Keep it in the subfamily: the conserved alphaherpesvirus US3 protein kinase

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The US3 protein kinase is conserved over the alphaherpesvirus subfamily. Increasing evidence shows that, although the kinase is generally not required for virus replication in cell culture, it plays a pivotal and in some cases an essential role in virus virulence in vivo. The US3 protein is a multifunctional serine/threonine kinase that is involved in viral gene expression, virion morphogenesis, remodelling the actin cytoskeleton and the evasion of several antiviral host responses. In the current review, both the well conserved and virus-specific functions of alphaherpesvirus US3 protein kinase orthologues will be discussed.

Introduction

Alphaherpesviruses represent the largest subfamily of the herpesviruses. The natural hosts of alphaherpesviruses vary from birds to humans. Like other herpesviruses, alphaherpesviruses establish lifelong latent infections in their natural host. Reactivation from latency upon specific, often stress- and/or immune suppression-related stimuli, may lead to recurrent disease symptoms. Three alphaherpesviruses have been described in humans: herpes simplex virus type 1 (HSV-1), HSV-2 and varicella-zoster virus (VZV). HSV-1 and HSV-2 are primarily associated with cold sores and genital lesions, but may rarely also cause devastating disease symptoms, including keratitis, blindness and encephalitis. VZV is the causative agent of chickenpox and shingles. A fourth alphaherpesvirus, herpes B virus, causes life threatening encephalitis in man, but its natural host is the macaque monkey. The alphaherpesviruses also comprise several important animal alphaherpesviruses. Pseudorabies virus (PRV) causes encephalitis, respiratory symptoms and abortions in swine. Bovine herpesvirus type 1 (BoHV-1) is the causative agent of infectious bovine rhinotracheitis in cattle and may also cause abortions. In horses, symptoms associated with equine herpesvirus type 1 (EHV-1) may vary from respiratory problems to paralysis and abortion. Marek’s disease virus (MDV) causes lethal infections in chickens, and, quite atypical for alphaherpesviruses, is a cancer-causing virus.

The US3 protein kinase is encoded by every alphaherpesvirus identified thus far, indicating that it is vital for viral fitness, although it is often not required for growth of the virus in cell culture (Frame et al., 1987; Hanks et al., 1988; Heineman et al., 1996; McGeoch & Davison, 1986; Purves et al., 1987). The US3 designation refers to the position of the gene in the prototypical herpes simplex virus genome, where it constitutes the third gene in the unique short segment. For most alphaherpesviruses, US3 orthologues are also referred to as US3, except for VZV, where ORF66 represents the US3 orthologue.

All alphaherpesvirus US3 orthologues have a kinase domain of 280–300 aa containing the ATP-binding domain and the catalytic active site (Fig. 1). The invariant lysine (K) in the former is critical for ATP binding and the invariant aspartic acid (D) in the latter is critical for catalytic activity. These critical invariant residues are often targets for mutagenesis in the construction of recombinant viruses and expression vectors encoding a kinase-inactive US3 mutant protein (Coller & Smith, 2008; Deruelle et al., 2007; Finnen et al., 2010; Kinchington et al., 2000; Ryckman & Roller, 2004). Importantly, some kinase-inactive US3 mutants may show an altered localization compared with the wild-type (WT) US3 protein (Coller et al., 2010). In such cases, it is crucial to investigate whether observed differences in activity between a WT and a kinase-inactive US3 protein are indeed due to the lack of kinase activity, rather than an altered subcellular localization. Amino acid sequence similarity between the US3 orthologues of different alphaherpesviruses is variable, and the similarity between the kinase domains is significantly higher compared with the complete US3 sequences (Table 1).

In contrast to other alphaherpesviruses, the US3 genes of PRV and HSV-1 both contain two transcriptional start sites and consequently encode two isoforms (Fig. 1). For HSV, the second start codon results in the formation of a minor truncated protein, designated US3.5 (405 aa, 50 kDa), which accumulates in cells infected with an ICP22null virus (Poon & Roizman, 2005). This truncated protein still retains kinase activity, but with altered functionality compared with US3 (481 aa, 70 kDa). Both US3 and US3.5 are able to mediate a post-translational modification of the histone deacetylases HDAC1 and HDAC2, but US3.5 lacks the anti-apoptotic activity of US3 and appears to be
less capable of mediating transport of progeny capsids across the nuclear envelope (Poon & Roizman, 2005; Poon et al., 2006b). HSV-1 US3 and US3.5 both cofractionate with mitochondria (Poon et al., 2006b). In PRV-infected cells, the presence of two start codons in the US3 gene leads to the expression of a minor long isoform (390 aa, 53 kDa), representing less than 5% of the total US3 protein in infected cells and a major short isoform (336 aa, 41 kDa) (van Zijl et al., 1990). Although both isoforms are expressed in infected cells, only the short isoform is incorporated into PRV virions (Klupp et al., 2001; Lyman et al., 2003; Zhang et al., 1990). The long PRV US3 isoform differs from the short one by the presence of an additional 54 aa N-terminal mitochondrial localization signal (Calton et al., 2004; Van Minnebruggen et al., 2003). This results in a drastically altered localization pattern of both isoforms: the long isoform is predominantly located in the mitochondria, the plasma membrane and the cytoplasm, whereas the short isoform is mainly located in the nucleus. These different subcellular localizations may correlate with different functions of US3 (Calton et al., 2004; Geenen et al., 2005).

Recombinant PRV or HSV viruses that either lack US3 or express a kinase-inactive US3 display only slightly reduced growth characteristics in most cell cultures but a strongly impaired virulence in pigs (PRV) and mice (HSV) (Coller & Smith, 2008; Inagaki-Ohara et al., 2001; Kimman et al., 1994; Meignier et al., 1988; Nishiyama et al., 1992; Purves et al., 1987; Reynolds et al., 2002; Ryckman & Roller, 2004; Sagou et al., 2009; Van den Broeke et al., 2009a). A recombinant VZV encoding a kinase-inactive ORF66 showed only a marginally reduced growth in a fibroblast cell line, but a severe growth and replication defect in primary human corneal stromal fibroblasts (Erazo et al., 2008). In addition, eliminating ORF66 expression or inactivating the kinase activity of ORF66 in VZV results in a substantial defect in human T-cell tropism, both in a SCID-hu mouse model of infection and in primary human T-lymphocytes (Moffat et al., 1998; Schaap-Nutt et al., 2006; Schaap et al., 2005; Soong et al., 2000).

Substrates

In vitro biochemical studies characterized a PRV US3 protein kinase minimal consensus phosphorylation sequence as \((R)_nX-(S/T)-Z-Z\), where \(n \geq 2\), \(S/T\) is the target site where either serine or threonine is phosphorylated, \(X\) can be absent or any amino acid, but preferably arginine, alanine, valine, proline or serine, and \(Z\) can be any amino acid except proline or an acidic residue. The optimal consensus sequence is similar except that \(X\) is not absent and \(n \geq 3\) (Leader, 1993; Leader et al., 1991; Purves et al., 1986). The phosphorylation target site specificity of HSV-1 and other alphaherpesvirus US3 kinases is similar to that of PRV US3 and protein kinase A (PKA), a cellular cyclic adenosine monophosphate (cAMP)-dependent protein kinase (Benetti & Roizman, 2004; Daikoku et al., 1993;
Alphaherpesvirus US3 protein kinase orthologues have been confirmed as substrates for PRV US3 (Van den Broeke et al., 2005; Grassi et al., 2003; Gwack et al., 2001; Hobbs & De Luca, 1999; Lu et al., 2003; Merezak et al., 2002; Poon et al., 2003). Phosphorylation of HDACs affects their enzymatic activity, cellular localization and protein–protein interactions (Gallinari et al., 2007). The US3 orthologues of HSV-1, HSV-2, PRV and VZV have all been reported to hyperphosphorylate HDACs. The Roizman laboratory first reported an HSV-1 US3-mediated post-translational modification of HDAC1 and HDAC2 in 2003. Infection and transduction assays showed that expression of US3 resulted in two HDAC1 and HDAC2 bands with slightly different electrophoretic mobility in Western blot. This post-translational modification correlated with a clearly reduced expression level of HDAC1, but not HDAC2 (Poon et al., 2003). Poon et al. (2006a) demonstrated that the kinase activity of US3 is required for the post-translational modification of HDAC1, suggesting that this post-translational modification represents phosphorylation. In addition, the kinase activity of US3 resulted in enhanced expression of transduced reporter genes (Poon et al., 2006a). Recent studies on VZV, PRV and HSV-1 indicate that hyperphosphorylation of HDACs is a conserved feature of US3 orthologues (Walters et al., 2009, 2010). Expression of either of these US3 orthologues results in hyperphosphorylation of HDAC1 and HDAC2, even in the absence of other viral proteins (Walters et al., 2009, 2010). Since ORF66 was not able to phosphorylate the kinase activity of US3 in infected cells, it appears that the observed ORF66-induced HDAC1 and HDAC2 phosphorylation occurs indirectly, probably via US3-mediated activation of a cellular kinase pathway (Walters et al., 2009). Indirect HDAC phosphorylation has also been suggested for HSV-1 and PRV US3 (Walters et al., 2010). HSV-1, VZV and PRV US3 all lead to phosphorylation of HDAC at a conserved serine residue in the C-terminal domain of the protein (Walters et al., 2009, 2010). Despite this similarity, some interesting differences have been observed as well. First, HSV-1 and VZV US3 orthologues are essential for virus-induced HDAC hyperphosphorylation, whereas this is not the case for PRV (Walters et al.,

### Functions

Alphaherpesvirus US3 protein kinase orthologues have been attributed multiple functions, which are reviewed below. A schematic overview is given in Fig. 2.

### Viral gene expression

Histone deacetylases (HDACs) deacetylate lysine residues on histone tails, resulting in chromatin condensation and repressed gene expression (Grozinger & Schreiber, 2002). Several reports have shown that herpesvirus gene expression depends on inhibition of HDACs (Danaher et al., 2005; Grassi et al., 2003; Gwack et al., 2001; Hobbs & De Luca, 1999; Lu et al., 2003; Merezak et al., 2002; Poon et al., 2003). Phosphorylation of HDACs affects their enzymatic activity, cellular localization and protein–protein interactions (Gallinari et al., 2007). The US3 orthologues of HSV-1, HSV-2, PRV and VZV have all been reported to hyperphosphorylate HDACs. The Roizman laboratory first reported an HSV-1 US3-mediated post-translational modification of HDAC1 and HDAC2 in 2003. Infection and transduction assays showed that expression of US3 resulted in two HDAC1 and HDAC2 bands with slightly different electrophoretic mobility in Western blot. This post-translational modification correlated with a clearly reduced expression level of HDAC1, but not HDAC2 (Poon et al., 2003). Poon et al. (2006a) demonstrated that the kinase activity of US3 is required for the post-translational modification of HDAC1, suggesting that this post-translational modification represents phosphorylation. In addition, the kinase activity of US3 resulted in enhanced expression of transduced reporter genes (Poon et al., 2006a). Recent studies on VZV, PRV and HSV-1 indicate that hyperphosphorylation of HDACs is a conserved feature of US3 orthologues (Walters et al., 2009, 2010). Expression of either of these US3 orthologues results in hyperphosphorylation of HDAC1 and HDAC2, even in the absence of other viral proteins (Walters et al., 2009, 2010). Since ORF66 was not able to phosphorylate HDAC1 or HDAC2 in an in vitro kinase assay, it appears that the observed ORF66-induced HDAC1 and HDAC2 phosphorylation occurs indirectly, probably via US3-mediated activation of a cellular kinase pathway (Walters et al., 2009). Indirect HDAC phosphorylation has also been suggested for HSV-1 and PRV US3 (Walters et al., 2010). HSV-1, VZV and PRV US3 all lead to phosphorylation of HDAC at a conserved serine residue in the C-terminal domain of the protein (Walters et al., 2009, 2010). Despite this similarity, some interesting differences have been observed as well. First, HSV-1 and VZV US3 orthologues are essential for virus-induced HDAC hyperphosphorylation, whereas this is not the case for PRV (Walters et al.,

### Table 1. Percentages of amino acid identity of different alphaherpesvirus US3 orthologues, based on the amino acid sequences of both the complete US3 ORF and the kinase domain only (in parentheses)

<table>
<thead>
<tr>
<th></th>
<th>HSV-1</th>
<th>HSV-2</th>
<th>VZV</th>
<th>PRV</th>
<th>BoHV-1</th>
<th>BoHV-5</th>
<th>EHV-1</th>
<th>EHV-4</th>
<th>MDV</th>
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<td>HSV-1</td>
<td>100</td>
<td>74 (84)</td>
<td>37 (45)</td>
<td>33 (40)</td>
<td>29 (43)</td>
<td>31 (42)</td>
<td>34 (41)</td>
<td>36 (43)</td>
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<tr>
<td>HSV-2</td>
<td>74 (84)</td>
<td>100</td>
<td>35 (44)</td>
<td>32 (40)</td>
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<td>31 (40)</td>
<td>34 (40)</td>
<td>36 (42)</td>
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<tr>
<td>VZV</td>
<td>37 (45)</td>
<td>35 (44)</td>
<td>100</td>
<td>29 (40)</td>
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<td>33 (43)</td>
<td>36 (45)</td>
<td>36 (44)</td>
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<tr>
<td>PRV</td>
<td>33 (40)</td>
<td>32 (40)</td>
<td>100</td>
<td>36 (45)</td>
<td>39 (48)</td>
<td>41 (48)</td>
<td>38 (46)</td>
<td>39 (48)</td>
<td>27 (37)</td>
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<tr>
<td>BoHV-1</td>
<td>29 (43)</td>
<td>30 (40)</td>
<td>41 (49)</td>
<td>33 (43)</td>
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<td>81 (89)</td>
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<td>29 (37)</td>
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<tr>
<td>BoHV-5</td>
<td>31 (42)</td>
<td>31 (40)</td>
<td>100</td>
<td>33 (43)</td>
<td>40 (48)</td>
<td>81 (89)</td>
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<td>40 (48)</td>
<td>39 (48)</td>
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<tr>
<td>MDV</td>
<td>31 (41)</td>
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<td>27 (37)</td>
<td>86 (91)</td>
<td>86 (91)</td>
<td>86 (91)</td>
<td>29 (37)</td>
</tr>
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Second, although HDAC inhibition increased plaquing efficiency of US3null PRV and VZV, this was not the case for HSV-1, suggesting virus-specific dependencies on HDAC hyperphosphorylation for efficient virus replication (Walters et al., 2010).

Further in support of a role for US3 in viral gene expression, recently, using a proteomic comparison of cells infected with WT PRV versus US3null PRV, US3 was found to affect expression levels of at least three viral proteins. US3 was associated with increased levels of the major DNA-binding protein UL29 and the large subunit of the ribonucleoside-diphosphate reductase UL39, but reduced levels of the DNA polymerase processivity factor UL42, suggesting a possible role for US3 in the viral DNA metabolism (Skiba et al., 2010).

Virion morphogenesis
Herpesvirus capsids are assembled in the nucleus. In order to reach the cytoplasm, where they obtain their final envelope at vesicles derived from the trans-Golgi network, they must cross the nuclear envelope. Herpesviruses appear to have evolved a unique pathway to accomplish this. First, capsids associate with the inner nuclear membrane, which probably requires local disruption of the lamin network. Second, they bud into the inner nuclear membrane, resulting in the formation of a primary enveloped particle in the perinuclear space. Finally, the envelope of the primary virion fuses with the outer nuclear membrane, releasing a naked capsid in the cytoplasm (Mettenleiter et al., 2009). US3 appears to fulfill several functions during this process, although neither of these may be absolutely essential, since alphaherpesvirus virion formation and egress do occur in the absence of US3, albeit with reduced efficiency. It is possible that cellular kinases may be able to compensate to some extent for the loss of US3 during virus nuclear egress.

Two conserved viral proteins are crucial for budding of capsids in the inner nuclear membrane: UL31 and UL34. UL34 is a type II membrane protein that interacts with UL31 to form a functional complex. Although not absolutely required for primary envelopment, US3 is necessary for the proper, homogeneous distribution of the UL31/UL34 complex along the nuclear membrane in both HSV-1- and PRV-infected cells. Indeed, UL31 and UL34 interact in discrete foci along the nuclear membrane in the absence of US3 (Kato et al., 2006; Klupp et al., 2001; Reynolds et al., 2001; Ryckman & Roller, 2004). This US3-mediated homogeneous distribution is possibly mediated by the UL13-induced phosphorylation of US3 since HSV-1 mutants with a deletion in either UL13 or US3 resulted in a similar phenotype concerning the localization of the

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**Fig. 2.** Overview of the different functions attributed to alphaherpesvirus US3 orthologues.
UL31/UL34 complex in the nuclear membrane (Kato et al., 2006). HSV-1 US3-mediated phosphorylation of both UL31 and UL34 has been demonstrated (Kato et al., 2005; Mou et al., 2009; Purves et al., 1991), but US3-mediated phosphorylation of UL31, rather than UL34, appears to regulate the localization of the nuclear envelopment complex (Mou et al., 2009; Ryckman & Roller, 2004). In contrast to HSV-1, PRV US3 does not phosphorylate UL34 in infected cells (Klupp et al., 2001). Although UL31 and UL34 are conserved in all herpesviruses (Mettenleiter, 2002), no role for ORF66 in the nuclear egress of primary VZV virions has been observed (Schaap et al., 2005).

In addition to regulating UL31 and UL34 localization during primary envelopment, expression of HSV-1 US3 at the inner nuclear membrane also leads to phosphorylation of lamina A/C, a constituent of the nuclear lamina, and emerin, an inner nuclear membrane protein interacting with a number of nuclear components, including lamins. US3-mediated phosphorylation of these proteins alters their localization and thus the architecture of the nuclear lamina. This probably facilitates access of nucleocapsids to the site of primary envelopment, i.e. the inner nuclear membrane (Leach et al., 2007; Morris et al., 2007; Mou et al., 2007).

The US3 protein kinase of HSV-1, PRV and MDV, but not of VZV, is also involved in de-envelopment of primary enveloped virions via fusion of the primary envelope with the leaflet of the outer nuclear membrane. Infection of cells with an HSV-1, PRV or MDV virus mutant lacking US3 leads to accumulations of primary enveloped virus particles in large invaginations of the inner nuclear membrane (Klupp et al., 2001; Reynolds et al., 2002; Ryckman & Roller, 2004; Schaap et al., 2005; Schumacher et al., 2005; Wagenaar et al., 1995). For HSV-1 and MDV, it has been shown that the US3 kinase activity is crucial for efficient virion de-envelopment since a kinase-dead US3 virus yielded a similar phenotype as observed for a US3 deletion mutant (Ryckman & Roller, 2004; Schumacher et al., 2008). Recently, it has been demonstrated for HSV-1 that US3-mediated phosphorylation of UL31 regulates this de-envelopment process (Mou et al., 2009). Alternatively, also for HSV-1, US3-mediated phosphorylation of the cytoplasmic domain of viral envelope protein gB on Thr887 has been implicated in gB-mediated fusion between the primary virion envelope and the outer nuclear membrane during nuclear egress (Farnsworth et al., 2007; Wisner et al., 2009).

Another role for US3 in correct assembly of new virions has been attributed to the VZV US3 orthologue ORF66. ORF66 directly phosphorylates and thereby regulates the subcellular localization of IE62, a major transcriptional regulatory protein and major tegument component. IE62 is located in the nucleus at early time points post-inoculation but becomes predominantly cytoplasmic at later time points due to ORF66-mediated phosphorylation of IE62 at Ser686. Cytoplasmic localization of IE62 is necessary for its incorporation into the tegument of progeny virions (Eisfeld et al., 2006; Kinchington et al., 2000, 2001).

**Cytoskeletal rearrangements**

For several alphaherpesviruses, the US3 protein has been described to induce drastic changes in the cytoskeleton of the host cell, in particular cell rounding due to stress fibre breakdown and often the formation of cellular protrusions. US3-mediated stress fibre disassembly has been reported for PRV, HSV-2, MDV and BoHV-1 (Brzozowska et al., 2010; Murata et al., 2000; Schumacher et al., 2005; Van Minnebrugge et al., 2003). US3-mediated cellular protrusions have been described for PRV, HSV-2 and BoHV-1 (Brzozowska et al., 2010; Calton et al., 2004; Favoreel et al., 2005; Finnen et al., 2010). These often heavily branched cell projections contain both actin and microtubules and reach several cell diameters of length. They establish intimate contacts with distant cells and are associated with enhanced virus spread. PRV particles have been reported to migrate in these projections with a net movement in the direction of the tip of the protrusion and were found to be able to enter the contacted distant cells, even in the presence of virus neutralizing antibodies (Favoreel et al., 2005). For PRV, HSV-2, BoHV-1, but not for MDV, the kinase activity of US3 is required for modulation of the actin cytoskeleton (Brzozowska et al., 2010; Finnen et al., 2010; Murata et al., 2000; Schumacher et al., 2008; Van den Broeke et al., 2009a). This points to differences or multiple effects of US3 on actin-regulating processes in the cell, especially since MDV US3, which does not require kinase activity for actin rearrangements, has been reported to induce stress fibre disassembly without mention of the formation of cell projections (Schumacher et al., 2005, 2008). Recently, for PRV, group A p21-activated kinases (PAKs) were found to mediate the US3-induced reorganization of the actin cytoskeleton. Group A PAKs are central regulators in the actin-controlling Rho GTPase signalling pathways. The PAK2 isoform appears crucial in US3-mediated stress fibre disassembly, whereas the PAK1 isoform is mainly involved in the US3-mediated protrusion formation (Van den Broeke et al., 2009b).

**Evasion of the antiviral response**

Herpesviruses achieve lifelong persistence in their hosts by establishing latency from which the virus can reactivate upon specific stimuli (Loch & Tampe, 2005; Pomeranz et al., 2005). In order to be able to spread during reactivation periods, herpesviruses have evolved diverse strategies to limit their recognition and subsequent elimination by the host’s antiviral responses. US3 appears to be involved in several of these evasion mechanisms.

**Anti-apoptotic properties.** Apoptosis of virus-infected cells may occur either as a direct response to viral infection or upon recognition of infected cells by immune effectors like cytotoxic T-lymphocytes (CTLs) or...
natural killer (NK) cells. Apoptosis of virus-infected cells early in the replication cycle is an intrinsic antiviral host defence mechanism eliminating virus-infected cells and thus blocking further virus spread (Koyama et al., 2000). As a consequence, several viruses, including herpesviruses, have developed strategies to interfere with apoptosis.

The US3 protein orthologues of several alphaherpesviruses have been shown to prevent apoptosis induced by infection and a variety of exogenous stimuli. The anti-apoptotic properties of US3 were first demonstrated for HSV-1, and ever since most research concerning the anti-apoptotic character of US3 has been done on the HSV-1 US3 orthologue. Leopardi et al. (1997) showed that US3 is required to protect cells from apoptosis induced by infection, since higher levels of apoptotic cells were observed with a US3-deficient HSV-1 compared with WT HSV-1 (Leopardi et al., 1997). These data were later extended by Jerome et al. (1999), who found that HSV-1 US3 efficiently protects infected cells from UV-induced apoptosis and partially protects infected cells from Fas- induced apoptosis (Jerome et al., 1999). Several possible mechanisms of HSV-1 US3-mediated protection from apoptosis have been reported. Ogg et al. (2004) demonstrated that transfection of US3-encoding plasmids protected cells from apoptosis triggered by the overexpression of pro-apoptotic proteins of the Bcl-2 family, and that this depended on the kinase activity of US3 (Ogg et al., 2004). Hence, since a kinase-intact US3 protein is able to protect cells from apoptosis in the absence of other viral proteins, US3 phosphorylates a cellular substrate(s) which either directly or indirectly prevents apoptosis. In this context, US3-mediated inhibition of Fas- or sorbitol-induced apoptosis was found to be associated with US3-mediated phosphorylation of Bad, thereby inactivating this critical component of the mitochondrial apoptotic pathway (Cartier et al., 2003a). Phosphorylation of this pro-apoptotic Bcl-2 family member at any of three regulatory serine residues (Ser112, Ser136 and Ser155) has previously been associated with the inactivation of this pro-apoptotic protein (Zha et al., 1996; Zhou et al., 2000). Homozygous mutant BadSS/A mice in which the three regulatory serine residues were changed to alanine are viable without gross abnormalities, but are hypersensitive to apoptosis induced by Fas and γ-irradiation, demonstrating the physiological significance of Bad phosphorylation at these residues for cell survival (Datta et al., 2002). However, HSV-1 US3 also blocks the pro-apoptotic activity of BadSS/A, suggesting that Bad phosphorylation at these regulatory sites is not an absolute prerequisite for the anti-apoptotic function of the HSV-1 US3 protein kinase (Benetti et al., 2003; Ogg et al., 2004). It may be worthwhile to examine whether US3 is able to phosphorylate BadSS/A.

It has also been shown that HSV-1 US3 mediates a post-translational modification of full-length Bad and thereby blocks caspase-mediated cleavage and concomitant activation of Bad (Benetti et al., 2003; Munger & Roizman, 2001). US3 may also inhibit apoptosis downstream of Bad since HSV-1 US3 is able to block apoptosis induced by overexpression of Bax, a factor downstream of Bad (Ogg et al., 2004). This may be explained by the ability of HSV-1 US3 to block proteolytic cleavage of procaspase-3 to active caspase-3, possibly by a US3-mediated phosphorylation of procaspase-3 (Benetti & Roizman, 2007).

In addition, HSV-1 US3 blocks CTL-mediated cell lysis by preventing cleavage of Bid by granzyme B. Since recombinant Bid is a US3 substrate in vitro, it is likely that US3- mediated phosphorylation of Bid blocks its processing by granzyme B (Cartier et al., 2003b).

Hence, it appears that HSV-1 US3 is able to protect cells from apoptosis induced by several stimuli in the absence of other viral proteins and that multiple mechanisms coexist.

The anti-apoptotic features of US3 have been confirmed to varying extent for different other alphaherpesviruses. For HSV-2, it has been shown that US3 renders cells less susceptible towards apoptosis induced by infection (Asano et al., 1999, 2000) and that US3 is both necessary and sufficient to block apoptosis induced by sorbitol (Hata et al., 1999; Murata et al., 2002). For VZV, the US3 orthologue ORF66 reduces the susceptibility of infected T-cells to virus-induced apoptosis (Schaap et al., 2005). PRV US3 renders cells less susceptible towards apoptosis induced by PRV infection (Geenen et al., 2005). In addition, PRV US3 also protects cells from apoptosis induced by several exogenous apoptotic stimuli such as sorbitol, staurosporine and overexpression of pro-apoptotic proteins of the Bcl-2 family (Geenen et al., 2005; Ogg et al., 2004). In line with data obtained for HSV-1, the kinase activity of the PRV US3 protein is required to protect cells towards infection- and staurosporine-induced apoptosis, and PRV US3 exerts its anti-apoptotic effects at least partly by mediating phosphorylation of the pro-apoptotic protein Bad (Deruelle et al., 2007). These data indicate that phosphorylation, and thereby inactivation, of Bad may be a conserved and potentially important aspect of the anti-apoptotic activity of US3 orthologues. Like its HSV-1 orthologue, PRV US3 is able to block apoptosis induced by overexpression of Bax, suggesting that PRV US3 can also block apoptosis downstream of Bad (Ogg et al., 2004). The US3 protein of MDV also possesses anti-apoptotic properties since transfection of US3 null MDV US3 has been shown to limit staurosporine-induced apoptosis and this was, like for HSV-1 and PRV, dependent on the kinase activity of US3 (Schumacher et al., 2008).

Although the anti-apoptotic properties of US3 have been clearly demonstrated for HSV-1, HSV-2, VZV, PRV and MDV, there is some controversy as to whether the US3 protein of BoHV-1 is anti-apoptotic. Like wild-type BoHV-1, US3null BoHV-1 was found to protect cells from sorbitol-induced apoptosis, which led to the suggestion that BoHV-1 US3 is not anti-apoptotic (Takashima et al., 1999). However, in the absence of US3, the time interval between infection and sorbitol treatment needed to be
larger to inhibit apoptosis (Takashima et al., 1999). One explanation for this observation may be the slower growth kinetics of US3null BoHV-1, which may delay the accumulation of anti-apoptotic proteins. Alternatively, this result may point to anti-apoptotic properties of US3. There is some additional, albeit indirect, support for the latter hypothesis. RK-13 cells infected with a recombinant BoHV-1 strain with a deletion/insertion in the gG(US4) gene show increased levels of apoptosis and reduced virus spread compared to cells infected with wild-type BoHV-1 (Nakamichi et al., 2001). This result may be an indirect indication that BoHV-1 US3 contains anti-apoptotic activity. Indeed, in PRV, it has been shown that deletion/insertions in the gG(US4) gene result in reduced expression of the upstream located US3 (Demmin et al., 2001). For PRV, like for BoHV-1, this resulted in reduced spread, which was not caused by the lack of gG but by the reduced levels of US3 (Demmin et al., 2001). In addition, further in line with a potential anti-apoptotic function of BoHV-1 US3, we recently found that the US3 protein of the closely related pathogen BoHV-5 protects cells from staurosporine-induced apoptosis (unpublished observations).

The prevention of apoptosis during early stages of infection is generally thought to enable the virus to replicate longer and hence result in a higher yield of progeny virus. In this context, the anti-apoptotic properties of the PRV, HSV and MDV US3 orthologues may correlate with the fact that a US3null or a kinase-dead US3 PRV, HSV or MDV reach lower end-point titres than the WT virus in several, but not all, cell types (Coller & Smith, 2008; Demmin et al., 2001; Deruelle et al., 2010; Kimman et al., 1994; Reynolds et al., 2002; Ryckman & Roller, 2004; Schumacher et al., 2008; Van den Broeke et al., 2009a; Van Minnebruggen et al., 2003). Very recently, PRV US3-mediated inhibition of apoptosis was found not to correlate with increased infectious virus production and virus spread (Deruelle et al., 2010). Hence, the lower virus titres and reduced spread that are frequently associated with a US3null or a kinase-dead US3 PRV appear to depend on effects of US3 other than its anti-apoptotic activity. However, these data do not exclude a possible role for anti-apoptotic viral proteins in increasing virus titres and virus spread since additional, yet unidentified, anti-apoptotic viral proteins may function earlier during infection and at least partly compensate for the loss of US3 due to redundancy (Deruelle et al., 2010).

**Interference with the interferon (IFN) system.** The production of IFNs, mainly type I (e.g. α/β) and type II IFN (γ), is a central immune response towards viral infections. The antiviral effect generated by IFN is both direct, through the induction of an antiviral state in neighbouring cells and indirect, through activation of different immune cells. Hence, it is not surprising that many viruses, including herpesviruses, have evolved mechanisms to interfere with the antiviral effects of IFNs by affecting either IFN production, IFN-signalling through the JAK/STAT pathway, and/or antiviral functions of IFN-induced proteins.

Piroozmand et al. suggested a role for HSV-1 US3 in overcoming an IFN-α-induced antiviral state. Using low m.o.i. infection assays, they showed that in the absence of US3, HSV-1 titres were more dramatically reduced in IFN-α-treated cells compared with wild-type virus. However, this difference disappeared when high m.o.i. infection assays were performed (Piroozmand et al., 2004). Using low m.o.i. virus plaque assays in two different cell types, we found no difference in IFN-α sensitivity between wild-type and US3null PRV (M. J. Deruelle and H. W. Favoreel, unpublished data). This indicates that viral protein(s) other than US3 are involved in the observed PRV-mediated counteraction of type I IFN-induced antiviral effects (Brukman & Enquist, 2006). More research is needed to clarify the ability of different US3 orthologues to reduce sensitivity of the virus towards the antiviral effects of IFN-α.

There is evidence for interference of US3 with two other main types of IFN, IFN-β and IFN-γ. The VZV US3 kinase orthologue ORF66 reduces IFN-γ signalling in infected T-cells by decreasing the level of STAT1 phosphorylation (Schaap et al., 2005). HSV-1 US3 is also involved in blocking IFN-γ-dependent gene expression by phosphorylating the α-subunit of the IFN-γ receptor (Liang & Roizman, 2008). HSV-1 US3 has also been suggested to antagonize the HSV-1-induced TLR3-mediated response by inhibiting IRF3 activation through an unknown mechanism, leading to an impaired IFN-β synthesis (Peri et al., 2008).

**Interference with MHC I cell surface expression.** In order to limit or delay elimination of infected cells by CTLs, many herpesviruses reduce MHC I cell surface expression by interfering with the cellular MHC I antigen processing pathway, a key component of the adaptive antiviral immune response (reviewed by e.g. Lilley & Ploegh, 2005; Loch & Tampe, 2005).

For BoHV-1, EHV-1, EHV-4 and PRV, but not VZV, the UL49.5 protein is able to downregulate MHC I cell surface expression by interfering with the peptide transport through the transporter associated with antigen processing (TAP) (Koppers-Lalic et al., 2005, 2008; Eisfeld et al., 2007). For VZV and PRV, the US3 orthologues appear to have the ability to interfere with MHC I cell surface expression (Abendroth et al., 2001; Deruelle et al., 2009; Eisfeld et al., 2007). For VZV, the viral US3 orthologue ORF66 was reported to mediate downregulation of MHC I from the cell surface upon transfection or transduction of human cell lines and CD3+ T-lymphocytes (Abendroth et al., 2001; Eisfeld et al., 2007). The exact mechanism of this downregulation of cell surface MHC I remains largely unclear, but appears to either involve delayed transport of the MHC I:peptide complex from the ER to the trans-Golgi network or retention of MHC I in the Golgi apparatus (Abendroth et al., 2001; Eisfeld et al., 2007).
Additional, unknown and both US3- and UL49.5-independent MHC I downregulation was also observed (Eisfeld et al., 2007). In addition, the contribution of ORF66 in MHC I cell surface downregulation appears to be mild (Eisfeld et al., 2007). Combined with the data on PRV US3 that indicate that its ability to downregulate cell surface MHC I is absent in different cell types (Deruelle et al., 2009), more research will be needed to clarify the biological significance of the interaction of US3 with the MHC I antigen presentation pathway.

**Inactivation of CTLs.** Different immune cells with the ability to kill virus-infected cells, including NK cells, lymphokine-activated killer cells, and CTLs, have been reported to become functionally impaired after cell-to-cell transmission of HSV (York & Johnson, 1993; Sloan et al., 2003). For CTLs, Sloan et al. (2003) reported that deletion of US3 impairs the ability of the virus to transmit this inactivation signal. Subsequent studies showed that the HSV-mediated inactivation of CTLs is due to an interference with T-cell receptor signal transduction, leading to selective production of interleukin-10, a cytokine that suppresses cellular immunity and favours viral replication (Sloan & Jerome, 2007; Sloan et al., 2006). However, it is unclear whether US3 has a direct role in this process. Alternatively, the contribution of US3 may be indirect, and for example lie in its general ability to promote spread of the virus from one cell to another.

**Reduction of viral antigen expression at the cell surface.**

Recently, it has been demonstrated for HSV-1 that US3 specifically phosphorylates gB on position Thr887 in its cytoplasmic domain, thereby affecting nuclear egress, subcellular localization of gB, and pathogenesis of HSV-1 in mice (Imai et al., 2010; Kato et al., 2009; Wisner et al., 2009). Besides its effect on nuclear egress, HSV-1 US3-mediated phosphorylation of gB on Thr887 also results in a downregulation of gB expression from the cell surface of infected cells. This may serve as an immune evasion mechanism since gB is a potent inducer of both the humoral and cellular immune response and also because lysis of HSV-infected cells by NK cells correlates with the level of gB expression at the cell surface (Kato et al., 2009). Endocytosis of gB has also been observed in PRV-infected cells and has also been suggested there to constitute an immune evasion mechanism (Favoreel et al., 1999, 2002; Van Minnebruggen et al., 2004).

**Conservation of US3 functions**

Despite a relatively low level of amino acid similarity (Table 1), alphaherpesvirus US3 orthologues show substantial functional conservation. Some functions of US3 are better conserved than others. This may either indicate differences in substrate specificity of different US3 orthologues or may suggest that the putative cellular components involved in less conserved US3 phenotypes may show considerable variation among species, or a combination of both. Unfortunately, the mechanisms behind most of the functions of US3 are still poorly understood, making it impossible at this stage to thoroughly assess these possibilities.

The finding that both HSV-1 and PRV US3 mediate their anti-apoptotic activity at least partly by phosphorylating the pro-apoptotic protein Bad (Cartier et al., 2003a; Deruelle et al., 2007) suggests that this US3 activity may be conserved over different alphaherpesviruses. This may correlate with the observations that the protein sequence of Bad is well conserved among different species and that most of the US3 orthologues show anti-apoptotic properties (Asano et al., 1999, 2000; Benetti & Roizman, 2007; Cartier et al., 2003a; Geenen et al., 2005; Jerome et al., 1999; Leopardi et al., 1997; Munger et al., 2001; Murata et al., 2002; Ogg et al., 2004; Schaap et al., 2005; Schumacher et al., 2008).

For PRV, PAKs, central molecules in actin-regulating Rho GTPase signalling pathways, have recently been identified as critical US3 substrates for the US3-mediated actin rearrangements (Van den Broeke et al., 2009b). Similar to Bad, PAKs are conserved proteins (Hofmann et al., 2004), again possibly being one of the reasons why these actin cytoskeletal rearrangements are conserved among different alphaherpesviruses (Brzozowska et al., 2010; Calton et al., 2004; Favoreel et al., 2005; Finnen et al., 2010; Murata et al., 2000; Schumacher et al., 2005; Van Minnebruggen et al., 2003). On the other hand, conservation of the cellular substrate does not completely explain the potential conservation of US3 functions. Indeed, whereas the kinase-activity of US3 is required for US3-mediated actin cytoskeletal rearrangements induced by PRV, HSV-2 and BoHV-1, these effects appeared to be independent of the kinase activity of US3 for MDV, thereby pointing to another (or an additional) mode of action (Brzozowska et al., 2010; Finnen et al., 2010; Murata et al., 2000; Schumacher et al., 2008; Van den Broeke et al., 2009a).

**Multiple functions of US3**

Probably due to their restricted genome size, many viruses evolved multifunctional proteins. The US3 protein kinase is a typical example of this viral space-saving strategy. Due to its kinase activity and apparent flexibility in substrate recognition sites (Mou et al., 2007), US3 is associated with a plethora of functions. It is conceivable that due to its large array of potential functions, not all phenotypes associated with US3 are of equal importance for the virus. One challenge in US3 research will therefore be to try to separate key biological functions of US3 from less important functions and perhaps potential artefactual side effects. One strategy to try to accomplish this will be to search for specific substrate recognition sites in US3. Mutations in these putative sites may interfere with selected US3 functions, enabling investigation of their contribution to the reduced virulence of US3null viruses.

Alphaherpesviruses may benefit from regulating the different activities of US3 according to the needs of the
moment and place. It is therefore possible that the activity of specific functions is regulated in space and time. For example, as mentioned above, PRV US3 is expressed as two isoforms, a long and a short isoform. The long isoform has been suggested to be more protective towards apoptosis of infected cells during late stages of lytic infection than the short isoform (Geenen et al., 2005). This may be due to both localization and timing of expression of this isoform. Unlike the short isoform, the long isoform localizes to mitochondria, which may aid in its ability to phosphorylate and thereby inactivate the pro-apoptotic protein Bad (Van Minnebruggen et al., 2003; Calton et al., 2004; Deruelle et al., 2007). In addition, whereas the short US3 isoform is expressed with early kinetics, the long isoform is expressed with late kinetics (Deruelle et al., 2009), correlating with the timing when it is needed to protect cells from apoptosis. Further along these lines, it is very well possible that specific functions of US3 particularly manifest in cell types in which these are beneficial to the virus. This correlates with the observation that some US3-dependent phenotypes are highly cell type dependent (Deruelle et al., 2009). It will therefore be interesting to analyse the different functions of US3 in important primary target cells, such as neurons, white blood cells and epithelial cells, organ cultures, and in vivo systems.

The multifunctionality of US3 indicates that the protein may be involved in several stages of the virus replication cycle. One aspect of the virus replication cycle in which US3 may participate and that has not been investigated thoroughly yet, is viral entry. Since US3 is a tegument component, the kinase is delivered in the cytoplasm during virus entry. It is conceivable that US3 phosphorylates cellular substrates during this early stage of infection. Although the absolute amounts of US3 protein delivered during virus entry are likely to be very limited, it may be sufficient to trigger specific cellular processes that may influence the viral replication cycle. It will be interesting to explore this largely uninvestigated aspect of US3.

Conclusion

The conservation of US3 over the entire alphaherpesvirus subfamily, combined with the plethora of functions associated with US3, underscores the importance of this viral protein kinase for alphaherpesvirus biology. More research is needed to decipher the substrates and mechanisms involved in the different US3-mediated effects, to identify the relative importance of individual US3-associated functions with regard to the importance of the kinase for virulence in vivo, and to investigate a potential role of US3 during virus entry. Seen the relatively low level of sequence similarity of US3 orthologues, compared with the relatively high level of functional similarity, specific mutagenesis of the limited conserved regions in the US3 sequence that do not belong to the core catalytic domains may reveal essential substrate interaction domains that are crucial for specific US3 functions. Such information will allow us to interfere more specifically with selected US3 functions in order to unravel their relative importance in alphaherpesvirus biology. In addition, combined with drug screening approaches, this information may lead to new antiviral strategies. Indeed, the importance of US3 in alphaherpesvirus virulence and the fact that US3 does not appear to be genetically closely related to any of the known cellular kinases, may make it an attractive candidate target for the development of antiviral drugs.

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