PI3K Signaling Regulates Rapamycin-Insensitve Translation Initiation Complex Formation in Vaccinia Virus-Infected Cells[∇]

Izabela Zaborowska and Derek Walsh*

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland

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How vaccinia virus (VV) regulates assembly of the host translation initiation complex eIF4F remains unclear. Here, we show that VV activated host PI3K to stimulate downstream mammalian target of rapamycin (mTOR), a kinase that inactivates the translational repressor 4E-BP1. However, although the mTOR inhibitor rapamycin suppressed VV-induced inactivation of 4E-BP1, it failed to inhibit eIF4F assembly. In contrast, PI3K inhibition in VV-infected cells increased the abundance of hypophosphorylated 4E-BP1 and disrupted eIF4F complex formation. PI3K signaling, therefore, plays a critical role in regulating protein production during VV infection, at least in part by controlling the abundance and activity of 4E-BP1.

The translation factor eIF4F recruits ribosomes to capped mRNAs to initiate protein synthesis. This multiprotein complex consists of eIF4E, the cap-binding subunit; eIF4A, an RNA helicase; and eIF4G, a large scaffolding protein on which the complex is built (6). A family of negative regulatory proteins, the eIF4E-binding proteins (4E-BPs), bind eIF4E and competitively inhibit its interaction with eIF4G (Fig. 1A). Phosphorylation of 4E-BPs results in the release of eIF4E, allowing it to interact with eIF4G and form an eIF4F complex. 4E-BPs are phosphorylated by mTOR, a kinase activated through upstream PI3K signaling, thereby promoting translation initiation in response to various extracellular stimuli (5).

Like all viruses, in the absence of its own translational machinery vaccinia virus (VV), a prototypical poxvirus (17), has evolved a range of strategies to prevent the host antiviral response from shutting down translation and to commandeer the cellular protein synthesis machinery to serve its own ends (4, 8, 12, 24, 28). VV has been shown to promote the formation of eIF4F complexes and inactivate the translational repressor protein 4E-BP1 in a rapamycin-sensitive manner (28), but how this is accomplished and whether these two events are linked in VV-infected cells remain unknown. Activation of signaling upstream of mTOR in cells infected with myxoma virus, a rabbit-specific poxvirus, has been shown to involve direct stimulation of Akt by the host range factor MT-5, and this occurs in a PI3K-independent manner (26, 32, 33). To determine if mTOR stimulation in VV-infected cells involved host PI3K activation, we first measured phosphorylation of the PI3K substrate Akt in uninfected and VV-infected cultures of serum-starved normal human diploid fibroblasts (NHDFs) (30) in the presence of specific inhibitors of PI3K or its downstream substrate mTOR. At 16 h postinfection (p.i.), increased phosphorylation of Akt was observed in VV-infected cells, as determined by Western blotting of whole cell extracts using phosphospecific antiserum, and this was inhibited by LY294002, a specific inhibitor of PI3K (Fig. 1B). Cellular PI3K

activity was also required for VV-mediated phosphorylation of subsequent downstream targets, including the mTOR substrates 4E-BP1 and p70S6 kinase (p70S6K) (Fig. 1B). The mTOR inhibitor rapamycin blocked VV-induced phosphorylation of 4E-BP1, as reported previously (28), together with phosphorylation of p70S6K, but did not affect phosphorylation of the upstream target Akt. This demonstrated that, unlike myxoma virus, VV requires host PI3K to regulate downstream signaling to mTOR. Stimulation of kinases outside of the PI3K pathway and known to be activated by VV, such as extracellular signal regulated kinase (ERK) (2), was only modestly affected by PI3K inhibition (Fig. 1B).

Although both drugs prevented VV-mediated phosphorylation of 4E-BP1, maintaining mock-infected phosphorylation levels or below, it was clear that hypophosphorylated 4E-BP1 accumulated to a much greater extent in LY294002-treated versus rapamycin-treated samples, despite the two drugs inhibiting phosphorylation of p70S6K equally (Fig. 1B and 2A). This occurred even in the presence of high concentrations of rapamycin. We therefore examined the effect of these inhibitors on the formation of eIF4F complexes in VV-infected cells. NHDFs were either mock infected or infected in the presence of LY294002, rapamycin, or a dimethyl sulfoxide (DMSO) solvent control. At 16 h p.i., soluble cell extracts were prepared and cap-binding eIF4E was recovered by 7-methyl GTP batch chromatography (28). Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with antisera against eIF4E, eIF4G, and 4E-BP1 to determine the composition of eIF4E-bound complexes in each sample (Fig. 2A). While release of the repressor protein 4E-BP1 and recruitment of eIF4G were evident in VV-infected cells in the presence of DMSO, despite retaining 4E-BP1 in complex with eIF4E to levels at or above those observed in mock-infected cells, as reported previously (28), rapamycin did not significantly alter amounts of eIF4G bound to eIF4E. However, inhibition of upstream PI3K signaling resulted in a robust recruitment of 4E-BP1 to eIF4E well in excess of that observed in either mock-infected or rapamycin-treated VV-infected cultures, and a corresponding robust reduction in the amount of eIF4G bound to eIF4E (Fig. 2A). Inhibition of VV-induced 4E-BP1 phosphorylation by rapamycin

* Corresponding author. Mailing address: National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. Phone: 353-1-7006260. Fax: 353-1-7005484. E-mail: derek.walsh@dcu.ie.

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FIG. 1. Vaccinia virus activates PI3K to phosphorylate downstream proteins that regulate translation. A. PI3K regulates mTOR and protein synthesis. Activation of PI3K stimulates a signaling pathway to mTOR, which in turn phosphorylates key regulatory components of the host cell translational control machinery. mTOR mediates phosphorylation of the eIF4F scaffolding protein eIF4G and p70S6 kinase (p70S6K) together with the translational repressor 4E-BP, releasing it from eIF4E. The points at which specific kinase inhibitors used in this study operate are illustrated. T-bars represent repression. Circled P's represent phosphorylation events. B. Serum-starved NHDFs were either uninfected or infected with 10 PFU VV (Western Reserve) per cell in the presence of equal volumes of DMSO, 40 μ M LY294002 (LY), or 2 μ M rapamycin (Rapa). 16 h.p.i. whole cell lysates were prepared and analyzed by Western blotting using the indicated antisera toward the phosphorylated forms of Akt (Ser473) (catalog no. 4058) and ERK (catalog no. 9101), or total forms of Akt (catalog no. 9272), p70S6K (catalog no. 9202) and 4E-BP1 (catalog no. 9452) from Cell Signal Technologies. Phosphorylation of 4E-BP1 and p70S6K is detected by mobility shift analysis with high-percentage SDS-PAGE gels. Hyper- and hypophosphorylated forms of each are indicated to the right. Host translation initiation factor eIF4E served as a loading control. The fold change (F.C.) in Akt and ERK phosphorylation over multiple experiments was quantified by densitometry and averaged and then presented relative to uninfected levels, arbitrarily set at 1, above a representative blot.

cin and more complete hypophosphorylation of this repressor by LY294002 were again evident in input samples. Examining its total abundance in low-percentage gels (30) clearly showed that although rapamycin prevented the degradation of 4E-BP1

FIG. 2. eIF4F complex formation in vaccinia virus-infected cells requires host PI3K signaling. A. Serum-starved NHDFs were infected with 10 PFU VV per cell in the presence of equal volumes of DMSO, 40 μ M LY294002 (LY), or 2 μ M rapamycin (Rapa). At 16 h p.i., soluble cell extracts were prepared and precleared before recovery of 7-methyl GTP binding eIF4E complexes. Washed beads were boiled in Laemmli buffer and samples analyzed by Western blotting using the indicated antisera. Fold change (F.C.) in eIF4G and 4E-BP1 bound to eIF4E was quantified by densitometry and presented relative to uninfected levels, arbitrarily set at 1. The need to saturate blots to observed levels of eIF4G in LY294002-treated samples prevented quantification on more linear exposures (-). B. Serum-starved NHDFs were infected as described for panel A. At 16 h p.i., whole cell lysates were run on 7.5% SDS-PAGE gels to prevent resolution of 4E-BP1 forms and total levels were determined by Western blotting with anti-4E-BP1 antiserum. Levels were quantified by densitometry, as described for panel A. Alternatively, samples were resolved by isoelectric focusing and blots were probed with anti-eIF4E antiserum. Migration of phosphorylated (P-4E) and hypophosphorylated (4E) eIF4E is indicated to the left. C. Serum-starved NHDFs were infected as described for panel A. At 16 h p.i., whole cell lysates were prepared and analyzed by Western blotting using antisera against the phosphorylated forms of eIF2 α (P-eIF2 α) or eIF4G (P-eIF4G) (Cell Signal Technologies), together with total eIF4G (T-eIF4G) (Ian Mohr, NYU School of Medicine). D. Serum-starved NHDFs were infected as described for panel A. At 16 h p.i., cultures were photographed by phase microscopy.

during VV infection, as reported previously (28), LY294002 actually caused an increase in the abundance of this repressor protein (Fig. 2B). 4E-BP1 phosphorylation and stability are intricately linked in both virus-infected and uninfected cells (3,

28, 30). Although this increase in repressor abundance may be directly linked to the degree to which 4E-BP1 becomes hypophosphorylated, and thus stabilized in these samples, the precise mechanism by which PI3K inhibition exerts these effects remains to be determined. In addition, phosphorylation of the cap-binding protein eIF4E, known to enhance VV replication (28), was also suppressed in LY294002-treated cultures (Fig. 2B). This was likely due to the collapse of eIF4F complexes, which prevents proper positioning of the eIF4G-bound kinase Mnk in proximity to its substrate, eIF4E, similar to previous reports (21, 27, 29).

Overall, these results demonstrated that PI3K activation was essential not only for VV-induced increases in eIF4F but also to prevent large pools of 4E-BP1 controlled by rapamycin-insensitive pathways from disrupting eIF4F complex formation, suggesting significant roles for mTORC1-independent processes in these events (9, 23). To investigate if other factors might contribute to the regulation of eIF4F in VV-infected cells, we also examined eIF4G phosphorylation, which is controlled by a number of kinases, including mTOR (11, 16, 22). Western blotting of cell extracts using antisera against the total or phosphorylated forms of eIF4G showed that while the abundance of eIF4G remained unaltered, VV clearly increased the accumulation of phosphorylated eIF4G (Fig. 2C). The PI3K and mTOR inhibitors prevented this stimulation to equal extents, maintaining phosphorylation at basal uninfected levels. This suggested that differences in eIF4G phosphorylation did not contribute to the dramatic decrease in eIF4F complexes observed in LY294002-treated VV-infected cells. The effects did not appear to be the result of cellular stress responses, as indicators including eIF4G cleavage and eIF2 α phosphorylation (12, 15) were not observed (Fig. 2C). The morphology of cultures (Fig. 2D) and formation of viral factories (not shown) showed that cells were indeed infected in the presence of LY294002, but a reduced cytopathic effect suggested that virus replication was significantly reduced, which may also explain the modest reductions in ERK activation observed in Fig. 1B.

We then determined rates of viral protein synthesis in NHDFs infected with VV in the presence of inhibitors. At 16 h.p.i., cultures were metabolically labeled with [^{35} S]methionine/cysteine for a 1-h period (28), and then whole cell extracts were resolved by SDS-PAGE and dried gels exposed to X-ray film. While rapamycin did not affect the synthesis of viral proteins, LY294002 suppressed translation rates, even at high input doses of virus (Fig. 3A). Previous studies of kinases that regulate the activity of individual translation factors as part of intact eIF4F complexes have shown that they have subtle stimulatory effects on translation that become apparent during low multiplicity infection and multiple rounds of virus replication (28, 30, 31). The low-level translation observed in LY294002-treated NHDFs despite dramatically reduced eIF4F likely reflects suggestions that VV mRNAs require but may have a reduced dependence upon eIF4F (1, 18, 25). Assembly of eIF4F during VV infection would therefore serve to maximize production of viral proteins. To this end, the abundance of viral antigens accumulated over the course of infection was dramatically reduced in LY294002-treated cultures but not in those treated with rapamycin, as determined by Western blotting with antiserum against VV (Fig. 3B). The PI3K inhibitor did not exert its effects by preventing entry of VV into cells, as

FIG. 3. Inhibition of PI3K suppresses the production of vaccinia virus proteins. A. Serum-starved NHDFs were infected in the presence of inhibitors, as in Fig. 2. At 16 h p.i., cultures were metabolically labeled with [^{35} S]methionine/cysteine for 1 h and then lysed in Laemmli buffer. Samples were resolved by SDS-PAGE, and fixed dried gels were exposed to X-ray film. Migration of molecular weight markers is indicated to the left. B. NHDFs infected in the presence of inhibitors as described for panel A were lysed in Laemmli buffer and then samples were resolved by SDS-PAGE and Western blotting was performed with antiserum against VV (Virostat, catalog no. 8101). Migration of molecular weight markers is indicated to the left. C. NHDFs were either pretreated with inhibitors for 1 h or left untreated, and they were then infected with 10 PFU VV per cell for 1 h. Virus was then removed and fresh medium containing inhibitors was added to both pretreated and untreated cultures. Whole cell extracts were made at 7 h p.i. and then analyzed by Western blotting as described for panel B. Panels show viral antigens detected in the lower section of blots as larger antigens are faint at this early point, but show the same effect.

the same results were observed if the inhibitor was added after removal of the viral inoculum (Fig. 3C). Finally, we measured the production of infectious virus in NHDFs infected for 18 h in the presence of inhibitors, harvesting virus by repeated freeze-thaw cycles and titration on BSC40 cells (28). Even at high input doses, production of infectious virus was reduced 48-fold by LY294002, while rapamycin repeatedly had modest but insignificant effects (Fig. 4). Given the role of PI3K signaling in various biological processes, it is important to note that the defects in VV replication observed in LY294002-treated

FIG. 4. PI3K is required for efficient production of infectious virus. Dishes (35 mm) of NHDFs were infected with 10 PFU VV per cell in the presence of DMSO, 40 μ M LY294002 (LY), or 2 μ M rapamycin (Rapa). At 18 h p.i., virus was harvested by freeze-thaw. Infectious virus was measured by serial dilution and plating on permissive BSC40 cells. Results are representative of the results from multiple independent experiments.

cultures are likely to be a result of the cumulative effect of diminished eIF4F and the inhibition of other, as yet undetermined PI3K-regulated functions in the VV life cycle. However, these findings identify novel mechanisms by which PI3K signaling regulates eIF4F activity in VV-infected cells.

A wide range of DNA viruses stimulate mTOR signaling to inactivate 4E-BPs in a rapamycin-sensitive manner (7, 10, 13, 14, 19, 28, 30, 31). However, rapamycin treatment frequently has little effect on viral protein synthesis or replication. In cells infected with human cytomegalovirus, 4E-BP1 phosphorylation is only partially sensitive to rapamycin and altered composition of Raptor/Rictor-containing mTOR complexes in infected cells explains this effect (9, 10, 31). Unlike HCMV infection, in cells infected with herpes simplex virus type 1 (HSV-1), phosphorylation and release of 4E-BP1 above mock-infected levels is completely blocked by rapamycin yet has little effect on eIF4F formation or virus replication (30). Although the presence of a virally encoded protein that directly stimulates eIF4F complex assembly (29) could potentially interfere with the ability of rapamycin to control translation in HSV-1-infected cells, the ineffectiveness of rapamycin has led to suggestions that 4E-BP abundance may be insufficient to regulate eIF4F, at least in NHDFs, and has no significant role in regulating virus replication. The effects of rapamycin observed here, using VV-infected NHDFs, were remarkably similar to those reported for HSV-1 infection, and it would be tempting to draw the same conclusions. However, the effects of PI3K inhibition demonstrate that rapamycin-insensitive signaling

mechanisms regulate both 4E-BP1 phosphorylation and abundance to suppress eIF4F formation in NHDFs infected with VV. These mechanisms may include mTORC2 or other rapamycin-insensitive complexes (9, 20, 23) and may have important regulatory functions in other biological situations that could be overlooked when using rapamycin alone to study 4E-BPs role in translation. As such, stimulation of eIF4F complex assembly, at least in VV-infected cells, requires inactivation of 4E-BP through PI3K signaling pathways that include both rapamycin-sensitive and -insensitive events.

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