Adenovirus-Induced Extracellular Signal-Regulated Kinase Phosphorylation during the Late Phase of Infection Enhances Viral Protein Levels and Virus Progeny

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Abstract

The Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling cascade enhances tumor cell proliferation in many cases. Here, we show that adenovirus type 5, a small DNA tumor virus used in experimental cancer therapy, strongly induces ERK phosphorylation during the late phase of infection. Pharmacologic inhibition of ERK phosphorylation reduced virus recovery by >100-fold. Blocking MEK/ERK signaling affected virus DNA replication and mRNA levels only weakly but strongly reduced the amount of viral proteins, independently of the kinases MNK1 and PKR. Hence, adenovirus induces the oncogenic Raf/MEK/ERK signaling pathway to enhance viral progeny by sustaining the levels of viral proteins. Concerning therapy, our results suggest that the use of Raf/MEK/ERK inhibitors will interfere with the propagation of oncolytic adenoviruses. (Cancer Res 2006; 66(3): 1282-8)

Introduction

Ras, Raf, mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinases (MEK), and exogenously regulated kinases (ERK) constitute a signaling pathway that enables malignant growth in a high proportion of human cancer species. Our knowledge on its role in virus infection is more limited, but some viruses have been shown to induce ERK phosphorylation upon virus adhesion or at other very early stages of infection (1). However, less is known about the activity of intracellular signaling in the later stages of virus infection and its contribution to virus replication. Intriguingly, influenza virus propagation largely depends on intact Raf/MEK/ERK signaling (2).

Adenovirus is a small DNA tumor virus expressing several oncoproteins that transform cells. These oncoproteins target the pRb and p53 tumor suppressors, but it is unknown whether and how the virus might affect Ras signaling and vice versa. Such knowledge would also be crucial for the development of oncolytic viruses that specifically replicate in cancer cells. Thus far, it was only reported that adenovirus induces ERK phosphorylation initially upon infection, independently of virus replication (3), and detailed analysis of Ras-induced signaling was only provided using nonreplicating adenovirus vectors (4). We sought to determine how intracellular signaling is affected through all stages of adenovirus infection, and whether the corresponding signaling pathways support the viral life cycle.

Materials and Methods

Cells and virus. NCI-H1299 cells (American Type Culture Collection, Rockville, MD) were maintained in DMEM containing 10% fetal bovine serum. Cells were treated with human leukocyte IFN-α (Sigma, St. Louis, MO) at 1,000 units/mL and kinase inhibitors LY294002 at 50 μmol/L, U0126 (both Calbiochem, La Jolla, CA), and CGP57380 (kindly provided by Dr. Hermann Gram, Novartis Pharma AG, Basel, Switzerland) at 10 μmol/L, or the corresponding volume of the solvent DMSO (Merck, Darmstadt, Germany). SL327 and PD098059 (Calbiochem) were used at 100 μmol/L or the indicated concentration. 309p virus was recovered from H1299 cells transfected with the genome of the commonly used Ad5 dl309 laboratory strain (5). Virus titers were determined by immunofluorescence as fluorescence forming units per cell as described previously (6).

Immunoblotting. Immunoblot analysis was conducted as described before, using whole-cell lysates (7). The following antibodies were used: peroxidase-coupled secondary antibodies (Fab fragments; Jackson, West Grove, PA), phospho-eIF2α[Ser^{52}], eIF2α (both Biosource, Camarillo, CA), β-actin (AC15; Abcam, Cambridge, MA), phospho-AKT[Ser^{473}], AKT (Cell Signaling, Beverly, MA), phospho-ERK1/2[Tyr^{204}] (E-4; Santa Cruz Biotechnology, Santa Cruz, CA), ERK1 (K-23; Santa Cruz Biotechnology), adenovirus E1A (M73; Calbiochem), adenovirus E2A (B6-8 obtained from J. Flint, Department of Molecular Biology, Princeton University, Princeton, NJ), adenovirus fiber (4D2; Neomarkers, Fremont, CA), lamin B1 (L-5; Zymed, South San Francisco, CA), β-tubulin (H-235; Santa Cruz Biotechnology), phospho-MNK1[Thr^{197}/Thr^{202}], phospho-S6[Ser^{235}/Ser^{236}], and phospho-eIF4E[Ser^{209}] (all three from Cell Signaling).

Reverse transcription-PCR. Reverse transcription and PCR were conducted as described previously (7). The following PCR program was used: 96°C for 3 minutes, and the indicated number of cycles at 96°C for 30 seconds, 57°C for 30 seconds, and 70°C for 30 seconds. For each gene, the following specific reverse transcription and reverse transcription-PCR (RT-PCR) primers were employed: E1A (only 13S and 12S transcripts), 20/25/30 cycles, reverse transcription, GGTGATGTCGGGCGTCTCAGG; forward, GCGGTTTCGCAGATTTTTCCCG; reverse, GCAGGCGCCATTTTAGGACG; E1A (all transcripts), same except forward, GCCACGGAGGTGTTATTACC; L5, 25 cycles, reverse transcription, GGCAAGCACCTC TGGCACGGCAAATACGG; forward, GCGGTTGAGGACAAACTCTTCGCG; reverse, GCGCTGCAGATGCTTCAACAGCACG; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 20 cycles, reverse transcription, GGTTCACACCCATGACGAACATG; forward, TGAAGGTCGGAGTCAACGGATTTGGT; reverse, GCAGAGATGATACCCTTTTGGCTC. To assess the levels of cytoplasmic mRNAs, cells were fractionated before RNA isolation as described previously (6).

Assessment of virus DNA levels. Infected H1299 cells were harvested followed by preparation of genomic DNA (Qiagen, Chatsworth, CA) and semiquantitative real-time PCR, using cycles at 96°C for 1 second, 62°C for 5 seconds, and 70°C for 10 seconds. The DNA amount was quantified at 89°C after every cycle. The following primers were used: forward,

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AGCGGGCACTCTTCCGTGG and reverse, GTCTGACGTCGCACACCTGGG. CP values describe the cycle of the amplification curve's changing point. Amplification of viral genomic DNA was assessed by calculating the difference of the CP values of the samples from 12, 18, and 24 hours to those from the corresponding 3-hour samples (Δcyc) and assuming the doubling of the PCR product with every cycle (DNA amplification = $2^{\Delta cyc}$).

Polyribosome analysis. Cell fractionation of infected H1299 cells was essentially conducted as described before (8). However, cytoplasmic extracts were obtained by dounce homogenization rather than addition of detergents. RNA was isolated using the Trizol-LS (Invitrogen, San Diego, CA) protocol for liquid samples, including the modified isopropanol precipitation step for polysaccharide containing solutions.

Results

Ras-dependent signaling pathways at late stages of adenovirus infection. To investigate the behavior of Ras-dependent signaling pathways during adenovirus infection, we measured the degree of ERK and AKT phosphorylation at different time points after infection. We infected H1299 cells with wild-type adenovirus 309p (5) and determined ERK and AKT phosphorylation by immunoblot analysis. Both kinases were increasingly phosphorylated after adenovirus infection (Fig. 1*A*). ERK phosphorylation increased 1 to 3 hours after infection, as expected from previous studies (1). Strikingly, following this first wave of ERK activation, ERK phosphorylation increased again at 12 hours and even more at 24 hours after infection to exceed the level of the first wave. This apparently biphasic increase in phosphorylation suggested that the second wave of phosphorylation may be linked to the onset of virus DNA replication and the entry into the late phase of the infectious cycle. To test the dependence of ERK phosphorylation on DNA replication, the infected cells were treated with cytosine-arabinoside (AraC), a known inhibitor of adenovirus DNA synthesis. As expected, AraC suppressed the expression of a late virus gene (*L5,* encoding the Fiber protein) but not that of an early gene (*E1A*). In parallel, AraC abolished the second wave of ERK phosphorylation but not AKT phosphorylation (Fig. 1*A*). We conclude that adenovirus promotes ERK signaling specifically in the late phase of infection.

Next, we employed pharmacologic inhibitors to block the major Ras-dependent signaling pathways and analyzed their effects on virus replication. We used LY294002 to specifically abrogate the activity of phosphatidylinositol-3 kinase (PI3K), the upstream kinase of AKT, and U0126 to block MEK1/2-mediated phosphorylation of ERK1/2. The inhibitors were added to the culture medium 3 hours after infection to ensure that the first wave of Ras activation, suspected to enable the efficient entry of the viral particles (1), was undisturbed. We confirmed the efficacy of the inhibitors in adenovirus-infected H1299 cells by immunoblot analysis of AKT and ERK phosphorylation (Fig. 1*B*) and then determined the virus yield. Although the MEK inhibitor, U0126, reduced the virus yield ~300-fold, the block of PI3K signaling decreased the virus titers only by one order of magnitude (Fig. 1*C*). Alternative inhibitors of ERK phosphorylation were then used in similar assays. PD098059, a weaker inhibitor of MEK than U0126 (9), only partially suppressed ERK phosphorylation in adenovirus-infected cells and was further attenuated by increased multiplicity of infection (Fig. 1*C*), again suggesting that adenovirus infection increases ERK phosphorylation activity. Nonetheless, PD098059 partially suppressed virus replication. An additional inhibitor of ERK phosphorylation, SL327, turned out to work more efficiently in this system (Fig. 1*D*), and its application reduced virus replication roughly 300-fold. Taken together, these results strongly suggest that MEK/ERK signaling is required for efficient formation of viral progeny.

Adenovirus life cycle depending on Ras signaling. We analyzed the effect of the signaling inhibitors on viral gene expression and DNA replication at different time points after infection. H1299 cells were infected, and signaling inhibitors were added as above. The accumulated virus DNA was then quantified by real-time PCR. Surprisingly, DNA replication was reduced only less than an order of magnitude by either inhibitor (Fig. 2*A*), suggesting that DNA replication is not primarily blocked by Ras signaling inhibition. Next, we analyzed viral gene products at the protein level. At 10 and 15 hours after infection, the expression levels of the adenoviral early proteins E1A and E2A were reduced by both inhibitors (Fig. 2*B*). However, at 24 hours after infection, U0126 but not LY294002 suppressed viral protein levels almost below detection limits. Adenovirus Fiber protein expression was suppressed by U0126 in parallel. In contrast, the levels of corresponding viral mRNA remained unaffected by inhibitor treatment, as determined by semiquantitative RT-PCR (Fig. 2*C*). Given the discrepancy between E1A mRNA and protein levels, we asked whether reduced protein levels caused by U0126 could result from decreased protein stability. We thus treated virus-infected cells with U0126 or DMSO solvent alone and blocked protein synthesis using cycloheximide after 24 hours. We determined the amount of E1A protein by immunoblot analysis at several time points after cycloheximide addition. Although total E1A protein levels were again markedly reduced after U0126 treatment, we did not observe any difference in the kinetics of E1A decay (Fig. 3*A*). We thus conclude that blocking MEK/ERK signaling does not alter E1A protein stability. We next asked whether U0126 might interfere with the nuclear export of the E1A transcripts, analogous to the inhibition of influenza virus RNP export in response to U0126 (2). We thus fractionated the infected and/or inhibitor-treated cells to separate the nuclei from the cytoplasmic content and determined the amount of E1A, L5, and GAPDH mRNA in the cytoplasmic fractions by semiquantitative RT-PCR (Fig. 3*B*). We observed equal levels of mRNAs in the cytoplasm, indicating that Ras signaling inhibitors do not impair mRNA export during adenovirus infection. Rather, it seems to interfere with virus protein synthesis. To investigate this, we determined the ribosome association of viral and cellular mRNAs by polyribosome analysis. Upon U0126 treatment, we observed a marked decrease of ribosome association with viral and cellular mRNAs (Fig. 3*C*), suggesting that MEK/ERK signaling is important for efficient translation within the infected cell.

Translational regulators in adenovirus replication. Ras signaling affects translation in several known ways (Fig. 4*A*), and we analyzed the involved factors to see whether they might be responsible for the reduction of adenovirus protein levels by U0126. One possible mechanism allowing Ras signaling to regulate translation occurs through the phosphorylation of ribosomal proteins. In particular, phosphorylation of the ribosomal protein S6 is long known to depend on active AKT and ERK signaling. Strikingly, adenovirus E1A has been shown to induce p70S6K an S6 kinase (10). We therefore tested whether Ras inhibitors were able to alter S6 phosphorylation in adenovirus-infected cells. As shown in Fig. 4*B*, phosphorylation of S6 is dramatically reduced by LY294002 but hardly affected by U0126 treatment and thus behaves in strong contrast to the observed effects on viral protein synthesis. Hence, S6 phosphorylation cannot be held responsible for impaired adenovirus growth under MEK inhibition. Second, ERK activates MNK1, a kinase that phosphorylates eIF4G and eIF4E, the cap-binding protein. Accordingly, we observed that adenovirus infection increased the levels of phosphorylated MNK1, whereas

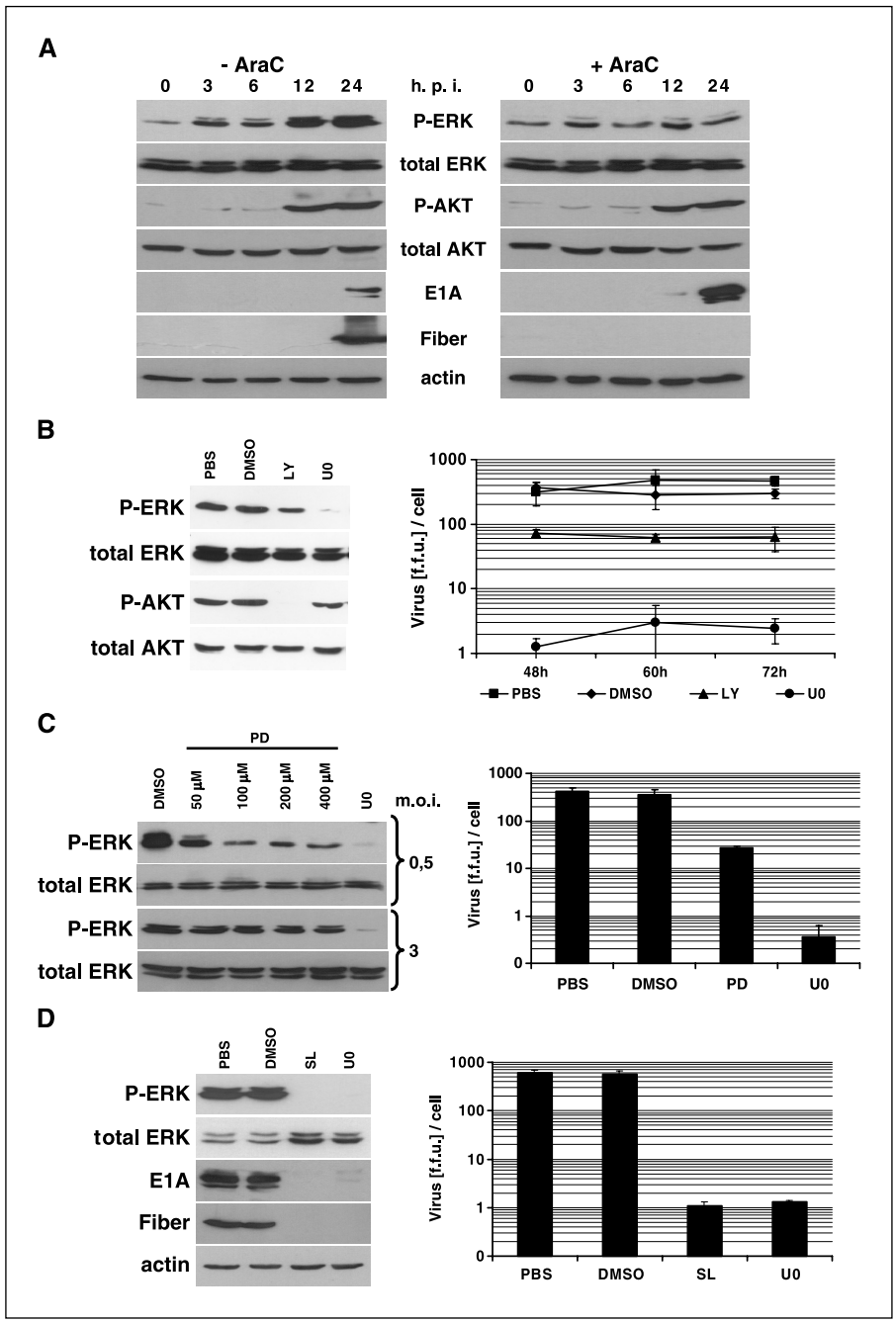

Figure 1. Mutual enhancement of adenovirus replication and Ras-dependent signaling. *A,* response of Ras-dependent signaling pathways to adenovirus infection. H1299 cells were infected with adenovirus 309p at a multiplicity of infection (*m.o.i.*) of 3 and treated with AraC (20 μg/mL) 30 minutes after infection where indicated. At the indicated time points, samples were taken and subjected to immunoblot analysis using antibodies that specifically recognize activating phosphorylations of ERK or AKT, or the indicated proteins. *B,* effect of Ras signaling inhibition on adenovirus yield. H1299 cells were infected with 309p at a multiplicity of infection of 3. Ras signaling inhibitors LY294002 (*LY*; 50 μmol/L) and U0126 (*U0*; 10 μmol/L), the DMSO solvent, or PBS (0.1%, v/v) were added to the culture medium 3 hours after infection. Twenty-four hours after infection, lysates were analyzed by immunoblot analysis with antibodies against the indicated proteins. Virus yields were determined after infection of H1299 cells with 309p at a multiplicity of infection of 0.5 (avoiding the possible overruling of regulatory mechanisms by exceedingly high multiplicity of infection). The indicated signaling inhibitors were added as above. At the indicated time points after infection, virus yield was determined. *Points,* average virus yield per cell from three independent experiments on a log scale; *bars,* SD. *C,* effect of MEK inhibitor PD098059 on adenovirus yield. Efficacy of PD098059 in infected cells was analyzed as in (*B*) and, after showing no effect on ERK phosphorylation, also at a multiplicity of infection of 0.5. MEK inhibitor U0126 or the DMSO solvent were added as controls. PD098059 was added 3 hours after infection at the indicated concentrations, DMSO at 0.8% (v/v), and U0126 as above. Twenty-four hours after infection, ERK phosphorylation was assessed by immunoblot analysis. Virus yield was assayed as in (*B*) after treatment with 400 μmol/L PD098059 or the controls as above. *D,* effect of MEK inhibitor SL327 on adenovirus yield. Efficacy of SL327 was assayed as in (*B*), applying SL327 at 100 μmol/L, U0126 as above, and the DMSO solvent or PBS at 1% (v/v) 3 hours after infection. Virus yields were assayed as in (*B*). *h.p.i.,* hours post infection.

U0126 (and, to a lesser extent, LY294002) reduced MNK1 phosphorylation (Fig. 4*B*). To assess whether this might be responsible for the reduction of viral proteins, we employed a specific inhibitor of MNK1, CGP57380 (ref. 11; kindly provided by Dr. Hermann Gram). As expected, CGP57380 reduced the phosphorylation of eIF4E even more strongly than U0126 but did not affect ERK phosphorylation (Fig. 4*C*). When virus replication and virus protein levels were determined as above, we found that CGP57380, unlike U0126, did not detectably influence either of them (Fig. 4*C*). Thus, reduced MNK1 activity is not responsible for the inhibitory effect of blocking ERK on adenovirus replication and protein levels. Although ERK also governs the phosphorylation and activity of elongation factor 2 (eEF2) in different systems (12), its phosphorylation was not found to be altered by U0126 in adenovirus-infected cells (Fig. 4*C*), suggesting that differential eEF2 phosphorylation is not the reason for decreased synthesis of virus proteins upon inhibition of ERK phosphorylation. Finally, another important regulator of translation in the context of virus infection is the cellular IFN-inducible kinase PKR, which upon activation is able to block translation through phosphorylation of the initiation factor eIF2α (13). Ras was reported to inhibit PKR (14). We therefore tested whether Ras inhibitors might alter eIF2α phosphorylation in adenovirus-infected cells. As shown in Fig. 3*D*, U0126 and LY294002 did not increase the amount of phosphorylated eIF2α, regardless of virus infection, IFN addition, or treatment with the PKR inducer polyIC. We conclude that eIF2α phosphorylation cannot be held responsible for the observed reduction in virus yield upon MEK inhibition.

Discussion

Signaling induced by virus infection. Our results show that adenovirus strongly enhances Ras signaling not only during the initial stages of infection but even more so in the late phase. A similar biphasic activation of ERK was previously observed during infection with influenza A virus (2) and coxsackievirus (15). The X protein from hepatitis B virus has been shown to induce ERK phosphorylation (16). Because E1A has been shown to mediate the nuclear translocation of phospho-ERK when transiently expressed (17), it might contribute to adenovirus-induced ERK signaling, but our studies using an inhibitor of viral DNA replication suggest that the late phase of the viral life cycle must be reached for full ERK phosphorylation.

Adenovirus not only induces ERK phosphorylation but also seems to depend on it. Most strikingly, the levels of adenovirus proteins are strongly reduced by inhibitors of ERK phosphorylation. Our results suggest that decreased translation is responsible. However, changes in the phosphorylation of MNK, PKR, S6, or eEF2 do not seem to mediate ERK-dependent translation of virus mRNA.

Oncolytic viruses. Viruses with a preference for cells with high Ras signaling may be useful in therapy. It has been proposed that

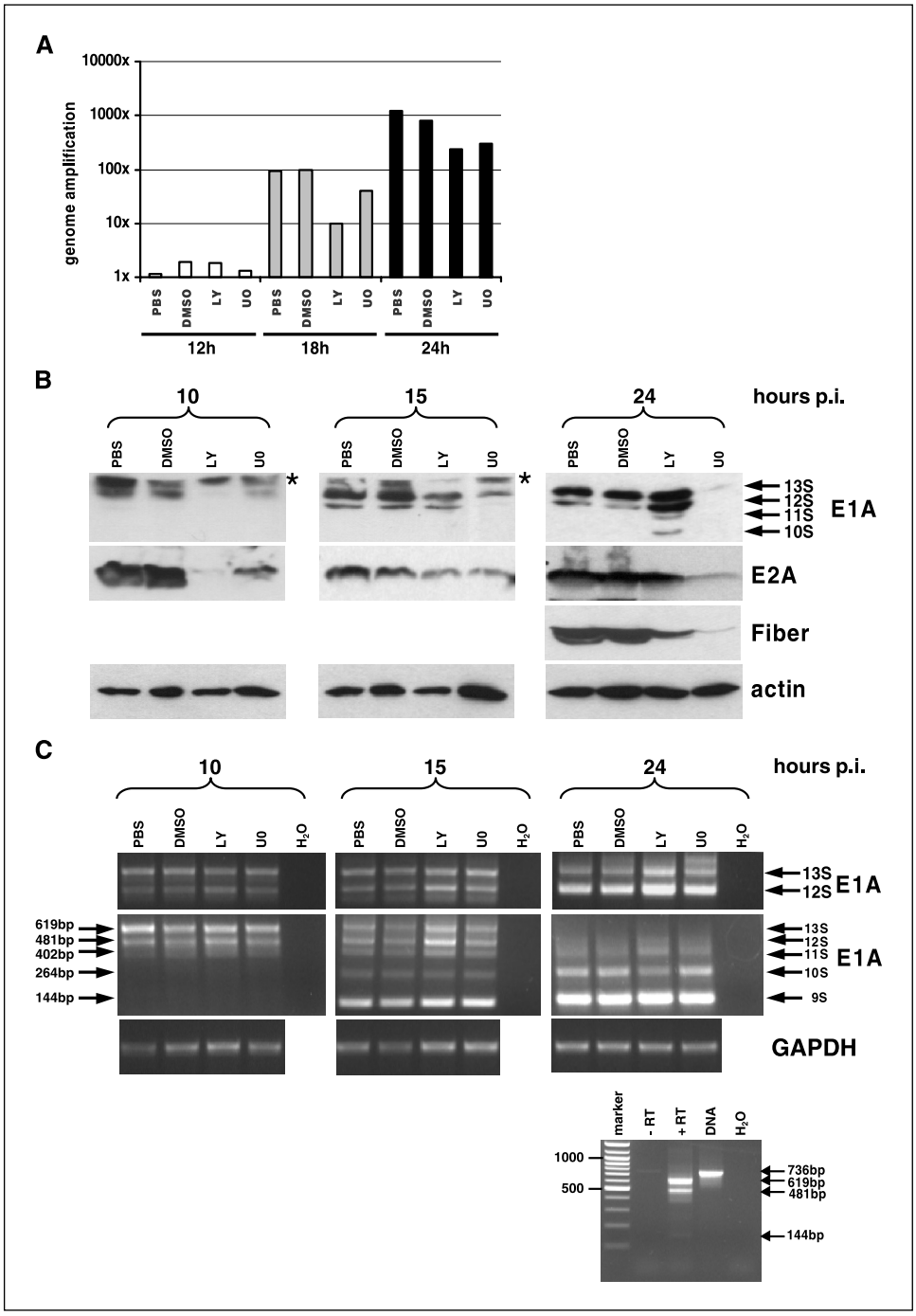

Figure 2. Effect of Ras signaling inhibitors on the viral life cycle. *A,* DNA replication. H1299 cells were infected with 309p at a multiplicity of infection of 0.5. The indicated signaling inhibitors were added as described above. After 3, 12, 18, and 24 hours, the amount of viral DNA was assessed by semiquantitative real-time PCR. Genome amplification was calculated as the relative increase of viral DNA content at every time point compared with the amount at 3 hours. *B,* protein synthesis. H1299 cells were infected and treated as above. After 10, 15, and 24 hours, cells were harvested, and immunoblot analysis was done with specific antibodies against the indicated proteins. **,* background band constantly seen on long exposures independently of infection. *C,* mRNA levels. H1299 cells were infected and treated as above. At 10, 15, and 24 hours post infection (*p.i.*), the relative amount of E1A and GAPDH transcripts was determined by semiquantitative RT-PCR analysis. Of note, LY294002 (*LY*) treatment altered the ratio between the different E1A splicing products; this may imply a role of AKT signaling in mRNA splicing, as recently reported for bovine papillomavirus (20). RNA of the 10-hour PBS sample with and without reverse transcription and genomic DNA were used as template in a control experiment to exclude DNA contamination. *U0,* U0126.

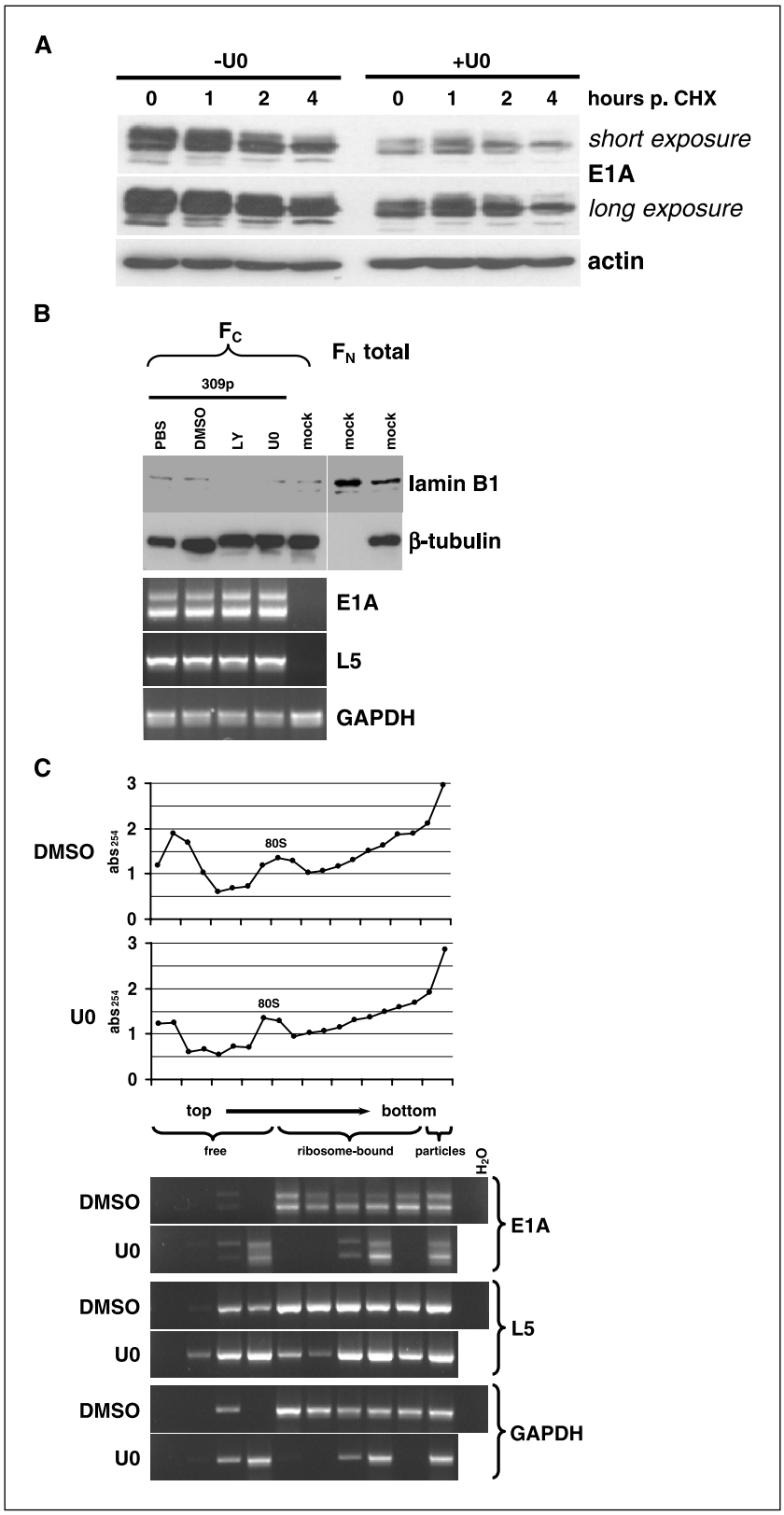

Figure 3. Effect of Ras signaling inhibitors on viral protein accumulation. *A,* E1A protein stability. H1299 cells were infected with a multiplicity of infection of 0.5 and treated with U0126 (*U0*; 10 μmol/L) or DMSO (0.1%, v/v) 3 hours after infection. After 24 hours, cycloheximide (*CHX*) was added to a final concentration of 50 μg/mL. Cells were harvested at 0, 1, 2, and 4 hours post cycloheximide (*p. CHX*) treatment, and lysates were subjected to immunoblot analysis with specific antibodies against the indicated proteins. *B,* mRNA export. H1299 cells were mock infected or infected as above and treated with LY294002 (*LY*; 50 μmol/L), U0126 (10 μmol/L), DMSO, or PBS (0.1% v/v each) or left untreated. After 24 hours, cells were harvested, and nuclei were separated from the cytoplasm by cell fractionation as described (6). Fractions from equal amounts of cells were analyzed by immunoblot analysis with specific antibodies against the indicated proteins and by RT-PCR analysis of E1A, L5, and GAPDH mRNA. *C,* translation. H1299 cells were infected and treated as in (*A*). Twenty-four hours after infection, the association of polysomes with the indicated mRNAs was assessed by RT-PCR analysis of RNA from fractions obtained by sucrose-gradient centrifugation of cytoplasmic extracts. The absorption profile of the fractions at 254 nm reveals the position of the 80S peak, which represents unbound ribosomes. Free mRNA molecules migrate above, and ribosome-bound mRNA migrate below the 80S peak.

viruses with particular sensitivity towards the IFN-responsive PKR enzyme may be used for selective targeting of tumor cells with active Ras, because Ras might lead to PKR inactivation (14). Consequently, an adenovirus recombinant without the PKR antagonist VAI (dVAI) was proposed for use in therapy (18). However, we could not observe any effect of Ras signaling on PKR activity. Instead, our findings suggest that adenovirus replication in general strongly depends on active Ras signaling. Considering its

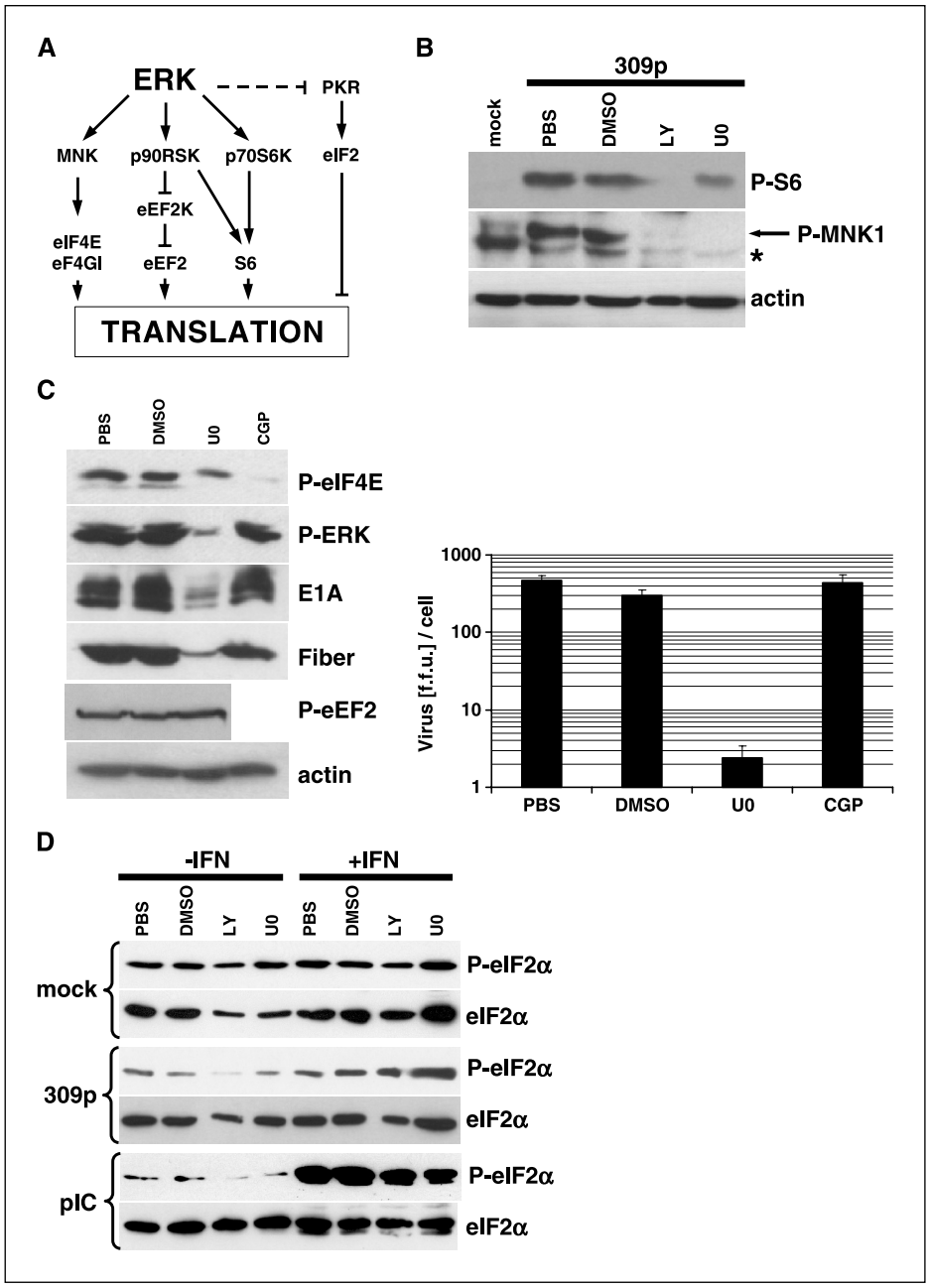

Figure 4. ERK-dependent regulators of translation during adenovirus replication. *A,* effect of ERK on translation. Schematic diagram of known links between ERK signaling and translation. *Arrows,* activating effects; *blunted arrows,* repressive effects. *Dashed arrow,* yet unknown mediators of ERK-dependent PKR repression. *eEF2K,* eEF2 kinase; *eIF2,* eukaryotic initiation factor 2; *eIF4E/G,* eukaryotic initiation factors E and G; *MNK,* mitogen-activated protein kinase interacting kinases; *p70S6K,* 70-kDa S6 kinases; *p90RSK,* 90-kDa ribosomal S6 kinase; *PKR,* protein kinase activated by double-stranded RNA; *S6,* ribosomal protein S6. *B,* phosphorylation of ribosomal protein S6 and MNK1 during adenovirus infection. H1299 cells were infected with 309p at a multiplicity of infection of 3 or mock infected. Ras signaling inhibitors LY294002 (*LY*), U0126 (*U0*), DMSO, or PBS were added as described above or cells were left untreated. Twenty-four hours after infection, the cells were harvested, and immunoblot analysis was done with specific antibodies against the indicated proteins. **,* background band. *C,* effect of MNK1 inhibition on adenovirus replication and yield. H1299 cells were infected with 309p at a multiplicity of infection of 3. Cells were treated with the MNK1 inhibitor CGP57380 at 10 μmol/L or U0126, DMSO, or PBS 3 hours after infection as described above. Twenty-four hours after infection, cells were subjected to immunoblot analysis with specific antibodies against the indicated proteins. To assess the effect of MNK inhibition on virus yield, H1299 cells were infected with a multiplicity of infection of 0.5 and treated as before every 24 hours. Seventy-two hours after infection, virus yield was determined as described above. *D,* effect of Ras signaling inhibitors on eIF2α phosphorylation. H1299 cells were infected with 309p at a multiplicity of infection of 3 or mock infected in the absence or presence of IFN-α. The indicated signaling inhibitors were added as described above. In addition, another set of cells was transfected with polyIC (2 μg/mL) using LipofectAMINE 2000 (Invitrogen). Cells were treated with interferon alpha (1,000 IU/mL) where indicated and subsequently exposed to Ras signaling inhibitors or DMSO as described above. Twenty-four hours after infection and 8 hours after transfection, respectively, cells were harvested, and immunoblot analysis was conducted with specific antibodies against the indicated proteins.

severe effect on viral protein synthesis, constitutively activated Ras may well contribute to override the translational block established by PKR during dVAI infection. However, because Ras signaling is actively triggered during adenovirus infection, it should be considered that oncolytic adenoviruses might by themselves induce the signaling pathway that their selectivity was built on.

It has been reported that the expression of the coxsackievirus-adenovirus receptor is up-regulated by Raf/MEK/ERK inhibitors, and the use of such inhibitors was suggested to increase the efficacy of replicating oncolytic adenoviruses (19). Our results argue against such an approach, whereas the initial infectivity might be enhanced by ERK inhibition, the production of progeny drops, and this will presumably lead to reduced intratumoral spread of the virus.

Pathogenesis and antiviral therapy. Adenovirus infections are mostly harmless but can occasionally cause serious threads, especially when inducing keratoconjunctivitis. Provided that the findings described here could be generalized to other adenovirus serotypes, pharmacologic inhibitors of Ras signaling might be useful not only against malignant but also against viral diseases.

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