Biochemical and Biophysical Research Communications **270,** 311–317 (2000)
doi:10.1006/bbrc.2000.2419, available online at http://www.idealibrary.com on IDEAL®

Inhibition of the 26S Proteasome Induces Expression of GLCLC, the Catalytic Subunit for γ-Glutamylcysteine Synthetase

Konjeti R. Sekhar,*[,1] Syrus R. Soltaninassab,*[,1] Michael J. Borrelli,† Zhi-Qi Xu,* Michael J. Meredith,‡ Frederick E. Domann,§ and Michael L. Freeman*[,2]

**Department of Radiation Oncology and Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232; †Department of Radiation Oncology, William Beaumont Hospital, Royal Oak, Michigan 48073; ‡Department of Oral Molecular Biology, School of Dentistry, Oregon Health Science University, Portland, Oregon 97201; and §Radiation Research Laboratory, University of Iowa, Iowa City, Iowa 52242*

Received March 2, 2000

The majority of short- and long-lived cellular proteins are degraded by the activities of the 26S proteasome, a large multi-catalytic protease. Its unique function places it as a central regulatory activity for many important physiological processes. Lactacystin is a very specific 26S proteasome inhibitor and represents an excellent tool for demonstrating that a pathway exhibits proteasome-dependent biochemical regulation. Exposure of HepG2 cells to lactacystin resulted in robust elevation of GLCLC mRNA levels, followed by an increase in GSH concentrations. GLCLC is the gene that encodes the catalytic subunit for γ-glutamylcysteine synthetase, the rate-limiting enzyme for the synthesis of glutathione (GSH). Inhibition of non-proteasome, protease activities did not induce GLCLC. Gel mobility shift assays and expression of CAT activity from heterologous reporter vectors identified Nrf2 mediation of the GLCLC antioxidant response element, ARE4, as the mechanism by which lactacystin induced GLCLC. These studies have identified 26S proteasome activity as a central regulatory pathway for glutathione synthesis.

Key Words: **glutathione; ARE/EpRE; transcription; proteasome; protein degradation; Nrf2; AP-1; GLCLC; gamma glutamycysteine synthetase.**

The majority of cellular proteins, both long and short lived are degraded by the 26S proteasome (1, 2), a large multicatalytic protease exhibiting 3 distinct proteolytic activities (reviewed in 3). The core 20S proteasome is a barrel shaped particle comprising 4 stacked rings that form a proteolytic chamber (reviewed in 1–3). In the chamber are chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide hydrolyzing activities. The 20S complex is capped at either one or both ends by 19S cap complexes. This 26S proteasome structure is ATP dependent and recognizes and degrades multi-ubiquinated proteins. Access to the inner chamber of the 20S particle is restricted to unfolded polypeptides. Unfolding of ubiquinated proteins is ATP dependent and mediated by the 19S cap structures. Misfolded proteins generated by heat shock or oxidative stress are also proteasome substrates (3, 4).

Many important physiological and pathophysiological processes are regulated by proteasome activity. Examples include cell cycle regulatory proteins (5), the inhibitor IκB (6), human papillomavirus oncogenesis (7), and ischemic renal failure (8). The demonstration that a process exhibits proteasome-dependent biochemical regulation has been enhanced by the development of specific proteasome inhibitors (9). One such inhibitor is the natural product lactacystin (10). Lactacystin is an inactive molecule that undergoes spontaneous conversion in aqueous solutions to the active proteasome inhibitor clasto-lactacystin β-lactone. Whereas lactacystin is impermeable to cell membranes, clasto-lactacystin β-lactone is permeable. The availability of intracellular clasto-lactacystin β-lactone is directly related to the intracellular concentration of glutathione (GSH) because the β-lactone reversibly reacts with GSH to form a thioester adduct. Formation of this adduct renders the β-lactone inactive.

Glutathione is a soluble antioxidant that participates in the reduction of peroxides, the detoxification and efflux of xenobiotics and acts as an intracellular redox buffer. The intracellular concentration ranges

[1] These two individuals contributed to an equal degree.

[2] To whom correspondence should be addressed at B902 TVC Radiation Oncology, Vanderbilt University School of Medicine, Nashville, TN 37232. Fax: 615 343-3061. E-mail: Michael.Freeman@mcmail.vanderbilt.edu.

0006-291X/00 $35.00

from 1 to 10 mM. GSH is synthesized in two sequential, ATP-dependent enzymatic reactions catalyzed by γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase (11). γGCS catalyzes the first and rate-limiting step in GSH synthesis. γGCS is composed of two subunits; catalytic (Glclc) and regulatory (Glclr). Reports have indicated that GLCLC transcriptional regulation can be mediated by several regulatory *cis* elements: ARE4 (12), AP-1 (13), NFκB (14) and MRE (15).

In this investigation, lactacystin was used to demonstrate that transcriptional regulation of GLCLC is regulated, in part, by proteasome function. Inhibition of proteasome function caused an increase in GLCLC mRNA expression that was regulated by Nrf2 activation of ARE4.

MATERIALS AND METHODS

DMRIE-C was purchased from Life Technologies. pBLCAT2 was purchased from ATCC. Lactacystin was purchased from Calbiochem. Azetidine carboxylate, and Calpain Inhibitor Peptide were purchased from Sigma. Aprotinin was purchased from ICN Biomedicals. Oct-1 and AP-1 oligonucleotides, as well as antibodies to Nrf2, c-Jun, and JunD were purchased from Santa Cruz.

HepG2 cells were maintained in Eagle's MEM medium supplemented with 10% fetal bovine serum and 1% sodium pyruvate.

Northern blot analysis. Total RNA was isolated using TRIzol reagent. The isolated RNA (20 μg per lane) along with a RNA ladder were fractionated by electrophoresis in a 1.1% agarose/2.2 M formaldehyde gel, blotted onto nitrocellulose membranes and baked. Prehybridization and hybridization were carried out at 42°C using a 265 bp ^{32}P labeled cDNA corresponding to GLCLC or 743 bp ^{32}P labeled cDNA corresponding to cyclophilin (16).

Glutathione analysis. Cells were washed twice with ice cold PBS and then scraped into ice cold 10% perchloric acid containing 15 nmol/ml γ-glu-glu as an internal standard. Quantitation of GSH was performed using the HPLC method described in (17).

Gel mobility shift assays. Nuclear extracts were obtained from serum starved HepG2 cells as described (18). Binding reactions were performed by adding 10 μg of nuclear extract to 0.6 ng of ^{32}P-labeled, double stranded oligonucleotide in binding buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.05% NP-40, 5 mM $MgCl_2$, 0.05 μg poly (dI-dC)) and incubating at 20°C for 15 min. Competition binding reactions were performed by addition of 50 fold excess of unlabeled probe. In some experiments, extracts were pretreated for 2 h at 4°C prior to binding reactions.

Transfection. A GLCLC ARE4 oligonucleotide (described in text) or a GLCLC AP-1 oligonucleotide (described in text) was subcloned into pBLCAT2 which contains a HSV thymidine kinase minimal promoter and expresses chloramphenicol acetyl transferase. CAT reporters were verified by DNA sequencing. Cells were transiently co-transfected with 0.5 μg pcDNA3.1/Myc-His/LacZ and 1 μg of the appropriate pBLCAT2 vector using DMRIE-C. After transfection cells were grown for 36 h, serum starved overnight, and then treated with lactacystin (5 μM). Cell extracts were prepared and assayed for β-galactosidase and CAT activity. Statistical analysis was performed using a Wilcoxon Signed Rank Test.

Construction of a Hsp27 expressing adenovirus vector. Adenovirus vectors for expressing Hsp27 were constructed by cloning the human HSP27 gene into the multiple cloning site of PCL-Neo (Promega). The expression cassette, under the control of the CMV promoter-enhancer, was then excised by restriction enzymes and subcloned into the adenovirus shuttle plasmid pE1sp1A. This construct was then co-transfected into 293 cells (calcium phosphate method) along with plasmid pJM17. Homologous recombination between these two plasmids produced a DNA construct containing a Hsp27 expression cassette. A plaque assay was used to isolate individual virus clones that were screened for their ability to produce Hsp27 in A549 human lung carcinoma cells and concomitant inability to replicate. A selected virus clone was amplified in 293 cells which were then lysed by freeze/thawing. The adenovirus was purified from the lysate by centrifugation at 60,000 ×g in a cesium chloride step gradient (1.1 g/ml, 1.3 g/ml, & 1.4 g/ml). The purified virus was dialyzed overnight in phosphate buffered saline. Sterile glycerol (final concentration = 10%) was added to the dialyzed virus preparation and aliquots were frozen at −70°C.

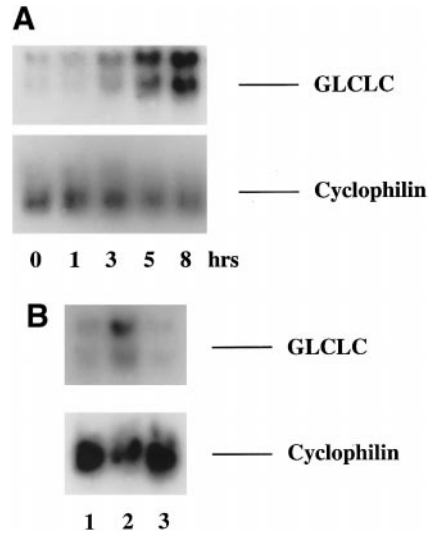

FIG. 1. Expression of GLCLC as assessed by Northern blotting. (A) Serum-starved HepG2 cells were exposed to 5 μM lactacystin for the indicated times. (B) Serum-starved HepG2 cells were exposed to 0 (lane 2) or 50 μg/ml of cycloheximide (lane 3) for 30 min prior to an 8 h exposure to 5 μM lactacystin (lanes 2 and 3). Lane 1 represents control.

RESULTS

Inhibition of Proteasome Function Induces GLCLC

Exposure to 5 μM lactacystin produced a rapid and robust increase in GLCLC mRNA levels (Fig. 1A). Similar results were observed in NIH 3T3 cells (data not shown). To ensure that cells treated with lactacystin were viable, HepG2 cells were exposed for 16 h to 5 μM lactacystin and then stained with trypan blue dye. Two hundred cells were counted. The examination revealed that 97% of cells were viable in that they excluded dye (data not shown).

The expression of GLCLC in cells exposed to lactacystin was compared to that produced by exposure to aprotinin, an inhibitor of serine proteases, or calpain inhibitor peptide. GLCLC and cyclophilin expression was determined by Northern blotting and quantitated by laser scanning densitometry (Table 1). Neither

TABLE 1

Expression of GLCLC Relative to Cyclophilin in HepG2 Cells

Treatment	GLCLC expression[+]
None	1.0
Lactacystin	4.5
Aprotinin	1.3
Calpain inhibitor	1.0

[+] HepG2 cells were exposed to the indicated inhibitor for 5 h and then RNA isolated. Laser scanning densitometry was used to quantitate GLCLC expression relative to cyclophilin in Northern blots.

aprotinin nor calpain inhibitor peptide affected expression of GLCLC.

To examine whether protein synthesis was required for lactacystin-dependent regulation of GLCLC mRNA expression, HepG2 cells were exposed to the protein synthesis inhibitor cycloheximide for 30 min prior and during an 8 h lactacystin treatment. As presented in Fig. 1B cycloheximide completely abolished the induction of GLCLC by lactacystin (compare lanes 2 and 3).

Next, GSH levels were measured in HepG2 cells exposed to 5 μM lactacystin for 8 h. Untreated cells contained 95 ($\pm$10) nmoles GSH/mg protein while cells exposed to 5 μM lactacystin contained 185 ($\pm$17), nmoles GSH/mg protein, an increase of approximately 2 fold.

The Increase in GSH and GLCLC Is Not Related to Expression of Hsp27

Proteasome function has been shown to regulate the stress-protein kinase p38 (19) and induce the synthesis of Hsp27 (20). Mehlen *et al.* (21) reported that overexpression of Hsp27 increased GSH levels in L929 cells and in NIH 3T3 cells. To investigate whether lactacystin-regulated expression of GLCLC could be a consequence of Hsp27 overexpression, HepG2 cells were mock infected, infected with an insertless adenovirus vector, with a human Hsp27 adenovirus expression vector, or with an adenovirus vector that expressed β-galactosidase. The MOI was adjusted so that 90% or more of the cells exhibited β-gal activity (data not shown). The data presented in Fig. 2A demonstrate that over expression of Hsp27 was achieved in HepG2 cells infected with the adenovirus expression vector. The data presented in Fig. 2B indicate that expression of GLCLC was not significantly affected in cells that overexpressed Hsp27. Specifically, quantitation by scanning densitometry indicated a $\leq$30% increase in GLCLC message compared to vector alone. This experiment also demonstrated that lactacystin-regulated expression of GLCLC remained unaltered under these conditions.

Increasing the Number of Proteins Targeted to the Proteasome Can Increase GLCLC Expression

The proteasome-degradation process can be stressed by exposing cells to amino acid analogs. Incorporation of analogs into polypeptides results in formation of non-native conformations that are ubiquitinated and undergo proteasome-dependent degradation (4, 22, 23). Thus, when cells are exposed to analogs, the number of proteins processed by the proteasome increases significantly.

HepG2 cells were exposed to the amino acid analog azetidine carboxylate, which is incorporated into proteins in the place of proline. After an overnight exposure, RNA was isolated and analyzed by Northern blotting (Fig. 3A). The data presented in this figure demonstrate that exposure to azetidine produced a dose dependent increase in GLCLC.

The transcription factor c-Jun is degraded by the 26S proteasome (24) and thus is a useful marker to deter-

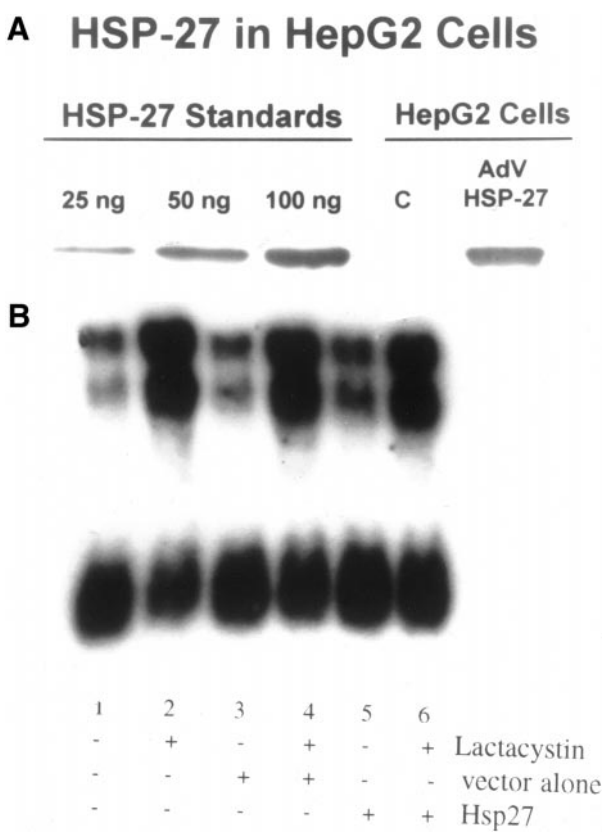

FIG. 2. Overexpression of Hsp27 does not affect expression of GLCLC. (A) Western blot illustrating Hsp27 expression in cells infected with vector alone (lane C) or in cells infected with the Hsp27 adenovirus expression vector (lane Adv Hsp-27). In addition, 25, 50, and 100 ng of purified Hsp27 was loaded into the gel for comparison (lanes Hsp-27 standards). (B) Northern blot illustrating GLCLC and cyclophilin expression. HepG2 cells were mock infected, infected with virus alone or with a Hsp27 adenovirus expression vector. After 36 h the cells were serum starved, and then exposed to 0 (lanes 1, 3, 5) or 5 μM lactacystin (lanes 2, 4, 6) for 8 h.

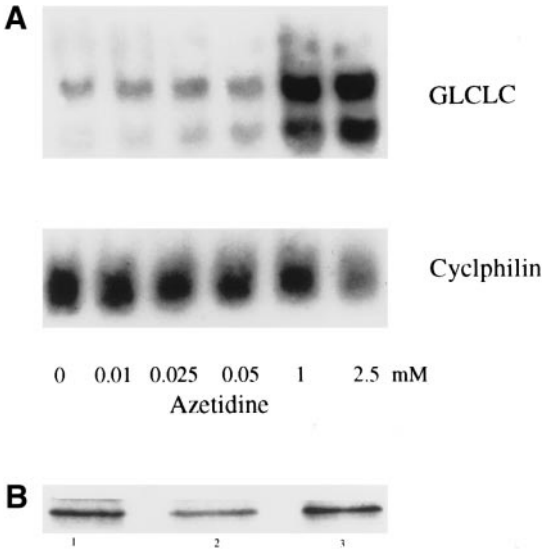

FIG. 3. Expression of GLCLC (A) and c-Jun (B) in HepG2 cells exposed to azetidine. (A) Northern blot illustrating expression of GLCLC in cells exposed overnight to the indicated azetidine concentrations. (B) Immunoprecipitation of c-Jun from cells labeled with ^{35}S methionine and immediately lysed (lane 1) or exposed to 0 (lane 2) or 2 mM azetidine (lane 3) for 1 h prior to lysis.

mine whether azetidine affected proteasome dependent degradation rates. The half life of c-Jun was examined in ^{35}S methionine pulse labeled HepG2 cells exposed to azetidine for 1 h. c-Jun was immunoprecipitated and quantitated by SDS-PAGE/autoradiography (Fig. 3B). Laser scanning densitometer analysis revealed that in control cells 56% of ^{35}S methionine labeled c-Jun had been degraded in 1 h (lane 2) compared to only 12% in cells treated with azetidine (lane 3).

Lactacystin and Azetidine Induce ARE4 Activity

HepG2 cells were treated with 5 μM lactacystin for up to 6 h and then nuclear lysates obtained. Gel shift mobility shift assays were performed using oligonucleotides encompassing the ARE4 element (12) or the AP-1 element located between bp -263 and -269 (13). The GLCLC regulatory sequences are as follows:

$ARE4_{wildtype}$ 5′-CCCC**GTGACTCAGCG**CTTTGT-3′
$ARE4_{m2}$ 5′-CCCC**GTGACTtgGCG**CTTTGT-3′
$ARE4_{m3}$ 5′-CCCC**GTGACTCAttG**CTTTGT-3′

ARE4 is located on the noncoding strand of promoter

$AP\text{-}1_{(-269\ to\ -263)}$ 5′-GAGTTCGTCAT**TGATTCA**AATAAT-3′

located on the coding strand of promoter

For comparison a consensus AP-1 site is also shown.

$AP\text{-}1_{consensus}$ 5′-CGCTTGA**TGACTCA**GCCGGAA-3′

As shown in Fig. 4A, proteasome inhibition increased ARE4 DNA binding activity and this could be abrogated with unlabeled oligonucleotide or oliognucleotide containing the m2 mutation (sequence shown above) but not the m3 mutation.

Nuclear extracts from cells treated with 5 μM lactacystin for 6 h were incubated with antibodies to c-Jun, JunD, or Nrf2 and then analyzed by GMSA. The data in Fig. 4B indicate that ARE4 DNA binding activity was disrupted by the Nrf-2 antibody but not antibodies to c-Jun or JunD.

In contrast to the results obtained using the ARE4 olionucleotide, proteasome inhibition did not affect the DNA binding activity of $AP\text{-}1_{-269\ to\ -263}$ (Fig. 5A). A single band that was not removed by cold competitor was observed, indicative of non-specific binding. In the same experiment, untreated nuclear lysate was mixed with an OCT-1 oligonucleotide as a positive control. Here OCT-1 specific DNA binding activity was ob-

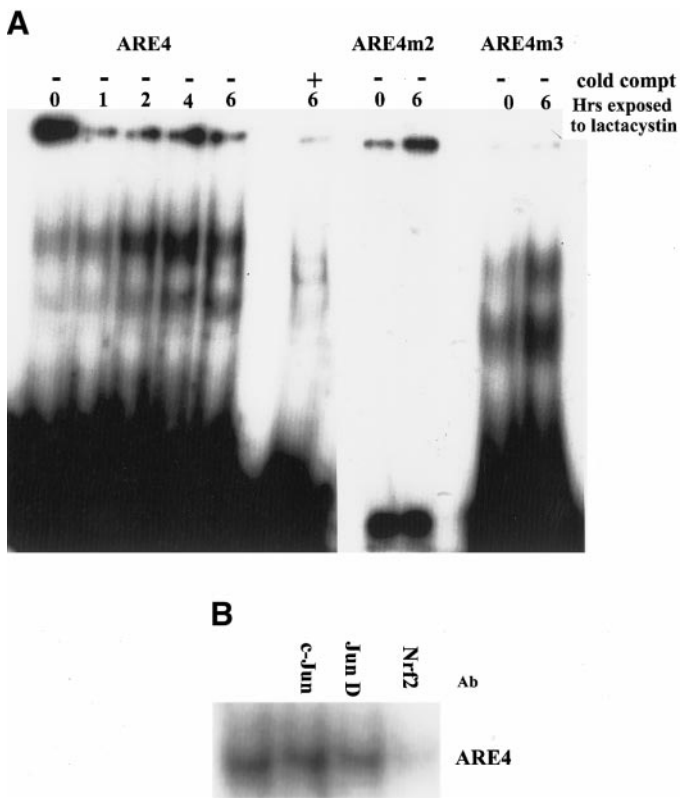

FIG. 4. (A) Gel mobility shift assay using wild-type or mutated ARE4 oligonucleotide. Serum-starved cells were exposed to 5 μM lactacystin for the indicated times. Nuclear lysates were then obtained and analyzed by gel mobility shift assay (10 μg/lane). The m2 and m3 mutations are described in the text. A 50 fold excess of cold competitor was added to extracts in lane marked with a (+). (B) Nrf2 antibodies disrupt ARE4 DNA binding activity. Serum-starved cells were exposed to 5 μM lactacystin for 6 h and nuclear lysates were obtained. The lysate (10 μg/lane) was incubated with antibodies to c-Jun, JunD, or Nrf2 for 2 h at 4°C prior to analysis by mobility shift assay.

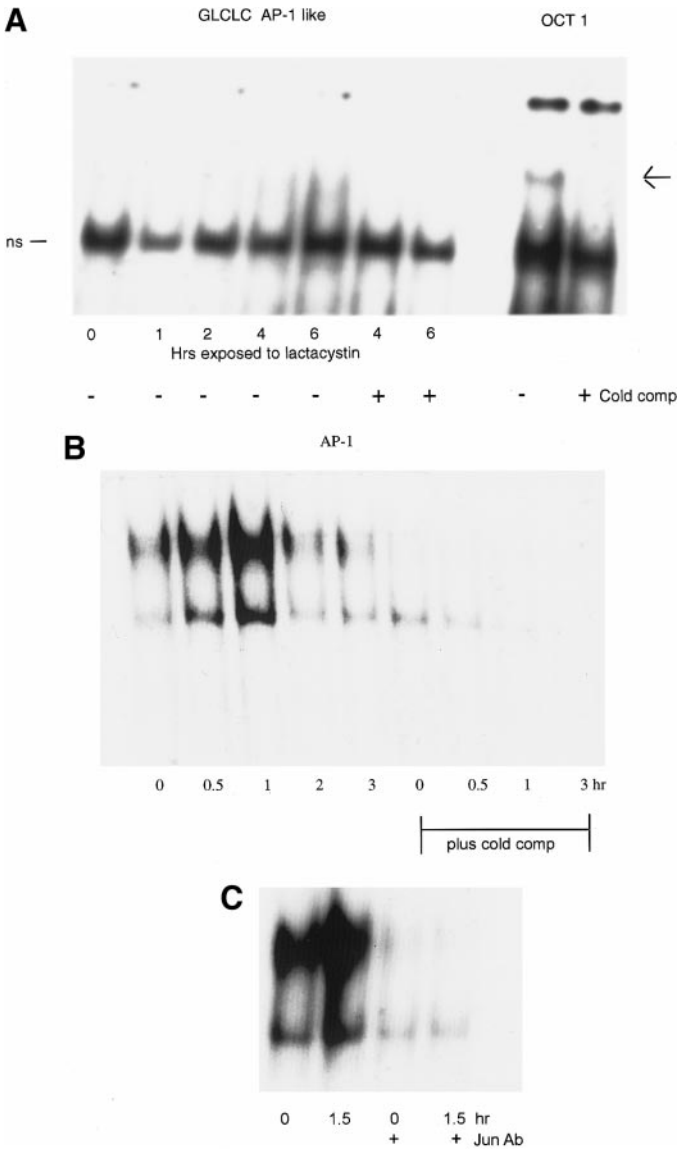

FIG. 5. Gel mobility shift assay using a GLCLC AP-1 oligonucleotide described in text, Oct 1 (Santa Cruz) or a consensus AP-1 oligonucleotide (Santa Cruz). Serum-starved cells were exposed to 5 μM lactacystin for the indicated times. Nuclear lysates were then obtained and analyzed by gel mobility shift assay (10 μg/lane). A 50-fold excess of cold competitor was added to extracts as indicated. Untreated cell lysate was used for the Oct 1 analysis. Nuclear extracts from HepG2 cells treated with lactacystin for 0 or 1.5 h were incubated with a Jun polyclonal antibody prior to EMSA. The antibody disrupts dimer formation.

served. These results are in contrast with those obtained when a true consensus AP-1 element was used (Fig. 5B). Rapid and robust DNA binding activity was observed and this could be competed away with cold competitor or with Jun antibodies (Fig. 5C).

ARE4 and the GLCLC AP-1 site were also compared to each other using heterologous reporter vectors (Table 2). HepG2 cells were transiently transfected with the reporter plasmid, pBLCAT2, containing a minimal Herpes simplex virus tk promoter, the coding region for bacterial chloramphenicol acetyltransferase and either a single GLCLC ARE4 site, the GLCLC AP-1 site or no insert at all. In addition, cells were also transiently transfected with pcDNA3.1/LacZ which constitutively expresses β-gal. CAT activity ($n = 3$) was determined from cell lysates and normalized for protein content of the lysate. Transfection efficiency was based on β-gal expression.

The results indicated that CAT expression was greatest in cells that had been transfected with plasmid pBLCAT2/ARE4. The GLCLC AP-1 sequences did not result robust CAT expression, agreeing with the GMSA experiments. Furthermore, treatment with either lactacystin or azetidine significantly increased ARE4-dependent gene expression but did not affect expression from the pBLCAT2/AP-1$_{-269\ to\ -263}$ vector.

These experiments illustrate two points. First, both reporter and GMSA experiments demonstrate ARE4 exhibited significant activity, in contrast to GLCLC AP-1 sequences. Second, ARE4 dependent activity could be enhanced by conditions which inhibited proteasome function.

DISCUSSION

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide involved in numerous cellular functions (for review see 25). Mammalian cells contain between 1 to 10 mM of glutathione located in subcellular pools. The majority of glutathione resides in the cytosol and nucleus in a reduced form (GSH) with only a small amount oxidized to GSSG. The ratio of GSH to oxidized GSSG is $\geq$10:1 (11). Approximately 10% of the glutathione is located in the mitochondria. In the endoplasmic reticulum the ratio of reduced to oxidized glutathione is 3:1 (26). Until recently, it was thought that ER pool of GSSG was a source of oxidizing equivalents which participated in disulfide bond generation. Now, however, the work of Cuozzo and Kaiser (27) has demonstrated protein thiol oxidation and GSSG formation

TABLE 2

Lactacystin Induces ARE4-Mediated Gene Expression

Vector	Treatment	CAT activity (±SD) relative to pBLCAT2
pBLCAT2/AP-1$_{-269\ to\ -263}$	None	1.7 (±0.2)
	5 μM lactacystin/5 h	1.7 (±0.2)
pBLCAT2/ARE4	None	5.5 (±0.6)
	5 μM lactacystin/5 h	13.3 (±0.3)*
	2.5 mM Azetidine/16 h	12 (±0.1)*

* Indicates that there is a statistically significant difference between untreated and treated samples ($p \leq 0.05$), as determined using a Wilcoxon Signed Rank Test.

in the ER is a consequence of Ero1 (endoplasmic reticulum oxidation 1) activity.

Disruption of glutathione metabolism can play a prominent role in the cascade of events leading to apoptosis (21–23), the progression of HIV (28), and in neurodegenerative diseases such as Parkinson's (29). Alterations in glutathione metabolism can contribute to the expression of resistance to certain chemotherapeutic drugs (reviewed in 26–28). Mulcahy *et al.* (30), Kurokawa *et al.* (31), and Ishida *et al.* (32) have directly demonstrated that over-expression of GSH can contribute to expression of drug resistance.

These pleiotropic effects produced by alterations in glutathione metabolism reflect the numerous physiological functions that involve glutathione. These include acting as a storage form of cysteine, providing reducing equivalents for many cellular reactions such as formation of deoxyribonucleotides, reduction of dehydroascorbate, leukotrienes, and detoxification of reactive oxygen species. GSH is also conjugated to many xenobiotics in Phase II detoxification reactions. Another important function for glutathione is to provide cellular redox buffering. This is a governed by the total glutathione concentration and the ratio of GSH to GSSG (33).

γ-Glutamylcysteine synthetase is the rate limiting enzyme in the synthesis of glutathione. The catalytic subunit of γGCS is GLCLC. Exposure to lactacystin produced a robust increase in GLCLC mRNA levels (Fig. 1) and in intracellular GSH levels. The increase in mRNA was not a consequence of message stabilization, as determined by measuring the half life of GLCLC mRNA (data not shown).

Recent investigations have provided data which indicate that several cis regulatory elements may contribute to the trancriptional regulation of GLCLC: MRE (15), NFκB (14), AP-$1_{-269\ to\ -263}$ (13), and ARE4 (also named EpRE4; 34). This current investigation has provided data demonstrating that ARE4 dependent expression of GLCLC can be regulated by proteasome function. Inhibition of proteasome activity, by definition, indicates that the NFκB site does not regulate expression under these conditions.

The GLCLC AP-1 site located between bp -269 and -263 was identified by Rahman *et al.* (13). They found that mutation of this site in the context of a 1050 bp promoter fragment inhibited transcriptional induction of heterologous CAT reporter vector induced by TNFα. They also compared the DNA binding activity of the GLCLC AP-1 sequence to a consensus AP-1 sequence using gel mobility shift assays. The two produced similar DNA binding activities. However, for this comparison, 25 μg of nuclear extract was reacted with the GLCLC AP-1 oligonucleotide while only 5 μg of extract was reacted with the consensus AP-1 oligonucleotide (13). This suggests that the GLCLC AP-1 element has 5 times less affinity than a consensus AP-1 element and is consistent with the data provided in Fig. 5 and Table 2. The GLCLC AP-1 sequence does not appear to exhibit significant heterologous DNA binding activity nor gene-dependent transcriptional activity. Whereas lactacystin treatment induced Jun-dependent DNA binding activity when a consensus AP-1 sequence was assayed (Fig. 5C), no induction was observed by the GLCLC AP-1 sequence. One interpretation of the results presented in Fig. 5, Table 2, and in Rahman *et al.* (13) is that the GLCLC AP-1 sequence can not act as a heterologous regulatory element in and of itself, but is part of an unidentified regulatory sequence.

Lactacystin-mediated induction of GLCLC was shown to be a consequence of Nrf2 binding to ARE4 (Fig. 4). This is consistent with the work of Wild *et al.* (34) who demonstrated that ARE4-mediated activity was regulated by Nrf2 in association with a Jun transcription factor, most likely JunD. Although not the focus of this investigation, expression of GLCLR, the regulatory subunit, is also regulated by Nrf2 (35). Therefore, it may be expected that treatment with lactacystin would induce both GLCLC and GLCLR and this account for the increase in GSH observed. However, even if GLCLR was not induced, increased expression of GLCLC by itself should be sufficient to account for the increase in GSH in HepG2 cells because in these cells Glclr synthesis exceeds that of Glclc (36).

Preliminary unpublished data from our laboratory suggests Nrf2 is a 26S proteasome substrate. HepG2 cells were exposed for 6 h to 0 or 5 μM lactacystin. Cell lysates were obtained and subjected to Western blotting with an Nrf2 antibody. The antibody recognized a immunoreactive 100 kDa band that was increased by the lactacystin treatment. No immunoreactive band was observed at 68 kDa. This is consistent with the observations of Mio *et al.* (37) who found that in vitro trancription/translation of human Nrf2 cDNA produced a immuno reactive band of 96 kDa rather than the predicted weight of 68 kDa, as measured by Western blotting. Mio *et al.* (37) attributed the discrepancy to an abundance of acidic residues that produced anomalous migration in SDS/PAGE. Venugopal and Jaiswal (38) transiently transfected HepG2 cells with a murine Nrf2 cDNA. Immunoprecipitation experiments revealed the presence of immuno reactive protein that exhibited sizes of 66 and 110 kDa. One interpretation is that the 66 kDa polypeptide represented the transfected murine Nrf2 while the 110 kDa polypeptides may have represented endogenous human Nrf2. This is based on the observation that murine cells exhibit a 66 kDa polypeptide that is immunoreactive with Nrf2 antibody (unpublished observations).

In summary, this investigation has demonstrated that ARE4 dependent expression of GLCLC can be regulated by proteasome activity. 26S proteasome-dependent degradation activity is central to a number of physiological processes (3, 9). These include

cell cycle progression (5), expression of stress induced trancription factors such as NFκB (6), and stress activated protein kinases JNK and p38 (19). It is important to note that the proteasome pathway is physiologically regulated, it is not a static system (3). The implication of proteasome regulation is that it provides a central mechanism for regulation of multiple pathways, including that for induction of glutathione synthesis.

ACKNOWLEDGMENT

This work was supported in part by a grant funded by USPHS Grants CA38079, CA73612, and CA60331.

REFERENCES

1. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) *Cell* **78,** 761–771.
2. Lee, D. H., and Goldberg, A. L. (1998) *Trends Cell Biol.* **8,** 397–403.
3. DeMartino, G. N., and Slaughter, C. A. (1999) *J. Biol. Chem.* **274,** 22123–22126.
4. Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998) *Cell* **92,** 367–380.
5. Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Mathias, N., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996) *Cell* **86,** 453–463.
6. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994) *Embo. J.* **13,** 5433–5441.
7. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990) *Cell* **63,** 1129–1136.
8. Takaoka, M., Itoh, M., Hayashi, S., Kuro, T., and Matsumura, Y. (1999) *Eur. J. Pharmacol.* **384,** 43–46.
9. Kirschner, M. (1999) *TCB* **9,** M42–M45.
10. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* **268,** 726–731.
11. Meister, A. (1988) *J. Biol. Chem.* **263,** 17205–17208.
12. Wild, A. C., Gipp, J. J., and Mulcahy, T. (1998) *Biochem. J.* **332,** 373–381.
13. Rahman, I., Antonicelli, F., and MacNee, W. (1999) *J. Biol. Chem.* **274,** 5088–5096.
14. Kondo, T., Higashiyama, Y., Goto, S., Iida, T., Cho, S., Iwanaga, M., Mori, K., Tani, M., and Urata, Y. (1999) *Free Radicals Res.* **31,** 325–334.
15. Gunes, C., Heuchel, R., Georgiev, O., Muller, K. H., Lichtlen, P., Bluthmann, H., Marino, S., Aguzzi, A., and Schaffner, W. (1998) *EMBO J.* **17,** 2846–2854.
16. Sekhar, K. R., Long, M., Long, J., Xu, Z. Q., Summar, M. L., and Freeman, M. L. (1997) *Radiat. Res.* **147,** 592–597.
17. Farris, M. C. a. R., D. J. (1983) Elsevier, North Holland Amsterdam.
18. Suzuki, Y. a. P., L. (1995) Redox Regulation of DNA–Protein Interaction by Biothiols, Vol. 252, Academic Press, New York.
19. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) *J. Biol. Chem.* **272,** 18033–18037.
20. Kim, D., Kim, S. H., and Li, G. C. (1999) *Biochem. Biophys. Res. Commun.* **254,** 264–268.
21. Mehlen, P., Kretz-Remy, C., Preville, X., and Arrigo, A. P. (1996) *EMBO J.* **15,** 2695–2706.
22. Knowles, S. E., Gunn, J. M., Hanson, R. W., and Ballard, F. J. (1975) *Biochem. J.* **146,** 595–600.
23. Seufert, W., and Jentsch, S. (1990) *EMBO J.* **9,** 543–550.
24. Salvat, C., Jariel-Encontre, I., Acquaviva, C., Omura, S., and Piechaczyk, M. (1998) *Oncogene* **17,** 327–337.
25. Lu, S. C. (1999) *FASEB J.* **13,** 1169–1183.
26. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* **257,** 1496–1502.
27. Cuozzo, J. W., and Kaiser, C. A. (1999) *Nat. Cell. Biol.* **1,** 130–135.
28. Jahoor, F., Jackson, A., Gazzard, B., Philips, G., Sharpstone, D., Frazer, M. E., and Heird, W. (1999) *Am. J. Physiol.* **276,** E205–E211.
29. Jenner, P., and Olanow, C. W. (1998) *Ann. Neurol.* **44,** S72–S84.
30. Mulcahy, R. T., Bailey, H. H., and Gipp, J. J. (1995) *Cancer Res.* **55,** 4771–4775.
31. Kurokawa, H., Ishida, T., Nishio, K., Arioka, H., Sata, M., Fukumoto, H., Miura, M., and Saijo, N. (1995) *Biochem. Biophys. Res. Commun.* **216,** 258–264.
32. Ishida, T., Nishio, K., Kurokawa, H., Arioka, H., Fukumoto, H., Fukuoka, K., Nomoto, T., Yokote, H., Hasegawa, S., and Saijo, N. (1997) *Int. J. Cancer* **72,** 865–870.
33. Freeman, M. L., Huntley, S. A., Meredith, M. J., Senisterra, G. A., and Lepock, J. (1997) *Cell. Stress Chaperones* **2,** 191–198.
34. Wild, A. C., Moinova, H. R., and Mulcahy, R. T. (1999) *J. Biol. Chem.* **274,** 33627–33636.
35. Moinova, H. R., and Mulcahy, R. T. (1999) *Biochem. Biophys. Res. Commun.* **261,** 661–668.
36. Soltaninassab, S. R., Sekhar, K. R., Meredith, M. J., and Freeman, M. L. (2000) *J. Cell. Physiol.* **182,** 163–170.
37. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9926–9930.
38. Venugopal, R., and Jaiswal, A. K. (1998) *Oncogene* **17,** 3145–3156.