Varicella-zoster virus (VZV), a member of the genus *Varicellovirus* within the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (Grose, 1981), is the causative agent of varicella during primary infection of the host and of herpes zoster upon reactivation from latency in sensory ganglia (Cohen, 1996). Open reading frame 9 (ORF9), a VZV-encoded late protein consisting of 302 aa, is a member of the highly conserved alphaherpesvirus UL49 gene family (Davison & Scott, 1986) and, as the orthologue of HSV-1 VP22, is believed to be a major constituent of the VZV virion tegument. VP22 has been extensively studied. The most abundant viral messages expressed during VZV infection. However, little is known concerning the function of ORF9 protein. Here, we found that transient expression of ORF9 fused to enhanced yellow fluorescent protein (EYFP) in COS-7 cells showed a predominantly cytoplasmic localization in the absence of other viral proteins. By constructing a series of ORF9 variants fused to EYFP, a *bona fide* bipartite nuclear localization signal of ORF9 was, for the first time, determined and mapped to aa 16–32 (RRKTPPSSGQYRTARR). Additionally, the nuclear export signal (NES) was identified and found to be in a leucine-rich region at aa 103–117 (LRHELVEDAVYENPL). Finally, ORF9 was demonstrated to be targeted to the cytoplasm through the functional NES by Ran and the chromosomal region maintenance 1-dependent pathway, and to the nucleus via an importin β-dependent pathway that does not require importin α5.

Varicella-zoster virus (VZV) open reading frame 9 (ORF9) mRNA is one of the most abundantly expressed messages during VZV infection. However, little is known concerning the function of ORF9 protein. Here, we found that transient expression of ORF9 fused to enhanced yellow fluorescent protein (EYFP) in COS-7 cells showed a predominantly cytoplasmic localization in the absence of other viral proteins. By constructing a series of ORF9 variants fused to EYFP, a *bona fide* bipartite nuclear localization signal of ORF9 was, for the first time, determined and mapped to aa 16–32 (RRKTPPSSGQYRTARR). Additionally, the nuclear export signal (NES) was identified and found to be in a leucine-rich region at aa 103–117 (LRHELVEDAVYENPL). Finally, ORF9 was demonstrated to be targeted to the cytoplasm through the functional NES by Ran and the chromosomal region maintenance 1-dependent pathway, and to the nucleus via an importin β-dependent pathway that does not require importin α5.
novel and adds to our knowledge about the subcellular localization of ORF9, however, the exact function of ORF9 in the different subcellular compartments during VZV infection or transfection remains unknown. It is likely that various dynamic interactions between ORF9 and different cellular proteins are required, because VP22 localized to the nucleus has been shown to associate with histones and nucleosomes and may have modulatory effects on virus replication (Ren et al., 2001) and transcription or replication of host cells, perhaps to prepare them for infection (Elliott & O’Hare, 2000). Additionally, the predominantly cytoplasmic localization has also been shown to be required for optimal protein synthesis at late time points of viral infection (Duffy et al., 2009). Accordingly, the cytoplasmic and nuclear localization of ORF9 must play an important role in this context, and this prompted us to identify the nuclear localization signal (NLS) and nuclear export signal (NES) of ORF9.

It is well known that NLSs are mainly composed of basic residues (Emmott & Hiscox, 2009). Sequence analysis using PSORT II (http://psort.hgc.jp/) (Nakai & Horton, 1999) predicted that ORF9 had two classical NLSs in the arginine-rich regions of ORF9, namely RRKTTPSYSGQY-RTARR (bipartite) at aa 16–32, and RKPK (pattern 4) at aa 272–275, which were designated NLS1 and NLS2, respectively. In order to determine whether one or both of these NLSs are functional, two deletion mutants lacking the corresponding NLS were made (Fig. 2a). As shown in Fig. 2(b), deletion of the region containing NLS2 (D271–302) had no effect on the nuclear targeting of ORF9–EYFP. In contrast, deletion of the N-terminal 32 aa containing NLS1 abrogated the nuclear targeting of ORF9–EYFP, which accumulated throughout the cytoplasm. These results clearly indicated that aa 1–32, but not aa 271–302, contain a functional NLS and are essential for nuclear localization of ORF9.

To determine whether the arginine-rich region (aa 16–32) functions as an NLS, we tested the ability of NLS1 to transport EYFP to the nucleus. As expected from the deletion-mutant analysis (Fig. 2b), ORF9 NLS1 fused with

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**Fig. 1.** Subcellular localization and time-course of expression of ORF9. (a) Schematic diagram of ORF9 fused with EYFP monomer, dimer and trimer at its C terminus and ORF9 fused with EYFP at its N terminus. (b) Subcellular localization of pORF9–EYFP, pEYFP–ORF9, pORF9–dEYFP, pORF9–tEYFP or pEYFP–N1 in COS-7 cells. (c) Time-course analysis of ORF9–EYFP expression. Each photomicrograph represents a vast majority of the cells, their having similar subcellular localization. Light-translucent pictures are introduced to show cellular morphology.
**Fig. 2.** Subcellular localization of the ORF9 deletion mutants. (a) Schematic representation of wild-type ORF9 protein and its N- and C-terminal deletion mutants fused with EYFP. (b) Subcellular localization of these ORF9 deletion mutants.
EYFP (aa 16–32–EYFP) showed significantly nuclear localization, whereas the fluorescence of aa 271–302–EYFP was identical to that of EYFP, thus confirming that NLS1, but not NLS2, was a functional NLS. To further confirm that the NLS1 is a bona fide bipartite NLS, plasmids encoding EYFP fused to three different peptides (aa 1–27, 1–32 and 21–210) were constructed (Fig. 2a). As shown in Fig. 2(b), aa 1–27 did slightly direct EYFP to the nucleus. In contrast, the fluorescence of aa 21–210–EYFP was evenly distributed throughout the cytoplasm and the nucleoplasm but not the nucleolus, indicating that both of the arginine-rich motifs RRK and RTARR were very important and were indispensable for the nuclear localization of ORF9. In addition, the fluorescence of aa 1–32–EYFP was identical to that of aa 16–32–EYFP, suggesting that aa 1–15 had no effect on the nuclear localization of NLS1. Taken together, these results indicated that the minimum sequence of ORF9 required for and capable of directing a heterologous protein into the nucleus is a 17 aa peptide, RRKTKTPSYGQYRTARR, which is a functional bipartite NLS that contains two arginine-rich motifs, RRK and RTARR.

It is well known that NESs consist of largely hydrophobic, leucine-rich sequences (Nakielny & Dreyfuss, 1999), which are critical for its nuclear export function (Görlich & Mattaj, 1996). Amino acid sequence analysis of ORF9 using the NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/) revealed that it contained two leucine-rich motifs that might represent putative NESs. To identify the minimum amino acid sequence within ORF9 responsible for its nuclear export function, leucine residues to yield103ARHEAVEDAVYENPL117, a new transferable NES activity and was sufficient to mediate the nuclear export of non-shuttling protein. Taken together, these pieces of evidence indicated that the minimum amino acid sequence required for the nuclear export of ORF9 is 103LRHELVEDAVENPL117.

The Ran protein has been shown to be required for classic NLS-dependent nuclear transport (Moore & Blobel, 1993) and the translocation of NES-containing proteins from the nucleus to the cytoplasm (Lindsay et al., 2001). Furthermore, most import and export processes studied so far require the Ran GTPase protein. To further explore the nuclear transport mechanism of ORF9, a dominant-negative Ran protein, Ran–GTP Q69L, which is deficient in GTP hydrolysis (Isegawa et al., 2008; Palacios et al., 1996), was introduced to determine whether Ran is required for the nuclear transport of ORF9. COS-7 cells were co-transfected with pRan–Q69L–ECFP and pORF9–EYFP and the subcellular localization of Ran–Q69L–ECFP and ORF9–EYFP was monitored. As shown in Fig. 3(a), co-transfection of Ran–GTP Q69L clearly blocked the nuclear export of ORF9, whereas ORF9 alone was predominantly targeted to the cytoplasm. This result demonstrated that the translocation of ORF9 from the nucleus to the cytoplasm is Ran dependent and requires GTP hydrolysis.

A well-characterized canonical NLS is recognized by members of the importin family of cellular transporters (Alvisi et al., 2008) such as the importin $z$–$\beta$ heterodimer, in which importin $z$ recognizes the NLS and importin $\beta$ facilitates the importin $z$–NLS interaction by mediating a conformational change in importin $z$ (Kobe, 1999). To identify the cellular receptor responsible for ORF9 nuclear targeting, two dominant-negative mutants of importin $z5$ and importin $\beta$ (also known as karyopherin $z1$ and karyopherin $\beta1$, respectively), which lack the ability to bind importin $\beta$ and Ran, respectively (Chi et al., 1997; Guo et al., 2010; Kutay et al., 1997; Reid et al., 2007), were introduced to determine whether they are required for the
nuclear transport of ORF9. Plasmids pORF9–EYFP or pORF9–mNES1–EYFP were co-transfected with pDN–kz1–ECFP or pDN–kβ1–ECFP into COS-7 cells, respectively, and their subcellular localization was monitored. As shown in Fig. 3(b), co-transfection of DN–kβ1–ECFP with ORF9–EYFP or ORF9–mNES1–EYFP significantly blocked the nuclear import of ORF9. However, dominant-negative importin α5 did not. These results demonstrated that the nuclear import of ORF9 is mediated by a classic importin β-dependent pathway that does not require importin α5.

Leucine-rich NESs have been identified in an increasing number of cellular and viral proteins executing quite varied biological functions. Most studies pertaining to nuclear export have implicated the chromosomal region maintenance 1 (CRM1) protein, a host cell protein that is a member of the importin/exportin family of nucleocytoplasmic transport factors and required for the nuclear export of proteins containing an NES (Fornerod et al., 1997). However, the antibiotic compound leptomycin B (LMB; Sigma), a potent and specific nuclear export inhibitor (Fukuda et al., 1997), can alkylate and inhibit CRM1. To investigate the nuclear export mechanism of ORF9, COS-7 cells transfected with pORF9–EYFP, pNES–EYFP (NES1) and positive control pRev–NES–EYFP were treated with LMB at a final concentration of 20 ng ml⁻¹ for 2.5 h. It was known that LMB treatment could almost completely abolish the nuclear export of pRev–NES–EYFP (Williams et al., 2008), and similarly the nuclear export activity of ORF9 and its NES were almost completely abolished by LMB treatment (Fig. 3c), indicating that the export of ORF9 protein is probably achieved through its direct interaction with the nuclear export receptor CRM1. Taken together, this is compelling evidence that the functional NES mediates the nuclear export of ORF9 from the nucleus to the cytoplasm through a CRM1-dependent pathway.

In conclusion, by constructing a series of deletion mutants fused with EYFP and fluorescence microscopy analysis, we identified an N-terminal arginine-rich NLS (16RRKTT-PSYSQYRTARR32) and an N-terminal leucine-rich NES (103LRHELVEDAVYENPL117) in VZV ORF9. However, some of the fusion proteins of ORF9 may diffuse through the nuclear pore complex (NPC) without active import and some others (of approx. 50–60 kDa) may be too big to diffuse across the NPC. Additionally, the ORF9 protein was demonstrated to be transported between the nucleus and the cytoplasm through a classical NES by a CRM1-dependent pathway and via a classic NLS by a Ran-dependent and importin β-dependent pathway that does not require importin α5.
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