GADD34 inhibits mammalian target of rapamycin signaling via tuberous sclerosis complex and controls cell survival under bioenergetic stress

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Abstract. Cells regulate the rate of protein synthesis during conditions of cell stress to adapt to environmental changes. However, the molecular interactions between signaling pathways controlling translation and the cellular response to stress remain to be elucidated. Here, we show that the expression of growth arrest and DNA damage protein 34 (GADD34) is induced by energy depletion and that the expression of this protein protects cells from apoptotic cell death. During conditions of cell stress, GADD34 forms a stable complex with tuberous sclerosis complex (TSC) 1/2, causes TSC2 dephosphorylation, and inhibits signaling by mammalian target of the rapamycin (mTOR). These findings demonstrate that crosstalk between GADD34 and the mTOR signaling pathways contributes to the response of the protein synthetic machinery to environmental stress. GADD34 may find clinical potential as a target drug for the treatment of mTOR-associated diseases.

Introduction

Protein synthesis is a major cellular process required for the regulation of growth, proliferation, differentiation and survival. Changes in gene expression patterns are necessary for the induction of these cellular processes. Among the processes that regulate gene expression, translation is the final step in the flow of genetic information. During conditions of cellular stress, cells must rapidly respond to changes in their physiological environment, and translational control allows more immediate and selective changes in expression than regulation at the level of gene transcription.

The best-characterized regulator of the translational machinery is target of rapamycin (TOR), which is an evolutionarily conserved serine/threonine protein kinase. The mammalian homologue of TOR, mTOR, stimulates protein synthesis by phosphorylating the major mTOR targets, ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein (4E-BP1). mTOR plays a pivotal role in controlling cell growth via its regulation of translation by phosphorylation of S6K and 4E-BP1 (1). Phosphorylation of S6K and 4EBP1 transduces nutrient and growth factor signals by modifying the rate of protein synthesis (2,3). *Tuberous sclerosis complex 1* (*TSC1*) and *TSC2* encode the tumor suppressors hamartin and tuberin, respectively, which are potent inhibitors of the mTOR pathway. Various types of cellular stress, such as glucose (3) or amino acid starvation (4) and hypoxia (5) activate the TSC1/TSC2 function and inhibit mTOR. TSC1 and TSC2 inhibit the mTOR function, leading to suppression of protein synthesis.

GADD34 belongs to a family of proteins whose expression is increased by growth arrest and DNA damage (GADD) (6). It is also a major regulator of translation during conditions of cell stress such as heat shock (7), virus infection (8), nutrient deprivation (9) and exposure to agents that cause improper folding of proteins in the ER (10). Cells respond to stress by turning off protein synthesis through phosphorylation of eukaryotic translation initiation factor 2α (eIF-2α) at serine 51 (11). Phosphorylated eIF-2α induces the expression of GADD34, which forms a functional complex with protein phosphatase 1 (PP1) to dephosphorylate eIF-2α, which leads to the restoration of protein synthesis (10). These processes appear to be critically involved in translational control during conditions of cell stress.

mTOR- and GADD34-mediated signaling pathways are thought to be independently regulated, and their integration

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during the regulation of translation under conditions of cell stress remains to be established. GADD34 expression is induced and mTOR signaling is inhibited in response to DNA damage, bioenergetic starvation or hypoxia (4-6,9,12,13), suggesting that signaling by these factors is coordinately regulated by stress and that it is involved in cellular adaptation to these conditions. In the present study, we aimed to clarify the integration of these signaling pathways during conditions of bioenergetic stress, and we have proposed a novel pathway mediated by GADD34, that inhibits mTOR signaling and maintains translation during conditions of bioenergetic stress.

Materials and methods

Materials. Anti-GADD34 (H-193), anti-TSC2 (C-20), anti-S6K (C-18), anti-eIF-2α (FL-315), anti-p53 (FL-393), anti-p21 (F-5) and anti-Myc (9E10) were obtained from Santa Cruz Biotechnology. Anti-HA (12CA5) was from Roche. Anti-Flag (M2) and anti-α-Tubulin (DM 1A) were obtained from Sigma. Anti-phospho-S^{51} eIF-2α was obtained from Biosource International. Anti-RB1 was from BD Pharmingen, and all other antibodies were from Cell Signaling. Rapamycin was from LC Laboratory, and cycloheximide was from Calbiochem. Full-length human GADD34 (GenBank accession no. BC003067) was obtained (Open Biosystems) and subcloned into Myc- or Flag-tagged pcDNA3. External and internal deletion mutants were generated by a combination between PCR-based manipulations and the appropriate external primers at the indicated amino acid positions in figures and digestions of restriction enzymes.

Cell culture, cell lines and transfection. Human embryonic kidney (HEK) 293 cells, and GADD34$^{-/-}$ and GADD34$^{+/+}$ mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). For glucose starvation, the cells were cultured with D-glucose-free DMEM or RPMI-1640 containing 25 mM HEPES and 10% dialyzed FBS (Invitrogen). Transfections were conducted using FuGENE6 (Roche Applied Science) or Lipofectamin 2000 (Invitrogen) according to the supplier's recommendations. TSC1$^{-/-}$ cells were derived from murine renal carcinoma. Stable TSC1-expressing cells were generated from TSC1$^{-/-}$ cells by transduction of the latter with a recombinant retrovirus expressing the human *TSC1* cDNA (GenBank accession no. NM_000368). GADD34-expressing cells were generated from TSC1$^{-/-}$ carcinoma cells, GADD34$^{-/-}$ and GADD34$^{+/+}$ MEFs by similar retroviral gene transfer. Cells were selected in the presence of blasticidin, and clones were isolated, expanded, and used in experiments.

Immunoprecipitation and immunoblot analysis. Cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% NP-40 and 1 mM Na_3VO_4 containing a mixture of protease inhibitors). The lysates were centrifuged at 20,000 x g for 10 min, and the supernatant was incubated with anti-Flag (Sigma), -HA (Roche), or -Myc (Sigma) immobilized beads for 2 h at 4˚C. The beads were washed three times with lysis buffer and boiled in the presence of SDS sample buffer. The protein complexes were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes and subjected to immunoblot analysis with the indicated antibodies.

Cell viability assay. Cell death was confirmed by trypan blue staining. Apoptosis was quantified using the annexin V-FITC apoptosis kit (MBL) according to the manufacturer's instructions. Briefly, MEFs were trypsinized (Invitrogen), pelleted by centrifugation, and re-suspended in annexin V binding buffer. FITC-conjugated annexin V (1 μl/ml) and propidium iodide (0.1 μg/ml) were added to cells and incubated for 15 min at room temperature in the dark. Analyses were performed on a FACSCaliber (Becton Dickinson). The data was analyzed with CellQuest software.

Cell cycle analysis. To analyze the cell cycle evaluated in control and GADD34-expressing cells, the distribution of DNA content was measured using the CycleTEST™ PLUS kit (Becton Dickinson). Flow cytometric data were collected on a FACSCaliber, and analyzed with CellQuest software.

RT-PCR. Total RNA was isolated from cells using TRIzol (Invitrogen) and first-strand cDNA was synthesized using Superscript III reverse transcriptase and Oligo-$d(T)_{12-18}$ primer (Invitrogen). The gene primer sequences were as follows: murine *Gadd34* forward, 5'-CTGCAAGGGGCTG ATAAGAG and reverse, 5'-AGGGGTCAGCCTTGTTTTCT; *Gapdh* forward, 5'-CATGACAACTTTGGCATTGTG and reverse, 5'-GTTGAAGTCGCAGGAGACAAC; human *GADD34* forward, 5'-GAATCAAGCCACGGAGGATA and reverse, 5'-CAGGGAGGACACTCAGCTTC; *GAPDH* forward, 5'-AACTTTGGTATCGTGGAAGGACT and reverse, 5'-CTGTAGCCAAATTCGTTGTCATAC. The PCR reaction consisted of 26 cycles for *GADD34* and *Gadd34* and 21 cycles for *GAPDH* and *Gapdh* using a 20-sec denaturation at 95˚C, 20-sec annealing at 55˚C and 30-sec extension at 72˚C.

Results

GADD34 protects cells against apoptosis caused by glucose depletion in a manner dependent upon mTOR. To clarify whether GADD34 is involved in the response to bioenergetic stress, we first examined the effect of glucose starvation on the expression of GADD34 in mouse embryonic fibroblast (MEF) and human embryonic kidney (HEK) 293 cells. Glucose starvation led to an induction of the expression of *GADD34* mRNA in both MEF (Fig. 1A) and HEK293 cells (data not shown), suggesting that GADD34 is involved in the energy sensor pathway. To investigate the functional significance of GADD34 induction in response to bioenergetic stress, we compared the effect of glucose starvation on wild-type (GADD34$^{+/+}$) and GADD34-deficient (GADD34$^{-/-}$) MEFs. Significant numbers of GADD34$^{-/-}$ MEFs died following depletion of glucose, whereas wild-type MEFs were unaffected (Fig. 1B and C). We next monitored the expression of caspase-3 in glucose-depleted, wild-type and GADD34$^{-/-}$ MEFs by immunoblot analysis. We detected the cleavage of caspase-3 in glucose-deprived GADD34$^{-/-}$ MEFs, but not in normal MEFs, demonstrating that GADD34$^{-/-}$ MEFs underwent

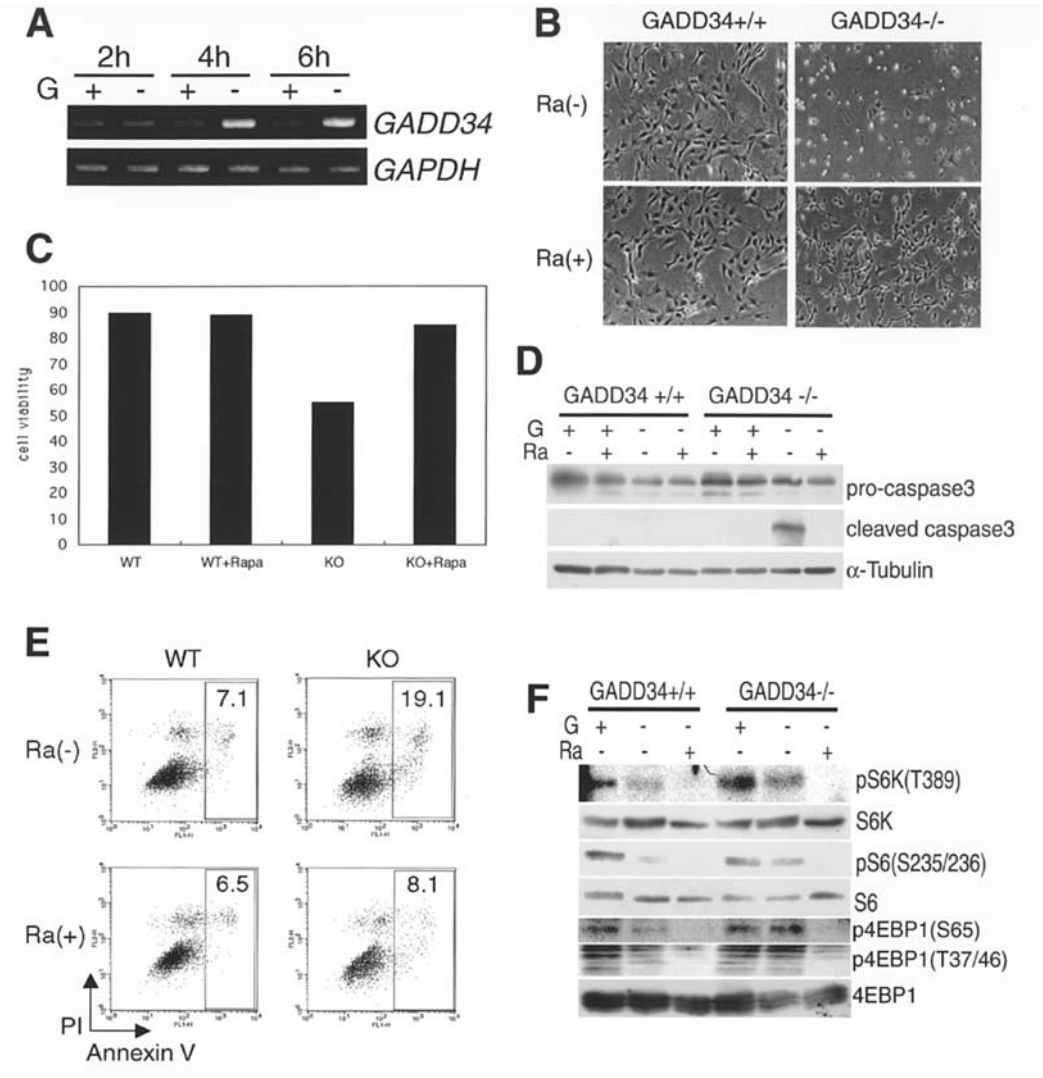

Figure 1. The induction of GADD34 protects cells against apoptosis caused by glucose depletion. (A) Glucose starvation induces the expression of GADD34 mRNA. Wild-type (GADD34$^{+/+}$) MEFs were cultured in 25 mM glucose (G^{+}) or glucose-free (G^{-}) medium for 72 h. *GADD34* mRNA was semi-quantified by RT-PCR. *GAPDH* mRNA was monitored as an internal control. (B) Significant GADD34-knockout (GADD34$^{-/-}$) cell death under glucose starvation was inhibited by rapamycin. GADD34$^{+/+}$ and GADD34$^{-/-}$ cells were cultured as (A) in the presence (Ra^{+}) or in the absence (Ra^{-}) of 20 nM rapamycin. Cells cultured for 72 h are shown. (C) Viability of GADD34$^{-/-}$ cells following glucose starvation was quantified by trypan blue staining. WT, wild-type MEFs; KO, GADD34-knockout MEFs. (D) Immunoblot analysis of caspase-3 in wild-type (GADD34$^{+/+}$) and knockout (GADD34$^{-/-}$) MEFs following glucose deprivation. The expression of α-Tubulin was monitored as an internal control. (E) Quantification of apoptotic cells in populations of wild-type (WT) and GADD34-knockout (KO) MEFs during glucose deprivation by flow cytometric analysis of annexin V-PI. Gated early and late apoptotic cell populations are shown as ratios (%) of the total cell population. (F) GADD34$^{-/-}$ cells exhibit high levels of mTOR activity without regard to glucose starvation. GADD34$^{+/+}$ and GADD34$^{-/-}$ cells were cultured as (B). The phosphorylation of S6K, S6 and 4E-BP1 was evaluated by immunoblot analysis of cell lysates with the indicated antibodies.

apoptotic cell death (Fig. 1D). Similarly, we observed a greater number of apoptotic cells in GADD34$^{-/-}$ MEFs than in wild-type cells during glucose deprivation by flow cytometric analysis of annexin V-PI (Fig. 1E). To note, rapamycin rescued GADD34$^{-/-}$ cells from death following glucose starvation (Fig. 1B-E), suggesting that the mTOR pathway is involved in the GADD34-mediated cellular response to bioenergetic stress.

To further evaluate the role played by the deregulation of mTOR signaling in the apoptotic death of energy-starved GADD34$^{-/-}$ MEFs, we investigated the effect of glucose starvation on mTOR signaling in GADD34$^{+/+}$ and GADD34$^{-/-}$ MEFs. As shown in Fig. 1F, glucose starvation inhibited the phosphorylation of mTOR targets S6K, S6 (a downstream target of S6K) and 4E-BP1 in GADD34$^{+/+}$ MEFs, whereas phosphorylation of these proteins was sustained in GADD34$^{-/-}$ MEFs treated in a similar fashion. Rapamycin inhibited the phosphorylation of S6K, S6 and 4E-BP1 in both GADD34$^{+/+}$ and GADD34$^{-/-}$ cells. These results suggest that apoptotic cell death caused by glucose starvation in GADD34$^{-/-}$ cells is due to deregulated signaling by the mTOR pathway, and that GADD34 may affect the signaling pathway upstream of mTOR.

GADD34 interacts with TSC1/2. GADD34 appears to play an inhibitory role in mTOR signaling, which is reminiscent of the effect of TSC1/TSC2 on mTOR signaling. Taken with the finding that GADD34, mTOR and TSC1/TSC2 are localized to the ER (14,15), this data suggests that GADD34 may

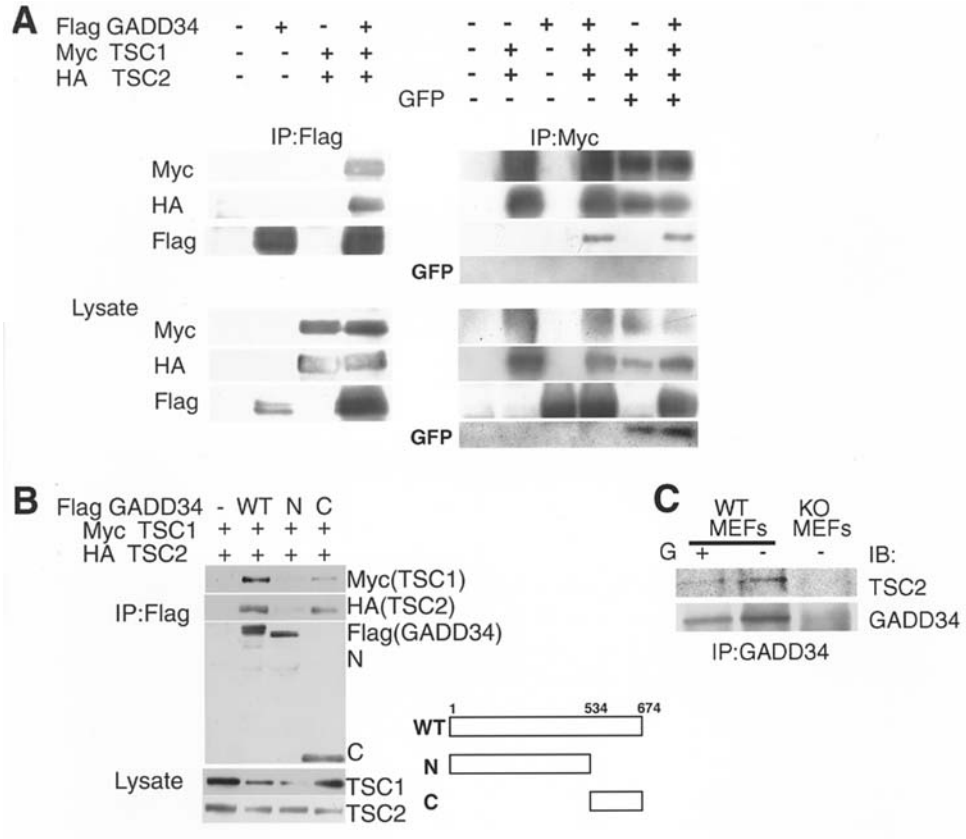

Figure 2. GADD34 interacts with TSC1/2. (A) HEK293 cells were transfected with Flag-GADD34 and/or Myc-TSC1/HA-TSC2 (see top). The cell lysates were immunoprecipitated with anti-Flag or anti-Myc antibodies. The immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis with the indicated antibodies. (B) The C-terminal region of GADD34 is required for association with TSC. HEK293 cells were transfected with full-length Flag-GADD34 (WT), or plasmids encoding either the N- (N) or C-terminal fragments of GADD34 (C) and Myc-TSC1/HA-TSC2. Cell lysates were immunoprecipitated with anti-Flag (M2; Sigma) immobilized beads, and the immunoprecipitates were blotted with the indicated antibodies. Schematic structures of the GADD34 fragments are indicated at the right. (C) Glucose-starvation enhances interaction between endogenous GADD34 and TSC. GADD34$^{+/+}$ and GADD34$^{-/-}$ cells were cultured in standard (G^+) or glucose-free medium (G^-) for 72 h. Protein complexes immunoprecipitated with the anti-GADD34 antibody were subjected to immunoblot analysis with antibodies against GADD34 and TSC2.

inhibit mTOR signaling via TSC1/TSC2. To clarify the relationship between GADD34 and TSC1/2, we evaluated the ability of GADD34 to interact with TSC1/TSC2. HEK293 cells were co-transfected with Flag-tagged GADD34 and Myc-tagged TSC1/HA-tagged TSC2. The cells were then lysed and the lysates were examined by immunoprecipitation and immunoblot analysis. Immunoprecipitation of cell lysates with the anti-Flag antibody and evaluation of the immunoprecipitates by SDS-PAGE followed by immunoblot analysis with antibodies to Myc, HA and Flag revealed that TSC1 and TSC2 coprecipitated with GADD34 (Fig. 2A). Reciprocal immunoprecipitation of the cell lysates with an anti-Myc antibody, followed by immunoblot analysis with anti-Flag and anti-HA antibodies, confirmed the association between GADD34 and TSC1/TSC2. A similar analysis of cell lysates from cells co-transfected with GFP, GADD34 and TSC1/TSC2, and immunoprecipitated using anti-Myc demonstrated that GFP was not involved in the complex between TSC1/TSC2 and GADD34 (Fig. 2A), suggesting that TSC1/2 and GADD34 associated in a specific manner.

We next examined which region of GADD34 is required for the association with TSC1/2. Cells were first co-transfected with Myc-TSC1, HA-TSC2 and either full-length Flag-GADD34 or plasmids encoding either the C- or N-terminal fragments of GADD34 (Fig. 2). Cell lysates were prepared and immunoprecipitated with the anti-Flag antibody and then subjected to immunoblot analysis with the anti-Myc, anti-HA or anti-Flag antibodies (Fig. 2B). The results demonstrated that the full-length and C-terminal fragments of GADD34 associated with TSC1/2, whereas the N-terminal fragment did not. In addition, glucose starvation strengthened the interaction between endogenous GADD34 and TSC2, whereas their interaction was weakly detected under non-starved conditions (Fig. 2C). These findings suggest that the induction of GADD34 expression in response to glucose starvation leads to the formation of a specific complex comprising GADD34 and TSC1/2. In addition, we demonstrated that this interaction requires the C-terminal fragment of GADD34 and that this interaction may be necessary for the inhibition of mTOR signaling.

GADD34 causes TSC2 dephosphorylation and inhibits signaling by mTOR. To evaluate the mechanism by which GADD34 inhibits mTOR signaling, we next evaluated the effects, under normal and glucose-deprived culture conditions, of the introduction of the *GADD34* gene into wild-type MEFs using a retroviral vector. The anti-TSC2 antibody recognizes both unphosphorylated and phosphorylated TSC2. A change

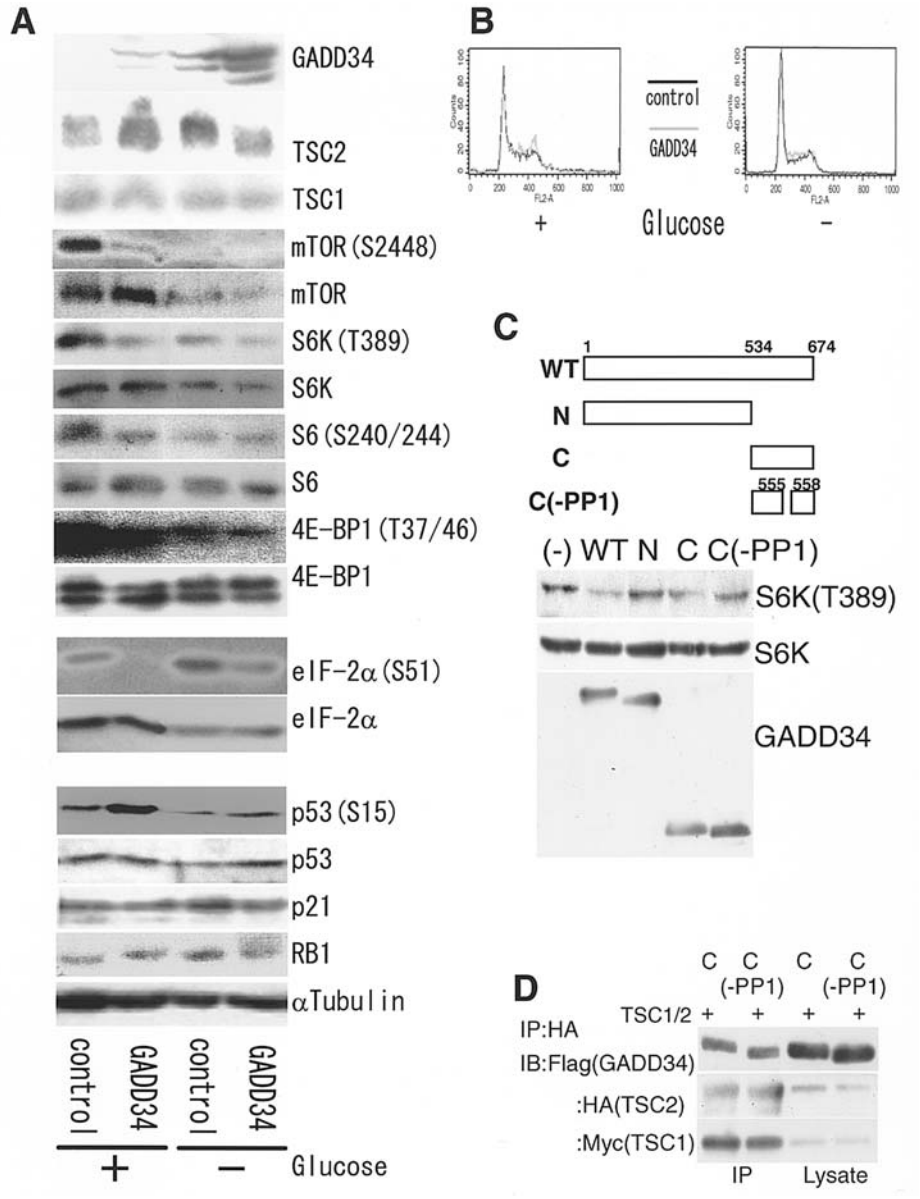

Figure 3. Retroviral-mediated overexpression of GADD34 leads to dephosphorylation of TSC2 and inhibits signaling by mTOR, but does not affect the cell cycle. (A) GADD34 inhibits mTOR signaling by dephosphorylating TSC2, particularly in glucose-deprived cells transduced with *GADD34*. The effect of retroviral gene transfer of *GADD34* into wild-type MEFs on the phosphorylation of mTOR, S6K, S6 and 4EBP1 was evaluated during culture in normal (+) and glucose-free (-) conditions. The expression and phosphorylation state of eIF-2α, p53, p21 and RB1 were also analyzed by immunoblot analysis using the indicated antibodies. (B) Flow cytometric analysis demonstrated that cell cycle phases are not significantly changed by forced expression of GADD34 or glucose deprivation. (C) The PP1 binding motif within the C-terminal fragment of GADD34 is required for the inhibition of S6K phosphorylation by GADD34. Immunoblot analysis was used to monitor the effect of expression of the various fragments of GADD34 on the phosphorylation of S6K at T389, using an antibody specific for phosphorylated S6K. Schematic structures of the GADD34 fragments transfected into the cells are shown in the upper panel. (D) The PP1 binding motif within the C-terminal fragment of GADD34 is not essential for binding to TSC1/2. HEK293 cells were cotransfected with plasmids encoding Myc-TSC1/HA-TSC2 and the Flag-GADD34 C-terminal domain with either an intact (C) or mutated (-PP1) PP1 binding motif. Cell lysates were immunoprecipitated with the anti-HA antibody and then subjected to immunoblot analysis using the indicated antibodies. Both intact C-terminal (C) and mutated C-PP1 fragments of GADD34 were able to bind to TSC1/TSC2, regardless of mutation of the PP1 binding domain.

in the phosphorylation status of the protein is indicated by a shift in its electrophoretic mobility. Overexpression of GADD34 led to TSC2 dephosphorylation as demonstrated by its electrophoretic shift-down, and inhibited the activity of the mTOR pathway, as evidenced by changes in the phosphorylation of mTOR, S6K, S6 and 4E-BP1. These effects were particularly pronounced in glucose-deprived cells transduced with exogenous *GADD34* (Fig. 3A). Thus, we have shown that GADD34 inhibits mTOR signaling by dephosphorylating TSC2.

Retroviral-mediated expression of GADD34 maintained a greater level of eIF-2α phosphorylation during glucose starvation than was the case under normal culture conditions (Fig. 3A), suggesting that expression of GADD34 was insufficient to dephosphorylate eIF-2α and to allow recovery of translation during glucose starvation. We further observed that the expression of p53, p21 and RB1 were unaffected by either retroviral expression of GADD34 or glucose-deprivation, indicating that none of these conditions has a significant effect upon the cell cycle. Furthermore, flow cytometric

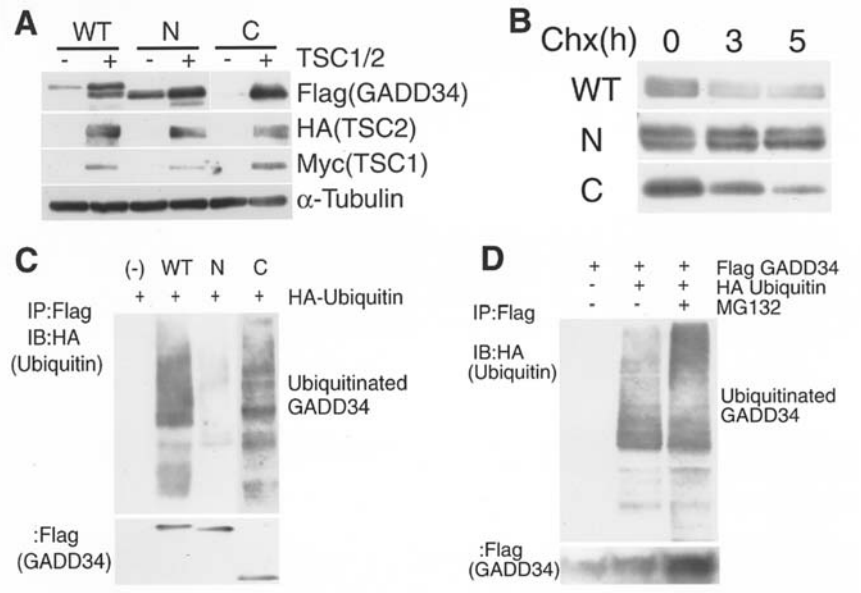

Figure 4. The expression of GADD34 is enhanced by coexpression of TSC1/TSC2. (A) Immunoblot analysis of the expression of GADD34 and TSC1/2 in HEK293 cells transfected with full-length (WT) or N- and C-terminal fragments of Flag-GADD34 (N, C) either with (+) or without (-) cotransfected Myc-TSC1/HA-TSC2. (B) Rapid turnover of GADD34 is mediated by a domain within the C-terminal region. HEK293 cells were transfected with plasmids encoding either full-length Flag-GADD34 (WT) or the indicated N- and C-terminal fragments (N, C). GADD34 stability was subsequently determined by immunoblot analysis after incubation with 300 μM cycloheximide (Chx) for the indicated periods of time (h, hours). (C) The C-terminal region of GADD34 is highly ubiquitinated. HEK293 cells were transfected with plasmids encoding HA-ubiquitin and either full-length or N- or C-terminal fragments of Flag-GADD34 (WT). Cell lysates were immunoprecipitated with the anti-FLAG antibody and subjected to immunoblot analysis with the anti-HA antibody to detect protein ubiquitination. (D) Treatment of transfected cells with a proteasomal inhibitor further enhances ubiquitination and expression of ectopic GADD34. HEK293 cells were transfected with plasmids encoding Flag-GADD34 and HA-ubiquitin or empty vector (-) in the presence or absence of 10 μM MG132 for 2 h before cell lysis. Cell lysates were immunoprecipitated with the anti-FLAG antibody and subjected to immunoblot analysis with the anti-HA antibody to detect protein ubiquitination.

analysis confirmed that none of these conditions has an impact on the cell cycle (Fig. 3B).

We further examined the effect of ectopic expression of GADD34 on the phosphorylation of S6K, a major downstream effector of mTOR in HEK293 cells (Fig. 3C). Overexpression of full-length GADD34 inhibited the phosphorylation of endogenous S6K at Thr389, indicating that GADD34 can inhibit mTOR signaling. To elucidate which region of GADD34 is required for this inhibition, we evaluated the effect of expressing the N- and C-terminal fragments of GADD34 on S6K phosphorylation (Fig. 3C). The C-terminal fragment of GADD34 inhibited S6K phosphorylation to a similar extent as the full-length protein, whereas the N-terminal fragment did not. We also examined whether deletion of the PP1 binding site within the C-terminal fragment of GADD34 affected its ability to inhibit S6K phosphorylation. Expression of the C-terminal fragment of GADD34 lacking the PP1 binding motif (C-PP1) did not inhibit S6K phosphorylation (Fig. 3C), despite the fact that C-PP1 was able to bind to the TSC1/TSC2 complex (Fig. 3D).

These findings indicate that GADD34 functions as a negative regulator of mTOR signaling by dephosphorylating TSC2 mediated by the interaction between its C-terminal region and TSC1/2, and that the PP1 binding site of GADD34 is critical for inhibition of the mTOR pathway.

GADD34 is highly susceptible to ubiquitin-proteasomal degradation, but is stable and functions when TSC is coexpressed. To evaluate the impact of the formation of a ternary complex between GADD34 and TSC1/TSC2 on mTOR signaling in stressed cells, we first evaluated the effect of TSC1/TSC2 expression on GADD34 protein stability. We observed an increase in the level of exogenous GADD34 expression in cell lysates prepared from HEK293 cells transfected with both full-length GADD34 and TSC1/TSC2, compared to the level observed in lysates prepared from cells transfected with GADD34 alone (Fig. 4A). To evaluate whether the increase in GADD34 stability is due to its interaction with TSC1/TSC2, we monitored the expression of the C- and N-terminal fragments of GADD34 in the presence or absence of coexpressed TSC1/TSC2. As was observed following expression of full-length GADD34, we found that coexpression of TSC1/TSC2 enhanced the stability of the C-terminal fragment of GADD34, which binds TSC1/TSC2. In contrast, coexpression of TSC1/TSC2 had only a modest effect upon the stability of the N-terminal fragment of GADD34, which is unable to bind TSC1/TSC2 (Fig. 4A). Transfection of cells with TSC1/TSC2 alone did not induce the expression of the endogenous GADD34 mRNA (data not shown), indicating that the increase in the level of GADD34 protein was mediated by an effect of the coexpressed TSC1/TSC2 at the translational and/or post-translational level, rather than at the level of GADD34 transcription. We next evaluated the stability of exogenous GADD34 and its fragments in the presence of cycloheximide (Chx). In the presence of Chx, the full-length and C-terminal fragments of GADD34 were less stable than the N-terminal fragment, indicating that the GADD34 protein is relatively unstable with a short half-life

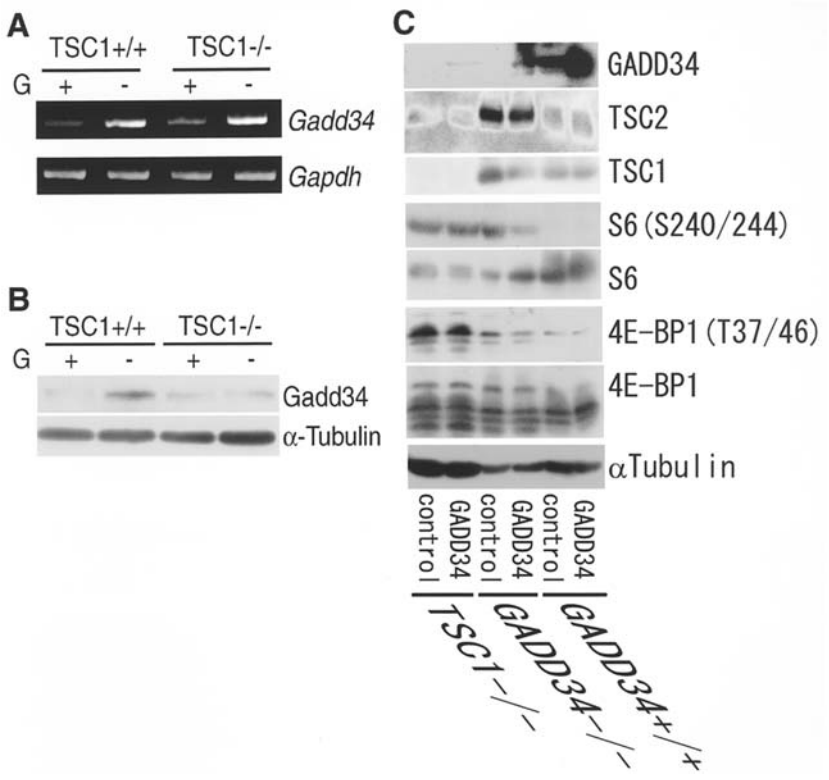

Figure 5. GADD34 stability and its effect upon mTOR signaling are dependent upon TSC1/2 expression in glucose-starved cells. (A) TSC exerted no effect upon the transcription of GADD34 during glucose starvation. *Gadd34* mRNA was monitored by semi-quantitative RT-PCR. (B) Enhanced expression of GADD34 was not observed in glucose-starved TSC1$^{-/-}$ cells. TSC1-deficient (TSC1$^{-/-}$) and TSC1-rescued (TSC1$^{+/+}$) cells cultured in glucose-free medium (G^{-}) for 24 h were subjected to immunoblot analysis for GADD34. (C) Ectopic GADD34 and stress-induced endogenous GADD34 cooperatively dephosphorylate TSC2 and suppress signaling by mTOR. TSC1-deficient carcinoma cells (TSC1$^{-/-}$), and both GADD34$^{-/-}$ and wild-type (GADD34$^{+/+}$) MEFs, which both express endogenous TSC1, were transduced with a retroviral vector expressing *GADD34*. The transfected cells were subjected to glucose starvation and components of the TSC-mTOR signaling pathway were evaluated by immunoblot analysis.

during normal culture conditions (Fig. 4B). These results suggest that GADD34 is stabilized through formation of a ternary complex with TSC1/TSC2.

To determine whether ubiquitin-dependent proteasomal degradation is involved in GADD34 degradation, we next examined the ubiquitination of full-length GADD34, as well as the N- and C-terminal fragments. Fig. 4C shows that both the full-length and C-terminal fragments of GADD34 were highly ubiquitinated, while the N-terminal fragment was not. The proteasomal inhibitor, MG132, enhanced GADD34 ubiquitination and further enhanced its stability (Fig. 4D). These results suggest that the instability of GADD34 results from its susceptibility to ubiquitin-proteasomal degradation and that coexpression of TSC1/2 can stabilize GADD34, probably due to its incorporation into a ternary complex with TSC1/TSC2. To evaluate further whether the endogenous TSC1/TSC2 complex stabilizes GADD34 expression, we monitored GADD34 expression in TSC1-deficient (TSC1$^{-/-}$) and TSC1-rescued (TSC1$^{+/+}$) mouse renal carcinoma cells under normal and glucose-starved culture conditions. We observed a similar level of GADD34 mRNA in both TSC1$^{-/-}$ and TSC1$^{+/+}$ cells in the absence of glucose in the media (Fig. 5A). However, the level of GADD34 protein was reduced in glucose-starved TSC1$^{-/-}$ cells compared to the level observed in glucose-starved TSC$^{+/+}$ cells, indicating that expression of TSC is critical to post-transcriptional stability of GADD34 in stressed cells. These findings indicate that GADD34 is unstable due to ubiquitin-proteasomal degradation, and that formation of a complex with TSC1/TSC2 stabilizes its expression during conditions of cellular stress.

Finally, we next evaluated whether exogenous expression of GADD34 suppresses mTOR signaling in a TSC-dependent manner. We used a retroviral vector to transduce *GADD34* into three different cell lines: TSC1-deficient carcinoma cells (TSC1$^{-/-}$), and GADD34$^{-/-}$ and GADD34$^{+/+}$ (wild-type) MEFs, which both express endogenous TSC1. Following transfection, we analyzed TSC-mTOR signaling by evaluating the phosphorylation status of S6 and 4E-BP1 by immunoblot analysis of lysates prepared from the transfected cells cultured in the absence of glucose. We detected only weak levels of GADD34 protein in the transfected TSC1$^{-/-}$ cells under these conditions. Notably, we observed higher levels of phosphorylation of S6 and 4E-BP1, suggesting that there was little inhibition of mTOR signaling in TSC$^{-/-}$ cells (Fig. 5C). In contrast, ectopically expressed and stress-induced endogenous GADD34 cooperatively dephosphorylated TSC2, as indicated by a significant shift-down in its electrophoretic mobility, and suppressed mTOR signaling in the presence of TSC1/TSC2, in either GADD34$^{-/-}$ or $^{+/+}$ cells (Fig. 5C). These results suggest that ectopically expressed GADD34 and stress-induced endogenous GADD34 were stabilized through an interaction with TSC, leading to dephosphorylation of TSC2, and suppression of mTOR signaling in glucose-starved cells.

Discussion

In the present study, we show that GADD34 protects cells from apoptosis caused by glucose depletion, and that this process involves the regulation of mTOR signaling. Glucose starvation induces apoptosis in TSC1- or TSC2-deficient cells. During glucose starvation, increased signaling by the mTOR pathway leads to cell death, and suppression of mTOR activity plays an important role in the regulation of cell survival (3). This study indicates that GADD34 contributes to the inhibition of mTOR signaling during glucose starvation.

In addition, we showed that the interaction between endogenous GADD34 and TSC1/TSC2 was enhanced by glucose starvation, and that this interaction was mediated in a specific manner by a domain within a C-terminal fragment of GADD34. GADD34 binds to the serine/threonine phosphatase PP1 through its C-terminal KVRF domain, recruits it to various molecules, and modulates their enzymatic activities (9,10).

In this study, we demonstrated that the TSC1/2 complex enhanced the interaction with GADD34 and also served as its target during glucose starvation. GADD34-bound PP1 was also required for mTOR inhibition, since the C-terminal mutant (C-PP1) lacking the PP1 binding motif had no inhibitory effects for S6K phosphorylation, but could still bind to the TSC1/2 complex. To note, the ternary complex of GADD34/TSC was stable in the presence of TSC and inhibited mTOR signaling during glucose starvation, although it was highly susceptible to ubiquitin-proteasomal degradation.

We also evaluated the translational machinery and the cell cycle involved in GADD34. During glucose starvation, GADD34 suppressed the translational machinery through both inhibition of TSC-mTOR and maintenance of eIF-2α phosphorylation, and did not positively affect cell cycle to escape the additional stresses. Nevertheless, the control of the translational machinery is complex and is mediated through various pathways. The activity of the translational machinery should be monitored by evaluation of both GADD34-eIF-2α and GADD34-mTOR pathways under the various conditions.

The TSC1/TSC2 complex is thought to inhibit the mTOR function by acting as a GTPase-activation protein via the TSC2 GAP domain (16), but how this is accomplished remains controversial. The crosstalk between GADD34 and mTOR proposed in the present study should be adequately discussed for both eIF-2α and mTOR pathways, and further study of this crosstalk should help to clarify how the translational machinery is controlled under conditions of cellular stress, such as bioenergetic starvation, DNA damage, and other infectious and metabolic stresses.

Various types of cellular stress, including DNA damage, nutrient or energy starvation and hypoxia inhibit mTOR signaling (3-5,13), but the mechanism(s) of this effect remains unknown. Since most of these types of cellular stress induce GADD34 expression (6,10,12), the inhibition of mTOR function by GADD34 might be fundamental to the cellular response to stress. Our findings provide a substantial advance in understanding the functional relationship between translational control and the cellular response to stresses. Altered signaling by mTOR is linked to several diseases, including cancer, diabetes and obesity (17-21). Regulation of mTOR signaling by GADD34 is of considerable interest in view of its potential for disease therapy. Consistent with the presented data, further clarification of the role of GADD34 in the mTOR pathway should contribute to progress in the treatment of such diseases.

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