Electron microscopy

HHV-6A-infected CBMCs were harvested on the fourth day after cell-to-cell infection by centrifugation at 2000×g, and then fixed with 4% paraformaldehyde plus 2.5 glutaraldehyde at room temperature for 1 h. Processing for electron microscopy (EM) was carried out as described previously (Mori et al., 2008).

Construction of the gQ1 deletion mutant and its revertant

The HHV-6A BAC DNA from *E. coli* DH10B was transformed into GS1783 *E. coli* using a Bio-Rad *E. coli* Pulser. Two-step Red-mediated mutagenesis was used for these recombinations, as described previously (Kato et al., 2008; Tischer et al., 2006). The experimental procedure is depicted in Fig. 4. First, we deleted N-terminal segment of the gQ1 gene, 150,281 bp to 149,196 bp, from the U1102 genome (PubMed accession NC_001664). Briefly, GS1783 containing HHV-6ABAC was cultured in LB medium containing 17 μg/ml chloramphenicol at 30 °C overnight. The overnight culture was then added to new LB medium containing 17 μg/ml chloramphenicol at a 1:30 ratio. The culture was continued until A_{600} reached 0.5–0.7. Then, the culture was transferred to a 42 °C water bath and shaken for 15 min at 170 rpm, which induced the expression of the Red recombination system. Finally, the bacteria were chilled in an ice bath for 20 min, and then harvested for the preparation of electroporation-competent cells, as described previously (Tanaka et al., 2003).

Next, 100 ng of the PCR product amplified from the plasmid pEP-KanS using the AgQ1deletion2F and AgQ1 deletion2R primers (shown in Table 1) was transformed into the GS1783 competent cells described above using the Bio-Rad *E. coli* Pulser. The bacteria were cultured at 30 °C for 1.5 h, and then plated onto LB agar plates containing 17 μg/ml chloramphenicol and 50 μg/ml kanamycin to select for *E. coli* clones harboring the kanamycin-resistance gene. The selected clones were confirmed by PCR using the appropriate primers (data not shown).

Next, the kanamycin-resistance gene was excised by expressing I-Sce1 restriction enzyme, which was induced by adding arabinose to the culture medium followed by induction of the Red recombination system. Briefly, 100 μl of an overnight culture of the GS1783 cells containing the kanamycin-resistance gene was inoculated into 2 ml of medium containing 17 μg/ml chloramphenicol. The bacteria were cultured for 2–4 h at 30 °C, and then 2% arabinose was applied. After another 1 h incubation, the culture was transferred into a 42 °C water bath and incubated for half an hour. The culture was then transferred to a 30 °C shaker and cultured with shaking as described above for 1.5 h before being transferred to agar plates containing 17 μg/ml chloramphenicol. Chloramphenicol-resistant, but kanamycin-sensitive clones were selected by plating single clones onto chloramphenicol- and chloramphenicol–kanamycin-containing plates. We named the resultant BAC, HHV-6ABACΔgQ1.

We next constructed a gQ1 deletion revertant. The deleted part of gQ1 was amplified from the U1102 genome using the ReF1 and ReR1 primers (shown in Table 1) and digested with BamH1. We also amplified the Kanamycin-resistance gene from pEP-KanS using the ReF2 and ReR2 primers (shown in Table 1) and digested it with BamH1. These two BamH1-digested DNA fragments were ligated and amplified with the ReF1 and ReR3 primers (shown in Table 1). Then, 100 ng of the PCR product was transformed into GS1783 *E. coli* electroporation-competent cells containing HHV-6ABACΔgQ1. The selected clones were confirmed by PCR by using the appropriate primers (data not shown). Next, the kanamycin-resistance gene was excised by expressing the I-Sce1 restriction enzyme followed by induction of the Red recombination system (as described above). Finally, the gQ1 deletion revertant (HHV-6ABACΔgQ1re) DNAs were isolated and confirmed by sequencing (data not shown).

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Characterization of varicella-zoster virus-encoded ORF0 gene—Comparison of parental and vaccine strains

Tetsuo Koshizuka [a], Megumi Ota [a,b], Koichi Yamanishi [a], Yasuko Mori [a,b,*]

[a] Laboratory of Virology and Vaccinology, Division of Biomedical Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[b] Division of Clinical Virology, Kobe University Graduate School of medicine, Kobe, Japan

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ABSTRACT

The varicella-zoster virus (VZV) Oka vaccine strain (vOka) differs from the parental strain (pOka) at several amino acid positions, but the mutations responsible for the attenuation of vOka have not been clearly defined. The ORF0 of vOka carries some of the mutations. Although we found that the ORF0 of both strains was incorporated into virus particles, the C-terminal region of vOka ORF0 was presented on the virion surface and was N-glycosylated, suggesting that the mutation in vOka ORF0 changes it into a novel envelope glycoprotein. In a mutant virus in which pOka ORF0 was replaced by vOka ORF0, the molecular weight of ORF0 was altered, but the plaque size was not. In addition, a pOka recombinant virus lacking the hydrophobic domain of ORF0 grew equally well as the wild-type virus, indicating that the mutation in ORF0 is not by itself sufficient for the attenuation of the vOka virus.

Introduction

Varicella-zoster virus (VZV) is a member of the human alphaherpesvirus subfamily and causes chickenpox (varicella) and shingles (zoster) (Cohen et al., 2007). Varicella is highly infectious. In children, it is associated with fever and generalized pruritic vesicular rash. The virus establishes latency in cells of the dorsal root ganglia during the primary infection. Herpes zoster is caused by the reactivation of VZV and is usually observed later in life.

To control VZV infection, a live attenuated vaccine (vOka) was developed from the Oka parental strain (pOka) (Takahashi et al., 1974). The attenuated vOka was obtained by multiple passages of pOka through human and guinea pig cells. The resulting attenuated strain became the first herpesvirus vaccine licensed for use in humans. Vaccination with vOka is efficient and causes few adverse events. Consequently, it is the only strain to be used worldwide (Arvin, 2001; Chaves et al., 2008). Although vOka's infectivity is attenuated in the skin zenograft SCID-hu mouse model (Moffat et al., 1998), it retains the capacity to downregulate the expression of major histocompatibility complex class I (Abendroth et al., 2001). The delay in the production of infectious vOka at the site of subcutaneous inoculation allows enough time for the induction of adaptive immunity. Despite its widespread use and effectiveness as a vaccine, however, the molecular mechanisms of vOka's attenuation are not well understood,.

A comparison of the pOka and vOka genomic sequences by Gomi et al. (2002) showed 42 mutated sites in vOka, with 20 amino acid substitutions in open reading frames (ORFs). In addition, Kemble et al. (2000) showed another amino acid substitution in ORF0. Therefore, a total of 21 amino acid substitutions are found in the ORFs of vOka. The sequence polymorphisms presented in Gomi et al. (2000) show only 15 of these 21 substitutions because vOka consists of a mixture of closely related viruses. The remaining 6 amino acid substitutions are located in ORF0, ORF6, and ORF62 and may contribute to the vOka attenuation.

The amino acid substitution in ORF0 is particularly interesting. ORF0 is encoded at the start site on the left side of the unique long (UL) region of the VZV genome. It is a positional and sequence homolog of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) UL56. Neither ORF0 nor UL56 has an N-terminal signal sequence, and both possess a transmembrane domain (TMD) at the C-terminus (Koshizuka et al., 2002). These are features of tail-anchored (TA) proteins (Borgese et al., 2003; High and Abell, 2004; Kutay et al., 1993; Wattenberg and Lithgow, 2001). The ORF0 protein of vOka possesses an extended C-terminal sequence because of a point mutation in its stop codon (Kemble et al., 2000). Finally, an ORF0-null VZV mutant, generated by using the bacterial artificial chromosome (BAC) system, shows severely retarded growth both in vitro and in vivo (Zhang et al., 2007).

In the present study, we investigated the role of ORF0 in VZV attenuation by preparing anti-ORF0 mono-specific antibodies in rabbits and characterizing the ORF0 protein. We found that ORF0 is incorporated into virus particles and that the extended C-terminal domain of

* Corresponding author. Laboratory of Virology and Vaccinology, Division of Biomedical Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Fax: +81 72 641/9812.
E-mail address: ymori@med.kobe-u.ac.jp (Y. Mori).

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vOka ORF0, but not the wild-type ORF0 of pOka, is N-glycosylated and presented on the surface of the virion.

Results

Construction of the ORF0 gene

The ORF0 protein is encoded by the nucleotide (nt) sequence from position 172 to 558 of the pOka genome. The stop codon of pOka ORF0 is at nucleotide (nt) position 559 of the pOka genome. The thymine at nt 559 of the pOka genome is replaced by a cytosine in the vOka genome (Fig. 1A, arrowhead), and the resulting protein indicates that the ORF0 gene lies between nt positions 172 and 834 of the vOka genome (Kemble et al., 2000). The ORF0 gene encodes a spliced mRNA in addition to an unspliced one (Fig. 1). Because both of the vOka ORF0 gene products are predicted to be translated, both variants were cloned into plasmids and sequenced.

The stop codon of the spliced vOka ORF0 was at nt position 767, and the splice junction was at nt positions 583 to 714. The stop codon of the unspliced ORF0 was at nt position 835. Two single nucleotide polymorphisms have been reported at positions 702 and 762 (Fig. 1A, open arrowheads) of the vOka genome. These are located within the ORF1 gene (Gomi et al., 2002) (Fig. 1A). In the spliced vOka ORF0, the polymorphism at 702 was spliced out, and the one at nt 762 caused a substitution of valine for alanine, at amino acid position 154 (Fig. 1B, vertical line). In the unspliced vOka ORF0, the nt mutations were silent and therefore did not affect the amino acid sequence. Therefore, the ORF0 protein of pOka contains 129 amino acids (aa); the spliced variant of vOka ORF0 contains 155 aa, and the unspliced one contains 221 aa (Fig. 1B). The molecular weight of each ORF0 protein predicted from the amino acid sequence was 14 kDa in pOka, 17 kDa for the spliced form in vOka, and 23 kDa for the unspliced form in vOka (Table 1).

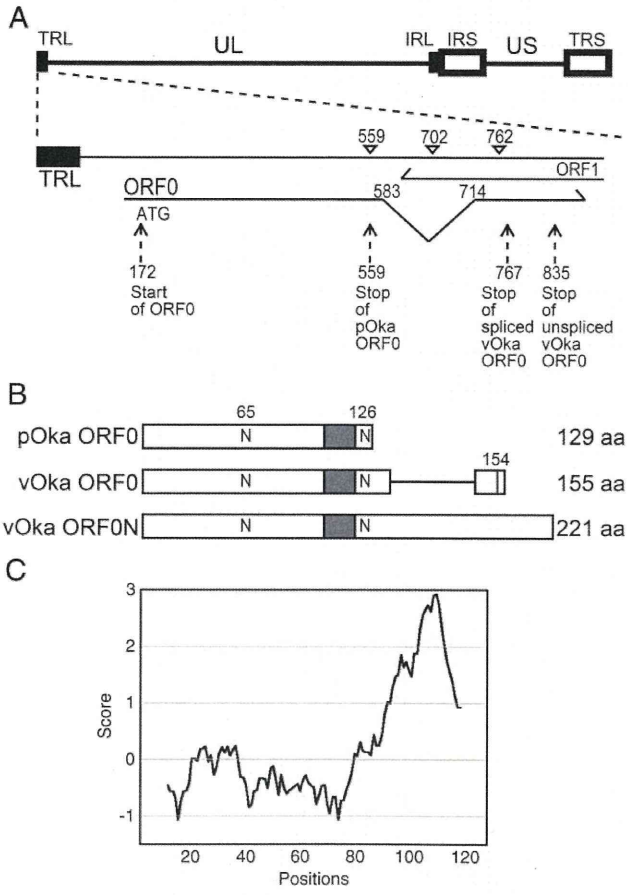

Fig. 1. Schematic representation of the VZV genome and ORF0 gene. (A) The VZV genome is shown with its unique long (UL) and unique short (US) regions. The start of the UL region is shown. The ORF0 and ORF1 mRNAs are illustrated, and their directions are indicated by arrows. Open arrowheads indicate the sites of single nucleotide polymorphisms in the vOka genome. The start and stop codons of ORF0 are indicated. The numbers indicate the nucleotide positions in the VZV genome. (B) The predicted ORFs of ORF0 are indicated by boxes. Gray boxes indicate the putative TMD. The putative N-glycosylation sites at N65 and N126 are denoted by "N." The vertical line indicates the amino acid polymorphism alanine or valine at amino acid position 154 of the vOka ORF0. The numbers indicate the amino acid positions. The amino acid length of the ORF encoded by each mRNA is given at right. (C) Kyte and Doolittle hydrophobicity plot of pOka ORF0. The window size used was 21 amino acids.

Table 1
The properties of ORF0 variants.

	Amino acids	Pattern of Western blotting	N-glycosylation	Intracellular localization
pOka ORF0	129	17 kDa	No	Plasma membrane
vOka ORF0 spliced	155	19–30 kDa Smeared	Yes	Plasma membrane
vOka ORF0 unspliced	221	20 kDa	No	Cytoplasm

Predicted C-terminal transmembrane domain and classification of ORF0

The ORF0 protein contains a C-terminal hydrophobic domain from amino acids 100 to 120 (Figs. 1B and C). We generated a Kyte and Doolittle (1982) hydrophobicity plot and applied the SOSIU algorithm (Hirokawa et al., 1998). The results indicated that ORF0 has a single TMD at its C-terminus but no N-terminal signal sequence (Fig. 1C). Since these are the defining characteristics of TA proteins (Koshizuka et al., 2002), the HSV-2 homolog of VZV ORF0 is a TA protein.

Expression of ORF0 proteins

To detect the ORF0 proteins, we generated rabbit antibodies against the N-terminal region of ORF0. On immunoblots, one of the

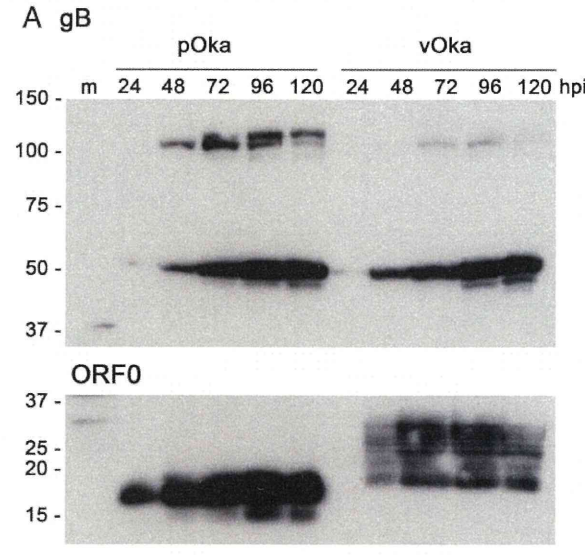

Fig. 2. Production of the ORF0 protein in VZV-infected cells. (A) MRC5 cells were mock infected (m) or infected with strain pOka or vOka by cell-to-cell spread and harvested at various times post-infection. Samples were separated by SDS–PAGE and analyzed by western blotting with an anti-gB (top) or anti-ORF0 (bottom) antibody. Molecular mass markers at left give values in kilodaltons (kDa). (B) Intracellular localization of ORF0 in VZV-infected cells. MRC5 or MeWo cells were mock infected or infected with strain pOka or vOka cell-free virus at moi 0.01. pOka- and vOka-infected cells were fixed at 48 hpi and 72 hpi, respectively, and subjected to confocal microscopic analysis. Scale bar: 15 μm.

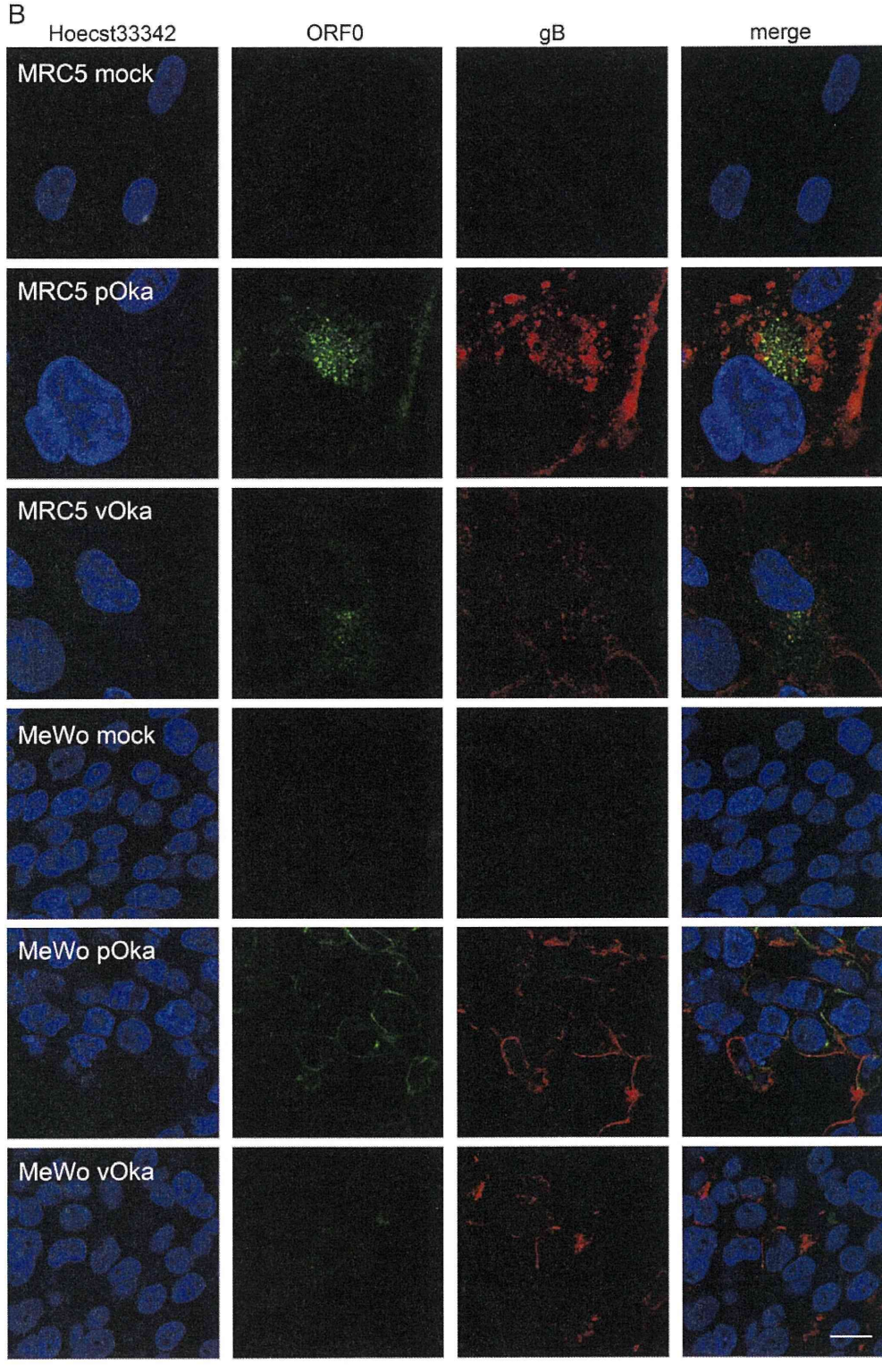

Fig 2 (*continued*).

resulting antisera specifically reacted with lysates from cells infected with either pOka or vOka (Fig. 2A). ORF0 was detected as a 17-kDa polypeptide in pOka-infected cells at 24 hpi and thereafter. In vOka-infected cells, the ORF0 was detected as a smeared band of 19- to 30-kDa polypeptides, beginning at 48 hpi.

Indirect immunofluorescence was used to determine the intracellular localization of ORF0 in VZV-infected cells (Fig. 2B). pOka- and vOka-infected cells were fixed, respectively, at 48 and 72 hpi. In MRC-5 cells infected with either virus, the ORF0 immunoreactivity accumulated at the perinuclear region of the cytoplasm and at the plasma membrane.

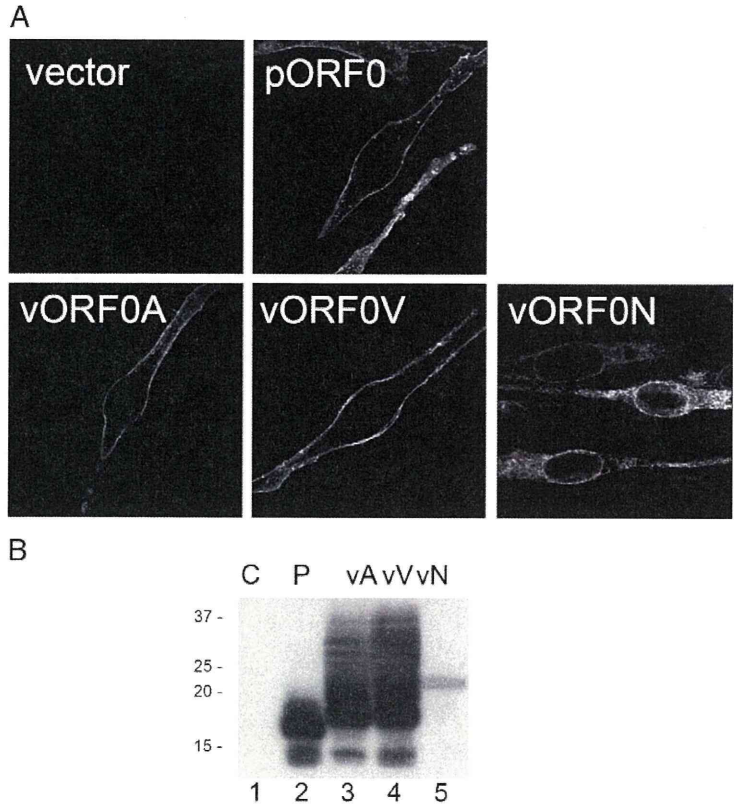

Fig. 3. Expression of ORF0 splice variants. (A) Intracellular localization of splice variants of ORF0. MeWo cells were transfected with the expression plasmid encoding pOka-ORF0 (pORF0), spliced vOka ORF0 with alanine at 154 (vORF0A), spliced vOka ORF0 with valine at 154 (vORF0V), or splicing-incompetent vOka ORF0 (vORF0N). Cells were stained with the anti-ORF0 antibody. (B) 293T cells were transfected with the expression plasmid encoding pOka-ORF0 (P: lane 2), spliced vOka ORF0 with alanine at 154 (vA: lane 3), spliced vOka ORF0 with valine at 154 (vV: lane 4), splicing-incompetent vOka ORF0 (vN: lane 5), or control (C: lane 1), and the cell lysates were separated by 13% SDS–PAGE and analyzed by western blotting with an anti-ORF0 antibody. Molecular mass markers are indicated at the left in kDa.

In MeWo cells infected with either pOka or vOka, the anti-ORF0 staining was marked at the plasma membrane and visible at the perinuclear region. In both cell lines, ORF0 partially colocalized with gB, a viral envelope glycoprotein (Fig. 2B).

Expression of vOka ORF0 splice variants

To determine whether the differing C-termini of the vOka ORF0 variants led to a difference in their intracellular localizations, we constructed the corresponding ORF0 expression plasmids and examined their expression in transfected cells (Fig. 3). The anti-ORF0 staining for pOka ORF0 (Fig. 3A, pORF0) was found predominantly at the plasma membrane as was the splice variant of vOka ORF0 with the alanine at aa 154 (Fig. 3A, vORF0A) and vORF0V, with a valine at aa 154 (Fig. 3A, vORF0V). The vOka ORF0 unspliced variant (vORF0N) was mislocalized, in that it was distributed throughout the cytoplasm (Fig. 3A).

To evaluate the contribution of the ORF0 C-terminus to each variant's band pattern on immunoblots, we used the lysates of transfected cells. pORF0 was detected as a 17-kDa polypeptide in these lysates (Fig. 3B, lane 2), as in the lysates of pOka-infected cells. Similarly, like the ORF0 of vOka-infected cells, vORF0A (Fig. 3B, lane 3) and vORF0V (Fig. 3B, lane 4) appeared as smeared bands. In contrast, vORF0N was detected as a sharp band of 20 kDa (Fig. 3B, lane 4). Therefore, the C-termini of the splice variants of vOka ORF0 caused the smeared band pattern.

N-linked glycosylation of vOka ORF0

To determine the specific cause of the smearing on the immunoblots, cells transfected with ORF0 plasmids or infected with VZV were

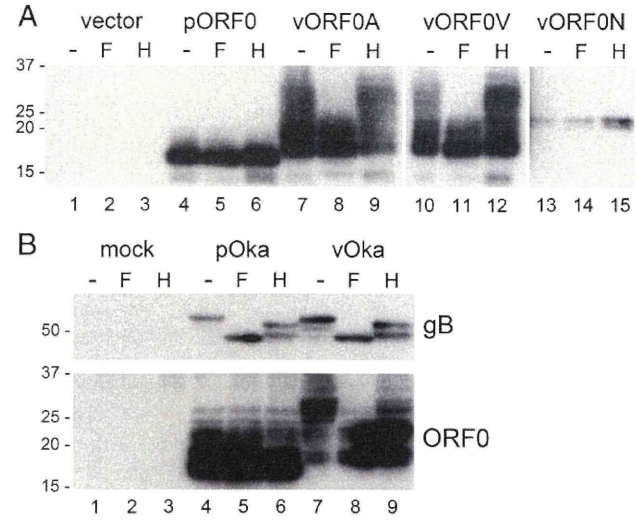

Fig. 4. N-linked glycosylation of vOka ORF0. (A) An expression plasmid encoding pOka-ORF0 (pORF0: lanes 4–6), spliced vOka ORF0 with alanine at 154 (vORF0A: lanes 7–9), spliced vOka ORF0 with valine at 154 (vORF0V: lanes 10–12) splicing-incompetent vOka ORF0 (vORF0N: lanes 13–15), or the control vector (vector: lanes 1–3) was transfected into 293T cells. The cell lysates were treated with PNGase-F (F: lanes 2, 5, 8, 11, 14), Endo-H (H: lanes 3, 6, 9, 12, 15), or without endoglycosidase (−: lanes 1, 4, 7, 10, 13). Samples were separated by SDS–13% PAGE and analyzed by western blotting. (B) VZV-infected cell lysates were analyzed with endoglycosidase. Lysates of mock-infected (lanes 1–3) or pOka (lanes 4–6)- or vOka (lanes 7–9)-infected cells were treated with PNGase-F (F: lanes 2, 5, 8), Endo-H (H: lanes 3, 6, 9), or without endoglycosidase (−: lanes 1, 4, 7). Samples were separated by SDS–PAGE and analyzed by western blotting.

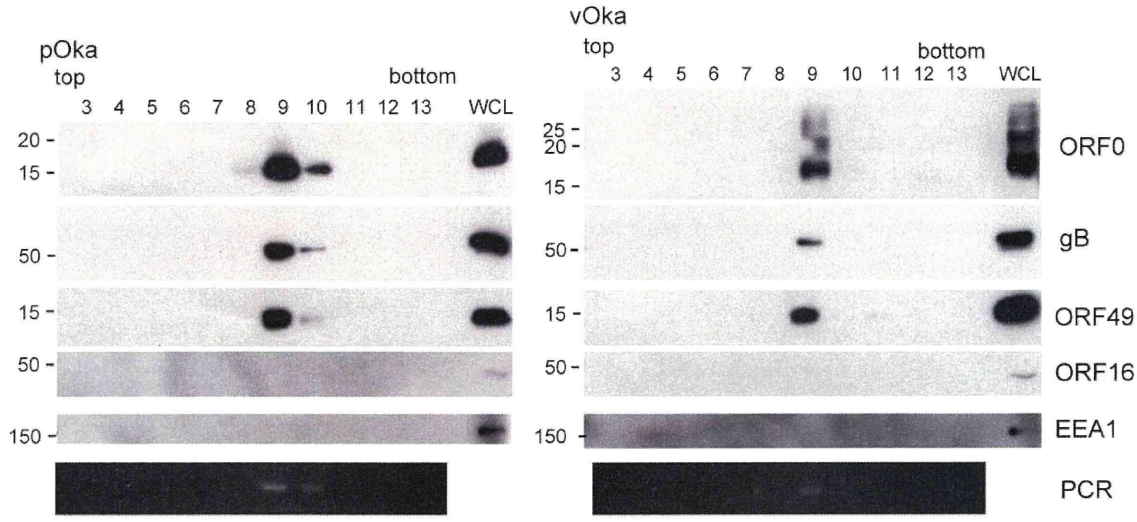

Fig. 5. The identification of ORF0 protein in VZV virus particles. (A) The virus particles of VZV were purified on a CsCl density gradient. The purified pOka (p) or vOka (v) virus particles were analyzed by western blotting with anti-gB, anti-ORF0, anti-ORF49, anti-ORF16, and anti-EEA1 antibodies. PCR was done as described in Materials and methods.

treated with endoglycosidases (Fig. 4). Although PNGase-F had no affect on the pORF0 band pattern (Fig. 4A, lane 5), both vORF0A (Fig. 4A, lane 8) and vORF0V (Fig. 4A, lane 11) migrated faster after the treatment. Endo-H did not change the band patterns of vORF0A or vORF0V (Fig. 4A, lane 9). However, the vORF0N band was not altered by either PNGase-F or Endo-H treatment (Fig. 4A, lanes 13–15). Likewise, in the lysates of VZV-infected cells, the bands for vOka ORF0 were smaller after PNGase-F treatment but not after Endo-H treatment (Fig. 4B, lanes 8 and 9). Thus, the vOka ORF0 protein translated from either splice variant is a glycoprotein modified with complex N-linked oligosaccharides.

ORF0 is incorporated into virus particles

To determine whether the ORF0 protein is a component of virus particles, pOka, and vOka virus particles were purified on a CsCl gradient and analyzed by western blotting (Fig. 5). The peak fraction that contained virus particles was determined by PCR (Fig. 5). As shown in Fig. 5, ORF0 proteins were detected in the peak fraction of both pOka and vOka. An envelope protein, gB, and a tegument protein, ORF49, which is the homologue of HSV UL11, were used as virus particle markers. In support of this finding, neither a non-structural viral protein, ORF16, which is the homologue of HSV UL42, nor a cellular protein, EEA1, was detected in the peak fraction. These results indicate that virus particles were purified successfully and that the ORF0 protein is a component of both pOka and vOka virus particles.

To determine the membrane orientation of the ORF0 proteins, purified VZV virus particles were treated with Proteinase K (ProK) in the presence or absence of detergent and then analyzed by western blotting (Fig. 6A). Proteins presented on the outside of virus particles, such the envelope glycoprotein gB, were sensitive to ProK both in the presence and absence of detergent (Fig. 6A, lanes 3, 4, 7, 8). The pOka ORF0 was sensitive to ProK only in the presence of detergent (Fig. 6A, lanes 3, 4), indicating that the N-terminus of pOka ORF0 was protected from ProK by the envelope and faced the tegument. The smeared bands of vOka ORF0 were sensitive to ProK in the absence of detergent (Fig. 6A, lane 7), and the vOka ORF0 band was shifted down to the molecular weight of pOka ORF0. The vOka ORF0 was also sensitive to ProK in the presence of detergent (Fig. 6A, lane 8). These results indicate that the C-terminus of the vOka ORF0 is presented on the surface of virus particles, but the N-terminus of both vOka and pOka ORF0 faces the tegument (Fig. 6B).

Construction of pOka and vOka recombinant viruses bearing each other's ORF0

To determine whether the C-terminus of the vOka ORF0 altered the growth of VZV, a recombinant pOka in which the native ORF0 was

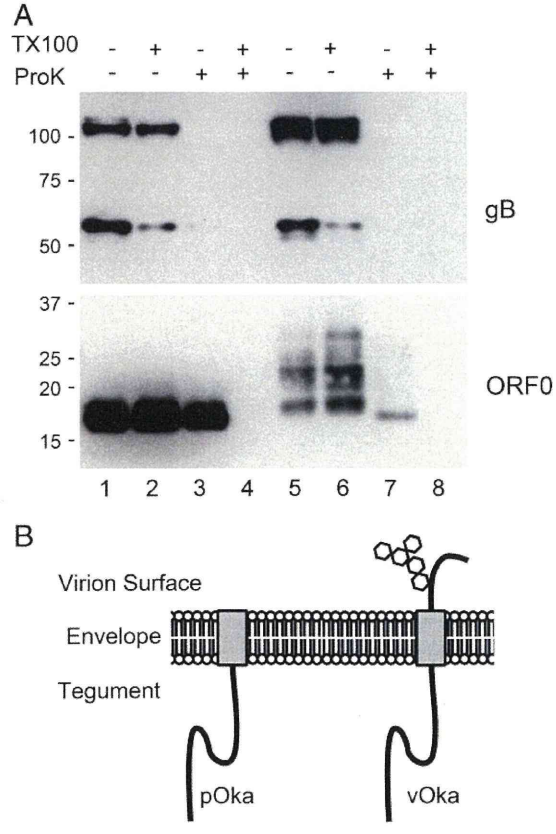

Fig. 6. Orientation of ORF0 in virus particles. (A) Purified virus particles were treated with ProK with or without detergent. The samples were separated by SDS–PAGE and analyzed by western blotting. Molecular mass markers are indicated at left. (B) Schematic representation of the membrane orientation of ORF0 proteins. The N-terminus of ORF0 faces the tegument of virus particles. The C-terminus of vOka ORF0 is presented on the surface of virus particles with N-linked glycosylation.

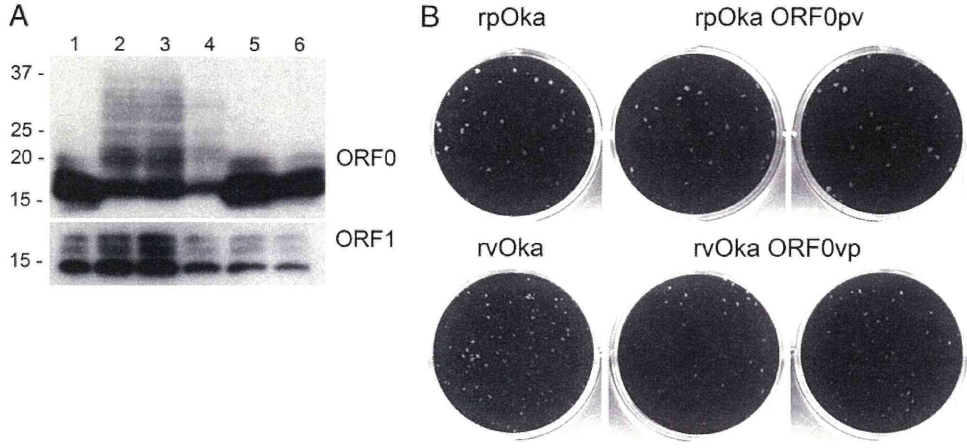

Fig. 7. Growth of ORF0 recombinant viruses and their intracellular protein expression. (A) MeWo cells were infected with rpOka, rpOka-vORF0, rvOka, or rvOka-pORF0 by cell-to-cell spread. Cell lysates were separated by SDS–PAGE and analyzed by western blotting. (B) MeWo cells infected with rpOka, rpOka-vORF0, rvOka, or rvOka-pORF0 were cultured for 1 week, fixed, and stained with 1% crystal violet.

replaced with that of vOka (rpOka-vORF0) and a recombinant vOka bearing the pOka ORF0 (rvOka-pORF0) were constructed by single-base substitution, as described in the Materials and methods. The successful substitution of the ORF0 stop codon was verified by sequencing, southern blotting (data not shown), and western blotting (Fig. 7A). There was no difference from wild-type VZV in the mean plaque size of either recombinant virus (Fig. 7B) in MeWo cells, indicating that the mutant C-terminus of the vOka ORF0 does not affect VZV growth.

To generate an ORF0-deficient virus, we constructed a recombinant virus rpOka-ORF0ΔTMD, which lacked the TMD of ORF0. No ORF0 protein was detected in rpOka-ORF0ΔTMD-infected cell lysates (Fig. 8A, ORF0). The ORF1 protein, which is a neighboring gene product of ORF0, was detected in the rpOka-infected cell lysates (Fig. 8A, ORF1), indicating that the neighboring gene was expressed normally. Viral gB was used as the marker of infection (Fig. 8A, gB). The amount of ORF0 RNA was decreased in the rpOka-ORF0ΔTMD-infected cells compared to the rpOka-infected cells (Fig. 8B). The growth of the rpOka-ORF0ΔTMD virus was not impaired in the plaque-formation assay using MeWo cells (Fig. 8C). Because the amount of ORF0 RNA was decreased in the rpOka-ORF0ΔTMD-infected cells by RT-PCR (Fig. 8B), the amount of ORF0 cDNA was compared with ORF1 cDNA by real-time PCR analysis. The copy number of ORF1 cDNA was set at 100%, and the abundance of ORF0 cDNA compared to ORF1 cDNA in the rpOka-infected cells was 97%, but it was 18% in the rpOka-ORF0ΔTMD-infected cells.

Discussion

The point mutation at the stop codon of pOka ORF0 caused the molecular weight differences in the ORF0 protein variant expressed by vOka. Because of this point mutation, the amino acid sequence at the C-terminus of vOka ORF0 was increased by 26 amino acids. When the stop codon was mutated in recombinant viruses, the molecular weight of the resulting ORF0 protein was changed to that of the vOka forms. These results indicate that the mutated C-terminal region of vOka ORF0 is responsible for its difference in molecular weight from pOka ORF0.

The N-terminus of the ORF0 protein was protected from ProK treatment, indicating that the N-terminus of the transmembrane domain faces the inside of the virus particle. Given the mechanisms of herpesvirus envelopment (Mettenleiter, 2002), the N-terminus of ORF0 must face the cytoplasm in infected cells and the inside of virus particles in virions. The C-terminus of vOka ORF0 was presented on the surface of virus particles, and it appears to be N-glycosylated at its C-terminus. The pOka and vOka ORF0 have two putative N-glycosylation sites at amino acid positions 65 to 67 and 126 to 128.

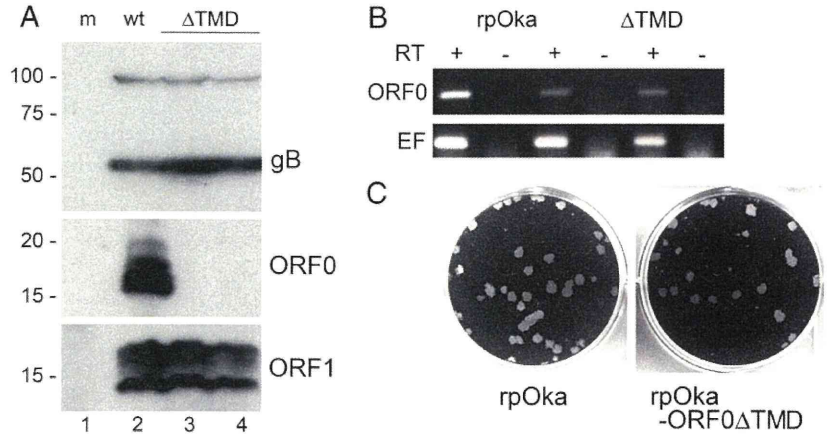

Fig. 8. Growth of ORF0-mutant-expressing virus. (A) MeWo cells were infected with the mutant virus or rpOkaORF0ΔTMD by cell-to-cell spread. Cell lysates were separated by SDS–PAGE and analyzed by western blotting. (B) RT-PCR. Total RNA was purified from virus-infected MeWo cells. After the cDNAs were synthesized with (+) or without (−) reverse transcriptase, VZV ORF0 and human elongation factor (EF) cDNAs were amplified by PCR. (C) MeWo cells infected with an rpOka ORF0 mutant lacking the TMD or rpOkaORF0ΔTMD, were cultured for 2 weeks, fixed, and stained with 1% crystal violet.

Although the pOka ORF0 possesses a putative N-glycosylation site at its C-terminus, it was not glycosylated, indicating that its C-terminus was inaccessible for glycosylation.

Given that the pOka and vOka ORF0s share their N-terminal and transmembrane sequences and that the N-terminus of both ORF0 proteins faces the cytoplasm of infected cells and the tegument of virus particles, the pOka and vOka ORF0s may interact with the same proteins and have similar N-terminally mediated functions. However, because of their different C-terminal sequences, their C-terminally mediated functions probably differ.

To date, the function of ORF0 remains unknown. The HSV-2 homolog of ORF0, UL56, which is mainly located at the perinuclear region of the cytoplasm (Koshizuka et al., 2002), associates with a ubiquitin E3 ligase, Nedd4, through the PY motif of UL56 (Ushijima et al., 2008). Because ORF0 also has a PY motif, it may also associate with Nedd4 family proteins. However, the intracellular localization of ORF0 differs from that of HSV-2 UL56, and therefore, ORF0 may possess one or more unique functions. In our experiments, viruses bearing the ORF0 stop codon mutants grew just as well as the wild-type virus, suggesting that the ORF0 mutation may not be related to the attenuation of vOka or that multiple point mutations of the VZV genome, including those in ORF0, may be necessary for the attenuation.

Similarly, even the complete removal of the ORF0 protein did not affect virus growth in our hands. A recombinant virus that lacked the TMD of ORF0 grew normally in MeWo cells, even though we confirmed that ORF0 was not expressed in them. This finding contradicts that of Zhang and colleagues, who reported that virus lacking the ORF0 gene showed severely retarded growth in vitro and in vivo (Zhang et al., 2007). However, in their study, Zhang et al. (2007) deleted the entire ORF0 sequence, whereas we removed only the TMD. Therefore, the discrepancy between our reports may be explained if the growth of the virus was affected by the deletion of the additional sequences rather than by the loss of ORF0 expression. The nucleotide sequences in the vicinity of the ORF0 gene may be important for DNA cleavage and/or packaging of the VZV genome because the ORF0 gene is located at the genomic terminus. Alternatively, the C-terminal region of ORF0 may not affect the viral growth.

Although weak RNA expression of the TMD-deleted ORF0 was detected by RT-PCR, the protein was not detectable by western blotting. The C-terminal TMD-deleted ORF0 protein should be detectable by the anti-ORF0 serum because this mono-specific serum was raised against the N-terminal region (amino acid positions 1–99) of the ORF0 protein. Therefore, this mutant ORF0 protein may not be expressed or may be expressed at an undetectable level in the recombinant virus-infected cells. Alternatively, the truncated ORF0 may be less stable than the wild-type protein, thus resulting in our inability to detect the protein.

A difference in the cellular localization patterns between the ORF0s of pOka and vOka might have suggested differing functions for these proteins. However, the ORF0 proteins of both pOka and vOka accumulated in the perinuclear region of the cytoplasm in infected MRC5 and MeWo cells, and both ORF0s partially colocalized with gB in infected MRC5 cells. Both ORF0s were located at the cell rim of infected MeWo cells, indicating that they were localized to the plasma membrane, like the ORF1 protein (Koshizuka et al., 2008). Thus, the localization patterns of the pOka and vOka ORF0 proteins did not differ substantially, and no clues to functional differences could be obtained from these data.

However, one way the function of the vOka ORF0 might be altered is through the N-linked glycosylation of its C-terminus, which we discovered because the bands of vOka ORF0 were smeared on immunoblots of infected and transfected cells. This novel modification might, in particular, affect vOka cell tropism. In the case of measles virus, the vaccine strain uses both SLAM and CD46 as receptors, whereas most wild-type strains use only SLAM (Dorig et al., 1993; Erlenhofer et al., 2002; Tatsuo et al., 2000). Point mutations in the hemagglutinin protein of the vaccine strain account for the measles virus's use of CD46 (Tahara et al., 2007). The C-terminus of vOka ORF0 may affect the attachment of vOka virus particles to target cells, by altering its interactions with cellular molecules. If this scenario is correct, antibodies raised against the C-terminus of vOka ORF0 may be responsible for the neutralization of the virus. In addition, some other post-translational modification(s) may contribute to changes in vOka ORF0 function. Even after the N-glycosylation was removed by digestion with PNGase-F, the band pattern of vOka ORF0 was still smeared. This issue will be addressed in future studies.

Materials and methods

Cells and viruses

MRC-5 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Human melanoma cells (MeWo) and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The VZV strains, pOka and vOka, were propagated in MRC-5 cells or MeWo cells. The preparation of cell-free viruses and the purification of virus particles from the infected MeWo cells were described previously (Koshizuka et al., 2008). Purified virus particles were analyzed by electron microscopy with negative staining, as described previously (Sadaoka et al., 2007).

Plasmids

The *Escherichia coli* expression plasmid pGEX-ORF0 was constructed by amplifying the domains encoding ORF0 codons 1 to 99 by PCR from the pOka genome and cloning the resulting DNA fragment into pGEX-4T-1 (GE Healthcare Biosciences, Piscataway, NJ) in frame with glutathione-*S*-transferase (GST). In pGEX-ORF0, the initiation methionine of ORF0 was altered to alanine. Total RNAs were extracted from pOka- or vOka-infected MeWo cells by TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's manual. The cDNA was prepared by reverse transcription (RT) with oligo(dT) using the Superscript III kit (Invitrogen). The DNA fragment of full-length ORF0 was amplified from pOka cDNA by PCR with primers ORF0-f1 (GGGGGCGGCCGCCATGGCGACCGTGCACTAC; *Not*I site is underlined) and ORF0-r3 (GGGGCTCGAGTCATGTAGTTGAGTTGGG; *Xho*I site is underlined). The DNA fragment was cloned into pCAGGS-MCS (Niwa et al., 1991) to generate pCAGGS-pORF0.

The vOka ORF0 was amplified from vOka cDNA by PCR with primers ORF0-f1 and ORF0-r4 (GGGGCTCGAGTCAGCATACAGAGCTTATGCAG; *Xho*I site is underlined), cloned into pCR2.1-TOPO (Invitrogen), and sequenced. Spliced vOka ORF0 cDNA was amplified by PCR with ORF0-f1 and ORF0-rvA (GGGCTCGAGTTATTCCACTGGTTGATGTCC; *Xho*I site is underlined) and cloned into pCAGGS-MCS to generate pCAGGS-vORF0A. The splicing donor site of unspliced vOka ORF0 was substituted by PCR with primers splice-mutF (CCGGAAGGGGAAGGCATTTATTCTCGCTTG) and splice-mutR (CAAGCGAGAATAAATGCCTTCCCCTTCCGG) to generate a splicing-incompetent mutant, pCAGGS-vORF0N.

Antibodies

The GST-ORF0 (amino acid positions 1–99) fusion protein was purified with glutathione sepharose beads (GE Healthcare Bioscience) and used as an antigen to generate rabbit anti-ORF0 mono-specific antibodies. The purification of anti-ORF0 was performed as described previously (Koshizuka et al., 2008). The anti-glycoprotein B (gB), anti-ORF16, and anti-ORF49 rabbit antibodies were described previously (Sadaoka et al., 2007). The anti-gB monoclonal antibody was described previously (Okuno et al., 1983). The anti-EEA1 (clone 14; BD Biosciences Pharmingen) monoclonal antibody was purchased.

Immunofluorescence assay

MRC-5 or MeWo cells were grown on coverslips and subjected to an immunofluorescence assay as described previously (Koshizuka et al., 2008).

Transfection

MeWo cells were transfected with the indicated plasmids by Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. 293T cells were transfected with the indicated plasmids by the calcium phosphate method. Ten micrograms of plasmid DNA was combined with 50 μl of 2.5 M $CaCl_2$ and 440 μl of sterile water. The calcium–DNA solution was mixed with 2× HEPES-buffered saline (140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.1). After incubation at RT for 30 min, the calcium phosphate–DNA suspension was transferred to the 293T cells. After another incubation, the medium was changed, and the 293T cells were harvested at 72 h post transfection.

Purification of virus particles

VZV virions were purified as described previously (Sadaoka et al., 2007). After the second Histodenz gradient purification, the virus particle-containing fraction was diluted with PBS and pelleted by centrifugation at 27,000 rpm for 2 h at 4 °C with a P40ST rotor (Hitachi). The pellet was suspended in PBS and layered onto a continuous 10–40% CsCl gradient, followed by centrifugation at 20,000 rpm for 48 h at 4 °C with a P40ST rotor (Hitachi). Fractions were collected from the bottom and analyzed by western blotting. Viral DNA was purified from fractions and analyzed by PCR using the primer pair ORF0-f1 and ORF0-r2 (GTCGACCTCACGGTACACAGCATTTGCG).

Protease assay

The protease assays were performed essentially as described previously (Koshizuka et al., 2002). Protease inhibitor cocktail (Sigma) was added to purified virus particles to prevent unexpected proteolysis. The purified virus particles were treated with ProK at a final concentration of 500 μg/ml in the presence or absence of 1% Triton X-100. After an incubation at 37 °C for 30 min, 2 mM PMSF was added to the samples to terminate the ProK reaction, and the samples were incubated on ice for 5 min. The samples were subjected to SDS–PAGE without boiling and analyzed by western blotting.

BAC mutagenesis

The recombinant pOka virus (rpOka) was constructed as previously reported (Nagaike et al., 2004; Yoshii et al., 2007). In brief, rpOka was reconstituted by transfecting MRC-5 cells with the pOka-BAC genome (pOka-BAC) (Nagaike et al., 2004; Yoshii et al., 2007). To remove the BAC sequence, the MRC-5 cells were first infected with a recombinant adenovirus, AxCANCre, which expresses the Cre recombinase (kindly provided by Dr. Y. Kawaguchi). The cells were then super-infected with the recombinant virus and cultured until plaques without GFP appeared (Kanegae et al., 1995; Tanaka et al., 2003).

To generate recombinant virus with the C-terminus TMD of ORF0 deleted (rpOkaORF0ΔTMD), nucleotide positions 457–589 of the pOka genome were replaced with a kanamycin resistance (Km^r) gene. The DNA fragments containing the Km^r gene were amplified with primers dORF0TMDfw407-456 (5′-ATGATGGAATCCACAGACTTCAGCTGGCTTTTCTAAGAATTCGCAAATGCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGCAAGCGAACCGGAATTGC-3′) and dORF0TMDrv590-541 (5′-TAAATACCTTCCCCTTCCGGACAGTAGTTTCATGTAGTTGAGTTGGGAGGGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCCTTTTTCAATTCAGAAGAACTC-3′). The DNA fragments were introduced by electroporation into *E. coli* DH10B harboring the pOka-BAC genome and pGETrec, which was a kind gift from Dr. P. A. Ioannou (Narayanan et al., 1999).

To generate recombinant viruses in which the ORF0 of vOka was replaced by the one for pOka and vice-versa, the stop codon of pOka ORF0 (at nucleotide position 560 of the VZV genome) was replaced with the codon for arginine, and the arginine codon of vOka ORF0 was replaced with the stop codon. These changes were performed using a two-step Red-mediated mutagenesis procedure (Tischer et al., 2006) in *E. coli* GS1783, essentially as described by Jarosinski et al. (2007). *E. coli* GS1783 harboring the pOka-BAC or vOka-BAC genome was used. The primer pairs for the amplification were as follows: ORF0pv1 (5′-GTTGCCGTTTTTCCCGAGGAACCTCCCAACTCAACTACACGAAACTACTGTCCGGAAGGGAGGATGACGACGATAAGTAGGG-3′) and ORF0pv2 (5′-AAGCGAGAATAAATACCTTCCCCTTCCGGACAGTAGTTTCGTGTAGTTGAGTTGGGAGGTCAACCAATTAACCAATTCTGATTAG-3′) or ORF0vp1 (5′-GTTGCCGTTTTTCCCGAGGAACCTCCCAACTCAACTACATGAAACTACTGTCCGGAAGGGAGGATGACGACGATAAGTAGGG-3′) and ORF0vp2 (5′-AAGCGAGAATAAATACCTTCCCCTTCCGGACAGTAGTTTCATGTAGTTGAGTTGGGAGGTCAACCAATTAACCAATTCTGATTAG-3′). The substitution site is underlined. The successful recombination was confirmed by PCR and southern blotting. MeWo cells were transfected with the recombinant virus genomes by using the Lipofectamine 2000 reagent.

Growth analysis of recombinant viruses

MeWo cells were infected with cell-free rpOka, rpOka-vORF0, rvOka, rvOka-pORF0, or rpOkaORF0ΔTMD viruses and incubated at 37 °C. The medium was replaced every 2 days, and the cells were stained with 1% crystal violet/70% ethanol for analysis (Yoshii et al., 2008).

RT-PCR and real-time PCR

Total RNA was isolated from the recombinant virus-infected MeWo cells using TRIzol (Invitrogen), and its concentration and purity were determined by spectrophotometry. The RNA integrity was verified by electrophoresis on 1.2% denaturing formaldehyde gels. One microgram of total RNA was transcribed using Superscript III reverse transcriptase (Invitrogen). An equal amount of cDNA (1/10 of the reaction) was used in each PCR. PCR was performed using GoTaq Green Master Mix (Promega). A primer pair for the human elongation factor gene was used as the control (EF-F: GCTCCAGCATGTTGTCACCATTC; EF-R: GGTGAATTTGAAGCTGGTATCTC). The ORF0 cDNA was detected using primers ORF0-f1 and ORF0-reverse (GTGCCATTCTCCACGTGAGG).

SYBR Green PCRs were performed in an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA) with specific primers for ORF0 (ORF0-f1 and ORF0-reverse:GTGCCATTCTCCACGTGAGG) and ORF1 (ORF1-1f: GAATTCGCGTCCAGGGTATCGGAGTA and ORF1-2r: CTCGAGTTAGCATAACCTGTCCATTTGCAT), according to the manufacturer's manual. The thermal profile for all SYBR Green PCRs was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. At the end of each SYBR Green PCR run, data analysis was performed by the SDS program version 1.4 (Sequence Detection Software; Applied Biosystems). Each sample was analyzed in duplicate.

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International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan) for the AxCANCre, and Dr. Jun-ichi Miyazaki (Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Osaka, Japan) for the pCAGGS.

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ORIGINAL ARTICLE

Improvement of the H5N1 influenza virus vaccine strain to decrease the pathogenicity in chicken embryos

Norikazu Isoda · Yoshihiro Sakoda · Masatoshi Okamatsu · Yoshimi Tsuda · Hiroshi Kida

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Abstract The avian influenza vaccine strain A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1) was found to be pathogenic in chicken embryos (CEs). In order to decrease the pathogenicity of Vac-1 in CEs, a series of reassortant viruses was generated between Vac-1 and A/Puerto Rico/8/1934 (H1N1) (PR8), and their pathogenicity and growth potential were compared in CEs. The results indicated that either the PB1 or PA protein was responsible for the pathogenicity of Vac-1 in CEs. The HA titers of the allantoic fluids of CEs inoculated with the recombinant H5N1 viruses, of which pathogenicity was lower than that of the recombinant Vac-1 prepared by reverse genetics in CEs, were equivalent to those of CEs inoculated with the recombinant Vac-1. One of the reassortant viruses, rg-PR8-PA/Vac-1 (H5N1), in which the PA gene was replaced with the corresponding gene of PR8, yielded allantoic fluids with the same HA titer as that of Vac-1, indicating that this reassortant should be a good candidate as an improved vaccine strain.

N. Isoda · Y. Sakoda · M. Okamatsu · Y. Tsuda · H. Kida (✉)
Laboratory of Microbiology,
Department of Disease Control,
Graduate School of Veterinary Medicine,
Hokkaido University, Kita-18 Nishi-9,
Kita-ku, Sapporo, Hokkaido 060-0818, Japan
e-mail: kida@vetmed.hokudai.ac.jp

Y. Tsuda · H. Kida
Research Center for Zoonosis Control,
Hokkaido University, Sapporo,
Hokkaido 060-0818, Japan

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have spread to 62 countries in Eurasia and Africa from Southeast Asia since 1996, and well over 400 million birds have died or been culled [27, 28]. This has greatly affected not only the poultry industry but also public health. H5N1 HPAI viruses infected 18 humans, and 6 died in Hong Kong in 1997 [2]. Since 2003, there have been 498 human cases of H5N1 virus infection, with 294 deaths in 15 countries as of May 6, 2010 [27]. "Stamping-out" is the basic measure for the control of HPAI. Vaccination may be an additional option when the disease spreads widely [18]. Inactivated H5 and H7 avian influenza virus vaccines have been prepared and evaluated by several research groups [9, 12, 20, 24, 25]. It is, therefore, important to assess the antigenicity, pathogenicity, and growth potential of the vaccine strains in chicken embryos (CEs).

CEs are currently used as the host in which influenza viruses can grow in sufficient amounts for vaccine production. It is well known that the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are responsible for extensive replication of influenza viruses in embryonated chicken eggs [1, 4, 7, 14, 15]. Amino acid substitutions in the vicinity of the receptor-binding site of HA are responsible both for growth potential in CEs and antigenicity [1]. After several passages in embryonated chicken eggs, influenza A and B viruses with amino acid substitutions in the vicinity of the receptor-binding pocket on the HA molecule show high growth potential [11, 15, 16]. The NA contributes to enhancement of virus yield in embryonated chicken eggs [7]. Amino acid substitutions in the HA and/or NA, however, lead not only to extensive growth but also to antigenic variation of the virus [15]. Internal proteins, such as PB2 and NP, which are components of the ribonucleoprotein (RNP)

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complex, may also contribute to the replication of influenza viruses in embryonated chicken eggs [16]. The pathogenicity of influenza viruses in CEs also affects the yield of the virus suspension, because embryo death makes it difficult to harvest infectious allantoic fluids due to postmortem change.

In a previous study, a non-pathogenic H5N1 reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1), was generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), both of which were isolated from migratory ducks in Asia [9]. Phylogenetic analysis of the H5 HA genes revealed that A/duck/Mongolia/54/2001 (H5N2) belonged to the Eurasian lineage [22]. In addition, antigenic analysis using a panel of monoclonal antibodies to the H5 HA proteins and antiserum to Vac-1 indicated that the HAs of HPAI viruses currently circulating in Asia were antigenically closely related to that of Vac-1 [22]. The inactivated vaccine prepared from Vac-1 was confirmed to be potent in a previous study [9]; however, the CEs in the eggs inoculated with Vac-1 died between 48 and 72 hours post-inoculation. Ideally, vaccine strains should be less pathogenic in chicken embryos for vaccine manufacture, although Vac-1 was defined as a nonpathogenic virus strain in 6-week-old chickens [9].

In the present study, it was revealed that the PB1 and PA proteins of Vac-1 influenza virus are responsible for pathogenicity in CEs. One of the reassortant viruses, rg-PR8-PA/Vac-1 (H5N1), in which PA gene was replaced with the corresponding gene of PR8, yielded allantoic fluids with the same HA titer as that of Vac-1, indicating that this reassortant should be a good candidate as a vaccine strain.

Materials and methods

Viruses

A/duck/Hokkaido/Vac-1/2004 (H5N1) was selected from reassortants between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), both of which were isolated from fecal samples from migratory ducks in Asia [9, 23]. The NA and NS gene segments of Vac-1 are derived from A/duck/Mongolia/47/2001 (H7N1), and the other 6 segments from A/duck/Mongolia/54/2001 (H5N2) [23]. Nucleotide and amino acid sequences of the eight genes of Vac-1 (H5N1) were submitted to the DNA Data Bank of Japan under accession numbers AB253760 (PB2), AB253761 (PB1), AB257726 (PA), AB263192 (HA), AB263193 (NP), AB263194 (NA), AB263195 (M), and AB263196 (NS) [9]. A/Puerto Rico/8/1934 (H1N1) was provided by St. Jude Children's Research Hospital, USA. PR8 and Vac-1 were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hours, and the allantoic fluids were then harvested.

Generation of recombinant viruses

A series of plasmids carrying the eight gene segments of PR8 was provided by Drs. E. Hoffmann and R. Webby, St. Jude Children's Research Hospital. The universal primer set for influenza A viruses was used for RT-PCR [6]. Each of the PCR products of the eight gene segments of Vac-1 was cloned into pCR 2.1 TOPO vector (Invitrogen). Eight segments of Vac-1 were cloned into a dual-promoter plasmid, pHW2000 [5]. The plasmids carrying either the PB1 or PA gene of PR8 or Vac-1 were used to construct chimeric gene segments. To exchange the PB1 genes between PR8 and Vac-1, the plasmids carrying the PB1 gene of either PR8 or Vac-1 were digested with *Mfe*I (Takara Bio; cleavage site, nucleotide position 719 in the PB1 gene) and *Bam*HI (Takara Bio; cleavage site, nucleotide position 1997 in PB1 gene), and both of their reading frames were divided into three fragments. The fragments were cloned, and the resulting 6 plasmids were designated as pHW-PB1/P719V (nucleotide position 1 to 719 of PR8, and the other region of Vac-1), pHW-PB1/V719P (nucleotide position 1 to 719 of Vac-1, and the other region of PR8), pHW-PB1/V719P1997V (nucleotide position 720 to 1997 of PR8, and the other regions of Vac-1), pHW-PB1/P719V1997P (nucleotide position 720 to 1997 of Vac-1, and the other regions of PR/8), pHW-PB1/V1997P (nucleotide position 1 to 1997 of Vac-1, and the other region of PR8), and pHW-PB1/P1997V (nucleotide position 1 to 1997 of PR8, and the other region of Vac-1). To exchange the PA gene between PR8 and Vac-1, plasmids carrying the PA gene of either PR8 or Vac-1 were digested with *Csp*45I (Toyobo; cleavage site, nucleotide position 706 in the PA gene) and *Sph*I (Takara Bio; cleavage site, nucleotide position 1240 in the PA gene), and both of their reading frames were divided into three fragments. The fragments were cloned, and the resulting 6 plasmids were designated as pHW-PA/P706V (nucleotide position 1 to 706 of PR8, the other region of Vac-1), pHW-PA/V706P (nucleotide position 1 to 706 of Vac-1, and the other region of PR8), pHW-PA/V706P1240V (nucleotide position 707 to 1240 of PR8, and the other regions of Vac-1), pHW-PA/P706V1240P (nucleotide position 707 to 1240 of Vac-1, and the other regions of PR8), pHW-PA/V1240P (nucleotide position 1 to 1240 of Vac-1, and the other region of PR8), and pHW-PA/P1240V (nucleotide position 1 to 1240 of PR8, and the other region of Vac-1).

Recombinant viruses were generated by reverse genetic methods according to Hoffman et al. [5]. Briefly, 293T cells and Madin-Darby canine kidney (MDCK) cells were cocultured in 35-mm dishes and transfected with 1 μg of each of the eight plasmids and 16 μl of TransIT-293T (Promega) in a total volume of 1 ml of OPTI-MEM (Gibco). After 30 hours, 1 ml of OPTI-MEM with 5 μg/ml

Table 1 MDTs of chicken embryos inoculated with wild-type and recombinant influenza viruses

Virus	Gene segment [a]								MDT ± SD (h) [b]
	PB2	PB1	PA	HA	NP	NA	M	NS	
wt-PR8	□	□	□	□	□	□	□	□	95.0 ± 9.8
wt-Vac-1	■	■	■	■	■	■	■	■	64.0 ± 11.9
rg-PR8	□	□	□	□	□	□	□	□	86.2 ± 13.2
rg-Vac-1	■	■	■	■	■	■	■	■	55.2 ± 5.9
rg-Vac-PB2-PB1-PA/PR8	■	■	■	□	□	□	□	□	※62.3 ± 8.6
rg-PR8-PB2-PB1-PA/Vac-1	□	□	□	■	■	■	■	■	※※79.1 ± 12.9
rg-Vac-1-HA-NA/PR8	□	□	□	■	□	■	□	□	88.0 ± 10.1
rg-PR8-HA-NA/Vac-1	■	■	■	□	■	□	■	■	62.2 ± 14.3
rg-Vac-1-NP-M-NS/PR8	□	□	□	□	■	□	■	■	※68.4 ± 11.4
rg-PR8-NP-M-NS/Vac-1	■	■	■	■	□	■	□	□	60.4 ± 9.6
rg-Vac-1-PB2/PR8	■	□	□	□	□	□	□	□	82.2 ± 3.9
rg-PR8-PB2/Vac-1	□	■	■	■	■	■	■	■	※※72.0 ± 15.5
rg-Vac-1-PB1/PR8	□	■	□	□	□	□	□	□	※53.3 ± 5.7
rg-PR8-PB1/Vac-1	■	□	■	■	■	■	■	■	※※88.0 ± 18.7
rg-Vac-1-PA/PR8	□	□	■	□	□	□	□	□	※69.6 ± 9.3
rg-PR8-PA/Vac-1	■	■	□	■	■	■	■	■	※※90.2 ± 18.1

[a] White; derived from A/Puerto Rico/8/1934 (H1N1), Black; derived from A/duck/Hokkaido/Vac-1/2004 (H5N1)

[b] Values significantly different from that of rg-PR8 at the 5% level are indicated by a single asterisk, and values significantly different from that of rg-Vac-1 at the 5% level are indicated by double asterisks

acetyltrypsin was added, and the cells were incubated at 35°C for 48 hours. One hundred μl of the supernatant was inoculated into 10-day-old embryonated chicken eggs, which were then incubated at 35°C for 48 hours.

Mean death time of CEs inoculated with recombinant influenza viruses

Wild-type or recombinant viruses generated by the reverse genetic method were inoculated into the allantoic cavities of ten 9-day-old embryonated chicken eggs at 100 times the 50% egg infectious dose (EID_{50}). All eggs were incubated at 35°C and observed for embryo death every eight hours until 120 hours post-inoculation. The mean death time (MDT) of the CEs was calculated as the mean hours until the death of all ten inoculated CEs. Differences in pathogenicity between rg-PR8 or rg-Vac-1 and the recombinant viruses were evaluated statistically using Student's t-test at the 5% level.

Comparison of the HA titer of recombinant viruses in embryonated chicken eggs

Each recombinant virus was inoculated into the allantoic cavities of six 9-day-old embryonated chicken eggs at a dosage of 100 EID_{50} and incubated at 35°C for 72 hours. Allantoic fluids of the eggs were collected every 6 or 12 hours starting at 24 hours post-inoculation. Dead CEs were not sampled further. An HA test was performed to assess the HA titer of each of the collected allantoic fluids. The HA titers of allantoic fluids of CEs at each time point were evaluated statistically using the Welch t-test at the 5% level [26].

Results

MDTs of CEs inoculated with reassortant influenza viruses

The MDT of CEs inoculated with rg-PR8 and rg-Vac-1 generated by reverse genetic methods was 86.2 and 55.2 hours, respectively (Table 1). Although the MDTs of these recombinant viruses were not equal to those of the respective wild-type viruses, significant differences were not found between the MDTs of wild-type and recombinant viruses. The MDT of either the wild-type or reassortant of Vac-1 was approximately 30 hours shorter than that of PR8. The MDT of CEs inoculated with rg-Vac-1-PB2-PB1-PA/PR8 (H1N1), which had PB2, PB1, and PA gene

Table 2 MDTs of chicken embryos inoculated with influenza viruses with a chimeric gene segment

Virus	Gene segment [a, b]				MDT ± SD (h) [c]
	PB2	PB1	PA	Other genes	
rg-PR8					86.2 ± 13.2
rg-Vac-1					55.2 ± 11.9
	1	240 665 758			
rg-PB1/P719V					69.0 ± 20.0
rg-PB1/V719P					77.6 ± 23.0
rg-PB1/V719P1997V					※※82.4 ± 17.4
rg-PB1/P719V1997P					※68.0 ± 16.2
rg-PB1/V1997P					58.6 ± 17.3
rg-PB1/P1997V					81.4 ± 21.3
			1 235 413 716		
rg-PA/P706V					※※86.0 ± 20.0
rg-PA/V706P					※53.0 ± 10.4
rg-PA/V706P1240V					※※82.4 ± 21.0
rg-PA/P706V1240P					85.0 ± 23.9
rg-PA/V1240P					69.6 ± 20.3
rg-PA/P1240V					※67.2 ± 14.2

[a] White; derived from A/Puerto Rico/8/1934 (H1N1), Black; derived from A/duck/Hokkaido/Vac-1/2004 (H5N1)

[b] Chimeric parts are described in Materials and methods

[c] Values significantly different from that of rg-PR8 at the 5% level are indicated by single asterisks, and values significantly different from that of rg-Vac-1 at the 5% level are indicated by double asterisks

segments from Vac-1 and the others from PR8, was 62.3 hours. The MDT of CEs inoculated with rg-PR8-PB2-PB1-PA/Vac-1 (H5N1), which had PB2, PB1, and PA gene segments from PR8 and the others from Vac-1, was 79.1 hours. The pathogenicity of PR8 in CEs increased upon recombination of these three gene segments from Vac-1, and conversely, that of Vac-1 decreased upon recombination of these gene segments from PR8. The MDTs of CEs inoculated with rg-Vac-1-HA-NA/PR8 (H5N1) and rg-PR8-HA-NA/Vac-1 (H1N1) were not significantly different from those inoculated with rg-PR8 and rg-Vac-1, respectively. It is thus postulated that the virus glycoproteins of Vac-1 are not responsible for pathogenicity in CEs. The MDT of CEs inoculated with rg-Vac-1-NP-M-NS/PR8 (H1N1), which had NP, M, and NS gene segments from Vac-1 and the others from PR8, was 68.4 hours. Although an aggravation of the pathogenicity of recombinants in CEs upon the introduction of the gene segments from Vac-1 was observed, no significant difference was found in the pathogenicity in CEs between rg-Vac-1 and rg-PR8-NP-M-NS/Vac-1. These results indicate that, although it is possible that some of these three genes should be responsible for pathogenicity, it is unlikely that they are responsible for the pathogenicity of PR8 or Vac-1 in CEs.

The present results of the MDT test indicate that the viral polymerase proteins of Vac-1 are responsible for pathogenicity in CEs. To identify the virus protein(s) responsible for the pathogenicity in CEs, 6 clones of single gene reassortant viruses were prepared, and the MDT of each recombinant virus was determined. The MDTs of CEs inoculated with recombinant viruses possessing 7 gene segments from Vac-1 and one from PR8 were significantly different from those of CEs inoculated with rg-Vac-1. In addition, for three recombinant PR8 viruses possessing 7 gene segments from PR8 and one from Vac-1, the MDTs of CEs inoculated with each of the recombinant viruses except rg-Vac-1-PB2/PR8 (H1N1) were significantly different from those of CEs inoculated with rg-PR8. Recombinant PR8 viruses with either the PB1 or PA gene of Vac-1 were more pathogenic in CEs than rg-PR8, and recombinant Vac-1 viruses with either of the genes of PR8 were less pathogenic in CEs than rg-Vac-1. These results indicate that the PB1 and PA proteins of Vac-1 are responsible for the pathogenicity in CEs.

Identification of amino acid region(s) responsible for pathogenicity in CEs

Chimeric genes of the PB1 and PA gene segments of either PR8 or Vac-1 were constructed to identify the amino acid residue(s) of the encoded proteins responsible for the pathogenicity in CEs on the basis of the MDTs (Table 2).

A mutant PR8 virus with the pHW-PB1/P719V1997P gene was more pathogenic in CEs than rg-PR8. On the other hand, a mutant Vac-1 with the pHW-PB1/V719P1997V gene showed lower pathogenicity in CEs than rg-Vac-1. Sequence analysis showed that the nucleotide region from 720 to 1997 in the PB1 gene encodes amino acids 240 to 665 of the PB1 protein, indicating that this region of the PB1 protein is responsible for the pathogenicity of Vac-1 in CEs. The MDT of CEs inoculated with rg-PA/P706V (H5N1), which had a chimeric PA gene from nucleotide position 706 to 1240, was 86.0 hours. The MDT of CEs inoculated with rg-PA/V706P (H1N1), which was also chimeric at the same residues, was 53.0 hours. Sequence analysis showed that the nucleotide region from 1 to 707 in the PA gene encoding amino acids 1 to 235 in the PA protein is responsible for the pathogenicity of Vac-1 in CEs.

Comparison of HA titers of recombinant viruses in embryonated chicken eggs

The HA titers of recombinant viruses in embryonated chicken eggs were compared. Each of the recombinant H5N1 viruses that showed low pathogenicity in CEs was inoculated into 9-day-old embryonated chicken eggs. The infectious allantoic fluids were collected every 6 or 12 hours starting at 24 hours post-inoculation. Maximum HA titers of the allantoic fluids of embryonated chicken eggs inoculated with rg-Vac-1 and those inoculated with the other recombinant viruses were between 128 and 512 (Fig. 1). HA titers of rg-PR8-PB1/Vac-1 (H5N1) at 48 and 72 hours post-inoculation were significantly lower than those of rg-Vac-1. On the other hand, HA titers of rg-PR8-PA/Vac-1 (H5N1) were not lower than those of rg-Vac-1 at any of the time points. These results indicate that, for the four H5N1 recombinant viruses generated from Vac-1, which are less pathogenic in CEs than Vac-1, the HA titer of rg-PR8-PA/Vac-1 (H5N1) in CEs stayed at a high level similar to that of Vac-1 for 72 hours after inoculation.

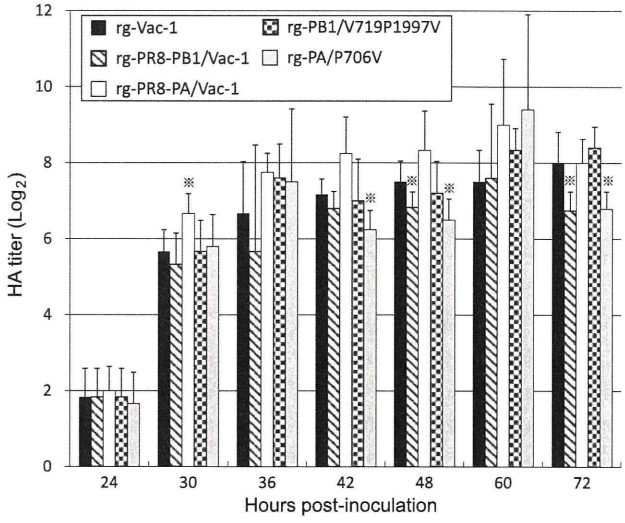

Fig. 1 Virus growth in chicken embryos. Each of six recombinant virus strains generated by reverse genetics was inoculated into six 9-day-old embryonated chicken eggs and incubated at 35°C for 72 hours. The allantoic fluids of the eggs inoculated with each virus were collected every 6 or 12 hours, starting at 24 hours post-inoculation, and samples were titrated by HA test. The mean (bar) and standard deviation (line) of the HA titer at each time point are shown. The differences in HA titer at each time point were evaluated statistically using the Welch t-test. An asterisk indicates a significant difference between the HA titers of the allantoic fluids of embryonated chicken eggs inoculated with rg-Vac-1 and those of each recombinant virus at the 5% level

Discussion

An influenza virus strain, A/duck/Hokkaido/Vac-1/2004 (H5N1), was generated by genetic reassortment between non-pathogenic H5N2 and H7N1 isolates from migratory ducks in order to prepare an inactivated avian influenza vaccine [9, 21, 23]. In the previous study, it was shown that the NA and NS gene segments of Vac-1 are derived from A/duck/Mongolia/47/2001 (H7N1), and the other segments from A/duck/Mongolia/54/2001 (H5N2) [23]. Although the pathogenicity of Vac-1 in CEs was not high (MDT = 64.0 hours), it should ideally be less pathogenic in CEs. In the present study, the contribution of the PB1 and PA proteins of the vaccine strains to pathogenicity in CEs was demonstrated. HA titers of the allantoic fluids of CEs inoculated with four of five recombinant viruses that were less pathogenic than Vac-1 were equal to that of rg-Vac-1 in CEs. The other recombinant virus, rg-PR8-PA/Vac-1 (H5N1), grew more efficiently than rg-Vac-1. Therefore, the present data provide information concerning how to establish a good vaccine strain with high growth potential and low pathogenicity in CEs.

Between PR8 and Vac-1, 10 amino acid differences were found from positions 240 to 666 in the PB1 protein, and seven amino acid differences were found from positions 1 to 235 in the PA protein (Table 3). In the present study, recombinant viruses with 10 and 7 amino acid substitutions in the PB1 and PA proteins, respectively, did not show significant differences in the MDT of CEs (data not shown). It has been shown that the virus polymerase proteins of avian influenza viruses are responsible for pathogenesis in different host animals [8, 13]. In the case of acquisition of pathogenicity in chickens by serial intracerebral passages, amino acid substitutions were identified not only in the HA but also in the internal proteins, including the PB1 and PA proteins [13]. The PB1 and PA proteins of H5N1 HPAI viruses were responsible for

Table 3 Amino acid differences in PB1 and PA proteins between PR8 and Vac-1

Virus	Amino acid at each position in the virus proteins																
	PB1 (from 240 to 665)										PA (from 1 to 235)						
	325	375	383	398	473	563	577	640	645	654	20	28	55	57	65	100	213
A/Puerto Rico/8/1934 (H1N1)	M	S	D	E	L	I	I	M	M	N	T	L	N	Q	L	A	K
A/duck/Hokkaido/Vac-1/2004 (H5N1)	I	N	E	D	V	R	L	V	V	S	A	P	D	R	S	V	R

lethality in mallard ducks, although the mechanism by which these two proteins play roles in pathogenicity has not been clarified [8]. It has been reported that strong interferon beta antagonism due to the accumulation of the virus NS1 proteins in the cytoplasm of infected cells is associated with high pathogenicity of the virus in avian hosts, including embryonated chicken eggs [10]. In the present study, it was considered that the virus pathogenicity in CEs may be associated with viral polymerase activity. However, we were not able to provide conclusive evidence relating to viral polymerase activity using a minigene luciferase assay and pathogenicity in CEs (data not shown). The association between the viral polymerase and pathogenicity in CEs needs to be clarified in a further study.

The crystal structure and function of the amino terminus of the PA protein have been analyzed [3, 17, 19, 29]. It has been shown that amino acid residues 1 to 209 in the PA protein contain the site of endonuclease activity [3]. The PB2 protein binds the 5' cap of host pre-messenger RNA that has been cleaved by the virus endonuclease, and the viral polymerase synthesizes viral messenger RNA. It has also been reported that amino acid residues 163 to 178 of the PA protein are directly or indirectly involved in complementary RNA promoter binding, suggesting a novel function for the PA protein in modulating promoter binding [17]. The sites responsible for these activities are located in the region of amino acids 1 to 235 in the PA protein.

In order to account for the defect in the avian influenza vaccine strain, the virus proteins responsible for pathogenicity in CEs were identified. Rg-PR8-PA/Vac-1 (H5N1), which shows lower pathogenicity in CEs than rg-Vac-1 and gives a high HA titer in CEs that is similar to that of Vac-1, was obtained as a new vaccine strain by using reverse genetics. The present data indicate that replacement of PB1 and PA genes of vaccine strains with those of the PR8 strain decreases their pathogenicity in CEs. This procedure could be applied for the establishment of influenza vaccine strains.

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Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory

Yoshihiro Sakoda [a], Sengee Sugar [b], Damdinjav Batchluun [b], Tseren-Ochir Erdene-Ochir [b], Masatoshi Okamatsu [a], Norikazu Isoda [a], Kosuke Soda [a], Hiroki Takakuwa [a], Yoshimi Tsuda [a], Naoki Yamamoto [a], Noriko Kishida [a,c], Keita Matsuno [c], Eri Nakayama [c], Masahiro Kajihara [c], Ayaka Yokoyama [c], Ayato Takada [c], Ruuragchaa Sodnomdarjaa [b], Hiroshi Kida [a,c,*]

[a] *Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*
[b] *State Central Veterinary Laboratory, Zaisan, Ulaanbaatar 210153, Mongolia*
[c] *Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan*

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ABSTRACT

H5N1 highly pathogenic avian influenza (HPAI) viruses were isolated from dead wild waterfowl at Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes in Mongolia in July 2005, May 2006, May 2009, July 2009, and May 2010, respectively. The isolates in 2005 and 2006 were classified into genetic clade 2.2, and those in 2009 and 2010 into clade 2.3.2. A/whooper swan/Mongolia/6/2009 (H5N1) experimentally infected ducks and replicated systemically with higher mortality than that of the isolates in 2005 and 2006. Intensive surveillance of avian influenza in migratory waterfowl flying from their nesting lakes in Siberia to Mongolia in every autumn indicate that HPAI viruses have not perpetuated at their nesting lakes until 2009. The present results demonstrate that wild waterfowl were sporadically infected with H5N1 HPAI viruses prevailing in domestic poultry in the southern Asia and died in Mongolia on the way back to their northern territory in spring.

Introduction

H5N1 highly pathogenic avian influenza (HPAI) virus infections have spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China (Smith et al., 2006; Xu et al., 1999). H5N1 virus infections have become endemic at poultry farms in some countries and cause accidental transmissions to humans, so H5N1 viruses are recognized as the most likely candidate for the next pandemic (Li et al., 2004; Peiris et al., 2007). The widespread presence of H5N1 HPAI viruses in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the transmission of viruses to wild bird populations. Domestic ducks and geese infected with HPAI virus shed progeny viruses in feces at the ponds in the farms, where migratory waterfowl visit. Thus, water-borne transmission easily occurs from domestic waterfowl to migratory waterfowl. In the past, such infections had been restricted to wild birds found dead in the vicinity of infected poultry farms, but there are concerns that infections of wild birds in which HPAI virus has caused mild or no clinical signs (e.g., ducks) could result in spread of the virus over large areas and long distances (Kim et al., 2009). Infections with HPAI viruses in many wild bird species at 2 waterfowl parks in Hong Kong were recorded in 2002 (Ellis et al., 2004) and further, more significant outbreaks in wild waterfowl were found at Lake Qinghai in Western China (Chen et al., 2005). H5N1 HPAI virus infections in poultry and wild birds now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could spread by migratory waterfowl to the west and south, since genetically closely related H5N1 viruses (clade 2.2) were isolated in several countries from 2005 to 2006 (Monne et al., 2008; Salzberg et al., 2007; Starick et al., 2008). From intensive surveillance in China, 2 antigenically distinct virus groups, clade 2.3.2 and clade 2.3.4, were characterized as the dominant isolates in wild birds (Kou et al., 2009; Smith et al., 2009).

A natural reservoir of influenza A virus is wild waterfowl (Kida et al., 1980, 1987; Webster et al., 1978). In previous studies, influenza A viruses of different subtypes were isolated from water of the lakes where migratory waterfowl nest in summer, even in autumn when waterfowl had left for the south for migration, suggesting that influenza A viruses are preserved in frozen lake water each year while

* Corresponding author. Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Sapporo 060-0818, Japan. Fax: +81 11 706 5273.
E-mail address: kida@vetmed.hokudai.ac.jp (H. Kida).

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Table 1
Identification of H5N1 isolates in Mongolia.

Date of isolation	Place	Isolates[a]	Amino acid sequence of HA cleavage site[b]	Intravenous pathogenicity index in chicken	Database accession no.
July, 2005	Khunt Lake, Bulgan	Bhg/Mongolia/1/05 (H5N1)	GERRRKKR/G	2.95	AB239300–AB239302, AB233319, AB239303–AB239306
	Erkhel Lake, Khuvsugul	Ws/Mongolia/3/05 (H5N1)	GERRRKKR/G	2.90	AB239307–AB239309, AB233320, AB239310–AB239313
May, 2006	Khunt Lake, Bulgan	Ws/Mongolia/2/06 (H5N1)	GERRRKKR/G	2.71	AB264769–AB264770, AB263751–AB263753, AB265202–AB265204
	Erkhel Lake, Khuvsugul	Cg/Mongolia/12/06 (H5N1)	GERRRKKR/G	2.80	AB284321–AB284328
May, 2009	Doityn Tsagaan Lake, Arkhangai	Ws/Mongolia/2/09 (H5N1)	RERRRKR/G	ND[c]	AB517665–AB517666
		Ws/Mongolia/6/09 (H5N1)	RERRRKR/G	2.97	AB520705–AB520712
		Ws/Mongolia/8/09 (H5N1)	RERRRKR/G	ND	AB517667–AB517668
July, 2009	Doroo Lake, Arkhangai	Bhg/Mongolia/X25/09 (H5N1)	RERRRKR/G	ND	AB521999, AB522000
		Bhg/Mongolia/X53/09 (H5N1)	RERRRKR/G	3.00	AB523764–AB523771
		Bhg/Mongolia/X54/09 (H5N1)	RERRRKR/G	ND	AB523366, AB523367
		Rs/Mongolia/X42/09 (H5N1)	RERRRKR/G	ND	AB523756–AB523763
		Rs/Mongolia/X63/09 (H5N1)	RERRRKR/G	ND	AB523368, AB523369
		Cg/Mongolia/X59/09 (H5N1)	RERRRKR/G	ND	AB522001, AB522002
		Cg/Mongolia/X60/09 (H5N1)	RERRRKR/G	ND	AB523772–AB523779
May, 2010	Ganga Lake, Sukhbaatar	Ws/Mongolia/1/10 (H5N1)	RERRRKR/G	3.00	AB569345–AB569352
		Ws/Mongolia/7/10 (H5N1)	RERRRKR/G	ND	AB569353, AB569354
		Ws/Mongolia/11/10 (H5N1)	RERRRKR/G	ND	AB569607, AB569608
		Ws/Mongolia/21/10 (H5N1)	RERRRKR/G	ND	AB569609, AB569610

[a] Abbreviated name of birds of each isolate: Bhg: bar-headed goose, Ws: whooper swan, Cg: common goldeneye, Rs: ruddy shelduck.
[b] A pair of dibasic amino acid residues was underlined.
[c] ND: not determined.

the waterfowl are absent (Ito et al., 1995; Okazaki et al., 2000). To monitor whether these HPAI viruses perpetuate in nature, virological surveillance of avian influenza has been carried out in the lakes in Mongolia where ducks congregate on their migration path from Siberia to the south since 2001.

In July 2005, May 2006, May 2009, July 2009, and May 2010, H5N1 HPAI viruses were isolated from whooper swans and other migratory waterfowl in Mongolia on the way back to their northern territory, although no outbreak was so far reported in poultry in Mongolia. In the present study, influenza A viruses isolated from dead waterfowl and fecal samples in the intensive surveillance of avian influenza in Mongolia were antigenically and genetically characterized. Pathogenicity of the isolated H5N1 viruses in chickens, pigs, and domestic ducks were investigated by experimental infection studies. The present results strongly support the notion that the global surveillance is essential to understand the ecology of influenza viruses for the control of influenza virus infection in birds and mammals.

Results

Isolation and identification of H5N1 HPAI viruses from dead waterfowl

Virus isolation was carried out for tissue samples of dead waterfowl, a bar-headed goose, whooper swan, common goldeneye, and ruddy shelduck, which were found at Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes in 2005, 2006, 2009, and 2010 (Table 1). In July 2005, H5N1 viruses were isolated from tissue homogenates and swab samples of a bar-headed goose and a whooper swan in Khunt and Erkhel Lakes. Similarly, H5N1 viruses were isolated from a whooper swan and a common goldeneye in May 2006 in Khunt and Erkhel Lakes. In May 2009, H5N1 viruses were isolated from 3 whooper swans in Doityn Tsagaan Lake. In late July 2009, H5N1 viruses were also isolated from dead wild birds, 3 bar-headed geese, 2 ruddy shelducks, and 2 common goldeneyes in Doroo Lake. In May 2010, H5N1 viruses were isolated from 4 whooper swans in Ganga Lake. From sequence analysis of the cleavage site of the hemagglutinin (HA), the C-terminus of HA1 had a pair of dibasic amino acid residues, which is a characteristic of HPAI viruses according to the manual of World Organization for Animal Health (OIE, 2009a). Furthermore, representative isolates of each year were highly pathogenic in chickens on intravenous inoculation and IVPIs of each isolate ranged from 2.71 to 3.00 (Table 1). Complete sequences of the HA, neuraminidase (NA), and other segments were deposited in the GenBank/EMBL/DDBJ as accession numbers described in Table 1.

Phylogenetic analysis of H5N1 isolates

The HA genes of H5N1 isolates were analyzed by the neighbor-joining method along with those of other H5 strains containing HPAI viruses recently isolated in the world (Fig. 1). The HA genes of the isolates in 2005 and 2006 were classified into clade 2.2, as Qinghai Lake-type viruses. Isolates from the same year, A/bar-headed goose/Mongolia/1/2005 and A/whooper swan/Mongolia/3/2005 (Ws/Mongolia/3/05), A/whooper swan/Mongolia/2/2006 (Ws/Mongolia/2/06) and A/common goldeneye/Mongolia/12/2006, were closely related and showed the highest homology. The 3 isolates in May 2009, 7 isolates in July 2009, and 4 isolates in May 2010 were classified into clade 2.3.2, the prototype of this clade was isolates from Hong Kong, China, and Vietnam in 2005. A/whooper swan/Mongolia/6/2009 (Ws/Mongolia/6/09) and other 13 isolates were closely related, having high homology with previous isolates from wild birds and chickens in Russia, China, Laos, and Japan.

Pathogenicity of H5N1 influenza viruses in pigs

To assess the pathogenicity of H5N1 isolates in pigs, each of Ws/Mongolia/3/05, Ws/Mongolia/2/06, and Ws/Mongolia/6/09 was inoculated intranasally at $10^{8.0}$ EID_{50} into two 4-week-old SPF pigs. Viruses were recovered from nasal swabs of all pigs infected with each H5N1 virus although apparent clinical signs were not observed in pigs for the 14 days study (Table 2). The periods of virus shedding in the pigs infected with Ws/Mongolia/2/06 were longer than in the pigs infected with Ws/Mongolia/3/05 or Ws/Mongolia/6/09.

Pathogenicity of H5N1 influenza viruses in ducks

To assess the pathogenicity of H5N1 isolates in ducks, each of Ws/Mongolia/3/05, Ws/Mongolia/2/06, and Ws/Mongolia/6/09 was