Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells

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Summary

Common human malignancies acquire derangements of the translation initiation complex, eIF4F, but their functional significance is unknown. Hypophosphorylated 4E-BP proteins negatively regulate eIF4F assembly by sequestering its mRNA cap binding component eIF4E, whereas hyperphosphorylation abrogates this function. We found that breast carcinoma cells harbor increases in the eIF4F constituent eIF4GI and hyperphosphorylation of 4E-BP1 which are two alterations that activate eIF4F assembly. Ectopic expression of eIF4E in human mammary epithelial cells enabled clonal expansion and anchorage-independent growth. Transfer of 4E-BP1 phosphorylation site mutants into breast carcinoma cells suppressed their tumorigenicity, whereas loss of these 4E-BP1 phosphorylation site mutants accompanied spontaneous reversion to a malignant phenotype. Thus, eIF4F activation is an essential component of the malignant phenotype in breast carcinoma.

Introduction

Genomic, transcriptional, and posttranslational regulatory mechanisms of oncogenesis are well established in the pathobiology of cancer. More recently, it has become evident that cancer is subject to translational control (De Benedetti and Harris, 1999; Zimmer et al., 2000; Hershey and Miyamoto, 2000). Protein synthesis is primarily regulated at the step of ribosome recruitment to the 5′ mRNA terminus (Hershey and Merrick, 2000; Raught et al., 2000; Dever, 2002). The principal mode of ribosome recruitment is association of the 43S ribosomal complex with the m^7G cap structure of transcripts and subsequent ribosome scanning toward an initiation codon (Kozak, 1978, 1989). In mammals, this association is mediated by a trimeric complex, termed eIF4F, which consists of the large scaffolding proteins eIF4Gs (eIF4GI and eIF4GII), the RNA helicase eIF4A, and the cap binding protein eIF4E (Gingras et al., 1999b; Hershey and Merrick, 2000). The major target for modulation of cap-dependent translation by environmental and metabolic signals is eIF4E. Extracellular stimuli resulting in cell cycle transit or survival regulate eIF4E abundance by Myc-mediated transcriptional transactivation (Rosenwald et al., 1993; Johnston et al., 1998) and positively modulate its activity by phosphorylation through the Ras/MAPK kinase cascade (Waskiewicz et al., 1997).

While phosphorylation of eIF4E changes eIF4F affinity for the transcript cap (Scheper et al., 2001), the primary regulation of assembly and integrity of eIF4F is exerted by three eIF4E binding proteins (4E-BPs), designated 4E-BP1, -BP2, and -BP3 (Lin et al., 1994; Pause et al., 1994). The predominant species in most cells is 4E-BP1. The 4E-BPs compete with the eIF4Gs for eIF4E using the same eIF4E binding motif (Tyr-X-X-X-X-Leu-0, where X varies and 0 indicates Leu, Met, or Phe) and inhibit formation of eIF4F by sequestering eIF4E in a translationally inactive complex (Mader et al., 1995). Growth and survival factors positively regulate assembly of the eIF4F complex by promoting sequential phosphorylation of the 4E-BPs on six serine/threonine sites through multiple Ras-dependent protein kinase cascades (Gingras et al., 1999a, 1999b, 2001; Mothe-Satney et al., 2000; Herbert et al., 2002). Hyperphosphorylated 4E-BP has a decreased affinity for eIF4E, resulting in its liberation to initiate translation.

SIGNIFICANCE

There is a large body of clinical data documenting that carcinoma of the breast, lung, colon, and head and neck contain pathologically increased levels of translation initiation factors. In this report, we show that sustained activation of the protein synthesis initiation machinery is sufficient to enable mammary epithelial cells to undergo clonal expansion and anchorage-independent proliferation and is necessary for breast cancer cells to persist and form tumors. These data establish hyperactivation of the translation initiation apparatus as part of the molecular strategy adopted by breast cancer cells to achieve autonomy and identify restoration of physiological translational control as a new therapeutic objective.

Elevated levels of eIF4E and eIF4GI are found in a broad spectrum of transformed cell lines and human cancers and is often associated with aggressive, poorly differentiated tumors (reviewed by DeBenedetti and Harris, 1999; Zimmer et al., 2000; Hershey and Miyamoto, 2000). Activation of eIF4F by ectopically expressing eIF4E or eIF4GI transforms rodent fibroblasts (Lazaris-Karatzas et al., 1990) and promotes development of lymphoma in the Eμ-Myc model of mouse B cell lymphoma (Wendel et al., 2004), and downregulation of eIF4F by increasing 4E-BP1 reverts the malignant phenotype of transformed rodent fibroblasts (Rousseau et al., 1996; Polunovsky et al., 2000).

Acquisition of autonomy for growth and survival signaling is a crucial hallmark of advanced cancer (Hanahan and Weinberg, 2000). In this connection, overexpressed eIF4E confers resistance to spontaneous and drug-induced apoptosis in a pleotropic manner, including pretranslational and translational activation of the apoptosis antagonist Bcl-X_L (Polunovsky et al., 1996; Tan et al., 2000; Li et al., 2003). Along these lines, wild-type 4E-BP1 sensitizes transformed rat fibroblasts to drug-induced apoptosis (Polunovsky et al., 2000), and mutant forms lacking key phosphorylation sites required for inactivation display significantly enhanced pro-apoptotic function (Li et al., 2002). Although the burden of evidence strongly suggests a link between the activity of the translation initiation apparatus and human malignancy, the data in humans remain inferential.

To directly examine the contribution of deregulated cap-dependent translation to the genesis and maintenance of human epithelial derived cancer, here we study the consequences of upregulating eIF4F in nontransformed human mammary epithelial cells and its inhibition in naturally occurring human breast carcinoma cells, and we establish a causal relationship between aberrant translational control and human breast carcinogenesis.

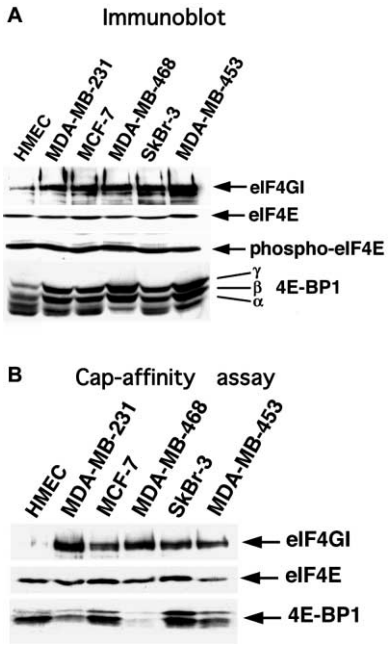

Figure 1. Analysis of the eIF4F translation complex in nontransformed and malignant mammary epithelial cells

A: Immunoblot showing steady-state levels of total and phosphorylated eIF4E, eIF4G1, and 4E-BP1 in cultures of actively proliferating nontransformed human mammary epithelial cells (HMECs, strain 184-A1) and in lines of breast carcinoma cells.

B: Examination of eIF4F integrity. Cell lysates were incubated with 7-methyl-GTP-Sepharose resin to capture eIF4E and its binding partners. Cap bound proteins were eluted with buffer containing 70 μM 7-methyl-GTP, subjected to SDS-PAGE, transferred to nitrocellulose, and probed for eIF4E, eIF4GI, and 4E-BP1.

Results

Breast carcinoma cells display a hyperactivated eIF4F complex

Initiation of cap-dependent translation is governed by several factors: the abundance and phosphorylation status of eIF4E, the balance between cellular levels of the eIF4Gs and 4E-BPs, and the phosphorylation status of the 4E-BPs. We set out to establish which of these events was associated with activation of cap-dependent translation in a series of breast cancer cell lines. Initially, we monitored expression levels of eIF4GI, eIF4E, and 4E-BP1, abundance of phosphorylated eIF4E, and the ability of eIF4E to associate with its binding partners in early passage human mammary epithelial cells (HMECs, strain 184, population passage number 12) and in HMECs immortalized by chemical mutagenesis (strain 184-A1, passage number 45) and found only marginal differences between these cell lines (data not shown). We concluded, therefore, that HMECs do not require changes in cap-dependent translation for immortalization.

Next we compared the pattern of eIF4GI, eIF4E, and 4E-BP1 expression between immortalized HMECs and a panel of breast carcinoma cell lines harboring diverse oncogenic alterations (Figure 1A). In contrast to previous reports (Kerekatte et al., 1995; Antony et al., 1996), there were no significant differences between nontransformed cells and carcinoma cell lines with regard to the levels or phosphorylation status of eIF4E; instead, expression levels of eIF4GI were substantially increased in all breast cancer cell lines tested. Examination of the status of 4E-BP1 revealed significant differences between immortalized HMECs and the breast carcinoma cell lines. In this analysis, the tendency of available anti-4E-BP1 antibodies to bind nonspecifically to many cellular proteins dictated a modification in the usual electrophoresis procedure (see Experimental Procedures). Cell extracts (50 μg/sample) were first boiled and cooled, allowing the majority of cellular proteins to precipitate (4E-BP1 is heat stable and remains in solution) before standard SDS-PAGE and immunoblotting procedures were carried out. This step precluded the use of the usual housekeeping proteins such as actin as loading controls. The dominant form of 4E-BP1 in nontransformed cells was the hypophosphorylated isoform α represented by two distinct bands. In marked contrast, cancer cell extracts manifested only one α band and were enriched with slow migrating forms of 4E-BP1 (isoforms β and γ). This upward mobility shift reflects extensive phosphorylation of 4E-BP1, which markedly reduces its ability to sequester eIF4E (Mothe-Satney et al., 2000; Gingras et al., 1999a). Thus, carcinoma cells harbor alterations that activate eIF4E by increasing its association with eIF4GI, thereby favoring formation of intact eIF4F.

To directly compare the integrity of eIF4F in nontransformed and malignant mammary epithelial cells, we assessed formation of the m^7GTP-eIF4E-eIF4G ternary complex in a cell-free test system. Aliquots of the crude cell extracts used for immunoblotting were incubated with agarose-immobilized m^7GTP cap ana-

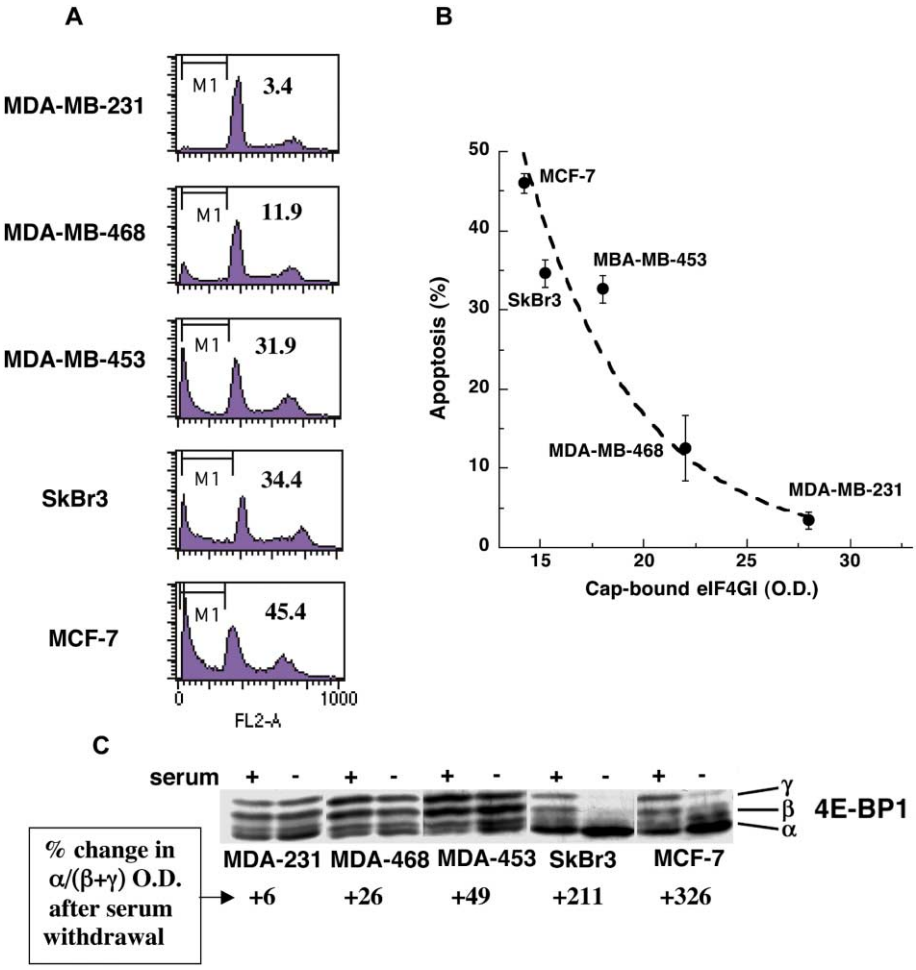

Figure 2. Evasion of intrinsic apoptosis by breast cancer cells is associated with increased eIF4F and constitutive phosphorylation of 4E-BP1

A and B: Breast carcinoma cells were cultivated in serum-deprived medium for 48 hr. Shown are a representative histogram of DNA content (**A**) and the relationship between apoptotic frequency and quantity of eIF4G1 associated with immobilized 7-methyl-GTP (**B**).

C: Western blot showing the 4E-BP1 isoform profile in apoptosis-sensitive (MCF-7, SkBr, MBA-MB-453) and apoptosis-resistant (MBA-MB-231, MBA-MB-468) breast carcinoma cells after being cultured in regular (+) or serum-deprived (−) medium for 48 hr.

log to capture eIF4E and its binding partners the eIF4Gs and 4E-BPs; the levels of cap bound eIF4E, 4E-BP1, and eIF4GI were analyzed by immunoblotting. The relative amounts of cap-captured eIF4GI in cell extracts can serve as a cumulative indicator of the integrity and functional potency of eIF4F, whereas the amounts of cap-associated 4E-BP1 estimate the negative impact of 4E-BP1 on eIF4F assembly. The cap bound fraction from HMECs contained significant amounts of 4E-BP1 and very little eIF4GI (Figure 1B), indicating that in nontransformed cells, cap-dependent translation is under strong 4E-BP1-mediated negative control. In contrast, the cap bound complexes from all carcinoma lines tested were enriched with eIF4GI, indicating that cancer cells existed in a translationally activated state, supporting the argument that breast adenocarcinoma cells acquire a set of metabolic and regulatory lesions that promote assembly of the eIF4F initiation complex.

Activation of eIF4F in breast cancer cells is associated with resistance to apoptosis

To evaluate the relationship between the status of eIF4F and resistance to intrinsic pro-apoptotic signaling, we quantified apoptosis in breast carcinoma cells in the absence of serum survival factors. Flow cytometric analysis of cell viability indicated that serum-deprived MDA-MB-231 and MDA-MB-468 cells were markedly more resistant to apoptosis than SkBr-3, MDA-MB-453, and MCF-7 (Figure 2A). The most resistant cell lines (MDA-MB-231 and MDA-MB-468) displayed the highest levels of intact eIF4F, and regression analysis of the data revealed an exponential relationship between resistance to apoptosis and the integrity of the eIF4F complex (Figure 2B).

Serum factors promote eIF4F assembly by stimulating pathways leading to phosphorylation of 4E-BPs (Gingras et al., 1999b). We assessed expression of 4E-BP1 isoforms in the presence or absence of serum and found a clear downward mobility shift in response to serum withdrawal in apoptosis-sensitive MCF-7 and SkBr3 cells and a bias toward hypophosphorylated species in serum-starved MDA-MB-453 cells, which exhibit a moderate susceptibility to apoptosis (Figure 2C). In contrast, MDA-MB-231 and MDA-MB-468, the cell lines least sensitive to apoptosis, did not change their pattern of 4E-BP1 expression, suggesting a constitutive mode of 4E-BP1 hyperphosphorylation. Thus, a sustained pattern of 4E-BP1 hyperphosphorylation and increased integrity of eIF4F in breast carcinoma cells are associated with decreased dependence on extracellular survival signals.

Activation of eIF4F enables immortalized mammary epithelial cells to form colonies on a plastic substratum and proliferate in an anchorage-independent fashion

Immortalized 184-A1 HMECs do not form full-size anchorage-dependent colonies on a plastic substratum or exhibit anchor-

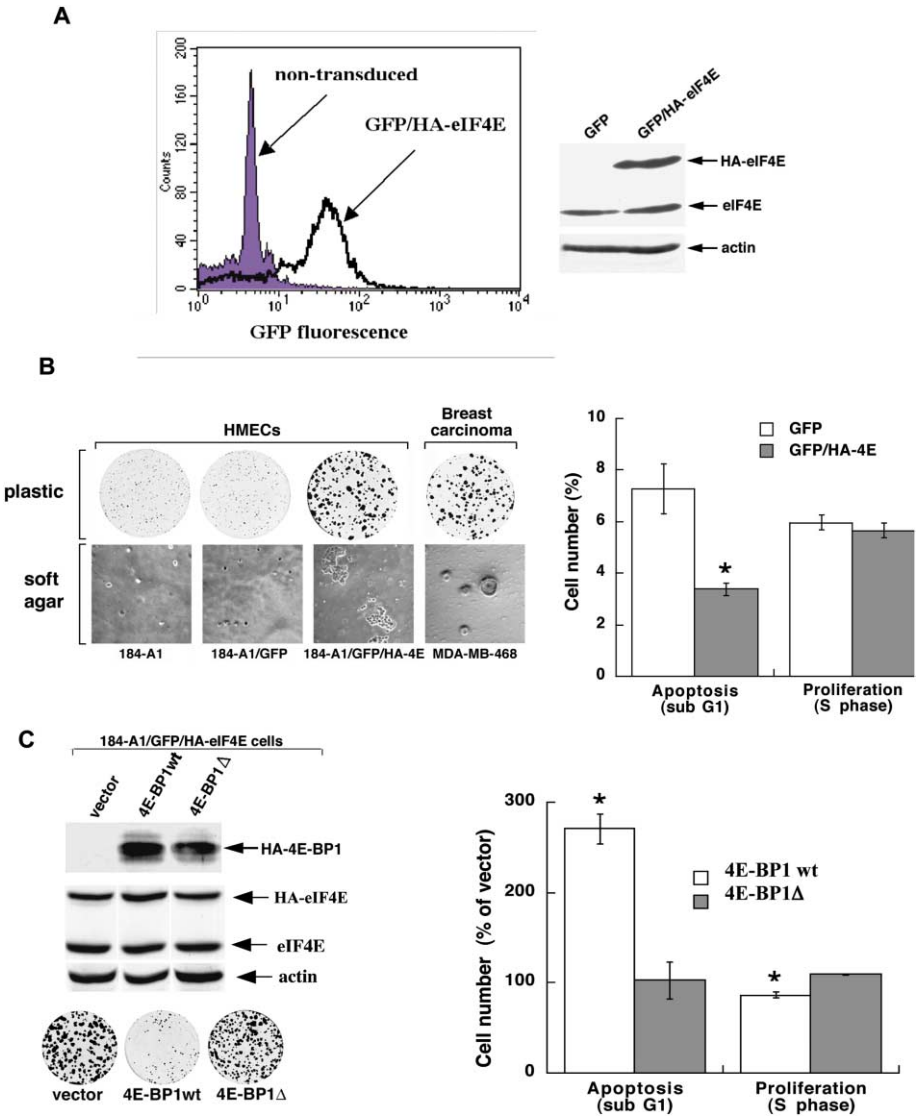

Figure 3. eIF4E confers HMEC with the ability to undergo anchorage-dependent and -independent clonal proliferation

A: Characterization of experimental cell lines. HMECs (strain 184-A1) were transduced with the MSCV-M1GR1 retroviral construct encoding GFP with or without 3HA-eIF4E, and subpopulations of cells expressing the highest levels of GFP were sorted by FACS. Shown are GFP fluorescence in parental HMEC 184-A1 cells and in sorted 184-A1/GFP/3HA-eIF4E cells (left) and eIF4E expression in sorted 184-A1/GFP and 184-A1/GFP/3HA-eIF4E cells.

B: Effect of overexpressed eIF4E on HMEC 184-A1 colony formation, cell cycle transit, and apoptosis. Left: Parental HMEC 184-A1 or those transduced with the indicated constructs were seeded at a density of 500 cells/well into 6-well clusters containing regular growth medium or seeded into soft agar (20,000 cells/well), with cultures continued for 2 weeks in MEBM. As a positive control, the growth pattern of MDA-MB-468 carcinoma cells is shown. Right: Exponential cultures of mock transduced or HA-eIF4E-expressing 184-A1 cells were analyzed by flow cytometry. Shown are the proportions of cells exhibiting hypodiploid (apoptotic) and S phase DNA content.

C: Functionally active 4E-BP1 reverses eIF4E-dependent alterations in cell viability and growth. 184-A1/3HA-eIF4E cells were stably transfected with empty pACTAG-2-*neo* or with pACTAG-2-*neo* encoding 4E-BP1wt or 4E-BP1Δ (which lacks the eIF4E binding site). Left: Shown are examples of a representative immunoblots and an anchorage-dependent clonogenic growth assay. Right: Flow cytometric analyses of DNA content (the means ± SD for two experiments) in 184-A1/3HA-eIF4E cells expressing 4E-BP1 wt or Δ. *$p < 0.001$ compared to mock transduced cells.

age-independent growth in soft agar. We therefore evaluated the impact of sustained eIF4F activation on these hallmarks of in vitro transformation. To achieve high levels of eIF4F activation in 184-A1 cells, we ectopically expressed eIF4E, its rate-limiting component. The procedure employed replication-defective retrovirus containing a green fluorescent protein (GFP) gene linked to a sequence encoding hemagglutinin (HA) epitope-tagged human eIF4E (HA-eIF4E). Cells expressing the highest levels of GFP (the brightest 10% of the GFP-positive cell population) were sorted using a FACSscan and propagated for further investigation (Figure 3A).

The results were striking. Introduction of HA-eIF4E not only enabled HMECs to form large colonies when adhering to a plastic substratum, but also conferred the ability to proliferate in soft agar (Figure 3B, left). Cytometric analysis of HMEC harboring ectopic eIF4E during exponential growth was carried out to define their cell cycle distribution and viability. Compared to mock transduced cells, there was a 50% decrease in the proportion of hypodiploid (apoptotic) cells in 184-A1/HA-eIF4E, whereas the proportion of actively proliferating cells was similar for the parental line and 184-A1/HA-eIF4E (Figure 3B, right). This indicated that the predominant impact of eIF4E was to reduce the frequency of apoptosis, rather than to increase cell proliferation. However, despite acquiring the capacity to grow in an anchorage-independent manner, the morphologic pattern of 184-A1/HA-eIF4E cell growth was profoundly different from that observed for breast carcinoma cells. MDA-MB-468 carcinoma cells formed dense multilayer ovoid and spheroid structures on a plastic substratum and in soft agar. In marked contrast, eIF4E-transduced HMECs formed flat colonies on plastic and monolayer aggregates in agar with coherent nests and trabeculae consisting of polygonal, closely attached cells with uniform nuclei. In addition, in stark contrast to highly tumorigenic MDA-MD-231 or MDA-MB-468 cells, 184-A1/HA-eIF4E cells did not form tumors in nude mice at least during 20 days of observation (not shown).

Increased expression of 4E-BP1 reverses the eIF4E-induced transformed phenotype in rodent fibroblasts, presumably by sequestration of overexpressed eIF4E (Rousseau et al., 1996). Consequently, we examined whether elevated 4E-BP1 can re-

store the nontransformed phenotype in HMECs ectopically expressing eIF4E. 184-A1/HA-eIF4E cells were transfected with a vector bearing a neomycin resistance cassette and sequences encoding HA-tagged wild-type 4E-BP1 or its 54–63 deletion mutant (4E-BP1Δ), which lacks the eIF4E binding site and is therefore unable to sequester eIF4E (Haghighat et al., 1995; Polunovsky et al., 2000). Both mock transfected 184-A1/eIF4E cells and cells bearing 4E-BP1Δ yielded full-size colonies on plastic after 14 days. In contrast, overexpression of wild-type 4E-BP1 markedly decreased the clonogenic potency of eIF4E-transduced cells. Cells co-expressing eIF4E and wild-type 4E-BP1 were unable to form large colonies on a plastic substratum (Figure 3C, left), failed to grow in soft agar, and displayed a modest but statistically significant reduction in the fraction of cells synthesizing DNA and a more than 2.5-fold increase in the frequency of apoptosis (Figure 3C, right). Thus, overexpressed eIF4E confers human epithelial cells with some properties characteristic of malignant conversion. These include resistance to intrinsic apoptosis and the ability to form anchorage-dependent and -independent foci—properties that were antagonized by functionally active 4E-BP1.

4E-BP1 phosphorylation status determines its potency in suppressing breast cancer cell viability, proliferation, and colony-forming ability

Breast carcinoma cells were characterized by activated eIF4F. Since 4E-BP1 decreased resistance to apoptosis and clonogenic capacity in HMECs with activated eIF4F, we reasoned that normalizing eIF4F in cancer cells by increasing 4E-BP1 should also attenuate these aspects of the malignant phenotype. However, when MDA-MB-231 and MDA-MB-468 cells were engineered to express high levels of wild-type 4E-BP1, there was only a marginal impact on apoptosis and clonogenicity. One explanation for this lack of sensitivity to the pro-apoptotic activity of 4E-BP1 was that in cancer cells, the activity of 4E-BP1 might be neutralized by extensive phosphorylation. We, therefore, examined whether pharmacological and/or genetic interventions that blocked 4E-BP1 phosphorylation would enhance the pro-apoptotic effect of overexpressed 4E-BP1 in cancer cells.

The multiple kinase cascades leading to 4E-BP1 phosphorylation include the Ras/PI3K/Akt/mTOR-mediated pathway (reviewed by Schmelzle and Hall, 2000; Rhoads, 1999; Raught et al., 2001). This pathway can be activated by extracellular growth factors through their cognate receptors, leading to phosphorylation of 4E-BP1 in a FRAP/mTOR protein kinase-dependent fashion (Gingras et al., 2001). We therefore examined the effects of 4E-BP1 phosphorylation on 4E-BP1-induced apoptosis in MDA-MB-231 and MDA-MB-468 cells, using the FRAP/mTOR inhibitor rapamycin as a tool to suppress 4E-BP1 phosphorylation. In order to minimize the effects of extracellular phosphorylation and survival signaling, cells were pre-incubated in serum-free medium for 24 hr. Surprisingly, immunoblot analysis revealed that these cell lines differ profoundly in their response to rapamycin. In MDA-MB-231 cells, rapamycin induced the expected 4E-BP1 mobility shift toward the faster migrating hypophosphorylated species. In contrast, there was no detectable effect of rapamycin on 4E-BP1 phosphorylation in MDA-MB-468 cells (Figure 4A).

To determine whether the ability of rapamycin to inhibit 4E-BP1 phosphorylation was associated with its ability to modulate 4E-BP1-induced apoptosis, MDA-MB-231 and MDA-MB-468 cells were engineered to express similar amounts of HA-4E-BP1 (Figure 4B). The resulting cells were serum-deprived in the presence or absence of rapamycin, and apoptosis was quantified. Rapamycin cooperated with ectopic 4E-BP1 in triggering apoptosis in MDA-MB-231 cells, where it was active in blocking 4E-BP1 phosphorylation. In marked contrast, rapamycin had no impact on apoptosis in MDA-MB-468 cells, in which 4E-BP1 is phosphorylated in a rapamycin-insensitive fashion. Thus, breast carcinoma cells may harbor alterations, which inactivate the pro-apoptotic activity of 4E-BP1 through hyperphosphorylation. These results support previous findings that resistance of human cancer cells to rapamycin can be associated with a decrease in the antitranslational potency of 4E-BP1 (Dilling et al., 2002).

The affinity of 4E-BP1 for eIF4E is governed by ordered phosphorylation of critical amino acid residues and regulated through FRAP/mTOR signaling (Mothe-Satney et al., 2000; Gingras et al., 2001). The presence of phosphates on sites T37 and T46, which are N-terminal to the eIF4E binding domain, is required for rapamycin-inhibitable phosphorylation of the C-terminal residues S65 and T70. To directly examine which of these phosphorylation sites on 4E-BP1 were required by breast cancer cells to suppress apoptosis, 184-A1 HMECs, MDA-MB-231, and MDA-MB-468 cells were infected with retroviral constructs encoding wild-type ($4E\text{-}BP1^{wt}$) or phosphorylation site mutant forms of HA-4E-BP1, and cells expressing identical amounts of GFP were sorted by FACS. Neither $4E\text{-}BP1^{wt}$ nor its mutants increased apoptosis in nontransformed HMECs (Figure 5A). In contrast, phosphorylation site mutants promoted apoptosis in both the MDA-MB-231 and MDA-MB-468 cell lines. The pro-apoptotic efficacy of 4E-BP1 mutants was site specific. In both carcinoma lines, expression of the A37/A46 and A70 mutants significantly increased apoptotic death, while mutating S65 was less effective. The cell lines differed in that rapamycin-sensitive MDA-MB-231 cells displayed increased susceptibility to mutating the T70 site, while the A37/A46 mutant was most active in promoting apoptosis in the rapamycin-resistant MDA-MB-468 cells. Analysis of proliferative activity also revealed suppression of cell cycle transit in cells expressing 4E-BP1 mutants, with the A37/A46 mutant exerting a modest but statistically significant antiproliferative effect in both cell lines tested (Figure 5B). Ectopic expression of phosphorylation site mutants of 4E-BP1 in breast carcinoma cells (MDA-MB-231 and MDA-MB-468) tended to reduce their ability to form anchorage-dependent colonies (Figures 5C and 5D), with the most profound effect observed with $4E\text{-}BP1^{A37/A46}$ in MDA-MB-468 cells and with $4E\text{-}BP1^{70}$ in MDA-MB-231 cells.

To determine whether the observed alterations in cell death, proliferation, and colony-forming capacity in response to each 4E-BP1 mutant was related to its potency in repressing the translational function of eIF4F, MDA-MB-468 cells were cotransfected with wild-type or a mutant form of 4E-BP1 along with a dicistronic reporter, which directs translation of Renilla luciferase in a strictly cap-dependent manner and mediates translation of firefly luciferase via an IRES (Poulin et al., 1998; Li et al., 2002). In accord with a potential causal link between the cell

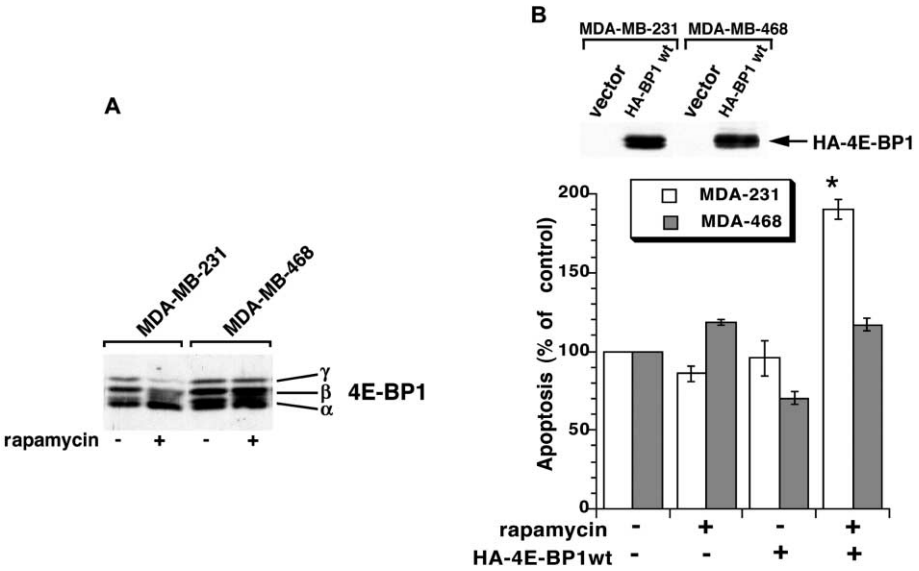

Figure 4. Concordance between 4E-BP1 phosphorylation and apoptosis in rapamycin-treated breast carcinoma cells

A: MDA-MB-231 and MDA-MB-468 cells were serum-deprived for 24 hr and continued in serum-free DMEM for 24 hr in the presence or absence of 100 nM rapamycin. Shown are immunoblots illustrating the phosphorylation status of 4E-BP1.

B: MDA-MB-231 and MDA-MB-468 cells were transduced with GFP/3HA-4E-BP1^{wt}, and subpopulations of each cell line expressing similar amounts of 3HA-4E-BP1 were isolated by GFP sorting and confirmed by HA-immunoblotting (top). Sorted cells were serum deprived in the presence or absence of rapamycin for 24 hr and apoptosis was quantified by FACS. The mean ± SD of three independent experiments is shown. *$p < 0.001$.

growth inhibiting and antitranslational activities of 4E-BP1, the most profound repression of cap-dependent translation observed with 4E-BP1$^{A37/A46}$ was associated with the greatest increase in apoptosis and the greatest decreases in colony formation and BrdU incorporation (data not shown).

To determine the maximum cell death response that could be attained by overexpression of 4E-BP1 in apoptosis-resistant cancer cells, MDA-MB-468 cells expressing 4E-BP1$^{A37/A46}$ were iteratively transduced with sequences encoding GFP/4E-BP1$^{A37/A46}$ with subsequent sorting of the brightest 10% of cells. After three rounds of GFP/4E-BP1$^{A37/A46}$ transduction, neither GFP nor HA-4E-BP1 expression levels could be elevated further (Figure 5E, top). Ectopic expression of maximum achievable levels of 4E-BP1$^{A37/A46}$ stimulated apoptosis in a dose-dependent manner, with a plateau observed at about 12%–14% (Figure 5E, bottom). This modest frequency of cell death could not be increased further by serum withdrawal or by combining serum withdrawal and rapamycin. Thus, increased expression of 4E-BP1 along with strategies that allow it to evade physiological mechanisms of 4E-BP1 inactivation sensitized a limited cohort of breast carcinoma cells to apoptosis. These findings suggest that the aggregate impact of relatively modest decreases in breast carcinoma cell viability and proliferation can lead to profound reductions in clonogenic capacity.

Tumorigenicity of breast carcinoma cells is inhibited by overexpressed 4E-BP1

When MDA-MB-468 cells were implanted into mammary fat pads of immunodeficient mice, they formed tumors with a latency period of 3 days and a diameter of 9.7 ± 0.8 mm (mean ± SEM) within 3 weeks (Figures 6A and 6B). In marked contrast, MDA-MB-468 cells expressing high levels of either 4E-BP1^{wt} or 4E-BP1$^{A37/A46}$ formed tumors with a much longer latency. Tumors formed by 4E-BP1^{wt} cells were first apparent at day 5, whereas 4E-BP1$^{A37/A46}$-expressing cells formed visible tumors only after 12 days. Control tumors grew at a rate 22.2 ± 1.5 mm^3/day from day 13 to day 21 after implantation, whereas during the same time period, tumors formed by 4E-BP1^{wt} cells grew 11.8 ± 1.2 mm^3/day and tumors arising from 4E-BP1$^{A37/A46}$ cells grew very slowly—only 3.5 ± 0.7 mm^3/day.

All tumors were excised after euthanasia on day 21. Histopathological analysis revealed that tumors formed by cells harboring 4E-BP1^{wt} or 4E-BP1$^{A37/A46}$ displayed increased cellular and nuclear pleomorphism and had fewer blood vessels than control tumors (Figure 6C). To evaluate cell population dynamics, tumor cell proliferative and apoptotic indices were quantified. Ectopic 4E-BP1 moderately suppressed tumor cell proliferative activity, as reflected by a 10% decrease in the number of Ki-67-positive cells and a 30% decrease in mitotic index (Figures 6C and 6D). To assess the frequency of apoptosis, tissue was analyzed using a TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. The apoptotic frequency was increased almost 7-fold in tumors arising from 4E-BP1^{wt} cells and about 2-fold in tumors formed by 4E-BP1$^{A37/A46}$ cells (Figures 6C and 6D). These data indicate that the major effect of 4E-BP1 on tumor growth is by promoting apoptosis. Our data also document a lesser but definite repression of tumor cell proliferation by 4E-BP1, as well as a possible negative effect of 4E-BP1 on angiogenesis.

These data also uncovered a striking contradiction. Wild-type 4E-BP1 displayed low-level proapoptotic activity in MDA-MB-468 cells in vitro but was highly potent in vivo. In contrast, 4E-BP1$^{A37/A46}$ manifested high pro-apoptotic and anticlonogenic potency in vitro but only modest activity in tumors. A clue to the significance of these findings came from an examination of tumor cell morphology. Cells ectopically expressing 4E-BP1$^{A37/A46}$ manifested a pattern of cell death that clearly differed from those in 4E-BP1^{wt} tumors. Most apoptotic cells in tumors formed by 4E-BP1^{wt} cells exhibited morphology typical for early stages of apoptosis. In contrast, the TUNEL-positive cells in tumors arising from 4E-BP1$^{A37/A46}$ cells contained predominantly compact apoptotic bodies—a morphological pattern typical of late-stage apoptotic degradation (Figure 6C). Based on this, we considered the possibility that a cohort of HA-4E-BP1$^{A37/A46}$ cells had already undergone apoptosis during the latent period and that the relapsed tumors arose from those cells that escaped

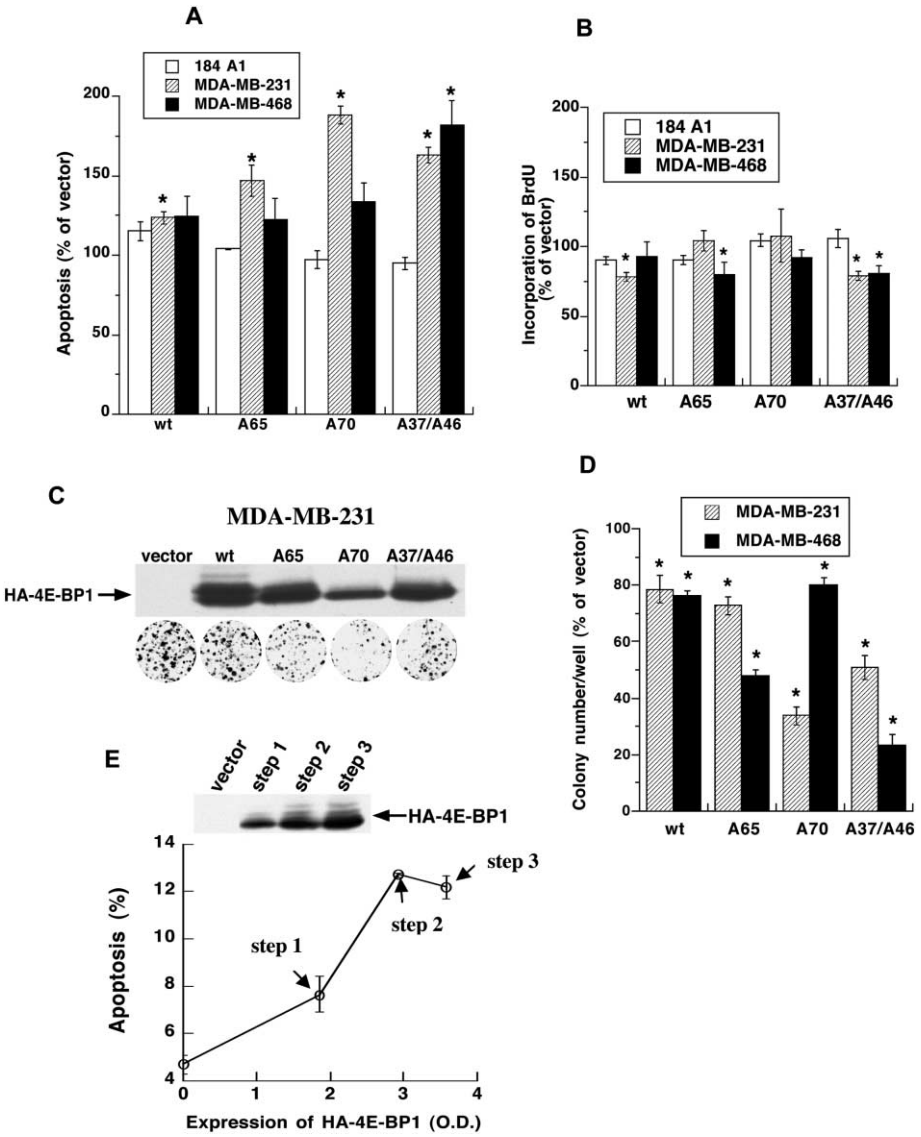

Figure 5. Mutating phosphorylation sites enhances the pro-apoptotic and antiproliferative activities of 4E-BP1 in breast carcinoma cells

A: Apoptosis in nontransformed HMECs and in breast carcinoma cells expressing wild-type or phosphorylation site mutants of 3HA-4E-BP1. Nontransformed HMECs (strain 184-A1) and breast carcinoma lines MDA-MB-231 and MDA-MB-468 were transduced with each GFP/3HA-4E-BP1 construct to be tested or empty GFP vector. Cells were sorted to express similar amounts of GFP and cultured for 48 hr in regular growth medium, and apoptosis was quantified by flow cytometry. *$p < 0.05$ compared to mock transduced cells.

B: Effect of wild-type and mutant 4E-BP1 on cell proliferation. 4E-BP1-transduced cells were exposed to 110 μM BrdU for 16 hr in regular growth medium. Incorporation of BrdU was quantified using the BrdU assay kit (Roche) according to the instruction provided by the manufacturer.

C: Effect of wild-type and mutant 3HA-4E-BP1 on colony-forming ability of stably transduced MDA-MB-231 cells. Expression of 3HA-4E-BP1 was confirmed by HA-immunoblotting (top), and 500 cells/well were seeded into 6-well clusters, with cultures continued for 2 weeks in regular growth medium. Colony formation was assessed by Coomassie blue staining of fixed cells (bottom).

D: Colonies formed by MDA-MB-231 and MDA-MB-468 cells stably expressing wild-type or mutant 3H-4E-BP1 (500 cells per plate) were quantified. The means ± SD for three experiments are shown. *$p < 0.05$ compared to mock transduced cells.

E: Relationship between expression levels of 4E-BP1$^{A37/A46}$ and apoptosis in MDA-MB-468 cells. MDA-MB-468 cells were iteratively transduced with GFP/3HA-4E-BP1$^{A37/A46}$ in a step-wise fashion. After each transduction step, the brightest 10% of cells were collected by using a FACS sorting procedure, and apoptosis frequencies were quantified by flow cytometry. Shown are HA-immunoblots after iterative transfer of GFP/HA-4E-BP1 into MDA-MB-468 cells (top) and apoptosis frequencies in transduced cells after cultivation in regular growth medium, presented as a function of 3HA-4E-BP1 expression level (mean ± SD, bottom).

apoptosis—presumably through the loss or inactivation of 4E-BP1$^{A37/A46}$—due to strong selective pressure against this pro-apoptotic protein.

Propagation of breast carcinoma cells selects for gain of apoptosis resistance and decreased expression of hypophosphorylated 4E-BP1

To assess the association between ectopic expression of 4E-BP1 and tumor cell fate, tumors formed by 4E-BP1^{wt} and 4E-BP1$^{A37/A46}$ cells were evaluated for HA-4E-BP1 expression by HA immunostaining. Both 4E-BP1^{wt} and 4E-BP1$^{A37/A46}$ tumors revealed high levels of ectopic 4E-BP1, predominantly in the regions where extensive cell death was observed (Figure 7A). Of note, the expression of HA-4E-BP1 in viable cells of 4E-BP1$^{A37/A46}$ tumors was markedly reduced compared with that found in tumors formed by 4E-BP1^{wt} cells. This suggested the possibility that the process of tumor formation indeed selected for cells that evade apoptosis and/or aggressively proliferate by loss of hypophosphorylated forms of 4E-BP1, and that the regrowth of 4E-BP1$^{A37/A46}$ tumors after a long latency resulted from loss of ectopic 4E-BP1 expression.

To examine this possibility experimentally, we traced expression of exogenous 4E-BP1^{wt} and the phosphorylation site mutants in long-term cultures of nontransformed HMECs and breast carcinoma cells. In HMECs, which resist the pro-apoptotic and antiproliferative effects of all forms of 4E-BP1 tested, expression levels of ectopic 4E-BP1 were unchanged during the entire 10 week period of observation (Figure 7B). In striking contrast, MDA-MB-468 cells revealed a dramatic reduction in steady-state levels of the mutant forms of ectopic 4E-BP1—and among these, 3HA-4E-BP1$^{A37/A46}$ was nearly absent after 7–8 weeks of cultivation (Figures 7B and 7C). The loss of ectopic 4E-BP1 expression in 3HA-4E-BP1$^{A37/A46}$-transduced breast cancer cells was closely associated with gain of resistance to apoptosis and restoration of tumorigenicity. Thus, inactivation of the anti-translational function of 4E-BP1 by its hyperphosphorylation

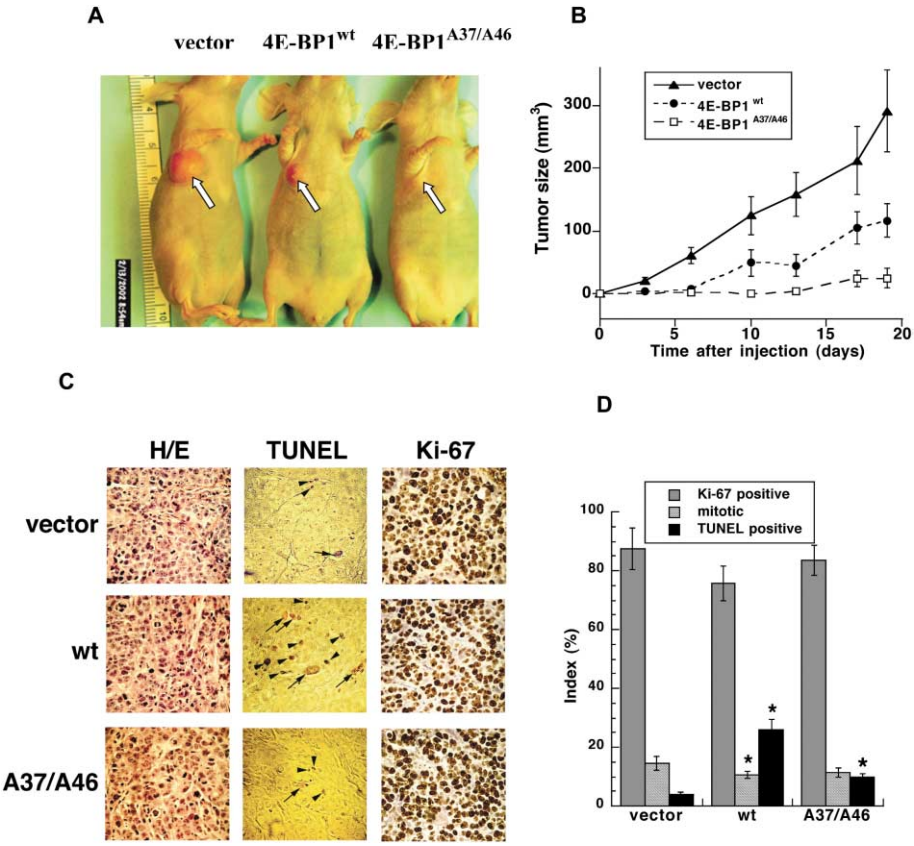

Figure 6. 4E-BP1 phosphorylation status determines its potency as an inhibitor of xenograft tumor growth

MDA-MB-468 cells (5×10^6) transduced with either 4E-BP1wt (4E-BP1^{wt}) or phosphorylation site double mutant (4E-BP1$^{A37/A46}$) were injected into the mammary fat pads of nude mice, and tumor growth was evaluated after the time intervals indicated.

A: Representative animals photographed at 21 days post injection. Arrows indicate loci where cells were injected.

B: Quantification of tumor volume. The average tumor volume for six animals from each cell line tested was determined (mean ± SE).

C: Histopathology of mouse fat pads after injection with 3HA-4E-BP1-expressing cells. Fat pad samples were excised at the time of euthanasia. Sections were stained with hematoxylin-eosin (H/E, left) to elucidate tumor morphology or underwent TUNEL staining to identify apoptotic cells (middle) and Ki-67 immunostaining to reveal proliferating cells (right). Representative microscopic fields are shown. Arrows point to apoptotic cells and arrowheads indicate apoptotic bodies.

D: Proliferative and apoptotic indices in tumors formed by 4E-BP1 and vector-transduced MDA-MB-468 cell lines. The results represent mean values ± SE from 500 microscopic fields of three tumors formed by cells expressing empty vector or 3HA-4E-BP1. *$p < 0.05$.

was an essential condition for efficient propagation of breast carcinoma cells in vitro and for their tumorigenicity in the murine xenograft model. These data are consistent with a scenario whereby expansion of transformed cells during breast tumorigenesis requires selection for durable activation of eIF4F as a part of the program acquired by cancer cells to propagate and persist.

Discussion

The existing paradigm for causality in cancer biology is that cancer cells can acquire oncogenic alterations in one of two ways. First, a gene directly controlling essential growth regulatory events can itself be mutated, as occurs with the Ras V12 mutations frequently found in lung cancer. Second, a group of genetic alterations—no one of which is obligatory—can act indirectly by modulating critical linchpin upstream regulators of an oncogene or a tumor suppressor gene. In the case of breast cancer, *ras* is mutated in only about 5% of cases (Bos, 1989). Nonetheless, breast cancer cells uniformly express activated Ras by virtue of harboring one or more alterations in upstream pathways converging on Ras (Olson and Marais, 2000). In this situation, mammary epithelial cells acquire deregulated (oncogenic) Ras—an essential component of the malignant phenotype—indirectly.

Does this same logic apply to the role of eIF4F integrity in malignant conversion of the mammary epithelium? A significant body of evidence links human cancer and activation of the cap-dependent translational machinery (reviewed by De Benedetti and Harris, 1999; Zimmer et al., 2000; Hershey and Miyamoto, 2000). Many known oncogenic factors are either upstream modulators of eIF4F integrity (e.g., HER-2/*neu*, EGF-R/*erb-b*, c-Myc, etc.), or downstream effectors of eIF4F function (e.g., cyclin D1, Bcl-X_L, etc.), or both (e.g., Ras) (Brown and Schreiber, 1996; Rhoads, 1999). Thus, in naturally occurring breast cancer, eIF4F can plausibly serve as an integrator and amplifier of a broad range of diverse proneoplastic signals emanating from mutated cancer-related genes.

These observations, however, leave open the key question of whether sustained activation of the translation initiation complex eIF4F in cancer cells is essential for expression of a transformed phenotype or whether it is a secondary consequence of the intrinsic need of cancer cells for increased protein synthesis. In this report, we show that sustained gain of eIF4F function causes a critical hallmark of cancer (i.e., self-sufficiency of human epithelial cells for clonogenic expansion) and that aberrant overexpression of eIF4F is essential for naturally occurring human breast carcinoma cells expressing diverse oncogenic alterations to persist and propagate in vitro and in vivo. We also discovered that the strategies used by cancer cells to maintain durable activation of eIF4F-mediated translation is more diverse than previously realized. In addition to increasing the expression levels of eIF4E, functional activation of eIF4F can occur by enhanced expression of eIF4GI and constitutive hyperphosphorylation of 4E-BP1. These data indicate that aberrant cap-dependent translational control is a causal factor in both the genesis and maintenance of the malignant phenotype in human mammary cancer.

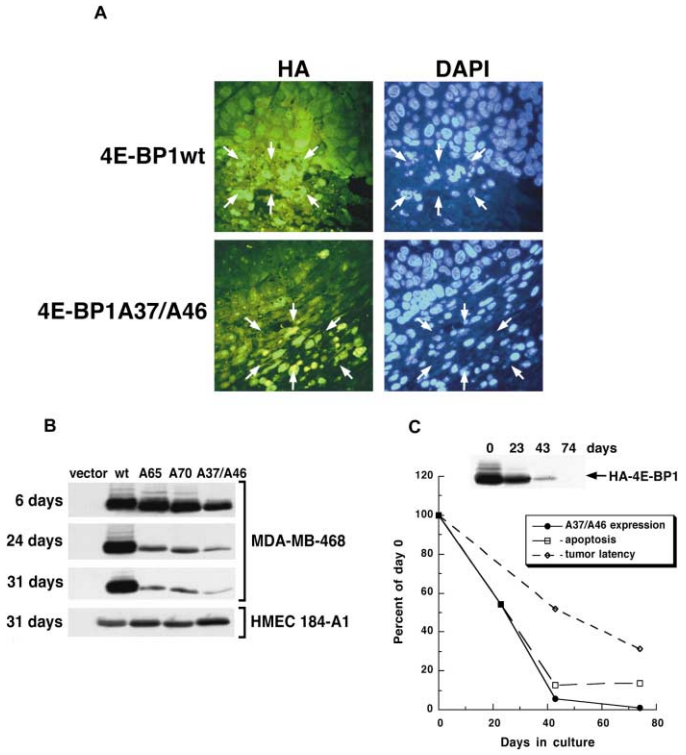

Figure 7. Loss of ectopic 4E-BP1 expression in breast cancer cells

A: Immunohistochemical analysis of 3HA-4E-BP1 in tumors formed by MDA-MB-468 cells expressing either 3HA-4E-BP1^{wt} or 3HA-4E-BP1$^{A37/A46}$. Sections of tumors from day 21 were probed with anti-HA antibody, and signal was visualized with FITC-conjugated secondary antibody. Following antibody staining, nuclei were counterstained with DAPI. Arrows indicate areas of extensive cell death with high levels of FITC fluorescence.

B: 3HA-4E-BP1 expression during in vitro cultivation of MDA-MB-468 and 184-A1 cells transduced with wild-type or phosphorylation site mutants of 3HA-4E-BP1. Extracts of transduced breast carcinoma cells and HMEC 184-A1 were analyzed by HA immunobloting at the indicated time intervals after transduction.

C: Loss of ectopic 4E-BP1 expression in 3HA-4E-BP1$^{A37/A46}$ transduced breast carcinoma cells is associated with gain of resistance to apoptosis and restoration of tumorigenicity. MDA-MB-468 cells transduced with HA-4E-BP1$^{A37/A46}$ were seeded in regular growth medium, cultivated for 7 days (day 0 in the plot, above), and subcultivated weekly thereafter at a 1:10 split ratio. After the indicated interval, cells were divided into three aliquots and were (1) examined for levels of 4E-BP1 by HA-immunoblotting, (2) subjected to flow cytometric quantification of apoptosis, or (3) injected into the mammary fat pads of nude mice (six animals, 5×10^6 cells/injection) to assay tumor latency (time interval after cell inoculation when tumors become visible in at least 50% of animals). The data from three independent experiments are presented normalized to the percentage of values observed for each parameter on day 0.

To our surprise, steady-state levels of eIF4E were not higher in breast carcinoma cells compared to nontransformed mammary epithelial cells. Although these results are in agreement with the findings of Raught et al. (1996) obtained in a murine model of breast cancer, they are at odds with earlier reports that human breast cancer cell lines overexpress eIF4E (Kerekatte et al., 1995; Antony et al., 1996). One reasonable explanation for this discrepancy is that eIF4E expression levels are extremely sensitive to proliferative status in nontransformed cells. Cells in midexponential growth, which was the case in our study, manifest levels of eIF4E comparable to cancer cells, whereas cells in any other growth state display much lower levels. In contrast, expression of eIF4E in cancer cells is constitutively high, independent of growth phase or density in culture. The growth state of nontransformed cells in previous studies was not specified, which we suspect accounts for the differences.

Our finding that 4E-BP1 hyperphosphorylation is part of the strategy used by breast carcinoma cell lines to upregulate eIF4F has not been previously observed in naturally occurring tumors. Similarly, while overexpression of the eIF4Gs is not widely reported in human tumors, in fact 30% of squamous lung carcinomas amplify the gene encoding eIF4GI (Brass et al., 1997). Noteworthy is the fact that, like breast carcinomas, lung squamous carcinoma cells do not overexpress eIF4E, whereas bronchoalveolar carcinomas overexpress eIF4E but not eIF4GI (Rosenwald et al., 2001). Thus, cancer cells utilize a large repertoire of mechanisms to promote the association of eIF4E with eIF4G.

Our findings support the idea of developing therapeutics that correct aberrant activity of eIF4F. One advantage of focusing attention on eIF4F as a novel target for anticancer therapy is its pleotropism, residing near the apex of many well-defined oncogenic growth and survival pathways. A second clear advantage is the potential for achieving a wide therapeutic margin. Since overexpressed 4E-BP1 does not preclude physiological function of normal cells (Polunovsky et al., 2000), therapeutic correction of aberrant cap-dependent translation has the potential to be well tolerated in patients with cancer. Finally, the availability of detailed crystal structural data (Marcotrigiano et al., 1997) may facilitate the development of small molecules that can compete with capped mRNAs for binding to eIF4E and therefore be used to titrate cap- dependent translation in cancer cells toward the normal (i.e., lethal) range.

Experimental procedures

Cell lines and constructs

HMEC strains 184 and 184-A1 were kindly provided by Martha Stempfer (Lawrence Berkeley National Laboratory). The 184 cell strain was derived from normal mammary tissue obtained at reduction mammoplasty (Stempfer, 1985). Cells of this strain have a finite growth potential in culture. 184-A1 cells were obtained as immortalized derivatives of 184 cells after treatment with benzo(a)pyrene (Stempfer, 1985). Both strains of HMECs were grown in chemically defined mammary epithelium basal medium (MEBM, Cambrex) supplemented growth factors. Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC) and maintained in regular growth medium (Dulbecco's modified Eagle's high glucose [4.5 g/l] medium [DMEM, Sigma] supplemented with 10% fetal calf serum [FCS, Sigma], 100 units/ml penicillin, 100 units/ml streptomycin, and 125 ng/ml amphotericin [GIBCO]).

Single and double 4E-BP1 phosphorylation mutants were described previously (Gingras et al., 1999a; Polunovsky et al., 2000). To obtain retroviral constructs expressing tagged translational regulators linked to the GFP reporter, both wild-type and mutant 4E-BP1 as well as human eIF4E sequences (Polunovsky et al., 1996; Tan et al., 2000) containing three amino-terminal hemagglutinin (HA) tags (Polunovsky et al., 2000) were excised from pACTAG-2-3HA using *Hind III* and *Apa I*. Resulting fragments were blunted using T4 DNA polymerase, and the gel-purified sequences were subcloned into the retroviral expression vector MSCV-M1GR1 (Clontech), which encodes GFP to identify successfully transduced cells. The resulting vectors were sequenced in their entirety, and the coding regions used in these experiments were found to be free of undesired mutations.

Cell transfection and retroviral transduction

For stable transfection, 5×10^5 184-A1 cells expressing ectopic eIF4E were seeded in 35 mm wells of 6-well clusters. After 24 hr, cells were transfected with 1 μg of the pACTAG-2 plasmid encoding the neomycin resistance

gene cassette and 3HA-tagged 4E-BP1 (either pACTAG *neo*/HA-4E-BP1^{wt} or pACTAG *neo*/HA-4E-BP1$^{\Delta}$) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated in the presence of Lipofectamine 2000 for 5 hr and switched to the appropriate growth medium containing 0.5 μg/ml G418 for 2 weeks prior to use.

For retroviral infection, cell monolayers (~50% confluent) were incubated in the presence of the retrovirus-containing supernatant and 8 μg/ml polybrene (Sigma) for 48 hr. Infection frequencies based on GFP expression were typically 25% to 30% for the HMEC 184-A1 strain, 95% to 98% for the MDA-MB-468 cell line, and 30% to 45% for the other carcinoma lines. Cell populations expressing different levels of the genes of interest were obtained by first sorting infected cells according to GFP intensity at 488 nm laser emission using a Becton Dickinson FACS DiVa (BD Biosciences) with a 530/28 optical filter, followed by immunoblot assessment of the sorted cell populations to assess expression of the gene of interest.

Immunoblot analysis

To analyze the components of eIF4F, cells were lysed by three freeze-thaw cycles, and equal amounts of cell extract protein per lane were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and identified using antibodies directed against eIF4E (mouse monoclonal antibody, 1:500, Transduction Laboratories), phospho-eIF4E (rabbit polyclonal antibody, 1:1000, Cell Signaling), and eIF4G1 (rabbit polyclonal antibody, 1:4000, developed in the laboratory of N.S.). Actin immunoblotting (mouse polyclonal antibody, 1:500, Sigma) was performed as a loading control.

To analyze endogenous and exogenous 4E-BP1, we followed established protocols (Pause et al., 1994; Gingras et al., 1999a) with minor modifications. Fifty micrograms of cell extract was boiled (100°C, 7 min) and non-heat-stable, precipitated cellular protein was removed by centrifugation (13,000 rpm for 10 min). This step precluded the use of the usual housekeeping proteins such as actin as loading controls. Resultant supernatant fluid (enriched for heat-stable 4E-BP1) was subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and identified using a rabbit polyclonal anti-4E-BP1 antibody (1:2500) developed in the laboratory of N.S. HA immunostaining was performed using a rat monoclonal antibody (1:2000, Roche).

In vitro cap-affinity assay

To assess the partitioning of eIF4E between its mutually exclusive binding partners—the repressor 4E-BPs and the initiation factor eIF4GI—we employed an in vitro cap-affinity assay performed as previously described (Polunovsky et al., 2000; Li et al., 2002).

Quantification of global cap-dependent translation

To quantify the global level of cap-dependent translation, we utilized a dicistronic reporter system (pcDNA-rLuc-polIRES-fLuc) as described previously (Li et al., 2002).

Anchorage-dependent colony formation

Cells were seeded into 6-well clusters at a density of 500 cells/well. Cultures were continued for 2 weeks, fixed with 4% formaldehyde, and stained with Coomassie Blue, and colonies with >50 cells were scored.

Anchorage-independent growth

Cells were seeded into soft agar at a density of 2×10^4 cells/well in 6-well clusters with a bottom layer of 1% SeaPlaque agarose (BioWhittaker Molecular Application, Rockland, ME) and a top layer of 0.6% SeaPlaque agarose in MEBM supplement with growth factors and antibiotics. Growth patterns were analyzed by microscopic observation after 2 weeks.

Quantifying DNA synthesis

10^4 cells/well were cultured (37°C, 10% CO_2, 16 hr) in 96-well clusters in complete medium containing 110 μM 5′-bromo-2′-deoxy-uridine (BrdU). Incorporation of BrdU into newly synthesized DNA was measured using a BrdU Labeling and Detection Kit III (Roche).

Flow cytometry

For quantification of DNA content, cultured cells were detached with trypsin, washed with PBS, fixed with ice-cold 70% ethanol, and resuspended in propidium iodide (PI) staining mixture as previously described (Polunovsky et al., 1996, 2000; Li et al., 2002). The percentage of cells with sub G1 and S DNA content was determined on a FACSCalibur flow cytometer (Becton Dickinson) with the CellQuest program.

Tumor formation, histology, immunohistochemistry, and TUNEL assays

5×10^6 MDA-468 cells were suspended in 100 μl of PBS and injected into the mammary fat pads of 4- to 5-week-old athymic female mice (BALB/CanNCr-nu). At the time of injection, aliquots of cells were set aside for immunohistochemistry and in vitro viability assays. Tumor growth was monitored by daily measurement. The time of initial tumor formation (the latency period) was defined as the interval after cell inoculation when tumors become visible in at least 50% of animals. Mice were sacrificed after 21 days of monitoring. Tumors were fixed in 4% paraformaldehyde at room temperature, and tumor specimens were sent to the University of Minnesota Histopathology Laboratory Research Service for routine histological analysis (H/E), TUNEL staining, and Ki-67 immunohistohemistry. To identify the HA tag by immunohistochemical staining, 4 μm sections were deparaffinized, boiled with antigen unmasking solution for 10 min, incubated with anti-HA antibody prelabeled with Alexa 488 fluorophore (Molecular Probes, 1:50) for 2 hr, and counterstained for 20 min with DAPI (Sigma).

Statistics

Statistical analyses were performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (S-PLUS Guide to Statistical and Mathematical Analysis, Version 4.0, Seattle, WA). A p value < 0.05 was considered significant. Results of flow cytometry were tabulated as the mean ± SD of 3–4 separate experiments. In each experiment, all conditions were examined in duplicate or triplicate. For analysis of tumorigenicity, mitotic and apoptotic indices are reported as the average number of events per 500× microscopic field (quantified in 10 fields), and tumor weights (MDA-MB-468/vector versus MDA-MB-468/4E-BP1^{wt} and MDA-MB-468/4E-BP1$^{A37/A46}$) were compared using a paired t test.

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