

# Herpes simplex virus type 1 disrupts the ATR-dependent DNA-damage response during lytic infection

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## Summary

Like other DNA viruses, herpes simplex virus type 1 (HSV-1) interacts with components of the cellular response to DNA damage. For example, HSV-1 sequesters endogenous, uninduced, hyperphosphorylated RPA (replication protein A) away from viral replication compartments. RPA is a ssDNA-binding protein that signals genotoxic stress through the ATR (ataxia telangiectasia-mutated and Rad3-related) pathway. The sequestration of endogenous hyperphosphorylated RPA away from replicating viral DNA suggests that HSV-1 prevents the normal ATR-signaling response. In this study we examine the spatial distribution of endogenous hyperphosphorylated RPA with respect to ATR, its recruitment factor, ATRIP, and the cellular dsDNA break marker,  $\gamma$ H2AX, during HSV-1 infection. The accumulation of these repair factors at DNA

lesions has previously been identified as an early event in signaling genotoxic stress. We show that HSV-1 infection disrupts the ATR pathway by a mechanism that prevents the recruitment of repair factors, spatially uncouples ATRIP from ATR and sequesters ATRIP and endogenous hyperphosphorylated RPA within virus-induced nuclear domains containing molecular chaperones and components of the ubiquitin proteasome. The HSV-1 immediate early protein ICP0 is sufficient to induce the redistribution of ATRIP. This is the first report that a virus can disrupt the usually tight colocalization of ATR and ATRIP.

Key words: Herpesvirus, ICP0, DNA-damage response, ATR, ATRIP, Hyperphosphorylated RPA, Phosphorylated H2AX, Chaperones, Proteasome

## Introduction

ATM (ataxia telangiectasia-mutated), ATR (ATM and Rad3-related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) are phosphoinositide-3-kinase-related protein kinases (PIKKs) that are rapidly activated in response to different forms of genotoxic stress. Through the phosphorylation of downstream targets, these stress kinases directly or indirectly activate proteins that participate in cell cycle checkpoints and DNA repair. If the DNA lesion is irreparable, then cells are eliminated through the induction of apoptosis (reviewed in Burma and Chen, 2004; Kurz and Lees-Miller, 2004; Shechter et al., 2004). Double-strand DNA breaks (DSBs) can result in the activation of DNA-PKcs leading to DNA repair via the nonhomologous end-joining (NHEJ) pathway (Burma and Chen, 2004) or in the activation of ATM leading to repair through the homologous recombination (HR) pathway (Kurz and Lees-Miller, 2004). ATR on the other hand, is activated by ssDNA intermediates that are generated by a variety of genotoxic assaults including those that cause replication fork perturbations. ATR also responds to DSBs but with slower kinetics than ATM. The ATR pathway also plays a crucial role in the maintenance of active, unperturbed replication forks (Shechter et al., 2004).

PIKK-mediated signal transduction involves the recruitment of the stress kinase to the site of DNA damage. PIKK recruitment is mediated through a conserved interaction motif

located within the C-terminus of interacting partners (Falck et al., 2005). ATM recruitment to DSBs is mediated through NBS1, a component of the MRE11-RAD50-NBS1 (MRN) repair complex (Falck et al., 2005). ATR is recruited to ssDNA through its tightly associated binding partner ATRIP, which can efficiently recognize replication protein A (RPA)-bound ssDNA (Ball et al., 2005; Falck et al., 2005; Zou and Elledge, 2003). DNA-PKcs recruitment to DSBs is facilitated by its interaction with the Ku80 subunit of the Ku70/Ku80 DNA end-binding complex (Falck et al., 2005). Nuclear events such as DNA replication and repair are commonly studied by monitoring the subcellular distribution of key proteins involved. For example, immunofluorescence (IF) microscopy of cells exposed to DNA-damaging agents have demonstrated that ATM and the MRN complex accumulate at DSBs as detected by staining for  $\gamma$ H2AX (phosphorylated histone H2AX) which is a sensitive marker for DSBs (Falck et al., 2005). It has been similarly shown that ATR, ATRIP and RPA accumulate at stretches of ssDNA generated in response to agents that induce DNA damage or replication stress (Ball et al., 2005; Barr et al., 2003).

As obligate intracellular parasites, viruses rely on and often manipulate host cell replication and repair factors presumably for their own benefit. Herpes simplex virus type 1 (HSV-1) is a large linear dsDNA virus that replicates in the nucleus of the infected cell within globular domains called replication

compartments (Quinlan et al., 1984). The first indication that HSV-1 activates a component of the cellular DNA damage response was the observation that lytic infection induces the phosphorylation of NBS1, a DNA damage signaling event that correlates with the recruitment of NBS1 to viral precursors of replication compartments (Wilkinson and Weller, 2004). The observation that replication compartments recruit components of the MRN complex (Taylor and Knipe, 2004; Wilkinson and Weller, 2004) suggests that the MRN complex is used by HSV-1 to promote viral DNA replication, perhaps for the formation of greater-than-unit-length concatemers (Wilkinson and Weller, 2004). Subsequent studies have demonstrated that HSV-1 infection induces an ATM-dependent activation of NBS1 and other downstream targets and that HSV-1 infection is somewhat compromised in cells deficient for Mre11 or NBS1 (Lilley et al., 2005; Shirata et al., 2005). These results support the suggestion that the MRN complex is important for efficient HSV-1 infection.

Although HSV-1 appears to activate components of the ATM-dependent signaling pathway, several lines of evidence suggest that HSV-1 also inactivates components of the NHEJ machinery. In some cell types the HSV-1-encoded immediate early protein ICP0 targets DNA-PKcs for degradation by the proteasome (Lees-Miller et al., 1996; Parkinson et al., 1999). In Vero cells, we have shown that the Ku80 subunit of the DNA-PK complex is not recruited to the earliest prereplicative sites for HSV-1 (Wilkinson and Weller, 2004). Furthermore, HSV-1 yields are actually increased in Ku70- or DNA-PKcs-deficient cell lines suggesting that NHEJ may have an inhibitory effect on infection (Parkinson et al., 1999; Taylor and Knipe, 2004). Thus, HSV-1 apparently uses ATM-mediated events while inactivating the NHEJ pathway. In this paper we extend these studies to the analysis of the subcellular ATR-mediated damage response during HSV-1 infection.

One predominant ATR signaling event for replication stress or DNA damage is the hyperphosphorylation of the 32-kDa subunit (RPA32) of the heterotrimeric RPA protein, which occurs in response to the accumulation of ssDNA. The hyperphosphorylation of RPA32, which is catalyzed by PIKKs, is thought to direct the role of RPA from DNA replication to that of repair (Vassin et al., 2004). Hyperphosphorylated RPA, which accumulates at ssDNA lesions serves as a marker for DNA damage and repair (reviewed by Binz et al., 2004) and has been shown to colocalize with ATR and Mre11 in nuclear foci following DNA damage (Robison et al., 2004; Wu et al., 2005). Two populations of RPA have been observed in HSV-1 infected cells. One RPA population, which can be detected with general anti-RPA antibodies, is found within viral replication compartments (Uprichard and Knipe, 1997; Wilcock and Lane, 1991; Wilkinson and Weller, 2004) and may act at viral replication forks. The other population of RPA is recognized by a phosphospecific anti-RPA antibody and represents uninduced, endogenous levels of hyperphosphorylated RPA that is sequestered away from replication compartments (Wilkinson and Weller, 2005). The sequestration of this uninduced population of hyperphosphorylated RPA (hereafter referred to as P-RPA) may be part of a global mechanism by which HSV-1 prevents triggering stress signals that could be deleterious to the progression of infection (Wilkinson and Weller, 2005). In this study, IF microscopy was used to show that HSV-1 infection prevents the ATR-dependent signaling of

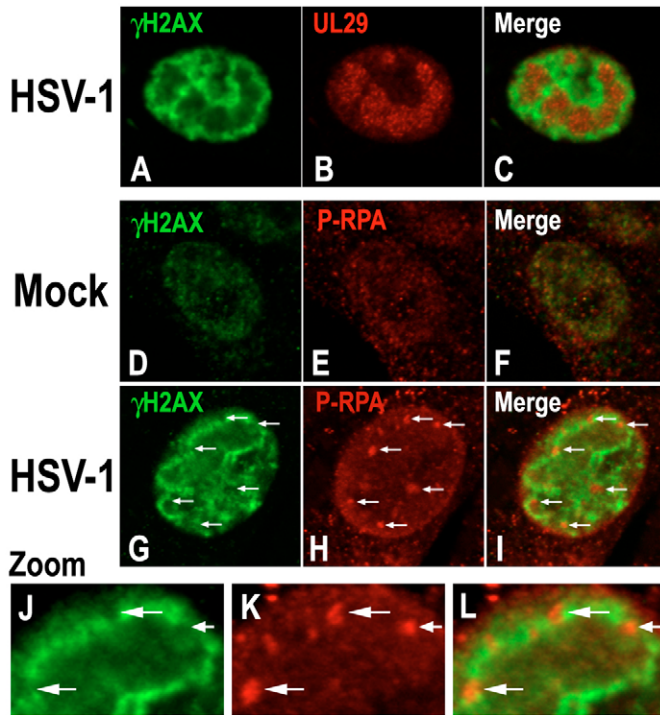
cellular replication stress or DNA damage by a mechanism that that spatially uncouples ATRIP from ATR and sequesters ATRIP and P-RPA within virus-induced domains enriched with molecular chaperones and the ubiquitin proteasome (VICE domains). We also show that the HSV-1 immediate early protein, ICP0, is sufficient to induce the relocalization of ATRIP.

## Results

### Subcellular distribution of $\gamma$ H2AX and P-RPA in HSV-1-infected cells

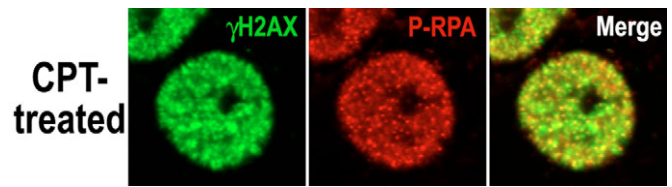
$\gamma$ H2AX and hyperphosphorylated RPA are commonly used as markers for DNA damage. The presence of  $\gamma$ H2AX is a sensitive indicator of DSBs within cellular chromatin, whereas hyperphosphorylated RPA accumulates at stretches of ssDNA generated from the processing of DNA lesions or perturbed replication forks.  $\gamma$ H2AX and hyperphosphorylated RPA are commonly found colocalized in DNA-damage-induced foci (Vassin et al., 2004; Wu et al., 2005). We first examined the localization of  $\gamma$ H2AX in relation to viral replication compartments as detected by staining for the HSV-1 ssDNA-binding protein, UL29. Double labeling of HSV-1-infected cells revealed an accumulation of  $\gamma$ H2AX in areas surrounding replication compartments (Fig. 1A-C). The marginalization of  $\gamma$ H2AX outside replication compartments is in agreement with previous reports describing the peripheral displacement of cellular DNA during HSV-1 infection (Monier et al., 2000; Simpson-Holley et al., 2004). Previous reports have demonstrated that the frequency and intensity of  $\gamma$ H2AX foci increase linearly with the amount of DNA damage sustained (Rogakou et al., 1999; Rogakou et al., 1998), with each discrete  $\gamma$ H2AX focus corresponding to one DSB (Sedelnikova et al., 2002). The accumulation of  $\gamma$ H2AX staining seen immediately surrounding replication compartments suggests that the marginated cellular chromatin has sustained significant DSBs during lytic infection. The marginalization of  $\gamma$ H2AX was observed at early times post infection when cells displayed small, developing replication compartments (data not shown). Although we cannot rule out the presence of undetectable amounts of  $\gamma$ H2AX at viral DNA, our results suggest that  $\gamma$ H2AX does not mark the occurrence of DSBs within the replicating HSV-1 genome. This is not surprising because replicating viral DNA is not found in an ordered nucleosomal form with only a fraction of the HSV-1 genomes associated with histones (Leinbach and Summers, 1980).

We next assessed the spatial relationship between P-RPA and  $\gamma$ H2AX in HSV-1-infected cells. Since the staining pattern of  $\gamma$ H2AX was shown to define the periphery of replication compartments (Fig. 1A-C), it was possible to characterize the localization of  $\gamma$ H2AX and P-RPA with respect to replication compartments simply by double staining for  $\gamma$ H2AX and P-RPA. Mock-infected cells typically displayed low levels of P-RPA distributed either in a diffuse or rough granular pattern within the nucleus as described previously (Wilkinson and Weller, 2005) (Fig. 1E). A low-level nuclear staining pattern for  $\gamma$ H2AX was also observed in mock-infected cells (Fig. 1D) with no significant co-staining of  $\gamma$ H2AX and P-RPA (Fig. 1F). Western analysis has demonstrated that productive HSV-1 infection does not induce the hyperphosphorylation of RPA (Wilkinson and Weller, 2005). Fig. 1G-I indicates that this endogenous P-RPA does not colocalize with the marginated



**Fig. 1.** Endogenous hyperphosphorylated RPA32 (P-RPA) does not mark sites of DSBs in HSV-1-infected cells. Vero cells were infected with HSV-1 (A–C,G–I) or mock-infected (D–F) and fixed with 4% PFA as described in the Materials and Methods. (A–C) HSV-1 infected cells were double labeled with mouse anti- $\gamma$ H2AX (green) and rabbit anti-UL29 (red) to detect the nuclear localization of DSBs with respect to HSV-1 replication compartments, respectively. The merged image shown in C indicates that  $\gamma$ H2AX clearly surround replication compartments. Mock-infected cells (D–F) and HSV-1-infected cells (G–I) were double labeled using mouse anti- $\gamma$ H2AX (green) and rabbit phosphospecific anti-P-RPA (red) to determine the nuclear localization of cellular DSBs and endogenous P-RPA, respectively. (J–L) Digital enlargements of an area of the infected cell nucleus show in G–I. Arrows indicate typical P-RPA foci that are exclusive of  $\gamma$ H2AX staining. Images were obtained at 100 $\times$  magnification with 2 $\times$  zoom.

$\gamma$ H2AX in HSV-1-infected cells. In fact, the two markers were consistently present at locations in the nucleus which are mutually exclusive of each other (Fig. 1J–L). This result was unexpected in light of previous demonstrations showing the colocalization of these two markers in repair foci following DNA damage (Vassin et al., 2004; Wu et al., 2005). To rule out the possibility that Vero cells were defective in the formation of this type of repair foci during the DNA damage response, we performed a control experiment in which cells were treated with 1  $\mu$ M camptothecin (CPT), a genotoxic agent known to induce the nuclear colocalization of hyperphosphorylated RPA and  $\gamma$ H2AX (Vassin et al., 2004; Wu et al., 2005). Indeed, CPT treatment of Vero cells induced the increased staining of hyperphosphorylated RPA and its accumulation with  $\gamma$ H2AX (Fig. 2) indicating that the segregation of P-RPA and  $\gamma$ H2AX into separate nuclear compartments during infection is not due to an inherent defect of Vero cells in the response to DNA damage. Thus, although Vero cells are competent to induce the colocalization of  $\gamma$ H2AX and hyperphosphorylated RPA into



**Fig. 2.** P-RPA and  $\gamma$ H2AX accumulate at camptothecin-induced DNA repair foci in Vero cells. Vero cells were treated with 1  $\mu$ M camptothecin (CPT) for 5 hours and prepared for IF analysis as described in the legend to Fig. 1. Cells were double labeled to detect the localization of  $\gamma$ H2AX (green) and P-RPA (red). The merged image shows a significant colocalization of the two signals (yellow). 100 $\times$  magnification with 2 $\times$  zoom.

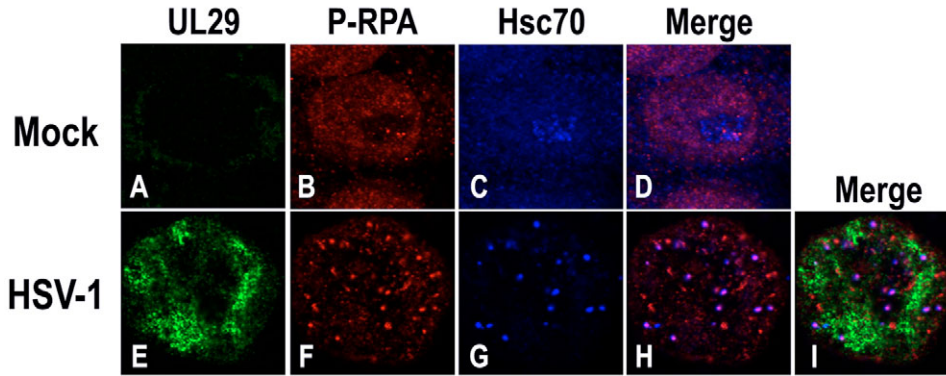
CPT-induced DNA repair foci (Fig. 2), endogenous P-RPA is actually excluded from sites staining for  $\gamma$ H2AX during HSV-1 infection (Fig. 1J–L). We conclude that HSV-1 sequesters endogenous P-RPA away from both replicating viral DNA and cellular DSBs.

#### P-RPA is present in HSV-1-induced foci called VICE domains

We and others have previously reported that HSV-1 infection causes the relocalization of cellular heat-shock proteins (Hsps) into domains that also contain components of the ubiquitin proteasome (Burch and Weller, 2004; Everett, 2000). We proposed that these virus-induced chaperone-enriched (VICE) domains represent a mechanism by which HSV-1 sequesters misfolded, modified or unwanted proteins. This sequestration may prevent cellular events, such as premature apoptosis, which would be catastrophic to the virus (Burch and Weller, 2004). The P-RPA staining pattern observed during infection exhibited a nuclear organization reminiscent of that observed for VICE domains (Burch and Weller, 2004; Burch and Weller, 2005; Everett, 2000). To confirm this, we examined cells that were triple-labeled with antibodies to UL29, Hsc70 (Heat shock cognate 70) and P-RPA to visualize replication compartments, VICE domains and endogenous P-RPA, respectively (Fig. 3). As previously described, HSV-1 infection results in the redistribution of Hsc70 from the nucleolus into VICE domains located adjacent to replication compartments (Fig. 3C,G) (Burch and Weller, 2004; Burch and Weller, 2005). A subset of P-RPA foci also colocalized with the Hsc70-staining VICE domains (Fig. 3E–I). Additional triple labeling experiments using antibodies directed against UL29, Hsc70 and  $\gamma$ H2AX indicate that VICE domains themselves are excluded from sites of cellular DSBs (Fig. 4). The cells shown in Figs 3 and 4 were extracted with Triton X-100 to remove the cytoplasm and nucleosolic proteins before fixation. Similar protein localization patterns were observed in cells that were fixed for detection of total proteins (results not shown).

#### HSV-1 infection induces the spatial uncoupling of the ATR-ATRIP complex

The accumulation of RPA on stretches of ssDNA (RPA-ssDNA) is thought to be the signaling intermediate that triggers the ATR-dependent response to replication stress or DNA damage (reviewed by Cortez, 2005). Upon activation, ATR is recruited to RPA-ssDNA by its binding partner, ATRIP (Zou and Elledge, 2003). To determine whether the ATR and ATRIP

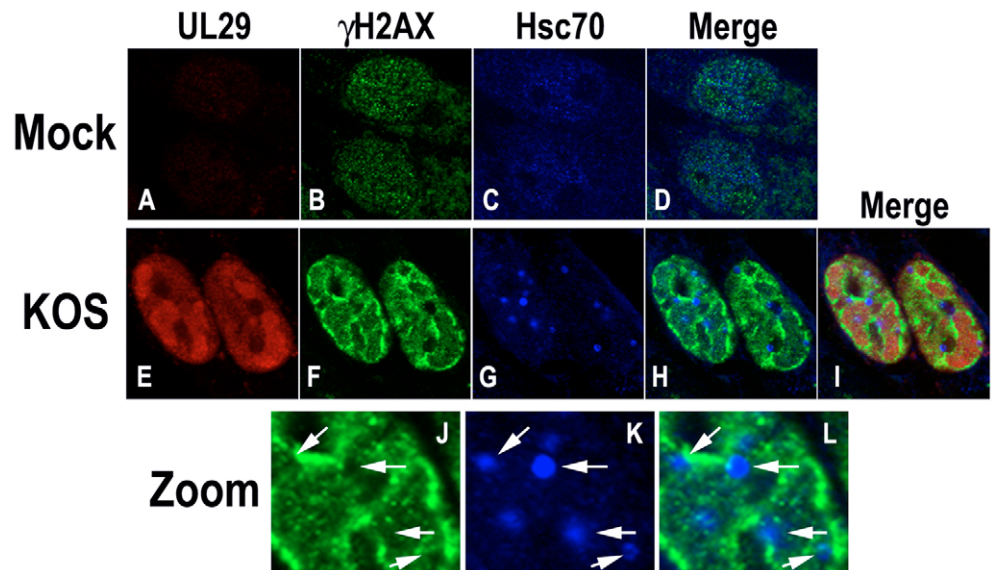


**Fig. 3.** Endogenous hyperphosphorylated RPA32 (P-RPA) is found within VICE domains during HSV-1 infection. Vero cells were mock-infected (A-D), or infected with HSV-1 (E-I). Pre-extracted cells were triple labeled with mouse anti-UL29 (green), rabbit anti-P-RPA (red) and rat anti-Hsc70 (blue) to detect the nuclear localization of replication compartments, endogenous P-RPA and VICE domains, respectively. (D,H) Merged images for P-RPA and Hsc70 with purple indicating the colocalization of the two proteins. (I) Merged image of UL29, P-RPA and Hsc70 for the HSV-1-infected cell. 100 $\times$  magnification with 2 $\times$  zoom.

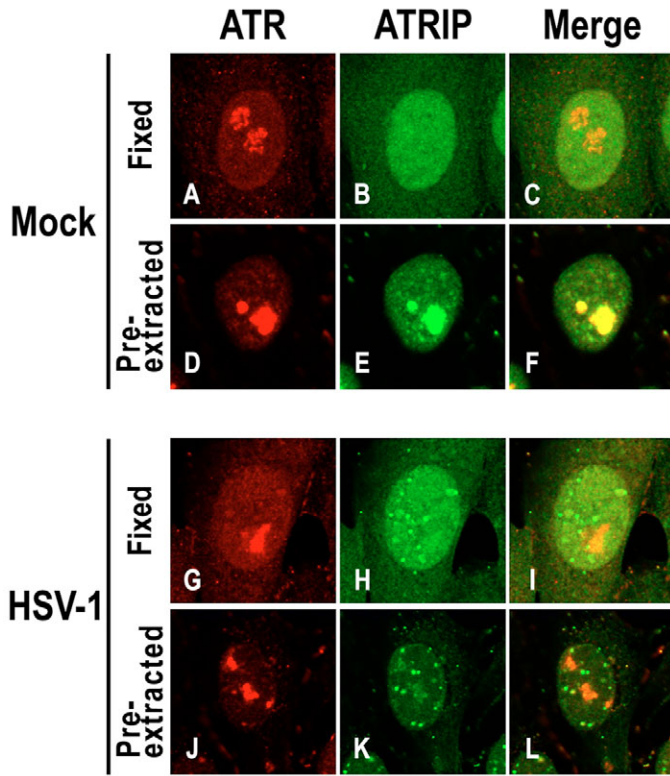
proteins are recruited to replication compartments or to P-RPA foci during HSV-1 infection, we examined their intracellular localization in cells fixed with 4% paraformaldehyde (PFA) to detect total protein populations or extracted with 0.5% Triton X-100. In situ detergent extraction allows for the visualization of the subpopulation of factors that are chromatin associated and which may otherwise be obscured by the nucleoplasmic pool. It is this chromatin-associated population that is generally considered to be relevant to DNA replication and repair (Andegeko et al., 2001; Dimitrova and Gilbert, 2000; Mirzoeva and Petrini, 2001; Vassin et al., 2004).

In mock-infected cells fixed with PFA, ATR was found to be predominantly nucleolar (Fig. 5A,C) whereas ATRIP was diffusely nuclear with some cytoplasmic staining (Fig. 5B,C). Some ATRIP staining was occasionally observed in the nucleolus of mock-infected cells (results not shown). Upon Triton extraction, mock-infected cells displayed strong signals for both ATR and ATRIP within the nucleolus (Fig. 5D-F). Despite reports suggesting that these proteins are found throughout the nucleus of unstressed cells (Itakura et al.,

2004a; Itakura et al., 2004b; Pichierri et al., 2003; Zou and Elledge, 2003), our finding that ATR and ATRIP colocalize in the nucleolus is consistent with a recent study that identified ATR and ATRIP as components of the nucleolar proteome (<http://www.lamondlab.com/NOPdb>) (Andersen et al., 2005). The nucleolar localization of ATRIP and ATR in Triton-extracted Vero cells was confirmed by double labeling uninfected cells with anti-ATRIP and anti-nucleolin (H-250) antibodies (results not shown). We next examined the spatial organization of ATR and ATRIP in HSV-1-infected cells (Fig. 5G-L). In PFA-fixed infected cells, the staining patterns for ATRIP and ATR were similar to those observed in mock-infected cells; although faint focal staining for ATRIP could occasionally be discerned within the diffuse nuclear background (Fig. 5, compare I with C). When cells were pre-extracted with Triton X-100, however, the nucleolar staining of ATR was retained in infected cells (Fig. 5J) whereas ATRIP was found to be distributed into nuclear foci (Fig. 5K). Thus, infection results in the redistribution of a detergent-resistant population of ATRIP from the nucleolus into nuclear foci (Fig.



**Fig. 4.** VICE domains are excluded from cellular DSBs during HSV-1 infection. Vero cells were mock infected (A-D), or infected with HSV-1, strain KOS (E-I). Pre-extracted cells were triple labeled with mouse anti-UL29 (red), rabbit anti- $\gamma$ H2AX (green) and rat anti-Hsc70 (blue) to detect the localization of replication compartments, cellular DSBs and VICE domains, respectively. Images were obtained at 100 $\times$  magnification with 2 $\times$  zoom. (J-L) Digital enlargements of an area of the cell shown on the left in panels F-H. Arrows indicate typical VICE domains that are exclusive of  $\gamma$ H2AX staining.



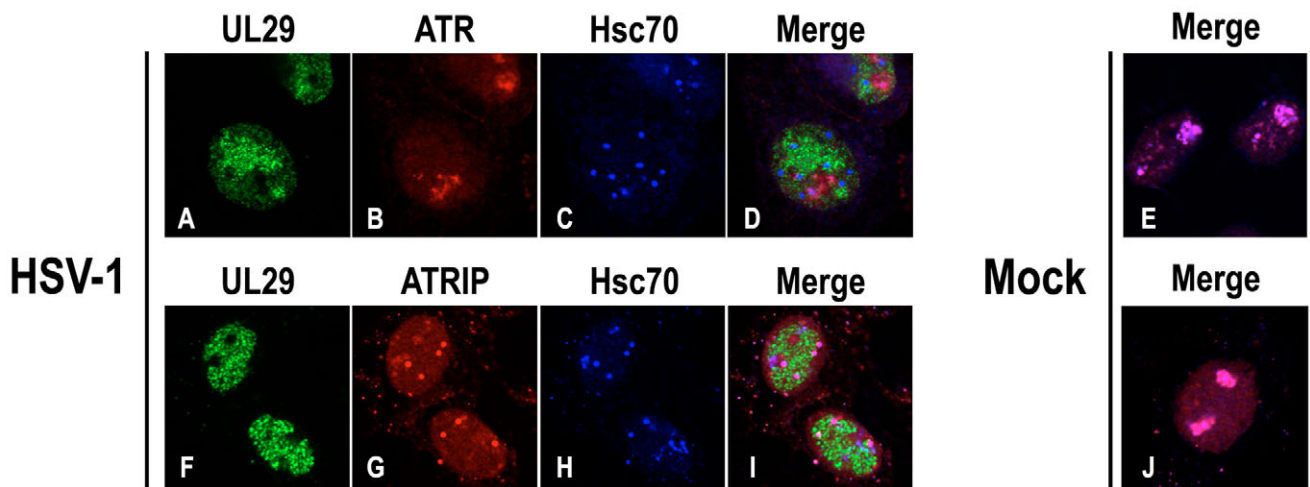
**Fig. 5.** The staining pattern observed for ATR/ATRIP is dependent on the method used for immunolabeling. Mock-infected (A-F) or HSV-1-infected (G-L) cells were either fixed in 4% PFA to visualize total cellular proteins (Fixed, A-C,G-I) or extracted with 0.5% triton X-100 to visualize chromatin-bound and/or matrix-associated proteins (Pre-extracted, D-F,J-L). Cells were double stained using rabbit anti-ATR (red) and mouse anti-ATRIP (green) antibodies. Yellow color indicates a colocalization of the two proteins in the merged images. 100× magnification with 2× zoom.

5, compare K with E). Although the cells presented in Fig. 5 were not stained for viral markers, parallel experiments in which infected cells were also stained with anti-UL29 indicate that >95% of cells were infected and that all cells that displayed a redistribution of ATRIP also possessed robust replication compartments (our unpublished results and Fig. 6). The redistribution of ATRIP, but not ATR, from the nucleolus, during HSV-1 infection is surprising in light of previous results describing the *in vivo* association of ATRIP with ATR and their colocalization into damage-induced foci in response to genotoxic assault (Zou and Elledge, 2003). We propose that HSV-1 spatially uncouples ATRIP from ATR as a mechanism to disrupt ATR-dependent cell-signaling events during lytic infection.

The nuclear ATRIP foci observed in pre-extracted HSV-1-infected cells resembled VICE domains (Fig. 5K). To determine whether the redistributed ATRIP is sequestered within VICE domains, we examined pre-extracted cells that were labeled using antibodies against UL29, Hsc70 and ATR or ATRIP (Fig. 6). These triple-labeling experiments confirm that, upon infection, ATR remains nucleolar and ATRIP is redistributed along with Hsc70 from the nucleolus into VICE domains located adjacent to replication compartments (Fig. 6). Furthermore, triple-labeling experiments using anti-ATRIP, anti-P-RPA and anti-Hsc70 antibodies confirm that ATRIP and a subpopulation of P-RPA are redistributed into VICE domains in HSV-1-infected cells (Fig. 7). Taken together these results suggest that HSV-1 may disarm cellular signals for genotoxic stress through the relocation of P-RPA and ATRIP. HSV-1 directs these two important components of the ATR-dependent DNA damage response away from sites of ongoing viral replication and sequesters them instead into VICE domains.

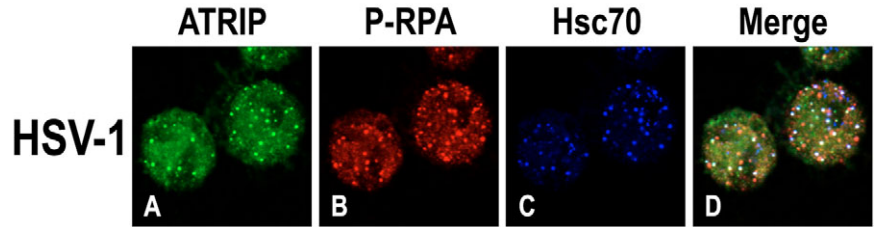
**ICP0 induces the redistribution of ATRIP in transfected cells**

The HSV-1 immediate-early protein, ICP0, is necessary for the

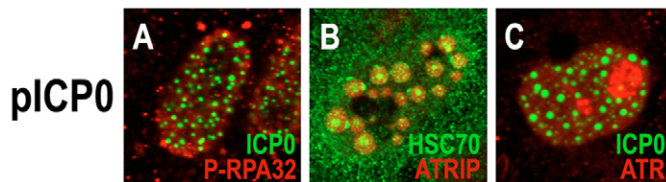


**Fig. 6.** ATR is retained in the nucleolus whereas ATRIP is redistributed to VICE domains during HSV-1 infection. Vero cells were infected with HSV-1 (A-D,F-I) or mock infected (E,J). Pre-extracted cells were triple labeled with mouse (A,E) or rabbit (F,J) anti-UL29 (green), rabbit anti-ATR (B,E; red) or mouse anti-ATRIP (G,J; red) and rat anti-Hsc70 (C,E,H,J; blue) to detect the localization of replication compartments, cellular DSBs and VICE domains, respectively. Lavender/pink color indicates the colocalization of ATR or ATRIP with Hsc70 in the merged images. 100× magnification with 2× zoom.

**Fig. 7.** ATRIP and P-RPA colocalize in VICE domains during HSV-1 infection. HSV-1-infected Vero cells were pre-extracted with 0.5% Triton X-100 as described in the Materials and Methods. Cells were triple labeled with mouse anti-ATRIP (green), rabbit anti-P-RPA (red) and rat anti-Hsc70 (blue). White foci shown in the merged image indicate the colocalization of the three proteins within VICE domains. 100× magnification with 2× zoom.



formation of VICE domains during infection (Burch and Weller, 2004). Furthermore, when cells are transfected with a plasmid expressing the *ICP0* gene, nuclear and cytoplasmic inclusions are observed that contain ICP0, Hsc70, conjugated ubiquitin and misfolded proteins (Burch and Weller, 2004; Everett, 2000; Lukonis and Weller, 1996). To determine whether ICP0 was sufficient for the redistribution of P-RPA and ATRIP, plasmids expressing ICP0 were used to transfect Vero cells which were then double labeled with antibodies against ICP0 and either P-RPA or ATR (Fig. 8A,C). Since both anti-ICP0 and anti-ATRIP are mouse monoclonal antibodies, the anti-ICP0 antibody could not be used in double-labeling experiments for detection of ATRIP; therefore, Hsc70 was used as a surrogate marker for the ICP0 inclusions (Burch and Weller, 2004). Transfected cells were double stained with antibodies against ATRIP and Hsc70. (Fig. 8B). Untransfected controls displayed staining patterns similar to that seen in mock-infected controls reported above (data not shown). Examination of cells transfected with the ICP0-expressing plasmid indicate that this viral protein is not sufficient for the redistribution of P-RPA into ICP0-containing nuclear inclusions (Fig. 8A) suggesting that cellular or viral factors other than ICP0 are needed for sequestering P-RPA. On the other hand, Fig. 8B shows a transfected cell displaying ATRIP in typical ICP0-induced nuclear inclusions, the interior of which stained for Hsc70 (Burch and Weller, 2004; Everett, 2000; Lukonis and Weller, 1996). Nuclear ATRIP inclusions were never seen in untransfected cells, suggesting that ICP0 is sufficient for its redistribution. Finally, the staining pattern of ATR remained nucleolar in ICP0-expressing cells (Fig. 8C).



**Fig. 8.** ICP0 is sufficient to redistribute ATRIP in transfected cells. Vero cells were transfected with an expression plasmid encoding the *ICP0* gene (pICP0) and fixed in 4% PFA as described in the Materials and Methods. (A) Merged image of a transfected cell double labeled with mouse anti-ICP0 (green) and rabbit anti-P-RPA (red). No colocalization of the two proteins was observed. (B) Merged image of a transfected cell double labeled with rat anti-Hsc70, a cellular marker that stains the interior of ICP0 inclusions (green), and mouse anti-ATRIP (red). Note the ATRIP-stained ICP0-like nuclear inclusions surrounding Hsc70. (C) Merged image of a transfected cell double labeled with mouse anti-ICP0 (green) and rabbit anti-ATR (red). No colocalization of the two proteins was observed. 100× magnification with 2× zoom.

Taken together, these results suggest that ICP0 is sufficient for the redistribution of ATRIP but is insufficient for the redistribution of P-RPA.

### Discussion

ATR is an essential signaling kinase that plays crucial roles in the regulation of DNA replication during an unperturbed cell cycle as well as in response to genotoxic stress. The ATR-ATRIP complex is thought to sense replication stress or DNA damage by recognizing persistent RPA-ssDNA intermediates that arise from many types of DNA lesions (reviewed by Shechter et al., 2004). The hyperphosphorylation of RPA on ssDNA is an additional signal for DNA damage (Vassin et al., 2004). We previously showed that productive HSV-1 infection does not result in the induction of RPA hyperphosphorylation; instead, endogenous hyperphosphorylated RPA is sequestered away from replication compartments into discrete nuclear foci (Wilkinson and Weller, 2004; Wilkinson and Weller, 2005). In this study, IF microscopy was used to examine the subcellular ATR response during HSV-1 infection. We found that: (1) although infection induced DSBs in cellular chromatin, as detected by  $\gamma$ H2AX staining, P-RPA did not accumulate at these breaks; (2) P-RPA was found within VICE domains which are enriched for cellular components involved in protein folding and degradation; (3) HSV-1 infection induced the spatial uncoupling of ATRIP from ATR and its redistribution from the nucleolus into VICE domains; (4) The HSV-1 immediate-early protein, ICP0 was sufficient to induce the redistribution of ATRIP. Taken together, these findings suggest that HSV-1 has evolved a strategy to disarm the ATR pathway during lytic infection.

**Dismantling of ATR signaling during HSV-1 lytic infection**  
One hallmark of the ATR-mediated DNA damage response is the colocalization of ATR, ATRIP, RPA and/or hyperphosphorylated RPA in nuclear foci where DNA repair is thought to take place (Cortez et al., 2001; Tibbetts et al., 2000; Vassin et al., 2004; Wu et al., 2005; Zou and Elledge, 2003). ATR stress signaling is dependent upon the recruitment of ATR to DNA which is mediated by ATRIP (Falck et al., 2005; Itakura et al., 2004a; Zou and Elledge, 2003). IF microscopic analysis has allowed us to demonstrate that crucial components of the ATR damage-response pathway are mislocalized within the infected cell nucleus. Although the general population of RPA can be found within replication compartments, possibly acting at unperturbed viral forks or replication intermediates (Uprichard and Knipe, 1997; Wilcock and Lane, 1991; Wilkinson and Weller, 2004), endogenous P-RPA is sequestered in VICE domains. Furthermore, in situ extraction of cells revealed that HSV-1 induces the uncoupling of the nucleolar population of ATRIP from ATR and redistributes

ATRIP into VICE domains. Although it is possible that some ATRIP is associated with ATR in the nucleosol of infected cells, several lines of evidence indicate that neither the nucleosolic nor the detergent-resistant fractions of ATR and ATRIP is activated during infection. (1) We have never observed either the induction of RPA hyperphosphorylation or the recruitment of ATR-ATRIP or P-RPA to replication compartments indicating that these signaling molecules are excluded from sites which contain viral DNA. (2) Using  $\gamma$ H2AX as a marker for the formation of DSBs, we demonstrated that ATR-dependent factors are not recruited to cellular DSBs that arise within marginated cellular chromatin of infected cells. (3) Human Chk1, a specific target of the activated ATR kinase is not phosphorylated during HSV infection; however Chk2, a target of the ATM kinase is phosphorylated (Lilley et al., 2005; Shirata et al., 2005) (our unpublished results). (4) Virus yield is unaffected in HSV-1-infected cells expressing a transdominant, kinase-dead mutant ATR (Lilley et al., 2005). Thus, we propose that HSV-1 avoids ATR-mediated signaling by preventing the recruitment of ATR-ATRIP and P-RPA to either viral or cellular DNA, uncoupling ATRIP from ATR and sequestering ATRIP and endogenous P-RPA within VICE domains.

#### VICE domains and sequestration of non-native proteins

During heat shock and other forms of stress such as infection, proteins become denatured or misfolded. The Hsc70/Hsp70 and Hsp90 multi-chaperone systems play important roles in targeting misfolded proteins to the ubiquitin proteasome for refolding or elimination (e.g. Connell et al., 2001; Demand et al., 2001; Doong et al., 2003). The redistribution of Hsc70, Hsp70, Hsp40, Hsp90, ubiquitylated proteins and the core catalytic complex of the proteasome into VICE domains in infected cells suggests that HSV-1 has evolved a mechanism to deal with misfolded and unwanted proteins (Burch and Weller, 2004; Burch and Weller, 2005; Parkinson and Everett, 2001). VICE domains are reminiscent of nuclear aggresomes, which form in response to misfolded proteins and contain heat-shock proteins and components of the ubiquitin proteasome (Anton et al., 1999; Fu et al., 2005). P-RPA and ATRIP may be targeted to VICE domains because they are recognized as misfolded, perhaps because they have been separated from their normal interaction partners as described below. Alternatively, the sequestration of P-RPA and ATRIP may reflect a more active targeting mechanism by which the virus removes particular cellular response signals. Thus, HSV-1 appears to manipulate not only the DNA damage response but also the unfolded protein response to its own advantage.

#### ICP0 plays a key role in disabling the ATR-dependent pathway

ICP0, which is an E3 ubiquitin ligase, plays a central role in dismantling the NHEJ pathway in some cell types by inducing the proteasomal-dependent degradation of DNA-PKcs during infection (Lees-Miller et al., 1996; Parkinson et al., 1999). We provide evidence here that ICP0 also plays an active role in dismantling the ATR pathway. ICP0 has been shown to localize at the nucleolus early upon infection possibly as part of a mechanism that induces the degradation or dispersal of nucleolar protein(s) (Morency et al., 2005). In this paper, we report that a significant population of ATR and ATRIP is

present in the nucleolus in uninfected Vero cells. We propose that ICP0 may dismantle ATR-dependent events by localizing to the nucleolus and inducing the uncoupling of the ATR-ATRIP complex. The uncoupling of ATRIP from ATR may expose hydrophobic residues resulting in its recognition by Hsc70/Hsp70. Since ICP0 can induce the redistribution of Hsc70/Hsp70 (Burch and Weller, 2004), it is possible that ATRIP is redistributed to VICE domains by virtue of its interaction with Hsp70 (Cortez et al., 2001). Although it is not clear how ICP0 could induce the uncoupling of the ATR-ATRIP complex, it is possible that the ubiquitin ligase activity targets either ATRIP itself or another cellular component responsible for maintaining the association of ATR and ATRIP for degradation.

When expressed in cells by transfection, ICP0 induces the formation of nuclear and cytoplasmic inclusions that contain ICP0, ubiquitylated proteins and Hsc70 (Burch and Weller, 2004; Everett, 2000). Misfolded viral proteins are also found within these ICP0-induced inclusions (Lukonis and Weller, 1996). The helicase-primase complex of HSV-1 is a heterotrimer whose full activity depends on the co-expression of all three subunits (UL5, UL8 and UL52). If one subunit is expressed without the other two in cells cotransfected with ICP0, that subunit is found within the ICP0 inclusions. Moreover, a subpopulation of the UL6 portal protein, which may need chaperone assistance during assembly into a portal ring, is also found within ICP0-induced inclusions (Lukonis and Weller, 1996). We proposed that proteins expressed without their normal interaction partners or that are misfolded might be sequestered in an ICP0-dependent fashion (Lukonis and Weller, 1996). In this paper we have demonstrated that ATRIP, which is spatially uncoupled from its binding partner during infection, is also found within ICP0-induced inclusions in transfected cells. Thus, it now appears that the ability of ICP0 to form nuclear inclusions around misfolded proteins in transfected cells and to induce VICE domain formation in infected cells may reflect an evolutionary strategy to take advantage of the host stress response to misfolded proteins. It is known that the accumulation of non-native proteins can trigger deleterious events such as apoptosis. Thus, the ability of ICP0 to sequester and potentially degrade non-native proteins may allow the virus to delay the induction of apoptosis at least until viral progeny are made. Experiments using ICP0 mutant viruses and proteasomal inhibitors are underway to characterize further the role of ICP0 in dismantling the ATR signaling pathway.

#### Both HSV-1 and adenovirus disarm components of the cellular DNA damage response

HSV-1 is not the only virus that disarms components of the cellular DNA damage response. Adenovirus type 5 (Ad5) infection inactivates the MRN complex by inducing the relocalization and degradation of one or more of the MRN subunits. Ad5 sequesters MRN subunits into nuclear foci located adjacent to viral replication centers as well as into cytoplasmic inclusion bodies (Araujo et al., 2005; Evans and Hearing, 2003; Evans and Hearing, 2005; Liu et al., 2005; Stracker et al., 2002). The MRN-containing nuclear foci in Ad5-infected cells are reminiscent of the HSV-1-induced VICE domains, and it will be of interest to determine whether they too are enriched for cellular components involved in protein

folding and degradation. The Ad5-induced, MRN-containing cytoplasmic inclusions, however, do appear to be aggregates enriched with chaperones and the ubiquitin proteasome (Araujo et al., 2005; Evans and Hearing, 2005; Liu et al., 2005), suggesting that both nuclear and cytoplasmic MRN-containing foci are part of a larger cellular response to the accumulation of misfolded proteins (reviewed by Garcia-Mata et al., 2002). Thus both adenoviruses and herpesviruses appear to disarm cellular responses to DNA damage through manipulation of the stress response and sequestration of repair factors. The fact that HSV-1 targets components of the ATR-response pathway whereas Ad5 targets the MRN pathway may reflect different DNA replication strategies used by these viruses (Wilkinson and Weller, 2004).

### HSV-1 infection appears to activate the ATM-dependent pathway while dismantling others

In HSV-1 infected cells,  $\gamma$ H2AX foci can be seen surrounding replication compartments in infected cells indicating the presence of DSBs; however, instead of being recruited to these DSBs in host chromatin, the NHEJ and ATR pathways are disrupted. On the other hand, HSV-1 appears to activate components of the ATM-dependent pathway (Lilley et al., 2005; Shirata et al., 2005; Wilkinson and Weller, 2004). The MRN repair complex and activated ATM, are apparently not recruited to cellular DSBs, but instead are found in viral replication compartments (Lilley et al., 2005; Shirata et al., 2005; Taylor and Knipe, 2004; Wilkinson and Weller, 2004). Thus, components of the ATM-pathway are activated and recruited to sites of viral DNA synthesis. Although ATR- and ATM-mediated pathways are related and both can be activated by similar genotoxic events, HSV-1 distinguishes between these two pathways, inactivating one and potentially using the other. The MRN complex and other downstream components of the ATM-mediated pathway may participate directly in HSV DNA synthesis, which we and others have suggested may involve recombination (reviewed by Wilkinson and Weller, 2003).

## Materials and Methods

### Cell lines, virus and infections

African green monkey kidney fibroblasts (Vero cells) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained as monolayers in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gemini Bio-Products, Woodland, CA), penicillin, streptomycin and amphotericin B (Invitrogen). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Strain KOS was used as the wild-type strain of HSV-1. For infections, subconfluent cells on coverslips were adsorbed for 1 hour with 10 particle-forming units of virus per cell, and incubated 5.5-6.0 hours postinfection. Mock infections were carried out in parallel.

### Transfections

pW3 (also called pICP0), which expresses ICP0 under its own promoter (Sacks and Schaffer, 1987) was used in transient transfection experiments. For each 35-mm dish, subconfluent cells on coverslips were transfected with 0.25  $\mu$ g pICP0 and 0.75  $\mu$ g pUC18 carrier DNA using Lipofectamine and PLUS reagents (Invitrogen) according to the manufacturer's instructions.

### Antibodies and reagents

Mouse monoclonal anti-UL29 antibody (39S) (Showalter et al., 1981) was obtained from ATCC. Rabbit polyclonal anti-UL29 (367) (Shelton et al., 1994) was supplied by William Ruyechan (University of SUNY, Buffalo, NY). Rabbit polyclonal anti-ATR and phosphospecific anti-phospho-RPA32 pSer4/pSer8 (BL647) antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Mouse monoclonal anti- $\gamma$ H2AX was purchased from Upstate (Lake Placid, NY). Mouse monoclonal anti-ATRIP was obtained from Cell Signaling Technology (Beverly, MA). Rat

monoclonal anti-Hsc70 was purchased from Stressgen Biotechnologies (San Diego, CA). Rabbit anti-nucleolin antibody (H-250) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-ICP0 antibody was obtained from the Goodwin Institute for Cancer Research (Plantation, FL). Secondary antibodies directed against mouse, rabbit or rat IgG and conjugated to Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 594 or Alexa Fluor 647 were purchased from Molecular Probes (Eugene, OR). Camptothecin was purchased from Sigma Aldrich (St Louis, MO).

### Labeling for indirect immunofluorescence microscopy

At 5.5 to 6.0 hours post infection or 16-18 hours post transfection, cells were briefly washed in PBS and processed for immunolabeling. For visualization of total cell proteins, cells were first fixed in 4% PFA then permeabilized for 10 minutes in 1% Triton X-100 in PBS. For the visualization of detergent-resistant or chromatin-associated nuclear proteins, an in situ extraction method that removes the cytoplasm and nucleosolic proteins was used. Cells were pre-extracted for 5 minutes on ice with 0.5% Triton X-100 in cytoskeletal buffer as described (Dimitrova and Gilbert, 2000) then fixed in 4% PFA for 10 minutes. After washing with PBS and blocking in 3% normal goat serum (NGS) in PBS, cells were incubated with primary antibodies diluted in 3% NGS for at least 30 minutes. Antibodies 39S, anti- $\gamma$ H2AX anti-ATR and anti-ICP0 were used at a concentration of 1:200 and antibodies 367, BL647, anti-Hsc70 and anti-ATRIP were used at 1:400. After thorough washing with PBS, cells were incubated with secondary antibodies diluted 1:200. After a final wash in PBS, coverslips were mounted in glycerol gelatin containing 2.5% DABCO (Sigma Aldrich) to retard photobleaching.

### Confocal microscopy

Cells stained for IF were examined using a Zeiss LSM 410 confocal microscope system equipped with an argon-krypton laser, an Axiovert 135 inverted microscope and a Zeiss  $\times$ 100 Plan Neofluar objective. Alexa Fluor 488 was excited at 488 nm. Alexa Fluor 546 or 594 excited at 568 nm and Alexa Fluor 647 at 647 nm. Appropriate emission filters were used for double- and triple-labeling experiments. Channels were scanned individually using settings established with control slides and images were merged by computer. To control for crossreactivity, samples were stained with one primary antibody and appropriate secondary antibodies. No overlap between the channels was observed for any of the samples at the settings used. Collected images were arranged using Adobe Photoshop 6.0.

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