Regulation of Antioxidant Metabolism by Translation Initiation Factor 2α

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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)2α and express a low amount of eIF2α. Sensitivity is restored when the clones are transfected with full-length eIF2α; transfection of wild-type cells with the truncated eIF2α gene confers resistance. The phosphorylation of eIF2α 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^{2+}. In contrast, the resistant clones maintain high GSH levels and show no elevation in peroxides or Ca^{2+} when stressed, and the GSH synthetic enzyme γ-glutamyl cysteine synthetase (γGCS) is elevated. The change in γGCS is regulated by a translational mechanism. Therefore, eIF2α 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 • eIF2α • resistance • glutamate

Introduction

Although programmed cell death (PCD)[1] 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 γ-glutamyl cysteine synthetase (γGCS), 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-hydroxydopamine, MPTP and MPP^+. 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^- (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^- 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^{2+} influx (Murphy et al., 1989; Li et al., 1997a,b; Tan et al., 1998a,b). PCD caused by oxidative glutamate

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[1]*Abbreviations used in this paper:* AD, Alzheimer's disease; DCF, dichlorofluorescein; eIF, translation initiation factor; GSH, glutathione; γGCS, 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 α subunit of the translation initiation factor 2 (eIF2α) 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 α 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 $tRNA_{met}$ when eIF2α 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 α 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. eIF2α 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^{2+} influx (Prostko et al., 1995; Srivastava et al., 1995; Reilly et al., 1998). Therefore, eIF2α 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 eIF2α protects nerve cells from oxidative stress–induced cell death by inhibiting GSH depletion and the increase in both ROS and intracellular Ca^{2+} that are normally seen in cells exposed to oxidative stress. These data demonstrate a unique role of eIF2α 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. eIF2α 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 indoacetoxymethylester (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 pcLXSN containing a cDNA library derived from the human embryonic lung cell line, MRC-5 (Somia et al., 1999). The library contained 2×10^6 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 5×10^5 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 μg 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 eIF2α (Research Genetics) was shown previously to recognize only phosphorylated eIF2α. 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-eIF2α antibody was used to determine the levels of total eIF2α in the HT22 cells and the resistant clones 8 and 15. The anti-eIF2α 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 γGCS 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 ~3.7 kb.

Transfection of Full-Length eIF2α into Clones 8 and 15

The full-length cDNA for eIF2α was obtained from Dr. Miyamoto (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) and was cloned into the pCLBABEpuro retroviral vector, a modified version of the pBABEpuro 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 eIF2a

The cDNA constructs for two mutants of eIF2α (S51A and S51D) were obtained from Dr. Kaufman (University of Michigan, Ann Arbor, MI) and subcloned into pCLBABEpuro. Retroviral vectors were made as described (Somia et al., 1999) with either pCLBABE-S51A, pCLBABE-S51D, or pCLBABEpuro alone. The viral vectors were used to infect HT22 cells, and infected cells were selected in 4 μg/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 [^{3}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 [^{3}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 [^{3}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 5×10^4 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 2×10^5 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 2×10^5 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^{2+} influx, respectively. Samples were prepared as described previously (Tan et al., 1998b).

Results

eIF2a 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 constitutes 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

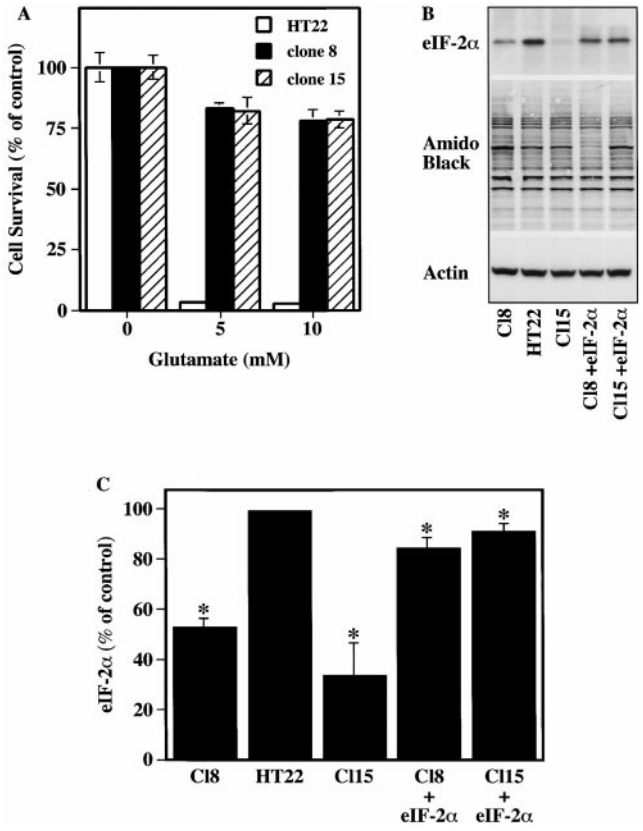

Figure 1. Clones 8 and 15 are resistant to glutamate and express low levels of eIF2α. Glutamate-resistant cells were cloned after infecting HT22 cells with a retroviral cDNA expression library. (A) Cell viability was measured using the MTT assay with wild-type HT22 cells (white bars), clone 8 (black 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 eIF2α protein than wild-type HT22 cells which are largely restored by the reintroduction of wild-type eIF2α. (B) eIF2α and actin protein levels were detected by Western blotting of cell lysates (25 μg) from wild-type HT22 cells, glutamate-resistant clones 8 and 15, and clones 8 and 15 transfected with wild-type eIF2α. 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 Cl8 + eIF2α 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 eIF2α in wild-type HT22 cells. Identical amounts (±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 ± SEM, $P < 0.05$).

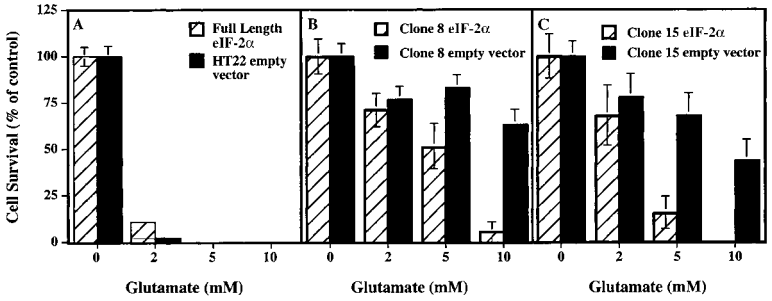

Figure 2. Glutamate-resistant clones 8 and 15 acquire glutamate sensitivity after transfection with full-length eIF2α. Wild-type HT22 cells and glutamate-resistant clones 8 and 15 were stably transfected with an expression construct of eIF2α. (A) The wild-type HT22 cells were unaffected by transfection of eIF2α. (B and C) Resistant clones 8 and 15 when transfected with the eIF2α 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 α subunit of eIF2 (eIF2α) 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_2O_2) and tert-butyl hydroperoxide but not to cell death inducers such as TNF-α, anti-FAS antibody, serum starvation, and glucose deprivation (data not shown).

Clones 8 and 15 Cause Glutamate Resistance by Lowering eIF2α Expression

As outlined previously, the introduction of the eIF2α 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 eIF2α 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 eIF2α cDNA fragment is altering eIF2α expression. Therefore, the two resistant clones and wild-type cells were assayed for eIF2α expression by Western blotting. Although the antibody used for these studies can identify the phosphorylated form of eIF2α (DeGracia et al., 1997), it recognizes both the dephosphorylated and phosphorylated forms of eIF2α in HT22 cells (see Materials and Methods). Using this antibody, it was found that both clones 8 and 15 express lower levels of eIF2α protein (Fig. 1, B and C). Similar results were obtained with another antibody against eIF2α (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 eIF2α expression. The gene fragments that were rescued from clones 8 and 15 are identical and contain a fragment of the eIF2α 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 eIF2α in the resistant clones is responsible for the resistance of the cells to glutamate, then the expression of full-length eIF2α should restore the sensitivity to glutamate. Transfection of full-length eIF2α 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 eIF2α 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 eIF2α cDNA (Fig. 2 A). This demonstrates that modulation of eIF2α expression has significant effects on glutamate toxicity in HT22 cells.

eIF2α Phosphorylation Also Mediates Glutamate Resistance

To confirm that the loss of eIF2α activity is linked to glutamate resistance, a second method was employed which utilizes a dominant negative approach to regulate eIF2α function. The phosphorylated form of eIF2α sequesters the guanine nucleotide exchange factor, eIF2β, resulting in a decrease in protein translation (Ernst et al., 1987). The S51D mutant of eIF2α mimics constitutive phosphorylation when serine 51 in eIF2α is replaced with an aspartic acid (Kaufman et al., 1989). The S51A mutant cannot be phosphorylated when serine 51 in eIF2α 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 eIF2α. To assay the effect of eIF2α phosphorylation on glutamate sensitivity, wild-type HT22 cells were infected with virus that contained either the S51D or

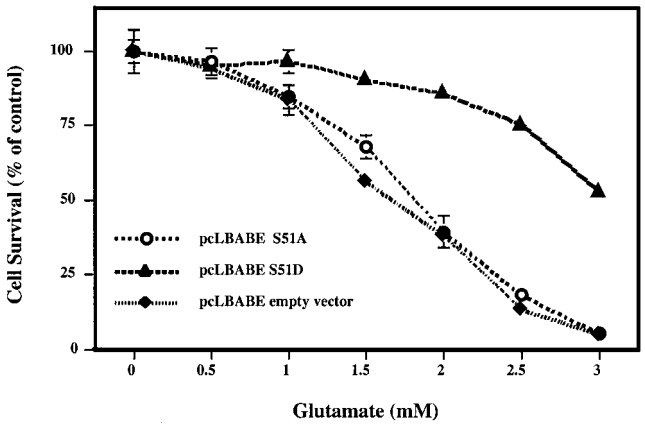

Figure 3. HT22 cells become glutamate resistant when the eIF2α S51D mutant is stably expressed. HT22 cells were infected with virus containing either the pCLBABEpuro empty vector (◆), the S51A mutant of eIF2α (-○), or the eIF2α S51D mutant (▲). Cell viability was measured by the MTT assay. Samples were prepared in triplicate ($n = 4$).

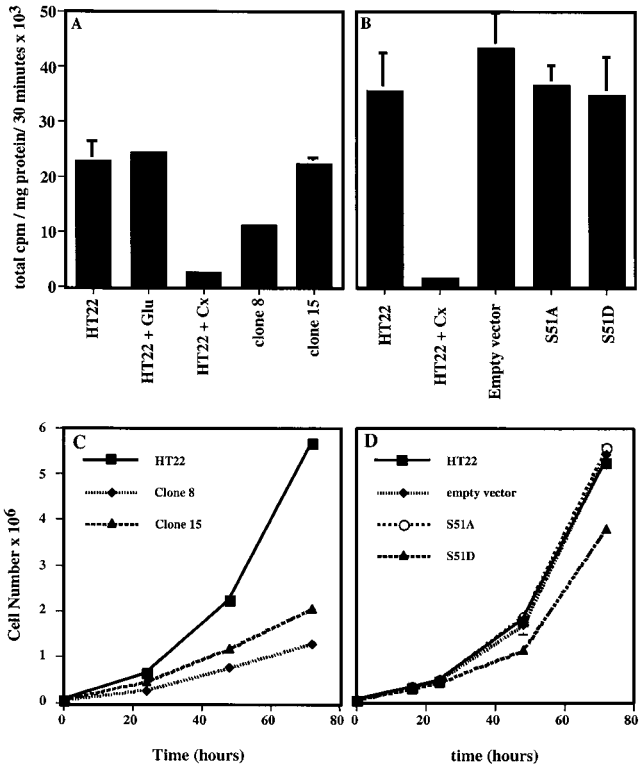

Figure 4. Effect of eIF2α expression on the rate of protein translation. Protein synthesis was measured by [^{3}H]leucine incorporation for 30 min and normalized to total protein. (A) Wild-type HT22 cells (HT22); cells exposed to 5 mM glutamate for 4 h (HT22 + Glu); cycloheximide (HT22 + Cx); and clones 8 and 15. (B) Wild-type HT22 cells and cells infected with either empty vector or the eIF2α phosphorylation mutants S51A or S51D. All assays were performed in triplicate (n = 2). (C) The growth rate is decreased when eIF2α 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 (◆); eIF2α mutant S51A (○); and mutant S51D (▲). Cell counts at all time points were done in triplicate, and the data are presented as the average ± SEM.

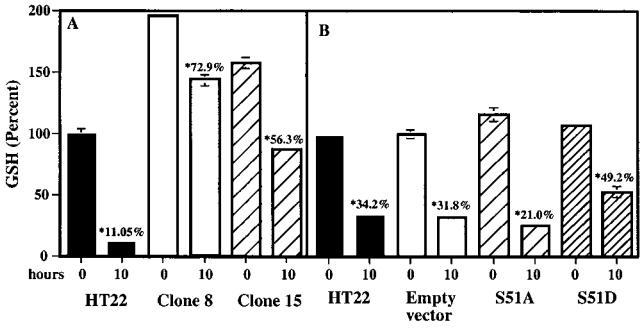

Figure 5. GSH levels in resistant cells. GSH levels were measured in control, untreated cells, and cells exposed to 5 or 2 mM glutamate for 10 h. (A) 5 mM glutamate. Clones 8 (white bars) and 15 (hatched bars) have higher basal GSH levels than wild-type HT22 and only deplete to 72 ± 4 and 56 ± 1% of 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 ~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).

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 eIF2α 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 eIF2α activity by protein phosphorylation can lead to glutamate resistance and that eIF2α phosphorylation may play an important role in cell death or survival after glutamate exposure. However, we could not directly assay eIF2α phosphorylation after glutamate exposure because none of the available antibodies immunoprecipitate or distinguish phosphorylated from unphosphorylated eIF2α in HT22 cells.

Changes in eIF2α Expression Do Not Affect Translation Rates but Do Slow Growth

To determine if eIF2α downregulation in the glutamate-resistant clones causes a decrease in protein synthesis, protein 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 eIF2α 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 eIF2α. 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 eIF2α 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 eIF2α 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 eIF2α expression or phosphorylation.

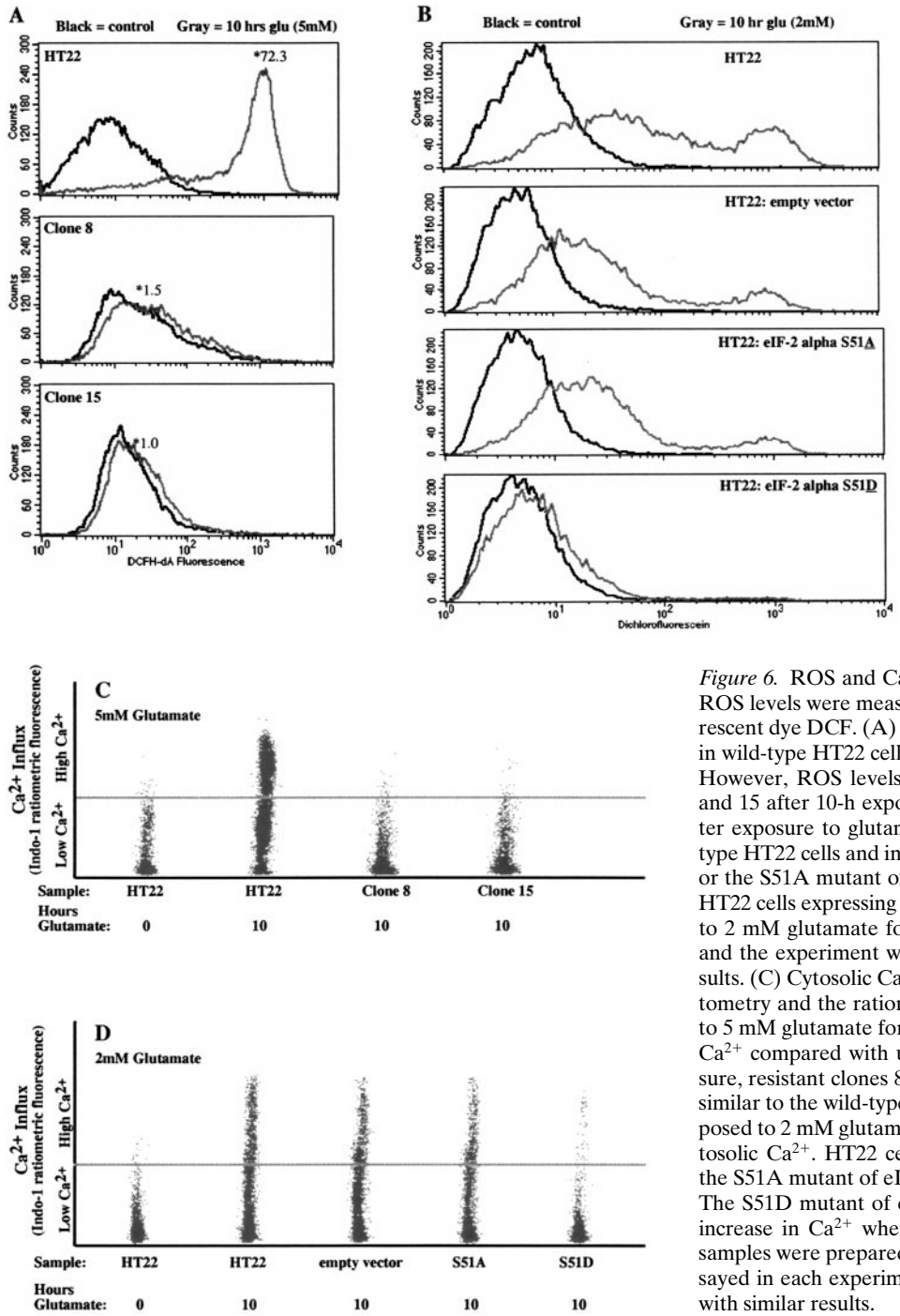

Figure 6. ROS and Ca^{2+} levels after exposure to glutamate. ROS levels were measured by flow cytometry using the fluorescent dye DCF. (A) ROS levels increase 72-fold (gray line) in wild-type HT22 cells exposed to 5 mM glutamate for 10 h. However, ROS levels do not increase in resistant clones 8 and 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 eIF2α. ROS levels do not increase in HT22 cells expressing the eIF2α 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^{2+} 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^{2+} compared with untreated cells. After glutamate exposure, resistant clones 8 and 15 maintain cytosolic Ca^{2+} 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^{2+}. HT22 cells infected with the empty vector or the S51A mutant of eIF2 also show similar increases in Ca^{2+}. The S51D mutant of eIF2α prevents the glutamate-induced increase in Ca^{2+} 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.

eIF2α Expression Alters Glutathione, ROS, and Ca^{2+} Responses to Glutamate

To understand the role of eIF2α 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–

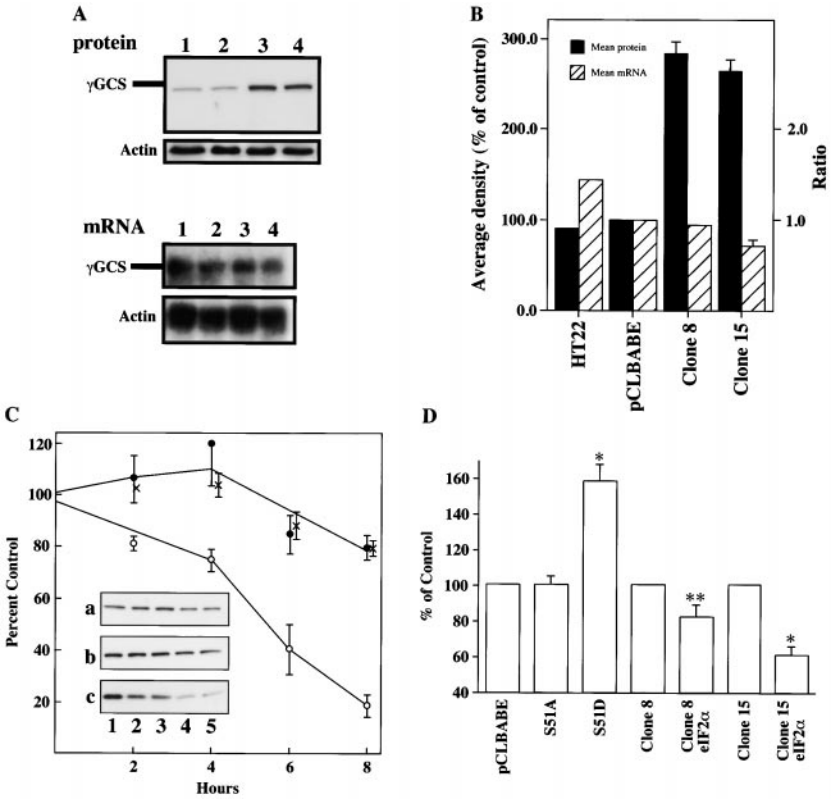

Figure 7. γGCS protein expression is regulated at the level of translation. (A) γGCS, actin protein, and mRNA expression was measured in wild-type HT22 cells (lane 1), cells infected with the empty vector (pCLBABE) (lane 2), and the resistant clones 8 (lane 3) and 15 (lane 4) by Western and Northern 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 γGCS in pCLBABE, 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 γGCS to actin is presented normalized to γGCS in pCLBABE as 1.0. The results were confirmed by reverse transcription PCR analysis (data not shown). (C) Proteolytic breakdown of γGCS and P27 in wild-type and resistant cells. HT22 cells and resistant clone 15 were treated with 100 μg/ml cycloheximide and the amount of γGCS and P27 was quantitated by Western blot at 2-h intervals. The values are normalized to 0 time and are the mean ± SEM of triplicate experiments. Inset, Western blots of γGCS, wild-type cells (a); γGCS, 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, γGCS, wild-type; ●, γGCS Cl 15; ○, P27. (D) Resistant clones 8 and 15 were infected with wild-type eIF2α, the wild-type HT22 clone was infected with S51A or S51D, and the levels of γGCS and actin were determined by Western blotting. The amounts of γGCS and actin were quantitated and the amount of γGCS was normalized to the actin loading control. In each set of cells, the transfected cells were then normalized to γGCS in their parental line pCLBABE, resistant clone 8 or 15, which was set at 100%. The data are presented as the mean ± 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 ~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^{2+} influx was measured in wild-type, resistant, and phosphorylation mutant-expressing cells. Ca^{2+} levels were determined by FACS® 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^{2+} than untreated controls, whereas resistant cell lines 8 and 15 maintained intracellular Ca^{2+} 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^{2+} levels increase significantly in the wild-type HT22 cells as well as the empty vector and S51A-expressing HT22 cells. The intracellular Ca^{2+} 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 eIF2α 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^{2+} normally associated with oxidative stress–induced cell death.

The Inactivation of eIF2α Upregulates γGCS Expression by a Translational Mechanism

Resistant clones 8 and 15 have decreased eIF2α 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 eIF2α protein levels and GSH production, the expression of the rate-limiting enzyme for GSH synthesis, γGCS, was examined in the wild-type cells and the resistant clones. Protein expression and mRNA levels of the catalytic subunit of γGCS were measured by Western and Northern blotting, respectively. Western blotting shows that the level of the catalytic subunit of γGCS is threefold higher in the resistant clones than in the wild-type HT22 cells (Fig. 7, A and B). In contrast, when both γGCS and actin mRNA were quantitated and their ratio normalized to cells expressing the empty pCLBABE retroviral vector, the amount of γGCS mRNA remained relatively constant (Fig. 7, A and B). To rule out the possibility that eIF2α activity changes the rate of γGCS 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, γGCS was degraded more slowly but at the same rate in resistant and wild-type cells. These results indicate that a decrease in eIF2α wild-type protein levels leads to an increase in production of the catalytic subunit of γGCS by a translational mechanism, resulting in significantly higher levels of GSH.

If eIF2α directly regulates γGCS 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 eIF2α to render them more sensitive to oxidative stress. Fig. 7 D shows that the levels of γGCS increased ~60% in cells transfected with S51D relative to wild-type cells. In contrast, the expression of γGCS decreased between 20 and 40% in the resistant clones 8 and 15 which already have a high level of γGCS protein when these clones were transfected with normal αIF2α (Fig. 7 B). These data, along with those presented above, strongly suggest that eIF2α expression and activity can directly modulate γGCS protein levels. It is also likely that the expression of additional proteins involved in the resistance to oxidative stress is regulated by eIF2α.

Discussion

The above data show that eIF2α plays a central role in programmed nerve cell death initiated by oxidative stress. Alterations in either the level of eIF2α 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 eIF2α in glutamate-induced cell death, followed by possible mechanisms that eIF2α could use to signal this type of cell death. The potential relevance of eIF2α nerve cell death in PD will also be discussed.

eIF2α 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 eIF2α from the retroviral library. The following evidence shows that eIF2α activity is required for cells to die via oxidative glutamate toxicity and other forms of oxidative stress: (a) eIF2α fragments rescued from the glutamate-resistant cells make wild-type cells resistant to glutamate upon reinfection; (b) Western blotting demonstrates that the eIF2α protein levels in the resistant clones are lower than in wild-type HT22 cells; and (c) eIF2α downregulation alone causes resistance to glutamate since clones 8 and 15, when transfected with full-length human eIF2α, become glutamate sensitive. Since eIF2α 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 eIF2α 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 eIF2α 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 eIF2α phosphorylation plays a key role in determining the fate of the glutamate-exposed HT22 cells is evident when the S51D mutant of eIF2α is expressed in the HT22 cells, resulting in glutamate resistance. The S51D mutant mimics a constitutively phosphorylated form of eIF2α 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 eIF2α or the phosphorylation mutants leads to overexpression of their respective transcripts but does not alter the overall levels of eIF2α protein (data not shown), the amount of eIF2α 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.

eIF2α Downregulation and the Constitutively Phosphorylated Form of eIF2α 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 eIF2α 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^{2+}. 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 eIF2α during times of stress signals the translation of specific proteins that increase cell survival. Since decreases in either eIF2α activity or protein levels both lead to an increase in GSH, we asked if the rate-limiting enzyme in GSH production, γGCS, was increased in the resistant cells compared with the wild-type HT22 cells. Fig. 7 shows that although the amount of γGCS is increased in the original resistant clones, the γGCS mRNA level remains constant and there is no difference in the rates of γGCS breakdown. In addition, γGCS is upregulated by the phosphorylation mutant, S51D, and downregulated by the introduction of additional eIF2α into the glutamate-resistant clones 8 and 15 (Fig. 7). These data show that eIF2α regulates γGCS expression by a translational mechanism. Amino acid starvation in *Saccharomyces cerevisiae* also causes eIF2α 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 eIF2α activity is low, leading to an increased production of γGCS to promote cell survival. In addition, it was recently shown that another form of stress, the unfolded protein response, causes the phosphorylation of αIF2α and the increased translation of activating transcription factor 4 (Harding et al., 2000).

eIF2α Plays a Unique Role in Programmed Cell Death

There have been several reports that positively link eIF2α to apoptosis: eIF2α phosphorylation by double-stranded RNA–activated protein kinase is the cause of cell death in TNF-α–stimulated cells (Srivastava et al., 1998), and eIF2α is cleaved by caspases after an increase in PKR kinase activity induced by TNF-α or poly(I):poly(C) (Satoh et al., 1999). However, HT22-resistant clones 8 and 15 are not resistant to TNF-α, indicating that they utilize a survival mechanism that is unique to oxidative stress. Ischemia and reperfusion in the rat brain also lead to eIF2α phosphorylation and cell death (DeGracia et al., 1997; Burda et al., 1998). In these cases, it was argued that death signals lead to eIF2α phosphorylation, protein synthesis shutdown, and cell lysis. In contrast, our data show that eIF2α phosphorylation protects cells from death. HT22 cells treated with thapsigargin, a substance shown to cause eIF2α 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 eIF2α phosphorylation determine whether eIF2α will be used to prevent or promote cell death. The above experiments link oxidative stress, GSH depletion, and the regulation of γGCS directly to eIF2α 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_2O_2 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, eIF2α, 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 eIF2α or phosphorylated eIF2α maintain high levels of GSH when stressed and do not die. These results point to a central role of eIF2α 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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