Glutamine Deprivation Induces the Expression of GADD45 and GADD153 Primarily by mRNA Stabilization*

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The expression of the growth arrest- and DNA damage-inducible genes, GADD45 and GADD153/CHOP (C/EBP-homologous protein), as well as GRP78 (glucose-regulated protein of 78 kDa) was examined in several human breast cell lines subjected to acute glutamine (GLN) deprivation. GLN deprivation caused rapid elevation of GADD45 and GADD153/CHOP mRNA levels in cells that were highly dependent upon GLN for growth and viability. Both GADD mRNAs were rapidly elevated up to several hundred-fold. In contrast, GRP78 expression was elevated by no more than 4-fold by GLN deprivation. The magnitude of GADD up-regulation roughly correlated with the extent of GLN dependence of each cell line. The levels of all three mRNAs were responsive to alterations of ambient GLN content in a physiologically relevant concentration range that corresponded to the affinities of cellular GLN transporters. Provision of GLN-derived metabolites partially inhibited the induction of GADD expression in GLN-deprived cells. Nuclear run-on assays and mRNA decay studies suggested that the primary mechanism leading to increased GADD mRNA levels was not transcriptional, but rather that GADD45 and GADD153/CHOP expression were up-regulated in response to GLN deprivation via marked stabilization of these mRNAs. These results suggest that the expression of GADD genes contributes to growth arrest and/or protection from metabolic damage during GLN-poor conditions.

GADD45 (growth arrest and DNA damage-inducible gene) is a p53-responsive gene encoding a protein that interacts with p21/WAF1/CIP1 as well as proliferating cell nuclear antigen (1–3). GADD153 encodes a C/EBP-homologous protein that binds to C/EBP isoforms, and these heterodimers bind novel DNA recognition sites (4, 5). Expression of GADD genes is induced by medium depletion and by genotoxic agents that cause DNA damage (6–9). Exogenous expression of GADD45 or GADD153 can cause antiproliferative effects (4, 10–12). GADD45 may inhibit cell proliferation by a mechanism involving its association with p21/WAF1/CIP1 (1, 3). Expression of both GADD45 and p21/WAF1/CIP1 are up-regulated by wild-type p53, and these proteins may play a causal role in the mechanism of growth repression by this tumor suppressor (2, 13–15). GADD153 expression is induced by genotoxins and inhibitors that block protein glycosylation and/or induce stress in the endoplasmic reticulum (ER) and may play a causal role in the induction of cell death following ER stress (16). Expression of exogenous GADD153 causes apoptosis in myeloblastic leukemia cells (12), and endogenous GADD153 expression has recently been linked to both leukemia cell apoptosis following etoposide treatment (17) and renal apoptosis following tunicamycin treatment (18).

GRP78 (glucose-regulated protein of 78 kDa or BiP) is a molecular chaperonin that aids in the synthesis and folding of glycoproteins in the ER (19). GRP78 expression is induced by glucose deprivation or any treatment that causes the accumulation of unfolded or malfolded proteins in the ER (20, 21). In fact, many treatments that lead to GRP78 expression (inhibition of protein glycosylation, glucose deprivation, and hypoxia) also induce GADD153 expression (16, 22, 23).

Recently, it was shown that essential amino acid deprivation induced GADD153 expression by both transcriptional and post-transcriptional mechanisms (24). In addition, GADD153 promoter activity was induced by starvation for both essential and nonessential amino acids (24). In contrast, little is known of what effects nutrient availability has on GADD45 expression. The amino acid GLN is a key regulator of cellular proliferation and survival, but there is no information regarding the mechanism by which GLN exerts this effect. In an attempt to gain insight into this phenomenon, we examined the expression of GADD45, GADD153, and GRP78 in subconfluent cultures of human breast cell lines subjected to acute GLN deprivation. The data presented here demonstrate that both GADD153 and GADD45 expression is controlled by GLN availability primarily through mRNA stabilization. Thus, GLN elicits a distinct molecular response, destabilization of GADD mRNAs. This response was particularly pronounced for GADD45 mRNA. Moreover, in a series of human breast cell lines, there is a direct correlation between the response of GADD mRNA expression to GLN deprivation and the GLN dependence of proliferation. The results suggest that the antiproliferative response to GLN starvation may involve induction of GADD45 and/or GADD153 expression by a mechanism that includes stabilization of this mRNA. This represents the first report of a potential molecular mechanism for the antiproliferative effects of glutamine deprivation.

EXPERIMENTAL PROCEDURES

Materials—All tissue culture media and medium supplements were purchased from Life Technologies, Inc. Amino acids and all biochem-

1 The abbreviations used are: ER, endoplasmic reticulum; GLN, glutamine; DMEM, Dulbecco’s modified Eagle’s medium; DRB, 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole; UTR, untranslated region; ARE, AU-rich element.
cals were obtained from Sigma or Fisher. HBL100, T47D, MB175, HS578Bst, SKBR3, and BT483 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). An additional description of these cell lines was published previously (25). TSE cells were established from a primary ductal breast carcinoma and kindly provided by Simon Powell (Massachusetts General Hospital, Radiation Oncology, Boston, MA). TSE.06 cells were obtained by adaptation of TSE cells to low GLN conditions. Tissue culture plasticware was purchased from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ), except for 24-well tissue culture plates, which were purchased from Costar Corp. (Cambridge, MA). CultuPlates™, UniFilter™ plates, and the 5% Carbonmicropate scintillation counter were obtained from Packard Instrument Company (Meriden, CT). L-Glutamic acid assay kits were purchased from Roche Molecular Biochemicals.

Cell Culture—Human cell lines were grown in 75-cm² T-flasks at 37 °C under a humidified atmosphere of 5% CO₂, 95% air. All of the cells, except TSE.06, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4 mM L-GLN, 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 10 µg/ml bovine insulin (complete DMEM). To obtain TSE.06 cells, TSE cells were serially passaged in T-75 flasks in medium containing diazoylated fetal bovine serum and progressively lower GLN concentrations. At each passage, confluent flasks were harvested, and half of the cells were seeded into a flask containing medium with a GLN concentration half that in the preceding culture. After approximately 4 months, cells growing in a GLN concentration of 0.06 mM were obtained and named TSE.06 cells. These cells were maintained in medium containing diazoylated fetal bovine serum and 0.6 mM L-GLN. For GLN deprivation and concentration-response experiments, cells were plated at various dilutions in 10-cm tissue culture dishes with complete DMEM and allowed to plate overnight (TSE, HBL100, T47D, and TSE.06) or for approximately 4 h (SKBR3 and BT483) before being rinsed twice with GLN-free medium (identical to complete DMEM, except supplemented with 10% diazoyl fetal bovine serum and no GLN) and incubated for 19 h before being fed with GLN-free media. The next day, equal volumes of GLN-free media containing amino acids or drugs as specified in the figure legends. Plating dilutions ranged from 1:3 (HBL100) to 1:6 (TSE) and were chosen to obtain cultures that were between 30 and 40% confluent at the initiation of GLN deprivation. SKBR3 and BT483 were allowed to plate for longer periods because they exhibit relatively slow attachment, spreading, and growth rates.

Cell Proliferation Assays—Subconfluent cells growing in complete DMEM were harvested, counted, and diluted in GLN-free medium. Cells were seeded in 96-well plates at approximately 4 × 10³ cells/well (HBL100) or 1 × 10⁴ cells/well (TSE) and allowed to attach overnight in GLN-free medium. The next day, equal volumes of GLN-free media supplemented with 2× concentrations of GLN were then added to wells so that the final GLN concentrations were between 0 and 4 mM. At the times indicated after feeding, relative cell densities were determined using the colorimetric tetrazolium-salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay as described under “Experimental Procedures.” Concentrations of GLN were as follows: 0 mM ([□]), 0.25 mM (○), 0.5 mM (○), 1.0 mM (○), 2.0 mM (○), and 4.0 mM (○).

modification of published methods (30) employing RNeasy™ total RNA extraction kits (Qiagen) to isolate nuclear RNA after in vitro transcription and ExpressHyb™ hybridization solution (CLONTECH) for capture reactions. For in vitro transcription reactions, 2–3 × 10⁶ nuclei were incubated for 30 min at 30 °C in transcription buffer (30 containing 250 µCi of [α-32P]UTP. Following chromatin disruption with high salt buffer, DNase treatment, and isopropyl alcohol precipitation, RNA was resuspended in 0.1% SDS, diluted with lysis buffer BRT (Qiagen), and then purified using the RNeasy™ total RNA extraction kit, following the manufacturer’s instructions. Transcripts were captured by linearized and denatured plasmids (described above for Northern blotting) immobilized on nylon membranes at 2 µg/µl. Capture membranes were blocked for 15–30 min in ExpressHyb™ hybridization solution at 65 °C and then contacted with 1 × 10⁶ cpm/ml of purified in vitro labeled RNA diluted in ExpressHyb™ hybridization solution for 30 h at 65 °C. After extensive washing at 65 °C with 1× SSC containing 0.1% SDS, membranes were treated with 50 µg/ml RNase A (Qiagen) for 30 min at room temperature and then washed at room temperature with 1× SSC containing 0.1% SDS. Captured transcripts were detected by contacting membranes to hypersensitive x-ray film (Reflection™, NEN Life Science Products), and autoradiographic images were quantified using a laser densitometer (Molecular Dynamics).

RESULTS

HBL100 and TSE Cells Are Highly Dependent upon Ambient GLN for Growth and Viability—Previous work from our laboratory examined the effect of medium GLN concentration on the growth and death of a series of human cell lines (25). For all these cell lines, proliferation and ultimate cell density were dependent on ambient GLN concentrations. Increased initial GLN concentrations supported faster growth rates and higher ultimate densities. The cell line TSE and HBL100 demonstrated the greatest dependence upon GLN for growth and viability. Growth and death curves of TSE and HBL100 cells are shown in Fig. 1. Exponentially growing cells were seeded at low density in GLN-free medium, allowed to attach overnight, and then fed with media containing various concentrations of GLN to obtain final GLN concentrations between 0 and 4 mM. Within 1 day after
feeding with GLN, cell densities began to increase in a GLN-dependent fashion. For HBL100 and TSE cells, GLN concentrations of 2.0 and 1.0 mM, respectively, were required to support maximal growth. The results shown are in keeping with the results of previous work, which determined that 0.8 and 0.6 mM GLN was required to support half-maximal growth rates of HBL100 and TSE cells, respectively (25). GLN deprivation caused the gradual death of these cells over the course of 5 days, as exhibited by a decline in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance and cell confluence, as well as the appearance of detached cells and debris.

**GADD45 and GADD153 mRNA Levels Are Rapidly and Dramatically Induced during GLN Starvation of HBL100 and TSE Cells**—Because TSE and HBL100 cells exhibited pronounced GLN dependence for growth and viability, these two cell lines were chosen to examine the induction of mRNAs corresponding to several stress response genes by acute GLN deprivation. Subconfluent cells were rinsed and fed with GLN-free medium, and the levels of GADD45, GADD153, and GRP78 mRNAs were determined in attached cells after 0 and 45 min and 1.5, 3, 6, 12, and 24 h of GLN deprivation (Fig. 2). GAPDH mRNA levels were also determined and used as a normalization factor to correct for RNA loading and transfer variations between samples. GLN deprivation caused a rapid and dramatic induction of both GADD mRNAs and a consistent, but lesser, induction of GRP78 mRNA. GADD mRNAs were appreciably elevated within 1.5 h of GLN starvation. In the case of HBL100 cells, the GAPDH mRNA-normalized level of GADD45 mRNA reached a maximum of 20-fold over the initial level at 12 h, and the level of GADD153 mRNA reached a maximum of 12-fold above the initial level at 6 h. In contrast, the GRP78 mRNA level peaked at only 1.8-fold greater than the initial level at 6 h. GAPDH mRNA levels were not consistently affected by GLN deprivation.

In TSE cells, GADD mRNAs were also discernibly elevated within 1.5 h of GLN starvation. In contrast to HBL100 cells, the level of all three mRNAs increased linearly with time in TSE cells, apparently not reaching maximum levels. At 24 h, GADD45 and GADD153 mRNA was increased by approximately 160- and 180-fold over the initial levels, respectively. GRP78 mRNA reached only 3.5-fold the initial level at 24 h. In contrast to HBL100 cells, the apparent level of GAPDH mRNA in TSE declined in a roughly linear fashion to approximately 31% of the initial value. Because of this, the increases in normalized GADD and GRP78 mRNA levels may overestimate the true induction of expression. Nonetheless, GLN deprivation caused the levels GADD45 and GADD153 mRNA to increase by at least 50- and 55-fold, respectively. Rinsing HBL100 or TSE cells with GLN-free medium and then feeding with medium containing a normal amount of GLN (4 mM) did not cause an observable induction of GADD mRNAs (see below, and data not shown).

**GADD45, GADD153, and GRP78 mRNA Levels Are Inversely Related to Ambient GLN Concentration and Coincide with GLN Dependence of Growth**—To determine the dose response of induction of these stress-response mRNAs, subconfluent HBL100 and TSE cells were rinsed with GLN-free medium and fed with media containing serial dilutions of GLN from 4.0 to 0.06 mM. At 24 h after feeding, levels of GADD45, GADD153, and GRP78 mRNAs were determined and normalized to GAPDH mRNA levels. Media containing reduced concentrations of GLN elicited a dramatic increase of both GADD mRNA levels and a small induction of GRP78 mRNA (Fig. 3). In the case of HBL100 cells, the GAPDH mRNA-normalized level of GADD45 mRNA was increased to a maximum of approximately 40-fold compared with the level in cells incubated with 4.0 mM GLN, and the level of GADD153 was increased to a maximum of approximately 20-fold. For both GADD mRNAs, maximum levels were observed in cells incubated with 0.5 mM GLN. Lower GLN concentrations actually caused a slight decrease of these mRNA levels from the maximum at 0.5 mM GLN. In contrast, GRP78 mRNA level was increased by a maximum of only 1.2-fold at 0.5 mM GLN. Interpolation of results suggested that half-maximal induction of these mRNAs occurs between 1.0 and 0.5 mM GLN, GAPDH mRNA levels were not consistently affected by GLN concentration.

In TSE cells, GADD45 mRNA induction was greatest, approximately 500-fold the level in 4 mM GLN-fed cells, at the lowest concentration of GLN (0.06 mM). The level of GADD153 mRNA also was greatest, approximately 270-fold, at 0.06 mM GLN. GRP78 mRNA levels were increased by a maximum of only 4-fold at 0.06 mM GLN. Interpolation of the results suggested that half-maximal inductions of these mRNAs occurs between 0.5 and 0.25 mM GLN. In contrast to HBL100 cells, GAPDH mRNA levels were affected by GLN deprivation. The apparent level of GAPDH mRNA in TSE cells was reduced in cultures fed with media containing less than 1.0 mM GLN, declining to approximately 38% of the level in 4 mM cells at 0.06 mM. Again, the increases in normalized GADD and GRP78 mRNA levels may therefore overestimate the true induction of expression, but GLN deprivation did cause the levels of GADD45 and GADD153 mRNA to increase by at least 190- and 100-fold, respectively.

It should be noted that rinsing of TSE or HBL100 cell with GLN-free medium and then feeding with GLN-replete medium did not cause any appreciable accumulation of GADD45, GADD153, or GRP78 mRNAs after 24 h. In addition, time course experiments were performed that examined the kinetics.
of induction for GADD45 and GADD153 mRNAs in TSE cells fed with media containing various GLN concentrations after being rinsed with GLN-free medium, and no transient induction of GADD mRNA levels was observed in cells fed with GLN-replete medium (data not shown).

Induction of GADD45 and GADD153 Levels Correlates with the GLN Dependence Exhibited by Several Human Breast Cell Lines—In a previous report, we characterized the GLN dependence and the GLN transporter characteristics for a series of human breast carcinoma cell lines (25). A direct correlation was found between two parameters: the GLN ED$_{50}$ for growth and the $K_m$ for GLN of the System ASC transporter. These represent the concentration of GLN required for half-maximal growth of each cell line and the concentration of GLN at which each cell line’s transporter is able to transport GLN at a half-maximal rate, respectively. The induction of GADD45, GADD153, and GRP78 by GLN deprivation was examined in several of these cell lines, including HBL100 and TSE cells as well as a derivative of TSE cells that was gradually adapted to growth in medium containing a low GLN concentration (0.06 mM), TSE.06 cells. These experiments were conducted in a fashion similar to those described above. Values for GADD45 and GADD153 mRNA inductions were obtained by quantitative Northern blotting of RNA obtained from cells subjected to GLN dose-response experiments and GLN deprivation time course experiments. In dose-response experiments, cells were incubated for 24 h in media containing 4, 1, 0.25, 0.06, and 0 mM GLN. In time course experiments, cells were fed with GLN-free medium and incubated for 0, 0.75, 1.5, 3.0, 6.0, 12, and 24 h (TSE and HBL100) or for 0, 3.0, 6.0, 12, and 24 h (all others). The results of this analysis are shown in Table I. Human breast cell lines that are less GLN-sensitive exhibited inductions of GADD45 and GADD153 mRNA levels by GLN deprivation that were less pronounced and occurred at lower GLN concentrations than the inductions in TSE or HBL100 cells. In addition, TSE cells adapted to grow in medium containing only 0.06 mM GLN (TSE.06 cells) no longer exhibited a striking response to GLN deprivation.

Transcription of GADD45, GADD153, and GRP78 Genes Is Increased by No More Than 2–5-Fold by GLN Deprivation—The large increase in GADD45 and GADD153 expression caused by GLN deprivation could be attributed to increased transcription rates, increased mRNA stability, or a combination of these mechanisms. In order to test the effect of GLN upon gene transcription, subconfluent TSE cells were rinsed and fed with GLN-free medium with or without the addition of 4 mM GLN, and nuclei were isolated 3 h after feeding. Capture membranes were hybridized with equal activities of purified RNA labeled in vitro with nuclei from GLN-deprived and GLN-fed cells. A representative experiment is shown in Fig. 4. Compared with GAPDH, the amount of radioactively labeled transcripts captured by immobilized GADD45, GADD153, and GRP78 cDNAs was relatively small. When the RNA was derived from nuclei of GLN-deprived cells, the amount of radioactivity captured by the GAPDH cDNA was slightly reduced, whereas that captured by GADD45, GADD153, and GRP78 cDNAs was increased by 4-, 3-, and 2-fold, respectively. This analysis was repeated three times.

### Table I

Comparison of GADD45 and GADD153 induction by GLN deprivation in human breast cell lines with varying GLN sensitivities

| Cell line   | Parameter | GLN ED$_{50}$ | Induction at ED$_{50}$ | Maximum induction | Time of maximum induction |
|-------------|-----------|---------------|------------------------|-------------------|---------------------------|
| HBL100      | Growth    | 0.8          | 0.39                   | 0.2               |                           |
|             | GLN uptake| 0.8          | 1.5                    | 0.5               |                           |
|             | GADD45    | 0.7          | 1.9                    | 0.8               | 12                        |
|             | GADD153   | 0.8          | 9.5                    | 12                | 6                         |
|             | GRP78     | 0.7          | 1.1                    | 1.8               | 6                         |
| TSE         | Growth    | 0.6          | 0.3                    | 0.25              | 24                        |
|             | GLN uptake| 0.25         | 250                    | 160               | 24                        |
|             | GADD45    | 0.3          | 135                    | 180               | 24                        |
|             | GADD153   | 0.3          | 0.3                    | 2.7               | 3.5                       |
| T47D        | Growth    | 0.1          | 4.1                    | 7.5               | 8                         |
|             | GLN uptake| 0.15         | 11.1                   | 14                | 37                        |
|             | GADD45    | 0.1          | 0.4                    | 7.5               | 8                         |
|             | GADD153   | 0.1          | 0.1                    | 14                | 37                        |
| SKBR3       | Growth    | 0.0^a        | 0.0^a                  | 0.0^a             | 2                         |
|             | GLN uptake| 0.0^a        | 0.15                   | 1.5               | 5                         |
|             | GADD45    | 0.1          | 1.2                    | 2                 | 4                         |
|             | GADD153   | 0.5          | 1.1                    | 1.4               | 1                         |
| TSE.06      | Growth    | 0.06         | 0.15                   | 0.06              | 5                         |
|             | GLN uptake| 0.19         | 0.1                    | 0.19              |                           |
|             | GADD45    | 0.06         | 0.5                    | 4                 | 24                        |
|             | GADD153   | 0.06         | 0.8                    | 13                | 24                        |
|             | GRP78     | ND^b         | ND                     | ND                | 1.4                       |

* a ED$_{50}$ for growth of 0.0 mM indicates a growth rate greater than 50% maximum in GLN-free medium.

* b ND, not determinable.
with similar results. The estimated increases in GADD45, GADD153, and GRP78 transcription rates by GLN deprivation varied but were always within 2–5-fold. This analysis suggested that GLN deprivation causes up-regulation of GADD45, GADD153, and GRP78 transcription. However, the marked increase in GADD45 and GADD153 mRNA levels observed cannot be fully attributed to this mechanism. In contrast, the increase of GRP78 mRNA levels may be due to this mechanism alone.

GLN Causes Rapid Degradation of GADD45 and GADD153 mRNA—In order to determine whether GLN may affect the stability of GADD45 and GADD153 mRNA, the decline of GADD45 and GADD153 mRNA levels in GLN-starved cells was examined following GLN repletion and/or the addition of two inhibitors of transcription, actinomycin D and DRB. In separate experiments, subconfluent TSE cells were starved for GLN overnight (approximately 18 h) to bring about the accumulation of high GADD45 and GADD153 mRNA levels. In the first experiment, GLN-deprived cells were then fed with GLN-free medium to which 0 or 4 mM GLN was added, and 5 μg/ml actinomycin D or 0.1% ethanol carrier. At the various times after feeding, relative mRNA levels were calculated by comparison of GAPDH-normalized values with the level observed in cells at time 0 (Fig. 5 A). GLN repletion elicited a rapid decline in GADD45 and GADD153 mRNA contents that was not greatly affected by actinomycin D. Half-lives of mRNA species were estimated by least-squares analysis of ln(GAPDH-normalized mRNA levels) versus time, obtaining a decay constant from the slope of the linear portion of each curve. After feeding with GLN, GADD45 mRNA levels decayed with apparent half-lives of 45 and 29 min with and without actinomycin D, respectively. Likewise, GADD153 mRNA levels in GLN-fed cells decayed with apparent half-lives of 36 and 26 min with and without actinomycin D. The estimated increases in GADD45, GADD153, and GRP78 transcription rates by GLN deprivation varied but were always within 2–5-fold. This analysis suggested that GLN deprivation causes up-regulation of GADD45, GADD153, and GRP78 transcription. However, the marked increase in GADD45 and GADD153 mRNA levels observed cannot be fully attributed to this mechanism. In contrast, the increase of GRP78 mRNA levels may be due to this mechanism alone.

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In contrast, when cells were fed with GLN-free medium, GADD45 mRNA levels were maintained both in the absence and presence of actinomycin D. In fact, except for the 8-h sample, GAPDH-normalized GADD45 mRNA levels seemed to increase slightly after feeding with GLN-free medium containing actinomycin D. This indicates that the decay rate of GADD45 mRNA was similar to that of GAPDH mRNA. In these cells, GAPDH mRNA half-life was estimated to be approximately 13 h. Comparing this figure to the half-life of GADD45 mRNA observed in the presence of GLN (45 min), it can be estimated that the decay of GADD45 mRNA was accelerated approximately 17-fold by ambient GLN. GADD153 mRNA levels were also maintained after feeding cells with GLN-free medium and ethanol carrier. When actinomycin D was included, the apparent half-life of GADD153 mRNA was 2.8 h. This contrasts with a half-life of 36 min observed in the presence of GLN and actinomycin D. Thus, the GADD153 mRNA half-life was accelerated approximately 5-fold by ambient GLN. In contrast, in the presence of actinomycin D, the decay of GRP78 mRNA accelerated by less than 2-fold by ambient GLN. The apparent half-life of GRP78 mRNA was 9.2 h in GLN-free medium and 4.8 h in the presence of GLN. This comparison, together with the expression of GRP78 mRNA observed in the ethanol control cultures, suggests that the effect of GLN upon GRP78 mRNA expression is equally attributable to transcriptional and post-transcriptional mechanisms.

Nearly identical results were obtained when this experimental procedure was repeated using DRB, rather than actinomycin D, to block transcription (Fig. 5B). This experiment confirmed the rapid decay of GADD45 and GADD153 mRNA levels in cells fed with 4 mM GLN (and treated with 0.1% MeSO carrier). In this case, GADD45 and GADD153 mRNA levels as well as GRP78 levels continued to increase for 8, 4, and 24 h after refedding the cells with GLN-free medium with MeSO carrier. The reason for this is not apparent, for the cells were starved for GLN for approximately 18 h prior to refedding, DRB effectively inhibited the further increase in these mRNA levels in continuously GLN-starved cells. After feeding with 4 mM GLN, GADD45 mRNA levels decayed with apparent half-lives of 53 and 26 min with and without DRB, respectively. Likewise, GADD153 mRNA levels in GLN-fed cells decayed with apparent half-lives of 38 and 28 min with and without DRB, respectively. Feeding with GLN and MeSO carrier caused GRP78 mRNA levels to transiently increase and then decrease to approximately 40% of the level at time 0. In the presence of 4 mM GLN and DRB, GRP78 mRNA decayed with an apparent half-life of 3.6 h. When cells were refed with GLN-free medium and DRB, the GADD45 mRNA levels were maintained and slightly increased relative to GAPDH mRNA levels. Again, this indicated that the decay rate of GADD45 mRNA was similar to that of GAPDH mRNA. In these cells, GAPDH mRNA half-life was estimated to be approximately 11 h. Comparing this value to the half-life of GADD45 mRNA in the presence of 4 mM GLN and DRB (53 min), it can be estimated that GLN feeding caused an approximately 12-fold increase in the GADD45 mRNA decay rate. In the presence of DRB, GADD153 mRNA decayed with an apparent half-life of 2.3 h when cells were refed with GLN-free medium. By comparison with the figure obtained in the presence of GLN and DRB (38 min), it is apparent that GLN caused an approximate 4-fold increase in the GADD153 decay rate. Under GLN-free conditions and DRB, GRP78 mRNA decayed with an apparent half-life of 6 h, which was approximately 65% greater than the half-life obtained in the presence of ambient GLN. Thus, all the findings obtained using actinomycin D to block transcription were confirmed by the use of DRB.

DISCUSSION

The data presented here describe a rapid and pronounced response to GLN starvation; namely, the induction of GADD45 and GADD153 mRNA expression. This response was particularly acute in two human breast cell lines (HBL100 and TSE), which exhibit relatively high rates of GLN utilization and extreme dependence upon GLN for growth and viability (25). HBL100 cells were originally obtained from a mother's milk shortly after giving birth (31). This cell line is considered to be untransformed but can become tumorigenic during extensive in vitro culturing (32). TSE cells were derived from the tumor of a ductal adenocarcinoma patient. TSE cells exhibit an epithelialoid morphology and form tumors in athymic nude mice (data not shown). The induction of GADD45 and GADD153 mRNA expression was lesser in breast cell lines that are less GLN-dependent. In general, the half-maximal inductions roughly coincided with GLN dependences for growth and GLN transporter affinities (Table I). This suggests that this response coincides with diminished GLN influx or, conceivably, a lack of GLN transporter occupancy. However, the results with TSE.06 cells suggest that transporter affinity does not determine the GLN concentration at which the response is half-maximal. TSE.06 cells exhibit very little GLN-dependence for growth but retain a GLN-transporter affinity that is not very different from that of the highly GLN-dependent parental TSE cells. The $K_m$ of the ASC GLN transporter expressed by TSE.06 cells was determined to be 0.19 mM, whereas that of the parental TSE cells is 0.39 mM. In contrast, adaptation to growth in low-GLN medium caused the GLN ED$_{50}$ for growth to be reduced by 10-fold in TSE.06 cells compared with parental TSE cells. Consequently, the half-maximal induction of GADD45 and GADD153 expression in TSE.06 cells was observed at approximately 0.06 mM GLN (Table I). By comparison with TSE cells, the induction of GADD mRNA expression by TSE.06 cells might be expected to be half-maximal at 0.3 or 0.4 mM if the GLN transporter affinity determined this response. In addition, the extent of GADD mRNA inductions are reduced by more than 1 order of magnitude in TSE.06 cells compared with the parent line.

Whether the up-regulation of GADD45 and/or GADD153 plays any causal role in the growth arrest of cell death that results when HBL100 and TSE cells are deprived of GLN has yet to be tested. There are indications that these GADD45 and C/EBP homologous proteins possess directly antiproliferative or apoptotic functions. Several previous studies have implicated GADD45 expression in the mechanism of growth arrest triggered by p53 expression (2, 10, 13–15). GADD45 expression has also been implicated in growth arrest and apoptosis (4, 11, 12). Conversely, GADD45 and/or GADD153 could possess functions analogous to traditional stress-response genes, serving to protect cells from stress-induced damage and/or aiding the recovery of normal cellular functions following stress. Indeed, growth arrest could itself serve as a protective mechanism, by conserving energy and halting DNA synthesis in a time of glutamine scarcity.

The effect of GLN deprivation on the expression of another stress-response gene, GRP78, was also examined. In contrast to GADD45 and GADD153 mRNA, GRP78 mRNA levels were increased only marginally in GLN-starved cells. This was surprising, given that many genotoxic agents that induce GADD153 expression have comparable effects on GRP78 expression (23, 33, 34). In particular, GADD153 and GRP78 are expressed in response to agents that interfere with glycoprotein expression, an observation that led to the conclusion that both genes respond primarily to ER stress (16). Moreover, exogenous expression of GRP78/Bip depressed the induction of GADD153 expression in response to several agents that cause ER stress as well as methyl methanesulfonate, an agent thought to
induce GADD153 expression by causing DNA damage (16). GADD153 expression is induced by deprivation of cells with several amino acids. Marten et al. (35) demonstrated the induction of GADD153 mRNA levels in rat hepatoma cells by deprivation of phenylalanine, methionine, leucine, and tryptophan. In these cells, tryptophan deprivation caused the greatest induction, which was approximately 40-fold. Bruhat et al. (24) demonstrated that leucine starvation caused a 30-fold up-regulation of GADD153 expression in HeLa, HepG2, and Caco-2 cells. These authors also demonstrated that deprivation of several amino acids caused GADD153 promoter activity to be increased by up to 8-fold in reporter-transfected HeLa cells. GLN deprivation caused GADD153 promoter activity to be increased by approximately 3-fold. This is consistent with the run-on transcription results (Fig. 4), which demonstrated that GLN increased transcription of the GADD153 gene in TSE cells by a similar amount. Likewise, GADD45 and GRP78 transcription were increased by 4- and 2-fold, respectively. Thus, the marked increase in GADD45 and GADD153 mRNA levels could not be solely attributed to increased gene transcription rates.

An examination of the decay of GADD45 and GADD153 mRNA in TSE cells suggested that GLN caused a rapid destabilization of these two transcripts (Fig. 5). The level of these mRNAs declined rapidly after GLN repletion, regardless of actinomycin D or DRB treatment. However, these mRNAs decayed relatively slowly when cells were fed with GLN-free media containing inhibitors of transcription. The effect of GLN upon mRNA decay rate was most pronounced for GADD45. The decay of this mRNA was accelerated by 12- and 17-fold by GLN. GLN had a lesser, but still pronounced, effect on GADD153 mRNA decay, accelerating its decay by 4- and 5-fold. GLN had a lesser effect on GRP78 mRNA decay, increasing its decay by less than 2-fold. Thus, the increases in GADD45, GADD153, and GRP78 mRNA levels produced by GLN deprivaton can be attributed to both increases in gene transcription rates and decreases in mRNA decay rates. In the case of GADD153 and GADD45, mRNA stabilization is the predominant cause of elevated mRNA levels. This is particularly true for GADD45 mRNA, which is dramatically stabilized in GLN-deprived cells.

Thus, the most striking effect of GLN deprivation observed in these cells is the pronounced inhibition of GADD45 mRNA decay. Computer analysis of the 3′-UTR of human GADD45 mRNA sequence shows that this message can assume a complex secondary configuration, which includes several stem-loop structures (data not shown). GADD45 mRNA stability may be affected by the activity of an RNA-binding protein, which interacts with one of these structures within the 3′-UTR of this mRNA. Several unstable mRNAs contain a sequence element known as an AU-rich element (ARE), which when bound by an ARE binding factor (AUF1) causes rapid degradation of these mRNAs (36). AREs contain multiple copies of the sequence AUUUUAU, and two groups have determined that the consensus ARE is composed of the sequence UUAUUAUUAU (37, 38). One copy of this element is only weakly destabilizing, but two or more repeats of this element cause pronounced destabilization. It should be noted that the 3′-UTR of GADD45 mRNA is relatively AU rich (68%). GADD45 does contain two AUUUA sequences in its 3′-UTR at bases 1060 and 1278. However, neither of these match the nine-base consensus; the upstream element is UUAUUAUAUAGA, and the downstream element is UUAUUAAUG. Thus, GADD45 contains only a single “ARE-like” element. We have not found any other known RPB recognition sequence element in the GADD45 3′UTR sequence.

The mechanism(s) by which GLN depletion and subsequent cellular stresses induce the transcription of and stabilize mRNAs for the growth arrest genes GADD45, GADD153, and GRP78 mRNAs remains to be determined. It is conceivable that completely separate mechanisms underlie these effects. Nonetheless, the antiproliferative nature of GADD45 and GADD153 suggests that the rapidly and dramatically up-regulated expression of these genes represents a potential molecular basis for the long observed yet poorly understood GLN dependence of mammalian cells in culture. Alternatively, the genes may serve to protect cells from the detrimental effects of GLN deprivation.

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