The EMBO Journal Vol. 22 No. 5 pp. 1180–1187, 2003

Stress-induced gene expression requires programmed recovery from translational repression

Isabel Novoa[1], Yuhong Zhang[1], Huiqing Zeng[1], Rivka Jungreis[1], Heather P.Harding[1,2] and David Ron[1,3,4,5]

[1]Skirball Institute of Biomolecular Medicine and the Departments of [2]Pharmacology, [3]Medicine and [4]Cell Biology, New York University School of Medicine, New York, NY 10016

[5]Corresponding author
e-mail: ron@saturn.med.nyu.edu

Active repression of protein synthesis protects cells against protein malfolding during endoplasmic reticulum stress, nutrient deprivation and oxidative stress. However, long-term adaptation to these conditions requires synthesis of new stress-induced proteins. Phosphorylation of the α-subunit of translation initiation factor 2 (eIF2α) represses translation in diverse stressful conditions. GADD34 is a stress-inducible regulatory subunit of a holophosphatase complex that dephosphorylates eIF2α, and has been hypothesized to play a role in translational recovery. Here, we report that GADD34 expression correlated temporally with eIF2α dephosphorylation late in the stress response. Inactivation of both alleles of *GADD34* prevented eIF2α dephosphorylation and blocked the recovery of protein synthesis, normally observed late in the stress response. Furthermore, defective recovery of protein synthesis markedly impaired translation of stress-induced proteins and interfered with programmed activation of stress-induced genes in the *GADD34* mutant cells. These observations indicate that *GADD34* controls a programmed shift from translational repression to stress-induced gene expression, and reconciles the apparent contradiction between the translational and transcriptional arms of cellular stress responses.

Keywords: protein phosphatases/protein synthesis/secretion/signal transduction

Introduction

Diverse upstream stress signals are coupled to a common effector step, the phosphorylation of serine 51 on the α subunit of the eukaryotic translation initiation factor 2 (eIF2). eIF2α phosphorylation inhibits nucleotide exchange on the eIF2 complex, attenuating translation of most mRNAs and reducing protein synthesis (Hinnebusch, 2000). This adaptation is believed to protect cells against potentially toxic malfolded or modified proteins that may accumulate under stressed conditions (Brostrom and Brostrom, 1998). eIF2α phosphorylation can also activate downstream gene expression programs by paradoxically promoting translation of special mRNAs encoding transcription factors such as yeast Gcn4p or mammalian ATF4 (Hinnebusch, 1996; Harding *et al*., 2000a). Phosphorylated eIF2α thus integrates diverse and seemingly unrelated forms of stress to initiate signaling in a common downstream stress-response pathway that we refer to as an integrated stress response.

The endoplasmic reticulum (ER) resident transmembrane eIF2α kinase PERK is activated by imbalance between the load of client proteins translocated into the ER lumen and the capacity of the organelle to process the load (so-called ER stress) (Kaufman, 1999; Mori, 2000; Patil and Walter, 2001). The resulting rapid decrease in protein synthesis reduces the load on the ER and plays an important role in cells' ability to resist ER stress (Harding *et al*., 1999, 2000b, 2001a; Scheuner *et al*., 2001). In the erythropoietic lineage, a different eIF2α kinase, HRI, is activated by lack of heme. The resulting decrease in globin synthesis prevents accumulation of toxic free globin chains and protects developing erythrocytes from destruction under conditions of limited iron availability (Han *et al*., 2001). In cells exposed to arsenite or cadmium, eIF2α phosphorylation and translation repression, which are effected by unknown mechanisms, minimize modification of newly synthesized proteins by the reactive transition metal (Brostrom *et al*., 1989; Brostrom and Brostrom, 1998). In all three examples, the phosphorylation of eIF2α protects the stressed cell from the toxic potential of its own modified or unfolded proteins.

The ER unfolded protein response (UPR) that activates PERK also induces transcription of genes whose products increase the organelle's capacity to process its client proteins (Kaufman, 1999; Mori, 2000; Patil and Walter, 2001). The implementation of this adaptation requires that the induced mRNAs be translated into proteins. Furthermore, signaling in two of the three pathways known to activate gene expression in the UPR requires new protein synthesis; both XBP-1, an effector of IRE1 signaling (Yoshida *et al*., 2001; Calfon *et al*., 2002), and ATF4, an effector of the eIF2α phosphorylation-mediated integrated stress response (Harding *et al*., 2000a; also see below) must be translated *de novo*. Some UPR-induced mRNAs have features that may protect them from translational repression (Macejak and Sarnow, 1990), and translation of some mRNAs, such as the *ATF4* mRNA, is even induced by modest levels of eIF2α phosphorylation (Harding *et al*., 2000a); however translation of most mRNA, including those of important ER chaperones like GRP78 (BiP) and GRP94 are repressed by severe ER stress (Harding *et al*., 2001b; and see below). Therefore, profound and persistent translational repression is seemingly at odds with cells' ability to effect a more long-term adaptation to ER stress. In arsenite-treated cells also, eIF2α phosphorylation-mediated shut down in protein synthesis should interfere with synthesis of new proteins

 Published by Oxford University Press

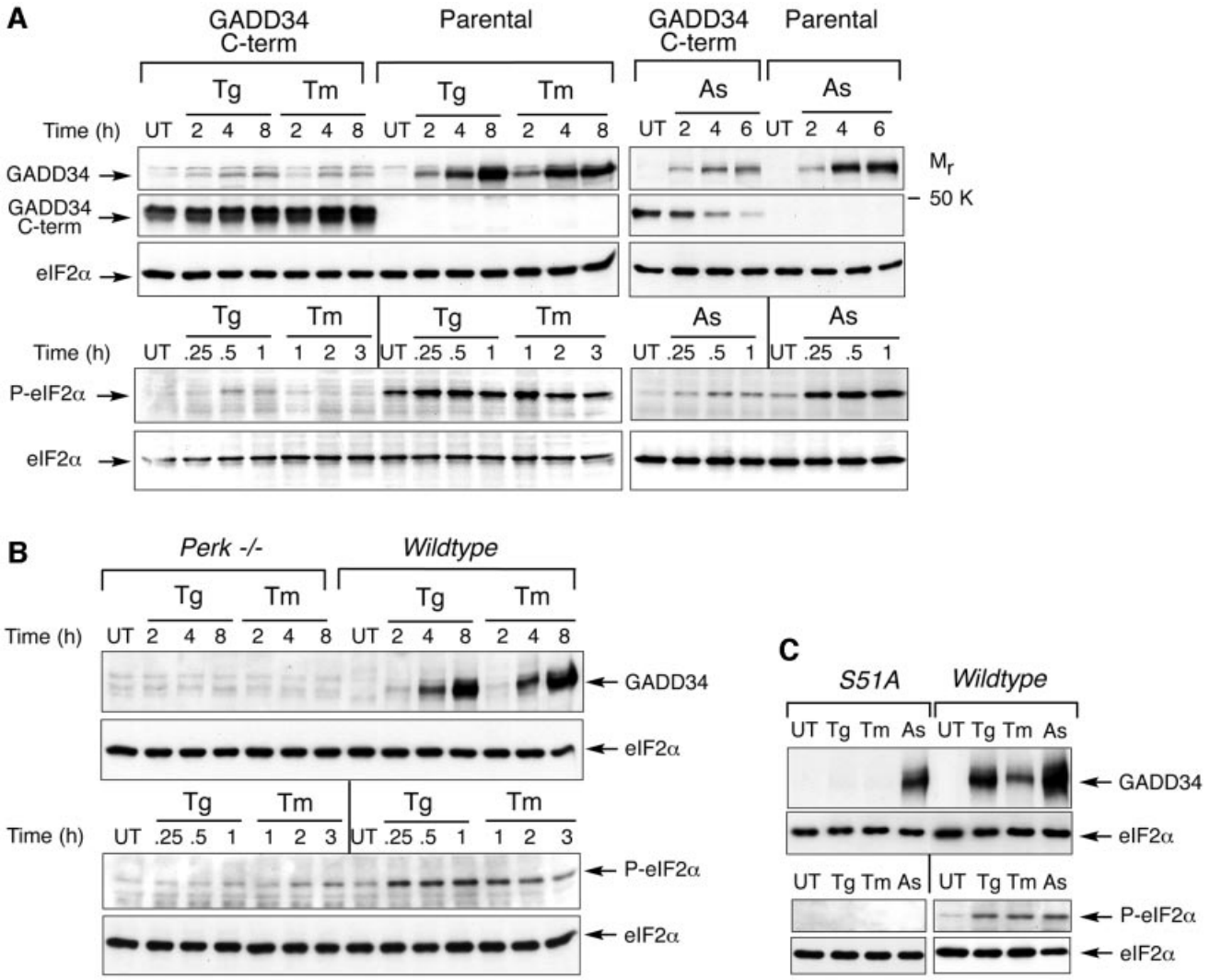


Fig. 1. eIF2α phosphorylation promotes GADD34 expression. (**A**) Immunoblot of GADD34, eIF2α phosphorylated on serine 51 (P-eIF2α) and total eIF2α from untreated (UT), thapsigargin (Tg)-, tunicamycin (Tm)-, or arsenite (As)-treated NIH-3T3 (Parental) cells or cells expressing a C-terminal fragment of GADD34 that constitutively dephosphorylates eIF2α (GADD34 C-term) (Novoa *et al*., 2001). (**B**) Immunoblot of GADD34, phosphorylated eIF2α and total eIF2α from untreated, thapsigargin and tunicamycin treated wild-type and *PERK–/–* mouse fibroblasts. (**C**) Immunoblot of GADD34 and total eIF2α from untreated (UT), thapsigargin (Tg; 8 h)-, tunicamycin (Tm; 8 h)- and arsenite (As; 4 h)-treated mouse embryonic fibroblasts with the indicated eIF2α genotype (upper panels). eIF2α phosphorylation was measured by immunoblot in the same cells following thapsigargin (Tg; 2 h), tunicamycin (Tm; 2 h) and arsenite (As; 2 h) treatment (lower panels).

that promote long-term resistance to the toxin (Levinson *et al*., 1980; Sok *et al*., 2001; and see below).

It has long been known that translational repression in response to both ER stress and exposure to arsenite is transient, and that expression of stress-induced mRNAs and their encoded proteins coincides with a phase of translational recovery (Brostrom *et al*., 1989; Brostrom and Brostrom, 1998; Kaufman, 1999). Yet the cellular mechanisms underlying translational recovery remain poorly understood.

GADD34 is a stress-induced gene encoding a regulatory subunit of a protein phosphatase 1 (PP1c)-containing complex that can dephosphorylate eIF2α *in vitro* and *in vivo* (He *et al*., 1996; Novoa *et al*., 2001). Herpes simplex virus has evolved a GADD34-like activity to evade the consequences of eIF2α phosphorylation that are induced by the eIF2α kinase PKR in virally infected cells (Roizman, 1999). *GADD34* is itself a target of the eIF2α phosphorylation-mediated integrated stress response (Novoa *et al*., 2001), suggesting that it may promote eIF2α dephosphorylation in stressed cells. Therefore, we decided to study GADD34's role in switching between an early phase of translational repression and a later phase of derepression in the course of stress responses.

Results

eIF2α phosphorylation plays a role in GADD34 induction by the integrated stress response

eIF2α phosphorylation at serine 51 promotes expression of stress-induced genes, such as the transcription factors ATF4 and CHOP (Scheuner *et al*., 2001). Expression of these genes, as well as GADD34, under conditions of ER stress or amino acid deprivation, requires activity of the

upstream eIF2α kinases PERK and GCN2 (Harding *et al*., 2000a; Novoa *et al*., 2001). To confirm that induction of GADD34 protein in stressed cells proceeds via signaling through eIF2α phosphorylation, we compared endogenous GADD34 protein levels in stressed cells that overexpress a C-terminal fragment of GADD34 that constitutively dephosphorylates eIF2α with their level in similarly stressed wild-type cells (Novoa *et al*., 2001). Inhibition of eIF2α phosphorylation attenuated endogenous GADD34 expression in cells treated with agents that cause ER stress (thapsigargin and tunicamycin) or oxidative stress (arsenite) (Figure 1A). *PERK–/–* cells that are impaired in eIF2α phosphorylation in response to ER stress also failed to express GADD34 protein when challenged with thapsigargin or tunicamycin (Figure 1B), as predicted by our previous studies (Novoa *et al*., 2001). In mutant mouse embryonic fibroblasts in which both alleles of eIF2α encode a mutant protein that can not be phosphorylated by stress-activated kinases (S51A substitution; Scheuner *et al*., 2001), activation of GADD34 by ER stress is abolished completely and activation by arsenite is attenuated (Figure 1C). These observations support a role for eIF2α phosphorylation in promoting expression of GADD34 protein in stressed cells.

Levels of phosphorylated eIF2α have been shown to decrease late during the stress response (Brostrom *et al*., 1989; Brostrom and Brostrom, 1998; Kaufman, 1999), but the underlying mechanism remained obscure. We decided to examine the temporal relationship between changes in eIF2α phosphorylation levels during an ER stress response and the activity of the upstream kinase PERK and expression of the putative stress-induced phosphatase GADD34. Cells were treated continuously with the ER stress-inducer thapsigargin, and the phosphorylation state of eIF2α was measured at intervals by immunoblot with an antibody specific to the phosphorylated form of the protein. Activation of the upstream eIF2α kinase PERK was monitored by following the characteristic shift in mobility of the phosphorylated active form of the protein by SDS–PAGE (Harding *et al*., 1999), whereas GADD34 expression in the same lysates was measured by immunoblot. eIF2α dephosphorylation was noted despite persistent activation of the upstream kinase PERK (Figure 2). GADD34 protein is expressed at very low levels in unstressed cells, and its induction by thapsigargin correlated temporally with the phase of the stress response in which levels of phosphorylated eIF2α are observed to decline (Figure 2). While the temporal profile of GADD34 expression did not obviously precede eIF2α dephosphorylation, the near coincidence of the two events is consistent with a role for GADD34 in modulating phospho-eIF2α levels.

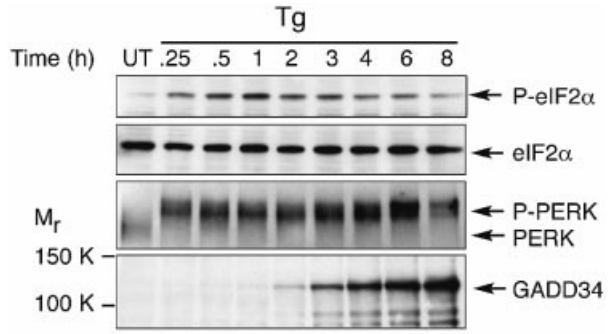


Fig. 2. Time course of GADD34 expression, eIF2α phosphorylation and PERK activation in ER stressed cells. Shown are immunoblots of P-eIF2α, total eIF2α, activated phosphorylated PERK (P-PERK), inactive PERK and GADD34 from untreated (UT) and thapsigargin (Tg)-treated NIH 3T3 cells.

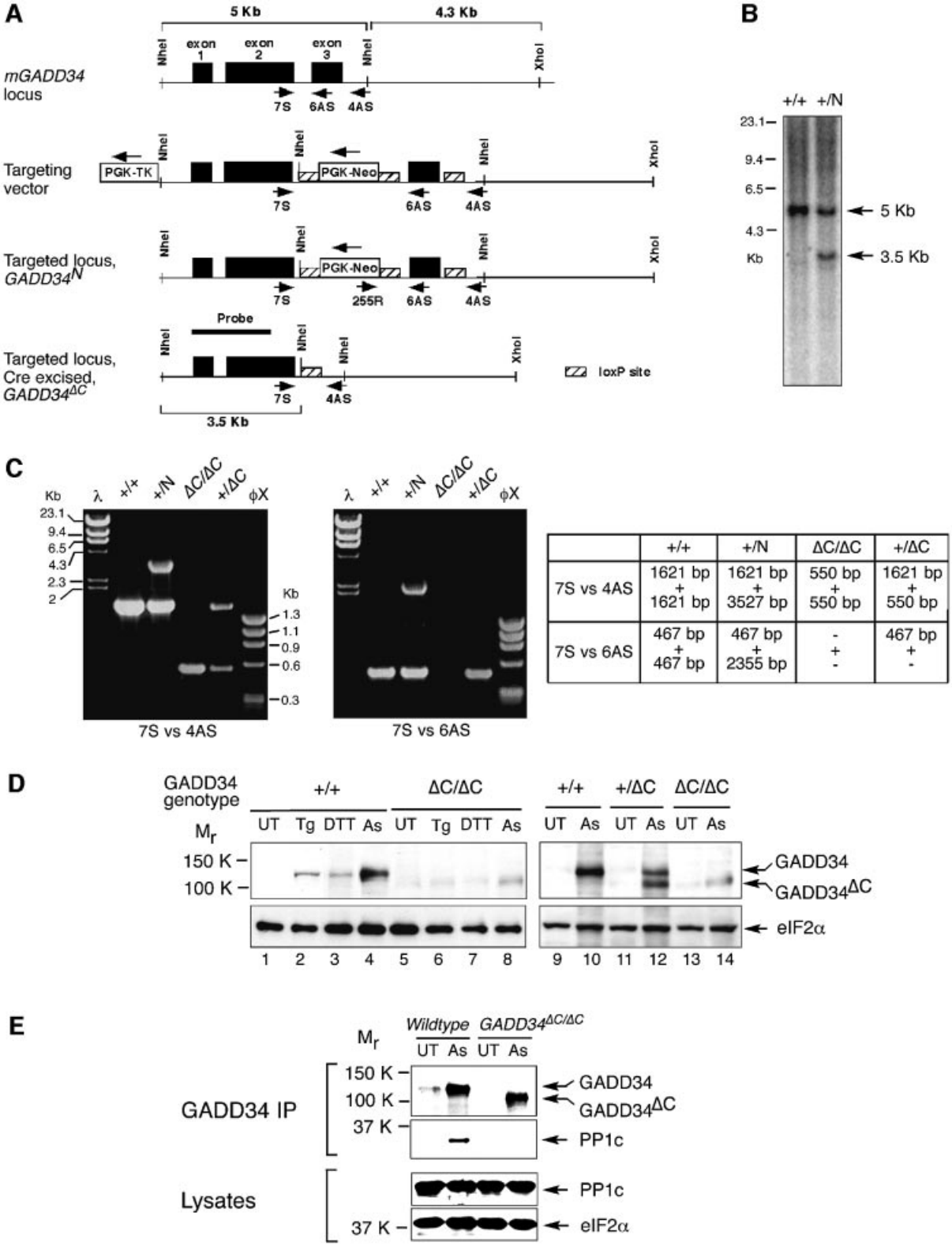
Generation of GADD34$^{\Delta C}$-targeted cells

To further explore the role of GADD34 in eIF2α dephosphorylation in stressed cells, we introduced a targeted mutation into the *GADD34* gene to delete the third exon, which encodes the domain of GADD34 protein (AA 549–657) that interacts with PP1c, the catalytic subunit of the phosphatase (Novoa *et al*., 2001). The targeting vector was designed to first introduce two *lox*P sites into the introns surrounding exon 3 and a neomycin resistance gene surrounded by loxP sites (Figure 3A). Mouse embryonic stem cell (ES) clones containing this *GADD34^{N}*-targeted allele were obtained after selection with G418 and gancyclovir, and their genotype was confirmed by PCR and Southern blot (Figure 3B and C). Cre recombinase was transiently expressed to isolate ES clones that excised exon 3 and the neomycin resistance cassette (*GADD34$^{\Delta C}$*; Figure 3C). The mutant allele was passed through the germline of chimeric mice, and homozygous mutant *GADD34$^{\Delta C/\Delta C}$* mouse embryonic fibroblasts were prepared from mutant embryos. Immunoblot analysis showed that the mutant allele encoded a truncated stress-induced protein, GADD34$^{\Delta C}$, of the predicted size, which was detected in the heterozygous (+/ΔC) and homozygous mutant cells (ΔC/ΔC) but was absent in the wild-type cells (Figure 3D).

To verify that the truncated protein encoded by the mutant allele lacked eIF2α phosphatase activity, we

Fig. 3. Targeted mutagenesis of *GADD34*. (**A**) Scheme of the genomic organization of mouse *GADD34* and targeting strategy to delete exon 3 encoding the PP1c-interacting domain (amino acid residues 549–657). From the top down: the wild-type locus; the targeting vector with the position of the oligonucleotides used to genotype the derivative alleles (arrows) and the *loxP*-sites (hatched rectangles) showing; the targeted *GADD34^{N}* locus before Cre-mediated excision of the Neo-cassette and exon 3; and the mutant *GADD34$^{\Delta C}$* allele after Cre-mediated excision of exon 3 and the Neo cassette. (**B**) Southern blot analysis of *Nhe*I-digested genomic DNA of the indicated *GADD34* genotypes. The position of the radiolabeled probe (*Hin*DIII cDNA fragment containing exon 1 and part of exon 2) and the predicted genomic *GADD34 Nhe*I fragments (5 and 3.5 Kb) are indicated in (A) above. (**C**) Detection of the various *GADD34* alleles by PCR. The primers 7S versus 4AS, and 7S versus 6AS are shown in (A). The table indicates the expected PCR products for each *GADD34* genotype. +, wild-type locus; N, targeted locus; ΔC, targeted locus after CRE-mediated excised of exon 3 and the neo cassette. (**D**) Immunoblot analysis of *GADD34* gene product in untreated (UT), thapsigargin (Tg)-, dithiothreitol (DTT)- and arsenite (As)-treated wild-type (+/+), *GADD34$^{\Delta C/+}$* and *GADD34$^{\Delta C/\Delta C}$* fibroblasts. eIF2α immunoblotting serves as a control for loading. (**E**) GADD34 and protein phosphatase 1 (PP1c) immunoblot of GADD34–PP1c complexes immunoprecipitated with anti-GADD34 antiserum from untreated (UT) and arsenite (As)-treated wild-type and *GADD34$^{\Delta C/\Delta C}$* cells (upper panels). Immunoblot of PP1c and total eIF2α in the lysate that served as the input for the GADD34–PP1c complex immunoprecipitation (two lower panels).

analyzed the interaction between GADD34$^{\Delta C}$ and the catalytic subunit of the protein phosphatase 1. Immunoprecipitation with GADD34 antibodies showed that GADD34$^{\Delta C}$ did not form a complex with PP1c (Figure 3E), a result consistent with previous observations whereby overexpressing the GADD34$^{\Delta C}$ truncated protein in transfected wild-type cells had no measurable impact on their stress response (Novoa *et al*., 2001). This, together with the normal stress response of heterozygous (+/ΔC) mutant cells (data not shown), suggests that the mutant



	+/+	+/N	ΔC/ΔC	+/ΔC
7S vs 4AS	1621 bp + 1621 bp	1621 bp + 3527 bp	550 bp + 550 bp	1621 bp + 550 bp
7S vs 6AS	467 bp + 467 bp	467 bp + 2355 bp	- + -	467 bp + -

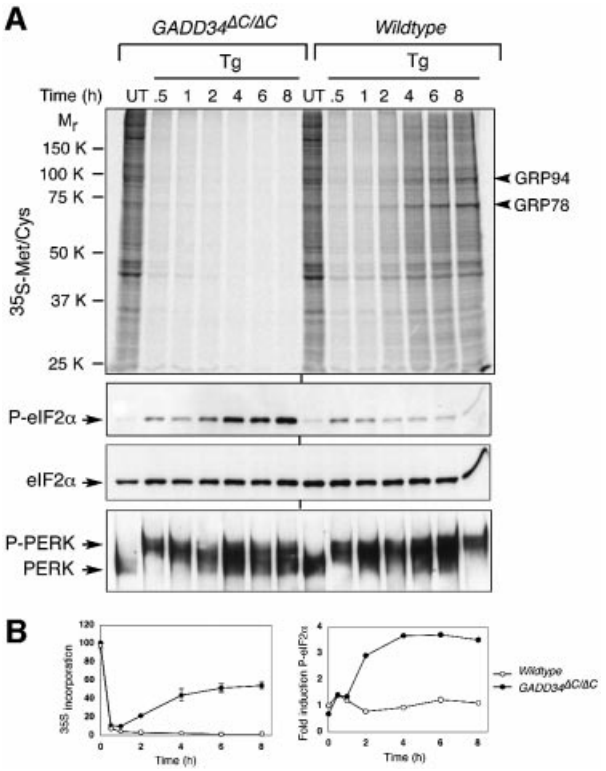


Fig. 4. GADD34 is required for eIF2α dephosphorylation and translational recovery during ER stress. (**A**) Upper panel, autoradiogram of an SDS–PAGE gel showing ^{35}S-methionine/cysteine incorporation into newly synthesized proteins in untreated (UT) and thapsigargin (Tg)-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts. Arrowheads to the right of the autoradiogram indicate the ER stress-inducible chaperones GRP78 and GRP94. Lower panels, immunoblots of P-eIF2α and total eIF2α, and immunoblots of PERK immunoprecipitates from the same cells. (**B**) Graphic presentation of ^{35}S-methionine/cysteine incorporation into newly synthesized proteins (left graph), and fold induction of phosphorylated eIF2α (right graph) in untreated and thapsigargin-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts. The level of ^{35}S incorporation in untreated cells is set at 100% whereas the signal of phosphorylated eIF2α from untreated wild-type cells is set as 1. Shown are mean ± SEM of experiments performed in duplicate and reproduced twice.

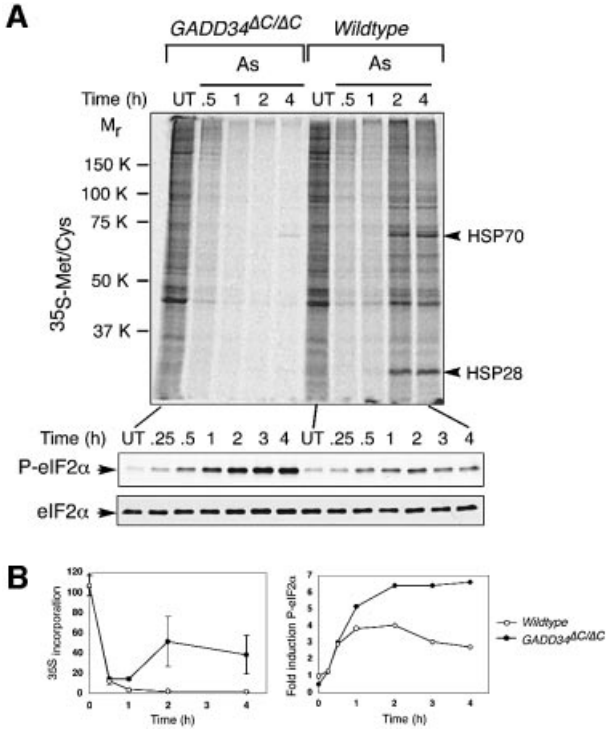


Fig. 5. GADD34 is required for eIF2α dephosphorylation and translational recovery during arsenite treatment. (**A**) Upper panel, autoradiogram of an SDS–PAGE gel showing ^{35}S-methionine/cysteine incorporation into newly synthesized proteins in untreated (UT) and arsenite (As)-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts. Arrowheads to the right of the autoradiogram indicate the arsenite-inducible chaperones HSP70 and HSP28. Lower panels, immunoblots of P-eIF2α and total eIF2α from the same cells. (**B**) Graphic presentation of ^{35}S-methionine/cysteine incorporation into newly synthesized proteins (left graph), and fold induction of phosphorylated eIF2α (right graph) in untreated and arsenite-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts. The level of ^{35}S incorporation in untreated cells is set at 100% whereas the signal of phosphorylated eIF2α from untreated wild-type cells is set as 1. Shown are mean ± SEM of experiments performed in duplicate and reproduced twice.

allele has pure loss-of-function features and is devoid of significant dominant-negative effects on the stress response.

GADD34 is required for eIF2α dephosphorylation and translational recovery in response to ER stress and oxidative stress

Next, we examined the impact of the *GADD34* mutation on eIF2α phosphorylation and protein synthesis in stressed cells. Wild-type and *GADD34*$^{\Delta C/\Delta C}$ mouse embryonic fibroblasts were continuously exposed to thapsigargin (an agent that causes persistent ER stress). At various time-points the rate of protein synthesis was measured by incorporation of radiolabeled methionine and cysteine into newly synthesized proteins, and eIF2α phosphorylation was analyzed in the same samples by immunoblotting with antibodies specific for the phosphorylated form of the protein.

In wild-type cells, thapsigargin treatment was associated with a profound but transient eIF2α phosphorylation and repression of new protein synthesis that was maximal between 0.5 and 2 h of treatment. This was followed by a partial decrease in eIF2α phosphorylation and partial recovery of protein synthesis later in the course of the stress response (Figure 4). These observations are consistent with previouse studies (Prostko *et al.*, 1993). Translation of the major ER stress-inducible chaperones, GRP78 and GRP94, occurred during this later phase of recovery from translational repression (Figure 4A). In contrast, thapsigargin-treated *GADD34*$^{\Delta C/\Delta C}$ cells experienced a progressive increase in eIF2α phosphorylation and persistent repression of protein synthesis, which interfered with translation of ER chaperones (Figure 4A and B). Activation of the stress-inducible kinase PERK was similar in both genotypes, suggesting that the mutation impacted on the dephosphorylation of eIF2α and not on stress-induced phosphorylation.

Similar differences between wild-type and mutant cells were observed in response to oxidative stress. In arsenite-treated wild-type cells, eIF2α phosphorylation was followed by dephosphorylation, translational recovery and synthesis of major stress-induced proteins HSP70 and HSP28, whereas persistent phosphorylation and translational repression were noted in arsenite-treated *GADD34*$^{\Delta C/\Delta C}$ mutant cells (Figure 5). The observations described above were made in mouse fibroblasts explanted

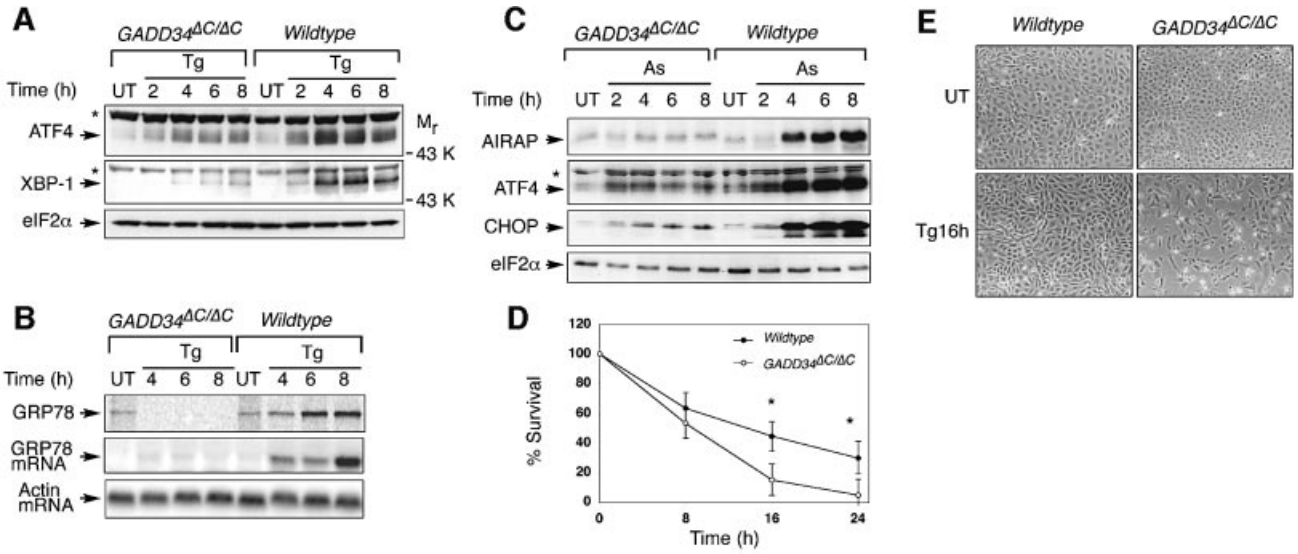


Fig. 6. *GADD34* promotes stress-induced gene expression. (**A**) Immunoblot of the UPR-induced transcription factors ATF4 and XBP-1 in untreated (UT) and thapsigargin-treated (Tg) wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts. The asterisks mark irrelevant proteins detected by the antisera. (**B**) Upper panel, autoradiogram of an SDS–PAGE gel of GRP78 immunoprecipitated from untreated (UT) and thapsigargin (Tg)-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts labeled with ^{35}S-methionine/cysteine. Middle and lower panels are northern blots of *GRP78* and β-*actin* mRNA from the same cells. (**C**) Immunoblot of the arsenite-induced proteins AIRAP (Sok *et al*., 2001), ATF4 and CHOP from untreated and arsenite-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts. (**D**) Survival of thapsigargin-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts, measured by their ability to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). Shown are the mean ± SEM of measurements carried out in duplicate on two different clones of cells from each genotype and reproduced 3 times. The asterisks denote $P < 0.0001$ by two-tailed *t*-test. 100% refers to MTT reduction by the untreated cells. (**E**) Photomicrographs of untreated and thapsigargin-treated wild-type and *GADD34*$^{\Delta C/\Delta C}$ fibroblasts.

from wild-type and mutant embryos. We observed similar effects of the GADD34 mutation in ER stressed and arsenite-treated ES cells (data not shown). These results show that GADD34-mediated eIF2α dephosphorylation plays a major role in stress responses as it is required for decreasing eIF2α phosphorylation levels late during the stress response. Inability to decrease the levels of phosphorylated eIF2α in the *GADD34* mutant cells impairs translational recovery and stress-induced protein synthesis.

GADD34-mediated eIF2α dephosphorylation is required for stress-induced gene expression

Expression of UPR target proteins is preceded by stress-induced transcription of their mRNAs (Kaufman, 1999; Patil and Walter, 2001). However, some of the transcription factors that mediate this induction, such as ATF4 and XBP-1, must be synthesized *de novo* (Harding *et al*., 2000a; Yoshida *et al*., 2001; Calfon *et al*., 2002). Accumulation of these upstream activators of stress-induced gene expression was impaired in *GADD34*$^{\Delta C/\Delta C}$ cells, most notably later in the course of the stress response (Figure 6A). Therefore, we wished to determine if GADD34-mediated eIF2α dephosphorylation also impacted on transcriptional induction of downstream UPR target genes. Northern blot analysis and pulse-labeling followed by immunoprecipitation showed that *GADD34*$^{\Delta C/\Delta C}$ cells were impaired not only in biosynthesis of GRP78 protein (Figures 4A and 6B) but also in induction of the encoding stress-induced mRNA (Figure 6B). In arsenite-treated *GADD34*$^{\Delta C/\Delta C}$ mutant cells, persistent repression of protein synthesis blocked the accumulation of arsenite-inducible proteins such as ATF4, CHOP and AIRAP (Figure 6C). Therefore, inability to restore protein synthesis in *GADD34*$^{\Delta C/\Delta C}$ cells affected not only the translation of the downstream targets of the stress response but also impaired activation of stress-induced gene expression programs.

The gene expression programs activated during stress responses impart stress resistance (Morimoto, 1998; Kaufman, 1999). Therefore, we compared the ability of wild-type and *GADD34*$^{\Delta C/\Delta C}$ cells to survive exposure to thapsigargin, an agent that causes ER stress. Mutant cells experienced decreased survival compared with wild-type cells (Figure 6D and E). Thus, *GADD34*, which is required for full induction of stress-induced gene expression, also protects stressed cells from death.

Discussion

The experiments described here, suggest a scenario whereby the integrated stress response, a signaling pathway activated by eIF2α phosphorylation of diverse causes, induces GADD34, which binds the catalytic subunit of a holophosphatase complex, directing it to phosphorylated eIF2α. GADD34-mediated eIF2α dephosphorylation thereby forms the effector arm of a negative-feedback loop that limits the impact of stress-activated eIF2α kinases such as PERK. Because of the inherent delay associated with induction of *GADD34* mRNA and translation of the protein, this negative-feedback loop has built-in latency that accounts for the biphasic profile of translational repression in stressed cells.

Previous studies have documented the ability of GADD34 to promote eIF2α dephosphorylation, and have revealed the role of stress-inducible eIF2α kinases in activating GADD34 gene expression (He *et al*., 1996; Novoa *et al*., 2001). The experiments described in this

paper establish a crucial role for GADD34-mediated eIF2α dephosphorylation in translational recovery during diverse stress responses. Analysis of *GADD34* mutant cells that are incapable of effecting such translational recovery have revealed, for the first time, the essential role this process plays in stress-induced gene expression. GADD34 activity thus reconciles the apparent contradiction between the translational and transcriptional arms of cellular stress responses.

As GADD34 is a target of the integrated stress response and the encoded protein is a proximal agent of translational recovery, it must be synthesized under relatively repressive conditions of eIF2α phosphorylation. The features that allow *GADD34* mRNA to be translated when eIF2α is phosphorylated are not known. The $GADD34^{\Delta C/\Delta C}$ cells also reveal some of the complexity involved in activating proximal targets of the integrated stress response. Accumulation of ATF4 protein is strictly dependent on stress-induced eIF2α phosphorylation and on the uORFs in its mRNA (Harding *et al*., 2000a; Novoa *et al*., 2001). Yet, in stressed $GADD34^{\Delta C/\Delta C}$ cells, ATF4 protein is expressed at lower levels than in the wild type (Figure 6A and C), despite very high levels of eIF2α phosphorylation in the mutant cells (Figures 4 and 5). This finding is consistent both with previous observations whereby uORFs afford but limit immunity from translational repression (Dever *et al*., 1993), and with the possible role of transcriptional feed-forward loops that reinforce ATF4 expression (Harding *et al*., 2000a) but are disrupted by the *GADD34* mutation. It is interesting to note that stressed $GADD34^{\Delta C/+}$ cells express more of the truncated protein encoded by the mutant allele than the homozygous mutant $GADD34^{\Delta C/\Delta C}$ cells (Figure 3D, compare lanes 12 and 14). This observation suggests that GADD34-mediated eIF2α dephosphorylation may also participate in a feed-forward loop that supports its own expression.

The significance of GADD34 as an agent of translational recovery is most apparent under conditions of severe stress, with high levels of eIF2α phosphorylation and marked repression of protein synthesis. Such conditions are found in ischemic neurons in developing brain infarcts (Doutheil *et al*., 1997; Kumar *et al*., 2001; DeGracia *et al*., 2002), and it is likely that under these circumstances GADD34 may play an important role in the ultimate recovery of protein synthesis. Another circumstance in which GADD34 may play an important role in translational recovery is during arousal from the torpor of hibernation, as the tissues of hibernating mammals exhibit very high levels of eIF2α phosphorylation and polysome deaggregation. Translational repression in hibernating animals is reversed immediately upon arousal in a process that may involve GADD34 (Frerichs *et al*., 1998; Connor *et al*., 2001). Our studies suggest that in these circumstances, GADD34 could have a profound impact on the outcome of the stress response, as reflected in the decreased survival of ER stressed $GADD34^{\Delta C/\Delta C}$ cells (Figure 6D and E).

Our experiments show that in stressed cells, GADD34-mediated translational derepression is important not only for translation of the downstream effectors of long-term adaptations to stress, but also for the expression of essential intermediates in the activation of stress-induced gene expression programs. However, it is important to remember that signaling through eIF2α phosphorylation also occurs in physiological circumstances, such as in the β cells of the endocrine pancreas during glycemic excursions (Harding *et al*., 2001a), or in the liver in the course of normal metabolism (Scheuner *et al*., 2001). Under these circumstances, activation of GADD34 may be important in modulating the intensity of the integrated stress response. Limiting activity in the integrated stress response may promote cell survival as at least one of the effectors of this response, the transcription factor CHOP, has been implicated in promoting programmed cell death in ER stressed cells (Zinszner *et al*., 1998; McCullough *et al*., 2001; Oyadomari *et al*., 2001). GADD34 is an essential component of a negative-feedback loop operating in the integrated stress response. The physiological impact of this loop is likely to depend on the severity and rate at which stress develops.

Materials and methods

ES cell culture and gene targeting

The mouse GADD34 gene was targeted in W4 ES cells by deleting the third exon encoding residues 549–657 that contains the PP1c binding domain required for phosphatase activity of the complex (Novoa *et al*., 2001). The 3577 base pair *Nhe*I–*Nde*I and 6078 base pair *Xma*I–*Xho*I fragments constituted the 5′ and 3′ homology arms of the targeting vector. A *PGK-Neo*^{r} cassette surrounded by *lox*P sites was inserted into the intron between exon 2 and 3, and a third *lox*P site was inserted 3′ to the third exon. ES clones with the appropriately targeted locus were then electroporated with a CRE-expressing plasmid to remove the *PGK-Neo*^{r} cassette and delete the third exon of the gene. Selected clones were injected into blastocysts for germline transmission. Mouse embryonic fibroblasts were obtained at day 13.5 from embryos parented by heterozygous mutant animals.

Cell culture

NIH3T3 cells, wild-type, *PERK–/–* (Harding *et al*., 2000b) and $GADD34^{\Delta C/\Delta C}$ mouse embryonic fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum, and eIF2α Ser 51 to Ala mutant mouse fibroblasts (Scheuner *et al*., 2001) were grown in DMEM supplemented with 10% fetal bovine serum, β-mercaptoethanol (0.55 μM) and non-essential amino acids (10 μM). The constitutively active GADD34 C-terminal protein fragment was expressed in cells by transduction of the A1 retrovirus (Novoa *et al*., 2001). Cells were treated with tunicamycin (2 μg/ml), thapsigargin (0.2 μM), DTT (1 mM) and sodium arsenite (80 μM), as described previously (Harding *et al*., 1999, 2000b).

Immunoblot, immunoprecipitation and northern blot

Cell extracts for immunoblotting and immunoprecipitation were obtained by lysis in 0.5% Triton X-100-containing buffer as described previously (Harding *et al*., 2000a). GADD34–PP1c complexes were immunoprecipitated with GADD34 antibodies bound to protein A–Sepharose beads after incubation with cell extracts containing 1 mg of protein as described previously (Novoa *et al*., 2001).

Newly synthesized proteins were labeled *in vivo* by a 15 min pulse of ^{35}S-Trans label (ICN; 50 μCi/ml) in DMEM lacking methionine but supplemented with 5% dialyzed calf serum. Cell extracts were prepared in 0.5% Triton X-100-containing buffer, and labeled proteins were resolved by SDS–PAGE and autoradiographed (Harding *et al*., 1999). Incorporation of ^{35}S-methionine/cysteine into newly synthesized GRP78 was revealed by immunoprecipitation, SDS–PAGE and autoradiography.

Rabbit polyclonal antibodies against GADD34 were prepared by immunization with a fusion protein of the N-terminal 536 residues of mouse GADD34 to glutathione S-transferase. PERK (Harding *et al*., 2000b), ATF4 (Vallejo *et al*., 1993), AIRAP (Sok *et al*., 2001) and CHOP (Ron and Habener, 1992) were detected as described previously. The antiserum to GRP78 was a gift from Gert Kreibich, and those to PP1c and XBP-1 were purchased from Santa Cruz Biotechnology. The phosphorylated form of eIF2α was detected with an antibody specific to the protein phosphorylated on serine 51 (Research Genetics), and total eIF2α was detected with a monoclonal mouse antibody (Scorsone *et al*., 1987).

RNA preparation and northern blot analysis of *GRP78* (BiP) and β-*actin* mRNA were carried out as described previously (Wang *et al*., 1996).

Acknowledgements

We thank David Levy, Robert Schneider and Joel Belasco for comments on the manuscript, NYU's Kaplan Comprehensive Cancer Center's transgenic mouse facility for producing chimeric mice, and Randal J.Kaufman and Donalyn Scheuner for generously providing the eIF2α^{S51A} mutant cells. This work was supported by NIH grants (ES08681 and DK47119). D.R. is an Ellison Medical Foundation Senior Scholar in aging.

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Received September 25, 2002; revised December 4, 2002; accepted January 14, 2003