Regulation of alphaherpesvirus infections by the ICP0 family of proteins

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Immediate-early protein ICP0 of herpes simplex virus type 1 (HSV-1) is important for the regulation of lytic and latent viral infection. Like the related proteins expressed by other alphaherpesviruses, ICP0 has a zinc-stabilized RING finger domain that confers E3 ubiquitin ligase activity. This domain is essential for the core functions of ICP0 and its activity leads to the degradation of a number of cellular proteins, some of which are involved in cellular defences that restrict viral infection. The article reviews recent advances in ICP0-related research, with an emphasis on the mechanisms by which ICP0 and related proteins counteract antiviral restriction and the roles in this process of cellular nuclear substructures known as ND10 or PML nuclear bodies. We also summarize recent advances in the understanding of the biochemical aspects of ICP0 activity. These studies highlight the importance of the SUMO conjugation pathway in both intrinsic resistance to HSV-1 infection and in substrate targeting by ICP0. The topics discussed in this review are relevant not only to HSV-1 infection, but also to cellular intrinsic resistance against herpesviruses more generally and the mechanisms by which viruses can evade this restriction.

Introduction

After a primary infection, herpesviruses establish life-long latent infections that periodically reactivate to enable transmission between individuals and ensure viral maintenance within the host population (Knipe et al., 2006). This review describes recent developments in studies on ICP0, a herpes simplex virus type 1 (HSV-1) protein that regulates both lytic and latent infection, and which mediates its roles by influencing several cellular pathways and proteins. All alphaherpesviruses that infect mammalian species express a member of the ICP0 family of proteins, which is defined by the presence of domain, known as a RING finger, that coordinates two zinc atoms by conserved cysteine and histidine residues (Fig. 1; Barlow et al., 1994). This domain confers E3 ubiquitin ligase activity to ICP0 and its viral orthologues (Boutell et al., 2002; Everett et al., 2010; Hagglund et al., 2002), mediating the ubiquitination and proteasome-dependent degradation of several cellular proteins during infection (Table 1). There is abundant evidence that ICP0-related proteins play central roles in alphaherpesvirus biology (Everett, 2006, 2011; Hagglund & Roizman, 2004; Smith et al., 2011). This article discusses recent advances in this complex and at times controversial subject area.

A brief summary of ICP0 biology during lytic infection

ICP0 is a 775-residue protein that is required for efficient lytic infection and productive reactivation from latency. The phenotype of ICP0-null HSV-1 mutants is most easily envisaged as a defect in the probability that a viral genome will commit a cell to lytic infection. Therefore, compared with wild-type (wt) virus, an ICP0-null mutant has a very high particle to p.f.u. ratio. The extent of this defect is cell-type dependent, being highest in human diploid fibroblasts (around 1000-fold), intermediate in many commonly used cell types (e.g. BHK, HeLa and Vero cells; 30–100-fold), and absent in certain cell lines (e.g. U2OS cells) (Everett et al., 2004; Yao & Schaffer, 1995). The defect is multiplicity dependent, so that even restrictive cell types can be infected efficiently if the amount of input virus is sufficiently high. These issues complicate the design and interpretation of experiments using ICP0 mutant viruses, as the results can be highly influenced by both cell type and input multiplicity. Experiments which measure consequences to the virus, such as the probability of commitment to lytic infection or viral gene expression, are best done at low multiplicity, based on titres in U2OS cells. Experiments that investigate consequences to the cell should be done at high multiplicity to ensure that all the cells are actively infected. This concept is based on the distinction between the direct effects of ICP0 itself, and the indirect effects resulting from a failure to commit to lytic infection and therefore defects in the expression of other viral proteins.

In low multiplicity infections of restrictive cell types the ICP0-null mutant genomes that fail to initiate a lytic infection are repressed and maintained in a quiescent state. These quiescent genomes can be de-repressed by subsequent
expression of ICP0 (reviewed by Efstathiou & Preston, 2005). Studies using animal models have indicated that ICP0-null mutant HSV-1 can establish and maintain latency, but reactivation in terms of the production of progeny virus particles is compromised (reviewed by Efstathiou & Preston, 2005; Nicoll et al., 2012). It might be expected that expression of ICP0 could be inimical to the establishment of latency by wt HSV-1, but there is good evidence that at least some transcription of the ICP0 gene has occurred in latently infected mouse neurones (Proença et al., 2008, 2011). The extent of ICP0 protein expression in this situation is unknown at present, and it is possible that any ICP0 that is expressed has reduced functionality (Chen et al., 2000). In the context of individual neurones, reactivation of limited viral gene expression can be detected in the absence of functional ICP0 but this does not lead to virus production (Thompson & Sawtell, 2006).

This brief summary of the phenotype of ICP0-null mutant HSV-1 illustrates the importance of ICP0 in both lytic and latent infection. The biochemical mechanisms that contribute to these phenotypes have been studied extensively. Fundamentally, the core activity of ICP0 resides in its E3 ubiquitin ligase function associated with its RING finger domain, because the absence of this activity renders the virus as defective as an ICP0-null mutant. Nonetheless, ICP0 has several other important motifs that are involved in substrate targeting of its E3 ligase activity, or in other functions (Figs 1c and 2). As a result, ICP0 has been implicated in the regulation of protein stability, chromatin modification, interferon-related pathways, the DNA damage response and a number of other diverse cellular pathways. The following sections will discuss advances in each of these aspects, but first it is necessary to consider an underlying theme in ICP0 biology, namely intrinsic resistance to virus infection.
Table 1. Cellular proteins known to be targeted for proteasome-dependent degradation by ICP0 in a RING finger-dependent manner during HSV-1 infection

<table>
<thead>
<tr>
<th>Protein</th>
<th>Localization/pathway</th>
<th>Phenotype during HSV-1 infection</th>
<th>Methodology</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PML isoform LSUMO-modified PML isoforms 1-10, SUMO-modified Sp100</td>
<td>Major ND10 constituent proteins</td>
<td>Inhibition of intrinsic antiviral immunity in response to herpes virus infection</td>
<td>Infection, transfection, inducible cell line</td>
<td>Chelbi-Alix &amp; de Thé (1999); Everett et al. (1998a); Müller &amp; Dejean (1999); Parkinson &amp; Everett (2000); Gu &amp; Roizman (2003); Everett et al. (2010); Boutell et al. (2011); Cuchet-Lourenço et al. (2012) Everett et al. (1998a); Boutell et al. (2011); Boyer-Guitaut et al. (2005)</td>
</tr>
<tr>
<td>High MW SUMO-1 and SUMO-2/3 conjugated proteins</td>
<td>Multiple</td>
<td>To be determined</td>
<td>Infection, inducible cell line, in vitro biochemistry</td>
<td>Lees-Miller et al. (1996); Parkinson et al. (1999) Chaurushiya et al. (2012); Lilley et al. (2010)</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA repair</td>
<td>To be determined</td>
<td>Infection</td>
<td></td>
</tr>
<tr>
<td>RNF8, RNF168</td>
<td>DNA repair</td>
<td>Inhibition of DNA repair. Potential role in inhibiting intrinsic antiviral immunity</td>
<td>Infection, transfection, in vitro biochemistry</td>
<td>Everett et al. (1999a); Lomonte &amp; Everett (1999); Lomonte et al. (2001); Lomonte &amp; Morency (2007); Morency et al. (2007); Gross et al. (2012)</td>
</tr>
<tr>
<td>CENP-A, CENP-B, CENP-C, CENP-I, CENP-H, CENP-N</td>
<td>Centromere/kinetochore assembly</td>
<td>Cell cycle arrest in pro-metaphase/interphase centromere damage response</td>
<td>Infection, transfection, inducible cell lines</td>
<td></td>
</tr>
<tr>
<td>USP7, USP7β</td>
<td>De-ubiquitinating enzyme</td>
<td>Reciprocal activities: USP7 stabilization of ICP0, ICP0 degradation of USP7.</td>
<td>Infection, in vitro biochemistry</td>
<td>Canning et al. (2004); Boutell et al. (2011); Antrobus &amp; Boutell, (2008)</td>
</tr>
<tr>
<td>IxBz</td>
<td>NF-κB transcriptional activation</td>
<td>To be determined</td>
<td>Transfection</td>
<td>Diao et al. (2005)</td>
</tr>
<tr>
<td>IFI16</td>
<td>Nuclear innate immune DNA sensor</td>
<td>Inhibition of IRF-3 signalling</td>
<td>Infection</td>
<td>Orzalli et al. (2012)</td>
</tr>
<tr>
<td>E2FBP1</td>
<td>Transcription</td>
<td>Relief of viral transcriptional repression</td>
<td>Transfection</td>
<td>Fukuyo et al. (2011)</td>
</tr>
</tbody>
</table>

The concept of intrinsic antiviral resistance

The concept of intrinsic antiviral resistance is highly relevant for an understanding of the ICP0 family of proteins. Also known as intrinsic antiviral defence or intrinsic immunity, this aspect of antiviral defence was first developed from the phenomenon of retroviral restriction (Bieniasz, 2004). In contrast to interferons and other cytokines in the innate immune system, which induce the expression of cellular proteins that impede virus infection (Goodbourn et al., 2000), intrinsic resistance is mediated by constitutively expressed cellular proteins that act within individual cells. General characteristics of intrinsic resistance include cell or species specificity, the potential to be overcome by high amounts of infecting virus (i.e. the system can be saturated), diversity in mode of action and stage of virus replication targeted and in many cases counteracting viral regulatory proteins. Thus, the outcome of infection can be determined by the balance between the activities of intrinsic resistance factors and the viral proteins that inactivate their effects. This broad definition is clearly consistent with the phenotype of ICP0-null mutant HSV-1.

The role of ND10 in intrinsic resistance

There is increasing evidence that the small nuclear substructures known as ND10 or PML nuclear bodies are involved in intrinsic resistance to herpesvirus infections (Everett & Chelbi-Alix, 2007; Geoffroy & Chelbi-Alix, 2011; Tavalai & Stamminger, 2008). The ND10 components that have been most studied in terms of intrinsic resistance are PML itself, Sp100, hDaxx and ATRX. Interest in the connections between ND10 and herpesvirus infections initially arose because of the observations that ICP0 first localizes to then disrupts ND10, and because the genomes of HSV-1 and several other DNA viruses become associated with ND10 proteins (Everett, 2001; Everett & Chelbi-Alix, 2007; Maul, 1998). ICP0 also localizes to and disrupts centromeres (see Table 1 for references) and at later times of infection begins to accumulate in the cytoplasm (Lopez et al., 2001), indicating its dynamic association with cellular compartments during the course of infection. The biochemical effects of ICP0 on PML and other ND10 components are summarized in a later section.
Advances in the understanding of the role of ND10 components in intrinsic resistance have come from the use of RNA interference (RNAi) technologies. ICP0-null mutant HSV-1 replicates more efficiently in cells depleted of PML (Everett et al., 2006), and PML is also involved in restriction of infections by human cytomegalovirus (HCMV) (Tavalai et al., 2006) and varicella zoster virus (VZV) (Kyratsous & Silverstein, 2009). PML is a complex protein that is expressed as several different isoforms (PML.I to PML.VI) that have distinct properties. In one assay system, ectopic expression of PML.I (the most abundant isoform) and PML.II, but not the other isoforms, partially reversed the effect of PML depletion on ICP0-null mutant HSV-1 infection efficiency (Cuchet et al., 2011). In another example, PML.IV was found to sequester progeny VZV capsids in nuclear 'cages' (Reichelt et al., 2011), and a later study made the important finding that PML restricts VZV pathogenesis in the context of human skin xenografts in the mouse (Wang et al., 2011). Several studies have reported that Sp100, hDaxx and ATRX also contribute to intrinsic resistance to various different herpesviruses (Adler et al., 2011; Cantrell & Bresnaham, 2006; Everett et al., 2008a; Full et al., 2012; Kim et al., 2011; Lukashchuk et al., 2008; Lukashchuk & Everett, 2010; Preston & Nicholl, 2006; Saffert & Kalejta, 2006; Tavalai et al., 2011; Tavalai et al., 2008; Tsai et al., 2011; Woodhall et al., 2006).

The increase in ICP0-null mutant HSV-1 replication efficiency in cells depleted individually of PML, Sp100, hDaxx or ATRX are modest (5–10-fold) compared with the full defect of the mutant virus. This might suggest that the roles of ND10 components are relatively minor compared with other means of host restriction, but it is also possible that these factors act in a cooperative manner. Indeed, depletion of PML and Sp100 simultaneously increases ICP0-null mutant HSV-1 replication to a greater extent than depletion of either protein alone (Everett et al., 2008a), and simultaneous depletion of PML, Sp100 and hDaxx increases ICP0-null mutant HSV-1 plaque formation by at least 50-fold (Glass & Everett, 2013). Analogous results were found in HCMV infection of cells depleted of both PML and hDaxx (Tavalai et al., 2008). Therefore it appears that the repressive effects of several ND10 components combine to impart a significant component of intrinsic resistance to HSV-1 replication. Also consistent with this hypothesis is the observation that HCMV proteins IE1 and pp71, which affect ND10 components PML/Sp100 and hDaxx/ATRX, respectively, can substitute almost completely for ICP0 in its core activities of stimulating lytic HSV-1 infection and de-repression of quiescent viral genomes (Everett et al., 2013).

ICP0-mediated inhibition of the recruitment of cellular repressors to HSV-1 genomes

One striking aspect of the behaviour of ND10 components in response to HSV-1 infection is their recruitment to sites that are closely associated with parental HSV-1 genomes (Everett & Murray, 2005). When the association between herpesvirus genomes and ND10 was first discovered, it was debated whether this reflected a positive or negative influence on viral infection (Maul, 1998). The evidence, particularly the RNAi-mediated depletion work summarized above, now indicates that it is a repressive cellular response to the entry of the viral genome into the nucleus. Recruitment of ND10 proteins to regions in close proximity to the HSV-1 genomes occurs very rapidly (Everett et al., 2007), but it is short lived and therefore hard to detect during wt virus infection. In the absence of ICP0, however, it is very prominent, particularly in cells at the edges of developing plaques. ICP0 inhibits the recruitment of several ND10 components, including PML, Sp100, hDaxx, ATRX and the SUMO family of proteins (Cuchet-Lourenço et al., 2011; Everett & Murray, 2005; Lukashchuk & Everett, 2010), and the ability of ICP0 to counteract this recruitment correlates well with inactivation of intrinsic resistance (Everett et al., 2009). The orthologues of ICP0 expressed by bovine herpesvirus type 1 (BHV-1), equine herpesvirus type 1 (EHV-1) and pseudorabies (PRV) also inhibit the recruitment of PML and hDaxx to the sites of ICP0-null mutant HSV-1 genomes, in these cases without major effects on the stability of PML (Everett et al., 2010). These findings suggest that the recruitment reflects a cellular response to viral infection that creates a repressive environment to restrict viral gene expression. Interestingly, repressed HSV-1 genomes in quiescently infected cultured cells are sequestered within enlarged, spherical ND10-like structures (Everett et al., 2007), and recent evidence has found that analogous sequestration occurs in latently infected mouse neurones (Catez et al., 2012).

Analysis of the features of PML, hDaxx and Sp100 that are required for their recruitment to sites associated with HSV-1 genomes found that the SUMO-interaction motifs (SIMs) of these proteins were in each case essential (Cuchet-Lourenço et al., 2011). PML.I can partially reverse the increase in ICP0-null mutant HSV-1 plaque formation in cells depleted of endogenous PML (Cuchet et al., 2011), whilst reconstitution of hDaxx-depleted cells with wt hDaxx completely restores full repression (Lukashchuk & Everett, 2010). SIM mutant versions of these proteins fail not only to be recruited to the viral genomes, but also to act as repressors (Cuchet-Lourenço et al., 2011; Lukashchuk & Everett, 2010). These data provide a link between the recruitment of these proteins to sites associated with HSV-1 genomes and their roles in intrinsic resistance. Note that, unlike the assembly of ND10 in uninfected cells, the recruitment of Sp100, hDaxx and ATRX to the virus-induced ND10-like foci is not dependent on PML (Everett et al., 2006, 2007, 2008a). The fact that PML, Sp100 and hDaxx are all independently recruited to viral genomes could explain why depletion of any individual one of these repressive proteins results in only a limited improvement in ICP0-null mutant plaque formation – the full effects of ND10-mediated repression appear to be dependent on the cooperative effects of multiple factors.

Identification of the cellular factors that are required for the initial nucleation of ND10 proteins at sites associated with HSV-1 genomes remains an important issue. Multiple
SUMO family members are present in the recruited foci, and in the case of SUMO-2/3 this recruitment is PML independent (Cuchet-Lourengo et al., 2011). Therefore, nuclear entry of the HSV-1 genome might stimulate a cascade of SUMO conjugation events that initiate formation of these foci through SUMO–SIM interactions. A prediction of this hypothesis is that SUMO conjugation is involved in the recruitment process and intrinsic resistance. Indeed, depletion of Ubc9, the SUMO E2-conjugating enzyme that is essential for SUMO modification, results in inefficient PML recruitment and a marked decrease in the effectiveness of intrinsic resistance to ICP0-null mutant HSV-1 (Boutell et al., 2011). A plausible mechanism by which ICP0 could inhibit ND10 component recruitment is presented in a later section.

**ICP0 and chromatin-related regulatory pathways**

The mechanisms by which viral transcription is repressed in the absence of ICP0 remain to be defined in detail. In recent years there have been substantial advances in studies on the role of chromatin structure in the regulation of HSV-1 and HCMV gene expression during both lytic and latent infection (reviewed by Knipe & Cliffe, 2008; Kutluay & Triezenberg, 2009). There was also a higher proportion of H3 with an active chromatin mark (acetylated on lysine residue 9) and a lower proportion with a repressive mark (trimethylated on lysine 9; H3K9me3) in the presence of ICP0 (Ferenczy & Deluca, 2009). Similar results were observed in HeLa cells, although in this case an increased level of acetylated H3 associated with the viral genome was linked to the activities of VP16 rather than ICP0 (Hancock et al., 2010).

ICP0 has the ability to induce de-repression of quiescent HSV-1 genomes harboured in cultured cells (reviewed by Efstathiou & Preston, 2005). There is a clear and important distinction between events during the initial stages of lytic infection (when nucleosome-free viral genomes might be assembled into repressed or active chromatin structures) and those during reactivation or de-repression, when a presumably compact, repressed genome takes on a more open chromatin configuration. One study found that ICP0-mediated de-repression occurred concomitantly with a reduction in H3K9me3 on viral promoters, although changes in histone loading and/or acetylation did not always correlate with the time-course or efficiency of viral gene expression in the presence of ICP0 (Ferenczy & Deluca, 2011). Another study found little overall change in histone occupancy but a substantial increase in levels of acetylated H3 during ICP0-mediated de-repression (Coleman et al., 2008). On the other hand, complementation of the expression defect of a VP16 activation domain mutant of HSV-1 by superinfection with HSV-2 (thus providing both VP16 and ICP0) did not cause reductions in histone loading or changes in their epigenetic marks (Kutluay & Triezenberg, 2009a). All of these studies concluded that ICP0-mediated reactivation does not necessarily correlate with reduced overall histone loading, and it appears that efficient ICP0-mediated reactivation can occur without major effects on histone occupancy or acetylation (Ferenczy & Deluca, 2011). As yet, there is little mechanistic information on how ICP0 might influence chromatin occupancy, and it remains possible that its affects are indirect.

(ii) ICP0 and histone deacetylase enzymes

One hypothesis to explain how ICP0 might activate gene expression is through inhibition of histone deacetylases (HDACs) to maintain viral chromatin in an active configuration. Whilst attractive because of the analogies with well understood cellular systems, there are several unresolved issues. In the absence of ICP0, the great majority of the HSV-1 genome (including most IE promoters in the most restrictive cell types) is very rapidly repressed. For HDACs to be a major factor in this repression requires that viral chromatin assembly be equally rapid. Whilst there is evidence for an early association of histones with viral DNA (reviewed by Nevels et al., 2011), the existing evidence suggests that histone loading is light during the early stages of infection of cultured cells, even when the viral genome is repressed. HDAC inhibitors do not complement ICP0-null mutant viruses in the most restrictive cell types, such as human diploid fibroblasts (Everett et al., 2008; Nicholl & Preston, 1996; Terry-Allison et al., 2007), although they can stimulate ICP0-null mutant HSV-1 gene expression and infection in some cell types, particularly neurones (Arthur et al., 2001; Danaher et al., 2005; Poon et al., 2006; Terry-Allison et al., 2007). On the other hand, ICP0 can be co-immunoprecipitated from

**Biological properties of ICP0**
cells expressing high levels of class II (but not class I) HDACs (Lomonte et al., 2004). Analogous studies with other alphaherpesviruses have reported interactions between ICP0 orthologues and HDACs, and/or stimulatory effects of HDAC inhibitors on viral gene expression (Wang et al., 2009; Zhang et al., 2006; Zhang & Jones, 2001). An important observation was that, unlike HDAC inhibitors, ICP0 does not increase the global levels of acetylated histone H4 (Lomonte et al., 2004). Taking all these observations into account, it appears that the hypothesis that the major pathway involved in ICP0 function is through HDACs cannot be upheld in its simplest form, at least in human diploid fibroblasts.

(iii) ICP0 and the CoREST/REST/HDAC1/LSD1 complex

Another mechanism by which ICP0 could influence viral gene expression through chromatin-related mechanisms is via interactions with chromatin modifying complexes. One prominent strand of research concerns the transcriptionally repressive REST/CoREST/HDAC1/LSD1 complex. This issue has stimulated much debate, involving a complex region of ICP0 that has been linked with several functions (Fig. 2). Initially, it was noted that ICP0 residues 537–613 (that include a low complexity serine/alanine-rich region) show similarity to the N-terminal part of CoREST. It was observed that, during HSV-1 infection, CoREST could be detected in ICP0 immune precipitates, and that at mid to late times of infection HDAC1 was displaced from the REST/CoREST/HDAC1 complex and CoREST was partly redistributed to the cytoplasm (although this latter effect occurred independently of ICP0) (Gu et al., 2005). Subsequently, GST pull-down assays identified a different region of ICP0 that could interact with CoREST in vitro. Given that GST fusions with ICP0 residues 543–768, 543–718 and 693–768 could bind to CoREST with similar efficiencies (Gu & Roizman, 2007), it appears that ICP0 sequences that are sufficient for interaction with CoREST lie between residues 693 and 718. Investigation of amino acid substitution mutations, however, identified a double mutation at residues 671 and 673 that reduced the interaction with CoREST, and an HSV-1 mutant (R8507) expressing this mutant form of ICP0 replicated less efficiently than the wt virus (Gu & Roizman, 2009). On the basis of these pieces of evidence, and the properties of HSV-1 recombinants expressing fragments of CoREST (Du et al., 2010; Gu & Roizman, 2007), it has been proposed that the biological functions of ICP0 during lytic and latent infection can be largely explained by its effects on the REST/CoREST/HDAC1/LSD1 complex.

This hypothesis is complicated by the number of functions attributed to overlapping segments of the relevant region of ICP0, including a region proposed to have ubiquitin ligase activity (HUL1; Hagglund & Roizman, 2002), sequences required for binding to USP7 and localization to ND10, and a set of sequences related to SIM (Fig. 2). The HUL1 activity could not be independently confirmed (Boutell et al., 2002; Vanni et al., 2012) and has not been further investigated. Given this complexity, mutation of sequences implicated in CoREST binding may also affect the efficiency of ICP0 localization to and effects on ND10. Indeed, mutant R8507 exhibits reduced rates of PML degradation (Gu & Roizman, 2009). A recent independent analysis of the ICP0 CoREST-binding mutant R8507 found only a small defect in plaque-forming efficiency, and the mutation did not reduce ICP0-mediated de-repression of gene expression in quiescently infected cells in terms of the proportion of reactivated genomes (although the total level of reactivated gene expression was less than that induced by the wt virus) (Ferenczy et al., 2011). Depletion of CoREST using stable RNAi neither improved plaque forming efficiency of ICP0-null mutant HSV-1 nor impacted on the replication efficiency of the wt virus (Everett, 2010). In contrast, it has been proposed that CoREST is required for efficient IE gene expression, but inhibitory for transcription of early genes (Zhou et al., 2011). Clarification of this discussion awaits independent analysis from other groups but, given that deletion of the whole CoREST-binding region impacts on ICP0 activity to a far lesser degree than deletion of the RING finger (Everett et al., 2009; Ferenczy et al., 2011), at present it appears that CoREST does not play a dominant role in regulating the onset of lytic HSV-1 infection or de-repression of quiescent viral genomes in cultured cells.

(iv) ICP0 and the hDaxx/ATRX chromatin modification/histone chaperone complex

The cellular proteins ATRX and hDaxx form another chromatin modifying complex that is influenced by ICP0 and several other herpesvirus regulatory proteins (Cantrell et al., 2002; Gu et al., 2007; Vanni et al., 2012). An important observation was that, unlike HDAC inhibitors, ICP0 does not increase the global levels of acetylated histone H4 (Lomonte et al., 2004). Taking all these observations into account, it appears that the hypothesis that the major pathway involved in ICP0 function is through HDACs cannot be upheld in its simplest form, at least in human diploid fibroblasts.

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et al. 2006). This complex not only interacts with HDACs and histones (Hollenbach et al., 2002; Xue et al., 2003), it also has histone chaperone activity (Drane et al., 2010; Lewis et al., 2010). There are strong indications, therefore, that these proteins could influence HSV-1 chromatin assembly or structure, which might explain their roles in intrinsic resistance and repression of viral transcription (see above). Consistent with this hypothesis, ATRX mRNA and protein stability are downregulated during HSV-1 infection through the combined activities of the viral microRNA miR-H1, the RNase activity of vhs, and the ubiquitin ligase activity of ICP0 (Jurak et al., 2012). Furthermore, exogenous expression of ICP0 displaces ATRX and hDaxx from an integrated and transcriptional repressed HCMV promoter-driven transgene array (Newhart et al., 2012). These data suggest that ICP0, either directly or indirectly, influences at least two cellular proteins implicated in heterochromatin assembly.

The E3 ubiquitin ligase activity of ICP0

The most prominent motif that ICP0 shares with its orthologues is an N-terminal C3HC4 RING finger domain (Fig. 1; Barlow et al., 1994; Everett et al., 1993, 2010). This domain confers E3 ubiquitin ligase activity (Boutell et al., 2002; Diao et al., 2005; Everett, 2000; Everett et al., 2010; Grant et al., 2012; Hagglund et al., 2002; Parkinson & Everett, 2001; Vanni et al., 2012; Walters et al., 2010) and is required for ICP0 to mediate the ubiquitination and proteasome-dependent degradation of a diverse range of cellular proteins (Table 1). Viruses that express ICP0 mutants with defective RING finger regions have replication defects equivalent to that of ICP0-null mutant HSV-1 (Everett, 1989; Everett et al., 2004; Lium & Silverstein, 1997) and fail to de-repress quiescent HSV-1 in cell culture (Everett et al., 2009; Ferenczy et al., 2011; Harris et al., 1989). The RING finger domain therefore plays a fundamental role in the overall biology of ICP0 during HSV-1 infection.

Whilst a detailed description of the ubiquitin-proteasome pathway is beyond the scope of this article (for a review, see Deshaies & Joazeiro, 2009), an understanding of the biochemical activity of ICP0 requires knowledge of the basic features of this pathway. E3 ubiquitin ligases provide the substrate specificity for the transfer of ubiquitin from E2 ubiquitin-conjugating enzymes onto target substrate proteins. This involves the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin to the side chain amino group of a lysine residue within the target protein. This initial modification (termed mono-ubiquitination) can, in itself, alter protein function and is often associated with intra-cellular signalling. Subsequent rounds of ubiquitin transfer onto lysine residues in the anchored ubiquitin molecule results in the formation of a poly-ubiquitin chain. As ubiquitin contains seven lysine residues, each of which can serve as an acceptor site for ubiquitin modification, a large number of structurally diverse chain types can be formed that differentially regulate many aspects of cell biology, including the cell cycle (Lys-11 linked chains), proteasome-dependent degradation (Lys-48 linked chains), as well as DNA repair and innate immunity (Lys-63 linked chains; reviewed by Trempe, 2011). Both substrate modification and poly-ubiquitin chain formation are mediated through multiple E3 ligase protein–protein interactions. These interactions are often transient due to the biochemical nature of substrate ubiquitination and chain elongation.

The biochemical specificity of ICP0

Like many RING finger ubiquitin ligases, ICP0 stimulates the formation of poly-ubiquitin chains and mediates the ubiquitination of substrates in a RING finger-dependent manner, both in vitro and in vivo (Boutell et al., 2002, 2005; Lilley et al., 2010; Vanni et al., 2012) (Table 1). In vitro assays provide model systems to test whether proteins that are degraded during infection are direct substrates of ICP0, as opposed to indirect phenotypes observed as a consequence of ICP0 activity on other proteins or pathways. Whilst in vitro studies can provide insight into the biochemical specificity of ICP0, these assays have limitations and require the careful use of controls and rate limiting amounts of enzymes in order to generate reliable results.

ICP0 stimulates the formation of unanchored poly-ubiquitin chains in vitro in the presence of two highly homologous E2 ubiquitin-conjugating enzymes, namely UBE2D1 (UbcH5a) and UBE2E1 (UbcH6) (Boutell et al., 2002; Vanni et al., 2012). Both of these E2s are recruited to intranuclear foci by ICP0 in a RING finger-dependent manner, demonstrating RING-dependent E2 cellular sequestration (Boutell et al., 2002). Exogenous expression of a catalytically inactive mutant of UBE2D1, but not UBE2E1, inhibits degradation of PML by ICP0 (Gu & Roizman, 2003). Therefore, the ICP0–UBE2D1 interaction is important for the biological role of ICP0 during infection, consistent with the observation that RNAi depletion of UBE2D1 enhances PML stability during wt HSV-1 infection (S. J. Griffiths and others, unpublished data). ICP0 may utilize more than one E2 enzyme during infection, as there are examples of other RING finger ubiquitin ligases that interact with multiple E2s (Markson et al., 2009; van Wijk et al., 2009). Analogous to other RING finger ubiquitin ligases (Deshaies & Joazeiro, 2009), ICP0 interacts with UBE2D1 via specific contact residues within the a-helix and loop regions of the RING domain (Vanni et al., 2012). Mutation of these residues not only inhibits its ubiquitin ligase activity in vitro, but also significantly impairs complementation of ICP0-null mutant HSV-1 and de-repression of quiescent HSV-1 in cultured cells (Vanni et al., 2012). These data are consistent with the inhibitory effects of proteasome inhibitors on ICP0-dependent stimulation of HSV-1 replication and reactivation
(Everett et al., 1998b). Consequently, small molecule inhibitors that block the ICP0–UBE2D1 interaction could make effective therapeutic antiviral agents.

**Mechanisms of substrate targeting by ICP0**

One of the most significant challenges in ubiquitin research is the identification of substrates that are specifically targeted by individual E3 ubiquitin ligases. Whilst advances in proteomic technologies have been very informative, they have also highlighted the complexity of genuine substrate identification (Bustos et al., 2012). For example, tandem affinity purification tagged ICP0 isolated from transfected 293T cells detected over 150 cellular interaction partners of ICP0, relatively few of which were identified as being degraded during HSV-1 infection (M. Chaurushiya and M. Weitzman, personal communication). ICP0 is therefore likely to interact with a number of proteins that form macro-molecular complexes and/or have large protein interaction networks. Some of these direct interactions may influence ICP0’s activity or substrate specificity, whilst others may be indirect binding partners that have no functional consequence on HSV-1 infection. It is becoming increasingly clear, however, that ICP0 can utilize multiple mechanisms, both direct and indirect, in order to target functionally relevant substrates for degradation (Fig. 3). The concepts discussed below are not necessarily mutually exclusive.

(i) **Direct substrate targeting**

The simplest model of substrate targeting involves direct protein–protein interaction between ligase and substrate through specific recognition motifs (Fig. 3a). Whilst this model is compelling, there are few documented examples of this mechanism, the clearest being that of ubiquitin-specific protease USP7. Like many ubiquitin ligases, ICP0 induces its own ubiquitination that decreases its stability (Boutell et al., 2002; Canning et al., 2004). ICP0 counteracts this activity by interacting with USP7 (Everett et al., 1999b), which in turn cleaves the ubiquitin moieties from ICP0. The consequent increase in stability of ICP0 is the most significant aspect of the ICP0–USP7 interaction, indicating a positive role for USP7 in HSV-1 infection (Canning et al., 2004). However, ICP0 has a reciprocal

![Fig. 3.](https://www.microbiologyresearch.org/journal/issue/472)

A diagrammatic representation of known or plausible substrate-targeting mechanisms employed by ICP0, as described in detail in the text: Ub (ubiquitin), E2 (ubiquitin-conjugating enzyme), Sub. (substrate), P (phosphorylated residue), S (SUMO-modified residue), Inter. Partner (interaction partner), SIM (SUMO-interaction motif), pSIM (phosphorylation-dependent SIM).
effect on USP7, resulting in USP7 ubiquitination and degradation in a manner dependent on the USP7 interaction motif within ICP0 (Boutell et al., 2005). The significance of this potential regulatory feedback loop remains to be fully elucidated, but the stabilization of ICP0 by USP7 is likely to be important when relatively low levels of ICP0 are expressed, for example during the initial stages of reactivation from latency. A second example of a direct substrate interaction involves PML-I (see SUMO-independent targeting section below).

(ii) Phospho-specific substrate targeting

Direct substrate interactions through simple binding motifs are unlikely to account for the diversity of identified ICP0 substrates. Phosphorylation plays a role in regulating ICP0’s ubiquitin ligase activity and substrate targeting (Boutell et al., 2008; Chaurushiya et al., 2012). An elegant example of ICP0 phospho-specific targeting (Fig. 3b) involves RNF8, a cellular RING-finger ubiquitin ligase implicated in DNA repair (Chaurushiya et al., 2012; Lilley et al., 2010). RNF8 contains an FHA (forkhead associated) domain that binds to a specific consensus motif within a number of DNA repair proteins that are phosphorylated in response to DNA damage (Mohammad & Yaffe, 2009). ICP0 mimics a cellular FHA phospho-binding site (67-pTELF-70; Fig. 1c). Phosphorylation of ICP0 residue threonine 67 by casein kinase I enables an interaction with RNF8, leading to RNF8 ubiquitination and degradation (Chaurushiya et al., 2012). Surprisingly, phosphorylation of this short motif also enables ICP0 to interact with a large number of other cellular proteins, including two other FHA domain containing proteins, Chk2 and Nbs1 (Chaurushiya et al., 2012). Whilst the significance of these additional interactions remains to be investigated, phospho-specific targeting provides a potential means by which ICP0 could influence the stability of multiple cellular proteins and/or pathways in a controlled manner.

Two other phosphorylation regions within ICP0, namely Phos-1 and Phos-2 (Davido et al., 2005), also influence ICP0’s ubiquitin ligase activity, including its ability to stimulate the formation of co-localizing conjugated ubiquitin (Phos-1) and the degradation of PML (Phos-2) in certain cell types (Boutell et al., 2008). Mutation of these domains also restricts HSV-1 pathogenesis and reactivation in an animal model (Mostafà et al., 2011). It is likely therefore that both viral and cellular kinases influence ICP0s ubiquitin ligase activity during lytic infection and/or viral reactivation. It is also possible that substrate phosphorylation affects ICP0 target specificity (Fig. 3c).

(iii) SUMO-dependent and -independent substrate targeting

One of the best characterized substrates of ICP0 is PML and its SUMO-modified forms (Chelbi-Alix & de Thé, 1999; Everett et al., 1998a; Müller & Dejean, 1999). However, only recently have the molecular mechanisms involved come to light (Boutell et al., 2011; Cuchet-Lourenço et al., 2012). ICP0 shares properties related to those of SUMO-targeted ubiquitin ligases (STUbLs), a class of enzymes that contain SIMs that mediate substrate interaction that is dependent on the SUMO conjugation status of the target protein (Fig. 3d and 3e; reviewed by Perry et al., 2008; Praefcke et al., 2012). Like ubiquitination, SUMO modification regulates many important cellular processes, including transcription, cellular localization, DNA repair and the cell cycle. STUbLs therefore have the potential to influence the activity of multiple regulators within a given pathway in a synchronized manner, without the need for independent protein interaction motifs.

Two recent reports have identified several potential SIMs, referred to here as SIM-like sequences (SLSs) within ICP0 (Boutell et al., 2011) and ORF61p (VICP0) (Wang et al., 2011), as well as related SLSs within other ICP0 orthologue proteins (Table 2). At least some of these sequences have been implicated in interactions between SUMO family members and ICP0 and ORF61p. For example, SLS4 of ICP0 interacts with SUMO-2 (Boutell et al., 2011), and because it lies immediately adjacent to the phosphorylated region known as Phos-2 (Fig. 1c), it is possible that it is an example of a phosphorylation regulated SIM (Stehmeier & Muller, 2009). Mutation of multiple SLSs within ICP0 reduced its ability to induce the degradation of SUMO-conjugated proteins in general, a phenotype shared by other ICP0 family members (Boutell et al., 2011). Simultaneous mutation of multiple SLSs also reduced ICP0-mediated complementation and reactivation efficiency, although mutation of individual motifs had little or no effect (Boutell et al., 2011). This raises the possibility of functional redundancy amongst the multiple SLSs within ICP0 or its orthologues. Similarly, mutation of SLSs within ORF61p reduced VZV replication in a human skin xenograft mouse model system, although no significant defect in replication was observed in cell culture (Wang et al., 2011). Collectively, these data suggest that SUMO-binding and SUMO-dependent substrate-targeting may be a conserved and biologically significant property of this family of viral ubiquitin ligases (Fig. 3d and 3e). This conclusion is consistent with the observation that ICP0 orthologue proteins can partially substitute for the functional activities of ICP0, despite not being able to recapitulate all of ICP0’s effects on ND10 individual constituent proteins (Everett et al., 2010).

At first glance these STUbL-like properties appear analogous to the activities of the mammalian STUbL RNF4, which has been shown to target SUMO-modified PML for degradation following arsenic oxide treatment in a SIM- and RING finger-dependent manner (Geoffroy et al., 2010; Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Weisshaar et al., 2008). There are, however, a number of important distinctions (Boutell et al., 2011). All nuclear PML isoforms contain the conserved RBCC (RING, B-box and coiled-coil) tripartite motif and SUMO modification sites encoded
within exons 1–6, followed by isoform-specific sequences encoded by downstream exons (Jensen et al., 2001). ICP0 induces the degradation of all SUMO-modified species of PML in an isoform-independent manner, consistent with its STUbL-like activities. In contrast to RNF4, however, ICP0 also degrades PML.I, the most abundant of the PML isoforms, regardless of its SUMO modification status (Boutell et al., 2011; Cuchet-Lourenc¸o et al., 2012). This activity is enabled by a direct and SUMO-independent interaction with PML.I via isoform-specific sequences encoded by exon 9 (Cuchet-Lourenc¸o et al., 2012). ICP0 therefore has two distinct mechanisms of targeting PML for degradation – SUMO-dependent targeting of all SUMO-modified PML isoforms and SUMO-independent targeting of unmodified PML.I.

It is unclear why ICP0 has evolved two mechanisms for targeting PML.I. One hypothesis involves the potential distinction between direct substrate targeting and the cellular localization of ICP0. Deletion of the C-terminal third of ICP0, which includes three SLSs, reduces its localization to ND10 (Cuchet-Lourenc¸o et al., 2012; Maul & Everett, 1994; Meredith et al., 1995). The ability of ICP0 to bind to multiple SUMO-modified proteins may therefore increase its localization to the SUMO-rich ND10 environment, thereby enhancing sequence-specific recognition of target proteins (such as PML.I) within these structures. Alternatively, ICP0 may have evolved dual targeting approaches in order to increase the diversity of its substrates or the efficiency with which they are ubiquitinated. This may be particularly important when ICP0 is in low abundance during the earliest stages of lytic infection or viral reactivation from latency.

(iv) Indirect substrate targeting

In this model, ICP0 might interact specifically with one protein in order to target a partner of that protein (or complex of proteins) for ubiquitination (Fig. 3f). Whilst this model is attractive, there is as yet little biochemical evidence to support it. However, ICP0 has been reported to interact with at least three other cellular RING finger ubiquitin ligases, namely RNF8, RNF168 and SIAH-1 (Chaurushiya et al., 2012; Lilley et al., 2010; Nagel et al., 2011). These interactions could expand the repertoire of ICP0s cellular substrates and/or mechanisms of substrate targeting.

**ICP0 and the DNA damage response**

An exciting development in ICP0 research concerns its effects on the recruitment of DNA damage response (DDR)
proteins to sites of damaged DNA. Such sites can be induced by irradiation, and in this case they are termed irradiation induced foci (IRIFs). DNA breaks are sensed by the MRN complex, comprising Mre11, Rad50 and Nbs1, which then recruits the ATM kinase, resulting in phosphorylation of histone H2AX and its accumulation in chromatin surrounding the break. This is followed by recruitment of Mdci, 53BP1, and other chromatin modification and repair proteins. Recruitment of 53BP1, which serves as a convenient marker for IRIFs, is dependent on two cellular RING finger ubiquitin ligases, RNF8 and RNF168. HSV-1 activates and interacts with the DNA damage response (Lilley et al., 2005; Mohri et al., 2011; Weller, 2010; Wilkinson & Weller, 2005, 2006), but the formation of IRIFs in HSV-1-infected cells is inhibited by ICP0 because it induces the degradation of both RNF8 and RNF168 (Lilley et al., 2010). ICP0-null mutant HSV-1 replicates more efficiently in cells lacking RNF8 and RNF168, implying that they are involved in a repressive response to HSV-1 DNA (Lilley et al., 2010, 2011). Furthermore, IRIF-like foci assemble in regions adjacent to HSV-1 genomes during the early stages of ICP0-null mutant, but not wt, HSV-1 infection (Lilley et al., 2011). H2AX and Mdci1 were shown to accumulate at these foci in the presence of ICP0, but proteins downstream of RNF8/ RNF168 did not (Lilley et al., 2011). The recruitment of DDR and ND10 proteins to regions close to the viral genomes are analogous in some respects, particularly with respect to the inhibitory effects of ICP0, but the former is not dependent on PML or other ND10 proteins, and the IRIF-like foci are spatially distinct from the corresponding ND10-like foci (Lilley et al., 2011).

Other aspects of ICP0

This section discusses briefly some other recent developments in the ICP0 field not considered above.

Interaction with the cellular E3 ubiquitin ligase SIAH-1

SIAH-1 is a cellular ubiquitin ligase that has many identified potential substrates, one of which is PML. A copy of a SIAH-1-binding motif (PXAXVXPXXR) occurs at residues 401–410 of ICP0 (Nagel et al., 2011). SIAH-1 co-immunoprecipitates with ICP0 and also co-localizes with ICP0 in infected cells. Deletion of the interaction region reduced the ability of plasmid expressed ICP0 to stimulate plaque formation of cotransfected ICP0-null mutant viral DNA (Nagel et al., 2011). This study raises the interesting possibility of viral and cellular E3 ubiquitin ligases acting in concert to regulate each other and their biological functions.

Interaction with transcription factor E2FBP1

Transcription factor E2FBP1 (ARID3a) enhances E2F1-mediated transcriptional activation, and also disperses ND10 by promoting the loss of SUMO-modified PML (Fukuyo et al., 2004). ICP0 and E2FBP1 interact when highly expressed in transfected cells, and E2FBP1 can be ubiquitinated by ICP0 in a RING finger-dependent manner (Fukuyo et al., 2011). High level expression of E2FBP1 caused decreased levels of ICP0 expression during HSV-1 infection, and therefore it was proposed that E2FBP1 has restrictive effects on viral replication that are countered by ICP0 (Fukuyo et al., 2011).

ICP0 in the tegument

Ever since ICP0 was identified as a component of the HSV-1 tegument (Yao & Courtney, 1992), there has been an intriguing question whether delivery of ICP0 into the cell via the particle itself could have a biological impact. Packaging of ICP0 requires its RING finger region and is dependent on VP22 (Delboy et al., 2010; Elliott et al., 2005; Maringer & Elliott, 2010; Potel & Elliott, 2005). A recent proposal is that virion-associated ICP0 is required for efficient delivery of the viral capsid to the nuclear periphery (Delboy & Nicola, 2011). Unequivocal confirmation of a role for virion ICP0 requires an experimental system in which normal levels of ICP0 can be incorporated into genotypically ICP0-null mutant virus particles. This is a technically challenging but worthwhile objective.

ICP0 and inhibition of innate immunity signalling pathways

ICP0-null mutant HSV-1 is sensitive to interferon (IFN) pre-treatment in cultured cells and is highly inhibited by the IFN system in vivo. ICP0 impedes IRF-3 signalling in some experimental systems, thereby counteracting the induction of IFN-stimulated gene expression that occurs in cells infected with ICP0-null mutant or replication defective HSV-1 mutants (reviewed by Sobol & Mossman, 2011). The ICP0 orthologues expressed by VZV and BHV-1 have also been shown to inhibit IRF-3-dependent signalling by inducing the degradation of IRF-3 and STAT-1 (Saira et al., 2007; Zhu et al., 2011). Depletion of STAT-1 or IRF-3 does not, however, increase the replication efficiency of an ICP0-null mutant HSV-1 in human fibroblasts (Everett et al., 2008b). Consequently, IRF-3 or STAT-1-dependent signalling is unlikely to contribute to the replication defect observed by an ICP0-null mutant HSV-1 in cell culture (Everett et al., 2008b). Furthermore, ICP0 expressed in an inducible cell line system is unable to counteract STAT-1- or IRF-3-dependent signalling pathways (Everett & Orr, 2009). A plausible explanation for the apparent conflict between these studies is that ICP0 may be unable to block IRF-3-dependent signalling when restricted to the nucleus (Paladino et al., 2010). However, a recent study made the intriguing observation that ICP0 induces the degradation of the nuclear DNA sensor IFI16, a protein implicated in IRF-3 activation following viral DNA entry into the nucleus (Orzalli et al., 2012). This exciting result promises further developments in the understanding of the interplay between ICP0 and innate immunity pathways.

ICP0 and VICP0 have also been linked to regulation of the NF-κB pathway, albeit in different ways. ICP0 impedes NF-κB...
signalling by promoting the degradation of the Toll-like receptor TLR2 (van Lint et al., 2010), and it also blocks the TLR pathway and NF-κB activation by a variety of stimuli (Daubeuf et al., 2009). VICP0 impedes NF-κB binding and signalling in dendritic cells in a RING finger-dependent manner at a stage downstream of IkBz phosphorylation but upstream of nuclear import of the NF-κB subunits p50 and p65 (Sloan et al., 2012). Taken together, these studies indicate that in addition to its nuclear functions, ICP0 family members also have cytoplasmic functions that impede cellular responses to virus infection. Whilst there is mounting evidence implicating a role for ICP0 in the inhibition of innate immunity signalling, further studies are required in order to determine whether these proteins are directly targeted for ubiquitination by ICP0.

Concluding remarks
Studies over the past three decades have shown that ICP0 directly influences, and in some cases inhibits, many fundamental cellular processes, including DNA repair, transcription, the cell cycle, and innate and intrinsic antiviral immunity. Recent work has begun to define the biochemical mechanisms by which these functions are achieved. As the number of pathways known to be influenced by ICP0 grows, it has become increasingly important to understand their relative contributions to the regulation of lytic infection and viral reactivation.

ICP0 uses multiple mechanisms to target a broad range of cellular substrates for degradation. Due to the variety of mechanisms employed, it is plausible that some of the cellular proteins degraded by ICP0 have little or no functional influence on the outcome of HSV-1 infection. The challenge, therefore, is to distinguish the substrates that are functionally relevant to the biology of HSV-1 from those that are innocent bystanders. This is particularly pertinent in light of the STUB1-like activity of ICP0 that leads to the degradation of numerous SUMO-modified proteins during infection. Whilst there is clear evidence that certain SUMO-modified ND10 proteins contribute to the repression of ICP0-null mutant replication, and that the SUMO modification pathway plays a role in mediating intrinsic immunity, it is likely that many of the SUMO-modified proteins that are degraded have little direct impact on the biology of HSV-1. It is of interest, however, that several viruses, including avian adenovirus (Boggio et al., 2004) and human papilloma virus (Heaton et al., 2011), also express proteins that interfere with SUMO modification. As SUMO conjugation regulates aspects of innate and intrinsic immunity, and also transcription (reviewed by Hay, 2005; Wimmer et al., 2012), one can speculate that SUMO modification could influence the antiviral activity of a number of cellular proteins. An understanding of the biochemical activity and substrate specificity of viral ubiquitin ligases like ICP0, or viral proteins that co-opt cellular ubiquitin ligase complexes during infection, may enable the development of novel avenues of therapeutic intervention.

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