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Adenoviral proteins mimic nutrient/growth signals to activate the mTOR pathway for viral replication

Clodagh O'Shea[1,*], Kristina Klupsch[1,3,4], Serah Choi[1,4], Bridget Bagus[1], Conrado Soria[1], Jerry Shen[2], Frank McCormick[1] and David Stokoe[1,*]

[1]Cancer Research Institute, University of California, San Francisco, CA, USA and [2]Onyx Pharmaceuticals, Richmond, CA, USA

Like tumor cells, DNA viruses have had to evolve mechanisms that uncouple cellular replication from the many intra- and extracellular factors that normally control it. Here we show that adenovirus encodes two proteins that activate the mammalian target of rapamycin (mTOR) for viral replication, even under nutrient/growth factor-limiting conditions. E4-ORF1 mimics growth factor signaling by activating PI3-kinase, resulting in increased Rheb.GTP loading and mTOR activation. E4-ORF4 is redundant with glucose in stimulating mTOR, does not affect Rheb.GTP levels and is the major mechanism whereby adenovirus activates mTOR in quiescent primary cells. We demonstrate that mTOR is activated through a mechanism that is dependent on the E4-ORF4 protein phosphatase 2A-binding domain. We also show that mTOR activation is required for efficient S-phase entry, independently of E2F activation, in adenovirus-infected quiescent primary cells. These data reveal that adenovirus has evolved proteins that activate the mTOR pathway, irrespective of the cellular microenvironment, and which play a requisite role in viral replication.

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Introduction

DNA viruses are obligate intracellular parasites whose lytic cycle is totally dependent on their ability to commandeer their host's replicative machinery. Therefore, DNA viruses encode proteins that subvert many of the same pathways that are deregulated in tumorigenesis. For example, in tumor cells, the Rb/p53 checkpoints are inactivated through mutations, while DNA viruses encode viral proteins to achieve the same end, for example adenovirus E1A and E1B-55K, respectively. Indeed, p53 (Linzer and Levine, 1979) and E2F (Kovesdi *et al*, 1986) were first discovered through the study of DNA viruses. An understanding of DNA viral proteins, the cellular pathways they perturb and their requisite role in adenovirus replication is therefore a fertile strategy for identifying novel cellular targets that may be perturbed in tumorigenesis. In this study, we use adenovirus to gain new insights into the regulation and functions of the mammalian target of rapamycin (mTOR), an important tumor target.

mTOR integrates nutrient and growth factor signals to regulate the translation initiation of mRNAs important for cell growth (Proud, 2002). Constitutive mTOR activation is thought to underlie the pathology associated with the inherited cancer syndrome tuberous sclerosis, a genetic disorder caused by inactivating mutations in either tuberin (TSC2) or hamartin (TSC1). TSC1 and TSC2 form a complex that inhibits mTOR, by decreasing Rheb.GTP levels through the GTPase-activating protein (GAP) activity of tuberin (Harris and Lawrence, 2003). mTOR plays a critical role in mediating growth factor/PI3-kinase signals that are commonly deregulated in tumorigenesis (Majumder *et al*, 2004; Wendel *et al*, 2004). Growth factors are thought to stimulate mTOR through activation of PI3-kinase and protein kinase B (PKB)/Akt, resulting in the phosphorylation of TSC2 and inactivation of the TSC1/TSC2 complex (McManus and Alessi, 2002). Another mechanism whereby growth factors may stimulate mTOR is through activation of phospholipase D (PLD), which produces phosphatidic acid (PA) that binds to mTOR and stimulates its kinase activity (Fang *et al*, 2001).

Amino acids (Hara *et al*, 1998), glucose (Patel *et al*, 2001) and ATP (Inoki *et al*, 2003b) also activate mTOR, albeit through less well-understood mechanism(s). Recently, the hyperstimulation of nutrient signaling pathways has been implicated in cancer. The genetic cancer syndrome Peutz–Jeghers disease is caused by inactivating mutations in LKB1, a protein kinase that regulates AMP kinase in response to AMP levels. Nutrient deprivation increases the AMP/ATP ratio in the cell, which activates AMP kinase, resulting in the phosphorylation of TSC2, thereby enhancing the ability of the TSC1/TSC2 complex to repress mTOR (Inoki *et al*, 2003b; Kimura *et al*, 2003). Thus, loss of LKB1 activity results in constitutive mTOR activation even upon nutrient withdrawal (Shaw *et al*, 2004).

Given the emerging importance of mTOR as a pivotal regulator of cell growth, understanding the mTOR signaling pathway may reveal new tumor targets. There is a profound functional overlap between DNA viruses and tumor cells with respect to the cellular pathways they each must perturb for their replication. Therefore, we investigated whether adenovirus has evolved viral proteins to activate PI3-kinase/mTOR for its replication in quiescent primary cells. This paper

*Corresponding authors. C O'Shea, UCSF Cancer Center, 2340 Sutter Street, Box 0128, San Francisco, CA 94115, USA. E-mail: oshea@cc.ucsf.edu or D Stokoe, Tel.: +1 415 502 2598; Fax: +1 415 502 3179; E-mail: stokoe@cc.ucsf.edu
[3]Present address: Cancer Research UK, 44 Lincolns Inn Fields, London WC2A 3PX, UK
[4]These two authors contributed equally to this work

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reveals a novel viral strategy to stimulate mTOR activity, even upon nutrient/growth factor deprivation, and which plays a requisite role in adenoviral replication.

Results

Adenovirus activates the PI3-kinase/mTOR pathway to replicate in primary cells

PI3-kinase/mTOR is important for the initiation of DNA synthesis. Therefore, we hypothesized that adenovirus may activate PI3-kinase/mTOR for its replication. We examined PI3-kinase activation in adenovirus-infected quiescent primary small airway epithelial cells (SAECs). PI3-kinase was activated in SAECs infected with wild-type (WT) adenovirus as demonstrated by a two- to three-fold increase in phosphatidylinositol (3,4) bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P3), but not in cells infected with a nonreplicating control virus that is defective for E1A expression (Figure 1A). These data demonstrate that the activation of PI3-kinase in adenovirus-infected primary cells occurs subsequent to E1A expression, and that viral binding/internalization is not sufficient. The phosphorylation (Figure 1B) and activation (data not shown, and Figure 2E) of the PI3-kinase downstream effector PKB (also known as Akt) and also the mTOR effector $p70^{S6K}$ were also induced in adenovirus-infected SAECs, while the total levels of these proteins were unaffected.

To test whether activation of PI3-kinase/mTOR is necessary for adenovirus replication in primary cells, we used the PI3-kinase/mTOR inhibitor LY294002 (Brunn *et al*, 1996). Figure 1B demonstrates that LY294002 prevented the induction of both PKB and $p70^{S6K}$ phosphorylation in adenovirus-infected SAECs. LY294002 was added 4 h postinfection (h.p.i.) and did not affect viral uptake, as evidenced by E1A expression (Figure 1D). However, the inhibition of PI3-kinase/mTOR activation by LY294002 resulted in a 10- to 50-fold reduction in the yield of infectious virus particles (Figure 1C). LY294002 also inhibited S-phase entry in infected cells, despite E1A-mediated activation of E2F-dependent transcription of both cellular and viral E2F targets such as cyclin A (Schulze *et al*, 1995) and E2A (Kovesdi *et al*, 1986), respectively (Figure 1D). Thus, activation of the PI3-kinase/mTOR pathway is necessary for S-phase entry and efficient viral replication in adenoviral-infected cells, independently of E2F activation.

E4-ORF1 is necessary and sufficient to activate PI3-kinase and PKB in adenovirus infection

We next investigated whether adenovirus encodes early viral proteins that activate PI3-kinase/mTOR for viral replication. Previous studies have implicated E4-ORF1 from Adenovirus 9, a related but distinct adenovirus to the Ad2/5 subgroup used in these studies, in the activation of PI3-kinase and PKB (Frese *et al*, 2003). Therefore, we investigated whether

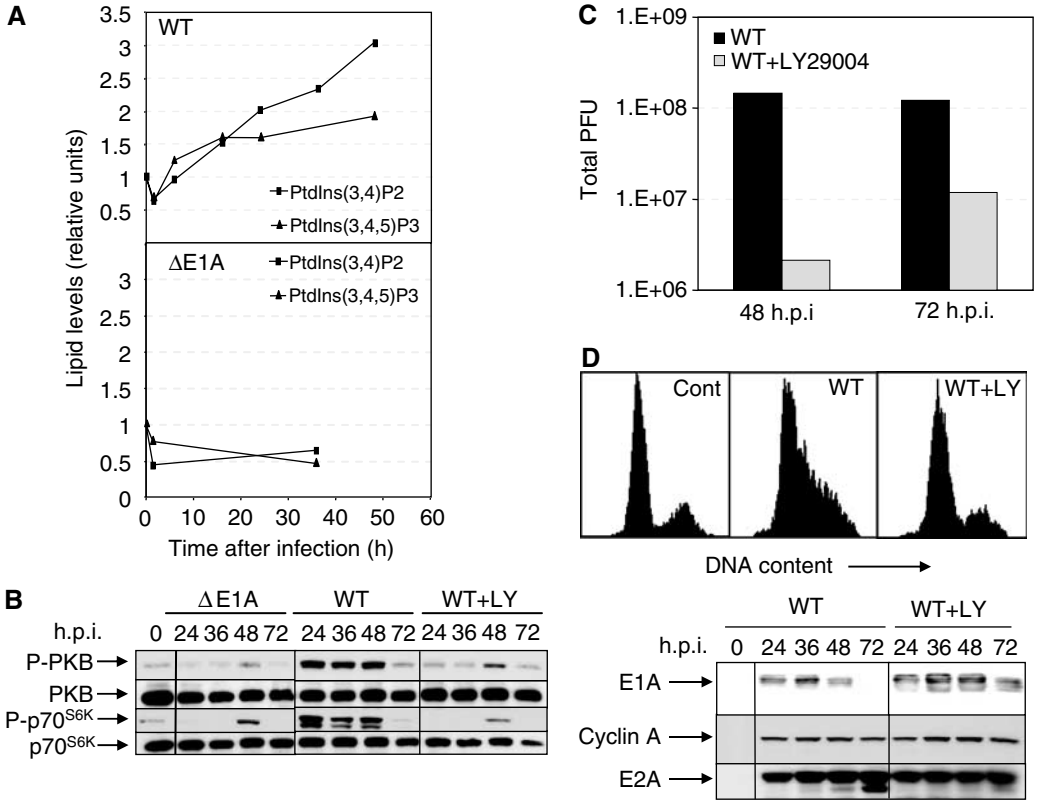

Figure 1 PI3-kinase/mTOR activation is required for S-phase entry and viral replication in infected SAECs. (**A**) Quiescent SAECs were infected with WT or ΔE1A viruses. PtdIns(3,4,5)P3 (triangles) and PtdIns(3,4)P2 (squares) levels were measured relative to total phospholipids, and normalized relative to uninfected cells. (**B**) Quiescent SAECs were infected with WT and ΔE1A viruses and incubated in the presence/absence of 40 μM LY294002 (added 4 h.p.i.). Total and phosphorylated PKB and $p70^{S6K}$ were analyzed by Western blotting. (**C**) WT virus production in SAECs, incubated in the presence/absence of 40 μM LY294002, was determined at 48 and 72 h.p.i. (**D**) Control-infected and WT-infected SAECs, incubated in the presence/absence of 40 μM LY294002, were stained with propidium iodide at 24 h.p.i. and analyzed by flow cytometry.

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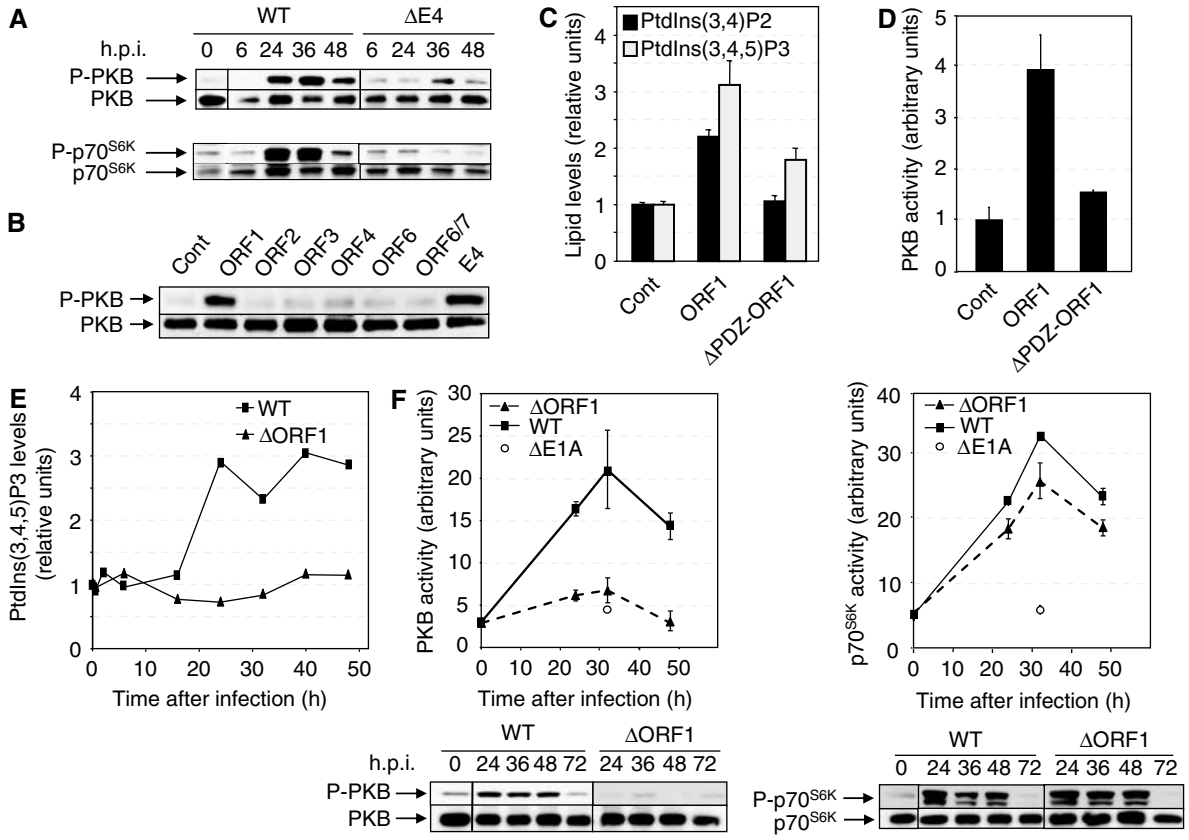

Figure 2 E4-ORF1 is necessary and sufficient for activation of PI3-kinase/PKB. (**A**) SAECs were infected with WT and ΔE4 adenoviruses and total and phosphorylated PKB and $p70^{S6K}$ analyzed by Western blotting. (**B**) U2OS cells were transfected with the indicated expression plasmids, harvested 24 h post-transfection, and total and phosphorylated PKB analyzed by Western blotting. (**C**) HCT-116 cells were transiently transfected with the indicated plasmids. At 24 h post-transfection, the cells were labeled with ^{32}P-orthophosphate and PtdIns(3,4)P2 levels (black bars) and PtdIns(3,4,5)P3 levels (gray bars) were measured. (**D**) HCT-116 cells were transiently transfected with the indicated plasmids and PKB activity measured. (**E, F**) PtdIns(3,4,5)P3 levels were measured in quiescent SAECs infected with WT and ΔE4-ORF1 viruses (E). PKB and $p70^{S6K}$ activity and phosphorylation were also examined (F).

E4-ORF1 could also underlie our observations outlined in Figure 1. Figure 2A demonstrates that SAECs infected with a ΔE4 adenovirus, a mutant virus that is defective for all six E4-ORFs (E4-ORF1, 2, 3, 4, 6, 6/7) (Halbert *et al*, 1985), failed to stimulate the phosphorylation of PKB or $p70^{S6K}$. The onset of E4 protein expression is also consistent with the time course of $p70^{S6K}$ activation in adenovirus-infected SAEs (Supplementary Figure 1). Therefore, to assess the ability of individual E4-ORFs to activate PI3-kinase, we transiently expressed each E4-ORF in HCT-116 cells (Figure 2B). E4-ORF1, but not other E4-ORFs, was sufficient to induce the phosphorylation of PKB. E4-ORF1 expression was also sufficient to increase PtdIns(3,4,5)P3 and PtdIns(3,4)P2 levels (Figure 2C) and activate endogenous PKB (Figure 2D). E4-ORF1 has a C-terminal PDZ domain-binding motif, a motif that has been previously linked to regulation of PI3-kinase activity (Wu *et al*, 2000). In contrast to WT E4-ORF1, a mutant that lacks the C-terminal PDZ domain-binding motif was unable to increase either PI3-kinase lipid products or PKB activity (Figure 2C and D). Therefore, similar to Ad9 (Frese *et al*, 2003), E4-ORF1 from Ad2/5 is sufficient to activate PI3-kinase.

To determine whether E4-ORF1 is necessary for activation of PI3-kinase and PKB in adenovirus infection, we utilized a mutant adenovirus containing an insertion in the E4-ORF1 gene that results in the expression of a protein that lacks the C-terminal 19 amino acids (Halbert *et al*, 1985), including the C-terminal PDZ domain-binding motif. Infection of SAECs with this mutant adenovirus (ΔE4-ORF1) failed to activate PI3-kinase, as evidenced by the lack of induction of PtdIns(3,4,5)P3 (Figure 2E). Consistent with this, the activation and phosphorylation of PKB was also almost completely abolished in SAECs infected with ΔE4-ORF1 compared to WT adenovirus (Figure 2F). However, in marked contrast to PKB, the activation and phosphorylation of $p70^{S6K}$ was only partially inhibited in ΔE4-ORF1-infected cells compared to WT virus (Figure 2F). Thus, adenovirus is able to induce $p70^{S6K}$ activation even in the absence of E4-ORF1-mediated activation of PI3-kinase and PKB. Taken together, we conclude that E4-ORF1 is necessary and sufficient for activation of PI3-kinase, but that $p70^{S6K}$ can still be activated via an alternative mechanism during adenovirus replication.

E4-ORF4 is necessary and sufficient to activate $p70^{S6K}$ upstream of mTOR and independently of PI3-kinase activation in adenovirus infection

As induction of $p70^{S6K}$ phosphorylation was completely abolished in SAECs infected with a ΔE4 adenovirus (Figure 2A), we reasoned that there may be an additional viral protein encoded by the E4 region that activates $p70^{S6K}$.

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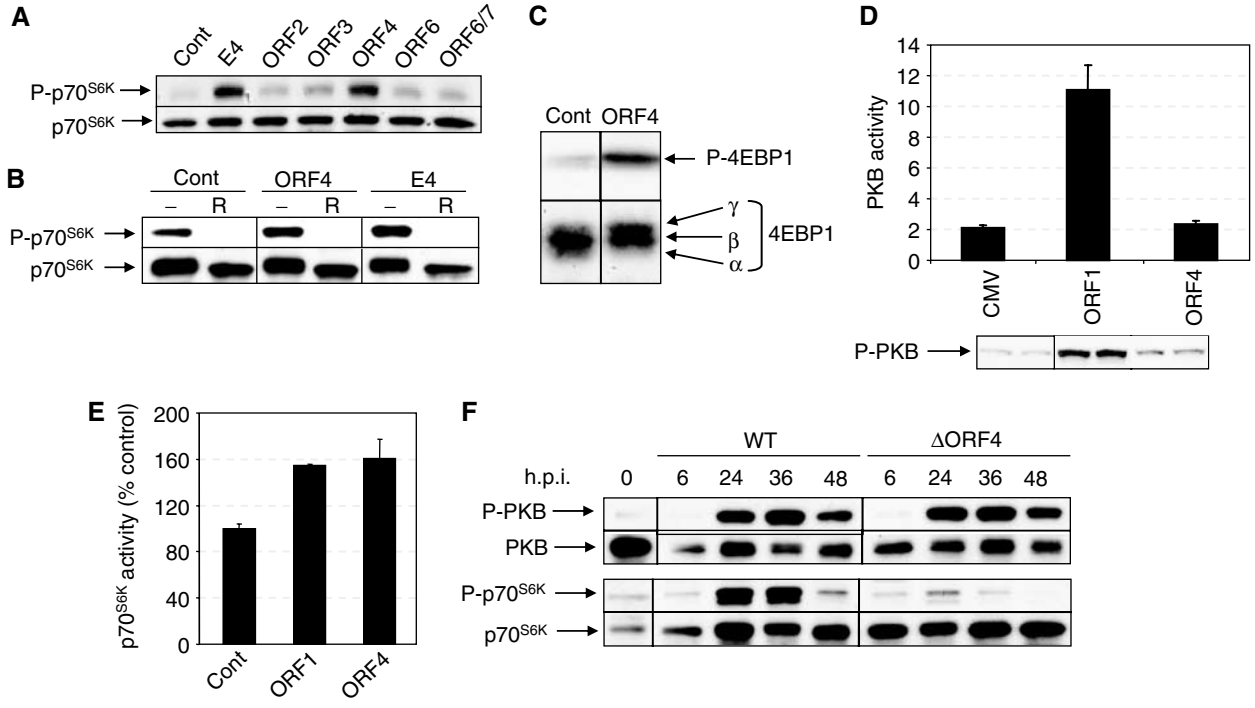

Figure 3 E4-ORF4 is necessary and sufficient for activation of $p70^{S6K}$ upstream of mTOR in adenovirus infection. (**A**) U2OS cells were transfected with the indicated plasmids. Lysates were analyzed for total and phosphorylated $p70^{S6K}$ by Western blotting. (**B**) U2OS cells were transfected with the indicated plasmids, and the lysates subjected to Western blot analysis using phospho-T389 $p70^{S6K}$ and total $p70^{S6K}$ antibodies. Where indicated, rapamycin (R) was added (50 nM) 6 h prior to harvesting. (**C**) U2OS cells were transfected with the indicated plasmids and the lysates subjected to Western blot analysis using phospho-S65 4EBP1 and total 4EBP1 antibodies. (**D**) HCT116 cells were transfected with the indicated plasmids. After 24 h, endogenous PKB activity was quantitated. A portion of the lysate was also examined for total and phosphorylated PKB by Western blotting. (**E**) HCT116 cells were transfected with the indicated plasmids. After 24 h, endogenous $p70^{S6K}$ activity was quantitated. (**F**) SAECs were infected with WT and ΔE4-ORF4 viruses and total and phosphorylated PKB and $p70^{S6K}$ analyzed by Western blotting.

Therefore, we examined $p70^{S6K}$ phosphorylation in U2OS osteosarcoma cells that had been transfected with individual E4-ORF expression constructs. Of the remaining five E4-ORFs, only E4-ORF4 was sufficient to induce $p70^{S6K}$ phosphorylation (Figure 3A).

To determine whether E4-ORF4 activates $p70^{S6K}$ upstream or downstream of mTOR, we examined if the induction of $p70^{S6K}$ phosphorylation by E4-ORF4 was sensitive to rapamycin, an mTOR inhibitor. Figure 3B demonstrates that both basal $p70^{S6K}$ phosphorylation and E4-ORF4-induced $p70^{S6K}$ phosphorylation were completely inhibited by rapamycin. We also examined whether E4-ORF4 affected additional mTOR targets, such as 4EBP1, an inhibitor of translation initiation. Figure 3C shows that expression of E4-ORF4 also resulted in phosphorylation of 4EBP1 at Ser65, an mTOR-dependent site (Gingras *et al*, 2001a). Although the best-understood mechanism underlying $p70^{S6K}$ activation is through PI3-kinase-mediated signaling to mTOR, E4-ORF4, unlike E4-ORF1, does not induce PKB phosphorylation (Figures 2B and 3D) or activation (Figure 3D). Nevertheless, either E4-ORF1 or E4-ORF4 was sufficient to increase $p70^{S6K}$ activity when transiently expressed in U2OS cells (Figure 3E). From this, we conclude that adenovirus encodes two viral proteins that are capable of activating $p70^{S6K}$ upstream of mTOR through independent mechanisms.

In Figure 2F, we demonstrate that PKB activation is almost completely ablated in ΔE4-ORF1-infected SAECs, while $p70^{S6K}$ activation is only partially impaired. Therefore, to determine whether E4-ORF4 is the critical viral protein necessary for activation of $p70^{S6K}$ in adenovirus-infected primary cells, we used a mutant adenovirus that encodes a 10-amino-acid deletion in E4-ORF4, ΔE4-ORF4 (*dl*359) (Halbert *et al*, 1985). SAECs infected with ΔE4-ORF4 were largely defective for $p70^{S6K}$ phosphorylation compared to WT virus-infected cells (Figure 3F). However, the induction of PKB phosphorylation by ΔE4-ORF4 infection was similar to WT virus. E4-ORF1-mediated activation of PKB most likely accounts for the small induction of $p70^{S6K}$ phosphorylation observed in ΔE4-ORF4-infected cells, as a ΔE4 virus is completely defective for any increases in $p70^{S6K}$ phosphorylation (Figure 2A). Taken together, these data demonstrate that E4-ORF4 is both sufficient and also the critical viral protein that is necessary for the activation of $p70^{S6K}$ in adenovirus-infected primary cells.

E4-ORF1 and E4-ORF4 cooperate to bypass the requirement for nutrients and growth factors to activate mTOR

Our finding that adenovirus encodes more than one viral protein capable of activating $p70^{S6K}$ suggests that E4-ORF1 and E4-ORF4 have nonoverlapping functions in stimulating mTOR activity depending on the cellular microenvironment. Therefore, to determine the ability of E4-ORF1 and E4-ORF4 to cooperate with each other, as well as with other known activators of mTOR, we deprived U2OS cells of both growth factors and nutrients and examined the ability of E4-ORF1

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and E4-ORF4 to maintain p70^{S6K} phosphorylation. As expected, transfer of U2OS cells to Dulbecco's phosphate-buffered saline (DPBS) completely abolished p70^{S6K} phosphorylation. Transfection of either E4-ORF1 or E4-ORF4 partially rescued p70^{S6K} phosphorylation under these conditions, whereas the expression of both E4-ORF1 and E4-ORF4, or the complete E4 region, was able to maintain robust p70^{S6K} phosphorylation even in DPBS (Figure 4A). The transfection efficiency in these experiments was approximately 40% as evidenced from control GFP-transfected cells. We also analyzed the ability of E4-ORF1 and E4-ORF4 to induce phosphorylation of 4EBP1 under these conditions. E4-ORF1 had a weak ability to maintain 4EBP1 phosphorylation in the absence of nutrients and growth factors. In contrast, E4-ORF4 alone showed a somewhat stronger protection, but again cooperated with E4-ORF1 to maintain 4EBP1 phosphorylation in DPBS (Supplementary Figure 2). From this, we conclude that E4-ORF1 and E4-ORF4 cooperate to induce mTOR activity in the absence of either nutrients or growth factors.

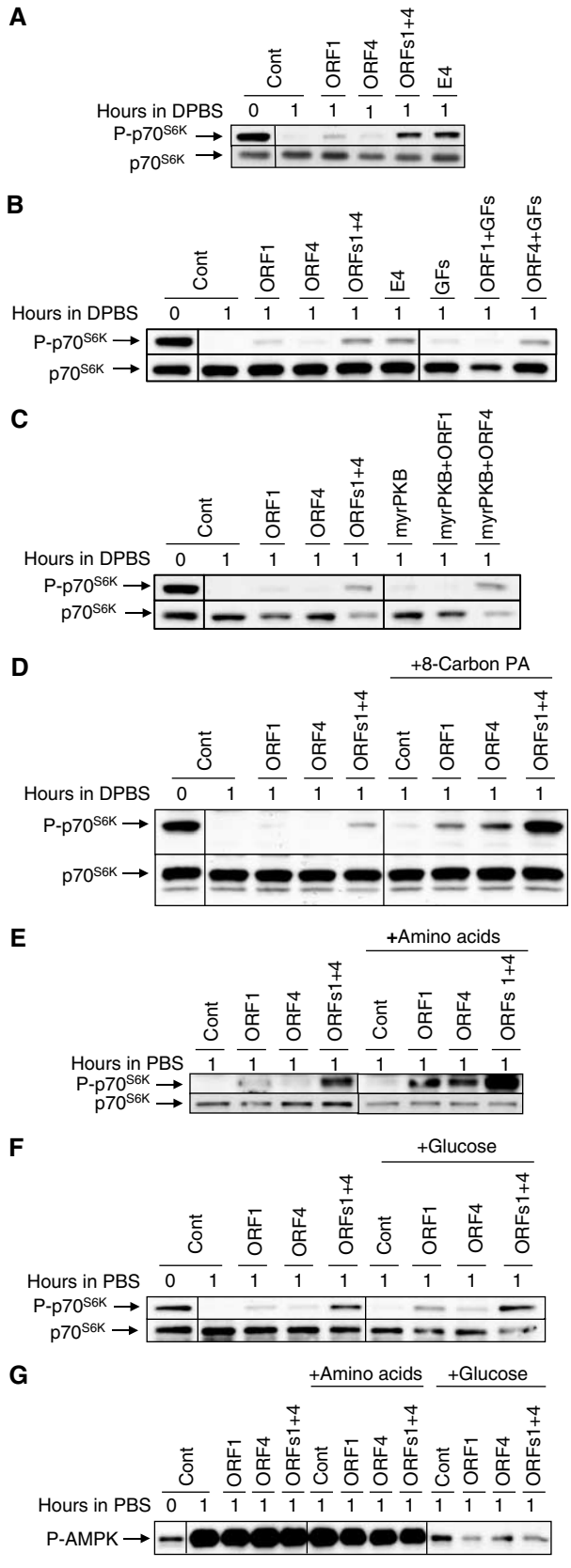

Figure 4 E4-ORF1 and E4-ORF4 cooperate to bypass the requirement for nutrients and growth factors to activate mTOR. (**A–G**) The indicated plasmids were transfected into U2OS cells in DMEM containing 10% FBS, serum withdrawn 18 h post-transfection and 3 h later either harvested (0 h time point) or transferred to DPBS for 1 h (1 h time point). Where indicated, growth factors (10 ng/ml insulin and 200 ng/ml EGF (GFs)), 100 μM PA, amino acids (to 1 × final) or glucose (4.5 g/l) were added to the DPBS for the 1 h incubation. The lysates were then subjected to Western blot analysis using phospho-T389 p70^{S6K}, total p70^{S6K} or phospho-T172 AMP kinase antibodies.

Figure 4A–C demonstrate that growth factors (GFs, a combination of EGF and insulin) stimulated p70^{S6K} phosphorylation to a similar extent as either E4-ORF1 or E4-ORF4 expression alone. However, while growth factors cooperated with E4-ORF4 in stimulating p70^{S6K} phosphorylation, they failed to cooperate with, and were redundant with, E4-ORF1 expression (Figure 4B). These data support the notion that growth factors and E4-ORF1 act in the same pathway to activate mTOR, while E4-ORF4 acts through an independent mechanism. Consistent with this, in Figure 4C, we demonstrate that an activated form of PKB cooperated with E4-ORF4, but not E4-ORF1, to stimulate p70^{S6K} phosphorylation in U2OS cells incubated in DPBS. From this, we conclude that E4-ORF1 acts in a growth factor signaling pathway and cooperates with E4-ORF4 to maintain p70^{S6K} phosphorylation in growth-limiting conditions.

We next tested whether E4-ORF1 or E4-ORF4 cooperated with PA in stimulating phosphorylation of p70^{S6K}. Figure 4D demonstrates that addition of short-chain (8C) PA alone caused a small increase in p70^{S6K} phosphorylation, and cooperated with either E4-ORF1 or E4-ORF4, or both. In addition, butanol, which diverts the production of PA, did not inhibit the ability of E4-ORF4 to stimulate p70^{S6K} phosphorylation (data not shown). Therefore, our data suggest that PA constitutes an independent input into mTOR activation distinct from E4-ORF1 or E4-ORF4.

Both amino acids (Hara *et al*, 1998) and glucose (Patel *et al*, 2001; Inoki *et al*, 2003b) are known to signal to mTOR independently of PKB activation. Therefore, we examined whether E4-ORF4 mimicked nutrient signaling to maintain

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p70^{S6K} phosphorylation in U2OS cells incubated in DPBS. E4-ORF1 cooperated with the addition of either amino acids or glucose to stimulate p70^{S6K} phosphorylation in cells transferred to DPBS for 1 h (Figure 4E and F). However, unlike E4-ORF1, E4-ORF4 cooperated with amino acids, but not glucose, to maintain p70^{S6K} phosphorylation in DPBS (Figure 4E and F). Although the cooperative effects in these experiments were small, they were nevertheless reproducible in four separate experiments. These data suggest that E4-ORF4 acts in the same pathway as glucose to activate mTOR.

AMP kinase is phosphorylated and activated upon nutrient withdrawal, due to increased AMP levels under these conditions, and acts to inhibit mTOR activation (Inoki *et al*, 2003b). Therefore, we examined the effects of nutrients and E4-ORF1/4 on AMP phosphorylation in DPBS. AMP kinase phosphorylation increased in DPBS as expected, which was prevented when glucose was re-added to the medium (Figure 4G). However, neither E4-ORF1 nor E4-ORF4 prevented AMP kinase phosphorylation upon nutrient withdrawal (Figure 4G). This suggests that if E4-ORF4 acts in the glucose signaling pathway, it must do so downstream of AMP kinase phosphorylation. Taken together, we conclude that E4-ORF1 and E4-ORF4 cooperate, and mimic growth factor and nutrient signals to activate mTOR under growth-limiting conditions.

E4-ORF1 and E4-ORF4 have differential effects on Rheb.GTP loading

The ability of E4-ORF1 and E4-ORF4 to maintain p70^{S6K} phosphorylation in cells incubated in DPBS is reminiscent of the effects of Rheb overexpression, which also bypasses the requirement for both nutrient and growth factor signals for mTOR activation (Garami *et al*, 2003; Zhang *et al*, 2003). Therefore, we examined whether the expression of both E4-ORF1 and E4-ORF4 could maintain p70^{S6K} phosphorylation to a similar level as Rheb overexpression in U2OS cells incubated in DPBS. Figure 5A demonstrates that p70^{S6K} phosphorylation was maintained to a similar level in U2OS cells transfected with both E4-ORF1 and E4-ORF4, or Rheb. Interestingly, Figure 5A also demonstrates that Rheb overexpression cooperated with E4-ORF1, but not E4-ORF4, to stimulate p70^{S6K} phosphorylation. Rheb is a small GTP-binding protein that activates mTOR only in the GTP-bound state. The GTP loading of Rheb is negatively regulated by the GAP activity of the TSC1/2 complex (Garami *et al*, 2003; Inoki *et al*, 2003a; Tee *et al*, 2003). Therefore, we examined whether E4-ORF1 or E4-ORF4 affected the ability of TSC1/2 to act as a GAP toward Rheb. E4-ORF1, but not E4-ORF4, antagonized the ability of TSC1/2 expression to decrease the GTP loading of Rheb (Figure 5B). This is consistent with the ability of E4-ORF1 to activate PKB, which is known to phosphorylate TSC2 and inhibit the TSC1/2 complex (McManus and Alessi, 2002). These data provide further evidence that E4-ORF4 activates mTOR through a parallel and independent pathway to E4-ORF1/growth factors/PI3-kinase. The lack of cooperation between E4-ORF4 and Rheb, compared to E4-ORF1 and Rheb, in stimulating p70^{S6K} phosphorylation is reminiscent of the inability of E4-ORF4 to cooperate with glucose. Taken together, our data suggest that E4-ORF4 may act in the same pathway as glucose and/or Rheb but has distinct effects from E4-ORF1/growth factors, which increase Rheb.GTP loading.

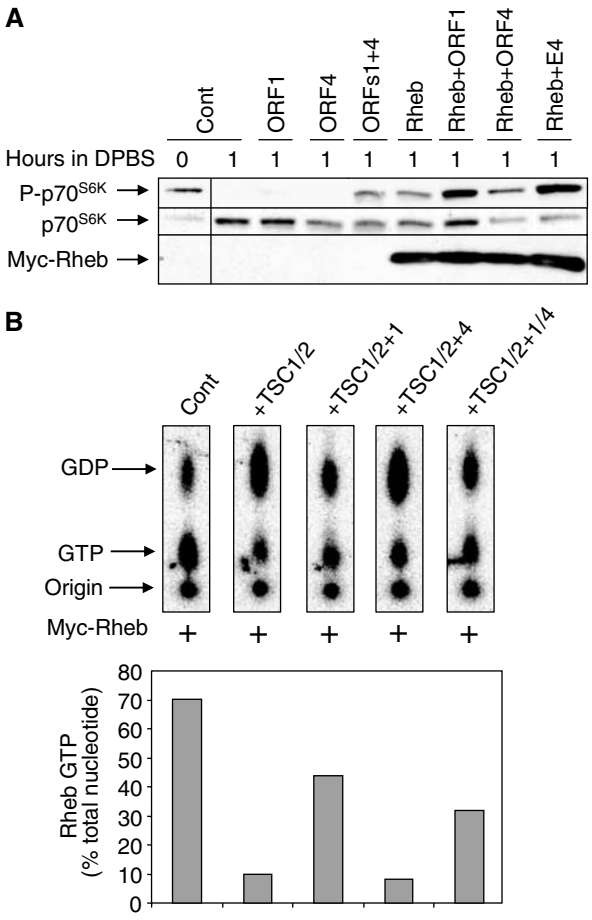

Figure 5 E4-ORF1 and E4-ORF4 have differential effects on Rheb.GTP loading. (**A**) U2OS cells were transfected with the indicated plasmids, serum withdrawn 18 h post-transfection and 3 h later transferred to DPBS for 1 h. The lysates were subjected to Western blot analysis using phospho-T389 p70^{S6K}, total p70^{S6K} and myc epitope. (**B**) U2OS cells were transfected with the indicated plasmids, and then labeled with 0.5 mCi/ml orthophosphate for 6 h. Rheb was immunoprecipitated through a myc tag, washed and the nucleotides associated with it analyzed by thin-layer chromatography. The ratio of GTP relative to GDP + GTP is indicated.

E4-ORF4 mutants that fail to bind the regulatory subunit of PP2A are defective for p70^{S6K} phosphorylation

E4-ORF4 is a 114-amino-acid polypeptide that is known to bind to the regulatory B subunit of the protein phosphatase 2A (PP2A) holoenzyme (Branton and Roopchand, 2001). PP2A exists as a trimeric complex consisting of one of two catalytic subunits, one of two regulatory A subunits and one of more than 20 B subunits, of which Bα is thought to associate with E4-ORF4 (Branton and Roopchand, 2001). Different regulatory subunits of PP2A are likely to control the activity, specificity and cellular localization of the holoenzyme. To determine whether the failure of the ΔE4-ORF4 virus to activate p70^{S6K} in infected SAECs (Figure 3F) can be attributed to defective PP2A binding, we cloned the E4-ORF4 gene expressed by this virus into an HA-tagged expression vector (termed 359). U2OS cells were transfected with WT and mutant forms of E4-ORF4 together with an EE-tagged form of PP2A Bα. In contrast to WT E4-ORF4, the 359 mutant of E4-ORF4 failed to bind and immunoprecipitate PP2A Bα (Figure 6A). Similarly, a 51A/54A point mutant of E4-ORF4,

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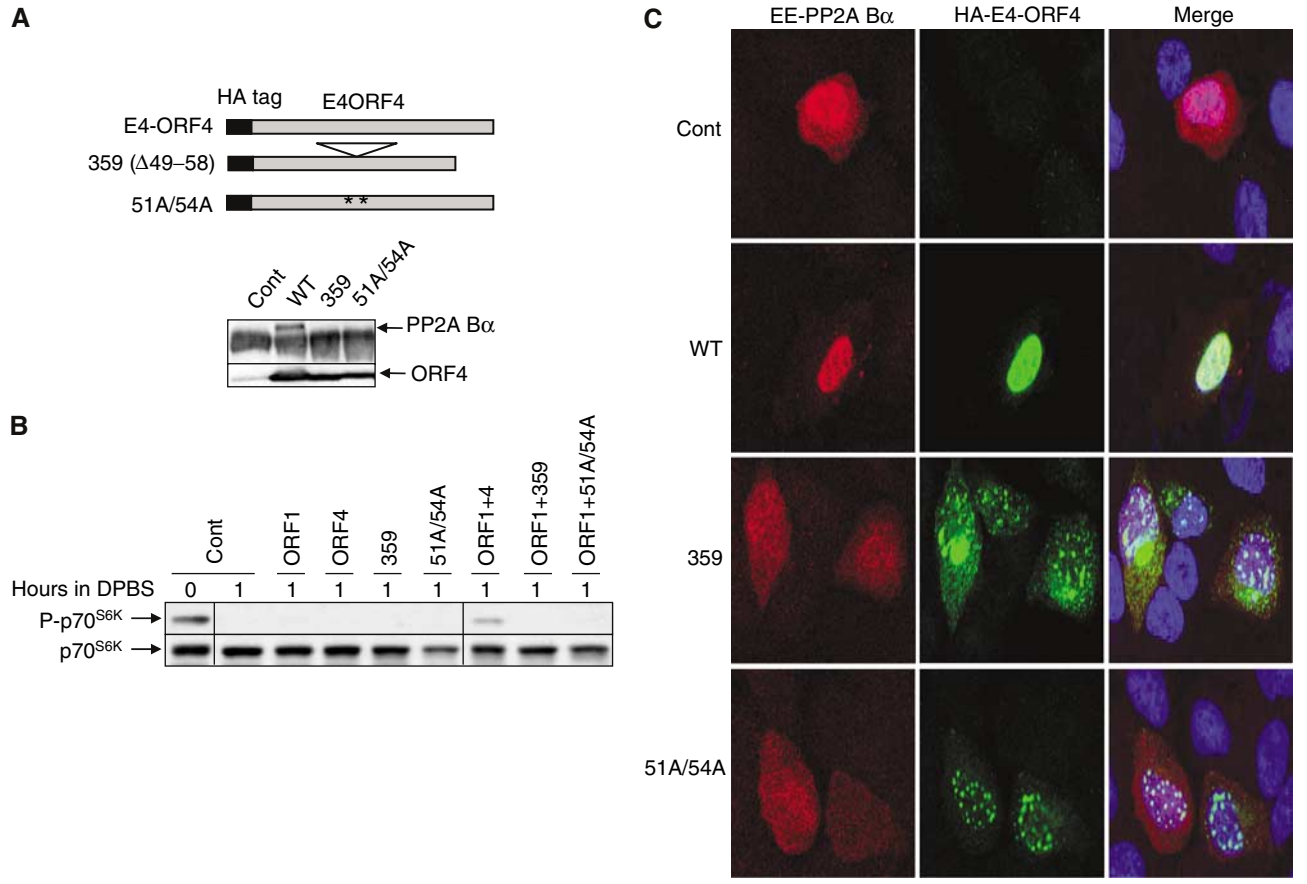

Figure 6 E4-ORF4 mutants that fail to bind and relocalize PP2A are defective for $p70^{S6K}$ phosphorylation. (**A**) U2OS cells were transfected with the indicated plasmids. WT and mutant E4-ORF4 proteins were immunoprecipitated through an HA tag, and Western blotted for EE-tagged PP2A Bα (upper panel) and HA-tagged E4-ORF4 (lower panel). The lower band present in the upper panel is the IgG heavy chain of the immunoprecipitating antibody. (**B**) U2OS cells were transfected with the indicated plasmids, serum withdrawn 18 h post-transfection and 3 h later transferred to DPBS for 1 h. Lysates were analyzed for total and phosphorylated $p70^{S6K}$ by Western blotting. (**C**) U2OS cells were transfected with the indicated plasmids and fixed 24 h later. PP2A Bα was visualized via an EE tag, and WT and mutant E4-ORF4 via an HA tag. Nuclei were counterstained with DAPI and images captured on a Zeiss Meta-scanning Confocal microscope.

which has previously been shown to be defective for PP2A binding (Marcellus *et al*, 2000), also failed to bind PP2A in this assay (Figure 6A). Figure 6B demonstrates that E4-ORF4 mutants that fail to bind to PP2A were unable to cooperate with E4-ORF1 to stimulate $p70^{S6K}$ phosphorylation in U2OS cells incubated in DPBS. We next analyzed the effect of WT and mutant E4-ORF4 on PP2A Bα localization. PP2A Bα exhibited a diffuse cytoplasmic/nuclear localization in transfected U2OS cells. However, when WT E4-ORF4 was coexpressed, PP2A Bα localized predominantly to the nucleus together with E4-ORF4. In contrast to WT E4-ORF4, the 359 and 51A/54A E4-ORF4 mutants were localized in distinct punctuate structures, and did not appear to affect PP2A Bα localization (Figure 6C). Taken together, these data suggest that the binding and relocalization of PP2A by E4-ORF4 may be part of the mechanism whereby adenovirus activates mTOR.

E4-ORF1 and E4-ORF4 are required for assembly of translation initiation complexes, DNA synthesis and viral replication

To determine whether E4-ORF1 and E4-ORF4 exert biologically relevant effects in activating mTOR in adenovirus-infected cells deprived of nutrients and growth factors, we examined $p70^{S6K}$ phosphorylation in WT adenovirus-, ΔE4-ORF1- and ΔE4-ORF4-infected U2OS cells upon transfer to DPBS. U2OS cells infected with WT adenovirus maintain $p70^{S6K}$ phosphorylation even in DPBS, whereas viruses expressing mutant E4-ORF1 or E4-ORF4 are unable to perform such a function (Figure 7A).

We next investigated whether the expression of E4-ORF1 and E4-ORF4 had functional consequences for the maintenance of cap-dependent translation in adenovirus replication. mTOR regulates cap-dependent translation, in part through the phosphorylation of 4EBP1. Unphosphorylated 4EBP1 inhibits eIF4E by preventing its binding to eIF4G, and subsequent association with capped mRNAs. Active translational complexes can be determined by measuring the levels of eIF4G associated with eIF4E precipitated through its binding to the capped mRNA mimic methyl^7-GTP Sepharose. Infection of nutrient- and serum-deprived U2OS cells with WT adenovirus resulted in high levels of eIF4G and low levels of 4EBP1 associated with methyl^7-GTP Sepharose. In contrast, higher levels of 4EBP1 and lower levels of eIF4G were associated with eIF4E in both ΔE4-ORF1 and ΔE4-ORF4 virally infected U2OS cells (Figure 7B). Taken together, these data demonstrate that both E4-ORF1 and E4-ORF4 are required for mTOR activation and protein

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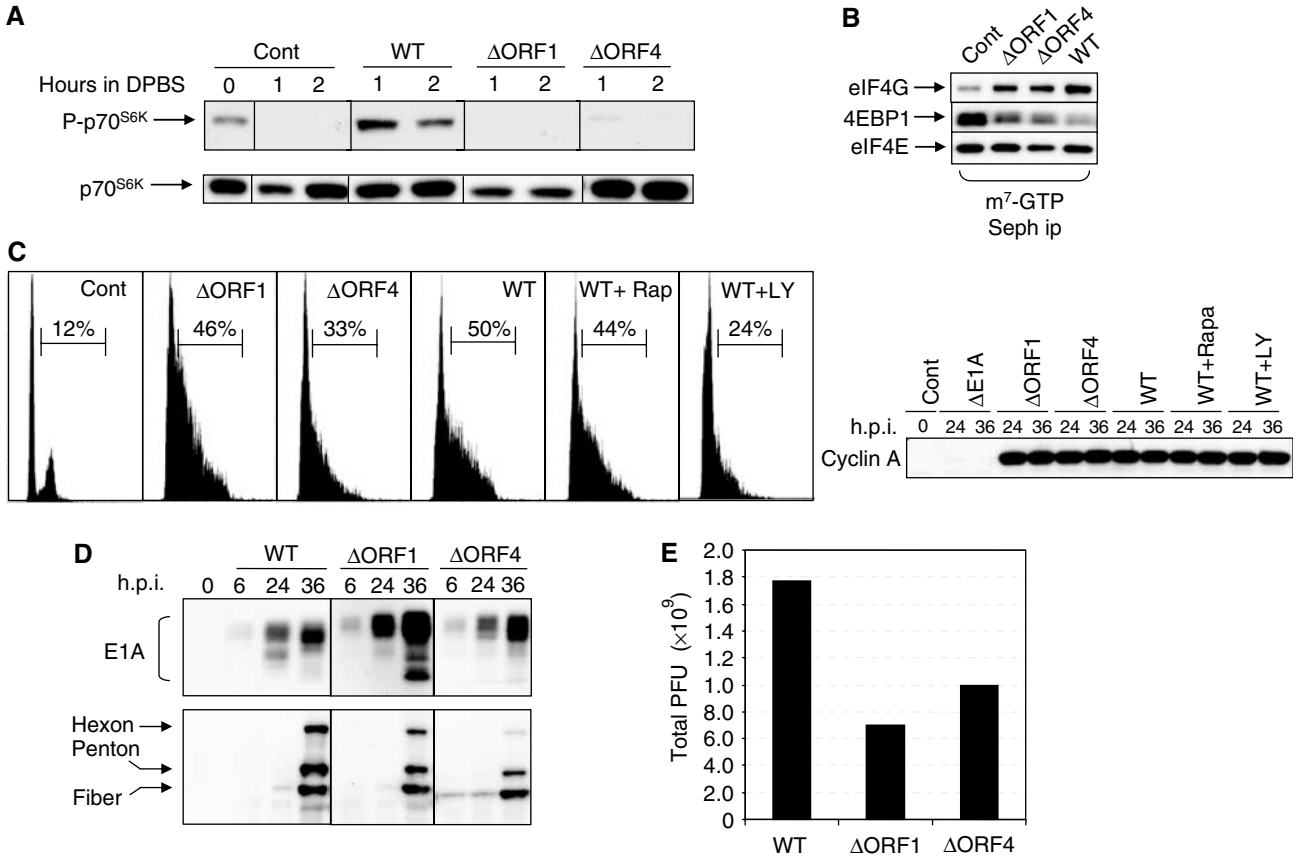

Figure 7 E4-ORF1 and E4-ORF4 are required for the assembly of translation initiation complexes, DNA synthesis and viral replication. (**A**) U2OS cells were infected with WT, ΔE4-ORF1, ΔE4-ORF4 and ΔE1A (mock) viruses. At 18 h.p.i., cells were serum deprived for 2 h, transferred to DPBS for the indicated times and total and phosphorylated $p70^{S6K}$ analyzed by Western blotting. (**B**) U2OS cells were infected with the indicated adenoviruses, transferred to PBS for 1 h at 18 h.p.i. and lysed. Methyl^7-GTP Sepharose was used to precipitate translation initiation complexes. Associated proteins were detected by Western blotting using antibodies against eIF4G, eIF4E and 4EBP1. (**C**) Quiescent SAECs were infected with the indicated viruses, stained with propidium iodide at 36 h.p.i. and analyzed by flow cytometry. The percentage of cells with a DNA content greater than $n = 1$ is indicated. Lysates were also examined by Western blotting for cyclin A. (**D**) Quiescent SAECs were infected with the indicated viruses. Lysates were analyzed by Western blotting for early (E1A) and late (hexon, penton and fiber) viral protein expression. (**E**) WT, ΔE4-ORF1 and ΔE4-ORF4 virus production was measured from infected SAECs harvested at 72 h.p.i.

translation initiation in virally infected cells under growth-limiting conditions.

We also examined whether loss of E4-ORF1 or E4-ORF4 functions had any consequences for viral replication under normal growth conditions. In Figure 1C and D, we demonstrate that the combined inhibition of both PI3-kinase and mTOR by LY294002 impaired S-phase entry and reduced virus production. However, the ΔE4-ORF1 and ΔE4-ORF4 viruses provide us with a genetic system with which to distinguish between the requirements for PI3-kinase/PKB and mTOR/$p70^{S6K}$ activation during adenoviral replication. Therefore, we determined the effects of ΔE4-ORF1 and ΔE4-ORF4 infection on S-phase entry and viral replication in quiescent SAECs. Both ΔE4-ORF1 and ΔE4-ORF4 viruses were less effective at promoting S-phase entry compared to WT adenovirus, although the ΔE4-ORF4 mutant virus was more severely affected (Figure 7C). Inhibition of $p70^{S6K}$ activity by rapamycin also impaired S-phase entry in WT adenovirus-infected quiescent SAECs (Figure 7C). The inhibition of S-phase entry by rapamycin was less than that observed with LY29004 in WT virus-infected cells, or indeed with the ΔE4-ORF4 mutant. However, rapamycin is not an ATP-competitive inhibitor and may not inhibit all mTOR functions (McMahon *et al*, 2002). Alternatively E4-ORF4 may activate pathways in addition to mTOR that are important for S-phase entry. Similar to Figure 1D, the defect in S-phase entry in ΔE4-ORF4 virus-infected cells is unrelated to the induction of E2F transcriptional activity, as evidenced by the induction of cyclin A. Finally, in ΔE4-ORF1- and ΔE4-ORF4-infected cells, both late viral protein expression (Figure 7D) and viral production (Figure 7E) were decreased by 50% compared to WT virus-infected cells. Taken together, these data suggest that mTOR activation is required for the initiation of S-phase entry in virally infected cells in addition to E1A-mediated activation of E2F, and plays an important role in viral replication.

Discussion

In this study, we use adenovirus to gain new insights into the regulation and functions of mTOR, an attractive therapeutic target. We reveal that adenovirus has evolved two proteins that function to activate mTOR, even under growth-limiting conditions, and which are required for efficient viral replication. One of these proteins, E4-ORF1, activates PI3-kinase, one of the best-characterized regulators of mTOR activity.

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However, an E4-ORF1 mutant virus that fails to activate PI3-kinase in infected primary cells is only partially compromised in its ability to activate $p70^{S6K}$. This led us to discover that adenovirus encodes an additional protein, E4-ORF4, which activates mTOR through a PI3-kinase/PKB-independent mechanism.

We were initially surprised to discover that adenovirus encodes two viral proteins that can activate mTOR. However, many of the pivotal growth-regulating checkpoints in the cell are regulated by multiple signals that exert integrated or additive effects upon critical downstream effectors. Although the transfer of cells to DPBS may mimic only the most severe pathological downregulation of mTOR signaling, overnight fasting is sufficient to downregulate dramatically mTOR activity and protein translation (Kimball *et al*, 2002), which, as we demonstrate here, could negatively impact adenoviral replication. Therefore, adenovirus may have evolved both E4-ORF1 and E4-ORF4 to ensure mTOR activation and viral replication even in fasted or starved cells.

The activation of mTOR is highly complex and regulated by multiple inputs emanating from growth factors, glucose, amino acids and PA. In our experiments, each of these factors provided independent inputs that were additive in their effects on mTOR activation. These results are consistent with a study that shows that serum and amino acids had independent effects in activating $p70^{S6K}$ in CHO-IR cells (Hara *et al*, 1998). However, this is in contrast to other experiments that suggest that the presence of nutrients is absolutely essential for the ability of growth factors to activate mTOR (Patel *et al*, 2001; Beugnet *et al*, 2003). One explanation for this apparent disparity could be that the relative importance of different inputs for mTOR activity may depend on the particular cell type and signaling pathways being assessed. Indeed, while germline mutations in PTEN, TSC1/2 or LKB1 all result in deregulated mTOR activity, they generate a very different tissue spectrum of precancerous lesions. The use of ΔE4-ORF1 and ΔE4-ORF4 adenovirus mutants is an unequivocal genetic demonstration that in infected SAECs, E4-ORF4 is the major mechanism whereby adenovirus activates mTOR/$p70^{S6K}$, while E4-ORF1/PI3-kinase plays a more minor role. A trivial explanation for the minor contribution of E4-ORF1, compared to E4-ORF4, to mTOR activation in infected SAEs may be their relative expression levels. However, a more intriguing explanation is that the signals generated by E4-ORF4 play a major role in mTOR activation in primary quiescent epithelial cells. Distinguishing between these possibilities may have important implications for understanding mTOR regulation in both normal and tumor cell growth and how to selectively target it for cancer therapy.

While growth factor signaling to mTOR is reasonably well understood, the nutrient signaling pathways remain largely undefined. E4-ORF4 is therefore a novel tool with which to probe the mTOR signaling pathway. Point mutants of E4-ORF4 that fail to bind to PP2A are defective for $p70^{S6K}$ activation. Therefore, the ability of E4-ORF4 to activate mTOR may be mediated through its binding and relocalization of the regulatory B subunit of PP2A. These data suggest a novel role for the PP2A heterotrimer in nutrient signaling upstream of mTOR. PP2A has previously been implicated in the activation of $p70^{S6K}$, but downstream of mTOR (Jiang and Broach, 1999; Peterson *et al*, 1999). Therefore, PP2A may act at multiple points in the PI3-kinase/mTOR pathway that could be modulated by different B subunit substrate specificities or signaling inputs. PP2A is also a critical cellular target of SV40 ST in *in vitro* transformation assays (Hahn *et al*, 1999) and PP2A A subunit mutations have been found in a subset of human tumors (Wang *et al*, 1998). Unlike ST, E4-ORF4 interacts with, but does not displace, the B subunit of PP2A. How E4-ORF4 and other different viral proteins modulate PP2A may yield important insights into PP2A's role in mTOR activation and tumorigenesis.

Using pharmacological inhibitors of the mTOR pathway and E4-ORF1/4 mutant viruses, we demonstrate that mTOR activation is required for the efficient initiation of S phase in adenovirus-infected quiescent primary cells, despite the induction of E2F transcriptional targets such as cyclin A. A requirement for $p70^{S6K}$ activation in S-phase entry has previously been demonstrated (Lane *et al*, 1993), and rapamycin arrests cells in the G1 phase of the cell cycle (Huang *et al*, 2001). Our data suggest that mTOR and the pocket proteins have nonredundant roles in regulating S-phase entry, and that mTOR acts downstream of E2F. Indeed, these results are reminiscent of the early work by Pardee and colleagues that defined a nutrient dependent checkpoint distal to the growth factor-dependent restriction point for S-phase entry (Yen and Pardee, 1978). We favor a hypothesis whereby mTOR regulates the translation, much like E2F regulates the transcription, of mRNAs important for DNA synthesis. Indeed, the translations of cyclin D1, myc and ornithine decarboxylase mRNAs are known to be dependent on mTOR activation (Gingras *et al*, 2001b), and could affect S-phase entry. Identifying the critical downstream effectors of mTOR could reveal important new targets for cancer therapy, especially given the emerging evidence that translation may be deregulated downstream of mTOR in many human tumors (Rajasekhar and Holland, 2004), and therefore, unaffected by rapamycin treatment. This was recently demonstrated experimentally in a murine model of B-cell lymphoma, in which tumors initiated by the expression of activated PKB responded to rapamycin treatment, while tumors driven by the expression of eIF4E were refractory to rapamycin (Wendel *et al*, 2004).

Nonreplicating adenoviruses (defective for the E1 region) are commonly used as gene expression/delivery vehicles, many of which contain the E4 region (He *et al*, 1998). However, given that the expression of E4 genes can activate the PI3-kinase/mTOR pathway, adenovirus vectors should perhaps be used with caution in the study of these pathways. E1A activates E4 gene expression via the cellular transcription factor E4F (Raychaudhuri *et al*, 1987). Although E1A is deleted in adenovirus expression vectors, and (at least in primary quiescent epithelial cells) this region is required for E4 region expression, in many transformed or cancer cell lines, E2F and E4F transcriptional activity is deregulated (Trimarchi and Lees, 2002). Indeed, in previous studies with PTEN, we have found that control delivery adenoviruses strongly activate PI3-kinase in U87-MG glioblastoma cells (Taylor *et al*, 2000). Therefore, we emphasize the use of caution, proper controls and preferably the use of E4-deleted adenoviruses in gene delivery experiments that analyze PI3-kinase and/or mTOR signaling.

In addition to their utility as tools with which to discover new tumor targets, viruses can also be used as selectively replicating oncolytic agents in cancer therapy. For example,

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deregulated E2F activity drives the selective lytic replication of a novel adenovirus, ONYX-411, in tumor cells (Johnson *et al*, 2002). The ΔE4-ORF1 and ΔE4-ORF4 viruses used in this study were originally characterized in HeLa cells where they were found to replicate at WT virus levels. However, HeLa cells express the HPV E6 protein that degrades TSC2 and activates mTOR (Lu *et al*, 2004). Here we show that the replication of both ΔE4-ORF1 and ΔE4-ORF4 viruses is compromised in primary SAECs, albeit to a minor extent. However, LY294002, which inhibits PI3-kinase/mTOR, results in, on average, a 15-fold defect in viral replication in infected primary cells, suggesting that a virus defective for both E4-ORF1 and E4-ORF4 may be similarly inhibited. This suggests that an E4-ORF1/E4-ORF4 mutant virus could be a novel and potentially efficacious oncolytic viral strategy for the treatment of tumors that have deregulated the growth factor/PP2A/mTOR pathway.

Materials and methods

Cells and culturing conditions

SAECs were obtained from Cambrex and rendered quiescent by growing them to 100% confluency followed by prolonged (8 days) incubation (O'Shea *et al*, 2004). HCT-116 and U2OS cells were cultured in McCoys and DME media, respectively, with 10% fetal calf serum (FCS). We define complete medium as small airway basal medium containing 'Singlequots' supplied by the manufacturer, or DMEM/McCoys in the presence of 10% FCS. Glucose and amino acids were added to DPBS at the same concentration used in DMEM.

Measurement of in vivo 3′ phosphorylated phosphoinositide lipids

Cells were washed and labeled for 3 h in 3 ml phosphate-free media containing 1 mCi [^{32}P]P_i. Lipids were extracted and deacylated as described previously (Auger *et al*, 1989) and separated using a Spherisorb S5 SAX column (Waters, Milford, MA). A gradient from 0 to 1.5 M NaH_2PO_4 was developed in 70 min, 0.5 ml fractions were collected and ^{32}P radioactivity was detected by Cerenkov counting.

Preparation of cell lysates and kinase assays

Cells were harvested directly into lysis buffer (0.5% NP-40, 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM $NaVO_4$ and protease inhibitor cocktail (Roche, Basel, Switzerland)/10 ml lysis buffer). Protein concentrations were determined and kinase assays performed as previously described (Taylor *et al*, 2000) using 50 μM GRPRTSSFAEG ('Crosstide') to measure PKB activity and 50 μM RRRLSS (S6 peptide) to measure $p70^{S6K}$ activity.

Virus isolation, propagation, infection and quantification

Viruses were propagated and titered as described previously (Johnson *et al*, 2002). Viruses used were as follows: WT is WtD (a hybrid adenovirus containing the left end of the Ad2 genome (approximately 12%) while the remaining 88% including the E4 region is derived from Ad5 (Barker and Berk, 1987)); ΔE4 (*dl366*), lacks all E4 sequences; ΔE4-ORF1 (*in351*), contains a 5 bp insertion in E4-ORF1 resulting in a frame shift and the deletion of the C-terminal 19 amino acids; and ΔE4-ORF4 (*dl359*), contains a 30 bp deletion in the E4-ORF4 region. All these mutants have been previously described (Halbert *et al*, 1985). SAECs were infected at a multiplicity of infection (MOI) of 10 and U2OS cells at an MOI of 30, which had been previously determined experimentally.

Rheb.GTP loading assay

Rheb.GTP loading was performed essentially as described by Bollag *et al* (1996). U2OS cells were transfected with myc-tagged Rheb either alone or in combination with GST-tagged TSC1 and TSC2, and HA-tagged E4-ORF1 and myc-tagged E4-ORF4. Cells were labeled with 0.5 mCi/ml orthophosphate for 6 h, and Rheb was immunoprecipitated through a myc tag. After washing, the nucleotides were eluted with EDTA, and separated by thin-layer chromatography in 1 M LiCl on PEI cellulose plates. The incorporated radioactivity into GDP and GTP was detected by phosphorimager analysis and quantitated using Imagequant.

PP2A immunoprecipitation and immunolocalization

U2OS cells were cotransfected with HA-tagged forms of E4-ORF4, 359 E4-ORF4 or 51/54 E4-ORF4 and EE PP2A Bα. For immunoprecipitations, transfected cells were lysed in 1% NP-40 buffer and normalized for protein content. E4-ORF4 was immunoprecipitated via HA and EE PP2A Bα/E4-ORF4 complexes examined by gel electrophoresis and Western blotting. For immunofluorescence, cells were fixed in 4% paraformaldehyde, permeabilized by incubation with 0.1% Triton/PBS for 4 min and blocked by incubation with 5% goat serum/PBS for 30 min. Cells were incubated with mouse anti-EE (a kind gift from Gideon Bollag) and rabbit anti-HA (Y-11, Santa Cruz Biotechnology) in 5% goat serum/PBS for 1 h. Primary antibodies were detected as described previously (O'Shea *et al*, 2004), and analyzed on a Zeiss Meta-scanning Confocal microscope.

Methyl^7-GTP immunoprecipitations

Infected cells were lysed in 0.5% NP-40 buffer and normalized for protein content. Equal protein was immunoprecipitated with methyl^7-GTP Sepharose beads (Amersham) for 1 h at 4°C. The beads were washed three times in lysis buffer and translation complexes were analyzed by gel electrophoresis and Western blotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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