Virology 425 (2012) 11–22

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Recruitment of host translation initiation factor eIF4G by the Vaccinia Virus ssDNA-binding protein I3

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ARTICLE INFO

Article history:
Received 1 August 2011
Returned to author for revision
9 December 2011
Accepted 23 December 2011
Available online 24 January 2012

Keywords:
Poxvirus
eIF4G
eIF4F
Translation initiation
I3

ABSTRACT

Poxviruses are large double-stranded DNA viruses that replicate exclusively in the cytoplasm of infected cells within discrete compartments termed viral factories. Recent work has shown that the prototypical poxvirus, Vaccinia Virus (VacV) sequesters components of the eukaryotic translation initiation complex eIF4F within viral factories while also stimulating formation of eIF4F complexes. However, the forces that govern these events remain unknown. Here, we show that maximal eIF4F formation requires viral DNA replication and the formation of viral factories, suggesting that sequestration functions to promote eIF4F assembly, and identify the ssDNA-binding protein, I3 as a viral factor that interacts and co-localizes with the eIF4F scaffold protein, eIF4G. Although it did not adversely affect host or viral protein synthesis, I3 specifically mediated the binding of eIF4G to ssDNA. Combined, our findings offer an explanation for the specific pattern and temporal process of eIF4G redistribution and eIF4F complex assembly within VacV-infected cells.

Introduction

Among the poxviruses that can infect humans, Variola Virus (VarV) is the causative agent of smallpox while Vaccinia Virus (VacV) was used as a vaccine in the eradication of smallpox and has become the laboratory prototype for the study of poxvirus infection (Moss, 2007). Both VarV and VacV belong to the *orthopoxvirus* genus, with close genetic, morphological and host range similarities. *Orthopoxviruses* are large, double-stranded DNA viruses whose genomes average 200 kbp, with VacV encoding approximately 200 genes. Poxviruses replicate exclusively in the cytoplasm of infected cells. After cell fusion and entry viral cores traverse the cytoplasm to specific sub-cellular sites that are the precursors of viral DNA synthesis. At early stages these cores rapidly produce and exude mRNAs due to the presence of a complete transcription system for early viral gene expression. As the infection proceeds the virus forms structures termed viral factories or replication compartments in the cytoplasm of the infected cell. Each incoming virus is capable of forming a unique replication compartment and these structures show a remarkable degree of self-sufficiency in terms of replicating viral DNA and transcription of the viral genome.

Despite this impressive compartmentalization and self-sufficiency poxviruses, like all viruses, remain dependent upon the host cell protein synthesis machinery to translate viral mRNAs into proteins (Walsh and Mohr, 2011). Similar to host messages, VacV mRNAs have a 7-Methyl GTP cap at their 5′ end (Boone and Moss, 1977; Martin and Moss, 1975; Venkatesan et al., 1980). Within the host cell a complex of eukaryotic translation initiation factors (eIFs), termed eIF4F plays a critical role in regulating the translation of capped messages ((Sonenberg and Hinnebusch, 2009) and Fig. 1A). eIF4F consists of eIF4E; a small cap-binding protein, eIF4A; an RNA helicase that facilitates ribosomal scanning and eIF4G; a large scaffolding protein on which the complex is built. In addition, polyA-binding protein (PABP) binds both the 3′ end polyA-tail of mRNAs and eIF4G, circularizing and stimulating the translation of mature messages ((Mangus et al., 2003; Sonenberg and Hinnebusch, 2009) and Fig. 1A). Finally, eIF4G also associates with the 40S ribosome through bridging interactions with eIF3 (Hinnebusch, 2006). eIF4F, therefore, plays a central role in recruiting the protein synthesis machinery to fully processed capped mRNAs to initiate their translation.

The assembly of eIF4F complexes is regulated, at least in part, by small eIF4E-binding proteins (4E-BPs). In their hypophosphorylated state, 4E-BPs act as translational repressors by competing with eIF4G for the same binding site on eIF4E (Fig. 1A). Signaling through the mTOR pathway results in 4E-BP phosphorylation, generating electrostatic repulsion that releases eIF4E to allow it to interact with eIF4G (Hara et al., 1997; Sonenberg and Hinnebusch, 2009; von Manteuffel et al., 1997). In contrast to many RNA viruses that inactivate eIF4F function, a growing number of DNA viruses are being found to stimulate the assembly of translation initiation complexes to maximize viral protein production (Arias et al., 2009; Castelló et al., 2009; Kudchodkar et al., 2004; McMahon et al., 2011; Moorman and

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doi:10.1016/j.virol.2011.12.022

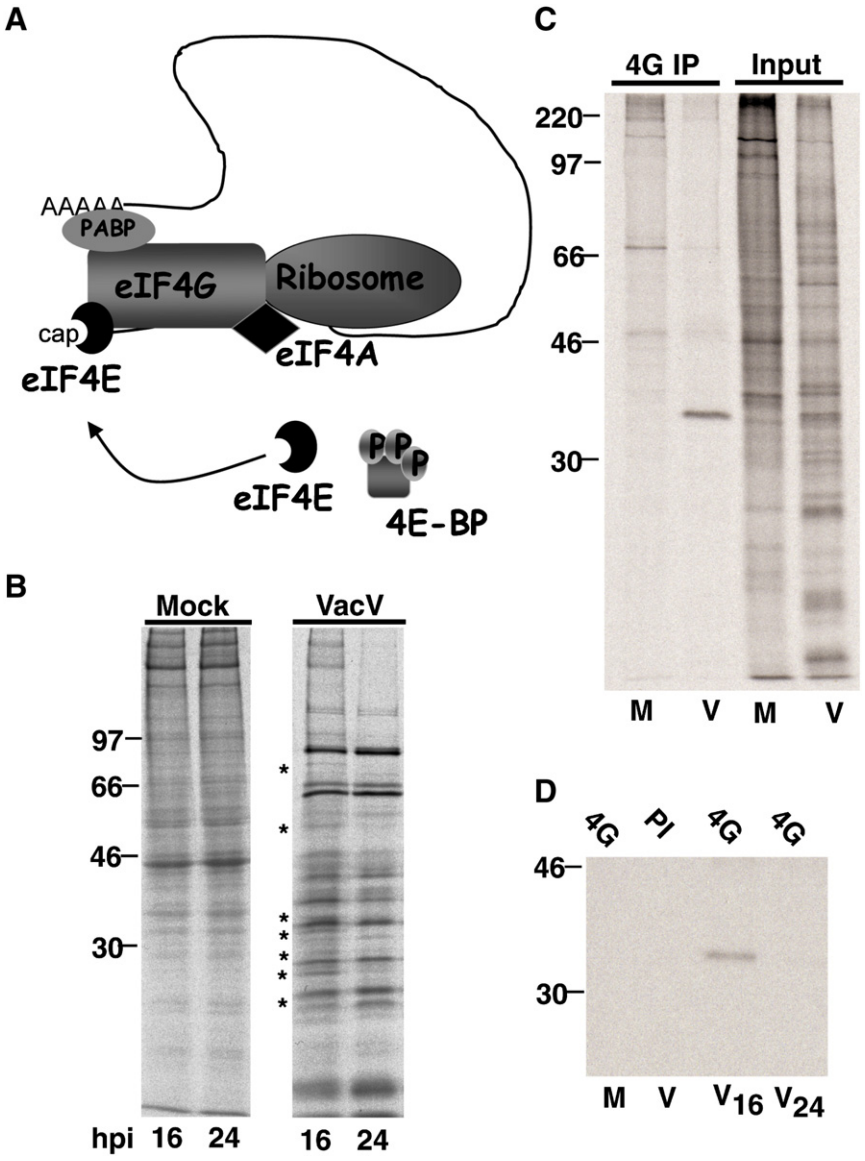

Fig. 1. A polypeptide synthesized in VacV-infected cells associates with eIF4G. (A) Recruitment of ribosomes to capped mRNAs by the translation initiation complex eIF4F. The 5′ 7-Methyl-GTP (cap) of mRNAs is bound by eIF4E, which in turn is bound by the scaffold protein, eIF4G. The RNA helicase, eIF4A also binds eIF4G to facilitate ribosome scanning. PolyA-binding protein (PABP) binds the n-terminus of eIF4G and the polyA-tail, bridging 5′ and 3′ ends of the mRNA. eIF4G indirectly recruits the 40S ribosome through interactions with eIF3. Formation of the eIF4F complex is repressed by small eIF4E-binding proteins (4E-BPs), which competitively inhibit eIF4E binding to eIF4G. In response to various stimuli mTOR signaling phosphorylates 4E-BPs, releasing eIF4E to join the eIF4F complex. (B) Serum starved NHDFs were mock-infected or infected with VacV at m.o.i. 10 for the indicated time in hours post infection (h.p.i.). 1 h prior to sample preparation cultures were metabolically labeled with ^{35}S-methionine/cysteine. Whole cell extracts were resolved by SDS-PAGE and fixed dried gels exposed to x-ray film. Migration of molecular weight markers (in kDa) is indicated to the left. Asterisks indicate proteins differentially-expressed at intermediate stage infection. (C) Serum starved NHDFs were mock-infected (M) or infected (V) at m.o.i. 5 for 15 h then metabolically labeled for 1 h. Soluble cell extracts were prepared and immunoprecipitated with anti-eIF4G antiserum. Immune-complex and input samples were resolved by SDS-PAGE and fixed, dried gels were exposed to x-ray film. Migration of molecular weight standards in kDa is indicated to the left. (D) Serum starved NHDFs were infected as described in C and metabolically labeled soluble cell extracts were prepared at 16 or 24 h.p.i. then immunoprecipitated with pre-immune serum (PI) or anti-eIF4G (4G) antiserum, as indicated. Immune complexes were resolved by SDS-PAGE and fixed, dried gels exposed to x-ray film. Migration of molecular weight markers in kDa is indicated to the left.

Shenk, 2010; O'Shea et al., 2005; Walsh, 2010; Walsh and Mohr, 2004; Walsh et al., 2005, 2008). Distinct from other mammalian DNA viruses, the *Poxviridae* and *Asfarviridae*, of which African Swine Fever Virus (ASFV) is the sole member, replicate exclusively in the cytosol of infected cells. Recent work has shown that in addition to stimulating eIF4F assembly both VacV and ASFV cause the redistribution of host translation factors to viral replication compartments (Castelló et al., 2009; Katsafanas and Moss, 2007; Walsh et al., 2008). How this rearrangement of the host translation system is accomplished remains unknown. Here, we show that eIF4F assembly is driven by events linked to viral DNA synthesis and factory formation and describe the identification of a VacV-encoded factor that associates with the eIF4F scaffold protein, eIF4G and recruits it to ssDNA.

Results

Identification of a VacV protein that binds eIF4G

Transformed or immortalized cell lines are frequently metabolically hyperactive, containing high basal levels of eIF4F and highly activated regulatory kinases that can mask the ability of some viruses to manipulate their activity. In contrast, the lowered metabolic state of serum-starved primary cells offers a means to study the ability of viruses to stimulate their host's translation system (Kudchodkar et al., 2004; Walsh and Mohr, 2004, 2006; Walsh et al., 2005), including both the redistribution and assembly of eIF4F complexes during VacV infection and the potential connections between these two

events (Walsh et al., 2008). To develop our understanding of how VacV recruits translation factors to viral factories we explored the possibility that a specific viral protein, or cellular protein induced during infection, might function through direct interaction with the eIF4F complex. To screen for such newly synthesized proteins we first took the approach of immunoprecipitating the scaffold protein, eIF4G from metabolically labeled uninfected and infected serum-starved primary Normal Human Diploid Fibroblasts (NHDFs). In NHDFs that were serum-starved for 5 days the viral lifecycle was protracted and reached an intermediate phase by 16 h.p.i., characterized by the incomplete inhibition of host protein synthesis and continued synthesis of mid-phase viral proteins that were not evident at late stages of infection (Moss, 2007) (Fig. 1B). Initially the intermediate phase of infection was screened by metabolically labeling uninfected and infected cultures from 15 to 16 h.p.i. then soluble cell extracts were nuclease treated using a cocktail of RNAse A, micrococcal nuclease and RNAse T1 previously shown to eliminate RNA-mediated interaction of proteins with eIF4G (Walsh and Mohr, 2006) and cleared with pre-immune serum. Anti-eIF4G immune complexes were recovered from cleared extracts then resolved by SDS-PAGE and fixed, dried gels were exposed to x-ray film, detecting proteins actively translated during the labeling period. In immune complexes from mock-infected samples eIF4G and a number of associated proteins were evident, while in VacV-infected samples these components were present but their intensity was decreased due to the partial suppression of host translation at this point (Fig. 1C). Notably, however, a single protein migrating at approximately 34 kDa was associated with eIF4G isolated from infected cell extracts that was not present in mock-infected samples. The signal intensity from this co-immunoprecipitating polypeptide declined by 24 h.p.i. (Fig. 1D), suggesting that it was either no longer associated with eIF4G at late stages of infection or no longer actively synthesized at this point.

To further explore the specificity of its interaction with eIF4G we then examined whether this protein was also evident in anti-PABP immune complexes. PABP is an abundant RNA-binding protein involved in a variety of cellular processes including the regulation of translation but exhibits a distinct distribution pattern from that of eIF4G in infected cells (Mangus et al., 2003; Smith and Gray, 2010; Walsh et al., 2008). Again, soluble cell extracts were prepared from metabolically labeled mock or VacV-infected NHDFs and nuclease-treated then immunoprecipitated with either anti-eIF4G or anti-PABP antiserum. When immune complexes were analyzed the ~34kDa protein was only readily detected in anti-eIF4G immune complexes (Fig. 2A). While the cap-binding protein, eIF4E was also readily recovered in association with eIF4G considerably less eIF4E was evident in anti-PABP immune complexes, despite efficient target antigen recovery (Fig. 2B). This suggested that only a relatively small amount of the total pool of PABP, an abundant multifunctional cellular protein, was associated with eIF4F complexes in these cells. Combined with the fact that in infected cells PABP remains largely excluded from factories (Walsh et al., 2008), this may also explain why PABP did not efficiently associate with the ~34kDa protein found associated with eIF4G. As such, the ~34kDa protein appeared to be specific in its interaction and did not randomly associate with general host factors that bind RNA.

To determine the identity of this protein soluble nuclease-treated cell extracts were prepared from mock and VacV-infected cells as described above then anti-eIF4G immune complexes were resolved by SDS-PAGE. When gels were silver-stained a single protein was evident in VacV-infected samples that were not present in mock-infected samples, migrating at approximately 34 kDa (Fig. 3A). This band was excised and analyzed by mass spectrometry. In various independent experiments an average of 4–6 peptides was identified, all of which corresponded to the VacV ssDNA-binding phosphoprotein I3 (GI no. 66275869) with sequence coverage ranging from 18.2 to 23%. I3 has previously been shown to be an early-to-intermediate stage protein whose synthesis declines at late stages of infection (Rochester and Traktman, 1998), in agreement with the ready detection of actively synthesized I3 bound to eIF4G at intermediate but not late stages of infection in Fig. 1D. It is also notable that VacV encodes a number of ssDNA- and RNA-binding proteins that are produced in infected cells (Moss, 2007) but which were not evident in eIF4G immune complexes (Fig. 1C), adding further support for the specificity of the I3 interaction suggested by eIF4G and PABP co-immunoprecipitation experiments.

To confirm the interaction of I3 with eIF4F by an alternative, non-immune-based assay we determined whether I3 was recovered with

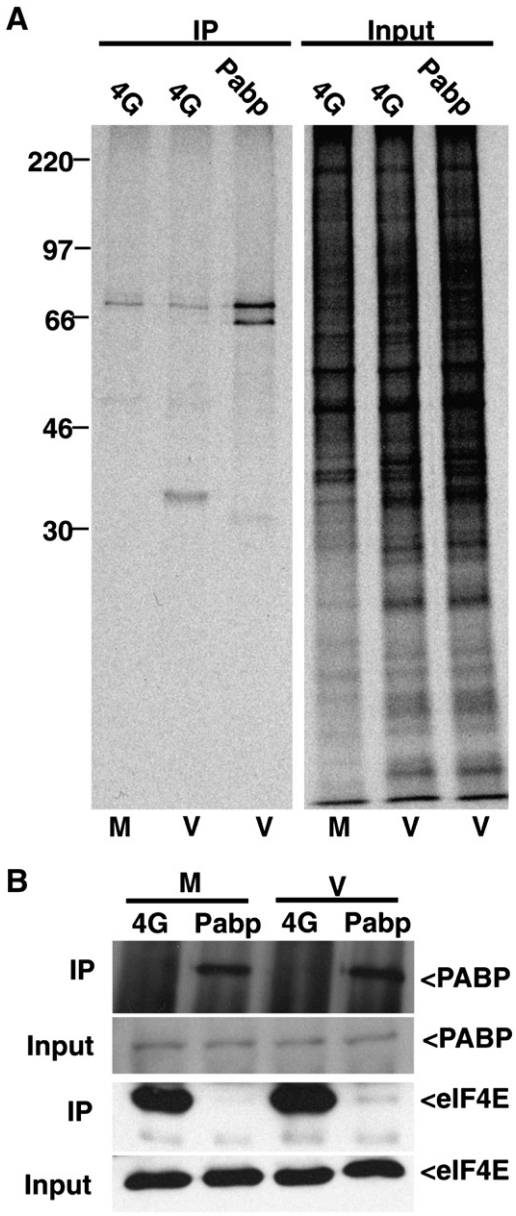

Fig. 2. The eIF4G-associated polypeptide in VacV-infected cells is not detected in PABP immune complexes. (A) Serum-starved NHDFs were mock-infected (M) or infected (V) and metabolically labeled soluble cell extracts were prepared 16 h.p.i. as described in Fig. 1. Extracts were immunoprecipitated with anti-eIF4G or anti-PABP antiserum. Immune-complex and input samples were resolved by SDS-PAGE and fixed, dried gels exposed to x-ray film. Molecular weight markers are to the left. (B) Serum-starved NHDF cells were infected at m.o.i. 10 for 18 h then soluble cell extracts were prepared and immunoprecipitated with anti-eIF4G or anti-PABP antiserum. Levels of PABP and eIF4E in immune complexes and input samples were examined by western blotting.

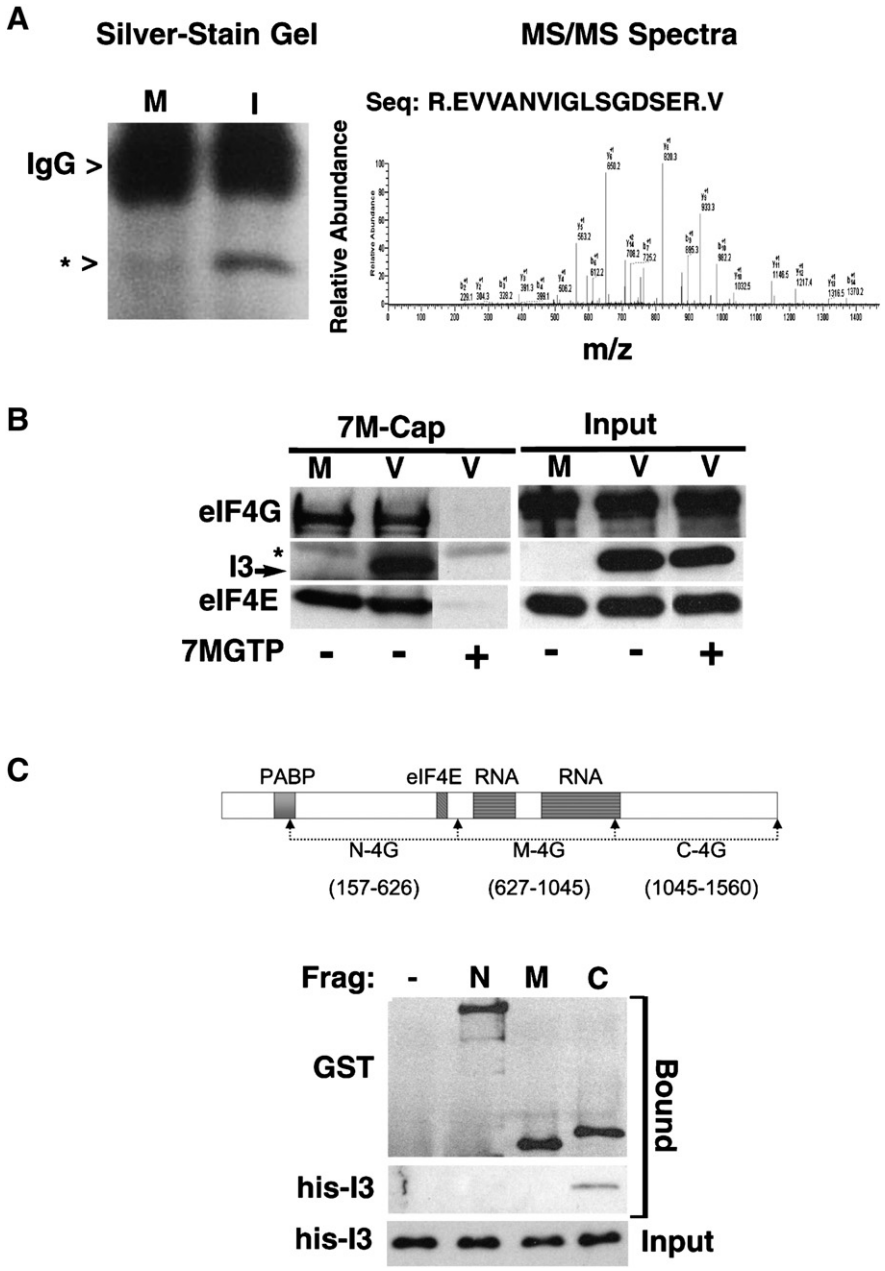

Fig. 3. Identification of the eIF4G-associated protein as VacV I3. (A) NHDFs were mock-infected (M) or infected (V) for 16 h. Soluble cell extracts were prepared and immunoprecipitated with anti-eIF4G antiserum. Immune-complexes were resolved by SDS-PAGE, gels were silver-stained and a specific band migrating at 34 kDa only present in infected samples was excised and identified by mass spectrometry. A representative image from silver-stained gels, with an asterisk indicating the 34 kDa protein in question, along with a sample MS/MS spectra of a peptide from I3 is shown. (B) 293 cells were mock-infected (M) or infected (V) at m.o.i. 5 for 16 h. Soluble cell extracts were prepared then eIF4E and associated proteins were recovered on 7-Methyl GTP resin in the presence (+) or absence (−) of competing free 7-Methyl GTP (3 mM). Resin-associated and input samples were analyzed by western blotting with the indicated antisera. All images were from the same blots and exposures, cropped to remove replicate experiments. A slower-migrating non-specific band, indicated with an asterisk, is evident in all cap-bound samples. (C) Schematic representation of the GST-tagged N-, M- and C-terminal regions of eIF4G used in binding assays. The location of binding sites for PABP, eIF4E and RNA binding domains within these fragments are indicated by the shaded boxes. The corresponding amino acid numbers of each fragment is given in brackets. Lower Panels: Binding reactions were performed between his-I3 and each of the GST-fused eIF4G fragments, together with dialysis buffer alone as a control (−). Glutathione-sepharose complexes were washed and boiled in Laemmli buffer then analyzed by western blotting using ant-GST and anti-histidine antibodies to detect eIF4G fragments and I3, respectively. Input samples demonstrate the presence of I3 in all reactions.

eIF4F that was captured from soluble cell extracts on 7-Methyl-GTP (cap) sepharose. 293 cells, which contain higher levels of eIF4F convenient for binding assays, were mock-infected or infected for 16 h then cap-binding eIF4F complexes were recovered from RNAse A-treated soluble cell extracts on cap-sepharose. When complexes were analyzed by western blotting I3 was readily detectable in infected cell samples and, together with the eIF4F components eIF4E and eIF4G, binding was competitively inhibited by the addition of free 7-Methyl GTP to extracts (Fig. 3B). This demonstrated that I3 associated with cap-binding eIF4F, even at the late stages of infection reached in 293 cells by 16 h. Finally, we examined whether I3 bound to eIF4G directly. To do this, his-tagged I3 and GST-tagged N-(aa157-626), M-(aa627-1045) or C-(aa1045-1560) terminal fragments of eIF4G (diagram Fig. 3C and (Cuesta et al., 2000)) were isolated from benzonase-treated BL21 cells. 200 ng his-I3 was incubated for 2 h with glutathione-sepharose with either dialysis buffer alone or

together with 1 μg of each GST-tagged eIF4G fragment then resin-bound complexes were washed and analyzed by western blotting. While each eIF4G fragment was equally recovered, with the slower mobility of N-4G through SDS-PAGE gels due to structural complexity in the N-terminal domain of eIF4G, I3 was found specifically bound to the C-terminal fragment of eIF4G (Fig. 3C).

Co-localization of I3 and eIF4G in infected NHDFs

Having established its interaction with eIF4G, we next examined whether I3 and eIF4G co-localized in infected cells. NHDFs were mock-infected or infected for 22 h, then fixed and analyzed by immunofluorescence using mouse anti-I3 and rabbit anti-eIF4G antibodies. In uninfected cells, no I3 staining was evident and eIF4G was detected in both the nucleus and the cytoplasm (Fig. 4, upper panel set), with notable concentrations in perinuclear regions similar to staining patterns reported by others (Willett et al., 2006). In infected cells, large viral factories were evident as Hoechst-stained structures in the cytoplasm that contained discrete concentrations of eIF4G, as reported previously (Katsafanas and Moss, 2007; Walsh et al., 2008). eIF4G present in factories co-localized with I3 (Fig. 4, middle panel sets). Similar patterns of co-localization were observed in >90% of infected cells. Notably, co-localization was not absolute in all cases (as evident in panel series 4), which may represent dynamic interactions between I3 and eIF4G or I3's multiple functions. However, in all cases there was a high degree of co-localization between I3 and eIF4G similar to the images presented.

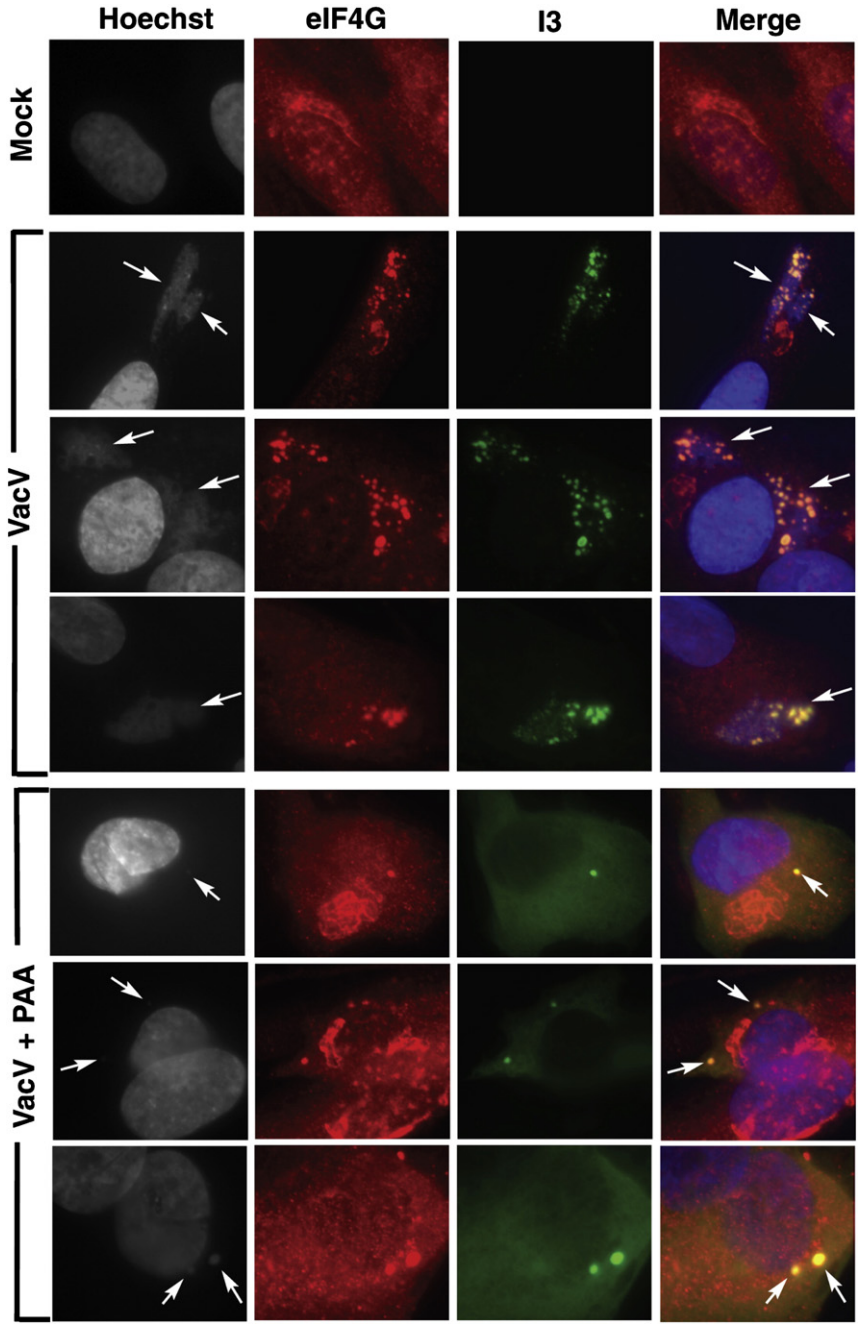

Fig. 4. Co-localization of I3 and eIF4G in infected NHDFs. NHDFs were either mock-infected (top panels) or infected with VacV at m.o.i. 5 for 22 h in the absence (middle three panels) or presence (lower three panels) of PAA. Cells were fixed and analyzed by immunofluorescence using rabbit anti-eIF4G (red) and mouse anti-I3 (green) antibodies. DNA was stained using Hoechst. Arrows indicate the location of viral factories (middle three panels) or sites of viral DNA that are precursors for factory formation (lower three panels).

Next we determined whether I3 and eIF4G co-localized prior to viral factory formation by inhibiting viral DNA synthesis using Phosphonoacetic Acid (PAA). It has been shown previously that in the presence of similar inhibitors, I3 is largely distributed throughout the cytoplasm but forms punctate structures that co-localize with DNA from incoming viral cores (Domi and Beaud, 2000; Rochester and Traktman, 1998; Welsch et al., 2003). These are precursors of viral factories and viral DNA can be faintly visualized by Hoechst staining. NHDFs were pre-treated with PAA then infected and analyzed by immunofluorescence as described above. In PAA-treated cultures small DNA-positive structures were evident near host nuclei (Fig. 4, lower panel sets). Both I3 and eIF4G were distributed throughout the cytoplasm, but both proteins formed punctate spots of co-localization with viral DNA. Notably, in many cells eIF4G-staining structures were evident near factories and nuclei, which may represent ER structures reported by others (Willett et al., 2006) or the presence of eIF4G in aggresomes, similar to those reported for other host proteins in VacV-infected cells (Paran et al., 2009). This suggested that I3 interacted with eIF4G prior to viral factory formation, in line with a potential role in eIF4G retention at sites of factory formation. To confirm this interaction, we examined the effects of PAA on I3 association with eIF4F complexes. 293 cells were used to recover eIF4F on cap-sepharose to avoid the complication of changes in eIF4F levels that occur in infected NHDFs. 293 cells were infected in the presence or absence of PAA then cap-bound proteins were recovered from soluble cell extracts on 7-Methyl GTP-sepharose. As illustrated in Fig. 5A, PAA treatment did not affect the accumulation of I3, characteristic of early-to-intermediate-phase VacV proteins (Rochester and Traktman, 1998) and did not adversely affect the binding of I3 to eIF4F. This demonstrated that I3 bound to eIF4F early in infection and prior to the onset of viral factory formation.

eIF4F assembly in VacV-infected NHDFs is sensitive to PAA

VacV causes increased eIF4F complex formation in normal human cells (Walsh et al., 2008) but the exact mechanism remains unclear. The translational repressor 4E-BP1 binds eIF4E at the same site as eIF4G, competitively inhibiting eIF4E:eIF4G interaction and suppressing eIF4F complex formation. Phosphorylation of 4E-BP1 by mTOR causes electrostatic repulsion and release of eIF4E. VacV activates mTOR signaling to promote phosphorylation and inactivation of the translational repressor, 4E-BP1 (Walsh et al., 2008; Zaborowska and Walsh, 2009). However, although inhibition of mTOR prevents VacV from inactivating 4E-BP1, its ability to enhance eIF4F complex formation is only partially reduced (Zaborowska and Walsh, 2009), suggesting that additional events contribute to eIF4F formation during infection. PAA, an inhibitor of viral DNA synthesis and factory formation, has been shown to dampen 4E-BP1 phosphorylation during VacV infection, leading to the suggestion that late gene products or viral factory formation are involved (Walsh et al., 2008). However, the full effects of this inhibitor on eIF4F complex formation have not been explored while its effects on 4E-BP1 phosphorylation appear to contradict a recent report that the PI3K–Akt–mTOR pathway is activated very early in infection (Soares et al., 2009). To address these issues we examined the effects of PAA on 4E-BP1:eIF4E binding and eIF4F assembly in resting primary cells in more detail. Serum-starved NHDFs were mock-infected or infected with VacV in the presence or absence of PAA and the levels of eIF4E-bound eIF4G and 4E-BP1 were examined by recovering eIF4E on 7-Methyl-GTP sepharose. As reported previously VacV infection stimulated 4E-BP1 phosphorylation (Walsh et al., 2008; Zaborowska and Walsh, 2009), evident as a decrease in antigen mobility through high percentage SDS-PAGE gels in input samples from infected cells (Fig. 5B). This was reflected in reduced binding of 4E-BP1 to eIF4E on 7-Methyl GTP resin, which was accompanied by an increase in the association of eIF4G with eIF4E, indicative of enhanced eIF4F formation. However, although PAA increased the mobility of 4E-BP1 through high-percentage gels in input samples (indicated with an arrow in Fig. 5B), suggesting 4E-BP1 dephosphorylation and similar to previous reports (Walsh et al., 2008), it did not prevent the release of 4E-BP1 from eIF4E on 7-Methyl GTP-sepharose (Fig. 5B). In addition, PAA had minimal effects on the phosphorylation of Akt on either Thr308 or Ser473 residues, the kinase that acts upstream to stimulate mTOR and 4E-BP1 phosphorylation during infection (Soares et al., 2009; Zaborowska and Walsh, 2009). Overall, this demonstrated that 4E-BP1 was released from eIF4E prior to viral DNA synthesis and suggested that the effects of PAA on 4E-BP1 phosphorylation, as measured by altered mobility through high-percentage gels, may simply reflect an altered rate of phosphate turnover without affecting actual release of the repressor from eIF4E. As such, 4E-BP1 is inactivated and released from eIF4E early in infection.

However, despite the release of 4E-BP1 from eIF4E PAA treatment did suppress the increase in eIF4G binding that occurred in VacV-infected cultures in the absence of this inhibitor (Fig. 5B). Although some increase in eIF4E:eIF4G binding was apparent, reflective of the release of 4E-BP1, the effects of PAA suggested that additional forces beyond the release of 4E-BP1 contributed to the increase in eIF4F levels observed in VacV-infected cells, possibly linked to the effects of PAA on the redistribution and concentration of factors within viral factories (Fig. 4).

I3 promotes the association of eIF4G with ssDNA

Given the association of I3 with eIF4F, we further explored how I3 might influence mRNA translation. To determine potential effects on host translation, the I3L gene was PCR-amplified from genomic viral DNA using primers that generated a C-terminally FLAG-tagged form of I3, which was ligated into a mammalian expression plasmid. Stable 293 empty vector or I3-expressing lines were generated by drug selection. Immunofluorescence using monoclonal anti-I3 antibody demonstrated that FLAG-I3 was predominantly cytoplasmic and expressed at detectable levels in approximately 60–70% of cells, while the ability of FLAG-I3 to associate with eIF4F was confirmed by recovering 7-Methyl GTP complexes from RNAse A-treated soluble cell extracts followed by western blot analysis (Fig. 6A). However, metabolic labeling showed no notable differences in the pattern or rates of cellular protein synthesis between control or FLAG-I3-expressing cultures (Fig. 6B). We also tested whether I3 affected translation of RNA isolated from VacV-infected cells in vitro. Rabbit reticulocyte lysates containing ^{35}S-Methionine were programmed with 10 μg total RNA from infected 293 cells in the presence of dialysis buffer or 200 ng purified his-I3 and incubated at 30 °C for 1 h. Samples were then boiled with an equal volume of 2× Laemmli Buffer and resolved by SDS-PAGE then gels were fixed, dried and exposed to x-ray film (Fig. 6C; upper panel) or analyzed by western blotting with anti-his antibody to confirm the presence of I3 in reactions (Fig. 6C; lower panel). The presence of I3 in reactions had no discernable effect on rates or patterns of viral protein synthesis. As such, I3 did not appear to adversely affect translation of either host or viral messages. However, it must be noted that I3 staining in FLAG-I3 lines was heterogenous and far lower than levels expressed during infection (not shown). As such, we cannot presently rule out the possibility that I3 influences translation when expressed at higher levels. It is also possible that I3 influences translation in the context of infection by retaining translation factors within viral factories. The very high affinity of I3 for ssDNA is thought to explain its retention in viral factories (Domi and Beaud, 2000; Rochester and Traktman, 1998; Tseng et al., 1999). We therefore examined the ability of I3 to recruit translation factors to ssDNA to determine whether its ssDNA-binding and eIF4G-binding functions were mutually exclusive or potentially contribute to a mechanism of sequestration. Soluble cell extracts were

prepared from FLAG- or FLAG-I3-expressing 293 cells then treated with RNAse A to prevent potential RNA:ssDNA bridging interactions. RNAse was then inactivated by the addition of RNAse inhibitor and precleared extracts were incubated with 5′ biotinylated ssDNA. Input DNA was recovered on anti-biotin antibody-conjugated sepharose and associated proteins were analyzed by western blotting. In control extracts a low level of eIF4G binding to either ssDNA or the sepharose resin was evident (Fig. 6D). However, in the presence of I3 significantly more (16-fold) eIF4G was recovered. In addition to eIF4G, higher levels of PABP were also bound in the presence of I3 (Fig. 6D), although background binding of PABP to ssDNA or the matrix complicated the interpretation of the precise degree to which PABP is recruited by eIF4G. However, as I3 does not appear to interact with PABP (Fig. 2), this demonstrated that I3 was capable of recruiting eIF4G and eIF4G-associated PABP to ssDNA. To confirm the specificity of factor recruitment by I3 the association of RBM3 was examined in the same samples. RMB3 is a cellular protein with both RNA and ssDNA-binding properties that has been shown to potentially interact with VacV A2 protein, but not I3 (Dellis et al., 2004; Derry et al., 1995). While RBM3 was found associated with ssDNA in both extracts its association was modestly reduced in I3 versus control extracts (Fig. 6D), possibly due to competition for binding from I3 itself. In addition to confirming the specificity of I3 function in eIF4G recruitment, this

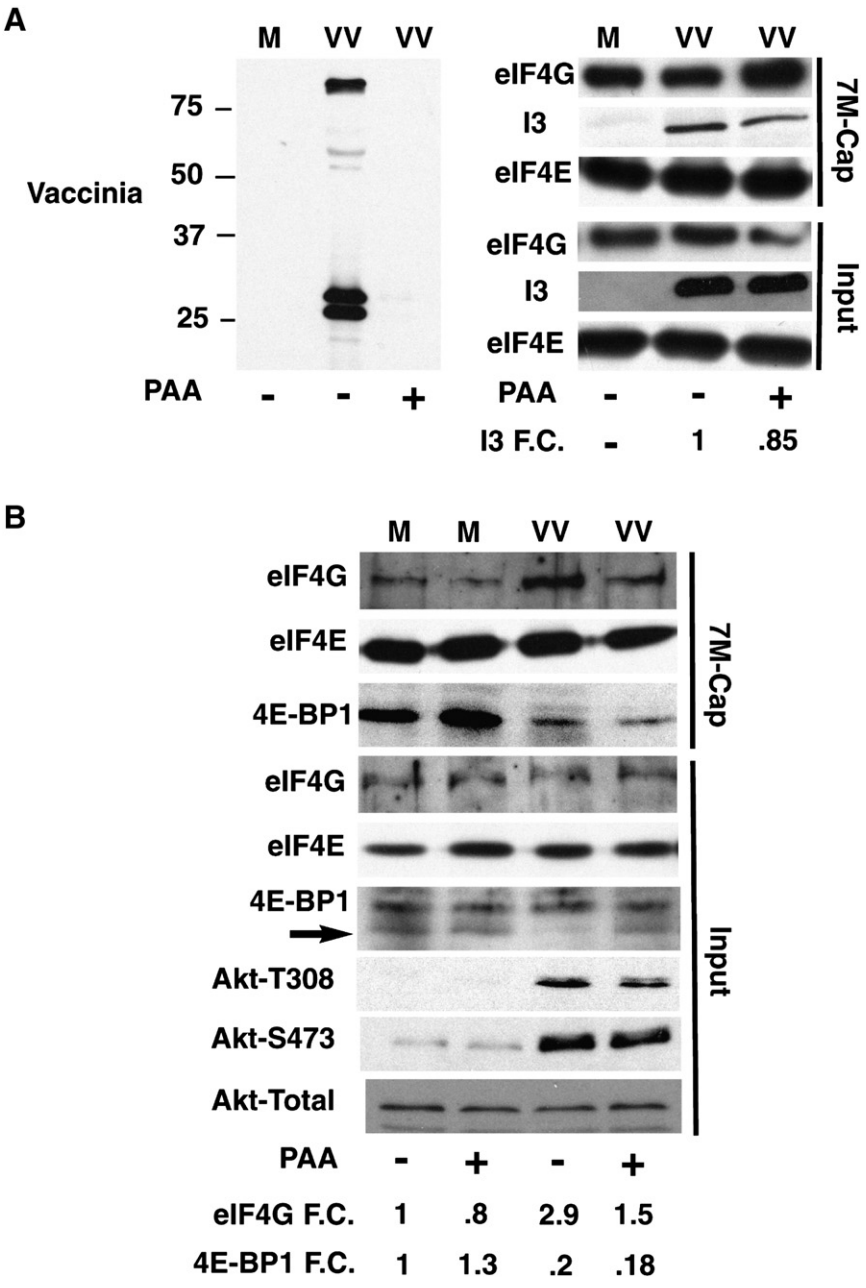

Fig. 5. I3 binding and eIF4F complex formation in PAA-treated cells. (A) 293 cells were mock-infected (M) or infected (V) in the presence or absence of PAA for 16 h then soluble cell extracts were prepared. Input samples were probed with anti-vaccinia virus antiserum to confirm the effectiveness of PAA treatment. Molecular weight markers (in kDa) are indicated to the left. 7-Methyl GTP-bound and input samples were prepared and analyzed by western blotting with the indicated antibodies (right panel). Densitometry was used to quantify the fold change (F.C.) in I3 binding to cap complexes in PAA-treated samples compared to untreated samples, arbitrarily set at 1. (B) Serum-starved NHDFs were mock-infected (M) or infected (V) at m.o.i. 10 in the presence or absence of PAA. 24 h.p.i. soluble cell extracts were prepared and precleared with sepharose 4B before cap-binding eIF4E and associated proteins were recovered on 7-Methyl GTP resin. Beads were boiled in Laemmli buffer and analyzed, together with input samples, by western blotting with the indicated antibodies. An arrow indicates the migration of hypo-phosphorylated 4E-BP1 in input samples. Densitometry was used to quantify the fold change (F.C.) in the association of eIF4G or 4E-BP1 with 7-Methyl GTP resin relative to mock-infected samples, arbitrarily set to 1.

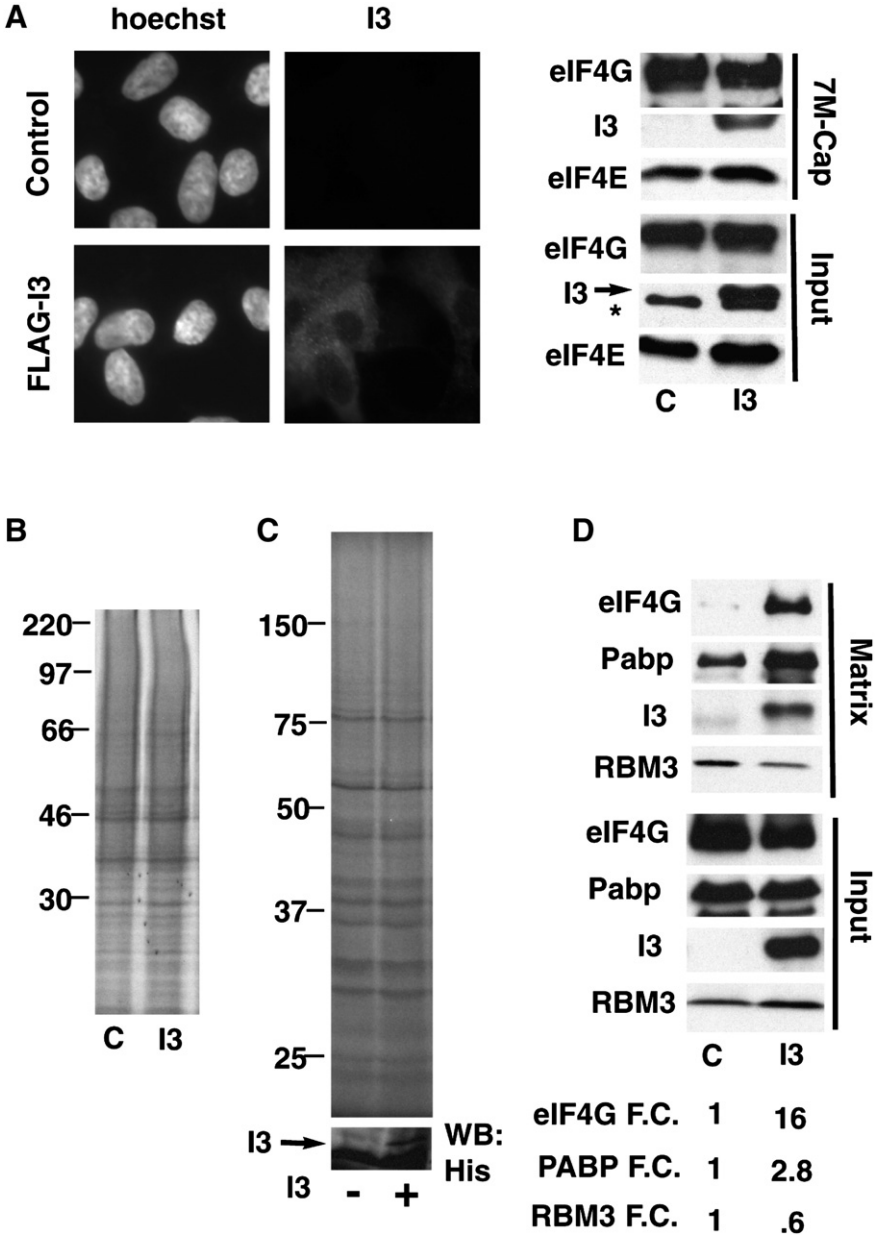

Fig. 6. I3 does not adversely affect host or viral mRNA translation but recruits eIF4G to ssDNA. (A) Left panel; Control empty-vector and FLAG-I3 cultures were fixed and analyzed by immunofluorescence using mouse anti-I3 antibody. DNA was stained using Hoechst. Right panel; soluble cell extracts were prepared from control (C) empty-vector or FLAG-I3 (I3) expressing 293 cells then precleared with Sepharose 4B. Extracts were incubated with 7-Methyl GTP-Sepharose 4B then resin-bound and input samples were analyzed by western blotting with the indicated antisera. Migration of I3 detected with anti-FLAG antiserum is indicated with the arrow (>). A non-specific band detected by this antiserum in input samples just below I3 is indicated by the asterisk. (B) Control (C) or I3-expressing (I3) cells were labeled with ^{35}S-Methionine for 60 min then lysed in laemmli buffer. Samples were resolved by SDS-PAGE and gels were fixed, dried and exposed to x-ray film. Migration of molecular weight standards (in kDa) is indicated to the left. (C) 10 μg total RNA isolated from VacV-infected cells was translated in rabbit reticulocyte lysates for 60 min the presence of dialysis buffer (−) or his-I3 (+). I3 was detected by using anti-histidine antibody. An intense background band from the reticulocyte lysate prevented longer exposures but his-I3 is clearly evident in samples. (D) Soluble cell extracts were prepared from FLAG- or FLAG-I3-expressing 293 cells. After RNAse A treatment and inactivation, extracts were precleared with anti-biotin antibody-conjugated sepharose. Precleared extracts were programmed with 7.5 μg biotin-conjugated ssDNA together with anti-biotin antibody-conjugated sepharose for 2 h. Immune-complexes and input samples were analyzed by western blotting with the indicated antisera.

also demonstrated that I3 was not simply stabilizing the input ssDNA in extracts and, thereby, non-specifically mediating the increased recovery of nucleotide-binding proteins in general.

Discussion

All viruses are dependent upon a host cell to provide the translational machinery needed for the synthesis of viral proteins. For both RNA and DNA viruses a key element in the battle to usurp host ribosomes is control of the translation initiation complex eIF4F (Walsh and Mohr, 2011). In addition to modulating cellular signaling pathways that regulate host translation factor activity, a growing number of viruses are being found to encode proteins that directly interact with the eIF4F complex. Among RNA viruses, rotavirus NSP3 protein (Groft and Burley, 2002; Montero et al., 2006; Piron et al., 1998; Vende et al., 2000) and the influenza virus proteins NS1 (Aragón et al., 2000; Burgui et al., 2003) and Polymerase (Burgui et al., 2007) bind the translation initiation factor eIF4G to mediate selective translation of viral messages or modulate host protein synthesis, although hantavirus N protein has recently been shown to take this approach even further and functionally replaces the eIF4F complex altogether (Mir and Panganiban, 2008). In the case of DNA viruses,

adenovirus-encoded 100 k protein binds eIF4G, evicting the eIF4E kinase Mnk from assembled complexes and facilitating translation of viral mRNAs via ribosome shunting (Cuesta et al., 2000; Xi et al., 2004). HSV-1 encodes another eIF4G-interacting protein, ICP6, which has homology to cellular chaperones and promotes the assembly of eIF4F complexes (Walsh and Mohr, 2006). In addition, HSV-1 encoded ICP27 interacts with the eIF4G binding protein, PABP to regulate translation of specific sub-sets of viral mRNAs (Ellison et al., 2005; Fontaine-Rodriguez and Knipe, 2008; Fontaine-Rodriguez et al., 2004; Larralde et al., 2006), while another HSV-1 function, VHS associates with eIF4A to regulate mRNA turnover (Feng et al., 2005). HCMV also encodes an ICP27-like protein, UL69 that associates with PABP and eIF4A (Aoyagi et al., 2010). As such, encoding factors that interact with the scaffold protein eIF4G or its binding proteins appears to be a common strategy employed by numerous viruses to enable direct manipulation of eIF4F function. Here we demonstrate that the I3 protein encoded by VacV also associates with eIF4G and can recruit it, along with PABP, to ssDNA.

Unlike many other mammalian DNA viruses, poxviruses and asfarviruses replicate exclusively in the cytoplasm of infected cells within specialized replication compartments. A number of cellular proteins have been shown to redistribute to viral factories during poxvirus infection while the localization of many others, including nucleotide-binding proteins, remains unaffected (Broyles, 1991; Castro et al., 2003; Hung et al., 2002; Katsafanas and Moss, 2007; Lin et al., 2008; Paran et al., 2009; Walsh et al., 2008). As such, the redistribution of factors during infection appears to be selective and, therefore, likely to be mediated by specific functions, as is the case for the recruitment of cellular topoisomerase II by VacV DNA ligase (Lin et al., 2008). A number of viral proteins produced early in VacV infection are synthesized in the cytoplasm but accumulate within viral factories as infection progresses. Among these, the association of VacV E3 protein with double-stranded RNA formed as a consequence of viral transcriptional read-through has been suggested to promote its accumulation within replication compartments (Katsafanas and Moss, 2007). Similarly, the formation of ssDNA in factories likely mediates the accumulation of I3 (Domi and Beaud, 2000; Rochester and Traktman, 1998; Welsch et al., 2003). The potential for these proteins to bind to specific host factors would provide a means for the virus to selectively organize the protein composition of viral factories, as opposed to a random diffusion of factors into these sites. As a virus-encoded ssDNA-binding protein it has been suggested that I3 may play a role in viral DNA replication, recombination or repair (Rochester and Traktman, 1998; Tseng et al., 1999). Indeed, a recent report has confirmed I3 functions in both replication and recombination (Gammon and Evans, 2009). Although I3 has been shown to interact with the viral ribonucleotide reductase (Davis and Mathews, 1993), other I3-interacting proteins have not been reported. However, BLAST and ELM (eukaryotic linear motif) analysis predicts that I3 contains various protein binding domains and target sites for phosphorylation by host kinases (unpublished observation). In line with this, previous studies have suggested that I3 is phosphorylated by a host, rather than viral, kinase(s) (Rochester and Traktman, 1998). Our findings provide the first evidence of I3 interaction with a host cell protein that exhibits the same pattern of redistribution upon VacV infection. As such, in addition to its role as a viral ssb and like many multifunctional viral proteins, I3 may also play an important role in sequestering host factors in poxvirus-infected cells, including those involved in translational control.

As the number of DNA viruses that are found to be capable of enhancing the levels of eIF4F in infected cells increases the mechanisms by which they accomplish this are just beginning to be elucidated. In the case of HSV-1, HCMV and VacV enhancement of eIF4G binding to eIF4E can occur in the absence of significant release of the repressor 4E-BP1, suggesting that these viruses can drive complex formation through additional mechanisms (Kudchodkar et al., 2004; Walsh and Mohr, 2004; Walsh et al., 2005; Zaborowska and Walsh, 2009). Conversely, in HSV-1-infected cells the release of 4E-BP1 alone does not appear to be sufficient to drive eIF4F complex formation in the absence of the viral eIF4F chaperone ICP6 (Walsh and Mohr, 2006). Similarly, we show that in VacV-infected cells stimulation of eIF4F complex formation is reduced in the presence of PAA despite the release of 4E-BP1 from eIF4E. This suggests that eIF4F assembly in VacV-infected cells also involves forces in addition to the release of 4E-BP1. VacV enhances eIF4F levels in cell types where initiation factor and eIF4F complex levels are low, likely by concentrating host translation factors within viral factories (Katsafanas and Moss, 2007; Walsh et al., 2008). A concentration-dependent process of eIF4F assembly in serum-starved NHDFs has also been proposed for human cytomegalovirus (HCMV) (Walsh et al., 2005). HCMV has not been shown to encode proteins that promote eIF4F formation and does not redistribute host translation factors. Instead, HCMV causes a robust increase in the steady-state levels of a number of host initiation factors, which appears to play a central role in fostering increased eIF4F formation during infection. In the absence of an increase in factor abundance during VacV infection (Walsh et al., 2008), creating local concentrations within viral factories likely achieves the same effect and alters the relative on-off rate of the eIF4E:eIF4G interaction, tilting the balance towards increased interaction. This is supported by the effects of PAA, which does not adversely affect I3 association with eIF4F or release of 4E-BP1 but suppresses initiation factor redistribution and eIF4F assembly (Fig. 5B) (Walsh et al., 2008). Its ability to recruit eIF4G to ssDNA suggests that I3 is likely to be involved in these events. However, due to the essential nature of I3, and similar to previous attempts (Rochester and Traktman, 1998), we have been unable to recover virus lacking the I3L gene to directly test this.

In addition to enhancing eIF4F levels in infected cells VacV also stimulates the recruitment of PABP, despite the fact that only relatively small amounts of the total pool of this protein are actually recruited into viral factories (Walsh et al., 2008). Here, immunoprecipitation-based approaches confirmed the increased formation of initiation complexes and recruitment of PABP, with more eIF4E detected in both eIF4G and PABP immune complexes recovered from infected compared to uninfected cell extracts, albeit modest at the intermediate point examined in this particular experiment. The inefficient recovery of eIF4E in association with PABP immune complexes suggests that only a limited fraction of the total cellular pool of this multifunctional protein is actually associated with translation initiation complexes, at least in NHDFs. Recruitment of this fraction of PABP into viral factories by virtue of its association with I3-bound eIF4F would explain why some PABP does redistribute to these structures (Walsh et al., 2008). It is likely that, similar to eIF4E, I3 is present in PABP immune complexes as part of the eIF4F-associated fraction of PABP but below detectable levels of this assay. Indeed, although I3 did not freely associate with PABP it did mediate recruitment of PABP to ssDNA in vitro, suggesting that I3 was not precluded from associating with, and could effectively recruit, PABP-bound eIF4G. This is in line with our finding that I3 binds the C-terminal region of eIF4G, while PABP associates with the N-terminus of eIF4G (Imataka et al., 1998). Overall, these findings provide insight into the mechanisms by which eIF4F is regulated during VacV infection and identifies a potential viral candidate for eIF4G sequestration in viral replication compartments.

Materials and methods

Cell culture and viruses

NHDF and 293 cells were cultured in DMEM containing 5% FBS as described previously (Walsh and Mohr, 2004; Zaborowska and Walsh, 2009). For serum starvation, confluent cultures of NHDFs were washed three times in PBS then cultured in DMEM containing

0.2% FBS for 5 days. Inhibitor treatment and metabolic labeling of cell cultures was performed as described previously (Walsh and Mohr, 2004; Walsh et al., 2008). VacV Western Reserve was a kind gift of Dr. Stewart Shuman, Memorial Sloan-Kettering, New York, and was propagated and titered on permissive BSC40 cells.

Cloning, plasmids and proteins

The I3L gene was amplified from VacV genomic DNA isolated as described previously (Roper, 2004). To generate FLAG-tagged I3L the following primers were used, containing EcoR1 and Sal1 restriction sites for cloning. The FLAG sequence is italicized.

Forward:
GCAACT GAATTC CGCCGCC ATG AGTAAGGTAATCAAGAAG
Reverse: GCAACT GTCGAC TCTAGT TCA *ctt gtc gtc atc gtc ttt gta gtc* TAC ATT GAA TAT TGG CTT TTC

The amplified PCR product was gel purified and digested with EcoR1-Sal1 then ligated into the EcoR1-Sal1 cloning site of the mammalian expression plasmid, pCI-Neo. Ligations were transformed into DH5α cells and individual colonies were isolated. Recovered plasmids were analyzed by restriction digestion then sequenced to ensure cloning fidelity. Stably-expressing 293 cells were established by transfecting cultures using lipofectamine (Invitrogen) followed by selection with geneticin.

To generate purified his-tagged I3 the I3L gene was PCR amplified using the following primers containing Not1 and Nde1 sites:

Forward: GCAACTCATATGAGTAAGGTAATCAAGAAG
Reverse:
GCAACTGCGGCCGCGACTAGTTCATACATTGAATATTGGCTTTTC

The PCR product was then digested with Not1 and Nde1 and ligated into Not1/Nde1 cloning sites in the bacterial expression plasmid, pET-15b. To purify protein, the pET-his-I3 plasmid was transformed into BL-21 cells (Invitrogen). Overnight starter cultures were used to inoculate 250 ml cultures grown at room temperature until O.D. 600 then induced with IPTG for 30–60 min. His-tagged I3 was purified by cell lysis followed by benzonase treatment and isolation on His-Talon columns (Clontech) according to the manufacturer's instructions. BL21 cells containing GST-tagged eIF4G fragments and their purification were as described previously (Cuesta et al., 2000; Walsh and Mohr, 2006). All proteins were dialyzed overnight, with buffer changes 2 h before and after the overnight dialysis, as described previously (Walsh and Mohr, 2006).

In vitro binding reactions and in vitro translation

For eIF4G:I3 binding reactions 20 μl p.b.v. glutathione-sepharose was washed 3 times in binding buffer [50 mM Hepes, pH 7.4, 100 mM NaCl. 0.5 mM $MgCl_2$, 2 mM EDTA, 2 mM Na_3VO_4, 25 mM glycerophosphate and complete mini EDTA-free protease inhibitor cocktail (Roche)] and blocked at room temperature for 1 h in binding buffer containing 5% BSA. Binding reactions were performed by rocking the blocked glutathione-sepharose with 1 μg GST-tagged eIF4G and 200 ng his-I3 in a 200 μl volume of binding buffer containing 1% BSA and 0.25% NP-40. Controls lacking proteins contained dialysis buffer.

In vitro translation was performed using rabbit reticulocyte lysates (Promega) in a total volume of 50 μl supplemented with 2 μl IVT grade ^{35}S-Methionine (Perkin Elmer), 200 ng I3 protein or equivalent volume of dialysis buffer as control together with 10 μg total RNA. Total RNA was isolated using Qiagen RNA spin kits from 10 cm^2 dishes of 293 cells that had been infected overnight with VacV at an m.o.i. 10.

Western blotting and immunofluorescence

Western blotting was performed as described previously (Walsh and Mohr, 2004). For immunofluorescence, cells were seeded on coverslips (Fisher) and treated as indicated. Cells were then fixed in 3.7% formaldehyde/PBS for 20 min, washed extensively with PBS and permeabilized in 0.1% Triton for 20 min. After washing, dishes were blocked in a mixture of 10% FBS and 0.25% Saponin in PBS for 40 min, then probed overnight at 4 °C with rabbit anti-eIF4G and mouse anti-I3 antibodies diluted in blocking buffer. Samples were washed three times with PBS-0.025% Saponin then probed for 40 min with Alexafluor 555-conjugated donkey anti-rabbit and Alexafluor 488-conjugated donkey anti-mouse secondary antibodies (Molecular Probes) in blocking buffer. Samples were washed three times PBS-0.025% Saponin, DNA was stained with Hoechst for 5 min and washed again in PBS-0.025% Saponin, followed by washes in PBS and water. Images were captured using a Zeiss Axioplan 2 microscope and imaging system.

Immunoprecipitation, 7-Methyl GTP chromatography and ssDNA binding assay

Anti-eIF4G and anti-PABP immunoprecipitation from metabolically labeled cell extracts was performed as described previously (Walsh and Mohr, 2006). Briefly, after labeling, soluble cell extracts were prepared in NP-40 lysis buffer (NLB) and centrifuged at 13,000 rpm for 10 min at 4 °C. Extracts were treated with a cocktail of nucleases (RNAse A, RNase T1 and Micrococcal Nuclease) then precleared and immunoprecipitated as described (Walsh and Mohr, 2006). For 7-Methyl GTP chromatography, 10^7 cells were washed in PBS then lysed in 600 μl NLB, treated with RNAse A and precleared with 80 μl packed-bed volume (pbv) BSA-blocked sepharose 4B for 1 h. Input samples were taken from precleared extracts and the remaining sample was incubated with 50 μl pbv BSA-blocked 7-Methyl GTP-Sepharose (Amersham) for 1 h then washed in NLB and boiled in Laemmli buffer. For ssDNA-binding assays, 3×10^7 cells were washed in PBS then lysed in NLB and centrifuged at 13,000 rpm for 10 min at 4 °C. Extracts were treated with RNAse A for 20 min at room temperature then treated with RNAse Inhibitor (Sigma-Aldrich) to stop the RNAse reaction prior to the addition of ssDNA. Extracts were precleared for 1 h with anti-biotin antibody conjugated sepharose 4B (Sigma Aldrich). Precleared extracts were then programmed with 7.5 μg phosphothioate-stabilized 5′-biotin conjugated ssDNA (sequence: ataattaataacaccatagaccaccgcccgaaggg) and 20 μl p.b.v. anti-biotin antibody-conjugated sepharose 4B for 2 h at 4 °C. Resin was recovered and washed extensively in NLB before boiling in Laemmli buffer.

Antibodies and chemicals

Anti-eIF4G antiserum was a kind gift of Ian Mohr, NYU Langone School of Medicine, New York, USA. Anti-PABP antiserum was a kind gift of Simon Morley, University of Sussex, UK. Rabbit anti-I3 antiserum was a kind gift of Jacomine Krijnse-Locker, EMBL Heidelburg, Germany. Mouse monoclonal anti-I3 was a kind gift of David Evans and Nicole Favis, University of Alberta, Canada. Anti-eIF4E antibody was from BD Transduction Laboratories. Anti-4E-BP1 and antibodies against the phosphorylated and total forms of Akt were obtained from Cell Signaling Technology. Anti-Vaccinia Virus antibody was from Virostat. Anti-FLAG and anti-histidine antibodies together with PAA were from Sigma-Aldrich.

Silver staining and mass spectrometry analysis

After resolution of samples by SDS-PAGE, gels were sequentially soaked for 10 min in 50% Methanol, 5% Methanol, 30 μM DTT, 0.1%

(w/v) $AgNO_3$, then visualized with 3% (w/v) Na_2CO_3. Bands were excised from silver stained gels and destained using a 1:1 mixture of 30 mM potassium ferricyanide/100 mM sodium thiosulfate solution. In-gel digestion using sequence grade trypsin (Promega) at a concentration of 12.5 ng/μl trypsin in 10 mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile was carried out overnight at 37 °C. Tryptic peptides generated were extracted and analyzed by LC-MS/MS using an LTQ ion trap mass spectrometer (Thermo Fisher Scientific). The data generated was searched using BioWorks 3.3.1 (Thermo Fisher Scientific) against a subset of human and viral proteins from the SwissProt database.

Acknowledgments

We thank Ian Mohr, Stewart Shuman, Simon Morley, David Evans, Nicole Favis and Jacomine Krijnse-Locker for kindly providing us with reagents. We would also like to thank Bernard Moss and Arban Domi for their advice and kind gift of the Vac-BAC/λ system used in our attempt to generate an I3L mutant. This work was supported by grants from the Health Research Board (RP/2007/52) to D.W. and from Science Foundation Ireland (07 IN.1 B1323).

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