Translational Control of the Antiapoptotic Function of Ras* 

Vitaly A. Polunovsky‡, Anne-Claude Gingras§, Nahum Sonenberg§, Mark Peterson‡, Annie Tan‡, Jeffrey B. Rubins‡, J. Carlos Manivel‡, and Peter B. Bitterman‡‡

From the ‡Department of Medicine and the ‡Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, Minnesota 55455 and the §Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada

Activated Ras has been shown to provide powerful antiapoptotic signals to cells through well defined transcriptional and post-translational pathways, whereas translational control as a mechanism of Ras survival signaling remains unexplored. Here we show a direct relationship between assembly of the cap-dependent translation initiation apparatus and suppression of apoptosis by oncogenic Ras in vitro and in vivo. Decreasing protein synthesis with rapamycin, which is known to inhibit cap-dependent translation, increases the susceptibility of Ras-transformed fibroblasts to cytostatic drug-induced apoptosis. In contrast, suppressing global protein synthesis with equipotent concentrations of cycloheximide actually prevents apoptosis. Enforced expression of the cap-dependent translational repressor, the eukaryotic translation initiation factor (eIF) 4E-binding protein (4E-BP1), sensitizes fibroblasts to apoptosis in a manner strictly dependent on its ability to sequester eIF4E from a translationally active complex with eIF4G1 and the co-expression of oncogenic Ras. Ectopic expression of 4E-BP1 also promotes apoptosis of Ras-transformed cells injected into immunodeficient mice and markedly diminishes their tumorigenicity. These results establish that 4E-BP1-dependent protein synthesis is essential for survival of fibroblasts bearing oncogenic Ras and support the concept that activation of cap-dependent translation by extracellular ligands or intrinsic survival signaling molecules suppresses apoptosis, whereas synthesis of proteins mediating apoptosis can occur independently of the cap.

Extracellular survival factors suppress the intrinsic apoptotic apparatus through cognate receptor kinases at the cell surface, which activate the proto-oncogene ras and a number of pleiotropic transcriptional and post-translational effector pathways (1). A major effector of Ras survival signaling is the serine/threonine protein kinase, Akt (2, 3). Transcriptional control is exerted by Akt-mediated phosphorylation of the Forkhead1 family transcription factor FKHRIL4(14) and the transcription factor nuclear factor-κB (5), which alter expression of apoptosis-related genes. Akt-mediated phosphorylation of the Bcl-2 family member Bad (6) and the cell death protease caspase-9 (7) is implicated in post-translational suppression of the intrinsic apoptotic machinery. Akt also regulates the FK506 binding-protein 12 (FKBP12)-rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR) (2), a kinase which functions in the control of translation by activating two components of the protein synthesis apparatus: (i) the initiation complex binding the 5′-mRNA cap and (ii) the 40 S ribosomal protein S6 kinase, p70S6K (8–11). However, data examining the importance of translational control in the mechanism of Ras survival signaling are lacking.

Translational control is usually exerted at the initiation step. In eukaryotes, the 5′-mRNA cap is bound by the eukaryotic translation initiation complex eIF4F, which consists of a bi-directional RNA helicase eIF4A, the docking protein eIF4G and the cap binding subunit eIF4E (12). A major target for regulation, eIF4E is considered to be rate limiting for translation initiation under most circumstances (13), and its up-regulation is associated with cell proliferation, suppression of apoptosis, and tumorigenicity (14, 15). The function of eIF4E is inhibited by members of the family of translational repressors, the eIF4E-binding proteins (4E-BPs, also known as PHAS) (13, 16). When hypophosphorylated, 4E-BPs compete with eIF4G for binding to eIF4E and sequester eIF4E in a nonfunctional complex. Upon hyperphosphorylation, 4E-BPs dissociate from the complex with eIF4E allowing it to form an active translation initiation complex (16, 17). To elucidate the role of translational control in Ras survival signaling, here we focus directly on the 5′-mRNA cap binding complex and examine the induction of apoptosis in cells transformed with oncogenic Ras after a generalized reduction in protein synthesis or after specific repression of cap-dependent translation initiation.

**EXPERIMENTAL PROCEDURES**

**Generation of Clones and Transient Transfection**—Cloned rat embryo fibroblasts (CREF) and CREF/RasV12 (a gift from A. De Benedetti) were subcloned and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The coding sequence of human 4E-BP1 was amplified by polymerase chain reaction and directionally cloned into the EcoRI and BamHI sites of the mammalian expression vector pSRQuro, a kind gift from Dr. P. Jolicoeur, Institut de Recherches Cliniques, Montreal. To generate the eIF4E binding site deletion mutant (amino acids 54–63; 4E-BP1-D), the 4E-BP1 coding sequence was cloned into the cytomegalovirus-based vector pACTAG-2, which was used as a template for polymerase chain reaction site-directed mutagenesis (18). Clones of CREF and CREF/RasV12 expressing wild type or mutant 4E-BP1 were generated using the FuGENE 6 (Roche Diagnostica) transfection technique. Selection of transfected cells was begun after 24 h with medium containing 1 μg/ml puromycin, and resistant clones were isolated after 12–16 days. CREF/RasV12s were also transiently transfected with a pACTAG-2 construct encoding hemo-gmaglutinin (HA)-tagged human 4E-BP1-wt, 4E-BP1-D, or a vector carrying only the HA tag. To detect the level of HA expression by flow

*This work was supported by NHLBI, National Institutes of Health-funded SCOR Grant 2P50-HL50152, a grant from the National Cancer Institute of Canada, a doctoral award from the Medical Research Council of Canada, and the M.D. Ph.D. program of the University of Minnesota Medical School. 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.

‡To whom correspondence should be addressed. Tel.: 612-624-0999; Fax: 612-625-2174; E-mail: bitte001@tc.umn.edu.

1The abbreviations used are: FRAP/mTOR, FKBP12-rapamycin-associated protein/mammalian target of rapamycin; eIF, eukaryotic translation initiation factor; 4E-BP, eIF4E-binding protein; CREF, cloned rat embryo fibroblasts; HA, hemo-gmaglutinin; BP1-wt, wild type 4E-BP1.
cytometry, cells were fixed with absolute ethanol and incubated for 16 h at 4 °C with mouse anti-HA IgG2a antibody (4 μg/ml, Roche Molecular Biochemicals) or with mouse isotype-specific IgG2a antibody (4 μg/ml, PharMingen) followed by incubation with fluorescein-conjugated antiserum to mouse IgG (1:4000) (20), and then stripped and probed a third time for eIF4GI (rabbit polyclonal antiserum 1:2500) (19), and stripped and probed for 4E-BP1 (rabbit polyclonal antiserum 1:2500) (19), and stripped and probed a third time for eIF4G (rabbit polyclonal antibody 1:4000) (20).

Apoptosis Assays—Frequency of apoptosis was quantified by flow cytometric analysis of the percentage of cells with hypodiploid DNA content. Adherent and nonadherent cells were pooled, washed in phosphate-buffered saline, and fixed with ice-cold 70% ethanol for at least 1 h. Fixed cells were washed and incubated in propidium iodide stain mixture 50 μg/ml propidium iodide, 0.05% Triton X-100, 37 μg/ml EDTA, 100 units/ml ribonuclease in phosphate-buffered saline). After incubation for 45 min at 37 °C, DNA content was determined by quantitative flow cytometry using standard optics of the FACScan flow cytometer (Becton Dickinson) and the CellQuest program.

Tumorigenicity Assay—Under sterile conditions, 3 × 10^6 cells in phosphate-buffered saline were injected into each flank of immunodeficient mice (Nu Nu, Harlan). CREF/RasV12 cells were injected into one flank of each pair. In the opposite flank, we injected cells from each of the four independently derived CREF/Ras/BP1-wt clones or as a negative control, untransformed CREF. Tumor formation was documented in the opposite flank of each pair. In the opposite flank, we injected cells from each of the four independently derived CREF/Ras/BP1-wt clones or as a negative control, untransformed CREF. Tumor formation was documented in the opposite flank of each pair.

Statistics—Results of flow cytometry were tabulated as the mean ± S.D. of two to five separate experiments. In each experiment, all conditions were examined in duplicate or triplicate. For analysis of tumorigenicity, mitotic and apoptotic indices represent the average number of events/×600 microscopic field (quantified in 10 fields); and tumor weights (CREF/RasV12 versus CREF/Ras/BP1 clones) were compared using a paired t test on a log scale.

RESULTS

Activation of Apoptosis in Ras-transformed Fibroblasts by Rapamycin—We used a cell system (21) in which constitutively expressed oncogenic RasV12 enables CREF to survive in otherwise lethal concentrations of cytostatic drugs (nongenotoxic, lovastatin; genotoxic, camptothecin, Fig. 1). The FRAP/mTOR inhibitor rapamycin completely abrogated Ras-dependent resistance to drug-induced cell death (Fig. 1b) and even when applied as a single agent, stimulated apoptosis in cells expressing activated Ras. This proapoptotic effect of rapamycin was not observed in nontransformed fibroblasts. These observations confirm a dual proapoptotic and antia apoptotic function for RasV12 (2) and implicate FRAP/mTOR in Ras-dependent rescue from both Ras-activated and drug-triggered apoptotic pathways.

When rapamycin was added to Ras-transformed cells, it caused a dose-dependent decline in protein synthesis, which paralleled its ability to sensitize cells to lovastatin-induced apoptosis (Fig. 1c). Of note, equipotent doses of the peptide elongation inhibitor cycloheximide actually blocked apoptosis (Fig. 1d), demonstrating that the execution of lovastatin-induced cell death requires global protein synthesis. These results suggest that a generalized inhibition of mRNA translation is not the means by which rapamycin exerts its proapoptotic effect, rather they point toward a selective inhibition of antia apoptotic mRNA translation or a mechanism independent of its ability to repress translation.

Activation of Apoptosis by 4E-BP1 in Fibroblasts Expressing Oncogenic Ras—FRAP/mTOR has a dual function in the regulation of translation. It stimulates protein synthesis by regulating ribosomal biogenesis through p70s6k (22) and specifically activates cap-dependent translation by phosphorylating the 4E-BPs (8–10). Our previous work linking suppression of apoptosis to the cap-dependent translation initiation apparatus (15) led us to explore whether the 4E-BPs modulate Ras-dependent viability and chemoresistance. CREF/RasV12 and CREF cells were transfected with BP1-wt linked to a puromycin selectable marker or with vector carrying only the selectable marker, and puromycin-resistant clones were isolated. Four CREF/Ras/BP1-wt and four CREF/BP1-wt clones were developed and assayed for steady state levels of 4E-BP1.
Translational Repressor 4E-BP1 Inhibits Ras Survival Signaling

FIG. 2. 4E-BP1 sensitizes Ras-transformed cells to apoptosis. Apoptosis was quantified by flow cytometry (a and b) and visualized by acridine orange staining (c and d). Apoptosis and immunoblot analysis of 4E-BP1 expression in clonal cell lines of CREF/Ras/V12 (a) and CREF (b) transfected with a construct encoding wild type 4E-BP1 are shown. Cells were cultured for 24 h in the presence (closed circles) or absence (open circles) of 5 μM lovastatin. Each point represents the mean ± S.D. (n = 3) (c and d). Micrographs of Ras-transformed (c) and nontransformed CREF (d) expressing wild type 4E-BP1 (×300) or puromycin vector (shown in the inset, ×75).

Under conditions in which expression of endogenous 4E-BP1 in all mock-transfected cells was undetectable, BP1-wt-transfected clones displayed a range of ectopic 4E-BP1 expression. Western blot analysis performed on total cellular extracts revealed human 4E-BP1 represented by hypo-(α), intermediate (β), and hyperphosphorylated (γ) forms (9, 16) (Fig. 2a), with the α form appearing as a doublet in some of the clones. Quantification of apoptosis by flow cytometry revealed that ectopic 4E-BP1 significantly increased the rate of spontaneous apoptosis in Ras-transformed cells in a dose-dependent manner. This 2–8-fold augmentation in basal apoptotic frequency was approximately doubled in the presence of lovastatin (Fig. 2a).

In sharp contrast to the results with transformed CREF/Ras/V12, 4E-BP1 did not activate apoptosis in nontransformed parental CREF lacking activated Ras (Fig. 2b). Whereas many cells comprising the CREF/Ras/BP1-wt clonal line displayed morphological hallmarks of apoptosis (Fig. 2c), ectopic expression of 4E-BP1 did not alter the morphology of CREF (Fig. 2d). Thus, ectopic 4E-BP1 shifted Ras signaling from suppression to induction of apoptosis.

Relationship between Apoptosis and Sequestration of eIF4E by 4E-BP1—To investigate whether the proapoptotic function of 4E-BP1 in CREF/RasV12 was directly related to its ability to sequester eIF4E from the translationally active eIF4E-eIF4GI complex, cellular extracts from each clonal line of CREF/Ras/BP1-wt were incubated with the cap analogue mGTP-agarose to capture eIF4E and its cellular binding partners. The levels of cap-bound eIF4E, 4E-BP1, and eIF4GI were quantified by immunoblotting and densitometry. Each CREF/RasV12/BP1-wt clone displayed eIF4E associated with significantly increased amounts of fast migrating, hypophosphorylated 4E-BP1 (Fig. 3a). Consistent with this, clones ectopically expressing 4E-BP1-wt generally manifested decreased amounts of eIF4GI bound to eIF4E. Although clone 2 revealed relatively high levels of eIF4GI, there was also an increased amount of eIF4E in the mGTP-captured material. Thus, the ratio of eIF4GI to cap analogue-bound eIF4E was significantly decreased in all 4E-BP1 clones tested, confirming the ability of overexpressed 4E-BP1 to inhibit assembly of the eIF4F translation pre-initiation complex. The apoptotic frequency in clones co-expressing activated Ras and 4E-BP1 was proportional to the amount of 4E-BP1 complexed with eIF4E (Fig. 3b) and was inversely related to the eIF4GI/eIF4E ratio (Fig. 3c), a relationship observed in the presence and absence of lovastatin. Thus, stimulation of apoptotic death by 4E-BP1 was a function of its activity in competitively displacing eIF4GI from eIF4E.

To determine whether the interaction of 4E-BP1 with eIF4E was a strict requirement for the proapoptotic function of 4E-BP1 in Ras-transformed cells, we utilized a 4E-BP1 deletion mutant (4E-BP1-Δ), which lacks the eIF4E binding site (18). Transient transfection of CREF/Ras/V12 with 4E-BP1-wt enhanced spontaneous apoptosis and sensitized cells to lovastatin in a manner similar to that observed in the stable CREF/Ras/V12/BP1-wt clones, suggesting that activation of apoptosis in 4E-BP1-transfected clones was not due to secondary genetic changes during clonal selection (Fig. 3d). In marked contrast, transient transfection with 4E-BP1-Δ had minimal effects on viability, despite similar levels of 4E-BP1 expression. Thus, the ability of 4E-BP1 to bind eIF4E was essential for its blockade of Ras-induced survival signaling.

Effect of 4E-BP1 on Apoptosis of Ras-transformed Fibroblasts in Vivo—Prior studies have shown that ectopic 4E-BP1 decreases the mitotic index and tumorigenicity of NIH 3T3 cells transformed with either eIF4E or src (23); apoptosis was not evaluated. To study all three parameters in Ras-transformed fibroblasts, we injected cells from the CREF/Ras/BP1-wt clonal line into immunodeficient mice. Tumors formed by each CREF/Ras/BP1 line tested were less than one-third the size of those formed by mock-transfected CREF/Ras V12, with less visible vascularity (Fig. 4, a and b). All CREF/RasV12 tumors contained cells forming ill-defined fascicles with ovoid nuclei and an elongated cytoplasm; apoptotic cells were rarely observed (Fig. 4c). In contrast, tumors formed by cells ectopically expressing 4E-BP1 displayed more nuclear pleomorphism and most microscopic fields contained scattered apoptotic cells (Fig. 4, d and e). Ectopic 4E-BP1 decreased the mitotic index of the tumor cells by approximately one-third and dramatically increased their apoptotic frequency by nearly 5-fold (Fig. 4f). Untransformed CREF did not form tumors. These findings establish that suppression of apoptosis in Ras-transformed cells in vivo depends in part on cap-dependent translation, a function that was not rescued by transcriptional or post-translational processes.

DISCUSSION

For nearly four decades, global translational control has been recognized as a fundamental regulatory process in biology (24). More recently, examples of selective control have emerged involving regulation at the translation initiation step, particularly in the integration of pleiotropic responses leading to differentiation,
proliferation, and survival (25, 26). Here we focus on the translational apparatus itself, examining initiation events involving the mRNA cap-binding protein eIF4E and its most abundant repressor, 4E-BP1. We find that concentrations of rapamycin that are known to inhibit 4E-BP1 phosphorylation and cap-dependent protein synthesis (10) sensitize fibroblasts carrying activated Ras to apoptosis, whereas nonselective inhibition of global protein synthesis by the peptide elongation inhibitor cycloheximide actually blocks apoptosis. We further show that enforced expression of 4E-BP1 in Ras-transformed fibroblasts lacking an eIF4E binding domain does not promote apoptosis. Shown are nonspecific green fluorescence (open histograms), expression of HA (closed histograms), and DNA content (shaded histograms) in CREF/RasV12 transfected with an empty HA vector, an HA-tagged wild type 4E-BP1, or an HA-tagged eIF4E binding site deletion mutant, 4E-BP1Δ. The results of a representative experiment are shown (three independent transfection experiments yielded similar results).

The downstream effector proteins linking the cap-dependent translation initiation apparatus to the apoptotic machinery and the precise mechanisms regulating the translation of their cognate mRNAs are unknown. Prior studies have identified
Translational Repressor 4E-BP1 Inhibits Ras Survival Signaling

The present findings add to our current understanding of cell biology by highlighting new regulatory events integral to cancer cell survival. Available data suggest that eIF4E is a powerful oncoprotein (14, 36), whereas its antagonist 4E-BP1 functions as a tumor suppressor gene (38, 39). Nonmalignant cells can apparently function over a wide range of 4E-BP1 expression. Its absence in knockout mice results in apparent phe-
notypic changes (38), and here we find that even dramatic overexpression in nontransformed fibroblasts is compatible with physiological function. Against the background of oncogenic Ras, however, 4E-BP1 exerts powerful control over cell growth, viability, and susceptibility to cytotoxic drugs. These findings suggest that translational repressors may constitute a significant component of the mammalian tumor surveillance system. In addition, our work identifies a novel mechanism whereby tumor cells bearing oncogenic Ras can acquire resistance to genotoxic and nongenotoxic therapeutic agents. Our data thus provide direct evidence linking the fundamental biological process of cap-dependent translation initiation with suppression of apoptosis by activated Ras.

Acknowledgments—We thank A. De Benedetti for cell lines and discussion, P. Jolicouer for the pSRa vector, J. Geagea for help cloning pSRa-BPI, J. Murray and Darlene Charkhbarane for technical assistance, the University of Minnesota Cancer Center Biostatistical Core for assistance in study design and data analysis, and B. Raught for critical review of the manuscript.

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