Defective translational control facilitates vesicular stomatitis virus oncolysis

Siddharth Balachandran[1] and Glen N. Barber[1,*]

[1]Department of Microbiology and Immunology and Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, Miami, Florida 33136
*Correspondence: gbarber@med.miami.edu

Summary

Vesicular stomatitis virus (VSV) exerts potent antitumor activity, although the molecular mechanisms underlying its oncolytic properties remain to be fully clarified. Here, we demonstrate that normally resistant murine embryonic fibroblasts are rendered highly permissive to VSV replication following cellular transformation, a progression that appears to compromise the antiviral effects of interferon (IFN). Subsequent studies revealed normal dsRNA-dependent protein kinase (PKR) activation and phosphorylation of eukaryotic initiation factor 2 (eIF2) α. Nevertheless, eIF2B-mediated guanine nucleotide exchange activity downstream of eIF2 was frequently aberrant in transformed cells, neutralizing eIF2α phosphorylation and permitting VSV mRNA translation. Thus, defects in translational regulation can cooperate with impaired IFN signaling to facilitate VSV replication, and may represent a common hallmark of tumorigenesis.

Introduction

VSV, the prototypic member of the family *Rhabdoviridae*, is an enveloped virus with a negative-stranded RNA genome that encodes five primary gene products. It was recently discovered that VSV possesses potent oncolytic activity in tissue culture cells and in vivo. We and others have since shown that VSV oncolysis proceeds via the activation of multiple caspase-dependent apoptotic cascades, and is effective against tumors exhibiting genetic lesions in several known oncogenic and tumor-suppressor pathways (Balachandran and Barber, 2000; Balachandran et al., 2001; Fernandez et al., 2002; Stojdl et al., 2000).

Our previous investigations have shown that both PKR and IFN act in concert to inhibit VSV replication in normal cells. PKR is activated by dsRNAs produced during viral replication, and acts early following VSV infection to inhibit translation of viral transcripts (Balachandran et al., 2000). PKR exerts its translation inhibitory effects by phosphorylating the α subunit of eIF2 on serine 51 (Clemens and Elia, 1997). eIF2, comprising three subunits, α, β, and γ, functions with methionyl-tRNA$_i$ and GTP to form the 43S preinitiation complex with the 40S ribosomal subunit. Following binding of mRNA and the 60S subunit to this complex, hydrolysis of eIF2.GTP occurs and eIF2.GDP is released. To participate in a subsequent round of translation initiation, eIF2.GDP must be converted to eIF2.GTP, a process carried out by the heteropentameric guanine nucleotide exchange factor (GEF) eIF2B. eIF2 with a serine 51-phosphorylated α subunit (αser51P) competitively inhibits eIF2B-mediated recycling of GDP for GTP on eIF2. Since eIF2 is generally found in excess of eIF2B, and as eIF2 (αser51P).GDP has an increased affinity for eIF2B, recycling of eIF2 and consequent rounds of translation initiation have been found to be inhibited by phosphorylation of only a small fraction of eIF2α (Clemens and Elia, 1997; Hershey, 1991; Kimball, 1999).

In addition to activating PKR, dsRNAs produced during viral replication are also potent inducers of Type I IFN synthesis (Barber, 2001; Stark et al., 1998). Type I (α/β) IFNs are a family of pleiotropic cytokines with powerful antiviral and immunomodulatory activity and are produced by most cells in response to viral infection (Stark et al., 1998). Following their production, type I IFNs act in an autocrine manner to induce the expression of several hundred genes that are required to fortify the antiviral state, as PKR by itself is not sufficient to thwart VSV infection in the absence of IFN signaling (Balachandran et al., 2000). Thus, PKR appears to primarily delay VSV mRNA translation long enough for IFN to be synthesized and establish a complete antiviral state.

Here, we confirm that the antiviral effects of IFN are compromised upon malignant transformation. We further demonstrate that translation control downstream of PKR activation and eIF2α phosphorylation is frequently disregulated in many transformed

SIGNIFICANCE

We have developed a fibroblast model system to dissect the molecular determinants of susceptibility to VSV, an oncolytic virus. We demonstrate that the antiviral effects of IFN, essential for inhibiting VSV replication, are commonly compromised in transformed cells, and may be a general feature of cellular transformation. However, defects in the IFN antiviral response could not fully explain the oncolytic activity of VSV. Subsequent analysis showed that defective control of mRNA translation initiation may play a role in permissiveness to VSV, and represent another feature of transformation. Aside from identifying key cellular pathways involved in facilitating viral oncolysis, our experimental data lay the framework for the intelligent design of future generations of genetically engineered oncolytic viruses with increased specificity and potency.

cells, can cooperate with attenuated IFN antiviral activity to facilitate VSV oncolysis, and may be a common feature of tumorigenesis.

Results

Transformed PKR$^{+/+}$ MEFs are permissive to VSV

We have previously shown that primary (129terSv × Balb/c) PKR$^{+/+}$ MEFs are essentially nonpermissive to VSV (Balachandran et al., 2000). In contrast, PKR$^{+/+}$ MEFs treated with neutralizing antiserum to IFN, or MEFs from PKR$^{-/-}$ mice, are very susceptible to VSV replication. Thus, MEFs of the 129terSv × Balb/c background are resistant to VSV unless either PKR activity or IFN signaling is ablated (Figures 1A, 1B, and 1E). We used these MEFs as a model system to study VSV oncolysis, since pooled populations of malignantly transformed isogenic cells could be readily obtained from these parental fibroblasts by serial passaging. Initially, we passaged primary MEFs following a 3T3 protocol and obtained several populations of immortalized cells that retained contact inhibition. Upon continuous passaging, however, such immortalized cells eventually gave rise to populations of rapidly growing highly refractile cells that displayed all the hallmarks of transformation, including greatly increased doubling times, lack of contact inhibition, ability to form colonies in soft agar, and potential to form tumors in nude mice (data not shown). Importantly, while the parental PKR$^{+/+}$ primary MEFs were resistant to VSV, their transformed counterparts became remarkably permissive to VSV replication and cytolysis (Figures 1C–1E). Similar results were obtained when comparing human fibroblasts transformed with genetically defined oncogenic elements (i.e., H-rasV12) with their normal parental counterparts (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/5/1/51/DC1 and Hahn et al., 1999).

Defective IFN-mediated antiviral activity in transformed cells

To examine the IFN system in these cells, we treated primary and transformed MEFs with synthetic dsRNA (poly [I:C]), or infected them with VSV, and measured the production of IFN over a period of 24 hr by standard inhibition of CPE assay. Both primary and transformed MEFs produced comparable amounts of IFN in response to poly (I:C) and after VSV infection (Figures 2A and 2B).

To examine whether IFN-triggered signal transduction was impaired in the transformed MEFs, reporter assays using constructs expressing firefly luciferase under the control of either a type I IFN stimulated response element (ISRE) or the type II IFN-responsive γ activated sequence (GAS) were performed next. Whereas primary MEFs responded to both mIFN-α/β and mIFN-γ, transformed MEFs were relatively unresponsive to both (Figures 2C and 2D). A lack of robust IFN-induced gene regulation in transformed MEFs was confirmed by immunoblotting for mIFN-α/β and mIFN-γ-inducible gene products STAT-1 and IRF-1 (Figure 2F). Accordingly, while the IFNs were very effective in inhibiting VSV production from primary cells, these cytokines were capable of only partial protection of the majority of transformed cells (Figure 2G). In general, we and others have found that IFN pretreatment reduces viral yield from numerous primary cells to virtually undetectable levels, whereas the same dose of IFN on transformed cells still results in between 1×10^3–1×10^8 plaque forming units (pfu)/ml progeny VSV virion production, depending on the cell line (Balachandran and Barber, 2000; Stojdl et al., 2000). It is therefore likely that defective IFN antiviral activity may be a common feature of transformed cells.

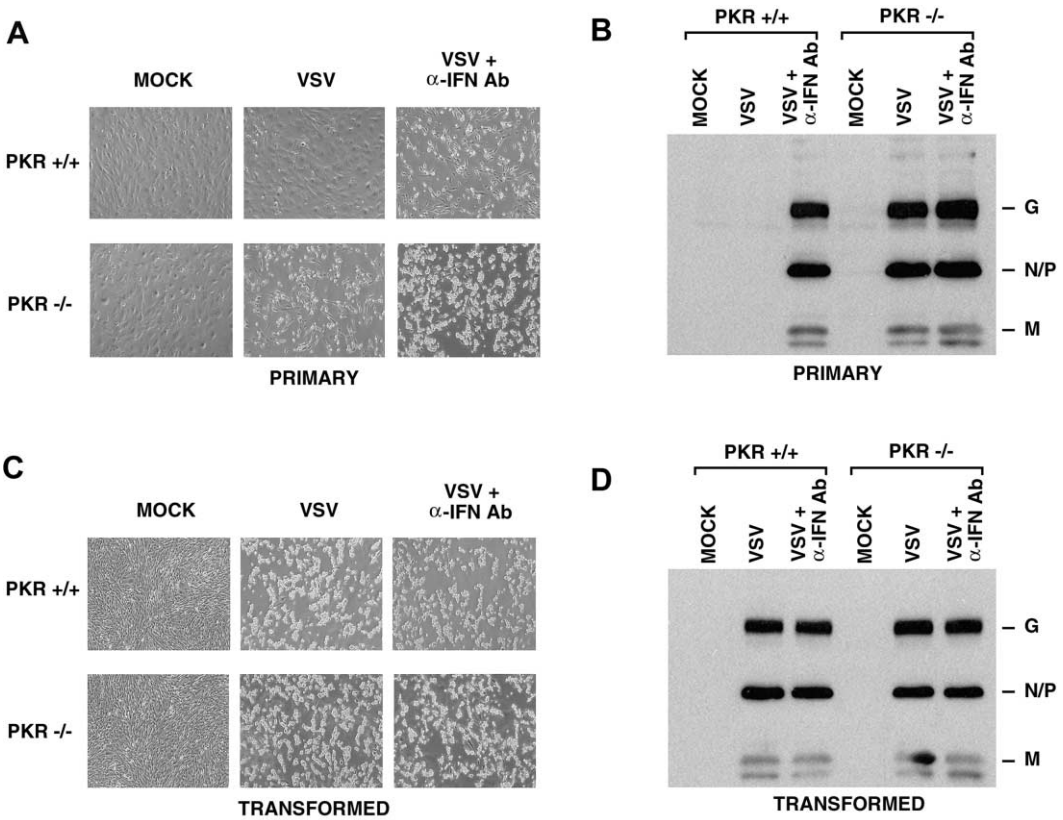
Kinetic analysis of VSV replication in primary and transformed cells

If defects in IFN action were solely responsible for the susceptibility of transformed MEFs to VSV, then it would be expected that ablation of IFN signaling in normal fibroblasts would result in these cells becoming susceptible to VSV infection, with kinetics and magnitude of replication comparable to those seen in transformed MEFs (as seen in Figure 1). We therefore infected primary or transformed PKR$^{+/+}$ MEFs with VSV in the presence or absence of potent anti-mIFN-α/β neutralizing antiserum, to eliminate autocrine IFN signaling, and measured viral replication by immunoblotting for viral proteins at various times p.i. However, while neutralization of IFN did compromise the host cell defense of primary MEFs, the kinetics of VSV replication in these cells were still significantly delayed when compared to transformed cells (Figures 3A and 3B). To recapitulate this finding in a different setting, we obtained immortalized STAT1$^{-/-}$ MEFs and transformed them by retroviral transduction of activated Ras (Ras^{G12V}). Viral replication in transformed STAT1$^{-/-}$ MEFs was then compared to their primary counterparts. Again we found that, despite a complete absence of IFN antiviral signaling in primary STAT1$^{-/-}$ cells, VSV replication still proceeded with slower kinetics in these cells compared to transformed STAT1$^{-/-}$ cells (Figure 3C). Next, we compared VSV replication in PKR$^{-/-}$ primary and transformed MEFs, and found no significant VSV replication in primary PKR-deficient fibroblasts until 24–36 hr p.i., whereas the transformed cells derived from them supported rapid and robust VSV replication within 2–4 hr p.i. (Figure 3D). Similar results were obtained when a panel of primary and transformed human cell types were compared (Figure 3E). Thus, while defective IFN action indeed contributes to VSV oncolysis, other factor(s) cooperate to facilitate robust VSV replication in transformed cells.

PKR activity and eIF2α phosphorylation in transformed MEFs

We sought to examine whether aberrancies in PKR signaling occurred in transformed MEFs predisposed to VSV oncolysis. In this light, enhanced Ras signaling via activation of the ERK pathway in transformed cells has been shown to facilitate viral oncolysis by abrogating PKR activity (Farassati et al., 2001; Strong et al., 1998). To examine whether Ras and ERK signaling play a role in cellular susceptibility to VSV, a panel of malignantly transformed human and rodent cell lines were treated with pharmacological inhibitors of Ras and ERK signaling. No significant decrease in VSV production was observed when these signaling cascades were inhibited (Supplemental Figure S3 at http://www.cancercell.org/cgi/content/full/5/1/51/DC1).

We next directly examined PKR activity and report no significant differences between primary and transformed cells in activation of PKR by either synthetic dsRNA (poly [I:C]) or VSV (Figure 4A). eIF2α phosphorylation on serine 51 in response to both poly (I:C) and VSV was also indistinguishable between cells resistant and susceptible to VSV (Figure 4A). Thus, the permissiveness of transformed MEFs to VSV does not appear to require either enhanced Ras and ERK signaling, or defective PKR activity and consequent eIF2α phosphorylation. Given



E

Genotype	Untreated	α-mIFN Ab
PKR +/+ Primary	1.1×10^4	3.8×10^6
PKR -/- Primary	8.1×10^7	9.8×10^8
PKR +/+ Transf.	8.6×10^8	1.2×10^9
PKR -/- Transf.	2.1×10^9	2.8×10^9

Figure 1. Primary MEFs are protected from VSV by PKR and autocrine IFN, but become susceptible upon transformation

A: Primary MEFs were infected with VSV (m.o.i. = 1) in the absence or presence of neutralizing anti-mIFN-α/β antiserum for 36 hr and photographed at 200× magnification.

B: Lysates prepared from cells treated as in **A** were analyzed for VSV replication by immunoblotting.

C: Transformed MEFs were infected with VSV (m.o.i. = 1) in the absence or presence of neutralizing anti-mIFN-α/β antiserum for 36 hr and photographed at 200× magnification.

D: Lysates prepared from cells treated as in **C** were analyzed for VSV replication by immunoblotting. Molecular masses (in kilodaltons [kDa]) are shown to the left of immunoblot exposures. Identities of individual VSV proteins are shown to the right.

E: Viral progeny yield from cells infected with VSV (m.o.i. = 10) in the absence or presence of anti-mIFN-α/β neutralizing antiserum was determined 48 hr p.i. Numbers represent mean PFU/ml virus of duplicate samples from two independent experiments.

these data, it was therefore plausible that elements downstream of PKR/eIF2α signaling might be defective in these cells.

Defective translation control downstream of eIF2α phosphorylation in transformed MEFs

The inhibition of translation by phosphorylated eIF2α is a major stress-responsive checkpoint employed by at least three kinases, in addition to PKR (Hinnebusch, 1994). Amino acid deprivation and malfolded proteins in the ER activate two of these kinases, general control nonderepressible (GCN)2 and PKR-like ER resident kinase (PERK), respectively (Hinnebusch, 1994; Ron, 2002). To determine whether amino acid starvation and ER stress resulted in eIF2α phosphorylation by their cognate kinases, primary and transformed MEFs were treated with the PERK activator tunicamycin or deprived of amino acids and assayed for eIF2α phosphorylation by immunoblotting. How-

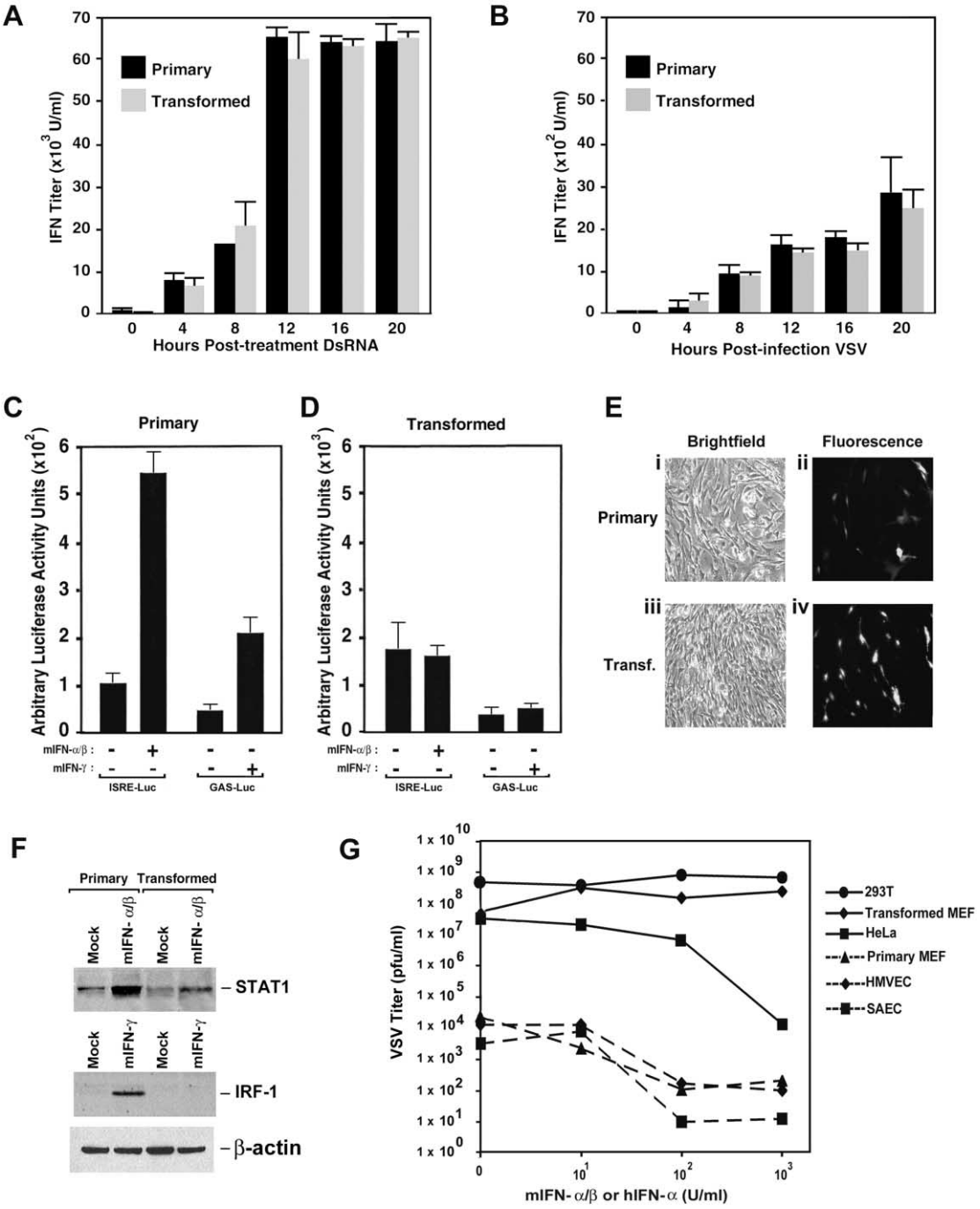


Figure 2. IFN signaling, but not production, is defective in transformed MEFs

A: Primary (black bars) or transformed PKR$^{+/+}$ MEFs (gray bars) were transfected with poly (I:C) (2 μg/ml) and IFN production was determined at the indicated times.

B: Primary (black bars) or transformed PKR$^{+/+}$ MEFs (gray bars) were infected with VSV (m.o.i. = 50) and IFN production was determined as in **A**. Error bars (in this and other experiments) represent standard error around the mean of triplicate samples from a representative experiment.

C: Primary PKR$^{+/+}$ cells were transfected with expression vectors encoding either ISRE-Luc and GAS-Luc, treated with IFN-α/β or IFN-γ respectively, and luciferase activity measured 18 hr posttreatment.

D: Transformed PKR$^{+/+}$ MEFs were treated as in **C**.

E: A plasmid encoding GFP was transfected into both primary (i and ii) and transformed (ii and iv) MEFs to show comparable transfection efficiencies of these cells.

F: MEFs were treated with either mIFN-α/β or mIFN-γ for 24 hr, after which lysates were examined for STAT1 and IRF-1 protein levels.

G: Transformed (solid lines) and primary (dashed lines) cell types were treated with the indicated amounts of either mIFN-α/β or human IFN-α (Hela, 293T, SAECs [small airway epithelial cells], HMVECs [human microvascular endothelial cells]) after which they were infected with VSV (m.o.i. = 10). 24 hr p.i., supernatants were collected and viral titers determined.

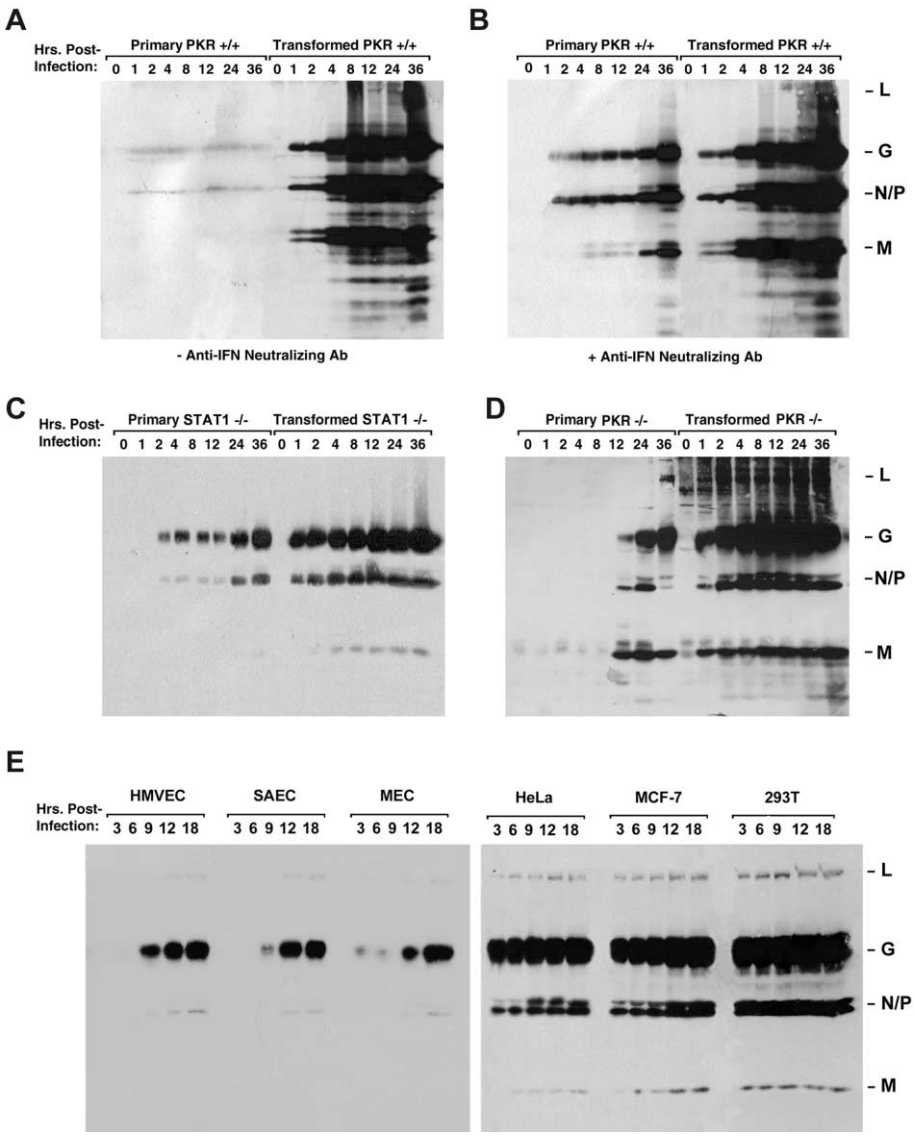


Figure 3. Kinetic analysis of VSV replication in primary and transformed MEFs

A: $PKR^{+/+}$ MEFs were infected with VSV (m.o.i. = 10). At the indicated times p.i., cells were lysed and VSV replication analyzed by immunoblotting.

B: $PKR^{+/+}$ MEFs were infected with VSV in the presence of neutralizing anti-IFN antiserum and analyzed as above for kinetics of VSV replication.

C: $STAT1^{-/-}$ MEFs were infected with VSV, and at the indicated times p.i., cells were lysed and VSV replication analyzed by immunoblotting.

D: $PKR^{-/-}$ cells were also analyzed as above for VSV replication by immunoblotting.

E: Three primary (HMVECs, SAECs, and mammary epithelial cells [MECs]) and three transformed (HeLa, MCF-7 and 293T) human cell types were infected with VSV and examined as above for VSV replication.

ever, neither PERK- nor GCN2-mediated phosphorylation of eIF2α was found to be significantly impaired in transformed cells (Figure 4B), indicating that stress-responsive eIF2α phosphorylation per se does not appear defective in transformed MEFs susceptible to VSV.

Next, we tested whether stress-specific phosphorylation of eIF2α in primary and transformed MEFs resulted in the inhibition of translation in these cells. A reporter plasmid encoding firefly luciferase under the control of a cytomegalovirus immediate early gene (CMV) promoter was therefore transfected into primary and transformed MEFs, prior to treatment with either poly (I:C), tunicamycin, or deprivation of amino acids. Changes in luciferase activity, reflective of the levels of luciferase protein, were used as an indicator of protein synthesis rates. After treatment with the eIF2α kinase activators, luciferase activity dropped to almost undetectable levels in primary MEFs. In contrast, despite identical kinetics and magnitude of eIF2α phosphorylation in primary and transformed cells (Figure 4B), virtually no decrease in luciferase activity was seen following treatment of transformed MEFs with any of the eIF2α kinase activators (Figure 4C). To confirm this finding, we measured protein synthesis rates by radiolabeled amino acid incorporation and obtained similar results, suggesting that translational control at the eIF2α checkpoint may be defective in transformed MEFs (Figure 4D). We surmised that this might be a result of aberrant eIF2B-mediated guanine nucleotide exchange on eIF2, since this is the primary reaction that is subject to control by eIF2α phosphorylation. If eIF2B were to somehow maintain elevated rates of guanine nucleotide exchange in transformed MEFs despite robust eIF2α phosphorylation by stress-specific kinases, it would remain possible that these cells could sustain normal rates of mRNA translation despite cellular stress. To test this hypothesis, we treated primary and transformed MEFs with various eIF2α kinase activators and compared the rates at which guanine nucleotide dissociation from eIF2 occurred. Indeed, we discovered that transformed MEFs have a greatly increased ability over primary MEFs to displace bound GDP from eIF2 (compare Figure 4E to 4F). Importantly, none of the eIF2α kinase

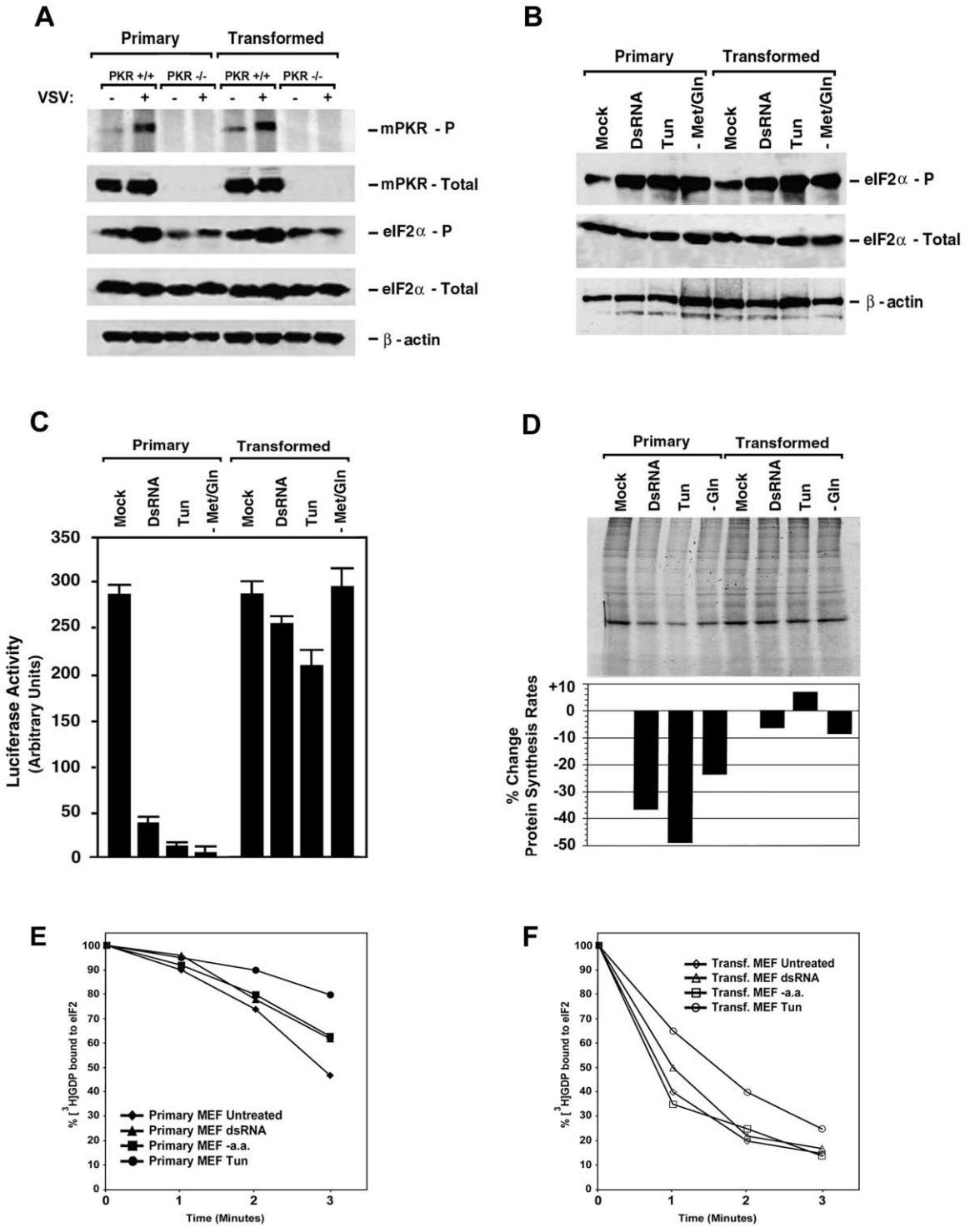


Figure 4. Defective translational control and disregulated eIF2B guanine nucleotide exchange activity downstream of PKR activation and eIF2α phosphorylation in transformed MEFs.

A: MEFs were either mock infected or infected with high m.o.i.s (200) of VSV and examined for PKR and eIF2α phosphorylation by in vivo kinase assay and immunoblotting using a eIF2α phosphoserine 51-specific antibody, respectively.

B: MEFs were treated with dsRNA (poly [I:C]) or tunicamycin, or deprived of amino acids for 3 hr, and examined for eIF2α phosphorylation by immunoblotting.

C: MEFs were transfected with a luciferase reporter construct, and treated with poly (I:C) or tunicamycin, or deprived of amino acids for 18 hr, after which luciferase activity was determined.

D: Cells treated as in **B**, except that only glutamine deprivation was used to activate GCN2, were pulsed for 30 min with [^{35}S]-methionine three hours posttreatment. Lysates from these cells were subjected to SDS-PAGE/autoradiography (top panel). The autoradiograph was densitometrically scanned, and the percent change in protein synthesis rates presented quantitatively (bottom panel).

activators used caused eIF2 nucleotide exchange rates in transformed cells to reduce to even the basal rates seen in primary MEFs cells (Figures 4E and 4F). Similarly, we found that human fibroblasts transformed with defined oncogenic elements displayed greater basal and post-eIF2α kinase-activated eIF2B GEF rates compared to their parental immortalized cells, despite normal eIF2α phosphorylation (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/5/1/51/DC1).

eIF2Bε can modulate translation and serve as a determinant of susceptibility to VSV

Since the eIF2B is present in rate-limiting amounts in most cells sensitive to eIF2α phosphorylation, an alteration in the levels and/or activity of this complex could putatively override the translational block imposed by phosphorylation of eIF2α (Kimball, 1999). We therefore sought to examine the levels of the five subunits of eIF2B by immunoblotting. While the levels of four of these subunits were approximately equivalent among pools of primary and transformed MEFs examined, there was a dramatic (>10-fold) increase in the levels of ε subunit in all transformed MEF populations examined, compared to their genotype-matched primary cell counterparts (Figure 5A).

As eIF2Bε is the catalytic subunit of eIF2B and has been shown to possess eIF2 guanine nucleotide exchange activity (with GDP exchange rates ~5%–10% that of the holo-complex [Fabian et al., 1997; Pavitt et al., 1998; Williams et al., 2001]), we hypothesized that an increase in the levels of this subunit could enhance translation rates, and possibly neutralize the consequences of eIF2α phosphorylation by increasing the rate of nucleotide exchange on eIF2 (Fabian et al., 1997). To study the effects of this subunit on translation, we subcloned the full-length rat eIF2Bε cDNA into a mammalian expression vector (Flowers et al., 1996). A variant of this subunit (called eIF2BεΔC) was generated by deleting its carboxy-terminal 188 amino acids, shown to be important for enzymatic activity and binding to eIF2 (Gomez et al., 2002; Figure 5B). In cotransfection experiments using these constructs in combination with an expression vector encoding firefly luciferase, we determined that eIF2Bε was able to moderately stimulate translation (~2-fold), while the eIF2BεΔC mutant suppressed translation by ~50% (Figure 5C). These results, taken together with a previous report showing that overexpression of *Drosophila* eIF2Bε in human 293T cells increases GEF activity on eIF2, would suggest that this subunit, by itself, can affect translation in mammalian cells (Figure 5C; Williams et al., 2001).

Full-length eIF2Bε, as well as eIF2BεΔC, was then retrovirally transduced into NIH3T3 cells to generate cells stably expressing these polypeptides. Immunoblot analysis revealed that cells obtained after retroviral transduction of eIF2Bε or eIF2BεΔC had similar levels of PKR, eIF2α, and the other eIF2B subunits, compared to cells transduced by control retrovirus, while levels of ectopically overexpressed eIF2Bε were similar to those seen in transformed MEFs (Figure 5D). These cells were next examined for susceptibility to VSV replication following infection with recombinant VSV expressing GFP (VSV-GFP). Despite the fact that immortalized NIH3T3 cells were already moderately permissive to VSV, we noticed that infected NIH3T3-eIF2Bε cells exhibited significantly greater CPE and GFP-fluorescence than control cells (Figure 5E). In contrast, infected NIH3T3-eIF2BεΔC cells manifested somewhat reduced CPE, and significantly decreased GFP fluorescence. In agreement with this, NIH3T3 cells overexpressing eIF2Bε produced approximately 8-fold more virus, while cells expressing the εΔC mutant reproducibly produced about 4- to 5-fold lower titers of progeny VSV than control cells 16 hr p.i. at an m.o.i. of 1 (Figure 5F).

Elevated levels of eIF2Bε are required for increased permissiveness of transformed MEFs and HeLa cells to VSV replication

We next employed RNA interference (RNAi) technology to examine whether the elevated levels of eIF2Bε found in transformed MEFs were required for their increased permissiveness to VSV. After three days of treatment with short interfering dsRNA duplexes (siRNA) directed toward eIF2Bε mRNA, we were able to detect an ~75% specific decrease in the levels of eIF2Bε protein (Figure 6A). We observed that cells transfected with siRNA to eIF2Bε, but not control cells, were almost completely protected against VSV-GFP-induced cytolysis 16 hr p.i., and produced approximately 10-fold less virus than control cells (Figures 6B–6D). To verify this finding, we used HeLa cells in which an siRNA specific for human eaIF2ε resulted in a reduction of endogenous pools of eIF2Bε protein to almost undetectable levels (Figure 6E). Progressively greater numbers (~20%–30%) of HeLa cells treated in this fashion underwent apoptotic cell death 4–5 days posttransfection, and most were dead by seven days posttransfection. (Figure 6F, data not shown). Remarkably, while HeLa cells treated with the transfection reagent or control siRNA alone manifested extensive CPE and widespread GFP expression upon infection with VSV-GFP, those treated with siRNA to eIF2Bε exhibited virtually no evidence of virus replication-induced CPE, even 36 hr posttransfection (Figures 6G and 6F). eIF2Bε siRNA-treated cells also showed an ~100-fold decrease in viral yield, compared to controls (Figure 6H).

Replicative abilities of recombinant VSV expressing eIF2Bε or eIF2εΔC

To further investigate the effects of eIF2Bε overexpression on VSV replication, we generated novel recombinant VSV expressing either full-length rat eIF2Bε, or the eIF2BεΔC variant (VSV-ε and VSV-εΔC, respectively) using described methods (Figure 7A; Lawson, 1995; Fernandez, 2002). Viruses produced in this fashion were plaque-purified, propagated on transformed BHK cells, and examined for expression of both recombinant and viral proteins by immunoblotting (Figure 7B). One-step growth curve analysis revealed that VSV-ε replicated to higher titers (~2 log) four hours p.i., compared to VSV-GFP, but then exhibited kinetics of replication virtually indistinguishable from VSV-GFP at later time points. VSV-εΔC, in contrast, grew with somewhat

E: Primary MEFs were treated with poly (I:C) tunicamycin or were grown in medium devoid of glutamine (−aa), and eIF2B guanine nucleotide exchange activity in these cells was determined 3 hr post treatment.

F: Transformed MEFs were treated and processed as in **E**. The amount of [^{3}H]GDP bound to eIF2 at the start of the reaction (time = 0 min) was arbitrarily set to 100%. Data shown are from one of three experiments with similar results.

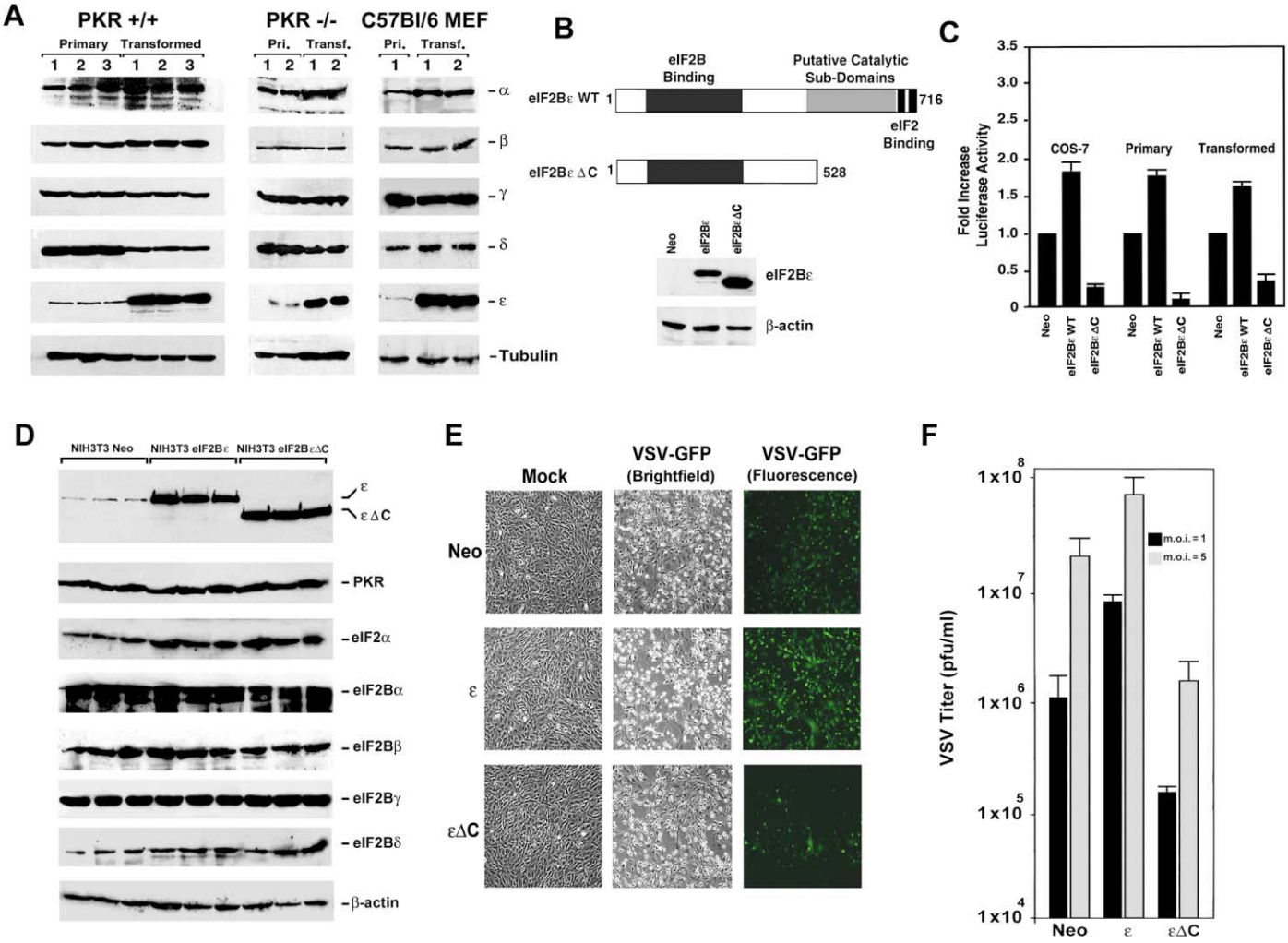


Figure 5. eIF2Bϵ is overexpressed in transformed MEFs and serves as a determinant of permissiveness to VSV

A: Lysates from pools of primary or transformed PKR$^{+/+}$, PKR$^{-/-}$, or C57Bl/6 fibroblasts were examined by immunoblotting for all eIF2B subunit levels. Numbers above lanes indicate independently derived pooled cell populations.

B: Schematic of eIF2Bϵ. The 716 amino acid rat eIF2Bϵ polypeptide has putative catalytic subdomains toward the carboxy end (light gray shading) and eIF2B-contact regions toward the amino terminus (dark gray shading). Two regions with a high concentration of aromatic and acidic residues, shown to be critical for eIF2 binding and found in the extreme carboxy terminus of the polypeptide, are also shown ([Asano et al., 1999], black shading). eIF2BϵΔC, lacking 188 C-terminal amino acids of the full-length protein, is also depicted. Expression was confirmed by immunoblotting (bottom panels).

C: These constructs were cotransfected with a luciferase reporter construct into the indicated cell types and luciferase activity was measured 18 hr later.

D: NIH3T3 cells expressing empty vector only (NIH3T3-Neo), eIF2Bϵ (NIH3T3-eIF2Bϵ), or eIF2BϵΔC (NIH3T3-eIF2BϵΔC) were created, and expression of all eIF2B subunits, PKR, and eIF2α were examined by immunoblotting.

E: Populations of these cells were infected with VSV-GFP (m.o.i. = 1), and photomicrographs (magnification 200×) taken 16 hr p.i.

F: Pools of control, eIF2Bϵ, or eIF2BϵΔC-expressing NIH3T3 cell populations were infected with VSV-GFP and viral progeny yield determined 16 hr p.i.

slower kinetics than VSV-GFP at later time points, and about 4-fold less ϵΔC-expressing virus was recovered from the culture supernatant 10 hr p.i. (Figure 7C).

Next, we examined whether viruses expressing eIF2Bϵ or eIF2BϵΔC were altered in their ability to replicate in normal cells. Indeed, we found that VSV-ϵ yielded about 5- to 6-fold more virus, compared to control VSV-GFP, from primary PKR$^{+/+}$ MEFs. In contrast, we were unable to detect any progeny virus yield from VSV-ϵΔC-infected primary MEFs (Figure 7Di). Nevertheless, overall viral yields were low, which we attributed to the antiviral effects of autocrine IFN. We therefore performed the experiment described above in the presence of neutralizing antiserum to Type I IFN. This time, greater differences were observed in the replicative abilities of these viruses in primary PKR$^{+/+}$ MEFs. Infection with VSV-ϵ yielded 10- to 15-fold more virus than infection with VSV-GFP, while VSV-ϵΔC replicated poorly in normal cells, producing ~2- to 3-fold fewer progeny virions than control VSV-GFP (Figure 7Dii).

Impaired control of translation downstream of eIF2α phosphorylation in tumorigenic human cells

Since VSV can replicate in the majority of transformed human cell lines, it was plausible that translational control at the eIF2α checkpoint might be defective in such cells. We therefore exam-

ined the ability of eIF2α kinase activators to inhibit ongoing translation in a panel of normal and transformed human cell types. While poly (I:C) or tunicamycin were both able to effectively inhibit translation in all normal cells, these agents were much less capable of decreasing translation rates in any of the four transformed cell lines tested at the doses used (Figures 8A and 8B, top panels). Thus, whereas transformed cells are capable of inhibiting translation following these stresses, they clearly do so to a lesser degree than do primary cells. Immunoblotting for phosphorylated and total eIF2α revealed that phosphorylation of eIF2α is not detectably compromised in any of these cells, and, indeed, in most other transformed human cell lines tested (Figures 8A and 8B, bottom panels, data not shown). We next examined the ability of lysates from various normal and tumorigenic human cell types to catalyze the displacement of [^{3}H]GDP loaded onto eIF2. Again, in general, transformed human cell lines displayed significantly higher rates of GDP displacement from eIF2 compared to normal human cells (Figures 8C and 8D).

We extended these findings by examining whether disregulation of eIF2B activity occurs with any regularity in primary human tumors. For these studies, we compared a panel of primary patient-derived Burkitt's B cell lymphomas to resting and lipopolysaccharide (LPS) activated CD19$^+$ B cells purified from human blood. Our initial analysis confirmed that these lymphomas were significantly more susceptible to VSV than either resting or activated normal B cells (Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/5/1/51/DC1). Once more, we found that the Burkitt's lymphoma samples all exhibited significantly greater basal rates of eIF2B-mediated guanine nucleotide exchange, compared to normal B cells (Figures 8E and 8F). Furthermore, while these GEF rates dropped in response to tunicamycin-triggered eIF2α kinase activation, they still remained higher than those found in similarly treated normal B cells.

Finally, to examine whether disregulated expression of eIF2Bε occurs in human tumors, we analyzed the transcriptional expression of eIF2Bε in a variety of matched normal and tumor tissue. This preliminary analysis indicated that eIF2Bε mRNA was overexpressed in a significant number of human tumor samples (Figure 8E). In a few cases, particularly in breast- and kidney-derived tumors, the expression of eIF2Bε was also observed to decreased, compared to normal tissue, although the reason(s) for this are presently unclear. Nevertheless, these data, taken in combination with the elevated eIF2B GEF rates seen in many transformed cell lines, indicate that disregulation of this translational checkpoint may be a common occurrence in human cancers.

Discussion

The emergence of VSV as a novel and potent oncolytic agent has made an investigation into the host cell determinants of permissiveness to this virus an important objective. In this study, we made use of a model system of primary and transformed MEFs, as well as of several normal and tumorigenic rodent and human cell lines, to show that both defective IFN action and impaired translational control can contribute to render cells highly susceptible to VSV. We found that defects in the control of translation were due, at least in part, to disregulation of signaling downstream of PKR activation and eIF2α phosphorylation, and involved markedly elevated rates of eIF2B-catalyzed dissociation of GDP from eIF2 in transformed MEFs.

A number of viruses have been shown to possess oncolytic activity, and although the mechanisms underlying their oncolysis remain unclear, a role for Ras-dependent signaling has been shown to dictate the oncolytic abilities of influenza virus, reovirus, and herpes simplex virus (HSV)-1 (Bergmann et al., 2001; Farassati et al., 2001; Strong et al., 1998). In our analyses, however, we were unable to implicate the Ras pathway in dictating permissiveness to VSV, indicating that this virus may exploit Ras-independent defects in host cell defense.

Although activation of PKR and consequent eIF2α phosphorylation occurred in transformed cells with kinetics and magnitude indistinguishable from normal cells, we report that the accompanying decreases in rates of mRNA translation seen in primary cells are not mimicked by their transformed counterparts. Translation in mammalian cells is a highly coordinated process, the regulation of which can be subverted during neoplastic transformation (Sonenberg, 1993). For example, the mRNA cap binding factor eIF4E is upregulated in several primary tumors and established cell lines, while the elongation factor eEF1A has been recently reported to be oncogenic and mutated in 25% of ovarian tumors (Anand et al., 2002; Zimmer et al., 2000). Importantly, disruption of the PKR-eIF2α checkpoint has also been shown to transform NIH3T3 cells, highlighting the importance of translation initiation control in growth (Sonenberg, 1993). While several tumorigenic cells appear to have readily detectable levels of functional PKR, they are capable of sustaining high levels of protein synthesis (Savinova et al., 1999). Consequently, these cells support VSV replication despite PKR activation. Our discovery that rates of eIF2B-catalyzed GDP-GTP exchange on eIF2 are greatly enhanced in transformed MEFs, coupled with the facts that eIF2Bε levels are increased in these cells, and that this subunit can, by itself, act as a determinant of permissiveness to VSV, demonstrate that disregulation of eIF2B can at least partially override the PKR-eIF2α checkpoint in mammalian cells. In conjunction with previous results, these data add eIF2B to the growing list of translation components disregulated during tumorigenesis (Kim et al., 2000). In this regard, a transformed hamster cell line was recently reported to display significantly elevated eIF2B activity compared to its normal counterpart, despite comparable eIF2α phosphorylation (Vojtechova et al., 2003). It is noteworthy that impaired translation control downstream of eIF2α phosphorylation in cancer cells would not only neutralize the growth-suppressive effects of PKR, but those of PERK and GCN2, as well. Since the latter two kinases repress translation following ER stress and amino acid deprivation, respectively, freedom from the consequences of their activation might confer a growth advantage, favoring rapidly proliferating cells in suboptimal conditions, particularly if default apoptotic pathways triggered (at least by PERK inactivation) are also compromised (Harding et al., 2000).

eIF2B is structurally the most complex GEF known, and the best studied mechanism of regulation of this factor remains competitive inhibition of its activity by eIF2(αser51P). However, in response to insulin and other mitogenic agents, other mechanisms are postulated to regulate eIF2B independent of eIF2α phosphorylation (Proud, 2001). Recent studies aimed at shedding light on these alternative cascades have identified at least four different kinases that can phosphorylate eIF2Bε in vitro.

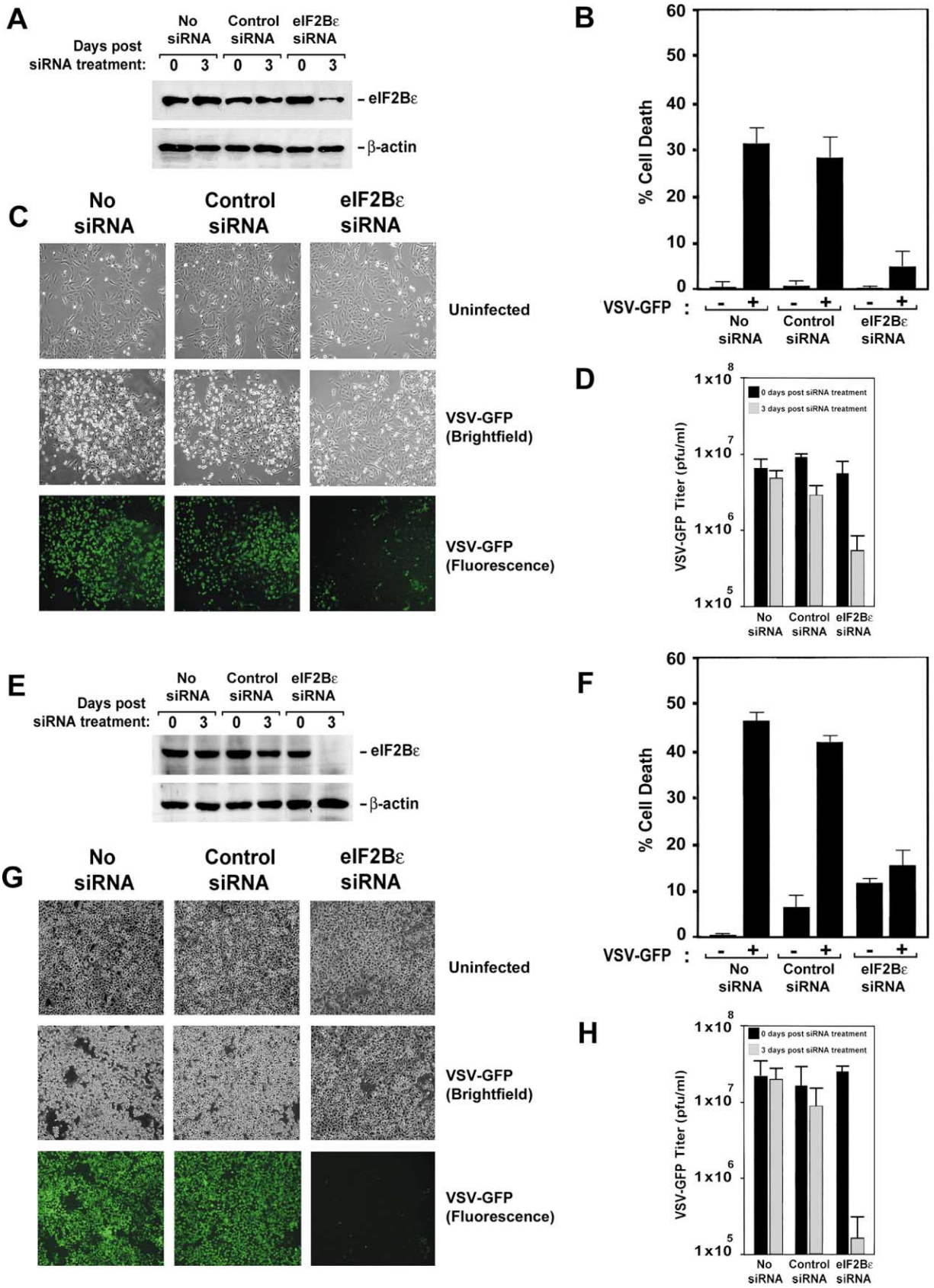


Figure 6. Sustained overexpression of eIF2Bε in transformed MEFs is required for their increased permissiveness to VSV

A: Transformed MEFs were either treated with the transfection reagent alone (mock), nonspecific siRNA (control), or siRNA to eIF2Bε, and eIF2Bε levels were examined by immunoblotting.

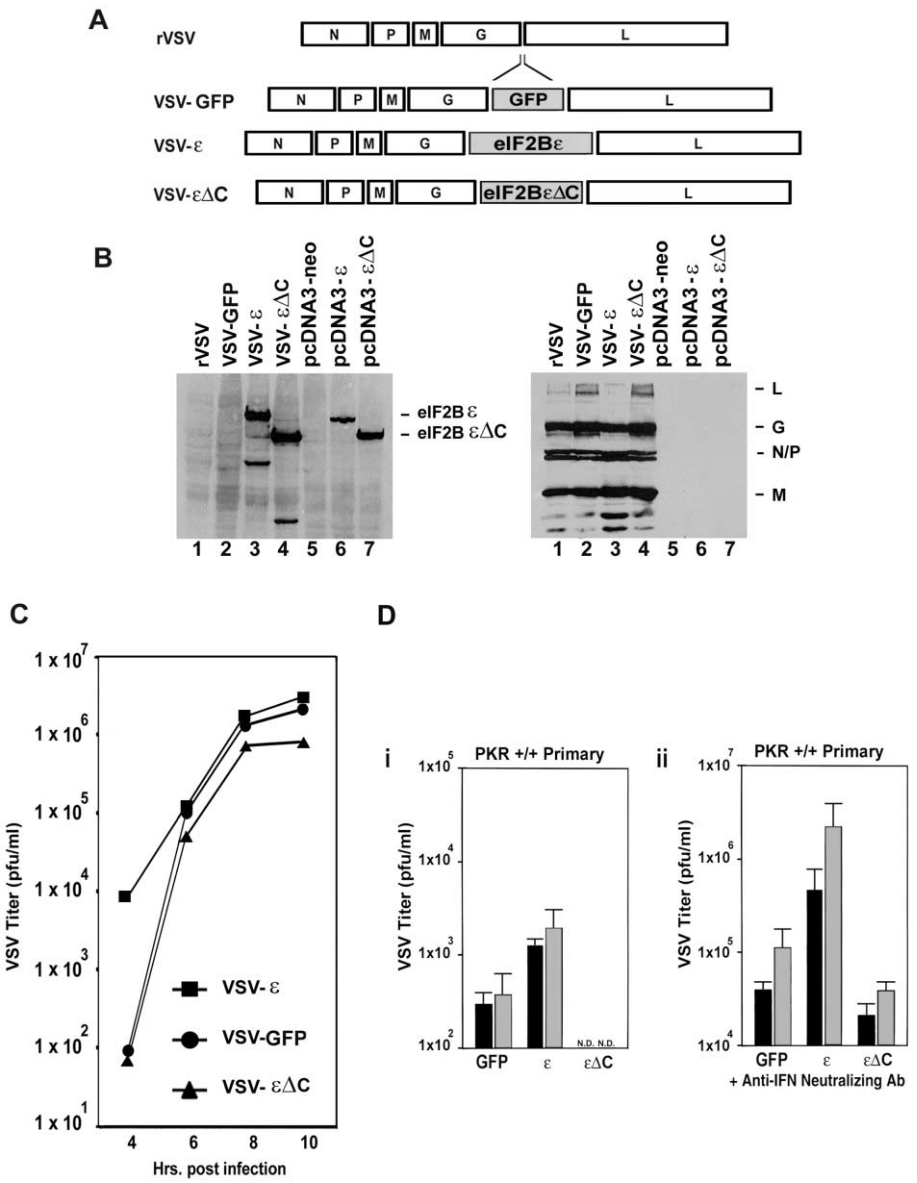


Figure 7. Recombinant viruses expressing eIF2Bϵ and eIF2BϵΔC

A: Schematic of recombinant viruses. The cDNAs encoding eIF2Bϵ and eIF2BϵΔC and GFP were inserted between the G and L genes of the VSV genome-encoding plasmid pVSVXN2.

B: BHK cells were infected with recombinant VSV expressing no transgene (rVSV), GFP (VSV-GFP), eIF2Bϵ (VSV-ϵ), or eIF2BϵΔC (VSV-ϵΔC), and expression of eIF2Bϵ and eIF2BϵΔC verified by immunoblotting using an antibody specific for rat eIF2Bϵ (lanes 1–4). 293T cells were transiently transfected with either pcDNA3.1Neo(−) (lane 5), or with pcDNA3.1Neo(−) encoding eIF2Bϵ or eIF2BϵΔC (lanes 6 and 7, respectively), and examined for eIF2Bϵ expression in parallel with the virally infected cell lysates. These samples were also examined for VSV proteins by immunoblotting using anti-VSV antiserum (right panel).

C: BHK cells were infected with the indicated viruses (m.o.i. = 0.01), and culture supernatants examined for viral progeny yield by standard plaque assay four hours p.i., and every two hours thereafter for ten hours.

D: These viruses were then used to infect (m.o.i. = 0.1 [black bars] or 1 [gray bars]) $PKR^{+/+}$ MEFs in the absence (i) or presence (ii) of anti-mIFNα/B neutralizing antiserum. Culture supernatants were examined for viral progeny yield 48 hr p.i. N.D. = none detectable.

Phosphorylation of eIF2Bϵ by CKI/CKII has been reported to stimulate eIF2B activity, while phosphorylation by GSK-3 has an inhibitory effect (Proud, 2001). Importantly, where examined in these studies, virtually no correlation was seen between eIF2α phosphorylation status and stimulation of eIF2B activity by growth factors and mitogens. Similarly, Sachs and colleagues have implicated eIF2B in the eIF2α-independent control of protein synthesis in response to butanol and other fusel alcohols, products of amino acid catabolism in yeast (Ashe et al., 2001). Pathways similar to the ones reported in these studies may play roles transformed cells, where robust mRNA translation is sustained despite normal eIF2α phosphorylation.

B: Transformed MEFs treated as above were infected with VSV 72 hr posttransfection. 16 hr p.i., cell viability was analyzed by Trypan blue exclusion.

C: Cells treated with siRNAs and subsequently infected with VSV-GFP as in **B** were photographed 16 hr p.i. (magnification 200×).

D: Viral titers from transformed MEFs treated with the indicated siRNAs and subsequently infected with VSV-GFP (m.o.i. = 0.1) was determined 16 hr p.i. by standard plaque assay. Virus infections were carried out 0 (black bars) or 3 (gray bars) days post siRNA treatment.

E: HeLa cells were treated with the transfection reagent alone (mock), nonspecific siRNA (control), or siRNA to eIF2Bϵ and eIF2Bϵ levels examined by immunoblotting.

F: HeLa cells treated as above were infected with VSV-GFP (m.o.i. = 0.1) 72 hr. posttransfection. 24 hr. p.i., cell viability was analyzed by Trypan blue exclusion.

G: Cells treated with siRNA and subsequently infected with VSV-GFP as in **E** were photographed 24 hr p.i. (magnification 200×).

H: Viral titers were determined 24 hr p.i. by standard plaque assay. Virus infections were carried out 0 (black bars) or 3 (gray bars) days post siRNA treatment.

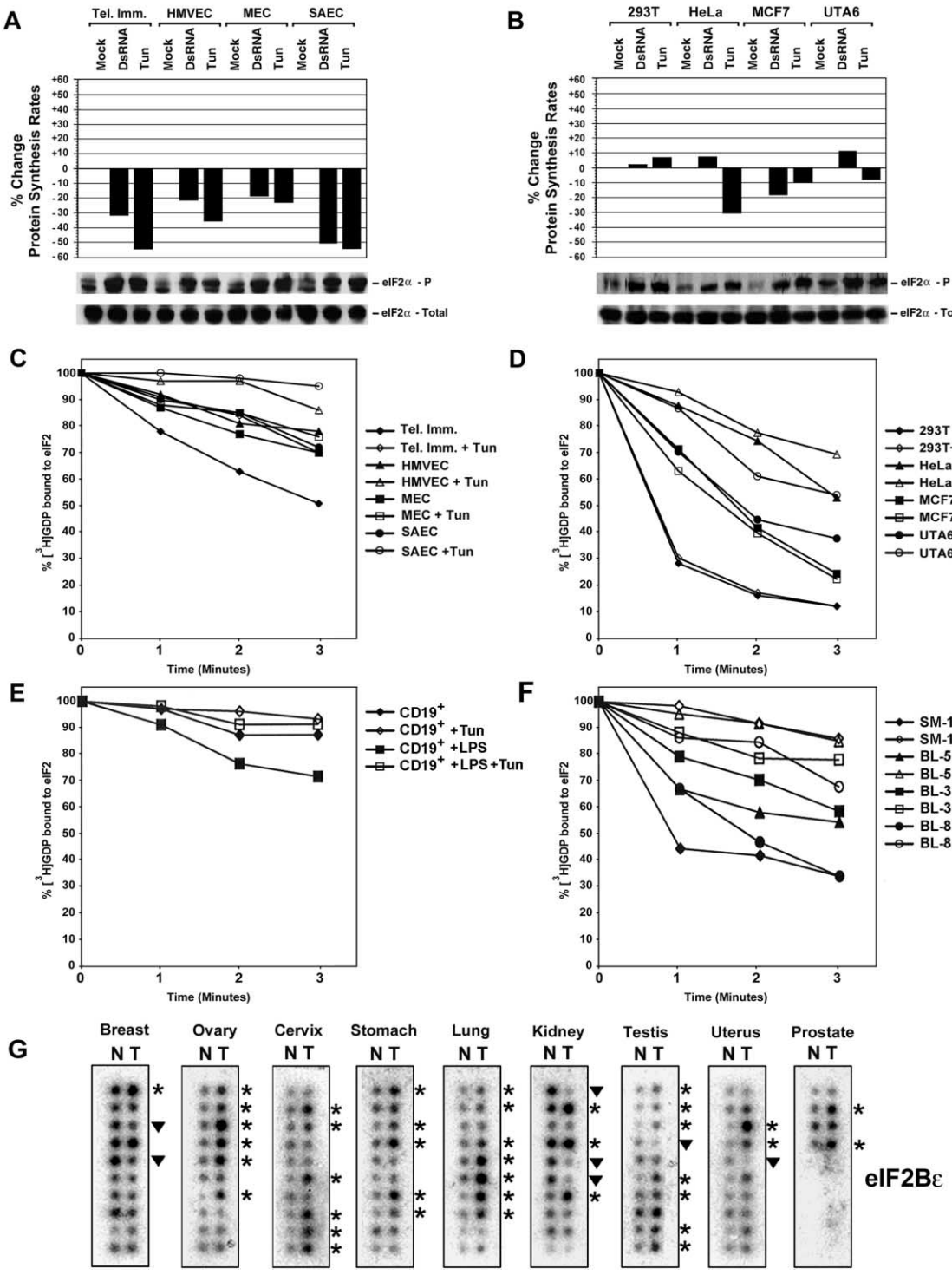


Figure 8. Defective translational control and disregulated eIF2B in transformed human cell lines and tumors

A: Four normal (HMVECs, MECs, SAECs, telomerase immortalized fibroblasts) human cell types were treated with either dsRNA (poly [I:C]) or tunicamycin for three hours, after which they were pulsed with [^{35}S]-labeled methionine and cysteine. Lysates from these cells were analyzed by SDS-PAGE autoradiography and quantitated by densitometry (top panel).

B: Four transformed (293T, MCF-7, UTA6, and HeLa) human cell types were processed as in **A**. These lysates were also immunoblotted against antibodies to eIF2α (total and phosphorylated, bottom panels of **A** and **B**).

C: HMVECs, MECs, SAECs, and telomerase-immortalized fibroblasts were treated with or without tunicamycin for three hours and subjected to eIF2B guanine nucleotide exchange reactions.

D: 293T, MCF-7, UTA6, and HeLa cells were treated with or without tunicamycin and processed as in **C**. The amount of [^{3}H]GDP bound to eIF2 at the start of the reaction (time = 0 min.) was arbitrarily set to 100%.

E: Resting or LPS (1 μg/ml) activated normal CD19^{+} human B cells were treated with or without tunicamycin for three hours, and subjected to eIF2B guanine nucleotide exchange reactions.

How might increased eIF2Bε levels compromise eIF2 (αser51P)-dependent control of translation initiation? Since eIF2B is the rate-limiting component of the guanine nucleotide exchange reaction on eIF2, the simplest explanation is that an increase in the levels of the catalytic subunit is, on its own, sufficient to mediate guanine nucleotide exchange on eIF2 independent of eIF2α phosphorylation. In vitro, the ε subunit by itself possesses 5%–10% guanine nucleotide exchange activity of the holocomplex, and overexpression of this polypeptide in 293 cells causes a marked increase in rates of guanine nucleotide exchange on eIF2 (Fabian et al., 1997; Williams et al., 2001). These findings support the idea that elevated levels of eIF2Bε levels can, to some extent, override the eIF2(αser51P) translational block. In this regard, the experimental data indicate that the eIF2Bε of higher eukaryotes may differ from their yeast homologs where simple eIF2Bε is not sufficient to overcome the potent growth-suppressive effects of eIF2α phosphorylation (Pavitt et al., 1998).

An alternative explanation for how eIF2Bε levels might result in an alleviation of the protein synthesis block imposed by eIF2(αser51P) is provided by the work of Hinnenbusch and colleagues who show that, in addition to existing as a pentameric holocomplex, the five eIF2B subunits can be separated into two distinct subcomplexes, each of which can independently bind eIF2 (Pavitt et al., 1998). The subcomplex containing eIF2B α, β, and δ binds more tightly to eIF2(αser51P) than to unphosphorylated eIF2 and exhibits no catalytic activity. In contrast, the subcomplex comprising eIF2B γ and ε manifests higher guanine nucleotide exchange activity than the wild-type eIF2B holocomplex and, surprisingly, is not inhibited by eIF2α phosphorylation (Pavitt et al., 1998). It is therefore conceivable that an increase in the levels of eIF2Bε can titrate eIF2Bγ away from the holocomplex and into this eIF2(αser51P)-nonresponsive catalytic subcomplex. An increase in the levels of this subcomplex could theoretically mitigate the normally growth-suppressive consequences of eIF2α phosphorylation.

Although we report an increase in the levels of eIF2Bε as a likely reason for the increased permissiveness of transformed MEFs to VSV, and for the hyporesponsiveness to eIF2α phosphorylation seen in these cells, it is conceivable that other mechanisms relieving eIF2(αser51P) mediated inhibition of eIF2B may be active in neoplastic cells. For example, elegant analyses in yeast have revealed that mutations in eIF2B which alleviate growth suppression induced by eIF2(αser51P) all map to the regulatory α, β, and δ subunits of eIF2B (Pavitt et al., 1997; Vazquez de Aldana and Hinnebusch, 1994). In fact, complete deletion in vivo of the yeast eIF2Bα homolog, or exclusion of this subunit from in vitro eIF2 GEF assays, completely alleviates suppression of eIF2B by eIF2α phosphorylation without affecting eIF2B GEF activity (Dever et al., 1993; Kimball et al., 1998). It is therefore possible that mutations and/or deletions analogous to those reported in these studies might also occur in transformed cells, rendering them insensitive to eIF2(αser51P). Further, since the activity of eIF2B can also be potently affected by phosphorylation and allosteric regulation of eIF2Bε, aberrancies at this level of regulation may also play roles in disrupting the eIF2α checkpoint following cellular transformation, and remain to be examined (Proud, 2001). In this regard, it is interesting to note that forced expression of high levels of eIF2Bε, unlike the case with inactive mutants of eIF2α and PKR, did not transform NIH3T3 cells, indicating that simply boosting the levels of this subunit does not disrupt cell growth control in the same manner as certain PKR and eIF2α variants (data not shown, Figure 5E).

The elucidation of the importance of both IFN and translation control in limiting VSV replication to transformed, but not normal, cells now allows the development of safer "smart" oncolytic versions of recombinant VSV expressing, for example, IFN-β, PKR, or eIF2εΔC, that will be severely compromised in their replication in normal cells, but retain oncolytic activity. For example, we have now generated recombinant VSV expressing IFN-β, and report that this virus displays greatly enhanced selectivity for transformed cells in tissue culture and in vivo (Obuchi et al., 2003).

In conclusion, we show that primary MEFs require both PKR and IFN signaling to thwart VSV infection, and neutralization of both of these pathways, but not either one singly, results in these cells becoming as permissive as transformed MEFs to VSV. Subsequently, we show that transformed cells possess defects in IFN antiviral activity, as well as in the PKR pathway predominantly downstream of eIF2α phosphorylation (Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/5/1/51/DC1). Thus, impairment of these key antiviral pathways may be a common occurrence in tumorigenesis, may predispose transformed cells to infection by VSV, and provide mechanistic insight into the oncolytic properties of this virus.

Experimental procedures

Antibodies and reagents

Polyclonal antiserum to VSV was obtained by immunizing Balb/c mice with wild-type VSV. Other antibodies used were as follows: anti-murine (m) PKR, anti-eIF2Bβ, anti-eIF2Bγ, anti-eIF2Bδ, anti-eIF2Bε (Santa Cruz Biotech.), anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho-eIF2α (Biosource International), anti-p21Ras (Oncogene Research Products), anti-mIFNα/β neutralizing antiserum (Research Disgnostics), anti-β-actin (Sigma), anti-eIF2Bα and anti-eIF2Bε polyclonal antisera (gifts of C. Proud), anti-eIF2α (gift of R. Jagus), and anti-β-tubulin (gift of H. Joshi). Chemicals and other biological reagents were obtained from Sigma, except tunicamycin, FTI-277, and PD98059 (Calbiochem-Novabiochem Corp.).

Cell lines

$PKR^{+/+}$ and $PKR^{-/-}$ (129 terSv × Balb/c) MEFs, $STAT1^{+/+}$ and $STAT1^{-/-}$ immortalized cell lines and MEFs (gift of J.Durbin), as well as their transformed counterparts, Balb-3T3 and Balb-Ras (gift of D. Faller), 293T (gift of P. Vertino), HeLa, BHK, C6 rat glioblastoma, MCF-7, and Cos-7 cells (ATCC) were maintained in DMEM supplemented with 10% FBS and antibiotics. NIH3T3 cells were maintained in DMEM supplemented with 10% FCS and

F: Four patient-derived Burkitt's lymphomas (SM-1, BL-3, BL-5, and BL-8) were treated with or without tunicamycin for three hours, and subjected to eIF2B guanine nucleotide exchange reactions.

G: The Cancer Profiling Array II blot (Clontech) containing pairs of normal (N) and tumor (T) tissue samples (each pair from an individual patient) spotted side by side was probed with a radiolabeled ~1.6 kb XhoI-HindIII derived fragment of the human eIF2Bε gene (Genbank accession number XM_029136) and exposed to X-ray film. The tissue of origin is indicated above each strip. Asterisks indicate sample pairs in which the eIF2Bε mRNA is upregulated, while solid triangles indicate sample pairs where eIF2Bε mRNA expression is reduced in tumor tissue, compared to the normal tissue.

antibiotics. Primary human cells (HMVECs, MECs, SAECs) were propagated in media as recommended (Clonetics/BioWhittaker, Inc.). Telomerase-immortalized human fibroblasts (Clontech, gift of W. Hahn), were grown in DMEM supplemented with 25% Medium 199, 10% FBS, and antibiotics. $CD19^+$ B cells were purified from human buffy coat using a positive selection technique, as per the manufacturer's instructions (Miltenyi Biotec Inc.), and cultured in RPMI supplemented with 10% FBS and antibiotics, with or with 1 μg/ml E. coli LPS for 24 hr before experiments. The Burkitt's lymphoma patient isolates (SM-1, BL-3, BL-5, and BL-8) were obtained following approval by the University of Miami Institutional Review Board/Medical Sciences Committee (courtesy of Dr. W. Harrington, Jr.; Lee et al., 1999).

Ectopic overexpression studies

Rat eIF2Bε cDNA in pBluescript II SK (−) (gift of S. Kimball, Flowers et al., 1996) was inserted between the NotI and HindIII sites of pcDNA3(−)Neo (Invitrogen). The eIF2BΔC mutant was generated by excising a NotI-BamHI fragment from full-length rat eIF2Bε cDNA, and was inserted between the NotI and BamHI sites of pcDNA3(−)Neo. LipofectAMINE PLUS (Invitrogen) or FuGENE 6 (Roche Diagnostics) reagents were used to transfect these and other constructs. To generate stable pools of NIH3T3 cells overexpressing either eIF2Bε or eIF2BεΔC, respective cDNAs were subcloned into the retroviral transduction vector pFB Neo (Stratagene). Recombinant eIF2Bε or eIF2BεΔC retroviruses obtained were used to infect NIH3T3 cells plated at a low density, and stable integrants were selected in 600 μg/ml G418. Independently obtained pooled populations of stable integrants were employed in all experiments to exclude clonal variations.

Metabolic labeling

Primary and transformed cells were transfected with dsRNA (poly[I:C], 2 μg/ml medium using the FuGENE 6 reagent, treated with tunicamycin (1 μg/ml), or grown in medium devoid of the indicated amino acid(s) for 3 hr, and pulsed with [^{35}S]-labeled methionine and cysteine for 30 min. Cells were immediately lysed in cold buffer I (20 mM Tris [pH 7.5], 400 mM NaCl, 50 mM KCl, 1% NP-40, 25 mM NaF, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 2 μg/ml aprotinin). Protein concentrations were determined by the Bradford assay using a commercial kit (Pierce), and approximately 50 μg protein per sample was resolved by SDS-PAGE and visualized by autoradiography.

Recombinant VSV

Plaque-purified VSV (Indiana strain) was used in all experiments, and titrated on BHK cells by standard plaque assay. To generate recombinant VSV expressing eIF2Bε or eIF2BεΔC, the polymerase chain reaction (PCR) was employed to introduce SalI and SpeI sites flanking the coding sequences of eIF2Bε and eIF2BεΔC. cDNAs were subsequently inserted into the compatible XhoI and NheI sites of pVSVXN2. Viruses were generated as described previously (Fernandez et al., 2002; Lawson et al., 1995).

eIF2B guanine nucleotide exchange assay

In vitro guanine nucleotide exchange reactions on lysates from treated and untreated cells were performed as described before, with the following exceptions. First, the concentration of protein in each lysate was determined prior to the initiation of the reaction, and all cell lysates were normalized to contain equivalent amounts of protein, and second, aliquots from the nucleotide exchange reaction were taken every minute (rather than every two minutes) for a total of three minutes and immediately filtered through nitrocellulose filters (Fabian et al., 1997).

RNA interference

siRNA duplexes to the murine eIF2Bε mRNA target sequence 5′-AAGUG GUGCCAUCCUACGUCC-3′ (Genbank accession number XM_148182) or to the human eIF2Bε mRNA target sequence 5′-AAGCAGUUCUGGUGGCC GAUA-3′ (Genbank accession number XM_209527) were commercially synthesized and employed in RNA interference experiments as per the manufacturer's instructions (Dharmacon Research, Inc.). All experiments were performed 72 hr posttransfection. As a control, nonspecific siRNA control duplex I was used.

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