Complementation of the human adenovirus type 5 VA RNAi defect by the Vaccinia virus E3L protein and serotype-specific VA RNAIs

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Human adenoviruses (HAdVs) encode for multifunctional non-coding virus-associated (VA) RNAs, which function as powerful suppressors of the cellular interferon (IFN) and RNA interference (RNAi) systems. In this study we tested the ability of various plant and animal virus encoded RNAi and IFN suppressor proteins to functionally substitute for the HAdV-5 VA RNAi. Our results revealed that only the Vaccinia virus (VACV) E3L protein was able to substitute for the HAdV-5 VA RNAi functions in virus-infected cells. Interestingly, the E3L protein rescues the translational defect but does not stimulate viral capsid mRNA accumulation observed with VA RNA. We further show that the E3L C-terminal region containing the dsRNA-binding domain is needed to enhance VA RNAi mutant virus replication. Additionally, we show that the HAdV-4 and HAdV-37 VA RNAi are more effective than the HAdV-5 VA RNAi in rescuing virus replication.

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Introduction

One of the fundamental pathways that a virus has to evade is the host cell antiviral response. The first line of antiviral defense mounted by the host is carried out by the interferon (IFN) and/or by the RNA interference (RNAi) systems. The antiviral IFN response depends on the activation of a cellular IFN signaling cascade, which triggers expression and activity of multiple interferon stimulated genes including the double-stranded (ds) RNA-activated protein kinase (PKR) (Sadler and Williams, 2008). In its enzymatically active form PKR phosphorylates the α subunit of eIF2α at serine 51 (Ser51) impairing the GDP-GTP cycling of eIF2 and causes a global inhibition of translation initiation, which would be detrimental for virus multiplication (Garcia et al., 2006). In the case of RNAi, the antiviral defense is initiated by viral double stranded RNA (dsRNA) replication intermediates, which are processed by the Dicer enzyme into small interfering RNAs (siRNAs). Subsequent incorporation of siRNAs into the RNA-induced silencing complex (RISC) gives rise to the activated RISC, which catalyzes viral RNA degradation and thereby blocks virus amplification (Voinnet, 2005). It is well established that the RNAi system can block amplification of multiple plant and invertebrate viruses (Ding and Voinnet, 2007; Szittya and Burgyan, 2013). In contrast, mammalian cells rely primarily on the antiviral IFN system to overcome virus infections. However, recent reports have also provided evidence that the RNAi system can function as an antiviral defense mechanism in mammalian cells (Li et al., 2013; Maillard et al., 2013).

To bypass the RNAi-mediated antiviral response, plant viruses encode for RNA silencing suppressor (RSS) proteins that target and modulate the functions of different RNAi components. This is well exemplified in the case of the Tomato bushy stunt virus (TBSV) P19 protein, which in its dimeric form interacts and sequesters siRNA duplexes from being incorporated into RISC (Danielson and Pezacki, 2013). Likewise, a very high affinity of the Rice hoja blanca virus (RHBV) NS3 protein towards siRNA duplexes blocks functional RISC assembly in plants (Hemmes et al., 2007).

Mammalian viruses encode for proteins that effectively antagonizes the IFN-induced antiviral response. Here the favorite target of intervention is the PKR enzyme. During most virus infections the latent PKR protein binds to dsRNA produced during virus multiplication (Dauber and Wolff, 2009). This binding results in a dimerization and autophosphorylation of the protein (Dey et al., 2005). Consequently PKR becomes active as a protein kinase phosphorylating the eIF2α substrate protein. To prevent the activated PKR from blocking viral protein synthesis, many viruses encode for proteins that have a suppressive effect on PKR activation/activity (Langland et al., 2006). For example, the Ebola virus (EBOV) VP35, the Influenza A virus NS1, and the Vaccinia virus (VACV) E3L proteins function as competitive dsRNA-binding proteins, blocking PKR dimerization and consequently PKR activation (Davies et al., 1993; Feng et al., 2007; Lu et al., 1995). In contrast, the Hepatitis C virus E2 and the Human immunodeficiency virus-1
(HIV-1) Tat proteins show homology to eIF2α and block the activated PKR from phosphorylating its natural substrate (Brand et al., 1997; Taylor et al., 1999). Interestingly, some of the PKR suppressor proteins described above (VP35, E3L, Tat) can also suppress the RNAi pathway in mammalian cells (Haasnoot et al., 2007). They are therefore regarded as potential mammalian virus-encoded RSS proteins (Bivalkar-Mehla et al., 2011).

In contrast to other mammalian viruses, human adenovirus (HAdV) and Epstein-Barr virus encode for short, highly structured non-coding RNAs that can counteract the host antiviral defense system (Steitz et al., 2011). A majority of HAdV serotypes encode for two virus-associated (VA) RNA genes, VA RNAI and VA RNAII. Both VA RNAs accumulate at similar low levels during the early phase of infection, whereas the VA RNAI becomes the predominant RNA expressed at the late phase of infection (Soderlund et al., 1976). VA RNAI binds with a high affinity to the PKR protein thereby preventing PKR dimerization and activation (Katze et al., 1987; Kitajewski et al., 1986a; McKenna et al., 2007). In contrast, VA RNAII shows little or no activity in comparison to VA RNAI as an RNA complementing HAdV growth (Ma and Mathews, 1993). The VA RNAs have also been shown to interfere with the RNAi system. For example, the VA RNAs act as competitive substrates suppressing Dicer processing of exogenous dsRNA into siRNA in HAdV-infected cells (Andersson et al., 2005). Also, VA RNAI blocks nuclear export of the Dicer mRNA, thereby reducing Dicer protein synthesis (Bennasser et al., 2011). Further, the VA RNAs are processed by the Dicer enzyme into viral small RNAs, the so-called mivaRNAs, and are efficiently incorporated into the RISC (Aparicio et al., 2006; Kamel et al., 2014; Xu et al., 2007). Collectively, these observations indicate that the VA RNAs may act as HAdV-encoded RSS molecules by suppressing the function of RNAi components in mammalian cells. Hence, the VA RNAs can be considered as multifunctional non-coding RNAs inhibiting both the IFN and the RNAi systems during lytic virus growth (Punga et al., 2013).

It is well established that the VA RNAs, and particularly VA RNAI is required to establish an efficient lytic adenovirus life cycle (Bhat and Thimmappaya, 1984; Fowlkes and Shenk, 1980; Kitajewski et al., 1986b; Thimmappaya et al., 1982). It is clear that the inhibitory effect of VA RNA on PKR activation is crucial for the virus but the significance of the suppressive effect of the VA RNAs on the RNAi is still unresolved. Interestingly, whereas majority of mammalian viruses have evolved proteins to counteract the host cell antiviral defense response, HAdV has evolved non-coding RNAs, the VA RNAs, to perform the same function(s).

Here we asked whether suppressor proteins encoded by other animal and plant viruses could substitute for VA RNAI during a lytic HAdV-5 infection. For this purpose we tested whether the plant TBSV P19 and RHBV NS3 proteins or the animal EBOV VP35 and VACV E3L proteins could functionally substitute for HAdV-5 VA RNAI during a lytic adenovirus infection. The results show that viral suppressor proteins, P19, NS3 and VP35, were unable to rescue VA RNAI mutant dl705 virus growth. In contrast, the VACV E3L protein partially rescued viral capsid protein synthesis and virus multiplication. Further functional analysis showed that the E3L C-terminal region containing the dsRNA-binding domain was critical for the rescue phenotype. Finally, we show that the HAdV-4 and HAdV-37 VA RNAI were more efficient to complement VA RNAI mutant virus replication compared to the native HAdV-5 VA RNAI.

**Results**

The VACV E3L protein can substitute for VA RNAI as an enhancer of adenovirus late protein synthesis

To determine whether the VA RNAI function(s) can be substituted by viral suppressor proteins known to interfere with the IFN and/or RNAi systems (Bivalkar-Mehla et al., 2011; Langland et al., 2006), HEK293 cells were transfected with increasing amounts of plasmids expressing Flag-epitope tagged TBSV P19, RHBV NS3, VACV E3L or EBOV VP35 proteins. At 24 h post-transfection (hpt) cells were infected with the VA RNAI deficient, dl705 virus (Bhat and Thimmappaya, 1984). After 22 h post-infection (hpi) cells were 35S pulse-labeled for 2 h, proteins were isolated and separated on SDS-PAGE followed by visualization of viral capsid protein accumulation by western blot analysis or the capsid protein synthesis by autoradiography (Fig. 1). We have previously shown that co-transfection of plasmid expressing simian adenovirus VA RNAI efficiently complement the VA RNAI mutant virus phenotype (Larsson et al., 1986). Thus, as expected cells transfected with the pHindB plasmid (expressing HAdV-2 VA RNAI and VA RNAI) significantly enhanced virus capsid protein synthesis.

**Fig. 1.** The VACV E3L protein can substitute for VA RNAI as an enhancer of adenovirus late protein synthesis. HEK293 cells were transfected with increasing amounts (2 and 4 μg) of plasmids encoding Flag-tagged P19, NS3, E3L, VP35 proteins and HAdV-2 VA RNA encoding plasmid pHindB. Transfected cells were further infected with VA RNAI deficient, dl705 virus and metabolically labeled with 35S-methionine/cysteine mix. Total protein extracts were resolved on SDS-PAGE and analyzed by western blotting (WB) and autoradiography (35S). Protein expression was detected using anti-capsid, anti-actin and anti-Flag antibodies. Hyphen (-) indicates uninfected, control plasmid (pcDNA3.1) transfected sample, letter “E” designates an empty, non-protein sample loaded lane on SDS-PAGE. Asterisk (*) specifies the migration of the viral capsid hexon protein. Black arrows specify the migration of indicated Flag-tagged proteins. Label P19* indicates migration of the potential P19 dimer protein. Relative quantification of the 35S-labeled hexon protein is shown below the image. The hexon protein 35S signal from the sample infected solely with dl705 was set as 1.
production in dl705-infected cells (Fig. 1, lanes 10 and 11). In contrast, transient overexpression of the P19, NS3 or VP35 proteins did not enhance capsid protein accumulation in dl705-infected cells (Fig. 1, lanes 2–5 and 8 and 9). This failure to complement the VA RNAI mutant phenotype was not due to a lack of expression of the Flag epitope-tagged proteins (Fig. 1, middle panel). Interestingly, overexpression of the VACV E3L protein stimulated the accumulation and synthesis of the viral capsid proteins, although not to the same level as pHindB co-transfection (Fig. 1, lanes 6 and 7 and 10 and 11). Quantification of the $^{35}$S incorporation into the hexon protein, which is the major viral capsid protein, indicated that pHindB co-transfection enhanced hexon protein synthesis approximately 3-fold better than the E3L protein.

To test whether the failure of the P19, NS3 and VP35 proteins to enhance capsid protein accumulation could be due to a lack of RNAi suppressor activity in HEK293 cells, we tested if the suppressor proteins could relieve siRNA-mediated firefly luciferase reporter gene silencing in the HEK293 cell line. In line with the previous reports (Fabozzi et al., 2011; Liu et al., 2012; Schnettler et al., 2008), the P19, NS3, and VP35 proteins enhanced firefly luciferase expression (Supplementary Fig. 1A), whereas the expression of the control renilla luciferase reporter gene was not enhanced (Supplementary Fig. 1B). Notably, the E3L protein elevated the expression of both reporter genes, suggesting that this protein might have a more general effect on reporter gene expression.

Taken together, our results indicate that the VACV E3L protein, at least partially can rescue the translational defect of virus capsid protein synthesis in dl705-infected HEK293 cells. The E3L C-terminal region is required to complement the VA RNAI mutant phenotype

The VACV E3L protein has a well-defined modular structure (Fig. 2A). The N-terminus of the protein contains a Z-DNA-binding domain needed for specific DNA sequence interaction (Kwon and Rich, 2005). Interestingly, the same domain has been shown to interact and inhibit the PKR enzymatic activity in some experimental systems (Romano et al., 1998; Thakur et al., 2014). The C-terminus of the E3L protein exhibits dsRNA-binding capacity (Chang and Jacobs, 1993). Mutating lysine 167 and arginine 168 (K167A/R168A) (hereafter referred as E3L(KRAA)) within this domain severely impair the dsRNA-binding capacity of the E3L protein (Ho and Shuman, 1996). Therefore, it became of interest to assess, which functional domain of the E3L protein was required for complementation of the dl705 mutant phenotype. For this purpose HEK293 cells were transiently transfected with plasmids encoding the E3L wild type or mutant proteins (Fig. 2A). After 24 hpt cells were infected with the dl705 virus. As a control, one plate of cells was infected with the wild-type dl705 virus, which expresses both VA RNAI and VA RNAII. At 22 hpi cells were $^{35}$S pulse-labeled and total proteins separated on an SDS-PAGE. As shown in Fig. 2B, co-transfection of a plasmid expressing the wild type E3L(wt) protein (lane 2) or the HAdV-2 VA RNAs (pHindB, lane 5) stimulated, as expected, capsid protein expression in dl705-infected cells. Expression of the HAdV-2 VA RNAs stimulated dl705 capsid protein expression almost to the same level as was seen in dl705-infected cells (Fig. 2B, lanes 5 and 7), whereas the E3L(wt) protein expression did not have such a drastic effect on capsid protein levels (Fig. 2B, lanes 2 and 7). The E3L mutant proteins either lacking the dsRNA-binding domain [E3L(1–100)] or containing the double mutation disrupting the dsRNA-binding activity [E3L(KRAA)], failed to enhance HAdV capsid protein expression (Fig. 2B, lanes 3 and 4). Notably, expression levels of the E3L(wt) and E3L(1–100) proteins were similar, whereas the E3L(KRAA) protein showed reproducibly a low expression in HEK293 cells (Fig. 2B, middle panel). Hence, failure of the E3L (KRAA) protein to enhance viral capsid protein expression might be due to reduced accumulation of this mutated E3L protein in dl705-infected cells.

It is likely that E3L(wt), like the VA RNAs, stimulates HAdV capsid protein expression by suppressing eIF2α phosphorylation. To test this hypothesis the samples shown in Fig. 2B were probed with antibodies recognizing phosphorylated eIF2α (P-Ser51) and total eIF2α. As shown in Fig. 2C, transient overexpression of E3L (wt), reduced eIF2α phosphorylation to the same level as did VA RNA co-expression (pHindB) (lanes 1 and 4). However, E3L(wt) or VA RNA overexpression did not reduce eIF2α phosphorylation to the same level as was seen in wild-type dl705-infected cells (Fig. 2C, lanes 1, 4, and 6). In agreement with the observed failure to rescue capsid protein accumulation (Fig. 2B), transfection of plasmids expressing the mutant proteins E3L(1–100) or E3L (KRAA) did not affect the P-Ser51 signal strength (Fig. 2C, lanes 2 and 3).

The VACV E3L protein functionally substitutes for VA RNAI in new virus progeny formation

A stimulatory effect on viral capsid protein synthesis does not necessarily translate into an enhanced virus production. Therefore, we tested whether substitution of VA RNAI with the E3L(wt) protein also enhanced formation of infectious dl705 virus particles. For this experiment HEK293 cells were transiently transfected with pHindB or plasmids encoding the E3L proteins shown in Fig. 2A. Following 24 hpt cells were infected with the dl705 virus and 40 hpi total cell lysates were titrated in 911 cells. The 911 cell line is used as a standard cell line for the titration and propagation of adenovirus (Fallaux et al., 1996). As shown in Fig. 3, transient expression of the E3L(wt) protein stimulated infectious dl705 virus production approximately 10-fold, whereas the E3L(1–100) or E3L (KRAA) proteins failed to improve the virus titer. It is noteworthy that the burst size of infectious virus in pHindB co-transfected cells was approximately 7-fold higher compared to the virus yield in E3L(wt) expressing cells. Taken together our results show that the VACV E3L protein, at least partially substitutes for VA RNAI in new adenovirus progeny formation. Further, our experiments indicate that the E3L C-terminal region containing the dsRNA-binding domain is required for this stimulatory effect on virus production.

Adenovirus dl705 infection alters the subcellular distribution of E3L

Previous experiments have shown that the E3L protein localizes both to the cytoplasm and the nucleus in transfected HeLa cells (Kwon and Rich, 2005). To determine the effect of HAdV infection on the localization of the E3L protein we investigated the subcellular distribution of the E3L(wt) protein at the late phase of a dl705 infection. For this purpose HeLa cells were transiently transfected with the plasmid encoding the Flag-tagged E3L(wt) protein. Transfected cells were infected with dl705, and 24 hpi cells were fixed and immunostained with a monoclonal anti-Flag antibody. As shown in Fig. 4A, the E3L(wt) protein showed both nuclear and cytoplasmic localization in uninfected HeLa cells. Interestingly, a dl705 infection resulted in an enhanced cytoplasmic staining of the E3L(wt) protein. This increase in cytoplasmic accumulation was further confirmed by a biochemical subcellular fractionation experiment (Fig. 4B, lanes 2 and 5).

We have repeatedly noted that the total levels of the E3L(wt) protein is higher in dl705-infected cells compared to uninfected cells (Fig. 4B, lanes 1 and 4). To determine whether this alteration in protein expression was due to changes in the E3L protein stability we tested the effect of a proteasome inhibitor on E3L abundance and
distribution. For this experiment, E3L transfected HeLa cells were treated with the proteasome inhibitor MG132 followed by separation into a cytoplasmic and a nuclear fractions (Fig. 4B). The biochemical fractionation was efficient as evidenced by the nuclear localization of the lamin B1 protein and the cytoplasmic distribution of the GAPDH protein, respectively. Similarly, the MG132 treatment resulted in the expected stabilization of the c-Myc protein (Fig. 4B, lanes 1 and 7). However, MG132 treatment did not enhance the E3L protein stability in HeLa cells (Fig. 4B, lanes 1 and 7). Taken together, our results indicate that the E3L(wt) protein has a preferred cytoplasmic localiza-

tion in dl705-infected cells.

E3L enhances adenovirus capsid protein synthesis by suppressing PKR phosphorylation of eIF2α

The E3L(wt) protein reduced eIF2α Ser51 phosphorylation in dl705-infected cells (Fig. 2C). However, the eIF2α Ser51 residue is the target for phosphorylation by multiple protein kinases including PKR, PERK, GCN2 and HI (Donnelly et al., 2013). Therefore, it became of interest to determine whether the reduced eIF2α phosphorylation in dl705-infected cells was due to E3L inhibition of the PKR function. For this experiment, we took advantage of a HeLa cell line where PKR expression has been reduced to less than 2% by a stable expression of a PKR specific shRNA (Zhang and Samuel, 2007). The PKR knockdown (PKRkd) and control (PKRkd–
cor) cell lines were transfected with E3L(wt) or pHindB plasmids for 24 h followed by dl705 infection for an additional 24 h. Similarly to the previous experiments (Figs. 1 and 2) E3L and VA RNA (pHindB) expression enhanced viral capsid protein synthesis also in PKRkd–
cor cells (Fig. 5A, lanes 6–8). However, viral capsid proteins accumulated to much higher levels in PKRkd cells, in agreement with the hypothesis that PKR has a negative effect on dl705 virus growth (Fig. 5A, lanes 2–4). Quantification of hexon protein synthesis confirmed that E3L(wt) and pHindB transfection elevated viral capsid protein synthesis in PKR expressing (PKRkd–
cor) but not in PKRkd cells (Fig. 5A). The same protein samples were also used to detect eIF2α Ser51 phosphorylation. In line with our previous data (Fig. 2), E3L(wt) or pHindB co-transfection reduced the eIF2α P-Ser51 signal in PKRkd–
cor cells (Fig. 5B, lanes 6–8), but not in PKRkd cells (Fig. 5B, lanes 2–4). Collectively, our data suggest that E3L-mediated eIF2α hypophosphorylation is mainly due to a

The E3L protein does not enhance viral late mRNA accumulation

Previous results have shown that the VA RNAs in addition to enhancing mRNA translation, via a suppression of the PKR function, also increase cytoplasmic mRNA abundance (Strijker et al.,
Complementation of dl705 virus replication by serotype-specific VA RNAi

The VA RNAs originating from different adenovirus serotypes display considerable variations in sequence and length, suggesting that they might have serotype-specific functions in infected cells (Ma and Mathews, 1993, 1996). Indeed, we showed recently that the VA RNAs from HAdV-4, HAdV-5, HAdV-11 and HAdV-37 are processed differently by the Dicer enzyme (Kamel et al., 2014). To determine if the VA RNAs from different serotypes are functionally redundant we tested whether VA RNAi from various serotypes were equally efficient in rescuing dl705 virus growth. For this experiment HEK293 cells were transiently transfected with plasmids expressing VA RNAi from HAdV-4, HAdV-5, HAdV-12 and HAdV-37, followed by dl705 virus infection. At 22 hpi cells were pulse-labeled and protein lysates were separated on an SDS-PAGE, followed by a visualization of viral capsid protein accumulation by western blot analysis and capsid protein synthesis by autoradiography. As shown in Fig. 7A, HAdV-4 and HAdV-37 VA RNAi had a more robust impact on capsid protein accumulation compared to the HAdV-2 VA RNAs (pHindB), HAdV-5 and HAdV-12 VA RNAi. In fact, HAdV-4 and HAdV-37 VA RNAi appear to complement the VA RNAi defect in dl705-infected cells more efficiently compared to the native HAdV-5 VA RNAi (Fig. 7A, lanes 2, 3 and 5). Northern blot analysis from the same experimental setup confirmed that similar levels of the HAdV-4, HAdV-5, HAdV-12 and HAdV-37 VA RNAi were expressed in the transfected HEK293 cells (Fig. 7B).

The different effects of the serotype-specific VA RNAs on viral capsid protein accumulation might be due to a variation in their impact on PKR-mediated eIF2α phosphorylation. To test this hypothesis we analyzed the eIF2α P-Ser51 phosphorylation status by western blotting in the same samples depicted in Fig. 7A. As shown in Fig. 7C, HAdV-4 and HAdV-37 VA RNAi expression reduced eIF2α P-Ser51 signal somewhat better compared to the other serotype VA RNAi (lanes 2–5).

To further confirm that HAdV-4 and HAdV-37 VA RNAi are effective in complementing dl705 virus growth, we measured infectious dl705 virus particle formation in VA RNAi plasmid transfected cells. For this experiment, HEK293 cells were transfected with plasmids expressing VA RNAi from HAdV-4, HAdV-5, HAdV-12 and HAdV-37. After 24 hpt cells were infected with the dl705 virus. The infected cells were harvested at 40 hpi, total cell lysate prepared and titrated in 911 cells. As shown in Fig. 7D, ectopic expression of the HAdV-4 and HAdV-37 VA RNAi were most effective in rescuing dl705 virus production. In contrast, HAdV-12 VA RNAi functioned poorly in all assays tested (Fig. 7).

Discussion

Here we have analyzed the ability of various RNAi and IFN suppressor proteins and serotype-specific VA RNAi to complement growth of HAdV dl705, a virus mutant defective in VA RNAi expression. Previous reports have shown that transient overexpression of the P19, NS3, VP35 and E3L proteins can suppress induced RNAi directed against reporter gene constructs in mammalian cells (Dunoyer et al., 2004; Haasnoot et al., 2007; Liu et al., 2012; Schnettler et al., 2008; Zhu et al., 2012). In addition, VP35, E3L and NS3 can complement the RNAi suppressor function of the HIV-1 Tat protein to support HIV-1 replication (Haasnoot et al., 2007; Schnettler et al., 2009). Since HAdV VA RNAi exhibits both IFN and RNAi suppressor activity (reviewed in Punga et al. (2013)), we tested whether transient overexpression of the P19, NS3, VP35, E3L proteins could substitute for VA RNAi in supporting dl705 virus replication. Plant virus proteins P19 and NS3, which lack IFN antagonistic activity (Schnettler et al., 2009), failed to complement dl705 virus growth whereas the VACV E3L protein showed a stimulatory effect on dl705 replication. Unexpectedly, the EBOV VP35 protein, which appears to use a similar strategy as the VACV E3L protein to counteract the antiviral IFN response (Feng et al., 2007; Schumann et al., 2009), failed to enhance viral capsid protein accumulation in dl705-infected cells.

Previous reports have shown that the E3L protein exhibits a dual inhibitory effect on PKR activity. Thus, a direct binding of PKR to the E3L N-terminal domain or a sequestration of dsRNA by the E3L C-terminal domain blocks PKR functions in different experimental systems (Chang and Jacobs, 1993; Romano et al., 1998; Sharpe et al., 1998). Our biochemical analysis suggests that the E3L C-terminal region containing the dsRNA-binding domain, and not the N-terminal PKR binding domain, is needed to enhance dl705 replication (Fig. 2). The E3L dsRNA-domain binds and thereby sequesters dsRNAs (Chang and Jacobs, 1993) needed for PKR activation. Thus, it is likely that the E3L protein blocks PKR activation in dl705-infected cells by sequestering the HAdV dsRNAs produced during a lytic infection (Maran and Mathews, 1988). It is important to mention that despite of our efforts, we were unable to express the E3L protein containing only the C-terminal dsRNA-binding domain (amino acids 100–190) in
Fig. 4. Adenovirus dl705 infection alters E3L subcellular distribution. (A) HeLa cells were transfected with 1 μg of E3L(wt)-Flag encoding plasmid followed by dl705 infection before the cells were subjected to indirect immunofluorescence analysis. The Flag-tagged E3L(wt) protein (red) and adenovirus E2-72K protein (green) were simultaneously stained and visualized. Nuclei were counterstained with DAPI (blue). Scale bars correspond to 10 μm. (B) Subcellular fractionation of dl705-infected and E3L(wt)-Flag expressing HeLa cells. Total (T), cytoplasmic (C) and nuclear (N) protein extracts were analyzed by western blotting. Detection of the Lamin B1 and GAPDH proteins was used to evaluate the efficiency of cytoplasmic and nuclear protein extraction. Detection of the c-Myc protein serves as control for MG132 treatment. The E3L protein was detected with an anti-Flag antibody. MG132 treatment (25 μM) was done 6 h before the cells were harvested. Relative accumulation of the Flag-tagged E3L protein in respective subcellular fractions is shown as the ratio to the total E3L protein. The Flag-tagged E3L signal in total lysates was considered as 1.
HEK293 cells. The reason for this failure remains unresolved, but considering also that the E3L(KRAA) protein showed reduced accumulation in the same cell line (Fig. 2B), it is possible that DNA sequence manipulations around the E3L C-terminal domain affect overall stability of the E3L protein.

The E3L protein expression enhances dl705 capsid protein accumulation, which coincides with a detectable reduction in 

\[ \text{eIF2} \alpha \text{Ser51 phosphorylation} \] (Fig. 2C). This change in phosphorylation was similar in cells expressing the VA RNAs, suggesting that both VA RNA and the E3L protein affect eIF2\(\alpha\) Ser51 phosphorylation to a similar extent in dl705 infected HEK293 cells. Despite this the E3L protein did not enhance dl705 virus replication to the same magnitude as did the ectopic expression of the HAdV-2 VA RNAs (pHindB, Fig. 3). A quantification of \(^{35}\text{S}\)-labeled hexon protein synthesis indicated that VA RNA co-expression increased hexon protein levels up to 4-fold better compared to cells expressing the E3L(wt) protein (Fig. 2B). This observation indicated that the effect of the VA RNAs on capsid protein synthesis and virus progeny formation in HEK293 cells might not only be due to a diminution in eIF2\(\alpha\) Ser51 phosphorylation. Indeed, a quantitative analysis of hexon mRNA levels indicated that ectopic VA RNA expression increased hexon mRNA abundance in dl705-infected HEK293 cells, an effect not seen with the E3L protein (Fig. 6). Thus, VA RNA, in contrast to E3L, appears to have two alternative mechanisms to stimulate HAdV growth; first VA RNA, like the E3L protein, rescues translation by reducing eIF2\(\alpha\) phosphorylation; second, the VA RNAs have a positive effect on viral late mRNA accumulation not seen with the E3L protein. Although the mechanism by which the VA RNAs enhance mRNA abundance is not known the effect has been reported previously. Thus, it has been demonstrated that transfection of a VA RNA expressing plasmid increases the steady state cytoplasmic mRNA abundance in HEK293 cells (Strijker et al., 1989; Svensson and

Fig. 5. E3L enhances adenovirus capsid protein synthesis by suppressing PKR phosphorylation of eIF2\(\alpha\). (A) PKR positive (HeLa\text{ld-}\text{con}) and PKR negative (HeLa\text{ld}) cells were transfected with 4 \(\mu\)g of E3L(wt) or VA RNA (pHindB) expressing plasmids followed by dl705 infection and \(^{35}\text{S}\)-metabolic labeling. Expression of PKR, Flag-tagged E3L(wt) and actin proteins in total cell lysates was detected by western blot analysis (WB) using the respective antibodies. The viral capsid proteins were detected by autoradiography (\(^{35}\text{S}\)). Asterisk (*) indicates the migration of viral hexon protein. The \(^{35}\text{S}\) labeled hexon protein signals were quantified and relative signal intensities are shown below the image. (B) Expression of the E3L (wt) protein specifically reduces eIF2\(\alpha\) Ser51 phosphorylation in HeLa\text{ld-}\text{con} cells. Total protein samples from HeLa\text{ld-}\text{con} and HeLa\text{ld} cells were analyzed by western blotting using anti-eIF2\(\alpha\) (Total) and anti-eIF2\(\alpha\) (P-Ser51) antibodies. Relative accumulation of P-Ser51 signal in total eIF2\(\alpha\) protein pool (P-Ser51/Total) is shown.

Fig. 6. The E3L protein does not increase hexon mRNA levels in dl705-infected cells. HEK293 cells, transfected with E3L(wt) or VA RNA (pHindB) expressing plasmids (2 and 4 \(\mu\)g), were infected with the dl705 virus for 24 h. Viral hexon and housekeeping HRPT1 mRNA levels were detected by qRT-PCR and hexon mRNA levels were normalized to HRPT1 mRNA levels. Hexon mRNA from the cells infected solely with dl705 was set as 1. Bars denote the mean ± standard deviation of a triplicate experiment.
Fig. 7. Complementation of dl705 virus replication by serotype-specific VA RNAI. (A) HAdV-4 and HAdV-37 VA RNAI enhance the viral capsid protein production in dl705-infected cells. Four micrograms of plasmids encoding VA RNAI from HAdV-4, HAdV-5, HAdV-12, HAdV-37 serotypes were transfected into HEK293 cells followed by dl705 infection and metabolic labeling. Total protein samples were analyzed by western blotting (WB) and autoradiography (35S). Hyphen (-) indicates uninfected sample or a sample transfected with an empty (pcDNA3.1) plasmid. As the control, cells were also infected with the wild type HAdV-5 (dl703) virus. Asterisk (*) indicates migration of the viral hexon protein. Relative quantification of 35S-labeled hexon protein is shown below the image. (B) Transient expression of serotype-specific VA RNAI. Total RNA was extracted from the HEK293 cells, which underwent the same experimental setup as in (A). Isolated RNA was analyzed by Northern blotting using γ-32P-ATP labeled probes against VA RNAI, VA RNAII, U6 snRNA. (C) The same protein lysates as shown in (A) were analyzed by western blotting using anti-eIF2α (Total) and anti-eIF2α (P-Ser51) antibodies. Relative accumulation of P-Ser51 signal in total eIF2α protein pool (P-Ser51/Total) is shown. The P-Ser51/Total eIF2α ratio in dl705 only infected sample was set as 1. (D) Improved formation of dl705 virus particles in serotype-specific VA RNAI expressing cells. Virus titers (FFU/ml) were determined in 911 cells after re-infection with HEK293 cell lysates transfected with indicated plasmids and infected with dl705 virus. Bars denote the mean virus titer (log10) ± standard deviation of the means.
The mechanism has not been thoroughly investigated although one of the original reports proposed that VA RNAI might stabilize ribosome bound mRNAs (Strijker et al., 1989), whereas the other concluded that the effect was mRNA length dependent (Svensson and Akusjarvi, 1990).

Interestingly, our results also demonstrated that HAdV-4 and HAdV-37 VA RNAI rescued dl705 growth more efficiently compared to the native HAdV-5 VA RNAI (Fig. 7D). Improved dl705 replication in HAdV-4 and HAdV-37 VA RNAI co-transfected cells was also accompanied by a reduction in eIF2α Ser51 phosphorylation (Fig. 7C). However, the difference in eIF2α Ser51 phosphorylation between the serotype-specific VA RNAs was not dramatic, implying that other activities coupled to VA RNAI, like the mRNA enhancer function, might contribute to the serotype-specific differences in VA RNAI function. A clear outlier among the tested VA RNAs was HAdV-12 VA RNAI, which was efficiently expressed (Fig. 7B), but did not enhance dl705 virus growth in HEK293 cells (Fig. 7D). This observation is in line with a recent report demonstrating that the HAdV-12 VA RNAI is a poor inhibitor of PKR activation in A549 cells (Wu et al., 2015).

A recent report showed that the HAdV-5 E1B-55K and E4orf6 proteins block activation of the PKR protein and maintain low levels of eIF2α Ser51 phosphorylation in virus-infected cells (Spurgeon and Ornelles, 2009). The Cul5-based ubiquitin ligation activity associated with the E1B-55 K/E4orf6 protein complex appeared to be necessary to prevent activation of PKR and phosphorylation of eIF2α during the late phase of HAdV-5 infection. However, the expression of the E1B-55K and E4orf6 proteins did not promote the cytoplasmic accumulation of VA RNAI and VA RNAII in these experiments (Spurgeon and Ornelles, 2009). Hence, it is possible that the eIF2α Ser51 phosphorylation is rigorously controlled by the expression and activity of several virus-encoded factors (E1B-55K, E4orf6, VA RNAI) during different phases of the HAdV infection.

Collectively, our results suggest that the VA RNAI-mediated suppression of the IFN-induced PKR pathway is more critical for support of virus multiplication compared to the RNAI suppressor activity associated with the VA RNAs. In contrast to our findings (Fig. 1), a recent report has suggested that the TBSV P19 protein can complement the RNAI suppressor function of the VA RNAs and stimulate VA RNA mutant virus growth (Rauschhuber et al., 2013). Our results do not exclude the RNAI suppressing function of the VA RNAs as a mechanism supporting virus growth (Andersson et al., 2005). However, our data clearly favor the conclusion whereby the main target for VA RNAI intervention is the IFN-induced PKR system. This is further supported by a report showing that PKR knockdown, but not Dicer knockdown, rescued growth of a VA RNAI deficient virus (Kamel et al., 2013). In addition, inhibition of PKR activity with the serine-kinase inhibitor 2-amino purine also functionally substitutes for VA RNAI (Cascallo et al., 2003).

Taken together, our data strongly suggest that the cellular IFN system is the major antiviral response that the adenovirus has to inhibit, and that its suppression is one of the main functions of the VA RNAI, at least in the cell lines tested.

Materials and methods

Plasmids

The original plasmids containing TBSV P19, RHBV NS3, EBOV VP35, VACV E3L cDNA sequences were kindly provided by Dr. Ben Berkhourt (Haasnoot et al., 2007; Schnettler et al., 2009). The corresponding cDNAs were cloned into pcDNA3.1 (Invitrogen) vector containing a Flag epitope tag sequence. The constructed plasmids express the P19, NS3, E3L and VP35 proteins as C-terminal Flag-tagged fusion proteins in mammalian cells. The plasmids encoding for the Flag-tagged E3L(KRAA) and E3L(1–100) proteins were generated using QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and by deletion PCR, respectively. The pHindB plasmid, which expresses HAdV-2 VA RNAI and VA RNAII, has been described previously (Svensson and Akusjarvi, 1984). The plasmids expressing VA RNAI from HAdV-4, HAdV-5, HAdV-12 and HAdV-37 serotypes were generated by PCR amplification of the respective viral DNA sequences and cloned into pcDNA5/FRT (Invitrogen) plasmid.

Cell lines, transfections and virus infections

Human embryonic kidney cell line 293 (HEK293) (Graham et al., 1977), human epithelial cervix carcinoma cell line (HeLa), and human embryonic retina cell line 911 (911) (Fallaux et al., 1996) were grown as monolayers in growth media containing Dulbecco’s modified Eagle medium (DMEM, Invitrogen), 10% fetal calf serum (FCS, PAA), 100 U/ml penicillin, 100 U/ml streptomycin (PEST, Gibco) at 37 °C in 7% CO2 containing cell incubator. HeLa PKRkd and PKRkd-con cell lines were kindly provided by Dr. Charles Samuel (Zhang and Samuel, 2007) and were maintained in growth media supplemented with 1 μg/ml puromycin (Sigma). Approximately 24 h before transfection seeds were seeded in 60 mm plates to reach a confluence of 60–70% at the time of transfection. Transient transfections were performed using TurboFect transfection reagent (Thermo Scientific) according to the manufacturer’s instructions. Transfected cells were further infected with dl703 or dl705 viruses 24 h post-transfection (hpt). The dl703 and dl705 viruses are derivatives of HAdV-5 where the VA RNA region was deleted and replaced with the corresponding region from HAdV-2. The wild-type virus (dl703) contains both VA RNAI and VA RNAII from HAdV-2 whereas the VA RNAI deficient virus (dl705) contains a deletion in the Box B element inactivating VA RNAI expression (Bhat and Thimmappaya, 1984). As the HAdV-2 and HAdV-5 genomes are closely related, the VA RNA sequences from both serotypes are almost identical (Ma and Mathews, 1996). Cells were infected at a multiplicity of 5 fluorescence forming units (FFU) per cell in 1 ml of infection media (DMEM +2% NCS +PEST) for 1 h at 37 °C. After 1 h of incubation the cells were washed twice with the growth media and replaced with fresh growth media for further incubation for 24 h.

35S-methionine/cysteine metabolic labeling

Approximately 22 h post-infection (hpi), the growth medium was removed, the cells washed once with PBS, and incubated in methionine/cysteine-free DMEM depletion media (Invitrogen), supplemented with 10% FCS, 1% PEST and 2% glutamine, for 30 min. After the depletion period, the cells were incubated in depletion media complemented with [35S] methionine/cysteine mix (30 μCi/ml; 2 ml/plate) for 2 h. After the pulse-labeling, cells were washed twice in PBS, collected into microcentrifuge tubes and processed as described below.

Total cell lysates and cell fractionation

Total cell lysates were prepared by incubating the collected cell pellets in RIPA buffer (25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, supplemented with protease inhibitor (Complete Mini EDTA-free, Roche Applied Sciences)) on ice for 15 min. The protein extract was clarified by centrifugation for 15 min and the protein concentration was determined by the Bradford assay (Bio-Rad). Cell fractionation
experiments were carried out using the PARIS™ kit (Life Technologies) according to the manufacturer’s instructions.

**SDS-PAGE and western blotting**

Equal concentrations of protein samples were separated on a 4–20% gradient precast (Bio-Rad) or on home-made 14% SDS-PAGE gels. Proteins were transferred onto Immobilon-FL western blot nitrocellulose membrane (Millipore) using Towbin transfer buffer (25 mM Tris, 192 mM Glycine). The membrane was blocked with Odyssey blocking buffer (LI-COR) for 1 h at room temperature and thereafter incubated with the primary antibody overnight at 4 °C.

***Next, the membrane was washed with PBST (PBS + 0.05% Tween 20) and incubated with the fluorescent secondary antibody (IRDye®680, LI-COR) for 45 min at room temperature. The PBST washed membranes were scanned with the Odyssey scanner (LI-COR). Scanned western blot images were quantified using Image Studio 2.1™ Software (LI-COR) according to the manufacturer's instructions. The following primary antibodies were used: anti-lamin B1 (1:4000; Abcam, ab16048), anti-Ad5 capsid (1:5000; Abcam, ab6982), anti-Flag (1:1000, Sigma, M2, F1804), anti-c-Myc (1:1000; Santa Cruz, sc-42), anti-actin (1:1000; Santa Cruz, sc-6251), anti-GAPDH (1:50,000; Ambion, Am4300), anti-elf2 (1:500, Abcam, ab26197), anti-elf2(phospho-S51) (1:500, Abcam, ab32157), anti-PKR (1:1000, Santa Cruz, sc-707). After immunoblotting the western blot membranes were exposed to a PhosphorImager screen (Fuji) and the 35S-signals quantitated using the Image One software (Bio-Rad).

**RNA extraction, northern blot analysis and qRT-PCR**

Total RNA was extracted using the TRReagent (Sigma) as previously described (Inturi et al., 2013). Ten micrograms of total RNA was separated on a denaturing 12% polyacrylamide gel, transferred to a Hybond N+ membrane (Amersham Biosciences). The γ-32P-ATP labeled probes were hybridized to the membrane as described previously (Kamel et al., 2014). After overnight hybridization in ULTRAhyb® buffer (Ambion), the membrane was washed three times for 10 min at 42 °C in 3 × SSC, 0.5% SDS buffer followed by a single wash with 1 × SSC, 0.5% SDS buffer for 15 min at 42 °C. Radioactive signals were detected and analyzed as described above. cDNA synthesis from total RNA samples was performed as published previously (Inturi et al., 2013). Purified RNA was DNaseI (RapidOut DNA Removal Kit; Thermo Scientific) using HOT FIREPol® expression was analyzed by quantitative reverse transcription PCR (qRT-PCR) on an Applied Biosystems 7900 system (Life Technologies) using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne). The HRPT1 primer sequences were obtained from RTPrimerDB database (http://medgen.ugent.be/rtpimerdb/), whereas hexon primer sequences were published previously (Inturi et al., 2013). Relative hexon mRNA expression was calculated after normalization to the HRPT1 mRNA levels using 2^−ΔΔCT method (Schmittgen and Livak, 2008).

**Indirect immunofluorescence assay**

The indirect immunofluorescence assay was carried out as previously described (Inturi et al., 2013). Briefly, HeLa cells were grown on fibronectin coated coverslips and transfected for 24 h with the indicated plasmids. Thereafter, the cells were infected with dl705 virus (5 FFU/cell) or mock infected (infection media without virus) for 1 h. Cells were fixed 24 hpi with 3% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBST (PBS + 0.01% Tween 20) for 15 min at room temperature. After blocking the cells with 2% BSA/PBST solution, the coverslips were subjected to immunofluorescence analysis using anti-Flag (M2, Sigma, 1:1000) and anti-E2A-72 K (kindly provided by Bruce Stillman) antibodies. Proteins were visualized with anti-FITC-conjugated anti-rabbit IgG (Sigma, F6005, 1:1000) and anti-TRITC-conjugated anti-mouse IgG (Sigma, TS539, 1:1000) secondary antibodies. Nucleus was detected by DAPI staining supplemented into the Fluoromount-G mounting media (Southern Biotech). Immunolabeled cells were visualized under a fluorescence microscope (Nikon eclipse 90i) and the images were analyzed with the NIS-elements (AR 3.10, Nikon) software.

**Virus titration**

HEK293 cells were transfected with respective plasmids followed by dl705 virus infection. Infected cells were harvested 40 hpi when the cells showed a clear cytopathic effect. Cell pellets were resuspended in 0.1 M Tris–HCl, pH 8.0, freeze-thawed three times, and the cell debris was removed by centrifugation. The virus containing supernatants were serially diluted 10-fold from 1:10^2 to 1:10^6 in growth media. An aliquot (10 µl) of each dilution was transferred to confluent monolayers of 911 cells seeded on 35 mm culture dishes. The infected 911 cells were incubated for an additional 36 h at 37 °C in a cell incubator. Thereafter, the infected 911 cells were extensively washed with PBS and fixed with 3 ml 95% methanol in PBS for 15 min at room temperature. After fixation, the cells were incubated with anti-adenovirus antibody (Millipore, MAB8052, 1:500) for 30 min at room temperature. The cells were washed with PBS before incubation with anti-FITC-conjugated anti-mouse IgG antibody (Sigma, F9006, 1:100) for 30 min at room temperature. Next, the cells were extensively washed in PBS and the positively stained cells were counted under a fluorescence microscope (Nikon eclipse 90i). The endpoint virus titers (FFU/ml) were set at the last dilution giving unequivocal fluorescent signal. The virus titers represent the mean ± SD of two independent experiments assayed in triplicate.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.07.002.

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