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Insights into the function of tegument proteins from the varicella zoster virus

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Chickenpox (varicella) is caused by primary infection with varicella zoster virus (VZV), which can establish long-term latency in the host ganglion. Once reactivated, the virus can cause shingles (zoster) in the host. VZV has a typical herpesvirus virion structure consisting of an inner DNA core, a capsid, a tegument, and an outer envelope. The tegument is an amorphous layer enclosed between the nucleocapsid and the envelope, which contains a variety of proteins. However, the types and functions of VZV tegument proteins have not yet been completely determined. In this review, we describe the current knowledge on the multiple roles played by VZV tegument proteins during viral infection. Moreover, we discuss the VZV tegument protein-protein interactions and their impact on viral tissue tropism in SCID-hu mice. This will help us develop a better understanding of how the tegument proteins aid viral DNA replication, evasion of host immune response, and pathogenesis.

varicella zoster virus, VZV, tegument

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Varicella zoster virus (VZV), which belongs to the alphaherpesvirus subfamily of the *Herpesviridae* family, causes chickenpox (varicella) in children during primary infection and can establish long-term latency in the dorsal root ganglia (DRG) or cranial nerve sensory ganglia [1]. Subsequent reactivation of latent VZV may cause shingles (zoster) [1,2]. In addition, VZV infection may lead to serious complications such as encephalitis, meningitis, myelitis, conjunctivitis, and postherpetic neuralgia, which is most common. The latter condition is a painful, refractory disease that can seriously affect a patient's quality of life [3–6]. VZV virions, like all the other herpesviruses, are composed of a DNA core, a capsid, a tegument, and an envelope (Figure 1). The innermost layer of these virions is a nucleocapsid containing the VZV DNA genome and the outermost layer is an envelope that is derived from the host cell membranes and contains viral envelope glycoproteins. Between the nucleocapsid and envelope is an amorphous protein layer known as the tegument. Herpesvirus tegument proteins have been shown to play a number of key roles during viral infection. These functions include the intracellular transport of virus particles [7–16], viral assembly, and egress [17–30], regulation of viral and host cell gene transcription and protein expression [31–43], as well as viral immune evasion [41,44–47].

The VZV genome is a double-stranded DNA molecule, which is approximately 125 kb in length, containing at least 70 open reading frames (ORFs) [1,48]. Although VZV has the smallest genome in the herpesvirus family, research on VZV protein functions and molecular mechanisms of VZV pathogenesis has lagged behind comparable research on other herpesviruses such as the herpes simplex virus-1

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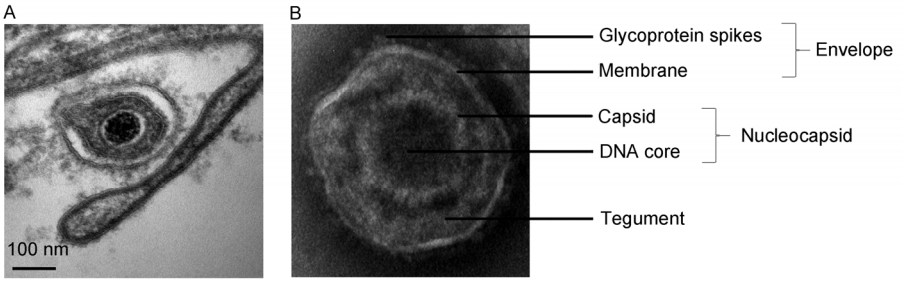

Figure 1 Electron micrograph of VZV. A, Extracellular VZV virion seen by TEM at a magnification of 30,000×. B, VZV virion structure.

(HSV-1) and the pseudorabies virus (PRV). One issue in analyzing the types and relative amounts of VZV tegument proteins is the strong cell-associated nature of VZV, which makes it very difficult to obtain mature VZV virions in sufficient quantity and purity for mass spectrometry analysis. Currently, both types and functions of VZV tegument proteins are mainly predicted from their HSV-1 homologues, which have already been identified and relatively well characterized (Table 1). Recent advances in bacterial artificial chromosome (BAC) technology have made genetic manipulation of the VZV genome more efficient, which greatly facilitates studies on functions of VZV genes and their products, virus-host interactions in VZV pathogenesis, as well as the design and development of new antiviral strategies and novel vaccines against VZV [49–51]. Additionally, *in vivo* studies of VZV pathogenesis have been aided by the development and use of the SCID-hu mouse model, which helps to overcome the strict species specificity of VZV and the corresponding lack of appropriate animal models for this research [50,51]. Current studies have shown that VZV tegument proteins also play important roles in VZV infection. Here we review recent progress in functional characterization of VZV tegument proteins as well as insights into the mechanism of VZV pathogenesis gained from these studies.

1 VZV immediate early (IE) protein within the tegument

The VZV genome encodes for at least three IE proteins, namely, IE4 (encoded by *orf4*), IE62 (encoded by *orf62* and *orf71*), and IE63 (encoded by *orf63* and *orf70*). *Orf62* and *orf63* in the internal repeat region of the genome are duplicated in the terminal repeat region as *orf71* and *orf70*, respectively [48]. VZV IE proteins IE4, IE62, and IE63 have all been identified as structural components of VZV virions and are likely located within the tegument layer according to protein content analysis of purified VZV virions [52,53]. In addition, VZV-encoded ORF61 protein is the functional homolog of HSV-1 IE protein ICP0, and thus, is a putative VZV IE protein. However, ORF61 is not virion-associated [52].

The interplay between IE proteins and other viral or cellular factors during VZV infection implicates VZV IE proteins as important regulators of viral and host gene transcription and expression [54–59]. In addition, VZV IE proteins are also involved in host immune evasion. For example, IE62 is capable of modulating host innate immune signaling by antagonizing activation of interferon response factor 3 (IRF3) [60]; IE63 can suppress the antiviral activity induced by interferon-α (IFN-α) via inhibition of phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) [61]. Thus, when VZV enters the cell, IE proteins in the tegument are presumed to be released into the cytoplasm along with the capsid and may be further transported into the nucleus. These proteins could then aid in host immune suppression as well as activation of viral gene transcription and expression, thereby promoting the efficient replication and cell-to-cell spread of VZV.

In early studies of mechanisms underlying VZV latency and reactivation, IE proteins IE4, IE62, and IE63 were found to be expressed and show a predominantly cytoplasmic distribution in latently infected neurons [62–64]. In contrast, IE proteins, except IE4, are distributed mainly in the nucleus of cells during lytic VZV infection. Therefore, it is speculated that these IE proteins are sequestered from the nucleus during VZV latency, thereby limiting their role in gene transactivation and VZV replication [64]. Furthermore, IE4 and IE63 were also found to be essential for the establishment of VZV latency in a cotton rat model infected with VZV gene deletion mutants [65,66]. However, the role of VZV IE proteins expressed during latency remains elusive.

VZV deletion mutants for *orf4*, *orf62/orf71*, and *orf63/orf70* fail to replicate in cell culture *in vitro*, in human skin organ culture (SOC) *ex vivo*, and in SCID-hu skin xenografts *in vivo*, respectively. Thus, these genes appear essential for VZV propagation [57,58,67,68]. Moreover, at least one copy of the duplicated IE62 or IE63 genes is required for VZV replication [57,68]. However, IE62 gene expression at an ectopic site in the VZV genome of a dual *orf62/orf71* deletion mutant permits viral replication in cell culture but fails to restore virus infectivity in human skin implants in SCID-hu mice. This indicates the existence of regulatory regions at the native site of *orf62* and/or *orf71*,

Table 1 VZV putative tegument proteins and their functions[a)]

VZV putative tegument proteins	HSV-1 ortholog/tegument proteins	Cell culture[#]	Predicted functions*	Identified functions
ORF3	UL55	Dispensable	Potential negative regulatory role in viral gene expression [31]	Not determined
ORF7[Φ]	UL51	Dispensable	Role in virion maturation and egress; role in cell-cell viral spread [25]	Role in cell-cell viral spread [107]
ORF8	UL50	Dispensable	dUTPase	dUTPase
ORF9[‡]	UL49	Essential	Role in viral protein synthesis and mRNA accumulation at early times of infection [37]; induces stabilization of microtulule [126]; potential role in virion envelopment and transcriptional regulation [24,32]	Role in virion nuclear egress and secondary envelopment [83,84]
ORF10[Φ]	UL48	Dispensable	α-transinducing factor; role in transactivation of immediate early gene expression [33,34]; role in virion maturation and egress [23]	Role in transactivation of immediate early gene expression [85,86]
ORF11[‡]	UL47	Dispensable	Role in gene expression [35]; role in virion maturation and egress [28]	RNA binding capacity; role in viral protein synthesis at early times of infection [76]
ORF12[‡]	UL46	Dispensable	Role in gene expression [35]; role in activation of Akt signaling [36]; potential role in virion tegumentation [30]	Role in activation of Akt signaling and regulation of cell cycle [90]; role in phosphorylation of ERK1/2 and inhibition of apoptosis [77]
ORF17[o]	UL41	Essential	Host shutoff protein	Host shutoff protein
ORF21	UL37	Essential	Inner tegument protein [9]; role in delivery of capsids to the nucleus [9]; role in virion maturation and egress [26]; role in activation of NF-κB signaling pathway [44]	Not determined
ORF22	UL36	Essential	Inner tegument protein [9]; role in delivery of capsids to the nucleus [9]; role in virion maturation and egress [27]; role in abrogation of IFN-β production [45]	Not determined
ORF36	UL23	Dispensable	Thymidine kinase	Thymidine kinase
ORF38	UL21	Essential	Roles in faciliating viral gene expression during early stages of infection [38]; possible role in capsid transport [13]	Not determined
ORF44	UL16	Essential	Potential role in viral genome packaging and virion maturation [29]	Role in the efficient production of infectious progeny virus [110]
ORF46	UL14	Essential	Minor tegument protein [9]; chapter-one-like activity [127]; roles in virion maturation and egress [14]	Not determined
ORF47[Φ]	UL13	Dispensable	Serine/threonine kinase; role in immune evasion [47]	Serine/threonine kinase; role in immune evasion [102]
ORF49[Φ]	UL11	Dispensable	Myristylated and palmitylated virion protein; roles in virion envelopment and egress [22]	Myristylated virion protein; role in the efficient production of infectious progeny virus [110]
ORF53	UL7	Essential	Palmitylated virion protein; potential role in mitochondrial function [128]	Not determined
ORF61[o]	ICP0	Essential	Transactivator or repressor; E3 ubiquitin ligase activity; mutiple roles in immune evasion [41]	Transactivator or repressor; E3 ubiquitin ligase activity [129]; mutiple roles in immune evasion [130,131]
ORF66[Φ]	US3	Dispensable	Inner tegument protein [9]; serine/threonine kinase; role in viral nuclear egress [19–21]; reorganizes the actin skeleton[132,133]; role in regulation of gene expression [42]; inhibits cell apoptosis [39,40]; role in immune evasion [46]	Serine/threonine kinase; modulates host immune response through downregulation of cell surface MHC-I [106]
ORF62/71[‡]	ICP4	Essential	Major transactivator; transcriptional repressor of viral immediate early gene [43]	Major transactivator; transcriptional repressor of viral immediate early genes; role in prevention of IFN production by inhibiting activation of IRF3 [60]
ORF64/69	US10	Dispensable	Not determined	Not determined

a) *, Functions of VZV putative tegument proteins were predicted based on charateristics of their homologous counterparts in HSV-1. #, Functions in cell culture were determined using VZV mutants null for genes encoding putative tegument proteins. o, the protein is proved not virion-associated. [Φ], the protein is proved virion-associated but its localization within the tegument of virion has not been confirmed. ‡, the protein is proved as a tegument protein in VZV.

which may affect the functions of adjacent genes [57]. In addition, it has been found that IE62 of parental Oka VZV (P-Oka) has stronger transactivation activity than that of the Oka vaccine strain (V-Oka), which is due to multiple mutations within *orf62* in the vaccine virus and may contribute to attenuation of V-Oka in skin [69–72]. Overall, the gene products of the major transactivator IE62, as well as its gene sequence and precise position in VZV genome play a pivotal role in VZV skin pathogenesis. In contrast, infectivity of a dual *orf63/orf70* deletion mutant can be restored to that of the wild type in culture as well as in skin and thymus/liver (T cell) xenografts in SCID-hu mice by introducing one copy of *orf63* into an ectopic site in the VZV genome [68]. Therefore, it is the gene product of *orf63* rather than its gene sequence at the native site that performs functions in VZV pathogenesis. Further studies were carried out with the aim of identifying functional domains of IE63. It was demonstrated that mutations of IE63 phosphorylation sites at serine 165 and 173 result in loss of VZV infectivity. On the other hand, a single alanine substitution at serine 181, serine 185, or threonine 171, which are also phosphorylation sites of IE63, impairs VZV replication both in culture and in skin implants in SCID-hu mice but has no effect on viral infectivity in T cells *in vivo* [73]. These data suggest that phosphorylation of IE63 by viral and/or cellular kinases is critical for VZV pathogenesis in skin, but the regulatory mechanism of IE63 phosphorylation and its function remain to be defined.

2 A conserved gene cluster of VZV containing *orf9* to *orf12*

VZV *orf9*, *orf10*, *orf11*, and *orf12* form a conserved gene cluster, which is located in the unique long region of the VZV genome [74]. Their homologous HSV-1 counterparts encode virus tegument proteins that are important for viral replication [75], and therefore proteins encoded by this gene cluster are putative VZV tegument proteins. To date, VZV ORF10 has been shown to be a component of the virus particle through western blot analysis of gradient-purified virions [53], while ORF9, ORF11, and ORF12 are detected in the tegument of virions using immunogold electron microscopy [74,76,77].

Orf9, which encodes a viral structural protein, is one of the most abundantly transcribed genes during VZV infection [78,79], and is essential for VZV growth both *in vitro* and *in vivo* [67,74]. However, the homologue of VZV *orf9* in HSV-1 and PRV is dispensable for virus replication [80,81]. Thus, it is likely that *orf9* differs from its homologues in its function during viral infection. Currently, VZV ORF9 has been reported to have several functions. For instance, ORF9 is predicted to orchestrate the assembly of VZV virus particles by recruiting VZV tegument proteins, including IE62, IE4, IE63, and ORF47 which form a complex with IE62 during viral infection, on the microtubule network [82]. ORF9 has also been shown to interact with VZV glycoprotein E (gE), which is not part of the ORF9-IE62-tubulin complex, but may be involved in secondary envelopment [74]. Lastly, ORF9 has been implicated in VZV formation and egress, and this appears to be mediated through VZV ORF47 kinase-dependent phosphorylation of ORF9 [83]. According to the latest report, deletion of the acidic cluster corresponding to amino acid (aa) 85 to 93 of ORF9, which contains the ORF47 phosphorylation consensus sequence, disrupts interactions between ORF9 and ORF47 as well as phosphorylation of ORF9. This deletion also causes the nuclear accumulation of ORF9 and ORF47, and most importantly, results in accumulation of primary enveloped VZV capsids in the perinuclear space due to a de-envelopment defect [84]. Therefore, it seems that ORF9 also regulates virus nuclear egress.

ORF10 is capable of transactivating the VZV IE62 promoter and plays a significant role in the initiation of VZV infection [85,86]. *Orf10* is not essential for VZV replication in culture and in T cells *in vivo*, but deletion of *orf10* impairs VZV replication in skin *ex vivo* and *in vivo* [67,87]. In addition, upstream stimulatory factor (USF), which is identified as a cellular transcription factor, binds specifically to the consensus sequence within the promoter region of *orf10* and is important for optimal transactivation activity of IE62. Deletion of the USF binding site in the *orf10* promoter impairs the virus infectivity in skin xenografts in SCID-hu mice, which is consistent with the phenotype of the full-length *orf10* deletion mutant [88]. Therefore, USF is a cellular determinant of VZV virulence, which is required for VZV replication in human skin *in vivo*. In short, ORF10 is involved in the regulation of viral and host gene expression, which is mediated by VZV IE62 and cellular USF, and is one of the determinants of VZV pathogenesis in skin *in vivo*.

Like *orf10*, *orf11* is not essential for VZV growth in culture, but deletion of *orf11* impairs viral infectivity severely in SCID-hu skin xenografts. Therefore, VZV ORF11 is also a determinant of VZV virulence in skin [74]. Further studies have been carried out to investigate the exact function of ORF11 in VZV infection. ORF11 was identified as a RNA binding protein with a conserved RNA-binding domain. However, knockout of this RNA-binding site does not affect VZV virulence both *in vitro* and *in vivo* [76]. Recently, ORF11 has been characterized to interact with ORF9, and mutation of the ORF9 binding site in ORF11 impairs VZV virulence in skin *in vivo* as severely as the deletion of *orf11* [89]. These data implicate that interactions between ORF11 and ORF9 play a key role in the function of ORF11 as a virulence determinant of VZV skin pathogenesis.

Orf12 is a non-essential gene for VZV growth both *in vitro* and *in vivo* [67,74], but ORF12 still contributes to optimal VZV infection. For example, ORF12 activates the PI3K/Akt/GSK-3β signaling pathway and thus regulates

host cell cycle progression [90]; ORF12 also activates ERK1/2 and p38 and inhibits apoptosis of the infected host cell [77]. In summary, *orf9-12* gene cluster plays a crucial role in VZV replication. *Orf9* is essential for VZV growth both *in vitro* and *in vivo*. Although *orf10* and *orf11* are dispensable for VZV replication in culture, they are both essential for VZV virulence in skin implants in SCID-hu mice. It is further demonstrated that USF binding to the *orf10* promoter and interactions between ORF9 and ORF11 are likely part of the mechanism underlying VZV pathogenesis in skin. On the other hand, ORF12, which is not essential for VZV replication, is able to activate certain cellular signaling pathways and promote VZV infection.

3 VZV serine/threonine kinases, ORF47 and ORF66

The VZV genome encodes two types of serine/threonine kinases, ORF47 and ORF66 [91]. Both proteins are known to be virion-associated and are putative VZV tegument proteins based on the characteristics of their homologues in the alphaherpesvirus subfamily [75,92,93]. *Orf47* and *orf66* are both dispensable for VZV growth in culture. However, the *orf47* deletion mutant fails to replicate both in skin and in T cells in SCID-hu mice, while knockout of *orf66* impairs VZV virulence in T cells but has almost no effect on virulence in skin *in vivo* [94–96]. In addition, mutation of the kinase motif of ORF47 and ORF66 produces phenotypes that are similar to their full-length gene knockout mutants [97,98]. Therefore, kinase activities of ORF47 and ORF66 are likely to be responsible for VZV virulence and tissue tropism.

To date, putative VZV tegument proteins ORF9, IE62, and IE63 are proven substrates of VZV ORF47 kinase [83,99]. As mentioned above, ORF47 binding and phosphorylation of ORF9 as well as IE63 is crucial for VZV replication [73,83,84]. On the other hand, although kinase activities of ORF47 are of great importance for VZV infection *in vivo*, protein-protein interactions and complex formation between ORF47 and IE62 have been proven essential for VZV replication in human skin *in vivo* in a further study [97,100]. ORF47 also phosphorylates the viral envelope protein gE [101]. When *orf47* is deleted, endocytosed gE will be phosphorylated by a cellular kinase, casein kinase II (CKII), lose its localization in the trans-Golgi network (TGN) and recycle to the plasma membrane. This is likely the cause of increased cell-to-cell spread and larger syncytia formation observed in cells infected with the *orf47* knockout mutant. Further, ORF47 also helps VZV to evade host immune responses through inhibition of IRF3 activation [102].

ORF66 phosphorylates VZV major transactivator IE62, which results in nuclear export and cytoplasmic accumulation of IE62 during late-stage VZV infection [103,104]. This is in contrast to ORF47-mediated IE62 phosphorylation, which does not affect IE62 nuclear localization [104]. Therefore, it is suggested that VZV ORF66 kinase can regulate IE62 nuclear functions by affecting IE62 subcellular localization. Both ORF66 and IE62 are expressed exclusively in the cytoplasm of infected neurons during latency, and thus, ORF66-mediated cytoplasmic sequestration of IE62 is predicted as one mechanism that inhibits IE62 transactivation activities and helps VZV stay dormant in neurons [105]. In addition, ORF66-mediated nuclear exclusion of IE62 is also required for virion incorporation of IE62 [93]. Further, ORF66 plays a role in VZV immune evasion. ORF66 can affect the intracellular transport of major histocompatibility complex class I (MHC-1) through the Golgi complex, leading to the downregulation of cell-surface MHC-1expression and thereby affecting viral antigen presentation [106].

4 ORF7, the first known determinant of VZV virulence in human neuronal cells

ORF7 is a virion-associated protein and may be a part of the tegument given its homologue protein UL51 in HSV-1 [75,107]. Like the HSV-1 *ul51* gene, *orf7* is dispensable for VZV growth in culture, however, ORF7 seems to affect VZV *in vitro* growth in a cell type-specific manner. For instance, deletion of *orf7* has no effect on VZV growth in a human melanoma cell line MeWo. Conversely, *orf7* knockout mutant produces smaller plaque sizes and slower growth kinetics than wild-type virus in a human diploid retinal pigment epithelial (RPE) cell line ARPE-19, suggesting defects in cell-to-cell spread [67,107]. Further, it is demonstrated that deletion of *orf7* impairs VZV virulence in *ex vivo* human skin, and thus, ORF7 is recognized as one of the virulence factors required for VZV skin infection [67]. Meanwhile, the *orf7* knockout mutant is severely impaired in its ability to grow in differentiated SH-SY5Y human neuroblastoma cells and normal human neuronal cells derived from human embryonic stem cells (hESC) *in vitro*. The same is true in human DRG *ex vivo* and *in vivo*, in contrast to the robust infection by wild type virus. Therefore, *orf7* is reported to be the first full-length gene responsible for VZV virulence in neuronal cells [107]. Subsequently, deletion of VZV *orf7* was shown not to prevent axonal infection and retrograde transport of virus particles to the neuronal somata in hESC-derived neurons *in vitro* [108]. However, viral and host gene transcription and protein expression have not yet been evaluated in these VZV-infected hESC-derived neuronal cells. Thus, it is unknown whether the *orf7* knockout mutant remains latent or begins to replicate and produce progeny viruses. In summary, although ORF7 has dual roles as virulence determinants in human skin and in DRG *in vivo*, the underlying mechanism has yet to be studied.

5 VZV ORF44 and ORF49

ORF44 and ORF49 are both putative tegument proteins of VZV according to homology-based predictions [75]. However, only ORF49 has been shown to be a component of VZV virions [109]. Like *orf7*, *orf49* is dispensable for VZV replication *in vitro* but has cell type-specific functions in cell-to-cell transmission of the virus. Deletion of *orf49* impairs viral spread in MeWo cells but not in a human embryonic lung fibroblast diploid cell strain, MRC-5, in comparison to the growth of wild type virus in both cell types. Therefore, ORF49 is regarded as a cell-tropic factor of VZV *in vitro* but the underlying mechanism remains elusive [109]. Moreover, an *orf49* knockout mutant also shows defective growth in SOC. Thus, it seems that ORF49 plays a significant role in VZV pathogenesis in human skin [67]. On the other hand, *orf44* appears to be essential for VZV replication, however, characteristics of ORF44 has not yet been reported [67].

Recently, ORF44 and ORF49 have been shown to interact and form a complex [110]. The binding motifs of these two proteins have been identified to be a single phenylalanine residue at position 129 for ORF44 and four amino acids from positions 41–44 in the carboxyl-terminal half of ORF49. The interaction between ORF44 and ORF49 is required for VZV infection *in vitro*, and a substitution of phenylalanine for alanine at position 129 of ORF44 causes lethal defects in viral replication, which is consistent with the phenotype of an *orf44* deletion mutant. Similarly, a VZV mutant with the aa 41–44 deletion shows defective viral growth in MeWo, which is comparable to that of an *orf49* knockout virus. However, this ORF49 mutant has not been evaluated in the SCID-hu mouse model, and thus, the role of ORF44 and ORF49 interaction in VZV pathogenesis *in vivo* has yet to be elucidated.

6 Other VZV tegument proteins of unknown function

As with the proteins mentioned above, ORF3, ORF8, ORF17, ORF21, ORF22, ORF36, ORF38, ORF46, ORF53, and ORF64/69 are all putative VZV tegument proteins, among which only ORF17 is characterized as the virion host shut off (VHS) protein but it is not associated with VZV virions [75,111]. In a comprehensive study of VZV virulence in MeWo *in vitro* and in SOC *ex vivo*, ORF3, ORF8, ORF36, and ORF64/69 were shown to be dispensable, while ORF21, ORF22, ORF38, and ORF53 were shown to be essential for VZV infection [67]. Apart from this study, there have been few reports regarding these proteins and research on their roles in VZV infection is still lacking. However, it should be noted that their homologous counterparts in HSV-1 have proved to be very important for viral replication and growth [9,13,14,26,27,31,38,44,45,127,128].

7 Conclusion and perspectives

VZV tegument proteins, like those of other herpesviruses, play multiple important roles at different stages of VZV infection, including regulation of viral and host gene expression, modulation of host immune response, and viral assembly and egress (Table 1). VZV tegument proteins are involved in a complicated network of interactions [112,113]. Some VZV tegument proteins have proved to be determinants of cell and tissue tropism and pathogenicity (Table 2).

Table 2 Tegument proteins required for VZV virulence in human skin, T cells and DRG *in vivo* in SCID-hu mouse model as well as their identified functional domains or motifs[a)]

Protein	Mutation description	Skin*	T cells*	DRG*
ORF7	Deletion	×	O‡	×
ORF10	Deletion	×	O	–
	Mutation of USF binding site	×	–	–
ORF11	Deletion	×	–	–
	Mutation of ORF9 binding site	×	–	–
ORF47	Deletion	×	×	–
	Mutation of kinase motif	×	×	–
ORF49	Deletion	×	–	–
ORF66	Deletion	O	×	–
	Mutation of kinase motif	O	×	–
ORF62/ORF71	Deletion of the duplicate genes with ectopic IE62 expression	×	–	–
ORF63/ORF70	Mutation of phosphorylation sites S181, S185 or T171	×	O	–

a) *, Determinants of VZV virulence in human tissues were screened out using recombinant VZV variants and the SCID-hu mouse model. ‡, Unpublished data from our lab. ×, no replication or impaired replication. O, no effect. –, not determined.

Nonetheless, only about half of these proteins have been studied to date, and great efforts are still required to characterize the functions of these proteins to improve our understanding of VZV pathogenesis.

VZV is highly homologous to HSV-1. Given this, the functions of VZV tegument proteins can be studied with particular reference to the research methods and experimental design for functional characterization of their HSV-1 counterparts. For example, the hESC-derived neuron model, together with time-lapse fluorescence microscopy, can be used to study the function of VZV tegument proteins in axonal infection and transport of virus particles. Similar systems have been widely used for functional studies of HSV-1 and PRV tegument proteins [8,15,16]. However, as mentioned above, only tegument protein ORF7 of VZV has been evaluated in this system [108]. Unlike HSV-1, VZV is highly cell-associated in cell culture and is highly species-specific so that research methods involving VZV have to be properly designed and executed to match its own characteristics. For example, in recent years, there has been concern about how tegument proteins become incorporated into the virion of herpesviruses. Although sufficient virions with high purity can be obtained and protein content in the tegument has been clarified in studies of HSV-1, the structure of the tegument appears amorphous using transmission electron microscopy (TEM) and cannot be determined at high resolution using cryo-electron tomography (cryo-ET) and cryo-electron microscopy (cryo-EM) due to the lack of radial symmetry [114–118]. However, accumulating evidence suggests that tegument proteins are incorporated into the virion orderly during assembly [18,119–124]. In addition, it is found that inner tegument proteins of HSV-1 and PRV recruit microtubule motors onto viral capsids and facilitate intracellular transport of virus particles [9,11]. As for VZV, it is very difficult to purify large numbers of mature infectious virus particles for these studies due to its cell-associated nature. Therefore, the structure and protein composition of the VZV tegument as well as the interactions between the virus particle and the host cell remain to be investigated.

Currently, the combination of VZV BAC technology and the SCID-hu mouse model has significantly advanced our understanding of VZV pathogenesis *in vivo*. Due to the relatively high availability of fetal human skin and the simplicity of its subcutaneous transplantation, functions of VZV genes and their products are studied more comprehensively in skin xenografts in SCID-hu mice. In contrast, the availability of fetal thymus and liver and DRG tissue is limited, and it is difficult to transplant these tissues under the kidney capsule in SCID-hu mice. Thus, the SCID-hu mouse model with thymus/liver or DRG xenografts has not been used as widely as that with skin xenografts in VZV studies. Moreover, human skin can be used for VZV infection by almost 10 days post-transplant in SCID-hu mice. In contrast, DRG can be used for infection at nearly one month after transplantation, while it takes at least 3 months before thymus/liver (T cells) xenograft can be used for VZV inoculation. So far, among VZV tegument proteins, only the two viral kinases, ORF47 and ORF66, have been shown to affect VZV T-cell tropism, while ORF7 is the first determinant of VZV virulence reported to affect viral neuroninvasion [94,107]. Thus, the function of many tegument proteins in VZV infection of T cells and neuronal cells remains to be determined.

Given that VZV tegument proteins are critical for VZV infection, they represent potential targets for VZV antiviral therapy. Although some of VZV tegument proteins are dispensable in culture, they are determinants of VZV tissue tropism and virulence *in vivo*. For example, deletion of VZV tegument ORF7 can impair viral virulence both in human skin and neurons, which may disrupt the ability of the virus to cause chickenpox and herpes zoster [67,107]. The current VZV vaccine is a live attenuated viral vaccine whose administration is generally regarded as safe [125]. However, the mechanism underlying attenuation of this vaccine virus remains unclear. Therefore, deletion of virulence determinants in VZV may generate a safer live vaccine candidate, which may have a significant impact on human lives and public health.

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