

# Regulation of Antioxidant Metabolism by Translation Initiation Factor 2 $\alpha$

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**Abstract.** Oxidative stress and highly specific decreases in glutathione (GSH) are associated with nerve cell death in Parkinson's disease. Using an experimental nerve cell model for oxidative stress and an expression cloning strategy, a gene involved in oxidative stress-induced programmed cell death was identified which both mediates the cell death program and regulates GSH levels. Two stress-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor (eIF)2 $\alpha$  and express a low amount of eIF2 $\alpha$ . Sensitivity is restored when the clones are transfected with full-length eIF2 $\alpha$ ; transfection of wild-type cells with the truncated eIF2 $\alpha$  gene confers resistance. The phosphorylation of eIF2 $\alpha$  also results in resistance to oxidative stress. In wild-type cells, oxidative

stress results in rapid GSH depletion, a large increase in peroxide levels, and an influx of Ca<sup>2+</sup>. In contrast, the resistant clones maintain high GSH levels and show no elevation in peroxides or Ca<sup>2+</sup> when stressed, and the GSH synthetic enzyme  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ GCS) is elevated. The change in  $\gamma$ GCS is regulated by a translational mechanism. Therefore, eIF2 $\alpha$  is a critical regulatory factor in the response of nerve cells to oxidative stress and in the control of the major intracellular antioxidant, GSH, and may play a central role in the many neurodegenerative diseases associated with oxidative stress.

**Key words:** oxidative stress • glutathione • eIF2 $\alpha$  • resistance • glutamate

## Introduction

Although programmed cell death (PCD)<sup>1</sup> is a widely used mechanism for sculpturing the developing nervous system, its inappropriate activation leads to premature nerve cell death in neuropathological disorders such as Alzheimer's disease (AD) (Yankner, 1996) and Parkinson's disease (PD) (Mochizuki et al., 1996). Nerve cell death in both PD and AD are thought to be linked to oxidative stress, as antioxidant systems are upregulated and there is extensive evidence for excessive lipid and protein peroxidation (Jenner and Olanow, 1996; Simonian and Coyle, 1996). Associated with oxidative stress is an early and highly specific decrease in the glutathione (GSH) content of the substantia nigra of PD patients (Perry et al., 1982; Sofic et al., 1992; Sian et al., 1994) which may precede the death of dopaminergic neurons (Dexter et al., 1994). In addition, the inhibition of  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ GCS), the rate-limiting step in GSH synthesis, results in the selective degeneration of dopaminergic neurons (Jenner and Olanow, 1996) and also potentiates the toxicity of 6-hydroxy-

dopamine, MPTP and MPP<sup>+</sup>. These data suggest that GSH and oxidative stress play pivotal roles in the pathogenesis of AD and PD.

There are several ways in which the concentration of intracellular GSH and the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non-receptor-mediated pathway which involves the glutamate-cystine antiporter, system Xc<sup>-</sup> (Bannai and Kitamura, 1980; Murphy et al., 1989; Sato et al., 1999). Under normal circumstances, the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc<sup>-</sup> antiporter in exchange for intracellular glutamate. Cystine is ultimately converted to cysteine and used for protein synthesis and to make the antioxidant GSH. However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is inhibited, and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of events which include the depletion of GSH, a requirement for macromolecular synthesis and caspase activity, lipoxygenase activation, soluble guanylate cyclase activation, reactive oxygen species (ROS) accumulation, and finally Ca<sup>2+</sup> influx (Murphy et al., 1989; Li et al., 1997a,b; Tan et al., 1998a,b). PCD caused by oxidative glutamate

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<sup>1</sup>Abbreviations used in this paper: AD, Alzheimer's disease; DCF, dichlorofluorescein; eIF, translation initiation factor; GSH, glutathione;  $\gamma$ GCS, gamma-glutamyl cysteine synthetase; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PCD, programmed cell death; PD, Parkinson's disease; ROS, reactive oxygen species.

toxicity has characteristics of both apoptosis and necrosis (Tan et al., 1998a) and has been well studied in primary neuronal cell cultures (Murphy and Baraban, 1990; Oka et al., 1993), neuronal cell lines (Miyamoto et al., 1989; Murphy et al., 1989), tissue slices (Vornov and Coyle, 1991), and in the immortalized mouse hippocampal cell line, HT22 (Li et al., 1997a,b; Tan et al., 1998a,b). HT22 cells lack ionotropic glutamate receptors but die within 24 h after exposure to 1–5 mM glutamate. Although the biochemical events have been well studied, little has been done to identify the transcriptional/translational changes that contribute to the glutamate-induced pathway of PCD. Changes in gene expression clearly play a role in the cell death cascade since macromolecular synthesis is required early in the death pathway (Tan et al., 1998a,b). Through the use of a genetic screen, we identified the  $\alpha$  subunit of the translation initiation factor 2 (eIF2 $\alpha$ ) as a gene whose expression is involved in oxidative stress-induced cell death and the regulation of intracellular GSH. eIF2 is a trimeric complex involved in the initiation of translation (Hershey, 1991; Pain, 1996). The complex is made up of three subunits designated alpha, beta, and gamma, and behaves in a manner analogous to the trimeric G-coupled proteins. The  $\alpha$  subunit dictates whether protein synthesis will or will not take place and is often referred to as the control point for protein synthesis. The eIF2 complex brings the 40S ribosomal subunit together with the initiating tRNA<sub>met</sub> when eIF2 $\alpha$  is bound to GTP. Upon hydrolysis of GTP to GDP, the complex is no longer active and protein synthesis is not initiated. GDP/GTP exchange takes place readily with the assistance of a guanine nucleotide exchange factor, eIF2B. However, when the  $\alpha$  subunit of eIF2 is phosphorylated on serine 51, a change in the conformation enables it to bind and sequester eIF2B, thus inhibiting GDP/GTP exchange and protein synthesis. eIF2 $\alpha$  phosphorylation takes place during ischemia (DeGracia et al., 1997; Burda et al., 1998), apoptosis (Srivastava et al., 1998; Satoh et al., 1999), viral infection (Samuel, 1993; Wek, 1994), and after Ca<sup>2+</sup> influx (Prostko et al., 1995; Srivastava et al., 1995; Reilly et al., 1998). Therefore, eIF2 $\alpha$  may have significant roles in the cell death process after oxidative stress that are separate from its known function as a regulator of protein synthesis. The experiments described below show that the downregulation or phosphorylation of eIF2 $\alpha$  protects nerve cells from oxidative stress-induced cell death by inhibiting GSH depletion and the increase in both ROS and intracellular Ca<sup>2+</sup> that are normally seen in cells exposed to oxidative stress. These data demonstrate a unique role of eIF2 $\alpha$  in oxidative stress-induced programmed nerve cell death, acting as a translational switch which dictates whether a cell activates a survival response or follows a cell death pathway. eIF2 $\alpha$  may therefore play a central role in neuropathologies involving nerve cell death which are associated with oxidative stress.

## Materials and Methods

The following chemicals were purchased from Sigma-Aldrich: puromycin, TCA, formic acid, GSH, GSH reductase, triethanolamine, sulfosalicylic acid, NADPH, BSA, glutaraldehyde, and L-glutamic acid (glutamate). The fluorescent probes 2',7'-dichlorofluorescein (DCF) diacetate and in-

doacetoxymethylester (Indo-1), pluronic F-127, and propidium iodide were obtained from Molecular Probes. The Coomassie Plus protein assay reagent and the SuperSignal substrate were both purchased from Pierce Chemical Co. Immobilon P was purchased from Millipore.

## Infection with the Retroviral cDNA Library

HT22 cells were infected with the retroviral vector pLXSN containing a cDNA library derived from the human embryonic lung cell line, MRC-5 (Somia et al., 1999). The library contained  $2 \times 10^6$  cDNAs, and the HT22 cells were infected with  $\sim 10^7$  virus particles. The cDNA library contains both sense and antisense sequences. The retrovirus stably integrates into the host cell's genomic DNA and expresses the cDNA inserted between its long terminal repeats. Clones containing genes that confer glutamate resistance were identified by selecting cells that survived in 10 mM glutamate. Genomic DNA from each clone was analyzed by PCR using primers that straddle the cDNA insert in the retroviral vector. The cDNA inserts were then subcloned and sequenced. Viral vectors were rescued from the clones by transfection with an ecotropic helper plasmid. These viral particles were collected from the media and used to infect the packaging cell line, PA317, which amplified the virus (Miller et al., 1993). The viral medium from the packaging cells was then used to infect wild-type HT22 cells in order to confirm that the cDNA was indeed able to make the HT22 cells resistant to glutamate.

## Immunoblotting and Northern Blot Procedures

Cells were plated at  $5 \times 10^5$  cells per 100-mm dish 12–16 h before use and lysed in sample buffer containing 3% SDS. Lysates were sonicated, protein concentrations were normalized using the Coomassie Plus protein assay reagent from Pierce Chemical Co., and 25  $\mu$ g protein was loaded per lane on 12% Tris-glycine SDS-PAGE gels (Novex). Gels were transferred onto Immobilon P membrane (Millipore) and blocked with 5% milk in TBS for 1 h at room temperature. An antibody against eIF2 $\alpha$  (Research Genetics) was shown previously to recognize only phosphorylated eIF2 $\alpha$ . However, in our hands the antibody recognized both phosphorylated and unphosphorylated protein when the Western blots and lysates were dephosphorylated with a mixture of bovine and calf intestine alkaline phosphatase. Blots were also probed with antibodies against both phosphorylated and total mitogen-activated protein kinase to confirm that proteins were completely dephosphorylated after treatment with the phosphatases. Therefore, this anti-eIF2 $\alpha$  antibody was used to determine the levels of total eIF2 $\alpha$  in the HT22 cells and the resistant clones 8 and 15. The anti-eIF2 $\alpha$  primary antibody was diluted into 5% BSA in TBS plus Tween 20 (TTBS) at 1:250 and placed on the blot overnight at 4°C. Blots were incubated with the secondary antibody, goat anti-rabbit IgG HRP conjugated (Bio-Rad Laboratories), for 1 h at room temperature at a dilution of 1:20,000 in 5% milk in TTBS. Blots were exposed to Eastman Kodak Co. X-OMAT Blue film for chemiluminescence using the SuperSignal substrate from Pierce Chemical Co.

Northern blots of the  $\gamma$ GCS catalytic subunit were done as described in the original paper in which cDNA clones were isolated (Gipp et al., 1992). Northern blots were done using a probe consisting of the COOH-terminal 387 amino acids of the protein which detected a single band of  $\sim 3.7$  kb.

## Transfection of Full-Length eIF2 $\alpha$ into Clones 8 and 15

The full-length cDNA for eIF2 $\alpha$  was obtained from Dr. Miyamoto (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) and was cloned into the pCLBAbE<sub>puro</sub> retroviral vector, a modified version of the pBAbE<sub>puro</sub> vector (Morgenstern and Land, 1990). This vector was then used for transfection with Lipofectamine (GIBCO BRL).

## Production of Retrovirus Expressing the Dominant Negative Mutants of eIF2 $\alpha$

The cDNA constructs for two mutants of eIF2 $\alpha$  (S51A and S51D) were obtained from Dr. Kaufman (University of Michigan, Ann Arbor, MI) and subcloned into pCLBAbE<sub>puro</sub>. Retroviral vectors were made as described (Somia et al., 1999) with either pCLBAbE-S51A, pCLBAbE-S51D, or pCLBAbE<sub>puro</sub> alone. The viral vectors were used to infect HT22 cells, and infected cells were selected in 4  $\mu$ g/ml puromycin (Sigma-Aldrich). The puromycin-resistant cells were tested for glutamate resistance by the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) cell death assay (Tan et al., 1998b).

## Translation and Degradation Assays

For translation assays, cells were labeled in 60-mm dishes with 500,000 cpm of [<sup>3</sup>H]leucine diluted in DME supplemented with 10% FBS for 30 min. The cells were then washed with ice-cold serum-free DME and lysed on the dish using 1 ml ice-cold 10% TCA plus 1 mM DTT and 1 mM cold leucine. Cellular protein was precipitated, dissolved in formic acid, and the [<sup>3</sup>H]leucine incorporation was determined by scintillation counting. The protein concentration was determined using the Coomassie blue plus protein reagent (Pierce Chemical Co.). The total counts per minute of [<sup>3</sup>H]leucine incorporated per milligram of protein for 30 min was calculated for each sample. Samples were prepared in triplicate. Protein degradation assays were done exactly as described elsewhere (Soucek et al., 1998). Cells were treated with 100 μg/ml cycloheximide, and protein abundance followed by Western blotting.

## Growth Assays

Five sets of triplicate dishes of cells were plated at  $5 \times 10^4$  in 35-mm dishes. The triplicate sets of each cell type were counted at 12, 24, 48, and 72 h after plating. Cells were dissociated using pancreatase (GIBCO BRL) for 15 min, resuspended in DME, and placed in Eppendorf tubes. Cells were counted directly on a Beckman Coulter counter after dilution in isotonic saline. The data are plotted as cell counts versus time in order to compare the growth rates for the different clones.

## GSH Assay

Total intracellular reduced GSH and oxidized GSH (GSSG) were measured as described previously (Tan et al., 1998b). In brief, cells were plated on 60-mm tissue culture dishes at  $2 \times 10^5$  cells per dish 12 h before adding 2–5 mM glutamate for 10 h and total GSH was assayed. Pure GSH was used to establish a standard curve.

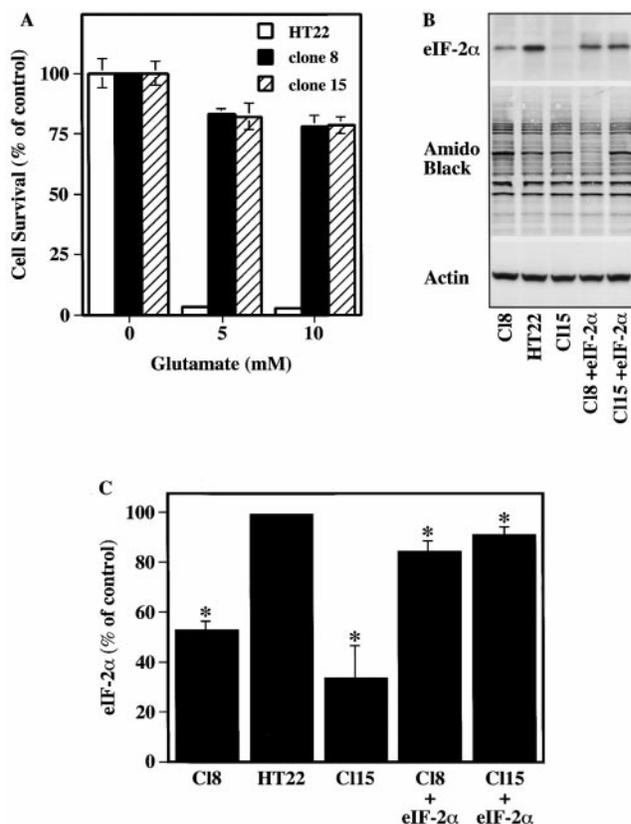
## Flow Cytometric Studies

Cells were plated on 60-mm dishes at  $2 \times 10^5$  cells per dish 12 h before adding 2–5 mM glutamate for 10 h. Samples were then labeled with the fluorescent dyes DCF and Indo-1 to determine ROS production and Ca<sup>2+</sup> influx, respectively. Samples were prepared as described previously (Tan et al., 1998b).

## Results

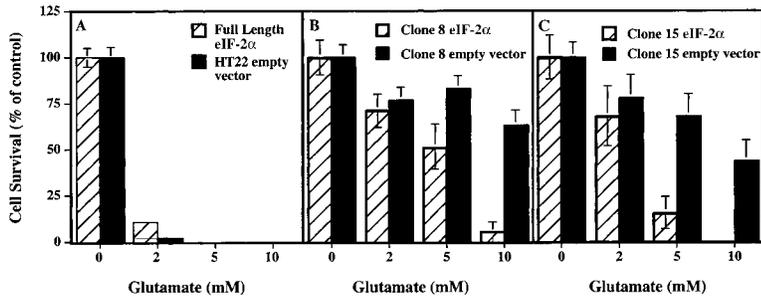
### eIF2α Is Involved in the Oxidative Glutamate Toxicity Pathway

Although a mechanistic outline of oxidative glutamate toxicity-mediated PCD has been developed (Li et al., 1997a,b; Tan et al., 1998a,b), very little is known about the changes in gene expression that are required for this pathway. To identify genes that may be involved in cell death or the protection from cell death, HT22 cells were infected with a cDNA expression library in a retroviral vector, and cells resistant to high concentrations of glutamate were selected. The retroviral library contained sense, antisense, and partial cDNA sequences. Therefore, glutamate resistance could be due to a sense cDNA which when overexpressed causes glutamate resistance. Alternatively, a transcript from an antisense cDNA could interfere with the expression of a gene normally required for cell death, or the product of a partial cDNA fragment may act in a dominant negative manner to block protein function. A fourth alternative is that during retroviral infection, a cDNA is inserted into the genome in a way that disrupts or upregulates the normal expression of a gene that is involved in glutamate-induced cell death. Finally, ploidy sometimes changes in the cells as they divide, and resistant cells may arise independently of the retroviral infection due to loss of chromosomes or chromosome fragments. This consti-



**Figure 1.** Clones 8 and 15 are resistant to glutamate and express low levels of eIF2 $\alpha$ . Glutamate-resistant cells were cloned after infecting HT22 cells with a retroviral cDNA expression library. (A) Cell viability was measured using the MTT assay with wild-type HT22 cells (white bars), clone 8 (black bars), and clone 15 (hatched bars) after a 24-h exposure to 0, 5, and 10 mM glutamate. Samples were measured in triplicate on 96-well plates ( $n = 10$ ). (B and C) Glutamate-resistant clones 8 and 15 have lower levels of eIF2 $\alpha$  protein than wild-type HT22 cells which are largely restored by the reintroduction of wild-type eIF2 $\alpha$ . (B) eIF2 $\alpha$  and actin protein levels were detected by Western blotting of cell lysates (25  $\mu$ g) from wild-type HT22 cells, glutamate-resistant clones 8 and 15, and clones 8 and 15 transfected with wild-type eIF2 $\alpha$ . Loading controls were actin (blot) and amido black staining of the blot. Note that the single band which is reduced in the stained blot in the C18 + eIF2 $\alpha$  lane is albumin from the serum in the growth medium which is somewhat variable because of washing. (C) The density of each protein band was measured using the program NIH Image, and the average density for each band was plotted relative to eIF2 $\alpha$  in wild-type HT22 cells. Identical amounts ( $\pm 5\%$ ) of actin in each lane served as loading controls. The experiment was repeated at least five times with similar results. \*Significantly different from HT22 wild-type controls (mean  $\pm$  SEM,  $P < 0.05$ ).

tutes a background level of naturally resistant clones in the genetic screen. HT22 cells were infected with the retroviral cDNA library and selected in 10 mM glutamate for 48 h, a condition where all of the cells normally die. The cDNA inserts in the pool of glutamate-resistant cells were rescued by remobilizing the vector (Miller et al., 1993), and a second round of infection and selection identified 12 genes that play a putative role in oxidative glutamate toxicity. Identical fragments (213 bp) of the gene encoding



**Figure 2.** Glutamate-resistant clones 8 and 15 acquire glutamate sensitivity after transfection with full-length eIF2 $\alpha$ . Wild-type HT22 cells and glutamate-resistant clones 8 and 15 were stably transfected with an expression construct of eIF2 $\alpha$ . (A) The wild-type HT22 cells were unaffected by transfection of eIF2 $\alpha$ . (B and C) Resistant clones 8 and 15 when transfected with the eIF2 $\alpha$  construct became glutamate sensitive as detected by the MTT assay after 24 h of exposure to 2, 5, and 10 mM glutamate. Samples were measured in triplicate ( $n = 3$ ).

the  $\alpha$  subunit of eIF2 (eIF2 $\alpha$ ) were identified in two separate clones. This gene was chosen for further study because of the requirement for protein synthesis in this form of cell death (Tan et al., 1998a,b). The subclones of HT22, designated clones 8 and 15, are extremely resistant to 10 mM glutamate and were maintained in the presence of 10 mM glutamate (Fig. 1 A). These clones are also resistant to other forms of oxidative stress, including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and tert-butyl hydroperoxide but not to cell death inducers such as TNF- $\alpha$ , anti-FAS antibody, serum starvation, and glucose deprivation (data not shown).

### Clones 8 and 15 Cause Glutamate Resistance by Lowering eIF2 $\alpha$ Expression

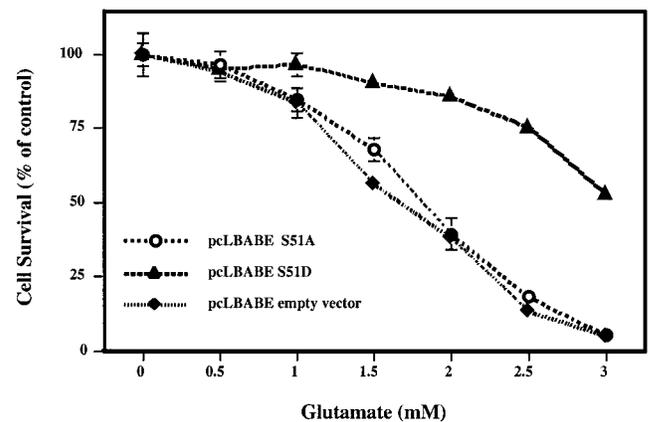
As outlined previously, the introduction of the eIF2 $\alpha$  gene fragment into clones 8 and 15 with the retroviral cDNA library could lead to stress resistance by one of several mechanisms. It is unlikely that the eIF2 $\alpha$  gene fragment is causing glutamate resistance by disrupting or upregulating a gene whose expression is involved in cell death because the same sequence generates glutamate resistance upon reinfection. This leaves the possibility that the eIF2 $\alpha$  cDNA fragment is altering eIF2 $\alpha$  expression. Therefore, the two resistant clones and wild-type cells were assayed for eIF2 $\alpha$  expression by Western blotting. Although the antibody used for these studies can identify the phosphorylated form of eIF2 $\alpha$  (DeGracia et al., 1997), it recognizes both the dephosphorylated and phosphorylated forms of eIF2 $\alpha$  in HT22 cells (see Materials and Methods). Using this antibody, it was found that both clones 8 and 15 express lower levels of eIF2 $\alpha$  protein (Fig. 1, B and C). Similar results were obtained with another antibody against eIF2 $\alpha$  (Ernst et al., 1987).

Since the retroviral expression library contained cDNAs in both the sense and antisense orientations as well as partial fragments of cDNAs, it is likely that an antisense fragment was expressed to downregulate eIF2 $\alpha$  expression. The gene fragments that were rescued from clones 8 and 15 are identical and contain a fragment of the eIF2 $\alpha$  cDNA from the 3' end of the full sequence (728–941 bp). Antisense gene fragments from cDNA libraries in retroviral vectors have been used previously to identify physiologically relevant genes (Gudkov and Roninson, 1997). If the downregulation of eIF2 $\alpha$  in the resistant clones is responsible for the resistance of the cells to glutamate, then the expression of full-length eIF2 $\alpha$  should restore the sensitivity to glutamate. Transfection of full-length eIF2 $\alpha$  human cDNA into both clones 8 and 15 restored glutamate sensitivity to both of the clones, whereas the empty vector had no effect (Fig. 2, B and C). The restoration of

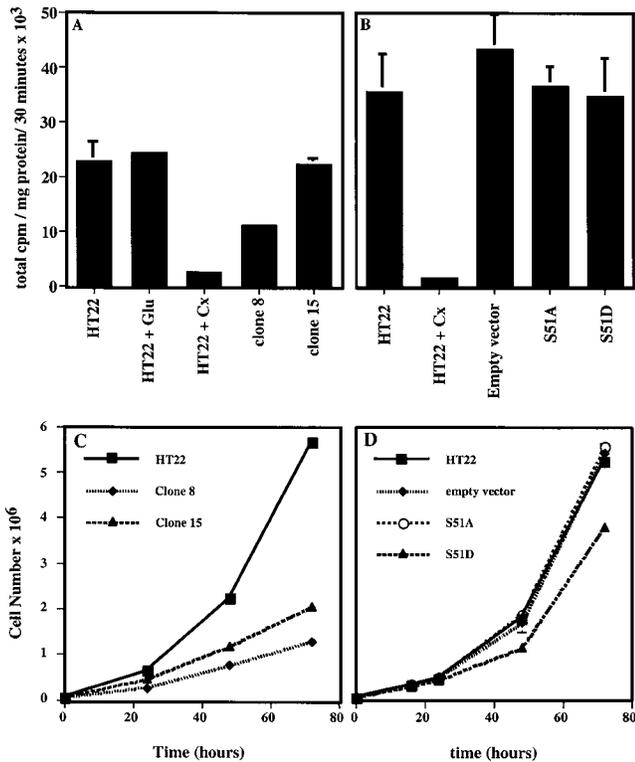
glutamate sensitivity is not, however, up to the level of wild-type cells at the highest glutamate concentrations, probably because it was only possible to elevate eIF2 $\alpha$  to 80–90% of its original level (Fig. 1, B and C). Wild-type HT22 cells remained sensitive to glutamate after being transfected with the full-length eIF2 $\alpha$  cDNA (Fig. 2 A). This demonstrates that modulation of eIF2 $\alpha$  expression has significant effects on glutamate toxicity in HT22 cells.

### eIF2 $\alpha$ Phosphorylation Also Mediates Glutamate Resistance

To confirm that the loss of eIF2 $\alpha$  activity is linked to glutamate resistance, a second method was employed which utilizes a dominant negative approach to regulate eIF2 $\alpha$  function. The phosphorylated form of eIF2 $\alpha$  sequesters the guanine nucleotide exchange factor, eIF2 $\beta$ , resulting in a decrease in protein translation (Ernst et al., 1987). The S51D mutant of eIF2 $\alpha$  mimics constitutive phosphorylation when serine 51 in eIF2 $\alpha$  is replaced with an aspartic acid (Kaufman et al., 1989). The S51A mutant cannot be phosphorylated when serine 51 in eIF2 $\alpha$  is replaced with alanine (Pathak et al., 1988). Thus, the S51D mutant inhibits protein synthesis while the S51A mutant prevents the shutdown of protein translation by the phosphorylation of eIF2 $\alpha$ . To assay the effect of eIF2 $\alpha$  phosphorylation on glutamate sensitivity, wild-type HT22 cells were infected with virus that contained either the S51D or



**Figure 3.** HT22 cells become glutamate resistant when the eIF2 $\alpha$  S51D mutant is stably expressed. HT22 cells were infected with virus containing either the pCLBABE empty vector ( $\blacklozenge$ ), the S51A mutant of eIF2 $\alpha$  ( $\circ$ ), or the eIF2 $\alpha$  S51D mutant ( $\blacktriangle$ ). Cell viability was measured by the MTT assay. Samples were prepared in triplicate ( $n = 4$ ).

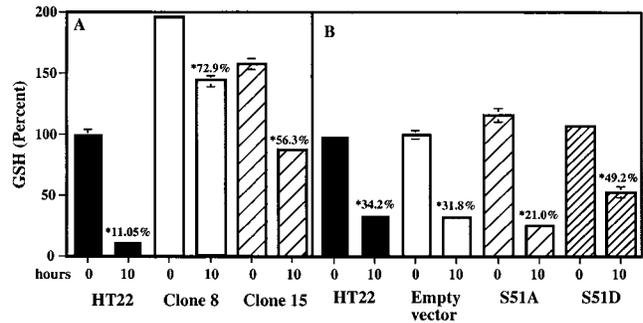


**Figure 4.** Effect of eIF2 $\alpha$  expression on the rate of protein translation. Protein synthesis was measured by [ $^3$ H]leucine incorporation for 30 min and normalized to total protein. (A) Wild-type HT22 cells (HT22); cells exposed to 5 mM glutamate for 4 h (HT22 + Glu); cycloheximide (HT22 + Cx); and clones 8 and 15. (B) Wild-type HT22 cells and cells infected with either empty vector or the eIF2 $\alpha$  phosphorylation mutants S51A or S51D. All assays were performed in triplicate ( $n = 2$ ). (C) The growth rate is decreased when eIF2 $\alpha$  is downregulated or phosphorylated in HT22 cells. Growth rates were measured by counting cells at 0, 24, 48, and 72 h after plating. Wild-type HT22 cells (■); resistant clone 15 (▲); and resistant clone 8 (◆). (D) HT22 cells (■); cells infected with empty vector (◆); eIF2 $\alpha$  mutant S51A (○); and mutant S51D (▲). Cell counts at all time points were done in triplicate, and the data are presented as the average  $\pm$  SEM.

S51A mutant or an empty vector, and the cells were tested for glutamate resistance. HT22 cells infected with virus containing the mutant S51D become more resistant to glutamate (Fig. 3). The S51A mutant of eIF2 $\alpha$  did not have any effect on the response of the cells to glutamate relative to empty vector (Fig. 3). These data show that the downregulation of eIF2 $\alpha$  activity by protein phosphorylation can lead to glutamate resistance and that eIF2 $\alpha$  phosphorylation may play an important role in cell death or survival after glutamate exposure. However, we could not directly assay eIF2 $\alpha$  phosphorylation after glutamate exposure because none of the available antibodies immunoprecipitate or distinguish phosphorylated from unphosphorylated eIF2 $\alpha$  in HT22 cells.

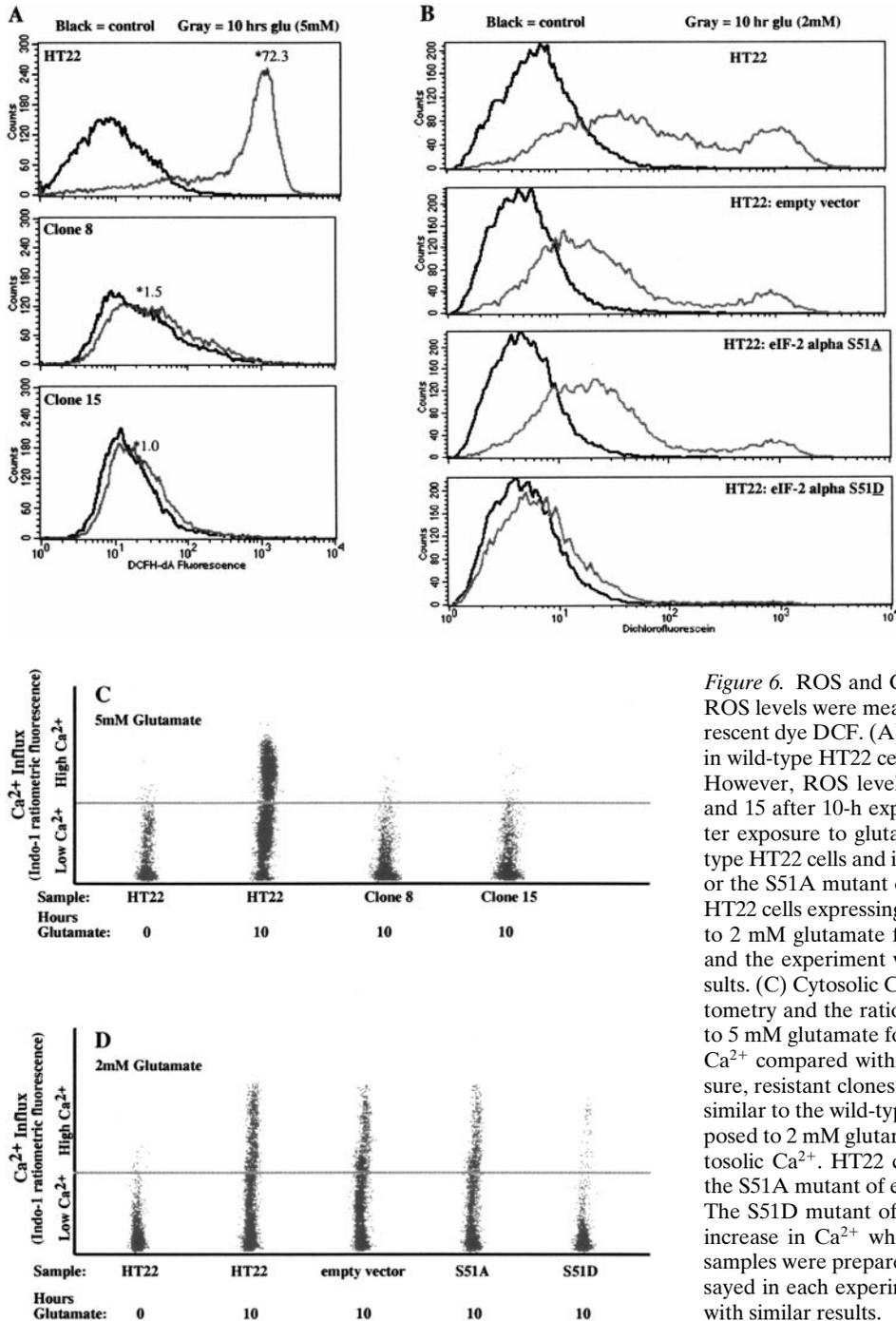
#### Changes in eIF2 $\alpha$ Expression Do Not Affect Translation Rates but Do Slow Growth

To determine if eIF2 $\alpha$  downregulation in the glutamate-resistant clones causes a decrease in protein synthesis, protein



**Figure 5.** GSH levels in resistant cells. GSH levels were measured in control, untreated cells, and cells exposed to 5 or 2 mM glutamate for 10 h. (A) 5 mM glutamate. Clones 8 (white bars) and 15 (hatched bars) have higher basal GSH levels than wild-type HT22 and only deplete to  $72 \pm 4$  and  $56 \pm 1\%$  of their basal GSH levels, respectively, with glutamate exposure. (B) 2 mM glutamate. Wild-type HT22 cells (black bars), empty vector infected cells (white bars), and S51A mutant infected cells (hatched bars) show GSH levels that are depleted to 20–30% of basal levels. In the S51D mutant-expressing cells (narrow hatched bars), GSH depleted to only  $\sim 50\%$  of the basal level. 100% GSH is defined as the GSH level assayed in the untreated control cells. The numbers above the glutamate exposed bars indicate the percentage of GSH relative to the basal level in the same cell line ( $n = 3$ ).

translation rates were measured in clones 8 and 15 as well as in cells expressing mutants S51A and S51D. By inhibiting translation with cycloheximide, HT22 cells are able to survive in the presence of glutamate for short periods of time (Tan et al., 1998a,b). Therefore, it was important to determine if the inhibition of translation is the sole mechanism by which clones 8 and 15 and the S51D mutant-expressing cell line become resistant to oxidative stress. To measure the rate of translation, cells were labeled with [ $^3$ H]leucine for 30 min and the total counts per minute of incorporated leucine per milligram of protein calculated. The rate of translation in clone 15 is the same as in wild-type HT22 cells, but it is reduced about twofold in clone 8 (Fig. 4 A). The rate of protein translation is unchanged in HT22 cells after infection with retrovirus containing the eIF2 $\alpha$  mutants (S51A and S51D) or empty vector (Fig. 4 B). Similarly, exposure of HT22 cells to glutamate during a 10-h time course does not lead to any significant changes in overall protein translation (data not shown). These data indicate that the inhibition of overall protein synthesis is not the mechanism underlying protection by eIF2 $\alpha$ . However, the translation rates do not reflect the growth rates for each clone, as the growth rate of the wild-type HT22 cell line is more than twofold faster than either clone 8 or 15 (Fig. 4 C). HT22 cells infected with the eIF2 $\alpha$  mutant S51D also have a slower growth rate than wild-type HT22 cells (Fig. 4 D) even though the protein translation rate of this mutant is the same as that in the wild-type cells (Fig. 4 B). In contrast, the S51A mutant has no significant effect on the translation rate (Fig. 4 B) or the growth rate (Fig. 4 D). These data show that changes in eIF2 $\alpha$  expression or activation by phosphorylation may lead to alterations in cell growth but not necessarily translation rates. However, it is possible that although the bulk of protein synthesis is not altered, the synthesis of specific proteins required for cell proliferation and cell death is regulated by altered eIF2 $\alpha$  expression or phosphorylation.

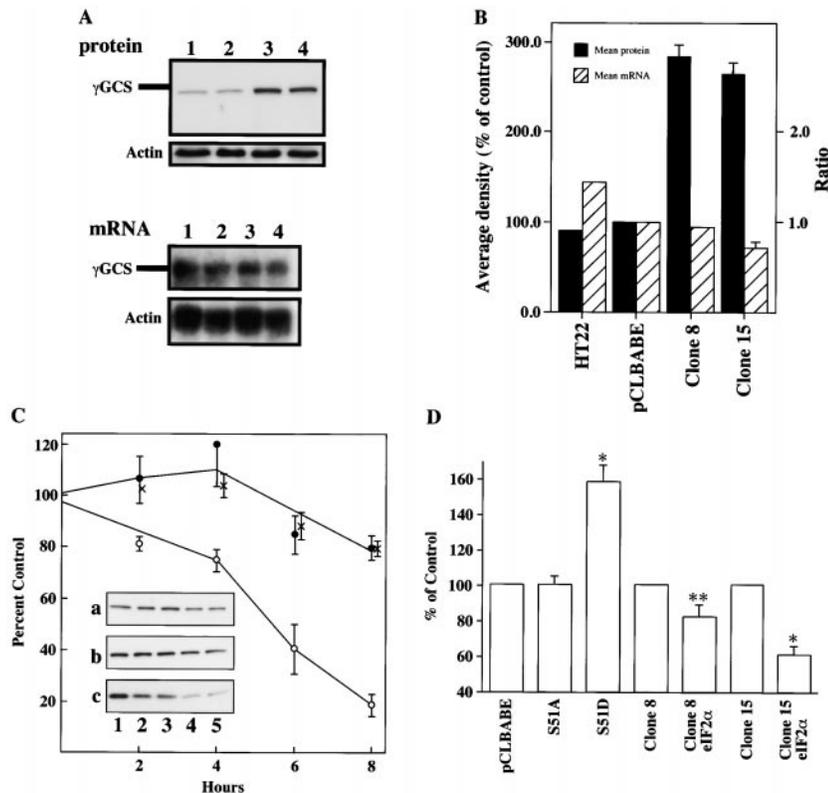


**Figure 6.** ROS and Ca<sup>2+</sup> levels after exposure to glutamate. ROS levels were measured by flow cytometry using the fluorescent dye DCF. (A) ROS levels increase 72-fold (gray line) in wild-type HT22 cells exposed to 5 mM glutamate for 10 h. However, ROS levels do not increase in resistant clones 8 and 15 after 10-h exposure to glutamate. (B) ROS levels after exposure to glutamate are increased (gray line) in wild-type HT22 cells and in HT22 cells infected with empty vector or the S51A mutant of eIF2 $\alpha$ . ROS levels do not increase in HT22 cells expressing the eIF2 $\alpha$  mutant S51D when exposed to 2 mM glutamate for 10 h. 10,000 live cells were assayed, and the experiment was repeated two times with similar results. (C) Cytosolic Ca<sup>2+</sup> levels were measured using flow cytometry and the ratiometric dye Indo-1. HT22 cells exposed to 5 mM glutamate for 10 h have a large increase in cytosolic Ca<sup>2+</sup> compared with untreated cells. After glutamate exposure, resistant clones 8 and 15 maintain cytosolic Ca<sup>2+</sup> levels similar to the wild-type untreated control. (D) HT22 cells exposed to 2 mM glutamate for 10 h have a large increase in cytosolic Ca<sup>2+</sup>. HT22 cells infected with the empty vector or the S51A mutant of eIF2 also show similar increases in Ca<sup>2+</sup>. The S51D mutant of eIF2 $\alpha$  prevents the glutamate-induced increase in Ca<sup>2+</sup> when stably expressed in HT22 cells. All samples were prepared in duplicate. 10,000 live cells were assayed in each experiment, and the study was repeated twice with similar results.

### *eIF2 $\alpha$ Expression Alters Glutathione, ROS, and Ca<sup>2+</sup> Responses to Glutamate*

To understand the role of eIF2 $\alpha$  in oxidative glutamate toxicity, several parameters of the glutamate response were measured in the resistant clones and the S51A and S51D mutant-expressing cell lines and compared with the wild-type HT22 cells. HT22 cells undergo a rapid depletion of GSH upon exposure to glutamate (Tan et al., 1998b). After 8 h of exposure to glutamate, GSH levels drop below 20% of their normal levels. Comparison of wild-type HT22 cells to glutamate-resistant clones 8 and 15 after 10 h of exposure to 5 mM glutamate revealed that

the GSH levels in the resistant cells do not go below 50% of the GSH levels in untreated resistant clones. Furthermore, before glutamate exposure, both cell lines have higher GSH levels than untreated wild-type HT22 cells (Fig. 5 A). The maximal difference in survival between the S51D mutant-expressing cell line and the control HT22 cells is detected at 2 mM glutamate (Fig. 3). When GSH levels in wild-type cells infected with the S51A or S51D mutants or the empty vector are measured after 10 h of exposure to 2 mM glutamate, the S51D mutant cell line shows a decrease to ~50% of the original level compared with the 70% decrease in the wild-type and empty vector-



**Figure 7.**  $\gamma$ GCS protein expression is regulated at the level of translation. (A)  $\gamma$ GCS, actin protein, and mRNA expression was measured in wild-type HT22 cells (lane 1), cells infected with the empty vector (pCLBABLE) (lane 2), and the resistant clones 8 (lane 3) and 15 (lane 4) by Western and Northern blot analysis, respectively. (B) The Western blot from A was analyzed using the program NIH Image to determine the densities of each band. The densities were measured in four experiments, averaged, and normalized first to actin and then to the level of  $\gamma$ GCS in pCLBABLE, set as 1.0. Actin served as a loading control and showed that there was an equal amount of protein in each lane. Northern blots were quantitated on a PhosphorImager. The ratio of the catalytic subunit of  $\gamma$ GCS to actin is presented normalized to  $\gamma$ GCS in pCLBABLE as 1.0. The results were confirmed by reverse transcription PCR analysis (data not shown). (C) Proteolytic breakdown of  $\gamma$ GCS and P27 in wild-type and resistant cells. HT22 cells and resistant clone 15 were treated with 100  $\mu$ g/ml cycloheximide and the amount of  $\gamma$ GCS and P27 was quantitated by Western blot at 2-h intervals. The values are normalized to 0 time and are the mean  $\pm$  SEM of triplicate experiments. Inset, Western blots of  $\gamma$ GCS, wild-type cells (a);  $\gamma$ GCS, clone 15 (b); P27, Cl 15

(c). Lanes 1, 2, 3, 4, and 5 are 0, 2, 4, 6, and 8 h after cycloheximide. x,  $\gamma$ GCS, wild-type; ●,  $\gamma$ GCS Cl 15; ○, P27. (D) Resistant clones 8 and 15 were infected with wild-type eIF2 $\alpha$ , the wild-type HT22 clone was infected with S51A or S51D, and the levels of  $\gamma$ GCS and actin were determined by Western blotting. The amounts of  $\gamma$ GCS and actin were quantitated and the amount of  $\gamma$ GCS was normalized to the actin loading control. In each set of cells, the transfected cells were then normalized to  $\gamma$ GCS in their parental line pCLBABLE, resistant clone 8 or 15, which was set at 100%. The data are presented as the mean  $\pm$  SEM,  $n = 4$ . \*Significantly different from parental cells ( $P < 0.01$ ); \*\*significantly different from parental cells ( $P < 0.05$ ).

infected cells. On the other hand, the S51A mutant cell line shows a decrease in GSH to  $\sim$ 20% of control levels (Fig. 5 B). This pattern of GSH depletion is consistent with the survival data which demonstrate that although the S51D-expressing HT22 cells are still healthy and dividing after 24 h of glutamate exposure, the other cell lines are dead (Fig. 3). HT22 cells exposed to glutamate for 10 h show a very large increase in ROS which follows the drop in GSH (Tan et al., 1998b). The fluorescent dye DCF was used to determine the levels of ROS production by flow cytometry in the resistant and mutant cell lines after exposure to toxic levels of glutamate. The level of ROS in wild-type HT22 cells after exposure to 5 mM glutamate for 10 h is increased  $>70$ -fold (Fig. 6 A). In contrast, the glutamate-resistant cell lines 8 and 15 do not show an increase in ROS above normal levels, and the cells survive and continue to divide (Fig. 6 A). When HT22 cells are exposed to 2 mM glutamate for 10 h, the cells die and there is an increase in ROS, although the DCF intensity is more diffuse than with 5 mM glutamate (Fig. 6 B). The same pattern of increased DCF is seen in cells expressing empty vector and the mutant S51A. However, HT22 cells expressing the mutant S51D have low levels of ROS and were able to survive glutamate treatment (Fig. 6 B). Finally, Ca<sup>2+</sup> influx was measured in wild-type, resistant, and phosphorylation mutant-expressing cells. Ca<sup>2+</sup> levels were

determined by FACS<sup>®</sup> analysis using the ratiometric dye Indo-1 (Tan et al., 1998b). After 10 h of exposure to 5 mM glutamate, HT22 cells have much higher levels of Ca<sup>2+</sup> than untreated controls, whereas resistant cell lines 8 and 15 maintained intracellular Ca<sup>2+</sup> levels similar to those of the wild-type HT22 cells (Fig. 6 C). The same experiment was performed on the phosphorylation mutant-expressing cell lines exposed to 2 mM glutamate. Ca<sup>2+</sup> levels increase significantly in the wild-type HT22 cells as well as the empty vector and S51A-expressing HT22 cells. The intracellular Ca<sup>2+</sup> level in the S51D-expressing cells remained similar to the HT22 cells that were not exposed to glutamate (Fig. 6 D). These data show that both the down-regulation of eIF2 $\alpha$  in clones 8 and 15 and the expression of the dominant negative phosphorylation mutant S51D all prevent the decrease in GSH and the increases in ROS and Ca<sup>2+</sup> normally associated with oxidative stress-induced cell death.

#### **The Inactivation of eIF2 $\alpha$ Upregulates $\gamma$ GCS Expression by a Translational Mechanism**

Resistant clones 8 and 15 have decreased eIF2 $\alpha$  activity and increased basal levels of GSH. Furthermore, the resistant clones and the cells expressing the phosphorylation mutant, S51D, maintain GSH levels 50% of their basal levels after glutamate exposure. To determine if there is a

causal relationship between eIF2 $\alpha$  protein levels and GSH production, the expression of the rate-limiting enzyme for GSH synthesis,  $\gamma$ GCS, was examined in the wild-type cells and the resistant clones. Protein expression and mRNA levels of the catalytic subunit of  $\gamma$ GCS were measured by Western and Northern blotting, respectively. Western blotting shows that the level of the catalytic subunit of  $\gamma$ GCS is threefold higher in the resistant clones than in the wild-type HT22 cells (Fig. 7, A and B). In contrast, when both  $\gamma$ GCS and actin mRNA were quantitated and their ratio normalized to cells expressing the empty pCLBACE retroviral vector, the amount of  $\gamma$ GCS mRNA remained relatively constant (Fig. 7, A and B). To rule out the possibility that eIF2 $\alpha$  activity changes the rate of  $\gamma$ GCS breakdown, resistant clone 15 and wild-type cells were treated with cycloheximide and the rate of protein loss followed by Western blotting. This method gives values of protein turnover identical to pulse-chase experiments (Soucek et al., 1998). The rapidly turned over cell cycle protein, P27, served as a positive control (Soucek et al., 1998). Fig. 7 C shows that in contrast to P27,  $\gamma$ GCS was degraded more slowly but at the same rate in resistant and wild-type cells. These results indicate that a decrease in eIF2 $\alpha$  wild-type protein levels leads to an increase in production of the catalytic subunit of  $\gamma$ GCS by a translational mechanism, resulting in significantly higher levels of GSH.

If eIF2 $\alpha$  directly regulates  $\gamma$ GCS expression, then its expression should be upregulated in wild-type cells made resistant by the S51D phosphorylation mutant and downregulated in the resistant cells which were transfected with wild-type eIF2 $\alpha$  to render them more sensitive to oxidative stress. Fig. 7 D shows that the levels of  $\gamma$ GCS increased  $\sim$ 60% in cells transfected with S51D relative to wild-type cells. In contrast, the expression of  $\gamma$ GCS decreased between 20 and 40% in the resistant clones 8 and 15 which already have a high level of  $\gamma$ GCS protein when these clones were transfected with normal  $\alpha$ IF2 $\alpha$  (Fig. 7 B). These data, along with those presented above, strongly suggest that eIF2 $\alpha$  expression and activity can directly modulate  $\gamma$ GCS protein levels. It is also likely that the expression of additional proteins involved in the resistance to oxidative stress is regulated by eIF2 $\alpha$ .

## Discussion

The above data show that eIF2 $\alpha$  plays a central role in programmed nerve cell death initiated by oxidative stress. Alterations in either the level of eIF2 $\alpha$  or its phosphorylation protect cells from glutamate-induced oxidative stress as well as other prooxidant agents. We will first discuss the evidence for the involvement of eIF2 $\alpha$  in glutamate-induced cell death, followed by possible mechanisms that eIF2 $\alpha$  could use to signal this type of cell death. The potential relevance of eIF2 $\alpha$  nerve cell death in PD will also be discussed.

### *eIF2 $\alpha$ Is Specifically Involved in Oxidative Glutamate Toxicity*

HT22 glutamate-resistant clones 8 and 15 were derived from a genetic screen after infection with a retrovirus-based cDNA expression library and selection with a high concentration of the prooxidant glutamate. Both clones contain an identical fragment of the gene for eIF2 $\alpha$  from the retroviral library.

The following evidence shows that eIF2 $\alpha$  activity is required for cells to die via oxidative glutamate toxicity and other forms of oxidative stress: (a) eIF2 $\alpha$  fragments rescued from the glutamate-resistant cells make wild-type cells resistant to glutamate upon reinfection; (b) Western blotting demonstrates that the eIF2 $\alpha$  protein levels in the resistant clones are lower than in wild-type HT22 cells; and (c) eIF2 $\alpha$  downregulation alone causes resistance to glutamate since clones 8 and 15, when transfected with full-length human eIF2 $\alpha$ , become glutamate sensitive. Since eIF2 $\alpha$  regulates the rate of protein translation and cell death requires protein synthesis, it is possible that the inhibition of cell death simply reflects a decrease in the rate of protein synthesis in the resistant cells. However, the decrease of eIF2 $\alpha$  in the resistant cells did not necessarily lead to a slower rate of protein synthesis. Although clones 8 and 15 are equally resistant to glutamate, only clone 8 has a rate of protein synthesis which is lower than that in the wild-type cells. In addition, cells infected with the eIF2 $\alpha$  phosphorylation mutant S51D, which also induces glutamate resistance, synthesize protein at a rate that is equal to that of the wild-type cells. These results indicate that a decrease in the rate of translation per se does not lead to glutamate resistance. Further evidence that eIF2 $\alpha$  phosphorylation plays a key role in determining the fate of the glutamate-exposed HT22 cells is evident when the S51D mutant of eIF2 $\alpha$  is expressed in the HT22 cells, resulting in glutamate resistance. The S51D mutant mimics a constitutively phosphorylated form of eIF2 $\alpha$  that cannot be dephosphorylated, such that it is able to sequester the guanine nucleotide exchange factor, eIF2B, and inhibit the initiation of protein synthesis (Ernst et al., 1987; Kaufman et al., 1989). Since the infection of HT22 cells with either eIF2 $\alpha$  or the phosphorylation mutants leads to overexpression of their respective transcripts but does not alter the overall levels of eIF2 $\alpha$  protein (data not shown), the amount of eIF2 $\alpha$  protein that is synthesized must be highly regulated. In contrast to our data, the S51D mutant causes apoptosis when transiently transfected into another cell line (Srivastava et al., 1998), presumably because it shuts down protein synthesis. However, in the HT22 cells expressing the S51D mutant, the cells maintain a normal protein synthesis rate, although the growth rate is slower than in the wild-type cells (Fig. 4 D). One explanation for how the HT22 cells infected with the S51D mutant are able to maintain reasonable translation and growth rates is that after infection with the S51D mutant, cells that greatly overexpress the mutant die, whereas the cells that mildly overexpress the mutant protein are able to survive at a slightly slower growth rate. This is likely because the infected cells become less resistant to glutamate with time. Therefore, they probably express sufficient amounts of the S51D mutant to survive glutamate exposure, but the cells that express the lower amounts of the mutant insufficient for survival in glutamate are eventually able to outgrow the other cells when not in the presence of glutamate.

### *eIF2 $\alpha$ Downregulation and the Constitutively Phosphorylated Form of eIF2 $\alpha$ Alter the Same Intermediates in the Cell Death Pathway*

The observation that the two glutamate-resistant clones selected by expression cloning and the overexpression of the phosphorylation mutant, S51D, produce similar changes in cell physiology during glutamate exposure fur-

ther supports the critical role of eIF2 $\alpha$  in the toxicity cascade. These cell lines all exhibit higher GSH levels than controls after glutamate exposure and lower levels of ROS and intracellular Ca<sup>2+</sup>. GSH levels in wild-type HT22 cells decline to <20% of controls after glutamate exposure, whereas GSH levels in both the resistant clones and the cells expressing the dominant negative S51D mutant drop to <50% of their basal levels. In contrast to control levels, this level of GSH is sufficient to maintain cell viability (Sagara and Schubert, 1998). The basal levels of GSH in the resistant clones were also higher than in the wild-type HT22 cells. It could be argued that the lower rate of translation and cell growth in the resistant cells frees up more cysteine, allowing them to maintain a higher basal level of GSH. However, clone 15 has a very high basal level of GSH but a normal rate of protein synthesis, suggesting that the resistant cells have higher GSH levels because they actively produce greater amounts of this antioxidant.

The above results suggest that the downregulation or phosphorylation of eIF2 $\alpha$  during times of stress signals the translation of specific proteins that increase cell survival. Since decreases in either eIF2 $\alpha$  activity or protein levels both lead to an increase in GSH, we asked if the rate-limiting enzyme in GSH production,  $\gamma$ GCS, was increased in the resistant cells compared with the wild-type HT22 cells. Fig. 7 shows that although the amount of  $\gamma$ GCS is increased in the original resistant clones, the  $\gamma$ GCS mRNA level remains constant and there is no difference in the rates of  $\gamma$ GCS breakdown. In addition,  $\gamma$ GCS is upregulated by the phosphorylation mutant, S51D, and downregulated by the introduction of additional eIF2 $\alpha$  into the glutamate-resistant clones 8 and 15 (Fig. 7). These data show that eIF2 $\alpha$  regulates  $\gamma$ GCS expression by a translational mechanism. Amino acid starvation in *Saccharomyces cerevisiae* also causes eIF2 $\alpha$  phosphorylation and leads to the selective translation of one specific transcription factor that signals the synthesis of amino acids so that the yeast can survive starvation (Samuel, 1993). A mechanism comparable to that employed by the yeast may be used in HT22 cells when eIF2 $\alpha$  activity is low, leading to an increased production of  $\gamma$ GCS to promote cell survival. In addition, it was recently shown that another form of stress, the unfolded protein response, causes the phosphorylation of  $\alpha$ IF2 $\alpha$  and the increased translation of activating transcription factor 4 (Harding et al., 2000).

### *eIF2 $\alpha$ Plays a Unique Role in Programmed Cell Death*

There have been several reports that positively link eIF2 $\alpha$  to apoptosis: eIF2 $\alpha$  phosphorylation by double-stranded RNA-activated protein kinase is the cause of cell death in TNF- $\alpha$ -stimulated cells (Srivastava et al., 1998), and eIF2 $\alpha$  is cleaved by caspases after an increase in PKR kinase activity induced by TNF- $\alpha$  or poly(I):poly(C) (Satoh et al., 1999). However, HT22-resistant clones 8 and 15 are not resistant to TNF- $\alpha$ , indicating that they utilize a survival mechanism that is unique to oxidative stress. Ischemia and reperfusion in the rat brain also lead to eIF2 $\alpha$  phosphorylation and cell death (DeGracia et al., 1997; Burda et al., 1998). In these cases, it was argued that death signals lead to eIF2 $\alpha$  phosphorylation, protein synthesis shutdown, and cell lysis. In contrast, our data show that eIF2 $\alpha$  phosphorylation protects cells from death. HT22

cells treated with thapsigargin, a substance shown to cause eIF2 $\alpha$  phosphorylation (Prostko et al., 1995), also leads to cell survival after glutamate exposure (data not shown). Finally, although it is generally assumed that any response to central nervous system injury is part of the cell death mechanism, it is equally likely that such a response is a component of a survival pathway (Maher and Schubert, 2000). Therefore, the nature of the stimulus and the extent of eIF2 $\alpha$  phosphorylation determine whether eIF2 $\alpha$  will be used to prevent or promote cell death. The above experiments link oxidative stress, GSH depletion, and the regulation of  $\gamma$ GCS directly to eIF2 $\alpha$  and programmed nerve cell death. Markers for both oxidative stress and the depletion of intracellular GSH are found in areas of central nervous system nerve cell death in PD (Sian et al., 1994). However, in both PD and AD large numbers of nerve cells do survive. It is therefore important to understand the mechanisms which lead to resistance to oxidative stress. In the brain, intracellular GSH is the single most important antioxidant, and GSH-peroxidase breaks down H<sub>2</sub>O<sub>2</sub> and a variety of organic peroxides, thus protecting cells from oxidative stress. The experiments presented here show that changes in the expression level or phosphorylation of a member of the protein translation complex, eIF2 $\alpha$ , can regulate the ability of a nerve cell to deal with oxidative stress. This appears to be primarily done through the regulation of GSH levels, as sustained GSH depletion is the initial event which triggers downstream events such as peroxide accumulation and ultimately cell death. Cells with low amounts of eIF2 $\alpha$  or phosphorylated eIF2 $\alpha$  maintain high levels of GSH when stressed and do not die. These results point to a central role of eIF2 $\alpha$  as a translational switch in the control of oxidative stress within the nervous system. They also suggest a possible therapeutic target for manipulating intracellular GSH levels.

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### *References*

- Bannai, S., and E. Kitamura. 1980. Transport interaction of L-cysteine and L-glutamate in human diploid fibroblasts in culture. *J. Biol. Chem.* 255:2372–2376.
- Burda, J., M. Martín, M. Gottlieb, M. Chavko, J. Marsala, A. Alcázar, M. Pavón, J. Fando, and M. Salinas. 1998. The intraschismic and early reperfusion changes of protein synthesis in the rat brain. eIF-2 $\alpha$  kinase activity and role of initiation factors eIF-2 $\alpha$  and eIF-4E. *J. Cereb. Blood Flow Metab.* 18: 59–66.
- DeGracia, D., J. Sullivan, R. Neumar, S. Alousi, K. Hikade, J. Pittman, B. White, J. Rafols, and G. Krause. 1997. Effect of brain ischemia and reperfusion on the localization of phosphorylated eukaryotic initiation factor 2  $\alpha$ . *J. Cereb. Blood Flow Metab.* 17:1291–1302.
- Dexter, D.T., J. Sian, S. Rose, J.G. Hindmarsh, V.M. Mann, J.M. Copper, F.R.

- Wells, S.E. Daniel, A.J. Lees, A.H. Schapira, et al. 1994. Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. *Ann. Neurol.* 35:38–44.
- Ernst, H., R. Duncan, and J. Hershey. 1987. Cloning and sequencing of complementary DNAs encoding the  $\alpha$ -subunit of translation factor eIF-2. *J. Biol. Chem.* 262:1206–1212.
- Gipp, J.J., C. Chang, and R.T. Mulcahy. 1992. Cloning and nucleotide sequence of a full-length cDNA for human liver  $\gamma$ -glutamylcysteine synthetase. *Biochem. Biophys. Res. Commun.* 185:29–35.
- Gudkov, A.V., and I.B. Roninson. 1997. Isolation of genetic suppressor elements (GSEs) from fragment cDNA libraries in retroviral vectors. *Methods Mol. Biol.* 69:221–240.
- Harding, H.P., I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, and D. Ron. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell.* 6:1099–1108.
- Hershey, J. 1991. Translational control in mammalian cells. *Annu. Rev. Biochem.* 60:717–755.
- Jenner, P., and C.W. Olanow. 1996. Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology.* 47:s161–s170.
- Kaufman, R.J., M.V. Davies, V.K. Pathak, and J.W. Hershey. 1989. The phosphorylation state of eucaryotic initiation factor 2 alters translational efficiency of specific mRNAs. *Mol. Cell. Biol.* 9:946–958.
- Li, Y., P. Maher, and D. Schubert. 1997a. Requirement for cGMP in nerve cell death caused by glutathione depletion. *J. Cell Biol.* 139:1317–1324.
- Li, Y., P. Maher, and D. Schubert. 1997b. A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron.* 19:453–463.
- Maher, P., and D. Schubert. 2000. Signaling by reactive oxygen species in the nervous system. *Cell. Mol. Life Sci.* 57:1287–1305.
- Miller, A., D. Miller, J. Garcia, and C. Lynch. 1993. Use of retroviral vectors for gene transfer and expression. *Methods Enzymol.* 217:581–599.
- Miyamoto, M., T.H. Murphy, R.L. Schnaar, and J.T. Coyle. 1989. Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. *J. Pharmacol. Exp. Ther.* 250:1132–1140.
- Mochizuki, H., K. Goto, H. Mori, and Y. Mizuno. 1996. Histochemical detection of apoptosis in Parkinson's disease. *J. Neurol. Sci.* 137:120–123.
- Morgenstern, J.P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587–3596.
- Murphy, T.H., and J.M. Baraban. 1990. Glutamate toxicity in immature cortical neurons precedes development of glutamate receptor currents. *Brain Res.* 57:146–150.
- Murphy, T.H., M. Miyamoto, A. Sastre, R.L. Schnaar, and J.T. Coyle. 1989. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron.* 2:1547–1558.
- Oka, A., M.J. Belliveau, P.A. Rosenberg, and J.J. Volpe. 1993. Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J. Neurosci.* 13:1441–1453.
- Pain, V. 1996. Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* 236:747–771.
- Pathak, V.K., P.J. Nielsen, H. Trachsel, and J.W. Hershey. 1988. Structure of the beta subunit of translational initiation factor eIF-2. *Cell.* 54:633–639.
- Perry, T.L., D.V. Godin, and S. Hansen. 1982. Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci. Lett.* 33:305–310.
- Prostko, C., J. Dholakia, M. Brostrom, and C. Brostrom. 1995. Activation of the double-stranded RNA-regulated protein kinase by depletion of endoplasmic reticular calcium stores. *J. Biol. Chem.* 270:6211–6215.
- Reilly, B., M. Brostrom, and C. Brostrom. 1998. Regulation of protein synthesis in ventricular myocytes by vasopressin. The role of sarcoplasmic/endoplasmic reticulum. *J. Biol. Chem.* 273:3747–3755.
- Sagara, Y., and D. Schubert. 1998. The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J. Neurosci.* 18:6662–6671.
- Samuel, C. 1993. The eIF-2 $\alpha$  protein kinases, regulators of translation in eukaryotes from yeasts to humans. *J. Biol. Chem.* 269:7603–7606.
- Sato, H., M. Tamba, T. Ishii, and S. Bannai. 1999. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem.* 274:11455–11458.
- Satoh, S., M. Hijikata, H. Handa, and K. Shimotohno. 1999. Caspase-mediated cleavage of eukaryotic translation initiation factor subunit 2 $\alpha$ . *Biochem. J.* 342:65–70.
- Sian, J., D.T. Dexter, A.J. Lees, S. Daniel, Y. Agid, F. Javoy-Agid, P. Jenner, and C.D. Marsden. 1994. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann. Neurol.* 36:348–355.
- Simonian, N.A., and J.T. Coyle. 1996. Oxidative stress in neurodegenerative diseases. *Annu. Rev. Pharmacol. Toxicol.* 36:83–106.
- Sofic, E., K.W. Lange, and P. Riederer. 1992. Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci. Lett.* 142:128–130.
- Somia, N., M. Schmitt, D. Vetter, D. Van Antwerp, S. Heinemann, and I. Verma. 1999. LFG: an anti-apoptotic gene that provides protection from Fas-mediated cell death. *Proc. Natl. Acad. Sci. USA.* 96:12667–12672.
- Soucek, T., R.S. Yeung, and M. Hengstschlager. 1998. Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. *Proc. Natl. Acad. Sci. USA.* 95:15653–15658.
- Srivastava, S., M. Davies, and R. Kaufman. 1995. Calcium depletion from the endoplasmic reticulum activates the double-stranded RNA-dependent protein kinase (PKR) to inhibit protein synthesis. *J. Biol. Chem.* 270:16619–16624.
- Srivastava, S., K. Kumar, and R. Kaufman. 1998. Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *J. Biol. Chem.* 273:2416–2423.
- Tan, S., M. Wood, and P. Maher. 1998a. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J. Neurochem.* 71:95–105.
- Tan, S., Y. Sagara, Y. Liu, P. Maher, and D. Schubert. 1998b. The regulation of reactive oxygen species production during programmed cell death. *J. Cell Biol.* 141:1423–1432.
- Vornov, J.J., and J.T. Coyle. 1991. Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. *J. Neurochem.* 56:996–1006.
- Wek, R.C. 1994. eIF-2 kinases: regulators of general and gene-specific translation initiation. *Trends Biochem. Sci.* 19:491–496.
- Yankner, B.A. 1996. Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron.* 16:921–932.