently showed that GLCLC is cleaved in acute myeloblastic leukemia cells undergoing etoposide-induced cell death (Siitonen et al. 1999). In the present study we report that caspase 3 dependent cleavage of GLCLC occured in many receptor driven and chemically induced models of apoptosis. Mutation of Asp 499 to Ala prevented GLCLC cleavage both in vitro and in vivo. Surprisingly, the intracellular depletion of GSH during apoptosis occured independent of GLCLC cleavage; furthermore, the cleaved protein was capable of binding with GLCLR to form an active holoenzyme. The functional effects of GLCLC cleavage are discussed.

MATERIALS AND METHODS

Chemicals and Reagents

All common laboratory reagents were purchased from Sigma (St. Louis, MO).

Animal Care and Dosing Protocol

Human *BCL-2* transgenic mice (C57/B6C3H background) were kindly provided by Dr. Stanley Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School). Genotyping of animals was performed using tail DNA with a PCR assay. Expression of BCL2 was under inducible control of a metallothionein promotor; animals were induced by addition of 25 mM $ZnSO_4$ to their drinking water for 5-7 days prior to dosing. Non-transgenic littermates were also given $ZnSO_4$ in their drinking water. Mice were dosed with 10 µg anti-Fas mAb (Jo2; Pharmingen, San Diego CA) in 100 µl sterile PBS by interperitoneal injection. Control mice were injected with PBS alone. Animals were sacrificed at 5h. post injection and liver samples were taken for immunoblotting and biochemical determinations.

Cell culture

Jurkat cells (clone E6.1) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan UT), 1% penicillin/streptomycin, 1 mM glutamine and 1 mM sodium pyruvate (Gibco-BRL, Milwaukie WI). MCF-7 cells transfected with the pcDNA3 vector alone or with pcDNA-caspase 3 were a gift from Vince Kidd (St. Jude's Research Hospital, Memphis TN). These cells were cultured in

DMEM media supplemented with 10% FBS, 1% penicillin/streptomycin, and 100 µg/ml geneticin (G418).

Plasmids and Bacterial Expression of Fusion Proteins

Murine GLCLC and GLCLR were subcloned into pET28a and pET21a from plasmids pCR3.1-GLCLC and pCR3.1-GLCLR (Reid et al., 1997a, 1997b) as described in Appendix E. pGEX-KG-caspase-3 was a gift from Dennis Templeton (Case Western Reserve University) and subcloned into the bacterial strain BL21(DE3) (Novagen) and GSH1$^-$ strain 821 (kindly provided by the *E. coli* Genetic Stock Center, New London CT). Proteins were expressed from single bacterial colonies grown on LB-agar plates supplemented with 100 µg/ml ampicillin, 30 µg/ml kanamycin sulfate, or both; plates were scraped into liquid Luria broth containing the same selective antibiotics, and grown for 16-20 h. at 25 °C. The bacteria were harvested by centrifugation and sonicated on ice in sonication buffer (20 mM Tris, 100 mM NaCl, pH 7.9) containing protease inhibitor mix (PI; 100 mg/ml Pefablock, 10 mg/ml N-α-Tosyl-L-Lysine chloromethyl ketone, 1mg/ml pepstatin A, 1 mg/ml aprotinin, and 1 mg/ml leupeptin; all from Boehringer-Manheim, Indianapolis, IN) at 1:1000 dilution. Extracts were clarified by centrifugation at 12,000 xG at 4°C, and 6-His proteins were collected on His-bind Resin (Novagen) as specified by manufacturer's instructions. Resin-bound proteins were washed with 40 mM imidazole and eluted with 400 mM imidazole in 20 mM Tris, 500 mM NaCl, pH 7.9. Protein quantitation was by the Bradford method (Bio-Rad, Hercules, CA). Resin-bound proteins were stored at -20°C in 20 mM Tris, 100 mM NaCl, 50% glycerol, pH 7.9.

Western blotting

Adherent cells were trypsonized and pelleted in media or PBS containing 10% FBS, then the pellets were resuspended in ice cold TES/SB (20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM boric acid, 1 mM L-serine, pH 7.4) containing protease inhibitors as above. Cells were sonicated, spun at 14000 rpm in a refrigerated microfuge, and the supernatants were used immediately or frozen at -80 °C. 50 µg of total cell protein per lane was separated by SDS-PAGE or by native gel electrophoresis on a 4-15% gradient gel (Bio-Rad) and transferred to PVDF membrane (Millipore). Protein loading and transfer were checked by staining the blots with Ponceau S (Sigma). Blots were stained with oligoclonal antisera raised against GLCLC or GLCLR (Thompson et al. 1999). Polyclonal anti-PARP antibody was from Upstate Biotechnology (Lake Placid, NY); mAb

raised against CPP32 was from Transduction Laboratories (Lexington KY). Staining was detected with HRP conjugated goat-anti-rabbit IgG (Boehringer) developed with ECL (Amersham, Arlington Hts., IL). Densitometry was performed using a Gel-Doc system and Molecular Analyst software (Bio-Rad).

GSH quantitation and GLCL activity assays

GSH in cell extracts or in media was measured by modification of the Teitze assay (Baker et al. 1990). Recombinant GLCL was assayed using a modification of the NADPH recycling assay of Seelig and Meister (Seelig and Meister 1985). Briefly, GLCL was eluted from His-bind resin (Novagen) with 400 mM imidazole buffer; eluted protein was quantified using the Bradford method (Bio-Rad) and diluted to 0.25 or 0.125 µg/ml in dH2O. This was added to prepared 96 well plates containing dilution series of cysteine and glutamate. The plates were pre-incubated at 37 °C for 5 min., then the reaction was started by addition of 4x assay buffer (400 mM Tris, 600 mM KCl, 20 mM ATP, 8 mM phosphoenolpyruvate, 8 mM EDTA, 80 mM $MgCl_2$, pH 8.0). Absorbence at 340 nm was monitored for 5 minutes, and initial rates of reaction were calculated from linear portions of the curves. Proteins from the diluted samples were then re-quantified by Western blotting followed by densitometry as described above. GLCL activity in cytosolic extracts was assessed using our previously described HPLC-based assay (Hamel et al. 1996, White et al. 1999).

Holoenzyme reconstitution and in-gel activity assays.

Histidine tagged full length or cleaved GLCLC was purified on Ni^{2+} chelate resin as described. Equal amounts of immobilized protein were incubated with untagged GLCLR in bacterial extract for 15, 30, or 60 minutes at 4 °C with constant agitation. The beads were precipitated and washed several times with 40 mM imidazole in His-bind buffer (20 mM Tris pH 7.9, 500 mM NaCl) and eluted with 400 mM imidazole in His-bind buffer. The eluted proteins were separated by native gel electrophoresis and either stained with Coomassie blue or subjected to an in gel activity assay. Aliquots of the eluted protein were also subjected to denaturing PAGE to assess association of GLCLR with the GLCLC-bead complex.

In-gel activity assays utilized the deposition of $CePO_4$ crystals in response to ATP hydrolysis (Seitz et al. 1991; Song et al. 1995; see also Appendix C). Eluted proteins

were separated by native PAGE on a 4-15% gel as described. The gels were equilibrated in GLCL cocktail (100 mM Tris, 10 mM $MgCl_2$, 10 mM ATP, 0.5 mM EDTA, pH 7.4) containing 15 mM glutamate for 10 min. at 37 °C. Reactions were started by replacement with GLCL cocktail containing 15 mM glutamate, 3 mM $CeCl_2$, and 5 mM cysteine, and the gels were incubated at 37 °C with shaking for 30-60 minutes. The deposition of $CePO_4$ was observed by transillumination on a Gel-doc (darkfield settings) using a MVI DarkLite trans-illuminating light source (Meridian, Kent WA) adapted to illuminate the gel, and the signal was quantified using Molecular Analyst software.

RESULTS

GLCLC is cleaved during apoptosis.

Apoptosis is characterized by the cleavage of a specific group of intracellular substrates; a recent estimate suggests a group of approximately 200 polypeptides is subject to caspase-mediated proteolysis (Nicholson 1999). Of the identified substrates for caspases, many are proteins which propagate the apoptotic signal, whereas others are characterized as playing critical roles in cellular homeostasis and damage repair (Stroh and Schulze-Osthoff 1998). Therefore we were greatly intrigued to discover that GLCLC, the catalytic subunit of the rate limiting enzyme in GSH synthesis, was a target of proteolysis during apoptosis (Siitonen et al. 1999). Western blots of cytosolic extracts from Jurkat cells treated with staurosporine, cyclohexamide, or UV irradiation to induce apoptosis showed that GLCLC was present in apoptotic cell in the 73 kDa full length form and also in a 60 kDa form (fig. 20a). The same was true for GLCLC from livers of mice treated with anti-Fas mAb, which induces fulminant hepatic apoptosis (fig. 20b). Anti-Fas treatment of mice overexpressing Bcl-2, which blocks hepatic apoptosis and spares the animals, also blocked the cleavage of GLCLC to the 60 kDa form. There was no change in the molecular weight of GLCLR in any of the model systems tested. Interestingly, the degree of GLCLC cleavage appeared to correlate with the processing of pro-caspase-3 into its active form (fig. 20a, fig 21a.).

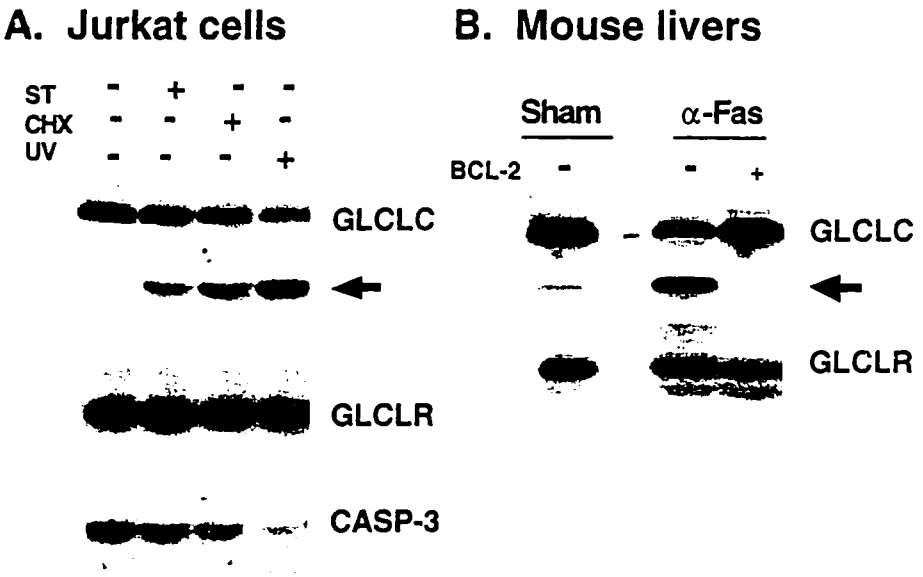

Figure 20. Cleavage of GLCLC during apoptosis. (A) Jurkat cells were treated with 100 ng/ml staurosporine, 20 μg/ml cyclohexamide, and 200 J UV irradiation for 8 h. as indicated. Extracts were separated by SDS-PAGE, immunoblotted, and stained for GLCLC, GLCLR, and pro-caspase-3. (B) Wild type mice and mice overexpressing Bcl-2 were treated with 10 μg anti-Fas mAb by IP injection; livers were harvested 5 h. later. Extracts were separated by SDS-PAGE, immunoblotted, and stained for GLCLC and GLCLR. *Arrows* indicate cleaved GLCLC.

GLCLC cleavage and GSH depletion in anti-Fas treated Jurkat cells.

One feature of apoptotic death in certain cell types is the dramatic depletion of intracellular GSH. This has been reported to occur through extrusion of reduced GSH, as well as increased utilization of GSH and NADPH due to excessive production of ROS (van den Dobbelsteen et al. 1996; Pierce et al. 2000). Treatment of Jurkat cells with anti-Fas mAb induced a time-dependent cleavage of GLCLC which correlated both with the processing of caspase-3 into its active form and the cleavage of poly-ADP ribose polymerase (PARP), a well documented caspase-3 substrate (fig. 21a). Furthermore, the intracellular GSH levels in these cells were diminished along a similar timecourse as GLCLC cleavage (fig. 21b). This correlation suggested that the cleavage of GLCLC might contribute to the depletion of GSH seen in apoptotic cell death.

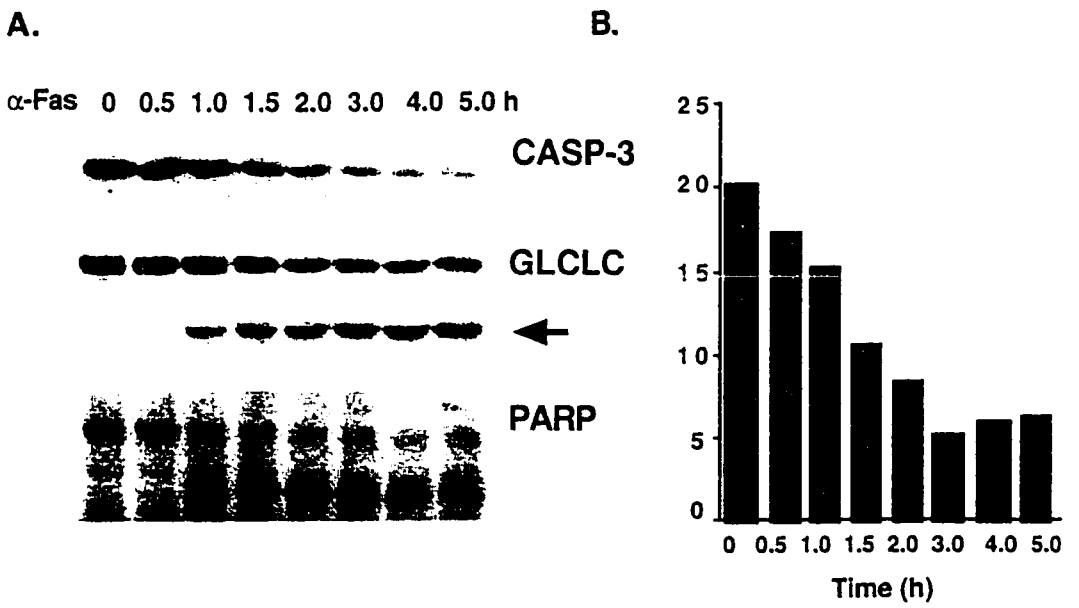

Figure 21. Timecourse of GLCLC cleavage and GSH depletion in anti-Fas treated Jurkat cells. Jurkat cells were treated with 100 ng/ml anti-Fas mAb for the indicated times. Cytosolic extracts were subjected to (A) immunoblotting for pro-caspase-3, GLCLC, and PARP protein levels; and (B) Tietze assay for total GSH.

GLCLC cleavage is caspase-3 dependent.

The kinetics of GLCLC cleavage during apoptosis suggested the possibility that the proteolysis was a caspase-mediated event. To test this hypothesis, we treated HeLa cells with the pan-caspase inhibitor Z-VAD-fmk, and stimulated apoptosis with TNF-α and cyclohexamide or anti-Fas mAb (fig. 22a). Treatment with Z-VAD-fmk, which completely inhibited the processing of pro-caspase-3 (bottom panel), and also blocked the cleavage of GLCLC. To test if GLCLC cleavage was specifically dependent on caspase-3, the MCF7 cell line, which does not normally express caspase-3, was employed. MCF7 cells transfected with vector alone or caspase-3 were treated with TNFα and cyclohexamide to induce apoptosis. Cleavage of GLCLC occurred only in the cells expressing caspase-3. Interestingly, in parallel experiments, GSH depletion during apoptosis was examined in caspase-3 transfected MCF7 cells and vector controls. There was no significant difference in the kinetics of GSH extrusion between these two cell types. This suggests that the depletion of intracellular GSH stores by active means is not dependent on caspase-3, and that the decrease in GSH levels is not due to cleavage of GLCLC (see Appendix E).

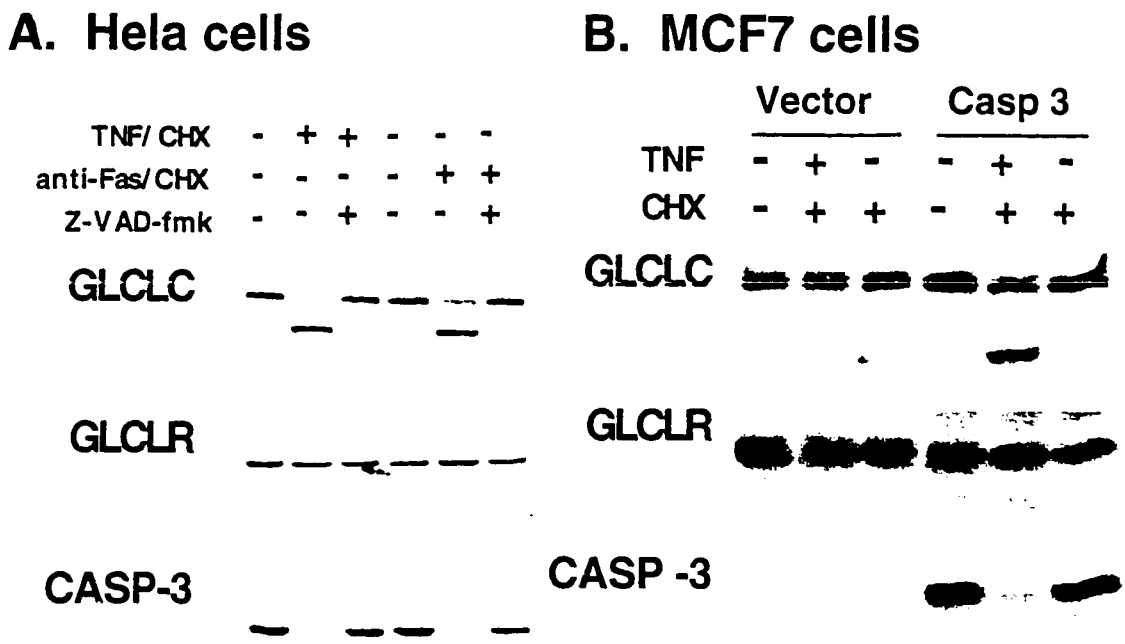

Figure 22. GLCLC cleavage is caspase-3 dependent. (A) HeLa cells were treated with ZVAD-fmk and 30 ng/ml TNFa plus 10 μg/ml CHX or anti-Fas mAb plus 10 μg/ml CHX for 12 h.; extracts were immunoblotted for GLCLC, GLCLR and caspase-3. (B) Vector transfected MCF7 cells (left lanes) or cells transfected with caspase-3 (right lanes) were treated with TNFa/CHX for 8 h. and proteins immunoblotted as described in (A). From Franklin et al., in preparation (Appendix E).

GLCLC is cleaved by caspase-3 at Asp^{499}

The experiments described above suggested that cleavage of GLCLC was dependent upon the activation of caspase-3. Furthermore, the recognition of cleaved GLCLC by our antisera suggested that the target of proteolysis was in the C-terminal end of the protein. Several consensus sequences for caspase-3 mediated proteolysis are present in this region of GLCLC. We employed site-directed mutagenesis to systematically test the potential cleavage sites. The sequence AVVD^{499}G was found to be the target of GLCLC cleavage during apoptosis, as only the mutant D499A and not the others prevented cleavage of in vitro translated GLCLC by apoptotic cell extracts (see Appendix E). To test whether GLCLC proteolysis was a caspase-3 mediated event, we utilized in vitro translated ^{35}S-labeled GLCLC (wild type and D499A) in a cleavage assay with bacterially expressed caspase-3. The wild type GLCLC was cleaved directly by caspase-3 into a 60 kDa fragment and a 13 kDa cleaved fragment, however no cleavage was seen in the D499A GLCLC (fig. 23a). Mutation of GLCLC at Asp^{499} also prevented its cleavage in apoptotic

cells. HeLa cells were transfected with His-tagged GLCLC (WT or D499A) and treated with TNFα/cyclohexamide(CHX) or Fas/CHX to induce apoptosis. Wild type GLCLC was cleaved in cells following both treatments, however the D499A GLCLC was not subject to proteolytic cleavage. In aggregate, these experiments showed that GLCLC was a bona fide target of caspase-3 in vitro, and that Asp^{499} is the site at which GLCLC is cleaved in apoptotic cells.

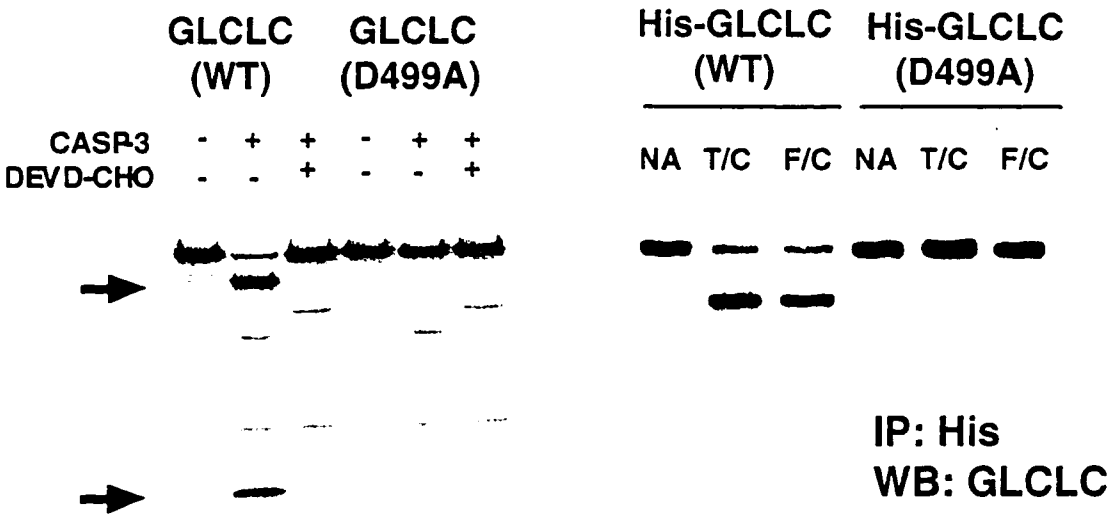

Figure 23. GLCLC is cleaved at Asp^{499}. (A) In vitro translated wild type and D499A GLCLC were subjected to in vitro cleavage by recombinant caspase-3. *Arrows* indicate location of the major cleavage products. (B) HeLa cells were transfected with 6His-tagged wild type and D499A GLCLC and treated with TNFa/CHX or anti-Fas/CHX as described in fig. 3. His-tagged proteins were purified from the cell extracts and immunoblotted for GLCLC. From Franklin et al., in preparation (Appendix E).

Cleaved GLCLC forms active holoenzyme with GLCLR.

The identification of Asp^{499} as the target site for caspase-mediated cleavage during apoptosis raised the possibility that the proteolytic event might alter GLCL function. Many of the proteins targets of caspases during apoptosis are inactivated by the cleavage, although there are also numerous examples of proteins which are activated by caspase mediated cleavage (Stroh and Schulze-Osthoff 1998, Nicholson 1999). There is remarkably little literature to indicate that caspase-mediated cleavage occurs without rendering some sort of functional change to the target protein. In the case of GLCLC, the cleavage site was found to be upstream of a cysteine residue (Cys^{553}) which had been described as important for the functional association between GLCLC and GLCLR. Tu

and Anders (1998b) described the site-directed mutagenesis of all cysteine residues in human GLCLC, and showed that only Cys553Leu resulted in an inability of GLCL to form a highly active holoenzyme. We therefore undertook to investigate the binding of cleaved GLCLC to GLCLR and the functional activity of a cleaved GLCL holoenzyme.

As the bacterially expressed Asp^{499} truncated protein was not stable, we utilized a bacterial co-expression system to generate cleaved GLCL, then incubated purified His-tagged cleaved or full-length GLCLC with bacterially expressed, untagged GLCLR to reconstitute GLCL holoenzyme. The associated GLCLR was thus pulled down with His-GLCLC as part of a holoenzyme complex. Purified proteins were eluted and subjected to native PAGE, followed by an in-gel assay for GLCL activity as described in Materials and Methods. The reconstitution/purification strategy for full length and cleaved GLCL holoenzyme is described in fig. 24.

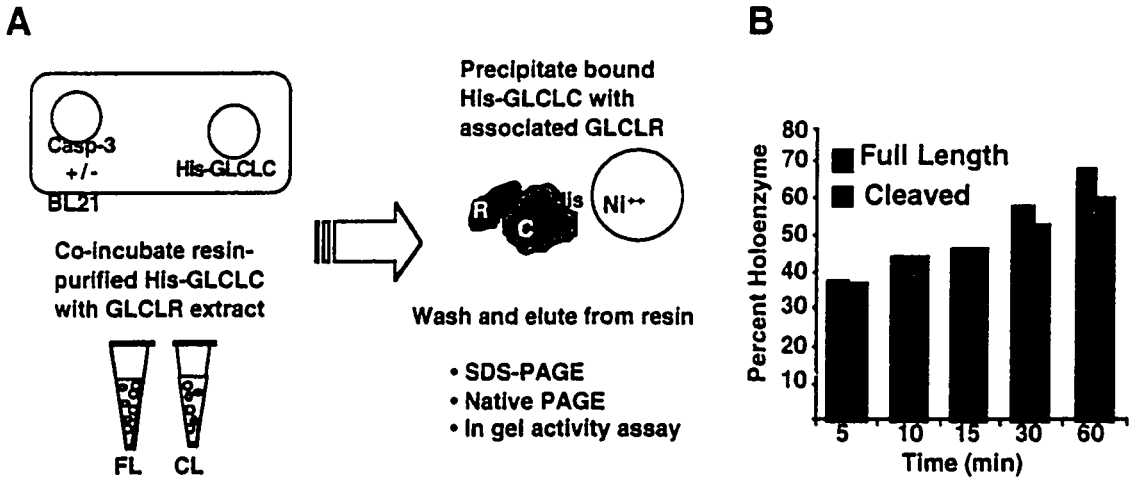

Figure 24. Holoenzyme reconstitution strategy for GLCL activity assays. (A) Holoenzyme reconstitution from purified full length and cleaved GLCLC. (B) Percentage of GLCLC in holoenzyme form, from densitometry of Coomassie-stained bands on native gels.

The reconstitution of GLCLC into holoenzyme with GLCLR was equivalent for the full length and cleaved forms of GLCLC (fig 24b). This suggests that the cleavage does not alter the binding of the two subunits. However, mutation of Cys^{553} did not prevent the physical association of GLCLC and GLCLR, rather it diminished the function of the holoenzyme thus formed (Tu and Anders 1998b). We developed an in gel activity assay to assess the relative function of the catalytic and holoenzyme forms of GLCL. Briefly,

proteins were separated by native PAGE on a 4-15% gradient gel, and the gel was incubated with GLCL substrates (ATP, cysteine, and glutamate) in the presence of $CeCl_3$. The generation of PO_4 was visualized as deposition of $CePO_4$ in coordination with the holoenzyme and GLCLC monomer bands in a Coomassie stained native gel. We found that GLCLC activity was much lower than that of the GLCL holoenzyme, and that increased incubation time for holoenzyme reconstitution (GLCLC + GLCLR; see fig. 24) resulted in increased holoenzyme activity (fig. 25). In addition, there was similar activity in the GLCL reconstituted from cleaved and full length GLCLC. However the cleaved form of GLCLC had a higher proportion of total protein in high molecular weight complexes than the full length form (fig 25, bottom 2 panels). The functional significance of these high molecular weight forms of cleaved GLCLC is unknown.

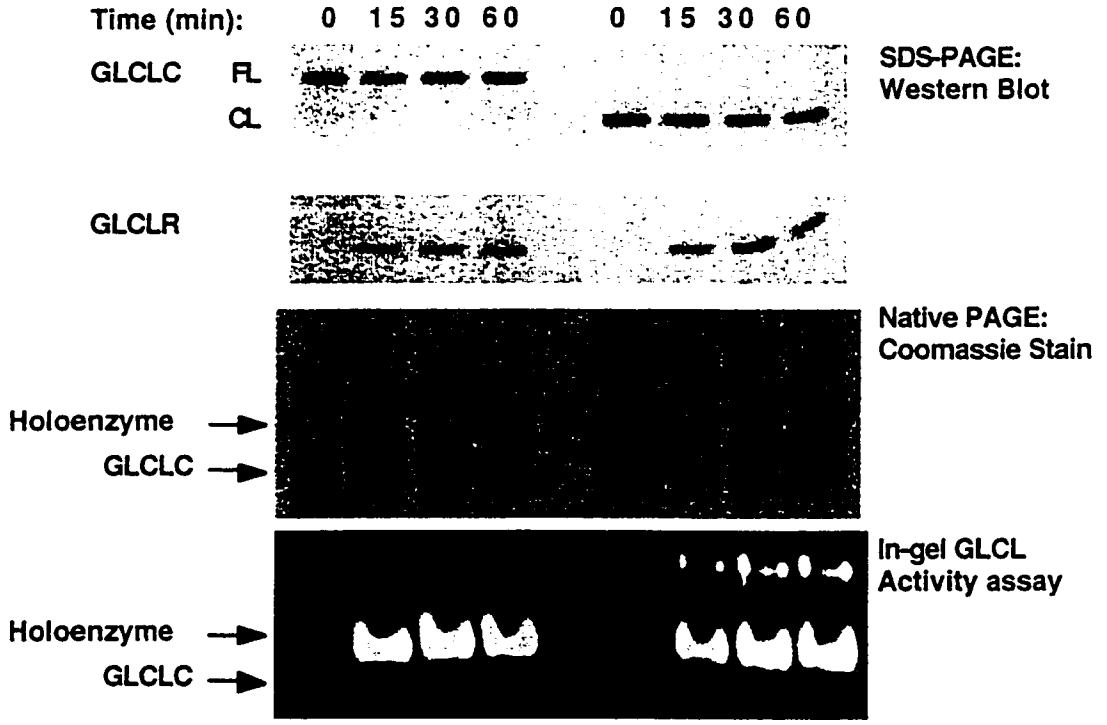

Figure 25. Holoenzyme reconstitution and in-gel assay for GLCL. Full length (left lanes) and cleaved GLCLC (right lanes) was co-incubated with GLCLR for the indicated times. Following purification of the GLCLC and associated GLCLR, the proteins were subjected to SDS-PAGE and immunoblotting for GLCLC and GLCLR (top panels), or native gel electrophoresis and staining with Coomassie blue (middle panel) or in-gel activity assay (bottom panel).

GLCL is activated in apoptotic cell extracts

To further assess the function of GLCL during apoptosis, we performed activity assays on the cytosols from apoptotic cells. Fig. 26 shows the results of two such assays from different apoptosis model systems. Both Jurkat cells treated with anti-Fas mAb, and AML12 hepatocytes treated with TNFα plus actinomycin D to induce apoptosis had increased GLCL activity following the apoptotic stimulus. The degree of upregulation is consistent with the response of GLCL to intracellular oxidative stress (see Chapter II). Interestingly, when size-exclusion chromatography was performed on extracts from anti-Fas treated Jurkat cells, the cleaved GLCLC fragment eluted ahead of the full length GLCLC fragment, indicating it was associated with molecular complexes of a larger size (fig. 27). This is also consistent with GLCL enzyme activation by oxidative stress. These results, taken together with the in-gel activity assay data, suggest that cleaved GLCLC can not only form active holoenzyme with GLCLR, but is also subject to post-translational mechanisms for upregulation of enzyme activity.

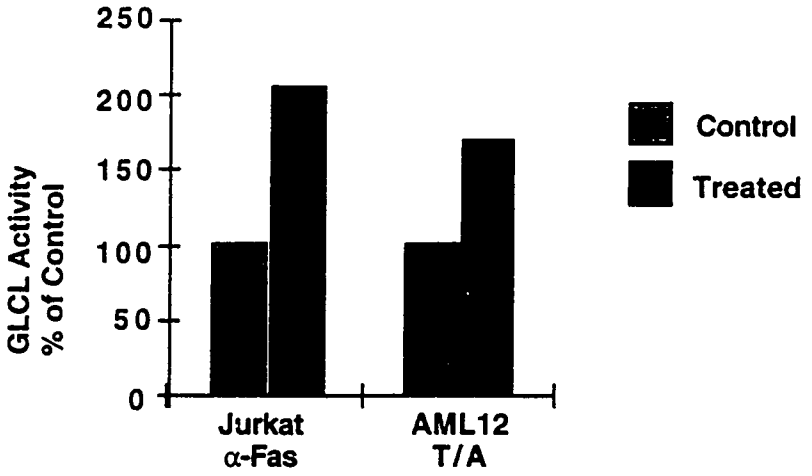

Figure 26. Upregulation of GLCL activity in apoptotic cells. Jurkat cells were treated for 8 h. with anti-Fas mAb, and AML12 cells were treated for 12 h. with TNFα plus actinomycin D to induce apoptosis. GLCL activity was assayed by the HPLC-based method for γ-GC formation (see Appendix B).

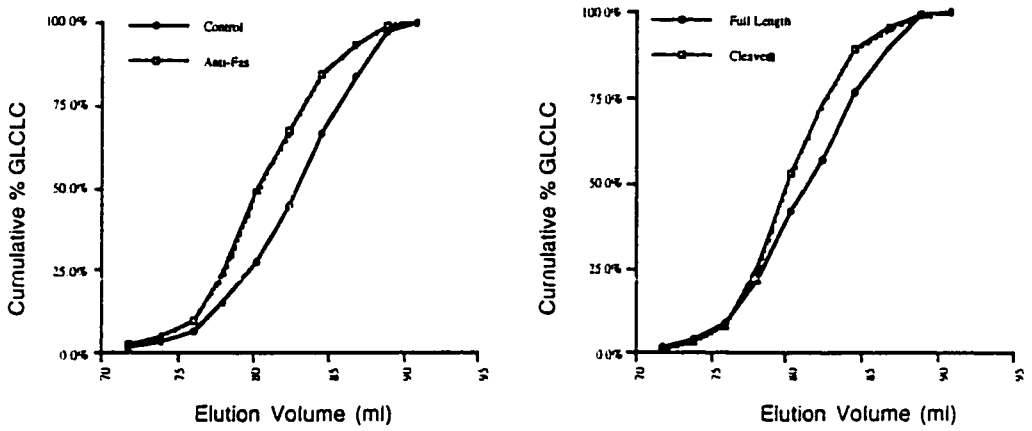

Figure 27. Size exclusion chromatography analysis of apoptotic Jurkat cells. Jurkat cells were treated for 12 h.with anti-Fas mAb, and extracts were subjected to SEC as described. A. Cumulative percentage of total GLCLC in fractions from control (circles) and anti-Fas treated (squares) Jurkat extracts. B. Comparison of cleaved vs. full length GLCLC in anti-Fas treated SEC fractions. Cumulative percentage of total full length (circles) and cleaved protein (squares) is shown.

DISCUSSION

The role of oxidative stress in apoptosis has been the subject of some controversy in recent years. Many inducers of apoptotic cell death cause a concomitant change in intracellular redox potential, and in numerous models the addition of antioxidants will block the apoptotic apparatus (Hall 1999). Ceramide, produced early in the apoptotic signaling cascade, can directly increase mitochondrial generation of ROS, and this effect is amplified by depletion of mitochondrial GSH (Garcia-Ruiz et al. 1997). The production of mitochondria-derived ROS also increases as mitochondrial function collapses; combined with the failure of cells to maintain GSH, NAD(P)H, and cardiolipin, this leads to the generation of oxidative stress in late apoptosis (Pierce et al. 2000). In addition, some cell types may actively push the balance toward a pro-oxidant state by exporting large amounts of reduced GSH early in apoptotis (van den Dobbelsteen et al. 1996).

It is unclear how these events play into the control of apoptosis, since several key mechanisms in the apoptotic process appear to require different redox states. For example, GSH was reported to inhibit neutral sphingomyelinase activity in vitro, and treatment with the GSH precursor N-acetyl cysteine prevented ceramide production in response to TNFα and IL-1b in astrocytes (Singh et al. 1998). Overexpression of GLCL suppressed TNFα induced apoptosis and inhibited NFκB and AP-1 signaling in hepatoma cells (Manna et al. 1999). In apoptotic thymocytes, GSH depletion was required for the

elevation of intracellular Ca^{2+} and subsequent endonuclease activation (Fernandez et al. 1995). In addition, the anti-apoptotic protein Bcl-2 has been shown to affect both the GSH content and subcellular distribution in cells, suggesting that part of its reported antioxidant effects are mediated through GSH (Meredith et al. 1998; Voehringer et al. 1998; Wright et al. 1998; Rimpler et al. 1999). On the other hand, caspases, which control both initiation and execution steps in apoptotic processes, require a reducing environment for maintenance of the active site cysteine (Stennicke and Salvesen 1997; Hampton et al. 1998). Some studies report that too much intracellular oxidation can interfere with apoptosis by inhibiting caspase activity, which either prevents death altogether or results in necrotic cell death, depending on the system studied (Boggs et al. 1998; Hampton and Orrenius 1998; Ueda et al. 1998; Hentze et al. 1999; Lee and Um 1999; Teramoto et al. 1999). More puzzling, whereas caspases require a reducing environment for activity, there are reports of caspase inhibition by both GSH and GSSG (Hentze et al. 1999; Watson et al. 1999). Such contradictory results suggest that the control of GSH level and redox status must be tightly regulated during apoptosis, or that different mechanisms for control of GSH prevail as the apoptotic process develops.

The cleavage of GLCLC from a 73 kDa full length form to a 60 kDa product may represent a mechanism for controlling GSH biosynthesis late in apoptosis. While the cleavage site occurs upstream of Cys^{553}, a residue identified as important for the formation of a highly active GLCL holoenzyme (Tu and Anders 1998b), the cleaved form of GLCLC can still bind GLCLR to produce an active holoenzyme. This suggests that the cleavage of GLCLC may exert subtle effects upon GLCL activity, which were not detected by the in-gel assay used to visualize holoenzyme reconstitution. For example, the cleavage may complement other post-translational modifications that occur in vivo but were not present in the in-vitro system. GLCL activity is modulated by intracellular oxidation state (see Chapter II). Increased GLCL activity in apoptotic cells could be due to ROS production following receptor engagement and/or mitochondrial dysfunction. Another consideration in the studies presented here is that both the in-gel reconstitution assay and the HPLC assay for cytosolic extracts were performed at saturating substrate conditions. Preliminary studies with cytosols from Jurkat cells treated with anti-Fas mAb and vector- and caspase-3 transfected MCF7 cells treated with TNFα/CHX showed decreased Km_{glu} in the apoptotic Jurkat cells and the caspase-3+ MCF7 cells, compared with untreated controls (data not shown). Interestingly, the apoptotic vector-transfected MCF7 cells, which lack

caspase-3, did not have altered kinetics compared with untreated controls, although they were shown to export GSH at a similar rate as the caspase-3+ cells. This suggests that GLCLC cleavage may alter GLCL function in a way that is distinguishable from the activation of GLCL by GSH depletion. Additional in vitro experiments must be performed to assess if cleavage of GLCLC directly alters the kinetic parameters of the GLCL holoenzyme, and studies with His-GLCLC transfected cells are needed to understand how GLCLC cleavage may synergize with other signals to activate GSH biosynthesis in vivo.

It is certainly possible that GLCLC is an innocent bystander in the apoptotic process. While the biology of some caspase substrates is understood, there are many targets of caspases whose function in apoptosis is unknown (Stroh and Schulze-Osthoff 1998). To directly assess the importance of GLCLC cleavage during apoptotic cell death, an in vivo model which replaces wild type GLCLC with the uncleavable GLCLC D499A mutant is needed. Future studies from our laboratories will address this question.

The fact that cleaved GLCLC bound GLCLR to form an active holoenzyme was a surprising result, given the body of literature suggesting that oxidative stress is an important feature in apoptosis. Data from studies employing pharmacological manipulation of intracellular GSH and transfection of GLCL have shown that high GSH levels or GLCL activity can inhibit the apoptotic process (Um et al. 1996; Deas et al. 1997; Singh et al. 1998; Hall 1999; Manna et al. 1999). Furthermore, reduced GSH is actively exported during apoptosis in many in vitro models (van den Dobbelsteen et al. 1996). However these studies, which have focused on the dying cells, were not designed to account for the effects of an apoptotic death on surrounding cells. Under most physiological conditions, apoptotic cell death occurs sparsely within a tissue. One function of apoptosis (as opposed to necrosis, or uncontrolled cell death) is to preserve the surrounding cells and to prevent inflammatory responses, thereby reducing the impact of cell death to a single, dying cell. There is good reason to believe that GSH export could be a mechanism for limiting the impact of apoptosis to the initially damaged cell. The production of ROS is greatly enhanced by the mitochondrial dysfunction that accompanies apoptotic cell death. If uncontrolled, this oxidative stress could influence signal transduction pathways in neighboring cells, leading to the activation of redox sensitive transcription factors such as NFκB. This may also be true of the macrophages

that scavenge apoptotic cells early in the process of cell death. Activation of the NFκB in scavenging macrophages could lead to the production of TNFα and other inflammatory cytokines. Local increases inflammatory cytokines and adhesion molecules could lead to infiltration of neutrophils, which are capable of perpetuating oxidative injury through the activation of myeloperoxidase and NADPH oxidase activities. Once an inflammatory response is initiated, it is difficult to control the redox environment of a tissue, and further production of ROS, necrotic cell death, and increasing inflammation can lead to tissue injury. A similar progression toward tissue failure is seen in ischemia/reperfusion injury. The biosynthesis of GSH, and its active export by dying cells, may serve to preserve the functional integrity of engulfing macrophages and surrounding cells, and ultimately to protect the tissue from any impacts of cell death.

NOTES TO CHAPTER III

The studies in this chapter were performed in collaboration with Dr. Chris Franklin of the UW Department of Pathology. Appendix E presents the paper we jointly prepared for submission, whereas this chapter focuses more on my contributions to the project. We also acknowledge the contributions of Dr. Robert Pierce, who helped with the animal studies and originally noticed the cleavage of GLCLC in his AML-12 model, and Collin White, who assisted with the HPLC assays for GLCL activity and worked on the development of the in-gel GLCL assay. Dr. Nelson Fausto and Dr. Terry Kavanagh provided direction and support for this project. Funding was provided by NIH grants CA75316, ES07032, CA23226 and CA74131, and ES04696, AG01751, and ES07033.

CHAPTER IV: SUMMARY OF RESULTS

The studies presented herein have detailed three mechanisms by which GLCL protein is regulated in human lymphocytes, under different conditions of cellular function. First, the protein levels of GLCLC and GLCLR are upregulated in response to receptor mediated and mitogen driven cell proliferation. There appears to be a multiplicity of pathways by which this induction can occur, depending on the responsiveness of the cell to a particular stimulation. CD3 stimulation is important for the upregulation of GLCLC and GLCLR in naive $CD4^+$ T cells; whereas CD28 stimulation appears to be more efficient for the upregulation of GLCL in previously activated, resting $CD4^+$ PHA blasts. Second, the activity of GLCL in lymphocytes exposed to oxidative stress is under post-translational control. The mechanism of upregulation of enzymatic activity involves a structural change which correlates with both the heterodimerization of GLCLC and GLCLR into a highly active holoenzyme, and their shift to a higher molecular weight fraction within the cell. The proximal mediator(s) of this change remain undefined. However, it appears that the formation of a disulfide bridge between GLCLC and GLCLR is not required for activation of the GLCL holoenzyme. Third, GLCLC is the target of caspase mediated cleavage during apoptotic cell death in lymphocytes and other cells examined. The sequence $AVVD^{499}G$ is a *bona fide* substrate for caspase-3 in vitro, and the cleavage of GLCLC in apoptotic cells requires active caspase-3 to occur. The 60 kDa fragment of GLCLC cleavage is stable and binds to GLCLR to form a functional holoenzyme. While preliminary studies hint at differences in function between the cleaved GLCL and the full length GLCL holoenzyme, further work must be done to define the kinetic parameters of cleaved GLCLC and its regulation in cells.

The variety of mechanisms for control of GLCL in lymphocytes is not surprising, given the body of literature which defines lymphocytes as very sensitive to redox changes in general, and GSH status in particular. As these cells contain less GSH than most tissues, and very little GLCL in their resting state, it seems clear that the mechanisms by which they control the production of GSH will figure largely into the signal transduction systems that govern their functional development. Several researchers have shown a connection between lymphocyte GSH content and defined human diseases. The high affinity antisera against GLCLC and GLCLR that were developed and characterized for the present work

will be useful in ongoing studies of GSH biosynthetic enzymes in lymphocytes from patients and healthy subjects.

The present work will also add to the literature on the basic biology of GLCL. The control of GLCL activation described herein is by mechanisms which may indeed apply to a variety of tissues. It appears that the upregulation of GLCL in response to mitogens is more closely linked with the proliferative response than with a particular lymphocyte signaling pathway. The activation of GLCL post-translationally occurred in many cell types besides lymphocytes, and formation of high molecular weight GLCL complexes was also seen in various tissues. Cleavage of GLCLC by a caspase-3 dependent mechanism occurred in all the models of apoptosis we tested and may provide an additional mechanism for control of GLCL activity during apoptosis. How GLCLC cleavage complements the activation of GLCL by intracellular oxidative stress remains to be seen.

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C u r r i c u l u m V i t a e

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2. Educational Background

1987. B.A. Biology. Bates College, Lewiston ME. Characterization of immunomodulatory effects of pokeweed antiviral protein.

2000. Ph.D. Toxicology. Department of Environmental Health, University of Washington, Seattle WA. Activation of glutamate-cysteine ligase in lymphocytes.

3. Employment

1985-1986. Student Intern. American Red Cross Blood Research Laboratories. Bethesda, MD. *In vivo* studies of MHC Class I tolerization as a strategy for allograft acceptance.

1988-1992. Research Assistant, Laboratory Instructor. Marine Research Laboratory and Environmental Studies Program, Bowdoin College, Brunswick, ME. Managed a teaching lab, directed laboratory and field research projects, and developed curriculum for a new course on environmental research methods.

1990-1993. Environmental Consultant (private contractor). Durham ME and Seattle WA. Conducted ecological research for natural resource damage assessments (NRDA) following marine oil spills.

1994-1997. Visiting Scientist/Collaborator. Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle WA. Studied vanadium-based phosphotyrosine phosphatase inhibitors and intracellular oxidation in lymphocytes. Developed and characterized polyclonal antisera against the antioxidant enzyme glutamate-cysteine ligase.

1997-2000. NIEHS Fellow. Environmental Pathology and Toxicology Training Program, University of Washington, Seattle WA. Conducted original research on the activation of glutamate-cysteine ligase in lymphocytes by protein induction, post-translational modifications and caspase-mediated cleavage.

4. Scholarships, Fellowships, Honors and Awards

1982-1987 Bates College, Tuition Scholarships

1983 Bates College, Dana Fellowship

1995 Society of Toxicology, Student Travel Award

1995 Gordon Research Conference, Student Travel Award

1997-2000, NIEHS Fellowship, Environmental Pathology and Toxicology Training Grant

1997 Oxygen Society, Young Investigator Award

1999 UW School of Public Health and Community Medicine, Outstanding Student Award

2000 Pacific Northwest Association of Toxicologists, Sheldon D. Murphy Award

5. Professional Activities:

Professional Memberships: American Association for the Advancement of Science, Society of Toxicology, Pacific Northwest Association of Toxicologists, The Oxygen Society, Washington State Public Health Association

1995-1997 Student Representative, Informatics Committee, UW Department of Environmental Health

6. Bibliography

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8. Jarvinen, K., P.P.Pietarinen-Runtti, K. Linnainmaa, K.O. Raivio, C.M. Krejsa, T.J. Kavanagh, and V.L. Kinnula. "Role of glutathione and manganese superoxide dismutase in oxidant and drug resistance of human mesothelioma and lung adenocarcinoma cells." Am. J. Physiol. Lung Cell Mol. Physiol. 278(4):L696-702.
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Reviews and Technical Reports

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2. Gilfillan, E.S., D.S. Page, and C.M. Krejsa. (1991) "Use of multivariate statistical techniques to follow community succession from oil impact to recovery in the field." Proc. 1991 Oil Spill Conf., American Petroleum Institute, Washington, DC.
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2. Krejsa, C.M., J.A. Ledbetter, and T.J. Kavanagh (1995) "Oxidant induced changes in red autofluorescence in lymphocytes and lymphoblastoid cell lines correlates with decreased glutathione." Toxicologist 15: 158a.
3. Krejsa, C.M., J.M. Esselstyn, J.A. Ledbetter, G.L. Schieven and T.J. Kavanagh. (1995) "The role of oxidative stress in the action of a peroxovanadium phosphotyrosine phosphatase inhibitor." Gordon Research Conference "Mechanisms in Toxicity", Meriden, NH.
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