Translation Factor eIF4E Rescues Cells from Myc-dependent Apoptosis by Inhibiting Cytochrome c Release*

Received for publication, August 28, 2002, and in revised form, November 15, 2002
Published, JBC Papers in Press, November 18, 2002, DOI 10.1074/jbc.M208821200

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Eukaryotic translation initiation factor 4E (eIF4E) markedly reduces cellular susceptibility to apoptosis. However, the mechanism by which the translation apparatus operates on the cellular apoptotic machinery remains uncertain. Here we show that eIF4E-mediated rescue from Myc-dependent apoptosis is accompanied by inhibition of mitochondrial cytochrome c release. Experiments achieving gain and loss of function demonstrate that eIF4E-mediated rescue is governed by pre-translational and translational activation of bcl-x as well as by additional intermediates acting directly on, or upstream of, the mitochondria. Thus, our data trace a pathway controlling apoptotic susceptibility that begins with the activity state of the protein synthesis machinery and leads to interdiction of the apoptotic program at the mitochondrial checkpoint.

The integration of extracellular information by a cell into a decision to live or die is of fundamental importance during development, wound healing, immune responses, and tumorigenesis (1, 2). Most studies addressing the regulation of apoptosis have focused on transcriptional control, signal transduction, and other post-translational regulatory events. More recently, it has become evident that apoptosis is also subject to translational control. Cap-dependent translation involves the assembly of initiation factors at the 5′ mRNA terminus to form the trimolecular cap binding complex, eIF4F. These factors include the cap-binding protein, eIF4E,1 an ATP-dependent RNA helicase, eIF4A, and an eIF4G polypeptide (eIF4GI or eIF4GII), which serves as a docking site for eIF4E and eIF4A (3). The function of eIF4E is negatively regulated by members of the translational repressor family, the eIF4E-binding proteins, that sequester eIF4E in a translationally inactive complex (4). Growth factors and other pro-survival stimuli promote phosphorylation of the eIF4E-binding proteins. Hyperphosphorylated eIF4E-binding protein has a decreased affinity for eIF4E, resulting in its liberation to initiate translation.

The activity of the cap-dependent translation initiation apparatus is a major determinant of cell fate during development (5, 6) and post-natal life (7). A constitutively active initiation apparatus sustains morphogenesis ex vivo (5), promotes cell cycle transit (8, 9), and leads to malignant transformation (9–12), whereas repression of aberrant translation initiation reverses oncogenesis (13–15). We have previously shown that apoptosis can be governed by the activity of eIF4E. Overexpression of eIF4E rescues cells from apoptosis (16, 17), whereas sequestration of eIF4E by overexpressed eIF4E-binding protein 1, triggers apoptosis and markedly diminishes tumorigenesis (18, 19).

The apoptotic program can be triggered through at least two distinct signaling pathways with the potential for cross-talk. One pathway, leading to activation of caspase-8, is triggered by ligation of specific cell surface death receptors, such as Fas/CD95 or tumor necrosis factor α receptor (20). The second pathway, initiated by various stressors such as cytotoxic drugs and radiation, is transduced through a caspase-2-mediated series of steps into mitochondrial release of cytochrome c (21). Subsequent formation of the apoptosome, a complex containing cytochrome c, adapter protein Apaf-1, and procaspase-9 leads to activation of caspase-9 (22). When activated, caspases-8 and -9 activate effectors caspases-3, -6, and/or 7, which in turn cleave critical cellular targets, resulting in death (23). Proteins of the Bcl-2 family tightly regulate mitochondrial release of cytochrome c. Proapoptotic proteins, such as Bid, Bax, Bad, and Bak, form pores in the outer mitochondrial membrane, whereas the anti-apoptotic proteins, Bcl-2 and Bcl-XL, inhibit pore formation (24, 25). In most cell types, these two pathways converge, and receptor-induced activation of caspase-8 also results in mitochondrial release of death promoters with subsequent activation of the apoptosome-dependent caspase cascade (26). Thus, the mitochondria integrate a variety of cell death signals, and the ability of Bcl-2/Bcl-XL to interdict apoptosis is one hallmark of mitochondrial involvement in the apoptotic pathway (27, 28).

Despite the strong connection between translational control and apoptosis, little is known about the underlying mechanisms. We have previously demonstrated that over-expressed eIF4E averts Myc-dependent apoptosis, at least in part, through a cyclin D1-dependent process (17). To provide further insight into the mechanism of eIF4E rescue, we examined which steps in the apoptotic cascade were blocked by eIF4E in rat embryo fibroblasts sensitized to apoptosis by constitutive expression of c-Myc. Here we show that cells rescued by eIF4E neither release cytochrome c from their mitochondria nor do they activate any downstream steps in the apoptotic cascade. A survey of Bcl-2 family members in rescued fibroblasts revealed most to be in the basal state. However, Bcl-XL was dramatically

* This work was supported by Department of Defense Grant BC98414 (to V. A. P.), National Institutes of Health Grant 2P50-HL50152 (to P. B. B.), and National Institutes of Health Grant HL 07741-07 (to S. L. and D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: eIF4E, eukaryotic translation initiation factor 4E; REF, rat embryonic fibroblasts; PBS, phosphate-buffered saline; PARP, anti-poly(ADP-ribose) polymerase; HPLC, high performance liquid chromatography; ASO, antisense oligodeoxynucleotide; UTR, untranslated region; IRES, internal ribosomal entry site; DAPI, 4,6-diamidino-2-phenylindole.

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Materials and Methods

Cell Lines—Cell lines were derived from rat embryonic fibroblasts (REF) as described (17). REF/Myc cells, which overexpressed c-Myc, were provided by Dr. Weinberg (Whitehead Institute, Cambridge, MA). REF/Myc/4E cells were generated by transducing REF/Myc with retrovirus CRE-BCS encoding wild-type murine eIF4E (retrovirus was kindly provided by P. Lebeaud, Massachusetts Institute of Technology, Cambridge, MA). Both REF/Myc and REF/Myc/4E cells were grown in complete medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 4.5 g/liter glucose, 100 units/ml penicillin, 100 units/ml streptomycin, and 250 mg/ml amphotericin).

Analysis of Apoptosis—Cells were subjected to proapoptotic conditions as specified in the text and figure legends. Both adherent and detached cells were collected, fixed in cold ethanol, washed with PBS, and stained with propidium iodide stain mixture (50 g/ml propidium iodide, 0.1% Triton X-100, 32 g/ml EDTA, 2.5 g/ml RNase in PBS) for 45 min at 37 °C. DNA content was determined by quantitative flow cytometry using the CellQuest program.

Immunoblot Analysis— Cultured cells were rinsed in PBS, trypsinized, and collected by centrifugation. Cells were resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton-X, 1% sodium deoxycholate) supplemented with protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, and 2 g/ml each of leupeptin, aprotinin, and pepstatin A) and microcentrifuged at 12,000 × g for 10 min, and the supernatant was retained. The protein concentration was measured using the Pierce BCA protein assay kit. 20 g of total protein was resolved by 15% SDS/PAGE, followed by immunoblotting with the following antibodies: anti-Bcl-XL (Trevigen), anti-Bcl-2 (Trevigen), anti-Bax (Santa Cruz Biotechnology), anti-Bad (Santa Cruz Biotechnology), anti-actin (Sigma), anti-tubulin (Sigma), anti-caspase-3 (Santa Cruz Biotechnology), anti-poly(ADP-ribose) polymerase (PARP, Upstate Biotechnology). Cytochrome c was analyzed by subjecting 50 g of cell free extract to 12% SDS/PAGE, transferred to nitrocellulose membranes, and incubated with mouse anti-cytochrome c antibody (BD Biosciences). Blots were incubated with an appropriate horseradish peroxidase-coupled secondary antibody and detected by ECL (Amer sham Biosciences) (2).

Caspase Activity Assay— Cells were subjected to proapoptotic conditions, and both detached and adherent cells were collected, incubated with lysis buffer, and centrifuged for 5 min at 10,000 × g.Active caspase-3 was measured using a CleavaLite caspase-3 activity assay kit (Chemicon). 50 g of lysate was added to a CleavaLite Renilla luciferase bioluminescent substrate that contains the caspase-3 cleavage site, DEVD. After incubation for 1 h at 37 °C, fresh luciferase substrate was added, and luminescence was read in a Lumat luminometer (EG&G Berthold). For caspase-9 activity, the cell lysate was centrifuged at 10,000 × g for 1 min, supernatant was collected, and caspase-9 activity was determined by cleavage of LEHD-p-nitroanilide using a caspase-9 colorimetric assay (R&D systems).

Determination of Cytochrome c Subcellular Distribution— Adherent and floating cells were pooled and suspended in mitochondrial buffer (250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, 20 g/ml aprotinin, 1 g/ml pepstatin A) and were then mechanically lysed using a loose-fitting Whirl Pak homogenizer, and centrifuged at 1000 × g for 10 min to remove nuclei and unbroken cells. The supernatant was centrifuged at 13,000 × g for 20 min to pellet the mitochondria. The resulting supernatant (cytosolic fraction) was centrifuged at 100,000 × g for 1 h. Samples were diluted to obtain a total protein concentration of 0.4 µg/ml, and cytochrome c concentration in each sample was determined by enzyme-linked immunosorbent assay using an R&D systems Quantikine murine cytochrome c immunoassay kit.

Measurement of Mitochondrial Membrane Potential—Cells were seeded on glass cover slips precoated with fetal calf serum and incubated (24 h at 37 °C) in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. Cultures were continued with or without 5 µM levamisole for an additional 24 h. Mitochondria were stained by exposing cells to 0.5 µg/ml rhodamine 123 dye for 45 min at 37 °C. Medium was changed to PBS immediately before analyzing the staining pattern of the cells.

Polyribosome Preparation—15 plates of actively proliferating cells in 100-mm dishes were treated with cycloheximide (100 µg/ml) for 5 min, harvested by trypsinization, and lysed as described (50) using 60 strokes in a Dounce homogenizer. Lysate was centrifuged at 10,000 × g for 10 min, and the nuclei pellet was removed. Cytoplasmic extract (1.5 mg measured at A260) was layered onto a 5-ml, 0.5-1.5 µm sucrose gradient. The sucrose gradients were centrifuged at 200,000 × g in a Beckman SW50 rotor for 90 min at 4 °C. The gradients were fractionated using an ISCO density gradient fractionator monitoring absorbance at 254 nm. Five 1-ml fractions were collected from each sample into tubes containing 100 µl of 10% SDS.

Quantification of bcl-x mRNA by Real-time PCR—The RNA from each fraction of the sucrose gradient was extracted using Tri-reagent (Sigma) and quantitated. RNA (5 µg) from each fraction was treated with DNase I (DNA-free T4, Ambion, Austin) according to the manufacturer’s directions. cDNA was synthesized from 2 µg of each RNA sample using a Taqman reverse transcriptase reagent kit (Applied Biosystems) primed with oligo-dT. Rat bcl-x DNA sequences for upper (5′-GGAGACCCCATGTTCCATCAAT-3′) and lower (5′-AGTGGCCCGCCCAAGG-AGAA-3′) primers and rat bcl-2 DNA sequences for upper (5′-CACCGTGGCATTCTCTTCTCCTCC-3′) and lower (5′-GACATCGGACGCTCTTG-3′) primers were synthesized and purified by HPLC. Real-time PCR was performed using a LightCycler FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals). The LightCycler PCR protocol consisted of a 10-min denaturation followed by 42 cycles of 95 °C for 10 s, 68 °C for 5 s, and 72 °C for 15 s. Reactions were set up as recommended by the manufacturer, optimized with 4 µg MgcI, with each primer at 1 µM. A portion of the cDNA reaction (9.6 µl) was used for amplification of each gradient fraction. Quantification of mRNA was carried out by comparison (linear interpolation) of the number of cycles to saturation in each sample, with the number of amplification cycles occurring in a standard containing a known amount of target mRNA (5 concentrations of the standard were used which spanned the range of values for the samples).

Northern Blot Analysis—Total RNA was isolated using an RNaseasy Total RNA kit (Qiagen, Santa Clarita, CA), and 15 µg RNA/lane was electrophoresed through 1% agarose, 2.2 M formaldehyde gel and transferred to nylon filters. A synthetic 40-mer oligonucleotide (5′-GGGTGT- CATTCAAGTTAGTGGCCATCAATTCGCGC-3′), which was designed specifically to recognize bcl-xL (2), was 3′-end labeled with 50 µCi of [α-32P]dATP (3000 Ci/mmol) using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals). A glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech, Palo Alto, CA) was randomly labeled with 50 µCi of [α-32P]dCTP (3000 Ci/mmol) and was used to probe Southern blots. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was purified using Sephadex G-25 spin columns (Roche Molecular Biochemicals). Hybridization was performed in 50% (v/v) formamide, 5× sodium chloride/sodium citrate (SSC), 1% SDS, 1× Denhardt’s, 10% dextran sulfate, and 250 µg/ml yeast RNA (Sigma) with either the 3′-end labeled bcl-xL oligonucleotide probe or the random primer-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe at 42 °C overnight. Blots were washed twice in 0.1× SSC, 0.1% SDS at 50 °C for 30 min and exposed to x-ray film.

Messenger RNA Stability—RNA stability was quantified as described previously (29). To assess stability of bcl-x mRNA, cells were treated with 5 µg/ml actinomycin D (Sigma) for the time intervals specified in the text and figure legends. Total RNA was isolated and analyzed by Northern blot.

Generation of REF/Myc/Bcl-XL Clonal Cell Lines—REF/Myc cells were stably transfected with a pAPuro vector containing an 800-bp fragment encoding wild type Bcl-XL (a gift from Dr. E. Prochownik, Children’s Hospital, Pittsburgh, PA) using the FuGENE 6 (Roche Diagnostics) transfection technique. Selection of transfected cells was begun after 24 h in complete medium containing 4 µg/ml puromycin. Resistant clones were isolated after 12–16 days.

Bcl-xL Antisense Oligonucleotides—Phosphorothioate antisense and nonsense DNA oligodeoxynucleotides were synthesized and purified by HPLC (Operon Technologies, Inc., Alameda, CA). An 18-mer antisense oligonucleotide sequence corresponding to the 3′ end of bcl-x mRNA and a control scrambled sequence were (a) antisense 5′-CCG GTT GCT AGA CAT C′3′ and (b) scramble 5′-CTG AAC GGA GAG ACC CTT-3′. Cells were seeded into chambers of 8-well glass chamber slides overnight and shifted to Dulbecco’s modified Eagle’s.
Prevention of Apoptosis by eIF4E

FIG. 1. eIF4E rescues cells from Myc-dependent apoptosis. REF/Myc and REF/Myc/4E cells were cultured in complete medium with or without 5 μM lovastatin for 24 h or in Dulbecco’s modified Eagle’s medium plus 1% fetal calf serum (FCS) alone, with 100 ng/ml tumor necrosis factor-α (TNF-α), or with 100 ng/ml Fas ligand (Fas-L) for 24 h. Apoptosis was quantified by flow cytometry. The data presented are the mean (±S.E.) of four independent experiments.

RESULTS

Increased Expression of eIF4E Inhibits Death Receptor-mediated and Stressor-induced Apoptotic Pathways—We previously reported that ectopic expression of eIF4E rescues REF harboring constitutively expressed c-myc (REF/Myc) from apoptosis triggered by either serum restriction (16) or cytotoxic stress (17). To evaluate whether eIF4E also suppresses death receptor-mediated apoptosis, we examined its impact on REF/Myc cell viability after treatment with tumor necrosis factor family ligands. When eIF4E was ectopically overexpressed in REF/Myc cells (REF/Myc/4E), apoptosis was inhibited in response to each apoptotic trigger tested (Fig. 1), indicating that eIF4E promoted suppression of the death receptor-mediated as well as the stress-induced apoptotic cascades. However, the magnitude of kill in response to tumor necrosis factor-α or Fas ligand was modest and not amenable to further study. In contrast, more than 64% of cells underwent apoptotic death in response to lovastatin, a value reduced to nearly the basal frequency by more than 64% of cells undergoing apoptotic death in response to lovastatin was modest and not amenable to further study. 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FIG. 2. eIF4E blocks PARP cleavage and suppresses caspase-3 and caspase-9 activation. A and B, immunoblot analysis of PARP and caspase-3. REF/Myc and REF/Myc/4E cells were cultured in complete medium with or without 5 μM lovastatin for 24 h. Cleavage of PARP (A) and activated caspase-3 (B) were detected by immunoblot analysis with tubulin shown as a loading control. C and D, analysis of caspase-3 and caspase-9 enzymatic activity. REF/Myc or REF/Myc/4E cells were cultured for the time interval indicated in the presence of 5 μM lovastatin. Caspase-3 like activity was detected using Cleav- aLiseTM caspase-3 activity assay kit (C), and caspase-9 like activity was detected with a colorimetric assay (D); results shown are representative of three independent experiments.

caspase-3 activation, whereas caspase-3 activity remained low in REF/Myc/4E cells throughout the entire interval of observation (Fig. 2C). Caspase-9 followed the same pattern. Activation was noted as early as 4 h in REF/Myc cells, reaching levels more than 3-fold that of control by 16 h (Fig. 2D). In REF/ Myc/4E cells caspase-9 remained in the inactive state.

Cytochrome c normally resides between the inner and outer mitochondrial membranes and is not usually found in the cytoplasm (30). Because cytochrome c is required for activation of caspase-9 (31), we examined the subcellular distribution of cytochrome c after exposure of cells to lovastatin. At base line, cytochrome c was detectable at low levels in the cytoplasm of REF/Myc cells, a value that increased more than 3-fold after
20 h of lovastatin treatment (Fig. 3A). In contrast, cytochrome c remained near basal levels in the cytoplasm of REF/Myc/4E cells after lovastatin treatment, a result corroborated by immunoblot analysis (Fig. 3B).

Mitochondrial inner transmembrane potential collapse frequently precedes cytochrome c release and caspase activation (32). To examine whether this was the case in our system, we treated cells with lovastatin for 24 h and measured the uptake of rhodamine 123, a cationic fluorophore that enters mitochondria in direct proportion to membrane potential (33). REF/Myc and REF/Myc/4E cells took up the dye similarly after lovastatin treatment, suggesting that the suppression of cytochrome c release in REF/Myc/4E cells was not due to eIF4E-induced alterations in mitochondrial membrane depolarization (data not shown).

Overexpressed eIF4E Selectively Stimulates Expression of Bcl-XL—Deregulated c-Myc mediates apoptosis in some transformed cell lines by selectively decreasing the mRNA and protein levels of the death antagonists Bcl-2 and Bcl-XL (34, 35). We therefore examined whether eIF4E had any effect on this interplay between c-Myc and the Bcl-2 family proteins. Immunoblot analysis demonstrated that overexpression of eIF4E resulted in a 7-fold increase of Bcl-XL protein in REF/Myc cells without significantly affecting the expression levels of Bcl-2, Bax, or Bad (Fig. 4).

Overexpression of eIF4E Increases Recruitment of Bcl-XL mRNA to Ribosomes—To examine whether overexpression of eIF4E increased cellular levels of Bcl-XL protein by direct translational activation, total RNA from REF/Myc and REF/Myc/4E was stratified by sucrose gradient centrifugation to separate translationally active transcripts (more bound ribosomes resulting in more rapid transit through the gradient) from less translationally active transcripts. The resulting fractions were subjected to quantitative PCR analysis for the bcl-XL transcript, comparing it to the bcl-2 transcript as a control. Examination of the absorbance pattern revealed a bias toward heavier polyribosomes, with an increased proportion of RNA in fractions 4 and 5 in cells ectopically expressing eIF4E (Fig. 5A). Real time PCR quantification indicated that the transcript for bcl-2 was distributed similarly in REF/Myc and REF/Myc/4E cells, with fraction 2 (the least translationally active) and fraction 5 (the most translationally active) containing similar amounts of transcript (Fig. 5B). In marked contrast, there was a significant increase in the quantity of bcl-XL mRNA appearing in heavy polyribosomes in REF/Myc/4E cells (Fig. 5C). The average number of bound ribosomes per bcl-XL transcript in REF/Myc/4E cells was 3.7 compared with 1.5 in REF/Myc cells, indicating a 2.5-fold increase in the rate of bcl-XL mRNA translation initiation (36, 37). Thus, one mechanism for the increase in Bcl-XL protein was direct translational activation of its mRNA.

Overexpressed eIF4E Increases Cellular Levels of Bcl-XL mRNA—A growing number of studies show that eIF4E directly or indirectly participates in a variety of pre-translational stages of protein synthesis including production and processing of mRNA and its nuclear-cytoplasmic transport (38). To gain insight into the influence of eIF4E on pretranslational events in the synthesis of Bcl-XL, we measured the abundance and stability of its mRNA. Quantitative PCR analysis demonstrated a dramatic effect of eIF4E on steady state levels of bcl-XL mRNA but not on the levels of bcl-2 mRNA (Fig. 6A). The level of bcl-XL mRNA was increased nearly 30-fold in REF/Myc/4E cells, a result supported by Northern blot analysis (Fig. 6B). The kinetics of bcl-XL mRNA degradation were only marginally altered by ectopic eIF4E (half-life of 3 h for REF/Myc and 2.5 h for REF/Myc/4E; Fig. 6C). These data indicate that in addition to direct translational activation, ectopic eIF4E stimulates synthesis of Bcl-XL by increasing the abundance of its transcript.
Decreasing Cellular Levels of Bcl-X L Reduces eIF4E-mediated Rescue from Apoptosis—To assess whether increased expression of Bcl-X L is required for the antiapoptotic function of eIF4E, REF/Myc/4E cells were treated with an antisense oligodeoxynucleotide (ASO) directed against human bcl-XL mRNA sequences in the predicted translation initiation region. A scrambled oligonucleotide served as a control. Incubation of cells with the ASO for 72 h significantly reduced the level of Bcl-X L protein, whereas the scrambled oligonucleotide had no effect (Fig. 7A). Continuation of these cultures for an additional 24 h in the presence ofLovastatin led to a significant increase in the apoptotic frequency of ASO compared with the scrambled oligonucleotide control (Fig. 7B). However, despite reduction of Bcl-X L in REF/Myc/4E by ASO below levels found in REF/Myc, the degree of apoptosis was only half (62.5% in REF/Myc versus 30% in ASO-treated REF/Myc/4E). These data indicate that increased expression of Bcl-X L is necessary for the full-scale antiapoptotic function of ectopic eIF4E and that Bcl-X L is not the sole determinant of eIF4E-mediated rescue from apoptosis.

Ectopic Expression of Bcl-X L Does Not Fully Reproduce eIF4E Rescue—To determine whether increased expression of Bcl-X L could substitute for eIF4E in the rescue of REF/Myc cells from apoptosis, we generated clonal lines of REF/Myc cells stably expressing a range of Bcl-X L protein. Twelve puromycin-resistant clones derived from REF/Myc cells transfected with a pAPuro/bcl-X vector were screened for levels of Bcl-X L expression. Two clonal cell lines were selected for further investigation (Fig. 8A), one line with a Bcl-X L level that closely matched the level of Bcl-X L expression in REF/Myc/4E cells (clone 7) and a second line in which the level of Bcl-X L was increased more than 5-fold (clone 13). To validate the function of our construct, we first examined the kinetics of cytochrome c release in the clonal lines by immunoblot and immunolocalization. Ectopic expression of
eIF4E completely suppressed cytochrome c release in the basal state and after lovastatin treatment (Fig. 8B). In the basal state, 15% of the cytochrome c in REF/Myc cells was in the cytoplasm, whereas after ectopic expression of Bcl-XL, virtually no cytoplasmic cytochrome c was detected. After treatment with lovastatin, levels of cytochrome c in the cytoplasm of REF/Myc cells were stable for 12 h, gradually increasing to more than 40% of total cellular cytochrome c by 24 h (Fig. 8B).

For the Bcl-XL clonal lines, there was a steady translocation of cytochrome c from the mitochondria to the cytoplasm beginning at 8 h and increasing up to 24 h. At all time points examined, the magnitude of cytochrome c release in cells overexpressing Bcl-XL was significantly less than that observed in REF/Myc, with a rank order of potency reflecting the expression level of Bcl-XL (Fig. 8B).

Morphological analysis showed a perinuclear granular pattern of cytochrome c in REF/Myc, REF/Myc/Bcl-XL 7, REF/Myc/Bcl-XL 13, and REF/Myc/4E cells in the basal state, consistent with a mitochondrial subcellular localization (Fig. 8C). After lovastatin treatment for 18 h, cytochrome c acquired a diffuse distribution (consistent with cytoplasmic localization) in REF/Myc and REF/Myc/Bcl-XL 7, and after 24 h a diffuse pattern was evident in REF/Myc/Bcl-XL 13. In contrast, the cytochrome c signal remained punctate in REF/Myc/4E cells for the 24-h observation interval. Consistent with these results, ectopic expression of Bcl-XL significantly attenuated apoptosis in REF/Myc cells (Fig. 8D). However, even the highest expressing clonal line manifested an apoptotic frequency of 30%, more than 2-fold that observed in REF/Myc/4E. These data indicate a qualitative difference between rescue by eIF4E and rescue by Bcl-XL.

**DISCUSSION**

To begin deciphering the rules governing translational control of cell death, in this report we examined which components of the apoptotic machinery were inhibited when apoptosis was suppressed by ectopic overexpression of eIF4E using Myc-dependent apoptosis in fibroblasts as a model. Here we show that eIF4E rescues cells by blocking release of cytochrome c from the mitochondria. Rescue by eIF4E was mediated in part by its ability to increase cellular levels of Bcl-XL, a key apoptotic antagonist. The eIF4E-induced increase in Bcl-XL was robust, occurring through at least two separate mechanisms, 1) direct

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**Fig. 7. Decreasing Bcl-XL to base-line levels does not fully restore the apoptotic response in REF/Myc/4E cells.** A, analysis of Bcl-XL protein levels after treatment with bcl-XL antisense oligodeoxynucleotides. REF/Myc/4E cells were treated with either 40 μM bcl-XL ASO or a scramble control (SCR) for 72 h. ASO was directed against human bcl-XL mRNA sequences in the predicted translation initiation region to inhibit synthesis of Bcl-XL protein, which was detected by immunoblotting. Expression of tubulin is shown as a loading control. B, apoptosis. REF/Myc/4E cells were treated as in A and cultured for an additional 24 h in the presence or absence of 5 μM lovastatin. The proportion of cells with hypodiploid DNA content was quantitated by flow cytometry.

**Fig. 8. Increasing steady state levels of Bcl-XL attenuates but does not eliminate apoptosis in REF/Myc cells.** A, steady state levels of Bcl-XL in clonal REF/Myc cell lines stably transfected with Bcl-XL. REF/Myc cells were stably transfected with Bcl-XL, and a series of clonal lines were developed. Shown is the immunoblot analysis of Bcl-XL protein in parental cells, clone 7 (chosen to closely match the Bcl-XL level in REF/Myc/4E) and clone 13 (chosen to have a level of Bcl-XL in great excess to that in REF/Myc/4E). B and C, subcellular distribution of cytochrome c. REF/Myc, REF/Myc/Bcl-XL 7, and REF/Myc/Bcl-XL 13 cells were cultured in complete medium with 5 μM lovastatin for the time intervals indicated. Shown are the relative amounts of cytosolic cytochrome c determined by immunoblot (B) and the cellular distribution of cytochrome c by immunofluorescence (nuclei stained with 4,6-diamidino-2-phenylindole; representative micrographs from three independent experiments are shown). The scale bar equals 20 μM (C). D, apoptosis. Clonal lines of REF/Myc cells ectopically expressing Bcl-XL or empty puromycin vector were cultured for 24 h in the presence or absence of 5 μM lovastatin. Apoptosis is shown for each cell line, quantified by flow cytometry.
translational activation and 2) increase in the abundance of the bcl-x<sub>L</sub> transcript. However, gain and loss of function experiments indicated that Bcl-X<sub>L</sub> did not fully account for the potent antiapoptotic activity of eIF4E. These observations indicate that the set point for cellular susceptibility to apoptosis can be governed by translational control.

The rate of protein synthesis is intimately connected with the process of programmed cell death. An ordered shut down of protein synthesis is one of the earliest events during apoptosis, and suppression of global translation can enhance apoptosis (37–40). Although maintenance of global translation tends to antagonize apoptosis, cap-dependent and IRES-driven modes of translation can impact cell death differently. One mediator of IRES-regulated translation, death-associated protein 5 (DAP5/p97/NAT1), for example, promotes cell death (39, 40). In contrast, eIF4E, the principal activator of cap-dependent translation, rescues cells from apoptosis (16–18).

Our findings that eIF4E modulates the mitochondrial checkpoint for apoptosis provides a glimpse into the mechanism by which the translational apparatus links survival signaling to the apoptotic machinery. We show that Bcl-X<sub>L</sub> is regulated by direct control its translation, a finding in accord with a recent publication reporting that fibroblast growth factor-2 translationally activates both Bcl-X<sub>L</sub> and Bcl-2 through a Ras/MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase)-dependent signaling pathway (41). In this regard, eIF4E is known to be activated in a MEK-dependent manner (42), thus defining a putative survival pathway from growth factor receptors through Ras activation of the translational apparatus to the Bcl-X<sub>L</sub>/Bcl-2-regulated apoptotic checkpoint.

What is the mechanism by which eIF4E selectively increases translation of the Bcl-X<sub>L</sub> transcript? It is generally agreed that activated eIF4E preferentially stimulates translation of those mRNA with a high degree of complexity in their 5′-untranslated region (5′-UTR), such as those with upstream open reading frames or a high GC-content (43, 44). One documented example of translational control of Bcl-2 family proteins through a 5′-UTR is the bcl-2 mRNA, which contains a 35-bp upstream open reading frame (45). The 5′-UTR of the bcl-x<sub>L</sub> message is relatively long and highly GC-rich (46), suggesting that it too may be a good candidate for eIF4E-mediated regulation, which we found to be the case.

Pretranslational stages of Bcl-X<sub>L</sub> production were also activated by overexpressed eIF4E, suggesting that transcriptional regulation, posttranscriptional maturation, and nuclear export of its mRNA may all be targets of translational control. Transcriptional control of bcl-x is an established mechanism for regulating cellular levels of Bcl-X<sub>L</sub> (47, 48). A number of transcription factors such as Stat5 (49, 50), NFκB (51), PAX3 (52), and Ets2 (53) may all be involved in regulating bcl-x expression. Although we speculate that eIF4E might increase the level of bcl-x<sub>L</sub> mRNA by activating translation of these factors or their regulators, detailed studies of the 5′- and 3′-UTR of the transcript will be needed to clarify the mechanism. Our attempts to separately quantify the impact of pre-translational and translational activation of Bcl-X<sub>L</sub> production by eIF4E using actinomycin D were unsuccessful due to the short half-life of its mRNA. It should be pointed out, however, that at least two transcription factors, c-Fos and c-Myc, are known to be targets for translation control (54). Future studies will undoubtedly expand the list.

Bcl-X<sub>L</sub> is not the sole mediator of eIF4E-dependent antiapoptotic signaling upstream of mitochondria, since gain of Bel-X<sub>L</sub> function could not reproduce the robust effects of eIF4E on apoptosis nor could loss of Bel-X<sub>L</sub> function completely abrogate eIF4E mediated rescue, thus supporting the available literature that translational control of apoptosis is mediated by a set of antiapoptotic effectors (24, 54, 55). Because the vast majority of mRNAs encoding growth factors, their cognate receptors and signal transduction pathways have long 5′-UTRs or contain upstream open reading frames, it is likely that a plethora of antiapoptotic pathways emanate from activated eIF4E. In this connection, we have previously documented that one downstream effector of eIF4E, cyclin D1, is required for eIF4E-mediated rescue from Myc-dependent apoptosis (17). Future investigations will be required to establish whether cyclin D1 and Bcl-X<sub>L</sub> lie on the same or different survival pathways downstream of eIF4E.

Our data help to clarify how c-Myc and eIF4E cooperate in tumorigenesis. Deregulated c-Myc triggers uncontrolled cell cycle progression and apoptosis, processes that have opposite effects on oncogenesis (56). In this regard, the oncogenic potential of c-Myc, which is masked by its own pro-apoptotic potency, requires an additional event that antagonizes apoptosis (57). Overexpressed eIF4E effectively cooperates with c-Myc in malignant conversion of rodent fibroblasts (10, 58). Our previous reports (16, 17) and present findings suggest that the impact of eIF4E on Myc-dependent oncogenesis is determined to a significant degree by its ability to suppress the mitochondrial events essential for Myc-activated apoptosis.

Thus, our data trace a novel pathway that controls cell susceptibility to apoptosis. The pathway originates at the protein synthesis machinery and leads to pretranslational and translational modification of the apoptotic program at the mitochondrial checkpoint. Our findings highlight the need for developing new discovery tools that accurately identify those transcripts that are translationally activated by eIF4E to mitigate a proapoptotic stress. In preliminary studies, combining polyribosome preparations to stratify mRNA by the number of bound ribosomes with gene expression microarray has begun to show promise. Independent of the identity of the entire set of translationally activated messages mediating eIF4E rescue, our data begin to explain how the translation initiation apparatus responds to suppression of apoptosis and promote oncogenesis, suggesting a plausible mechanism to explain the oncogenic synergy between eIF4E and a variety of pre-neoplastic alterations that promote both uncontrolled cell cycle progression and cell death.

Acknowledgments—We thank Dr. Janet Dubinsky (Department of Neuroscience, University of Minnesota) for helping to detect the mitochondrial transmembrane potential using rhodamine 123 dye. We also thank Drs. Nahum Sonenberg, Ronald Jemmerson, and Timothy Behrens for invaluable help and discussions and Dr. Edward Prochownik (Children’s Hospital, Pittsburgh, PA) for the pAPuro/bcl-X vector.

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