JOURNAL OF VIROLOGY, Mar. 2009, p. 2406–2416 Vol. 83, No. 6
0022-538X/09/$08.00+0 doi:10.1128/JVI.01972-08

E4orf1 Limits the Oncolytic Potential of the *E1B-55K* Deletion Mutant Adenovirus▽

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Received 18 September 2008/Accepted 29 December 2008

Clinical trials have shown oncolytic adenoviruses to be tumor selective with minimal toxicity toward normal tissue. The virus ONYX-015, in which the gene encoding the early region 1B 55-kDa (*E1B-55K*) protein is deleted, has been most effective when used in combination with either chemotherapy or radiation therapy. Therefore, improving the oncolytic nature of tumor-selective adenoviruses remains an important objective for improving this form of cancer therapy. Cells infected during the G_1 phase of the cell cycle with the *E1B-55K* deletion mutant virus exhibit a reduced rate of viral late protein synthesis, produce fewer viral progeny, and are less efficiently killed than cells infected during the S phase. Here we demonstrate that the G_1 restriction imposed on the *E1B-55K* deletion mutant virus is due to the viral oncogene encoded by open reading frame 1 of early region 4 (*E4orf1*). *E4orf1* has been reported to signal through the phosphatidylinositol 3′-kinase pathway leading to the activation of Akt, mTOR, and p70 S6K. Evidence presented here shows that *E4orf1* may also induce the phosphorylation of Akt and p70 S6K in a manner that depends on Rac1 and its guanine nucleotide exchange factor Tiam1. Accordingly, agents that have been reported to disrupt the Tiam1-Rac1 interaction or to prevent phosphorylation of the ribosomal S6 kinase partially alleviated the *E4orf1* restriction to late viral protein synthesis and enhanced tumor cell killing by the *E1B-55K* mutant virus. These results demonstrate that *E4orf1* limits the oncolytic nature of a conditionally replicating adenovirus such as ONYX-015. The therapeutic value of similar oncolytic adenoviruses may be improved by abrogating *E4orf1* function.

Conditionally replicating adenoviruses are a novel class of biological agents used to treat cancer (57). The *E1B-55K* deletion mutant virus ONYX-015, originally known as *dl*1520 (4), is one of the first of such agents (7). H101 is another *E1B-55K* deletion mutant adenovirus that is being used for tumor therapy in China (30, 78). We previously reported that cells infected during the G_1 phase of the cell cycle with *E1B-55K* deletion mutant adenoviruses exhibit a reduced rate of viral late protein synthesis, produce fewer viral progeny, and are less effectively killed than cells infected during S phase (34, 35, 66). These observations indicated that the *E1B-55K* deletion mutant virus ONYX-015 is restricted in cells infected in G_1. This restriction is significant because a large fraction of cells within a tumor exist in the G_1 phase of the cell cycle (71). Here we show that the G_1 restriction imposed on the *E1B-55K* deletion mutant virus is due to the viral oncogene encoded by open reading frame 1 of early region 4 (*E4orf1*).

The *E4orf1*-encoded protein is a small adapter molecule that associates with PDZ domain-containing proteins including MUPP1, PATJ, MAGI-1, ZO-2, and Dlg1 (46). PDZ domain-containing proteins often serve as scaffolds for the assembly of signaling complexes at the plasma membrane (64). Through its association with PDZ domain-containing proteins, the *E4orf1*-encoded protein promotes signaling through the phosphatidylinositol 3′-kinase (PI3-kinase) pathway to effectors such as protein kinase B (Akt), the mammalian target of rapamycin (mTOR), and the S6 ribosomal protein kinase (p70 S6K) (27, 54). Through these effectors, PI3-kinase alters protein synthesis and cell survival (21, 28). *E4orf1* is the principal oncogenic determinant of species D adenovirus type 9 (42). The transforming ability of *E4orf1* can be blocked by the PI3-kinase inhibitor LY249002 (27). However, phosphorylation of p70 S6K can also proceed by pathways that are independent of PI3-kinase or Akt. For example, the Rho-like GTPase Rac1 can activate p70 S6K (17). Rac1 is itself regulated by cellular factors to which it binds, including the Rac1-specific guanine nucleotide exchange factor T-cell lymphoma invasion and metastasis 1 protein (Tiam1). Tiam1 and the neural tissue-associated F-actin-binding protein neurabin II or spinophilin recruit p70 S6K into a complex containing Rac1, resulting in increased phosphorylation of p70 S6K (12, 36, 50). Interestingly, both Tiam1 and neurabin II are PDZ-containing proteins. These observations provided a potential basis by which *E4orf1* may modulate protein synthesis and cell survival.

In this report, we show for the first time that *E4orf1* restricts the abilities of the *E1B-55K* deletion mutant virus to produce viral progeny, to direct viral late protein synthesis, and to kill tumor cells. Drugs that are reported to prevent phosphorylation of p70 S6K or to disrupt the interaction between Tiam1 and Rac1 increase the cell-killing ability of the *E1B-55K* deletion mutant virus to nearly the same level observed for an *E1B-55K*/*E4orf1* double mutant and the wild-type virus. By uncovering a role for *E4orf1* in the course of a lytic adenovirus infection, this study presents novel genetic and pharmacolo-

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▽ Published ahead of print on 7 January 2009.

gical means by which the effectiveness of replicating oncolytic adenoviruses can be improved.

MATERIALS AND METHODS

Cell culture and cell viability. Cervical carcinoma-derived HeLa and adenovirus *E1*-transformed 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. Non-small-cell lung carcinoma-derived H1299 cells and glioblastoma-derived U87 and U251 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were maintained and studied at 37°C in a humidified atmosphere with 5% CO_2. Cell culture media, cell culture supplements, and serum were obtained from Life Technologies (Gaithersburg, MD). Because the metabolic activity of adenovirus-infected cells appears to vary with the genotype of the infecting virus (data not shown), surrogate measures of viability that monitor mitochondrial activity such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay were found to be inappropriate. For that reason, cell viability was measured by trypan blue dye exclusion (65).

Cell cycle synchronization. Synchronously dividing cells were obtained by a combination of mitotic detachment and hydroxyurea block as originally described in reference 15 with the modifications described in references 35 and 53. Cells synchronized by this method displayed synchrony (>80% S-phase content at the appropriate time) for three cycles. Synchronously dividing cells were infected as the cells passed through the indicated stage of the cell cycle, during the first hour of S phase (early S phase) or during the first 3 h of G_1 (early G_1). S-phase-enriched populations of cells were produced by one of two means with comparable outcomes. First, cells were treated with 1 mM hydroxyurea (Sigma, St. Louis, MO) in normal growth medium for 24 h; upon replacement of the medium with normal medium or virus infection medium, the cells resumed cycling from the G_1/S border. Alternatively, cells were exposed to uracil arabinoside, which was previously reported to increase the S-phase content of leukemic cells (16). Cells were cultured in normal medium with 0.2 to 0.4 mM uracil arabinoside (ICN/MP Biomedical, Solon, OH). After 48 h, the medium was replaced with virus in infection medium. The fraction of cells in S phase was determined at the time of infection by propidium iodide staining and DNA analysis by flow cytometry as described previously (35, 53). Each method yielded a population of viable HeLa cells with at least 80% of the cells in S phase.

Viruses. The wild-type virus used for these studies was *dl*309 (44). *E1B* mutant viruses included *dl*338 (56a), *dl*1520 (4), and *dl*110 (2). The *E4or1*/*E4orf2* double-mutant virus and the *E1B-55K*/*E4orf1*/*E4orf2* triple-mutant virus *dl*1018 were described previously (9). Additional mutant viruses included *E4orf1* and *E4orf2* mutants *in*351 and *in*352, respectively (37). The *E1B-55K*/*E4orf1* mutant virus MAT2 was created by recombination of the *E1B-55K* mutation of *dl*1520 with the *E4orf1* mutation of *in*351 as described in reference 66. *E1B-55K*/*E4orf2* mutant virus 223 was created by similar methods to include the *E1B-55K* deletion mutation of *dl*1520 and the *E4orf2* mutation of *in*352. An E1 deletion mutant adenovirus vector expressing the T17N dominant-negative form of Rac1 (see, for example, reference 14) was purchased (Cell Biolabs, Inc., San Diego, CA). Viruses were propagated by using 293 cells (45). The titer of each viral preparation was determined by plaque assay and tested by a fluorescent focus assay for infectivity with antibodies to E1A or the E2A DNA-binding protein (33). Infections were performed at a true multiplicity of 20 to 30 (35).

Flow cytometry. DNA content was determined by flow cytometry with a FACScalibur instrument (Becton Dickinson and Co., Franklin Lakes, NJ) and CellQuest (Becton Dickinson) or ModFit software (Verity Software House, Topsham, ME) as described in references 35 and 53.

Transmission electron microscopy to measure viral progeny. At least 100 cells per experiment were scored for the presence or absence of progeny virus particles by transmission electron microscopy as described previously (35, 66).

Plaque assays for viral yields. Virus yield was determined by plaque assay with 293 cells as described previously (35, 53).

Viral late protein synthesis. Infected cells were pulse-labeled for 1 h with ^{35}S-labeled amino acids (Tran^{35}S-label; MP Biomedicals, Costa Mesa, CA) at various times after infection. Cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the viral late proteins were quantified by phosphorescence imaging with a Molecular Dynamics PhosphorImager instrument and ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting. Protein from equivalent numbers of cells were separated by SDS-PAGE, transferred to a nitrocellulose support, and analyzed by immunoblotting as described previously (65). Monospecific primary antibodies (p70 S6K, phosphospecific p70 S6k [Thr389], Akt, phosphospecific Akt [S473], phosphospecific p70 S6K [S235/236], Rac1) were obtained from Cell Signaling Technology (Danvers, MA) and used according to the manufacturer's instructions. Polyclonal rabbit antiserum specific for adenovirus was kindly provided by Arnie Berk (University of California at Los Angeles) and used at a dilution of 1:10,000. Immune complexes were visualized with horseradish peroxidase-conjugated secondary antibody from Jackson ImmunoResearch Laboratories (West Grove, PA) and the SuperSignal chemiluminescent substrate from Pierce (Rockford, IL).

Statistics. Log-transformed values were compared by the two-tailed *t* test with the Holm correction for multiple comparisons or Tukey's honest significant difference algorithm. Cell viability was analyzed by logistic regression with a quasibinomial model to determine the half-life and 95% confidence interval (CI). Values are presented with the mean and the standard deviation, standard error of the mean, or 95% CI as indicated. *P* values of less than 0.05 were considered significant.

RESULTS

The *E1B-55K* deletion mutant adenovirus ONYX-015/*dl*1520 is restricted in cells infected in G_1. Cells infected during the G_1 phase of the cell cycle with *E1B-55K* deletion mutant viruses produce fewer viral progeny, synthesize viral structural proteins at a reduced rate, and are less efficiently killed than cells infected during the S phase (34, 35, 66). Consequently, compared to cells infected in the S phase, *E1B-55K* deletion mutant viruses such as ONYX-015 can be viewed as G_1 restricted. This restriction is significant because a large fraction of the cells within a tumor exist in the G_1 phase of the cell cycle (71). To elucidate the basis for this restriction, synchronously dividing HeLa cells were infected with the wild-type virus *dl*309, the *E1B-55K* deletion mutant virus *dl*1520, or the *E1B-55K*, *E4orf1*, and *E4orf2* triple-mutant virus *dl*1018 and evaluated by electron microscopy for viral progeny. Virtually all of the wild-type virus-infected cells contained progeny virus, whereas more S-phase cells infected with the *E1B-55K* single-mutant virus contained viral progeny than did cells infected during G_1. Surprisingly, the G_1 restriction was absent in cells infected with *dl*1018 (Fig. 1A). These results suggest that *E4orf1*, *E4orf2*, or both restrict the production of *E1B-55K* mutant viral progeny in cells infected in G_1.

To determine if *E4orf1* or *E4orf2* was responsible for the G_1 restriction, additional *E1B-55K* deletion mutant viruses with mutations in the *E4orf1* or *E4orf2* gene were created and their replication was evaluated. The wild-type virus replicated to nearly equivalent levels irrespective of the phase of the cell cycle, whereas the *E1B-55K* deletion mutant virus *dl*1520 produced more viral progeny in cells infected during S phase than during G_1 (Fig. 1B). Replication of the *E1B-55K*/*E4orf2* double-mutant virus 223 was indistinguishable from *dl*1520; both viruses produced more progeny in cells infected during S phase than in those infected during G_1. By contrast, the *E1B-55K*/*E4orf1* double-mutant virus MAT2 and the *E1B-55K*/*E4orf1*/*2* triple-mutant virus *dl*1018 produced statistically equivalent ($P > 0.2$) amounts of viral progeny from cells infected in the S phase and from cells infected in G_1 (Fig. 1B). These results demonstrate that *E4orf1* acts in G_1 cells infected with the *E1B-55K* deletion mutant virus to restrict virus production.

***E4orf1* restricts viral late protein synthesis.** To determine if *E4orf1* restricts viral late protein synthesis in cells infected in G_1, synchronously dividing cells were infected at either G_1 or S phase with the viruses indicated in Fig. 2. The infected cells were pulse-labeled with radioactive amino acids, and the newly synthesized viral late proteins were visualized by gel electro-

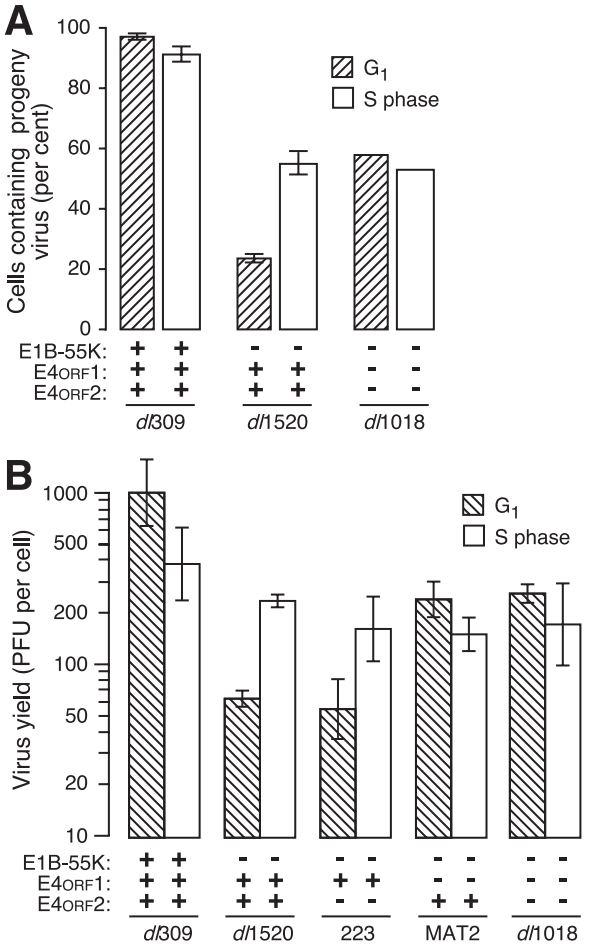

FIG. 1. *E4orf1* imposes a G_1 restriction on virus production directed by *E1B-55K* deletion mutant adenoviruses. Synchronously dividing cultures of HeLa cells were generated by mitotic detachment and HU treatment as described in Materials and Methods. Cells were infected at early G_1 (hatched bars) or early S phase (light bars) with the indicated viruses. (A) Infected cells were harvested after 32 h, and at least 100 infected cells from multiple samples were evaluated by transmission electron microscopy for the presence of progeny viral particles. The percentages of cells containing progeny viral particles from four (*dl*309 and *dl*1520) and two (*dl*1018) independent experiments are shown with the standard deviation indicated by error bars. (B) Synchronously dividing HeLa cells were generated and infected as in panel A with the viruses indicated in panel B. After 72 h, the yield of progeny virus was determined by plaque assay and is presented as the infectious virus per infected cell. Results from three independent experiments are represented as the mean with standard error of the mean. The status of the relevant viral gene is indicated below each bar.

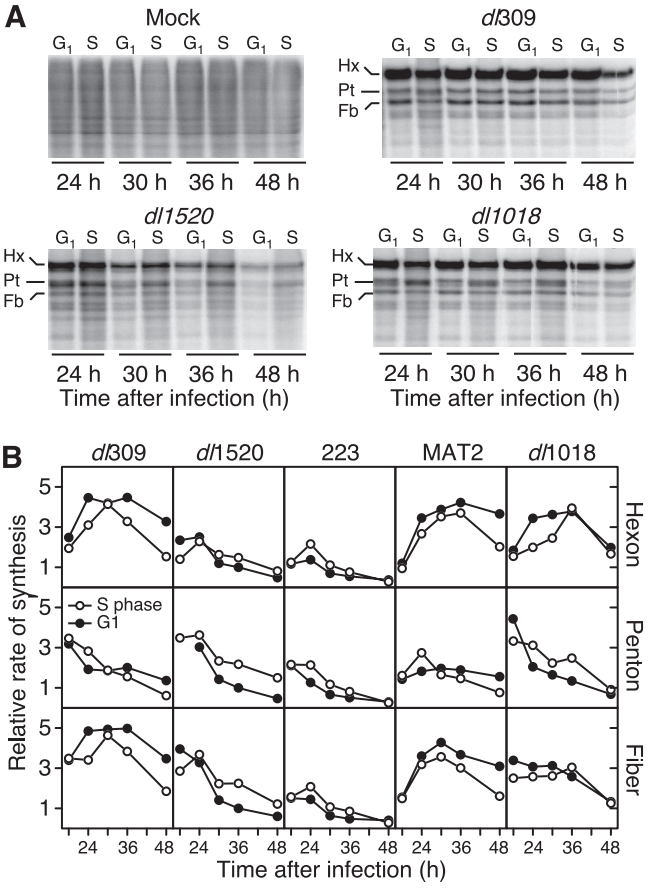

FIG. 2. *E4orf1* imposes a G_1 restriction on viral late protein synthesis directed by *E1B-55K* deletion mutant adenoviruses. (A) Synchronously dividing cultures of HeLa cells were generated by mitotic detachment and HU treatment and mock infected or infected with the indicated viruses at early G_1 or early S phase. Infected cells were pulse-labeled for 1 h with ^{35}S-labeled amino acids at 24, 30, 36, and 48 hpi. Labeled proteins were separated by SDS-PAGE, and the newly synthesized protein was visualized and quantified by phosphorescence imaging. Representative gels are shown with the viral late structural proteins hexon, penton, and fiber indicated as Hx, Pt, and Fb, respectively. (B) The relative rates of synthesis of the viral hexon, penton, and fiber proteins were determined by pulse-labeling as indicated for panel A. The rate of synthesis of each protein was normalized to the rate measured for *dl*1520 at 36 hpi in cells infected in G_1. G_1 rates are shown with closed circles, and S-phase rates are shown with open circles.

phoresis and phosphorescence imaging. Representative images are shown in Fig. 2A. Quantitative phosphorescence imaging confirmed that wild-type virus *dl*309 directed the synthesis of more viral late protein than *E1B-55K* single-mutant virus *dl*1520 or *E1B-55K*/*E4orf2* double-mutant virus 223 (Fig. 2B). As reported previously, the wild-type virus directed more viral late protein synthesis in cells infected in G_1 than in cells infected in the S phase, whereas *dl*1520 directed the synthesis of more viral late proteins in cells infected in the S phase than in cells infected in G_1 even though *dl*1520 blocked host protein synthesis in cells infected in the S phase less effectively than in cells infected in G_1 (35). The cell cycle dependence and temporal pattern of protein synthesis were the same for both 223 and *dl*1520. Strikingly, the time courses of viral late protein synthesis directed by wild-type virus *dl*309, *E1B-55K*/*E4orf1*/*2* triple-mutant virus *dl*1018, and *E1B-55K*/*E4orf1* double-mutant virus MAT2 were nearly identical (Fig. 2A and B). Protein synthesis directed by these mutant viruses resembled that of the wild-type virus in that the rate of viral late protein synthesis in cells infected in G_1 nearly always exceeded the rate of synthesis in cells infected in the S phase. These results show for the first time that *E4orf1* restricts viral late protein synthesis during an *E1B-55K* deletion mutant virus infection and suggest that the characteristic defect in viral late gene expression observed for *E1B-55K* single-mutant viruses such as ONYX-015

(see, for example, references 26 and 76) can be alleviated by abrogating *E4orf1* function.

To determine if *E4orf1* restricts viral late protein synthesis only in the absence of *E1B-55K*, additional viruses bearing mutations in the *E1B-55K*, *E4orf1*, or *E4orf2* gene were evaluated with asynchronously dividing cells. Because we observed the greatest differences in rates of viral late translation around 36 h postinfection (hpi), the rate of synthesis of the hexon, penton, and fiber proteins measured in each experiment was normalized to the mean rate of synthesis measured at 36 hpi for the *E1B-55K*/*E4orf2* double-mutant and *E1B-55K* single-mutant viruses. Differences among the rates of viral late protein synthesis were modest at 24 hpi (Fig. 3B). By contrast, at 36 hpi, the rates of viral late protein synthesis directed by the *E1B-55K* single-mutant-like viruses were substantially reduced (6-fold, range of 3- to 14-fold in 24 experiments) compared to other viruses. By 48 hpi, the rate of late protein synthesis directed by the wild-type virus had dropped to a level similar to that of the *E1B-55K* single-mutant-like viruses ($P > 0.3$). Strikingly, the rate of protein synthesis directed by *E1B-55K*/*E4orf1* double-mutant virus MAT2 or *E4orf1* single-mutant virus *in*351 remained significantly higher than those of the *E1B-55K* single-mutant-like viruses and the wild-type virus ($P < 0.01$ and $P < 0.014$, respectively). This trend was observed for infected cells evaluated as late as 72 hpi (data not shown) and is represented schematically in Fig. 3C.

***E4orf1* restricts the cell-killing potential of *E1B-55K* deletion mutant virus ONYX-015.** To determine if *E4orf1* also restricts the cytolytic nature of the *E1B-55K* deletion mutant virus, HeLa cells were infected with the same viruses analyzed in Fig. 3 and cell viability was determined over the course of 7 days. Logistic regression was used to determine the time required to kill one-half of the infected cells ($t_{1/2}$) and 95% CI. As expected, wild-type virus *dl*309 killed more quickly ($t_{1/2}$ = 3.9 ± 0.3 days) than *E1B-55K* single-mutant virus *dl*1520 ($t_{1/2}$ = 6.4 ± 0.4 days) or *E1B-55K*/*E4orf2* double-mutant virus 223 ($t_{1/2}$ = 6.2 ± 0.5 days). Surprisingly, both the *E1B-55K*/*E4orf1* double-mutant virus and triple-mutant virus *dl*1018 killed cells more quickly ($t_{1/2}$ = 4.9 ± 0.2 and 5.0 ± 0.2 days, respectively) than either single-mutant virus *dl*1520 or *E1B-55K*/*E4orf2* double-mutant virus 223. These findings confirm that the presence of *E4orf1* limits the cytolytic properties of the *E1B-55K* deletion mutant virus in HeLa cells.

HeLa cells express the human papillomavirus type 18 (HPV-18) E6 protein, which, like the *E4orf1*-encoded protein, targets several PDZ domain-containing proteins (31, 46). However, results obtained with HeLa cells were replicated with non-HPV-transformed cells, including the lung carcinoma-derived H1299 cell line and p53-positive and -negative glioblastoma-derived cell lines (data not shown). Thus, it seems unlikely that any shared targets of the *E4orf1* and HPV-18 E6 proteins influenced the apparent restriction imposed by *E4orf1* on virus production, viral late protein synthesis, and cell killing. These results led us to investigate possible *E4orf1*-related signals that are involved in the restriction of the *E1B-55K* mutant virus.

Akt and p70 S6K are phosphorylated in the presence of the PI3-kinase inhibitor LY294002 in adenovirus-infected cells. The *E4orf1*-encoded protein stimulates signaling through PI3-kinase to targets such as Akt, p70 S6K, and mTOR (27, 54). Because these PI3-kinase targets can regulate cellular translation (11, 21, 28), we first evaluated the contribution of the PI3-kinase pathway to viral late protein synthesis. The PI3-kinase inhibitor LY294002 has been reported to interfere with the ability of *E4orf1* to activate the PI3-kinase pathway. We therefore expected that LY294002 would mimic the absence of *E4orf1* during a *dl*1520 infection and increase the rate of viral late protein synthesis. Contrary to our expectation, viral late protein synthesis decreased in a concentration-dependent manner in *E1B-55K* deletion mutant virus-infected HeLa cells

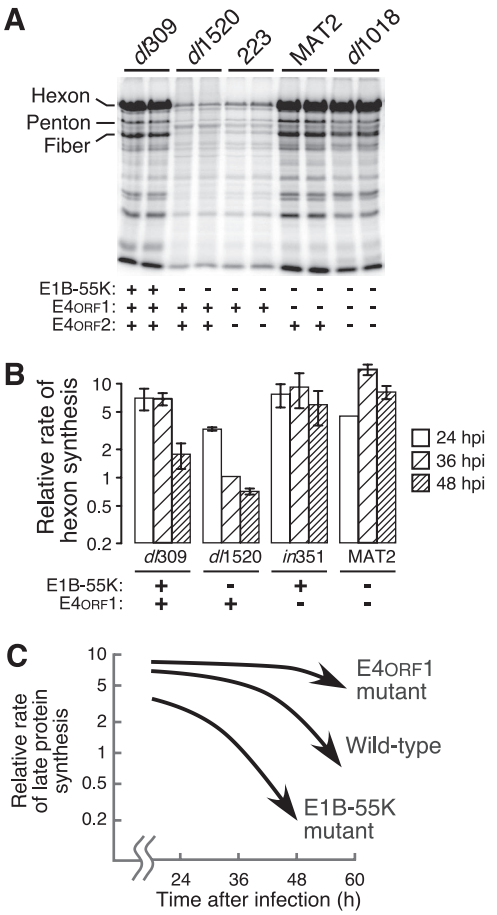

FIG. 3. *E4orf1* restricts viral late protein synthesis in adenovirus-infected cells. (A) Duplicate cultures of asynchronously dividing HeLa cells were infected with the indicated viruses and pulse-labeled with ^{35}S-labeled amino acids at 36 hpi. Labeled proteins were separated by electrophoresis and visualized by phosphorescence imaging. The late structural proteins hexon, penton, and fiber are indicated. The status of the relevant viral gene is indicated below each lane. (B) Asynchronously dividing HeLa cells were infected with the indicated viruses and pulse-labeled for 1 h with ^{35}S-labeled amino acids at 24, 36, and 48 hpi. Radioactivity incorporated into the hexon protein was quantified by phosphorescence imaging. This value was normalized to the amount of radioactivity incorporated into hexon in *dl*1520 virus-infected cells at 36 hpi. The values shown are the mean of 9 to 23 independent infections with the standard error of the mean indicated. The status of the relevant viral gene is indicated below each virus. (C) The relative rate of late protein synthesis directed by representative viruses at late times after infection is schematically represented for an *E4orf1* deletion mutant virus, an *E1B-55K* deletion mutant virus, and the wild-type virus. *E4orf1* restricts viral late protein synthesis at all times of infection in the *E1B-55K* mutant background and at very late times in the context of a wild-type virus infection.

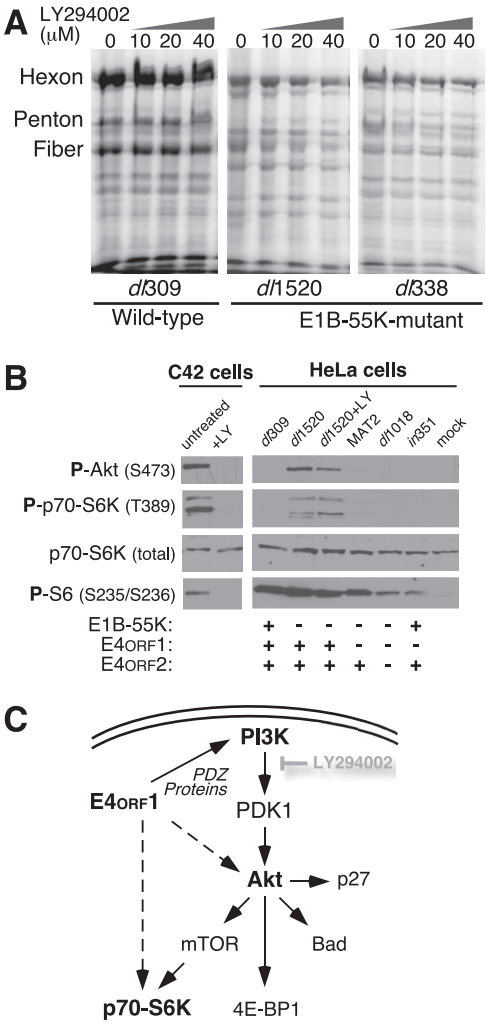

FIG. 4. The PI3-kinase inhibitor LY294002 diminishes viral late protein synthesis and does not reduce Akt and p70 S6K phosphorylation in adenovirus-infected cells. (A) Asynchronously dividing HeLa cells were infected with either the wild-type virus *dl*309 or the *E1B-55K* deletion mutant virus *dl*1520 or *dl*338. Four hours after infection, LY294002 was added at the indicated concentration. At 36 hpi, the cells were pulse-labeled with ^{35}S-labeled amino acids and the labeled proteins were visualized by SDS-PAGE and phosphorescence imaging. (B) Akt and p70 S6K are phosphorylated in *dl*1520 virus-infected cells. The C4-2 prostatic cancer cell line with constitutive activation of the PI3-kinase pathway was left untreated or exposed to 50 μM LY294002 (LY) for 36 h before cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose, and sequentially analyzed by blotting with antibodies specific for phospho-Akt (Ser473), phospho-p70-S6K (Thr389), total p70-S6K, and phosphoribosomal protein S6 (serines 235 and 236). Asynchronously dividing HeLa cells were mock infected or infected with the indicated viruses and exposed to 50 μM LY294002 at 4 h after infection. At 36 hpi, cells were harvested and protein was separated by SDS-PAGE, transferred to nitrocellulose, and analyzed with the indicated antibodies. The status of the relevant viral gene is indicated below each lane.

exposed to LY294002 (Fig. 4A), as well as in human cells of other origins, including lung carcinoma and glioblastoma (data not shown). Thus, it seems unlikely that *E4orf1* restricts viral late protein synthesis by stimulating PI3-kinase activity.

Phosphorylation of the PI3-kinase downstream effectors Akt and p70 S6K was evaluated with phosphospecific antibodies to serve as an indicator of activation (61). Phosphorylation of Akt, p70 S6K, and S6 in C4-2 cells, which contain a constitutively activated PI3-kinase pathway (77), was largely eliminated by 50 μM LY249002 (Fig. 4B). In contrast to C4-2 cells, HeLa cells do not contain measurably phosphorylated Akt, p70 S6K, and S6. Therefore, any increase in the phosphorylation of these proteins upon infection would be due to viral activity. Interestingly, HeLa cells infected with *E1B-55K* single-mutant virus *dl*1520 showed significant phosphorylation of Akt and p70 S6K at 18 to 24 hpi in comparison to the wild-type virus or other viruses lacking *E4orf1* (Fig. 4B). The difference in Akt phosphorylation between *dl*1520 and wild-type virus-infected cells was less dramatic by 36 hpi (data not shown). Nonetheless, in *dl*1520 virus-infected cells, LY294002 reduced but did not eliminate the phosphorylation of Akt at Ser473. Additionally, and in contrast to C4-2 cells, LY294002 did not alter the total amount of phosphorylated p70 S6K in *dl*1520 virus-infected HeLa cells; rather, LY294002 appeared to alter the distribution of phosphorylated forms recognized by the antibody to phospho-Thr389 (Fig. 4B). Similar effects were seen in *dl*1520 virus-infected H1299 and U87 cells (data not shown). To verify that these observations were indeed due to *E4orf1*, we generated *E4orf1*-expressing H1299 cells. The constitutive level of Akt phosphorylation was elevated in the *E4orf1*-expressing cells. Treatment of these cells with LY294002 did not eliminate the phosphorylation of Akt, thus recapitulating the observation in *dl*1520 virus-infected cells (data not shown). These results led us to conclude that if *E4orf1* contributes to the phosphorylation and activation of p70 S6K and Akt in *dl*1520 virus-infected cells, it may act through both PI3-kinase-dependent and -independent pathways.

Involvement of Rac1 in *E4orf1*-induced phosphorylation of p70 S6K and perhaps Akt. The small GTPase Rac1 can contribute to the phosphorylation of p70 S6K (17). The activity of Rac1 is governed by binding partners such as Tiam1 and neurabin II. Tiam1 is a widely distributed guanine nucleotide exchange factor that is specific for Rac1 (50). Neurabin II has been reported to bind both Tiam1 and p70 S6K (12, 13). Intriguingly, both Tiam1 and neurabin II contain PDZ domains. Because the *E4orf1*-encoded protein is able to bind to many PDZ domain-containing proteins, the *E4orf1*-encoded protein could possibly interact with Tiam1, neurabin II, or both and promote the phosphorylation of p70 S6K through Rac1 (Fig. 5A).

To determine if Rac1 contributes to the phosphorylation of p70 S6K in adenovirus-infected cells, phosphorylation at Thr389 was compared among *dl*1520- and MAT2-infected cells following exposure to the broad-spectrum inhibitor of p70 S6K phosphorylation HA1077 (22) or the specific inhibitor of the Tiam1-Rac1 complex NSC23766 (29). S-phase cells infected with *dl*1520, which do not exhibit the characteristic G_1 restriction, contained dramatically less phosphorylated p70 S6K at both 24 and 36 hpi than did untreated *dl*1520 virus-infected cells (Fig. 5B). Similarly, MAT2-infected cells, which show no evidence of the G_1 restriction, contained less phosphorylated p70 S6K than did untreated *dl*1520 virus-infected cells. In *dl*1520 virus-infected G_1 cells, the inhibitor of p70 S6K phosphorylation HA1077 reduced the amount of phosphorylated p70 S6K detected at 24 hpi, with less of an impact at 36 hpi. A

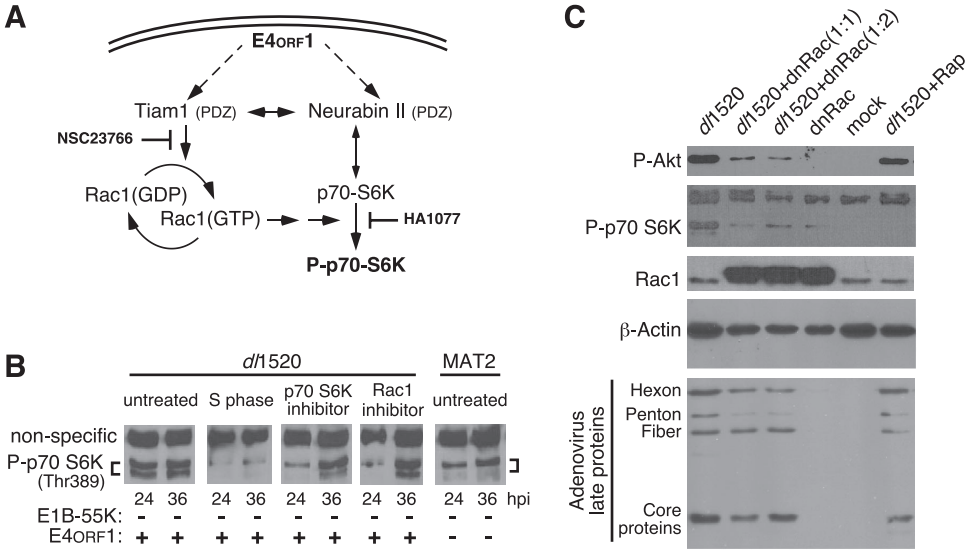

FIG. 5. *E4orf1*-induced phosphorylation of p70 S6K and Akt may involve Rac1. (A) Schematic representation of a potential signaling pathway initiated by *E4orf1* through the PDZ domain-containing proteins Tiam1 and neurabin II. Solid arrows identify known pathways and interactions. (B) HeLa cells were blocked at the G_1/S border by exposure to HU for 24 h, released from the block, and infected 1 h later (S phase) or infected as an asynchronously dividing culture with the *E1B-55K* single-mutant virus *dl*1520 or the *E1B-55K*/*E4orf1* double-mutant virus MAT2. At 4 hpi, the growth medium was replaced with fresh medium or with medium containing the p70 S6K inhibitor HA1077 at 50 mM or the Tiam1-Rac1 inhibitor NSC23766 at 40 μM. At 24 and 36 hpi, cellular proteins were analyzed by immunoblotting for phosphorylation of Thr389 on p70 S6K. A nonspecific cross-reacting protein is indicated which served as a loading control. The status of the relevant viral gene is indicated below each lane. (C) Asynchronously dividing HeLa cells were mock infected or infected with a nonreplicating adenovirus vector expressing dnRac1 (dnRac) at a multiplicity of 20 (1:1) or 40 (1:2). After 24 h, the cells were again mock infected or infected with the *E1B-55K* deletion mutant virus *dl*1520 at a multiplicity of 20. After 1 h, rapamycin (Rap) was added to 50 nM to one culture. Cells were harvested 36 h after infection with *dl*1520. Infected cell proteins were separated by SDS-PAGE and analyzed by immunoblotting with monospecific antibodies (phospho-Akt, phospho-P70 S6K, Rac1, β-actin) or polyclonal serum specific for the adenovirus late proteins.

similar pattern was observed for infected cells exposed to the Tiam1/Rac1 inhibitor NSC23766 at 40 μM. In these studies, the pattern of p70 S6K phosphorylation observed in MAT2-infected cells was more similar to that observed in drug-treated *dl*1520 virus-infected cells than in nontreated *dl*1520 virus-infected cells. These results support the notion that p70 S6K may be differentially phosphorylated by a Tiam1/Rac1-related pathway in *E1B-55K* deletion mutant virus-infected cells.

To further investigate the contribution of Rac1 to the phosphorylation of Akt and p70 S6K, as well as to viral late protein synthesis, *dl*1520 virus-infected cells were coinfected with an adenovirus vector expressing dominant-negative Rac1 (dnRac1). Levels of β-actin were reduced by dnRac1 (Fig. 5C). A comparable effect was observed in THP1 cells expressing a similar construct (70), most likely reflecting a role for Rac1 in modulating the actin cytoskeleton. Phosphorylation of both Akt and p70 S6K was reduced by dnRac1 (Fig. 5C). HeLa cells infected with *dl*1520 were also treated with rapamycin to determine if the rapamycin-sensitive form of mTOR was responsible for the increase in Akt and p70 S6K phosphorylation. In agreement with the work of Sarbassov and associates (60), prolonged exposure of HeLa cells to rapamycin eliminated p70 S6K phosphorylation but had no effect on Akt phosphorylation (Fig. 5C).

Notably, even though both dnRac1 and rapamycin reduced or eliminated p70 S6K phosphorylation, neither substantially affected the accumulation of viral late proteins in cells infected with the *E1B-55K* deletion mutant virus *dl*1520 (Fig. 5C). In contrast to dnRac1 or rapamycin, HA1077 and NSC23766 produced a slight increase in viral late protein synthesis directed by the *E1B-55K* mutant virus. HA1077 elicited a 1.4-fold increase ($P < 0.001$, $n = 9$) in the rate of hexon synthesis, while the Tiam1/Rac1 inhibitor NSC23766 increased hexon synthesis by 1.7-fold over that in nontreated cells ($P < 0.0001$, $n = 11$). Neither drug affected the rate of hexon synthesis in MAT2-infected cells (data not shown), nor were these inhibitors able to recapitulate the high levels of hexon synthesis observed in MAT2-infected cells at 36 h (1.7-fold compared to 10-fold). These results provide additional support for the idea that *E4orf1* promotes the phosphorylation of p70 S6K and Akt in a Rac1-dependent manner but that this is not likely the mechanism by which *E4orf1* restricts late protein synthesis.

The PI3-kinase/Tiam1-Rac1 signals initiated by *E4orf1* promote survival in adenovirus-infected cells. The results shown in Fig. 5 led us to infer that the regulation of viral late translation is largely independent of the phosphorylation status of p70 S6K and signaling through PI3-kinase. However, signaling through the p70 S6K (38), PI3-kinase (21, 28), and Rac1 (23, 43) pathways also promotes cell survival. Consequently, we tested the possibility that *E4orf1* restricts the cell-killing potential of the *E1B-55K* deletion mutant virus through the aforementioned pathways. We first measured the impact of *E4orf1* on the cell-killing potential of the *E1B-55K* mutant virus in four cell lines derived from tumors of three origins. Cell viability was measured after infection with either the *E1B-55K* single-mutant virus *dl1520* or the *E1B-55K*/*E4orf1* double-mutant virus MAT2. The double-mutant virus MAT2 killed these tumor cells more effectively than did the *E1B-55K* single-

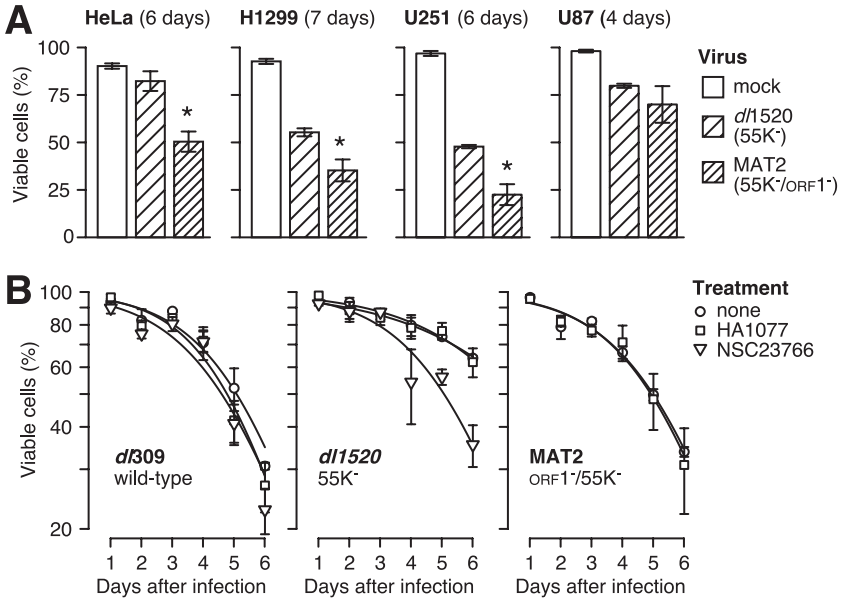

FIG. 6. *E4orf1* restricts the tumor cell-killing potential of *E1B-55K* mutant virus *dl*1520 (ONYX-015) in a Tiam1/Rac1-dependent manner. (A) Cell lines derived from human cervical carcinoma (HeLa), large-cell lung carcinoma (H1299), and malignant glioblastoma (U251, U87) were mock infected or infected with the indicated viruses at a multiplicity sufficient to infect all of the cells. The viability of the cells was determined by trypan blue dye exclusion on the indicated day after infection. Values represent the mean of three independent infections with the standard error of the mean. The asterisk identifies values for MAT2-infected cells that are significantly ($P < 0.05$) less than the viability of *dl*1520 virus-infected cells. (B) HeLa cells were infected with the wild-type virus (*dl*309), the *E1B-55K* mutant virus (*dl*1520), or the *E1B-55K*/*E4orf1* double-mutant virus (MAT2) at a multiplicity of 20 and left untreated (circles) or were adjusted to 50 mM HA1077 (squares) or 40 μM NSC2366 (triangles) at 4 hpi. The fraction of viable cells was determined daily for 6 days after infection by trypan blue dye exclusion. The plotted values represent the mean of three independent infections with the standard error of the mean indicated by error bars. The lines represent best-fit curves determined by logistic regression with a quasibinomial model.

mutant virus (Fig. 6A). This result indicates that *E4orf1* indeed restricts the cell-killing potential of the *E1B-55K* deletion mutant virus.

Since Fig. 5 indicates that *E4orf1* promotes the phosphorylation of p70 S6K through Tiam1/Rac1, we tested the hypothesis that inhibiting the phosphorylation of p70 S6K and/or disrupting the Tiam1-Rac1 interaction would increase the cell-killing potential of the *E1B-55K* single-mutant adenovirus. Cells were infected with the wild-type virus, the *E1B-55K* single-mutant virus, or the *E1B-55K*/*E4orf1* double-mutant virus and treated with the vehicle control, HA1077, or NSC232766. The fraction of viable cells was determined by trypan blue dye exclusion, and the half-life of the infected cells was estimated by regression analysis. The viability of mock-infected cells was unaffected by the drugs used in these experiments (data not shown). HeLa cells infected with the wild-type virus died at comparable rates, irrespective of drug treatment ($t_{1/2}$ = 4.6 to 5.1 days). Untreated cells infected with the *E1B-55K* single-mutant virus died more slowly ($t_{1/2}$ = 7.0 ± 0.6 days) than wild-type virus-infected cells ($t_{1/2}$ = 5.1 ± 0.4 days), as reported previously (33, 65). Treatment of *dl*1520 virus-infected cells with HA1077 had no significant effect on the rate of HeLa cell death ($t_{1/2}$ = 7.2 ± 1.1 days), although HA1077 did increase the rate at which *dl*1520 virus-infected H1299 or U87 cells died (data not shown). The Tiam1/Rac1 inhibitor NSC232766 accelerated the rate of cell killing mediated by the *E1B-55K* single-mutant virus ($t_{1/2}$ = 5.1 ± 0.4 days) to that measured for the *E1B-55K*/*E4orf1* double-mutant virus MAT2 ($t_{1/2}$ = 5.1 ± 0.4 days) and the wild-type virus. Cell killing by the *E1B-55K*/*E4orf1* double-mutant virus was not affected by treatment with HA1077 ($t_{1/2}$ = 7.2 ± 1.1 days) or NSC232766 (data not shown). From these results, we inferred that *E4orf1* acts through Tiam1-Rac1 to promote the survival of adenovirus-infected cells. These signals limit the cytolytic potential of the *E1B-55K* single-mutant adenovirus ONYX-015 (*dl*1520) and are expected to impose similar restrictions on other replication-competent oncolytic adenoviruses such as H101 as well.

DISCUSSION

The *E1B-55K* deletion mutant adenovirus ONYX-015 and, more recently, H101 have been used to treat cancer. ONYX-015 is more effective in combination with other therapies than as a single agent (30, 78). Therefore, improving the oncolytic nature of this single agent should improve its value as a cancer therapy. We previously reported that *E1B-55K* deletion mutant adenoviruses replicated more effectively in cells infected during the S phase than in cells infected during G_1 and that the mutant virus killed cells infected in the S phase more effectively than cells infected in G_1 (33, 66). Because most of the cells in a tumor are in G_1 (71), we chose to examine more closely the basis for the relative G_1 restriction imposed on the *E1B-55K* deletion mutant virus. In this report, we show that *E4orf1* limits viral progeny production, viral late protein synthesis, and cell killing. Consequently, conditionally replicating viruses such as ONYX-015 that contain an intact *E4orf1* gene suffer from an intrinsic limitation to their oncolytic nature.

This report also highlights the possibility that other viral genes contribute to the oncolytic nature of replication-competent adenoviruses in ways that have not been recognized previously.

Among many activities attributed to the *E1B-55K* protein, promoting viral late gene expression is key to a productive infection (reviewed in references 6 and 26). In the infected cell, the *E1B-55K* and *E4orf6* proteins form an E3 ubiquitin ligase that includes cullin 5 (Cul5), elongins B and C, and Rbx1 (39, 58). Because viral late gene expression was reduced by proteasome inhibitors (19) or by expression of a dominant-negative Cul5 variant (76), the *E1B-55K* protein appears to promote late gene expression by the targeted degradation of a protein. The identity of this protein (or proteins) remains elusive. Cellular proteins known to be targeted by the adenovirus ubiquitin ligase are central to the cellular response to DNA damage. These targets include p53 (39, 58), Mre11 (67), and DNA ligase IV (3). Woo and Berk have noted that components of the nonsense-mediated mRNA decay pathway, which also participate in mRNA export, are targets of the DNA damage response (76). These investigators suggested that the inhibition of viral late mRNA export observed in cells infected with the *E1B-55K* mutant may stem from a failure to suppress signaling from cellular kinases that sense DNA damage. However, *E4orf6* mutant viruses that failed to direct the degradation of Mre11 were not defective for late gene expression (8), indicating that targets other than Mre11 may be important in this regard. Recently, additional targets of the adenovirus ubiquitin ligase with no obvious role in the cellular DNA damage response have been identified. These include the nonstructural Rep52 and capsid proteins of adeno-associated virus type 5 (52). In view of the diverse requirements for the recruitment of specific substrates by the viral ubiquitin ligase (49, 63), it would not be surprising that additional cellular targets of the adenovirus ubiquitin ligase remain to be identified and that one of these may be critical for viral late gene expression.

Like the adenovirus *E1B-55K*- and *E4orf6*-encoded proteins, the human immunodeficiency virus type 1 (HIV-1) *Vif*-encoded protein forms an E3 ubiquitin ligase with Cul5, elongins B and C, and Rbx1. The HIV ubiquitin ligase directs the degradation of apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G, or APOBEC3G (5). APOBEC3G serves as an antiviral effector toward a wide variety of viruses and retroelements (40, 41). Several investigators have suggested that the Cul5-based adenovirus ubiquitin ligase targets antiviral effectors to promote efficient adenovirus replication (8, 74, 76). Further support for this notion may be inferred from the overlapping functions of the *E1B-55K*/*E4orf6*-encoded protein complex and the *E4orf3*-encoded protein. Like the *E1B-55K*/*E4orf6*-encoded protein complex, the *E4orf3*-encoded protein suppresses the DNA damage response, albeit by mislocalizing components of the MRN complex (1, 24, 68). Recently, the *E4orf3*-encoded protein has been shown to explicitly disable the interferon-mediated antiviral response by mislocalizing the cellular proteins Daxx and PML (72, 73). For these reasons, we favor the idea that cellular effectors directly engaged in the antiviral response are targeted by the *E1B-55K*/*E4orf6*-encoded protein complex.

It is tempting to speculate about possible connections among the cellular DNA damage response, the postulated antiviral activity targeted by adenovirus ubiquitin ligase, and G_1 restriction. We reported that *E1B-55K* and *E4orf6* deletion mutant adenoviruses replicate less effectively in cells infected during G_1 than in S phase (34, 35). If the *E1B-55K*/*E4orf6*-encoded ubiquitin ligase must suppress an antiviral activity for efficient virus replication, it stands to reason that this antiviral activity is less pronounced in cells infected in the S phase. Because cells engaged in DNA synthesis must tolerate partially replicated DNA, single-stranded DNA, and transient DNA strand breaks, sensors that elicit an antiviral state in response to aberrant DNA may be subdued in S-phase cells compared to G_1 cells. Consequently, viruses that are crippled in the ability to suppress the DNA damage response are predicted to be less restricted following infection of an S-phase cell. Because the *E4orf3* single-mutant virus is not G_1 restricted (34, 66), we anticipate that the *E4orf3* targets Daxx and PML do not impose the G_1 restriction observed in *E1B-55K* mutant virus-infected cells.

In this report, we show that disruption of *E4orf1* alleviates the apparent G_1 restriction imposed on *E1B-55K* mutant viruses (Fig. 1). *E4orf1* may contribute to the development of an antiviral state that must be suppressed by the adenovirus ubiquitin ligase. Because cells infected with the *E1B-55K*/*E4orf1* double-mutant virus synthesize wild-type levels of viral DNA (M. Thomas and D. Ornelles, unpublished observations), it seems unlikely that the act of viral DNA synthesis could serve as the only trigger of the antiviral response. *E1B-55K*/*E4orf1* double-mutant viruses also direct the synthesis of wild-type levels of viral proteins (Fig. 2 and 3). Since the cytoplasmic levels of viral late mRNA remained the same in single- and double-mutant virus-infected cells (unpublished observations), we concluded that this effect was due to increased translational efficiency of viral late mRNA. Perhaps the postulated antiviral activity that is elicited by *E4orf1* and suppressed by the adenovirus ubiquitin ligase limits viral progeny production at a posttranslational step. Finally, because neither of the two *E4orf1*/*E1B-55K* double-mutant viruses replicated to the same level as the wild-type virus (Fig. 1), it seems likely that the *E1B-55K*-encoded protein may contribute to the production of viral progeny independently of its role in promoting viral late gene expression.

The role of *E4orf1* during a productive adenovirus infection remains poorly understood. *E4orf1* mutant viruses were reported to be indistinguishable from the wild-type virus when studied in established tumor cell lines (10, 37). We also observed that *in*351 replicated in HeLa cells to levels equivalent to that of the wild-type virus *dl*309 (data not shown). However, *E4orf1* may be important to virus replication in quiescent human cells, where it enables adenovirus to mobilize the translational machinery in nutrient-deprived cells (54, 55). Somewhat at odds with this notion, we show that *E4orf1* limits viral late translation. Our findings indicate that *E4orf1* counteracts the ability of the *E1B-55K*-encoded protein to promote viral late gene expression. Other ostensibly opposing activities among adenovirus products exist. For example, the *E1A*-encoded proteins stabilize p53 (47, 48) while the *E1B-55K*- and *E4orf6*-encoded proteins promote p53 degradation (51, 59). Some of these opposing activities may reflect a switch in the viral replication program, as is the case with the *E1B-19K*-encoded protein, which promotes cell survival at early times, while the E3 adenovirus death protein promotes death at late

times (75). It appears that *E4orf1* and *E1B-55K* exert opposing effects at the same time. Systems that incorporate opposing activities to maintain homeostasis are poised to respond rapidly to changes in the environment. Perhaps the integration of *E4orf1* into a signal transduction cascade impinging on translation permits a more rapid response than could be achieved by changes at the level of transcription.

The *E4orf1*-encoded protein is a small polypeptide that occurs as monomers or trimers with distinct functions and binding partners associated with each form (18). The transforming ability of *E4orf1* maps to the PDZ domain-binding element at the carboxy terminus of the protein. The ability to bind cellular PDZ domain-containing proteins is necessary for the *E4orf1*-encoded protein to transform cultured cells in a PI3-kinase-dependent manner (27, 32). One consequence of increased signaling through the PI3-kinase pathway by *E4orf1* is the activation of Akt, mTOR, and p70 S6K. Our results suggest that *E4orf1* may also elicit phosphorylation of both Akt and p70 S6K in a manner that depends on Rho-like GTPase Rac1. Because neither the PI3-kinase inhibitor LY294002 nor the suppression of Rac1 activity overcame the restriction to viral late protein synthesis, *E4orf1* may regulate late protein synthesis through a pathway that has not yet been described. Intriguingly, the *E4orf1*-encoded protein was reported to bind a 70-kDa cellular phosphoprotein independently of its PDZ domain-binding element (18). It will be of interest to determine if this 70-kDa protein contributes to the ability of *E4orf1* to restrict viral late protein synthesis.

Although signaling through the PI3-kinase pathway did not appear to be the means by which *E4orf1* restricts late protein synthesis, this activity may restrict virus-mediated cell killing. Accordingly, exposure to the PI3-kinase inhibitor LY294002 increased the cytolytic nature of the *E1B-55K* deletion mutant virus (data not shown). Moreover, pharmacological agents known to disrupt the Tiam1-Rac1 interaction increased the cytolytic nature of the *E1B-55K* deletion mutant virus to that of the *E1B-55K*/*E4orf1* double-mutant virus (Fig. 6). Thus, *E4orf1* signaling to Tiam1/Rac1 may limit the cytolytic nature of replication-competent oncolytic adenoviruses such as ONYX-015. Another limitation of ONYX-015 as a single agent for cancer therapy includes the limited spread of this virus throughout the tumor (57). Because *E4orf1* restricts both virus production (Fig. 1) and the synthesis of viral structural proteins (Fig. 2 and 3), *E4orf1* may further limit the oncolytic potential of the *E1B-55K* deletion mutant virus by reducing the yield of progeny virus in the tumor. Although it remains to be determined if the *E1B-55K*/*E4orf1* double-mutant virus spreads more effectively in a tumor model than the *E1B-55K* deletion mutant virus, this seems likely in view of the pivotal role *E4orf1* plays in determining so many of the characteristics of this virus.

Other viruses initiate signaling cascades through the Tiam1/Rac1 pathway. The EBNA3C latent protein of Epstein-Barr virus is an oncoprotein that targets the cellular protein Nm23-H1, which in turn prevents Nm23-H1 from limiting tumor metastasis (69). Nm23-H1 negatively regulates Tiam1 and inhibits Rac1 activation in vivo (56). The HIV-1 Nef protein stimulates activation of Vav1 (25), which is a Tiam1-like guanine nucleotide exchange factor (20, 62). Nef activation of Vav1 increases levels of virus transcription and replication (25). Because viruses as diverse as Epstein-Barr virus and HIV target pathways involving Tiam1/Rac1, it seems likely that this represents an important cellular target for additional viruses. In the case of the *E1B-55K* mutant adenovirus, disabling this cellular target appears to increase virus replication, increase the potential for virus spread, and increase virus-mediated cell death. Targeting the Tiam1/Rac1 pathway during oncolytic virus therapy may be especially effective because elevated Tiam1 and Rac1 activities have been associated with more aggressive cancers and a poor clinical outcome (29). Thus, targeting the Tiam1/Rac1 pathway in conjunction with oncolytic adenovirus therapy may improve the cancer cell-killing potential of the *E1B-55K* deletion mutant virus and be an effective multipronged approach to cancer therapy.

ACKNOWLEDGMENTS

We thank Gary Ketner (Johns Hopkins University) and Tom Shenk (Princeton University) for providing viruses used in this study. We thank Arnie Berk (University of California at Los Angeles) for providing *dl*1520 and for the generous gift of adenovirus-specific polyclonal antiserum. We thank Ron Javier (Baylor College) for providing *E4orf1* plasmids, as well as for valuable discussions. We also acknowledge the anonymous reviewers of the manuscript for their insightful comments. We thank Ken Grant of the Micromed facility of the Comprehensive Cancer Center of Wake Forest University for assistance with electron microscopy.

Cell culture reagents were provided by the Cell and Virus Vector Core Laboratory of the Comprehensive Cancer Center of Wake Forest University, which is supported in part by National Cancer Institute grant CA12197. Michael A. Thomas was supported in part by National Research Service award F31 CA11020 from the National Institutes of Health. This work was supported by Public Health Service grants CA77342 and CA77342S1 from the National Cancer Institute to David Ornelles.

REFERENCES

1. **Araujo, F. D., T. H. Stracker, C. T. Carson, D. V. Lee, and M. D. Weitzman.** 2005. Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. J. Virol. **79:**11382–11391.
2. **Babiss, L. E., and H. S. Ginsberg.** 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. J. Virol. **50:**202–212.
3. **Baker, A., K. J. Rohleder, L. A. Hanakahi, and G. Ketner.** 2007. Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation. J. Virol. **81:**7034–7040.
4. **Barker, D. D., and A. J. Berk.** 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology **156:**107–121.
5. **Barraud, P., J. C. Paillart, R. Marquet, and C. Tisne.** 2008. Advances in the structural understanding of Vif proteins. Curr. HIV Res. **6:**91–99.
6. **Berk, A. J.** 2005. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. Oncogene **24:**7673–7685.
7. **Bischoff, J. R., D. H. Kirn, A. Williams, C. Heise, S. Horn, M. Muna, L. Ng, J. A. Nye, A. Sampson-Johannes, A. Fattaey, and F. McCormick.** 1996. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science **274:**373–376.
8. **Blanchette, P., K. Kindsmuller, P. Groitl, F. Dallaire, T. Speiseder, P. E. Branton, and T. Dobner.** 2008. Control of mRNA export by adenovirus E4orf6 and E1B55K proteins during productive infection requires E4orf6 ubiquitin ligase activity. J. Virol. **82:**2642–2651.
9. **Bridge, E., and G. Ketner.** 1990. Interaction of adenoviral E4 and E1b products in late gene expression. Virology **174:**345–353.
10. **Bridge, E., S. Medghalchi, S. Ubol, M. Leesong, and G. Ketner.** 1993. Adenovirus early region 4 and viral DNA synthesis. Virology **193:**794–801.
11. **Buchkovich, N. J., Y. Yu, C. A. Zampieri, and J. C. Alwine.** 2008. The TORrid affairs of viruses: effects of mammalian DNA viruses on the PI3K-Akt-mTOR signalling pathway. Nat. Rev. Microbiol. **6:**266–275.
12. **Buchsbaum, R. J., B. A. Connolly, and L. A. Feig.** 2003. Regulation of p70 S6 kinase by complex formation between the Rac guanine nucleotide exchange factor (Rac-GEF) Tiam1 and the scaffold spinophilin. J. Biol. Chem. **278:**18833–18841.
13. **Burnett, P. E., S. Blackshaw, M. M. Lai, I. A. Qureshi, A. F. Burnett, D. M. Sabatini, and S. H. Snyder.** 1998. Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton. Proc. Natl. Acad. Sci. USA **95:**8351–8356.

14. **Burstein, E. S., D. J. Hesterberg, J. S. Gutkind, M. R. Brann, E. A. Currier, and T. L. Messier.** 1998. The ras-related GTPase rac1 regulates a proliferative pathway selectively utilized by G-protein coupled receptors. Oncogene **17:**1617–1623.
15. **Cao, G., L. M. Liu, and S. F. Cleary.** 1991. Modified method of mammalian cell synchronization improves yield and degree of synchronization. Exp. Cell Res. **193:**405–410.
16. **Chandrasekaran, B., R. L. Capizzi, T. E. Kute, T. Morgan, and J. Dimling.** 1989. Modulation of the metabolism and pharmacokinetics of 1-β-D-arabinofuranosylcytosine by 1-β-D-arabinofuranosyluracil in leukemic mice. Cancer Res. **49:**3259–3266.
17. **Chou, M. M., and J. Blenis.** 1996. The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1. Cell **85:**573–583.
18. **Chung, S. H., R. S. Weiss, K. K. Frese, B. V. Prasad, and R. T. Javier.** 2008. Functionally distinct monomers and trimers produced by a viral oncoprotein. Oncogene **27:**1412–1420.
19. **Corbin-Lickfett, K. A., and E. Bridge.** 2003. Adenovirus E4-34kDa requires active proteasomes to promote late gene expression. Virology **315:**234–244.
20. **Crespo, P., K. E. Schuebel, A. A. Ostrom, J. S. Gutkind, and X. R. Bustelo.** 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature **385:**169–172.
21. **Datta, S. R., A. Brunet, and M. E. Greenberg.** 1999. Cellular survival: a play in three Akts. Genes Dev. **13:**2905–2927.
22. **Davies, S. P., H. Reddy, M. Caivano, and P. Cohen.** 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351:**95–105.
23. **Deshpande, S. S., P. Angkeow, J. Huang, M. Ozaki, and K. Irani.** 2000. Rac1 inhibits TNF-α-induced endothelial cell apoptosis: dual regulation by reactive oxygen species. FASEB J. **14:**1705–1714.
24. **Evans, J. D., and P. Hearing.** 2005. Relocalization of the Mre11-Rad50-Nbs1 complex by the adenovirus E4 ORF3 protein is required for viral replication. J. Virol. **79:**6207–6215.
25. **Fackler, O. T., W. Luo, M. Geyer, A. S. Alberts, and B. M. Peterlin.** 1999. Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. Mol. Cell **3:**729–739.
26. **Flint, S. J., and R. A. Gonzalez.** 2003. Regulation of mRNA production by the adenoviral E1B 55-kDa and E4 Orf6 proteins. Curr. Top. Microbiol. Immunol. **272:**287–330.
27. **Frese, K. K., S. S. Lee, D. L. Thomas, I. J. Latorre, R. S. Weiss, B. A. Glaunsinger, and R. T. Javier.** 2003. Selective PDZ protein-dependent stimulation of phosphatidylinositol 3-kinase by the adenovirus E4-ORF1 oncoprotein. Oncogene **22:**710–721.
28. **Fruman, D. A., R. E. Meyers, and L. C. Cantley.** 1998. Phosphoinositide kinases. Annu. Rev. Biochem. **67:**481–507.
29. **Gao, Y., J. B. Dickerson, F. Guo, J. Zheng, and Y. Zheng.** 2004. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc. Natl. Acad. Sci. USA **101:**7618–7623.
30. **Garber, K.** 2006. China approves world's first oncolytic virus therapy for cancer treatment. J. Natl. Cancer Inst. **98:**298–300.
31. **Glaunsinger, B. A., S. S. Lee, M. Thomas, L. Banks, and R. Javier.** 2000. Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. Oncogene **19:**5270–5280.
32. **Glaunsinger, B. A., R. S. Weiss, S. S. Lee, and R. Javier.** 2001. Link of the unique oncogenic properties of adenovirus type 9 E4-ORF1 to a select interaction with the candidate tumor suppressor protein ZO-2. EMBO J. **20:**5578–5586.
33. **Goodrum, F. D., and D. A. Ornelles.** 1998. p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. J. Virol. **72:**9479–9490.
34. **Goodrum, F. D., and D. A. Ornelles.** 1999. Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. J. Virol. **73:**7474–7488.
35. **Goodrum, F. D., and D. A. Ornelles.** 1997. The early region 1B 55-kilodalton oncoprotein of adenovirus relieves growth restrictions imposed on viral replication by the cell cycle. J. Virol. **71:**548–561.
36. **Habets, G. G., E. H. Scholtes, D. Zuydgeest, R. A. van der Kammen, J. C. Stam, A. Berns, and J. G. Collard.** 1994. Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell **77:**537–549.
37. **Halbert, D. N., J. R. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. **56:**250–257.
38. **Harada, H., J. S. Andersen, M. Mann, N. Terada, and S. J. Korsmeyer.** 2001. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc. Natl. Acad. Sci. USA **98:**9666–9670.
39. **Harada, J. N., A. Shevchenko, D. C. Pallas, and A. J. Berk.** 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J. Virol. **76:**9194–9206.
40. **Holmes, R. K., M. H. Malim, and K. N. Bishop.** 2007. APOBEC-mediated viral restriction: not simply editing? Trends Biochem. Sci. **32:**118–128.
41. **Izumi, T., K. Shirakawa, and A. Takaori-Kondo.** 2008. Cytidine deaminases as a weapon against retroviruses and a new target for antiviral therapy. Mini-Rev. Med. Chem. **8:**231–238.
42. **Javier, R. T.** 1994. Adenovirus type 9 E4 open reading frame 1 encodes a transforming protein required for the production of mammary tumors in rats. J. Virol. **68:**3917–3924.
43. **Jeong, H. G., H. J. Cho, I. Y. Chang, S. P. Yoon, Y. J. Jeon, M. H. Chung, and H. J. You.** 2002. Rac1 prevents cisplatin-induced apoptosis through down-regulation of p38 activation in NIH3T3 cells. FEBS Lett. **518:**129–134.
44. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17:** 683–689.
45. **Jones, N., and T. Shenk.** 1978. Isolation of deletion and substitution mutants of adenovirus type 5. Cell **13:**181–188.
46. **Lee, S. S., B. Glaunsinger, F. Mantovani, L. Banks, and R. T. Javier.** 2000. Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. J. Virol. **74:**9680–9693.
47. **Li, Z., C. P. Day, J. Y. Yang, W. B. Tsai, G. Lozano, H. M. Shih, and M. C. Hung.** 2004. Adenoviral E1A targets Mdm4 to stabilize tumor suppressor p53. Cancer Res. **64:**9080–9085.
48. **Lowe, S. W., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. **7:**535–545.
49. **Luo, K., E. Ehrlich, Z. Xiao, W. Zhang, G. Ketner, and X.-F. Yu.** 2007. Adenovirus E4orf6 assembles with Cullin5-ElonginB-ElonginC E3 ubiquitin ligase through an HIV/SIV Vif-like BC-box to regulate p53. FASEB J. **21:**1742–1750.
50. **Mertens, A. E., R. C. Roovers, and J. G. Collard.** 2003. Regulation of Tiam1-Rac signalling. FEBS Lett. **546:**11–16.
51. **Moore, M., N. Horikoshi, and T. Shenk.** 1996. Oncogenic potential of the adenovirus E4orf6 protein. Proc. Natl. Acad. Sci. USA **93:**11295–11301.
52. **Nayak, R., K. D. Farris, and D. J. Pintel.** 2008. E4Orf6-E1B-55k-dependent degradation of de novo-generated adeno-associated virus type 5 Rep52 and capsid proteins employs a cullin 5-containing E3 ligase complex. J. Virol. **82:**3803–3808.
53. **Ornelles, D. A., R. N. Broughton-Shepard, and F. D. Goodrum.** 2007. Analysis of adenovirus infections in synchronized cells. Methods Mol. Med. **131:** 83–101.
54. **O'Shea, C., K. Klupsch, S. Choi, B. Bagus, C. Soria, J. Shen, F. McCormick, and D. Stokoe.** 2005. Adenoviral proteins mimic nutrient/growth signals to activate the mTOR pathway for viral replication. EMBO J. **24:**1211–1221.
55. **O'Shea, C. C., S. Choi, F. McCormick, and D. Stokoe.** 2005. Adenovirus overrides cellular checkpoints for protein translation. Cell Cycle **4:**883–888.
56. **Otsuki, Y., M. Tanaka, S. Yoshii, N. Kawazoe, K. Nakaya, and H. Sugimura.** 2001. Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. Proc. Natl. Acad. Sci. USA **98:**4385–4390.

56a. **Pilder, S., M. Moore, J. Logan, and T. Shenk.** 1986. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol. **6:**470–476.

57. **Post, D. E., F. R. Khuri, J. W. Simons, and E. G. Van Meir.** 2003. Replicative oncolytic adenoviruses in multimodal cancer regimens. Hum. Gene Ther. **14:**933–946.
58. **Querido, E., P. Blanchette, Q. Yan, T. Kamura, M. Morrison, D. Boivin, W. G. Kaelin, R. C. Conaway, J. W. Conaway, and P. E. Branton.** 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev. **15:** 3104–3117.
59. **Querido, E., R. C. Marcellus, A. Lai, R. Charbonneau, J. G. Teodoro, G. Ketner, and P. E. Branton.** 1997. Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J. Virol. **71:** 3788–3798.
60. **Sarbassov, D. D., S. M. Ali, S. Sengupta, J. H. Sheen, P. P. Hsu, A. F. Bagley, A. L. Markhard, and D. M. Sabatini.** 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell **22:**159–168.
61. **Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini.** 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science **307:**1098–1101.
62. **Schuebel, K. E., N. Movilla, J. L. Rosa, and X. R. Bustelo.** 1998. Phosphorylation-dependent and constitutive activation of Rho proteins by wild-type and oncogenic Vav-2. EMBO J. **17:**6608–6621.
63. **Schwartz, R. A., S. S. Lakdawala, H. D. Eshleman, M. R. Russell, C. T. Carson, and M. D. Weitzman.** 2008. Distinct requirements of adenovirus E1b55K protein for degradation of cellular substrates. J. Virol. **82:**9043–9055.
64. **Sheng, M., and C. Sala.** 2001. PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. **24:**1–29.
65. **Shepard, R. N., and D. A. Ornelles.** 2004. Diverse roles for E4orf3 at late times of infection revealed in an E1B 55-kilodalton protein mutant background. J. Virol. **78:**9924–9935.
66. **Shepard, R. N., and D. A. Ornelles.** 2003. E4orf3 is necessary for enhanced S-phase replication of cell cycle-restricted subgroup C adenoviruses. J. Virol. **77:**8593–8595.

67. **Stracker, T. H., C. T. Carson, and M. D. Weitzman.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418:**348–352.
68. **Stracker, T. H., D. V. Lee, C. T. Carson, F. D. Araujo, D. A. Ornelles, and M. D. Weitzman.** 2005. Serotype-specific reorganization of the Mre11 complex by adenoviral E4orf3 proteins. J. Virol. **79:**6664–6673.
69. **Subramanian, C., M. A. Cotter II, and E. S. Robertson.** 2001. Epstein-Barr virus nuclear protein EBNA-3C interacts with the human metastatic suppressor Nm23-H1: a molecular link to cancer metastasis. Nat. Med. **7:**350–355.
70. **Sumita, C., M. Yamane, T. Matsuda, M. Maeda, T. Nariai, Y. Fujio, and J. Azuma.** 2005. Platelet activating factor induces cytoskeletal reorganization through Rho family pathway in THP-1 macrophages. FEBS Lett. **579:**4038–4042.
71. **Tay, D. L., P. S. Bhathal, and R. M. Fox.** 1991. Quantitation of G_0 and G_1 phase cells in primary carcinomas. Antibody to M1 subunit of ribonucleotide reductase shows G_1 phase restriction point block. J. Clin. Investig. **87:**519–527.
72. **Ullman, A. J., and P. Hearing.** 2008. Cellular proteins PML and Daxx mediate an innate antiviral defense antagonized by the adenovirus E4 ORF3 protein. J. Virol. **82:**7325–7335.
73. **Ullman, A. J., N. C. Reich, and P. Hearing.** 2007. Adenovirus E4 ORF3 protein inhibits the interferon-mediated antiviral response. J. Virol. **81:**4744–4752.
74. **Weitzman, M. D., and D. A. Ornelles.** 2005. Inactivating intracellular antiviral responses during adenovirus infection. Oncogene **24:**7686–7696.
75. **Wold, W. S., A. E. Tollefson, and T. W. Hermiston.** 1995. E3 transcription unit of adenovirus. Curr. Top. Microbiol. Immunol. **199**(Pt. 1)**:**237–274.
76. **Woo, J. L., and A. J. Berk.** 2007. Adenovirus ubiquitin-protein ligase stimulates viral late mRNA nuclear export. J. Virol. **81:**575–587.
77. **Wu, H. C., J. T. Hsieh, M. E. Gleave, N. M. Brown, S. Pathak, and L. W. Chung.** 1994. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int. J. Cancer **57:**406–412.
78. **Yu, W., and H. Fang.** 2007. Clinical trials with oncolytic adenovirus in China. Curr. Cancer Drug Targets **7:**141–148.