A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells

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We have previously demonstrated that the release of hepatitis E virus (HEV) from infected cells depended on ORF3 protein, which harbours one or two PSAP motifs. To elucidate the PSAP motif(s) in the ORF3 protein during virion egress, five PSAP mutants derived from an infectious genotype 3 cDNA clone of pJE03-1760F/wt that can grow efficiently in PLC/PRF/5 cells were analysed. Four mutants, including mutLSAP, mutPSAL, mutLSAL (the substituted amino acids in the authentic PSAP motif are underlined) and mutPLAP/PSAP (the changed amino acid in the additional PSAP motif is underlined) generated progenies as efficiently as the wild-type virus. Conversely, the HEV RNA level in the culture supernatant of mutPLAP/LSA RNA-transfected cells was significantly lower than in cells transfected with the wild-type RNA, similar to an ORF3-null mutant. Consistent with the ORF3-deficient mutant, the mutPLAP/LSA mutant with no intact PSAP motifs banded at 1.26–1.27 g ml⁻¹ in sucrose, and was captured by anti-ORF2, but not by anti-ORF3, with or without prior treatment with detergent (0.1 % sodium deoxycholate). The absence of the ORF3 protein on the mutant particles in the culture supernatant was confirmed by Western blotting, despite the expression of ORF3 protein in the RNA-transfected cells, as detected by immunofluorescence and Western blotting. Therefore, at least one of the two intact PSAP motifs in the ORF3 protein is required for the formation of membrane-associated HEV particles possessing ORF3 proteins on their surface, thus suggesting that the PSAP motif plays a role as a functional domain for HEV budding.

INTRODUCTION

Hepatitis E virus (HEV), a member of the genus Hepevirus in the family Hepeviridae, is the causative agent of acute hepatitis E, which occurs in many parts of the world (Emerson & Purcell, 2006; Harrison, 1999; Okamoto et al., 2003; Worm et al., 2002). HEV is a non-enveloped virus and its genome comprises positive-sense ssRNA of approximately 7200 nt, which is capped and polyadenylated (Kabrane-Lazizi et al., 1999; Tam et al., 1991). The genome consists of a 5′ UTR, three ORFs (ORF1, ORF2 and ORF3) and a 3′ UTR (Emerson & Purcell, 2006). ORF1 encodes non-structural proteins including the helicase and RNA-dependent RNA polymerase (Agrawal et al., 2001; Koonin et al., 1992). All but the 5′ terminus of ORF3 is overlapped by ORF2, and the ORF2 and ORF3 proteins are translated from the same bicistronic subgenomic RNA (Graff et al., 2006). The ORF2 protein is the viral capsid protein, while the ORF3 protein is a small protein of only 113 or 114 aa. It was suggested that the ORF3 protein acts as an adaptor to link the intracellular transduction pathways, reduce the host inflammatory response and protect virus-infected cells (Chandra et al., 2008). Four major genotypes (genotypes 1–4) of HEV have been identified in mammals. Genotypes 1 and 2 have caused outbreaks of hepatitis E as water-borne epidemics, while genotypes 3 and 4 have been found in sporadic cases of acute hepatitis E that were most likely zoonotic in origin because genotypes 3 and 4 HEVs infect not only humans, but also swine, and rarely, other non-primate mammals (Meng, 2010; Okamoto, 2007). At least three genotypes of avian HEV have been identified from chickens worldwide, but these genotypes only share approximately 50 % nucleotide sequence identity with mammalian HEVs (Meng, 2010; Bilic et al., 2009).

Recently, using faecal suspensions or serum samples with high HEV loads originally obtained from hepatitis patients who contracted imported or domestic infection of genotypes 1, 3 or 4 HEV as inocula, we developed an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5) and a lung cancer cell line (A549), which yielded an HEV load of up to 10⁸ copies ml⁻¹ in the culture supernatant, and successfully propagated multiple
generations of serial passages in culture supernatant (Tanaka et al., 2007, 2009; Lorenzo et al., 2008; Takahashi et al., 2007, 2010). In addition, we constructed a full-length infectious cDNA clone (pJE03-1760F/wt) of a genotype 3 HEV that can grow as efficiently as the faeces-derived virus in both PLC/PRF/5 and A549 cells (Yamada et al., 2009b). Our previous study using an ORF3-deficient cDNA clone of pJE03-1760F/wt revealed that the ORF3 protein is essential for virion egress from infected cells (Yamada et al., 2009a). Furthermore, the ORF3-null mutant produced virus particles with the same buoyant density (1.26–1.27 g ml\(^{-1}\) in sucrose) as faecal HEV, which were clearly different from the cell culture-generated wild-type virions and those in the circulation of infected individuals, which banded at 1.15–1.16 g ml\(^{-1}\) and are intimately associated with lipids and the ORF3 protein (Yamada et al., 2009a; Takahashi et al., 2008b, 2010). These observations suggest that the acquisition of host-cell membrane on the surface of the virions is dependent on the expression of the ORF3 protein.

It was previously reported that cellular Tsg101 binds to the PSAP motif located within the ORF3 protein, and that substitutions in the PSAP sequence abolish the ability of the protein to bind Tsg101 (Surjit et al., 2006). The PSAP motif is conserved in all HEV isolates, including avian HEV (see Fig. 1). Tsg101 has been identified as a critical cellular protein required for budding of enveloped viruses, i.e. human immunodeficiency virus type-1 (HIV) and Ebola virus, from the plasma membrane (Garrus et al., 2001; Martin-Serrano et al., 2001). Therefore, in this study, we investigated the function of the PSAP motif in the ORF3 protein in the release of virus by using various site-directed mutants derived from the pJE03-1760F/wt cDNA clone, which has two PSAP motifs in the ORF3 protein, and a robust cell-culture system for HEV.

**RESULTS**

The PSAP motif in the ORF3 protein is conserved in all known HEV strains

The PSAP motif between amino acid residues 95 and 98 of the ORF3 protein was found to be conserved among all known HEV strains including avian HEV strains (Fig. 1). Interestingly, several isolates belonging to genotype 3, including strain JE03-1760F, possessed one additional PSAP motif between residues 86 and 89, located N-terminal to the other motif. Therefore, for simplicity in this manuscript, the PSAP motif located at residues 86–89 was tentatively designated the ‘first PSAP motif’ and that located at residues 95–98 as the ‘second PSAP motif’.

Analysis of HEV variants with mutations in the second PSAP motif of the ORF3 protein

To examine whether the second PSAP motif in the ORF3 protein is essential for the release of HEV virions from infected cells, we carried out site-directed mutational analysis using pJE03-1760F/wt, an infectious cDNA clone of genotype 3 HEV (Fig. 2a). First, amino acid substitutions were introduced into the second authentic PSAP motif in ORF3 to construct three PSAP variants with a mutation from Pro to Leu at the first amino acid residue (mutLSAP) or at the fourth amino acid residue (mutPSAL), or with a dual mutation from Pro to Leu at the first and fourth residues (mutLSAL). Although ORF3 overlaps ORF2, these mutations do not change the ORF2 amino acid sequence in any of the three variants. RNA transcripts of pJE03-1760F/wt cDNA clone, and two PSAP motifs in the ORF3 protein, and a robust cell-culture system for HEV.

**Fig. 1.** Alignment of the partial amino acid sequences of the ORF3 protein of mammalian (genotype 3, aa 82–102; genotypes 1, 2 and 4, aa 83–103) and avian (aa 54–74) HEV strains. Representative HEV isolates with a different amino acid sequence within the indicated area were adopted, and their GenBank accession numbers are indicated in the parentheses. Asterisk indicates an HEV sequence derived from a rabbit.

Table: Analysis of HEV variants with mutations in the second PSAP motif of the ORF3 protein

<table>
<thead>
<tr>
<th>JE03-1760F</th>
<th>First</th>
<th>Second</th>
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<tbody>
<tr>
<td>Genotype 1</td>
<td>NPSDH</td>
<td>R-NPSDH</td>
</tr>
<tr>
<td>Genotype 2</td>
<td>NO-Q-GHLV</td>
<td>EIR</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>V-Q-VH</td>
<td>V-VH</td>
</tr>
<tr>
<td>Avian 54</td>
<td>SQVH</td>
<td>H-GHL</td>
</tr>
<tr>
<td>Genotype 1</td>
<td>TQ-SGALNAPRE</td>
<td>--I-Q</td>
</tr>
<tr>
<td>Genotype 2</td>
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<tr>
<td>Genotype 3</td>
<td>TQ-SGALNAPRE</td>
<td>--LP</td>
</tr>
</tbody>
</table>


Mutation at the first residue: mutLSAP (Pro to Leu) at amino acid 86
Mutation at the fourth residue: mutPSAL (Pro to Leu) at amino acid 89
Mutations at both residues: mutLSAL (Pro to Leu) at amino acid 86 and 89

These results suggest that the acquisition of host-cell membrane on the surface of the virions is dependent on the expression of the ORF3 protein.
RNA-transfected cells during the observation period of 6–28 days post-transfection (p.t.) (Fig. 2b). A gradual decrease in HEV load was observed in the culture medium of the ΔORF1 mutant RNA-transfected cells, serving as a negative control, whose titre probably reflects residual amounts of the introduced RNA transcripts in the culture medium.

The culture supernatants of wild-type RNA- or mutant RNA-transfected cells were subjected to equilibrium centrifugation in a sucrose density gradient. Three mutants (mutLSAP, mutPSAL and mutLSAL) in the culture supernatant exhibited a peak density of 1.16 g ml⁻¹, similar to the wild-type virus (Fig. 2c). When the peak fraction of the wild-type virus and these three mutants in the culture supernatant of transfected cells was subjected to immunocapture RT-PCR assays, essentially no virus was captured by a mouse mAb against ORF2 protein (anti-ORF2 mAb) (H6225) and anti-ORF3 mAb (TA0536) (Table 1). However, upon prior treatment with 0.1 % sodium deoxycholate, the binding efficiency of these three mutants increased to 61.8–90.8 % for the anti-ORF2 mAb and to 68.9–73.6 % for the anti-ORF3 mAb, similar to the wild-type virus (85.2 and 75.2 %, respectively). These results suggest that the properties of these particles generated by transfection of mutant RNAs are similar to those of wild-type particles in cell culture, i.e. released particles are likely to be associated with lipids and the ORF3 protein.

Analysis of HEV variants with mutation in the first or both PSAP motifs in the ORF3 protein

As shown in Fig. 1, strain JE03-1760F has two PSAP motifs in the ORF3 protein. We next constructed HEV clones with amino acid substitutions in the first PSAP motif only (mutPLAP/PSAP), or in both the first and second PSAP motifs (mutPLAP/LSAL) of the ORF3 protein (Fig. 2a); these mutations do not affect amino acid sequences of the ORF2 protein. The HEV RNA level in the culture supernatant of the mutPLAP/PSAP RNA-transfected cells increased gradually from 6 days p.t., reaching 1.7 × 10⁷ copies ml⁻¹ on 28 days, although the level was approximately 0.5-fold lower than that of cells transfected with the wild-type RNA during 6–28 days p.t. (Fig. 3a). In contrast, the introduction of amino acid substitutions in both PSAP motifs (mutPLAP/LSAL) significantly reduced the virus yield in the culture supernatant, similar to an ORF3-deficient variant, ΔORF3 (Fig. 3a). To clarify whether the increase in viral RNA shown in Fig. 3(a) is related to an increase in cells expressing viral protein, immunocapture RT-PCR assay was performed using an anti-ORF2 mAb (H6225) capable of capturing HEV particles, after treatment with 0.1 % sodium deoxycholate. The genomic RNAs of captured wild-type, mutPLAP/PSAP, mutPLAP/LSAL and ΔORF3 HEV particles were detectable at 4 days p.t., and the RNA levels increased gradually thereafter, in a similar manner to that indicated in Fig. 3(a) (Fig. 3b); the culture supernatant of the ΔORF1 RNA-transfected cells...
tested negative for particle-associated HEV RNA throughout the observation period. The particles in the culture supernatant of the mutPLAP/PSAP RNA-transfected cells peaked at $1.16 \text{ g ml}^{-1}$, as did the wild-type virus, while the particles generated in the culture supernatant of the mutPLAP/LSAL RNA-transfected cells banded at $1.26–1.27 \text{ g ml}^{-1}$, similar to those in the DORF3 RNA-transfected cells (Fig. 3c).

Immunofluorescence assays (Fig. 3d) and Western blotting (Fig. 3e) revealed the expression of the ORF3 protein in the mutPLAP/LSAL RNA-transfected cells, indicating that an anti-ORF3 mAb used in the present study is reactive even with the mutated ORF3 protein, despite the presence of one or two amino acid mutations in each of the two PSAP motifs, and that at least one of the two PSAP motifs in the ORF3 protein is indispensable for the formation of membrane-associated HEV particles.

**Characterization of the mutPLAP/LSAL particle in the culture supernatant**

To examine whether the ORF3 protein associates with viral particles, Western blot analysis was performed using the culture supernatant of transfected cells. In the cells transfected with wild-type virus, the ORF3 protein was clearly detected in the culture supernatant (Fig. 3f). In contrast, ORF3 protein expression was not detectable in the culture supernatant derived from DORF3 RNA- and mutPLAP/LSAL RNA-transfected cells. Immunocapture RT-PCR was then performed for the culture supernatant in the peak fraction obtained from sucrose density-gradient centrifugation (Table 1). Viral particles in the culture supernatant of the mutPLAP/LSAL RNA-transfected cells were efficiently captured by an anti-ORF2 mAb, but not by anti-ORF3 mAb, similar to what was observed in the supernatant of the DORF3 RNA-transfected cells, with or without prior treatment with 0.1 % sodium deoxycholate.

**PSAP motifs are important for efficient virion egress**

The intracellular viral RNA was serially quantified by two distinct real-time RT-PCR methods: the ORF1 and ORF2/3 probes are capable of detecting genomic RNA only (Fig. 4a) and both genomic and subgenomic RNAs (Fig. 4b), respectively. The HEV RNA level detectable by both the ORF1 probe and the ORF2/ORF3 probe in the mutPLAP/LSAL RNA-transfected cells was as high as that in the wild-type RNA- and DORF3 RNA-transfected cells at 8, 16 and 24 days p.t., and increased in the same manner over time. Next, to examine the efficiency of virion egress in PLC/PRF/5 cells inoculated with the cell

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Input (copies per well)</th>
<th>Captured HEV per input (%)</th>
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<tbody>
<tr>
<td></td>
<td>mAb H6225 (anti-ORF2)</td>
<td>mAb TA0536 (anti-ORF3)</td>
</tr>
<tr>
<td><strong>Without pre-treatment with detergent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJE03-1760F/wt (1.16 g ml$^{-1}$)</td>
<td>1 300</td>
<td>2.5</td>
</tr>
<tr>
<td>mutLSAP (1.16 g ml$^{-1}$)</td>
<td>700</td>
<td>2.7</td>
</tr>
<tr>
<td>mutPSAL (1.16 g ml$^{-1}$)</td>
<td>1 200</td>
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<td>440</td>
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<td>mutPLAP/PSAP (1.16 g ml$^{-1}$)</td>
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<td>230</td>
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</tr>
<tr>
<td><strong>With pre-treatment with detergent‡</strong></td>
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</tr>
<tr>
<td>pJE03-1760F/wt (1.16 g ml$^{-1}$)</td>
<td>1 100</td>
<td>85.2</td>
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<tr>
<td>ΔORF3 (1.27 g ml$^{-1}$)</td>
<td>370</td>
<td>92.3</td>
</tr>
</tbody>
</table>

*Viruses were derived from the culture supernatant of transfected cells (RNAs of pJE03-1760F/wt, mutLSAP, mutPSAL, mutLSAL, mutPLAP/PSAP, mutPLAP/LSAL or ΔORF3) at 28 days p.t., and peak fractions with the indicated sucrose density (see Fig. 2c and Fig. 3c) were subjected to immunocapture RT-PCR.†mAb against hepatitis B virus pre-S2 protein (Okamoto et al., 1985).‡Prior to performing the immunocapture RT-PCR assay, 6 μl of the sucrose fraction was mixed with 60 μl of 0.11 % sodium deoxycholate and incubated at 37 °C for 2 h, then was diluted 1 : 10 with PBS containing 0.1 % BSA.
culture-generated wild-type virus or ORF3 variants, the culture supernatant of the wild-type RNA-, mutPLAP/LSAL RNA- or ΔORF3 RNA-transfected cells were inoculated into the PLC/PRF/5 cells. When wild-type HEV was inoculated into the cells, HEV RNA was first detected in the culture supernatant on day 8 and reached \(4.0 \times 10^3\) copies ml\(^{-1}\) on day 12 (Fig. 4c). In contrast, low levels of background RNA at \(<10^2\) copies ml\(^{-1}\) were seen in the culture supernatants of cells inoculated with the mutPLAP/LSAL or ΔORF3 virus during the course of 8–12 day experiment (Fig. 4c), probably due to cell death as described in detail in our previous paper (Yamada et al., 2009a). Notably, no significant difference in the intracellular HEV RNA level was observed upon inoculation of the wild-type virus and its ORF3 variants (Fig. 4d). When the amount of adsorbed virus was measured by quantitative RT-PCR targeting the ORF2/3 region 1 h after inoculation, the HEV RNA level in the cells inoculated with the mutPLAP/LSAL virus was higher than wild-type (\(1.3 \times 10^3\) vs \(1.7 \times 10^2\) copies per \(10^5\) cells), similar to the ΔORF3 virus (\(1.9 \times 10^3\) copies per \(10^5\) cells), thus suggesting that the adsorption and entry of the mutPLAP/LSAL and ΔORF3 viruses are more efficient than wild-type virus.

**Interaction of the ORF3 protein with Tsg101 in cultured cells**

To investigate the direct interaction of the ORF3 protein with Tsg101 and loss thereof with the PSAP mutations, we used co-immunoprecipitation to show ORF3–Tsg101 interaction in cells inoculated with wild-type or mutant (mutPLAP/LSAL) virus in the culture supernatant, or transfected with RNA transcripts of wild-type or mutant cDNA clone. However, in the present study, co-immunoprecipitates of ORF3–Tsg101 proteins were not detectable in the infected or transfected PLC/PRF/5 cells, probably due to insufficient expression levels of either or both of Tsg101 and ORF3 proteins to permit detection by Western blotting. Then, with the advent of an expression plasmid for the full-length ORF3 protein (aa 1–113) of the JE03-1760F strain, pCI-HEVORF3/wt (Takahashi et al., 2008b), a mutant plasmid pCI-HEVORF3/mutPLAP/LSAL was generated. In the PLC/PRF/5 cells transfected with pCI-HEVORF3/mutPLAP/LSAL, the ORF3 protein was expressed as efficiently as the wild-type ORF3 protein (Fig. 5a). The extracts of PLC/PRF/5 cells transfected with pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL were immunoprecipitated with goat anti-Tsg101 polyclonal antibody and the resulting immunoprecipitates were sub-
jected to Western blotting by using either anti-Tsg101 mAb or anti-ORF3 mAb. As shown in Fig. 5b, cellular Tsg101 was immunoprecipitated with goat anti-Tsg101 in both cells expressing the wild-type ORF3 protein and those expressing the mutPLAP/LSAL ORF3 protein, while it was not immunoprecipitated with normal goat IgG, thus representing the specificity of the goat anti-Tsg101 antibody. The immunoprecipitation with goat anti-Tsg101 antibody for cell extracts expressing wild-type ORF3 protein co-immunoprecipitated ORF3 protein detectable by Western blotting with anti-ORF3 mAb (Fig. 5c). In contrast, the immunoprecipitation for the extracts from cells expressing the mutPLAP/LSAL ORF3 protein did not show any co-immunoprecipitates of the ORF3 protein, suggesting the loss of interaction between Tsg101 and ORF3 proteins due to inactive PSAP motifs in the mutated ORF3 protein.

**DISCUSSION**

In the present study, we demonstrated that the ORF3 protein, with at least one of the two PSAP motifs, is required for efficient release of virions from cultured cells. Furthermore, an intact PSAP motif of the ORF3 protein was found to be essential for egress of HEV particles associated with lipids and the ORF3 protein. These results suggest that the PSAP motif(s) of the HEV ORF3 protein is a functional domain for virion egress.

While this manuscript was being prepared for submission, Emerson et al. (2010) reported that release of genotype 1 HEV from cultured cells depends on the ORF3 protein and requires an intact PXXP motif in the ORF3 protein. With the advent of an infectious cDNA clone of genotype 1 HEV (the Sar55 strain), they confirmed our previous study conducted by using a genotype 3 infectious cDNA clone of the JE03-1760F strain, reporting that the ORF3 protein is essential for virion egress from infected cells, that the ORF3 protein is present on the surface of HEV particles released from infected cells, and that the HEV particles released from infected cells are lipid-associated (Yamada et al., 2009a). Genotypes 2 and 4 HEVs have not yet been examined for the function of the ORF3 protein on virion egress by using infectious cDNA clones. However, given their conservation, and since the association of HEV virions of genotypes 1, 3 or 4 in blood circulation with ORF3 protein and lipids on the surface has been noted (Takahashi et al., 2010), it is very likely that the function...
of the ORF3 protein related to HEV morphogenesis is common to all HEV strains, irrespective of genotype.

A Pro-rich sequence is present in the C-terminal region of the ORF3 protein in all mammalian and avian HEV strains (Fig. 1). Although two PSAP motifs are present in the ORF3 protein of only a limited number of genotype 3 HEV strains, including JE03-1760F, the present study revealed that not only the second, authentic PSAP motif but also the first, additional PSAP motif in the ORF3 protein can be involved in virion egress. The mutPLAP/PSAP variant with a Ser to Leu mutation in the first PSAP motif, which is identical to the majority of reported genotype 3 HEV strains (Fig. 1), generated progeny slightly less efficiently (approx. 0.5-fold) than the wild-type virus (Fig. 3a), despite the presence of an intact sequence in the second, authentic PSAP motif, suggesting that the first/additional PSAP motif is capable of enhancing virion release from infected cells. The Pro-rich sequence of PSAPPLPP (aa 95–102 in genotype 3) in the C-terminus of the ORF3 protein differ slightly by genotype. Genotype 1 HEVs have a conserved PSAPPLPP sequence (aa 96–102: aa 103 has His in place of Pro), with two PXXP motifs (aa 96–99 and 99–102). In recent work by Emerson et al. (2010), the second Pro in the PSAPPLPP sequence was substituted with Glu or Leu, to yield PSAQ/LPLP (changed amino acids underlined). Their study indicated that the PSAQPLP and PSALPLP mutants did not promote virus egress. However, it remained unclear whether either of the PSAP and PLLP motifs or both is important for egress of genotype 1 HEV. Genotypes 2 and 4 HEVs have in common a Pro-rich sequence of PSAPPLPP, with three PXXP motifs (aa 96–99, 99–102 and 100–103). In contrast, the fourth or fifth Pro residue is changed to His or Arg, respectively, in some genotype 4 HEV isolates, suggesting that a PSAPP sequence, which is conserved in all HEV isolates including avian HEV strains, is important for virion egress from infected cells. Although mutLSAL and mutPLAP/LSAL both have two intact PXXP motifs of aa 86–89 and 99–102, only mutLSAL produced progeny as efficiently as the wild-type strain (Fig. 2b), while mutPLAP/LSAL was unable to release virions into culture medium upon inoculation of the mutant virus (Fig. 4c). These results indicate that the PXXP motif of aa 99–102 is not essential for virion release, and suggest that other amino acids between the first and fourth Pro residues of the PXXP motif are also important for efficient virion egress. Based on the high conservation of the PSAP motifs among all mammalian and avian HEV strains, it is very likely that an intact PSAP motif in the ORF3 protein, whether it is located in the original site (aa 95–98 in genotype 3 HEV) or in the additional site (aa 86–89), is capable of promoting efficient virion release from infected cells.

The present study also demonstrated that the PSAP motif in the ORF3 protein is involved in the formation of membrane-associated HEV particles possessing ORF3 proteins on the surface. Consistent with an ORF3-deficient mutant, the mutPLAP/LSAL mutant virus with no intact PSAP motifs in the culture supernatant of RNA-transfected cells banded at 1.26–1.27 g ml\(^{-1}\) in sucrose (Fig. 3c), and was captured by anti-ORF2 mAb, but not by the anti-ORF3 mAb, with or without prior treatment with detergent (sodium deoxycholate) (Table 1). The absence of ORF3 protein on the mutant particles in the culture supernatant was confirmed by Western blotting (Fig. 3f), despite the fact that ORF3 protein expression was detected in the RNA-transfected cells by both immunofluorescence (Fig. 3d) and Western blotting (Fig. 3e) assays, similar to the wild-type virus. These results indicate that an intact PSAP motif in the ORF3 protein plays a pivotal role in the release of HEV particles having lipid-associated membranes and ORF3 protein.

Recent studies have revealed that viral matrix proteins play critical roles during the later stages of virus budding in many enveloped RNA viruses, including retroviruses, rhabdoviruses, filoviruses and orthomyxoviruses; these viral proteins possess a so-called late (L)-domain containing PT/SAP, PXXY and YXXL, which are critical motifs for efficient budding (Bouamr et al., 2003; Ciancanelli & Basler, 2006; Göttingler et al., 1991; Harty et al., 1999, 2000; Wirblich et al., 2006). The PTAP motif was first identified in HIV Gag and has been reported to bind to Tsg101, which functions in vacuolar protein sorting (Garrus et al., 2001). This interaction between Gag and Tsg101 is required for HIV budding. It has also been reported that Tsg101 binds to the PSAP motif of HEV located within the ORF3 protein (Surjit et al., 2006). The present preliminary study with a co-immunoprecipitation procedure showed the direct interaction of the ORF3 protein with Tsg101 and loss thereof with the PSAP mutations in PLC/PRF/5 cells transfected with a full-length wild-type (pCI-HEVORF3/wt) or mutant (pCI-HEVORF3/ mutPLAP/LSAL) ORF3 expression plasmid (Fig. 5). Taken together, it is very probable that the ORF3 protein promotes budding of membrane-associated HEV particles by recruiting Tsg101. To understand the precise steps of HEV budding, the cellular signalling pathways involving Tsg101 and other related host factors in relation to the release of HEV virions need to be explored in future studies.

In conclusion, our data provide evidence of the functional role of the PSAP motif(s) in the ORF3 protein for efficient egress of HEV virions from infected cells. In addition, it was found that at least one of the two PSAP motifs is required for the formation and release of membrane-associated HEV particles possessing the ORF3 protein on their surface. Further studies are warranted to elucidate how and why HEV acquires the lipid-associated membrane and the ORF3 protein when it leaves cells, despite this virus being a non-enveloped virus.

**METHODS**

**Cell culture.** PLC/PRF/5 cells (ATCC no. CRL-8024) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) heat-inactivated FCS, 100 U penicillin G ml\(^{-1}\), 100 μg streptomycin
ml⁻¹ and 2.5 μg amphotericin B ml⁻¹ (growth medium), at 37 °C in a humidified 5% CO₂ atmosphere, as described previously (Tanaka et al., 2007).

**HEV cDNA clones.** pJE03-1760F/wt (GenBank accession no. AB437316) is a full-length infectious cDNA clone of a genotype 3 HEV obtained from a faecal specimen containing a high load of wild-type HEV (strain JE03-1760F; 2.0 × 10⁷ copies ml⁻¹), which can replicate efficiently in PLC/PRF/5 cells (Yamada et al., 2009b). For comparison, a derivative ORF3-deficient mutant (pJE03-1760F/ΔORF3, GenBank accession no. AB437317) whose initiation codon of the ORF3 gene was mutated to GCA (Ala) (Yamada et al., 2009a), was used. As a negative control, an ORF1-deficient mutant genome (pJE03-1760F/ΔORF1, GenBank accession no. AB437319) containing a frameshift mutation in ORF1 (Yamada et al., 2009b) was also used.

**Site-directed mutagenesis.** To construct variants of pJE03-1760F/wt with mutations in the PSAP motifs in the ORF3 protein (see Fig. 2a), an AflI–Xbal fragment of pJE03-1760F/wt, which contains the ORF3 and ORF2 coding regions of the genome and poly(A) tail sequence (polyAT7b) (Yamada et al., 2009b), was subcloned into a pT7 Blue T vector (Novagen). Site-directed mutagenesis was carried out by inverse PCR according to the method described previously (Sasaki et al., 2001), using the obtained subclone of pJE03-1760F/wt, a high-fidelity DNA polymerase (KOD-Plus; Toyobo), and appropriate oligonucleotide primers. The primer pairs used for the construction of five PSAP mutant plasmids (pT7-mutLSAP, pT7-mutPSAP, pT7-mutEDPSAP, pT7-mutEDPSAL, and pT7-mutEDPSAL) are shown in Supplementary Table S1 (available in JGV Online). To construct variants of pJE03-1760F/wt or its variants at 28 days p.t. were subjected to equilibrium centrifugation in a sucrose density gradient as described previously (Yamada et al., 2009a). The gradients were fractionated, and the density of each fraction was measured by refractometry.

**Immunocapture RT-PCR assay.** The immunocapture RT-PCR assay was performed as described previously (Takahashi et al., 2008a), with or without prior treatment with 0.1% sodium deoxycholate (Wako) at 37 °C for 2 h. An anti-ORF2 mAb (H6225) (Takahashi et al., 2008a) and an anti-ORF3 mAb (TA0536) (Takahashi et al., 2008b) were used in this study. As a negative control, a mAb against hepatitis B virus pre-S2 protein (no. 5520) (Okamoto et al., 1985) was used.

**Western blotting.** Transfected cells were lysed in lysis buffer [50 mM Tris/HisCl (pH 8.0), 1% NP-40, 150 mM NaCl and protease inhibitor cocktail (Sigma)], and proteins in the cell lysates (5 μl of the lysate corresponding to 2.5 × 10⁴ cells) or the culture supernatants (10 μl of the supernatant corresponding to 2.0 × 10⁴ copies) were separated by SDS-PAGE on 12% gels. The proteins were blotted onto a PVDF membrane, immunodetected with an anti-HEV ORF3 mAb, and then visualized by chemiluminescence as described previously (Yamada et al., 2009b).

**Virus inoculation.** Monolayers of PLC/PRF/5 cells in a six-well plate were inoculated with 10⁵ copies of HEV progenies in the culture supernatant of transfected cells. After incubation at room temperature for 1 h, the cells were washed with PBS and 2 ml of maintenance medium was added to each well, and then the cultures were incubated at 35.5 °C. The maintenance medium consisted of 50% DMEM and 50% Medium 199 (Invitrogen) containing 2% FCS, 30 mM MgCl₂, 100 U penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 2.5 μg amphotericin B ml⁻¹. After incubation at 37 °C for 2 days, the culture medium was replaced with 2 ml of maintenance medium and incubated at 35.5 °C. Each day, half of the culture medium (1 ml) was replaced with fresh maintenance medium. The collected culture medium was centrifuged at 1300 g at room temperature for 2 min and the supernatant was stored at -80 °C until use.

**Virus adsorption assay.** The virus adsorption assay was performed as described previously (Yamada et al., 2009a). Briefly, after washing PLC/PRF/5 cells in each well of a six-well plate with PBS, virus (10⁵ copies) in culture supernatant was inoculated onto monolayers of the cells. After incubation at room temperature for 1 h, cells were washed with PBS, trypsinized, and collected by centrifugation at 100 g at room temperature for 5 min. After removal of the supernatant, the
cell pellet was resuspended in TRIzol Reagent and stored at \(-80^\circ C\) until virus titration.

**Expression plasmid construction and co-immunoprecipitation.**

To construct a mutant plasmid pCI-HEVORF3/mutPLAP/LSAL with mutations in the two PSAP motifs, similar to mutPLAP/LSAL (Fig. 2a), site-directed mutagenesis was carried out by inverse PCR using pCI-HEVORF3/wt as a template, which is an expression plasmid for the full-length wild-type ORF3 protein (aa 1–113) of the JE03-1760F strain (Takahashi et al., 2008b). PLC/PRF/5 cells were transfected with pCI-HEVORF3/wt, pCI-HEVORF3/mutPLAP/LSAL or pCI vector (Promega) using TransIT-LT1 reagent (Mirus Bio). At 48 h after transfection, co-immunoprecipitation assay was carried out using Immunoprecipitation kit (Protein G) (Roche). After clarification by brief centrifugation, lysates were used for immunoprecipitation with goat anti-Tsg101 polyclonal antibody (Santa Cruz Biotechnology) or normal goat IgG (MP Biomedicals). Immunoprecipitated protein, cellular Tsg101, was separated by 10% SDS-PAGE, followed by Western blotting with an anti-Tsg101 mAb (Santa Cruz Biotechnology). Co-immunoprecipitated proteins (pCI-HEVORF3/wt, pCI-HEVORF3/mutPLAP/LSAL or pCI vector) were separated by 15% SDS-PAGE, followed by Western blotting with an anti-ORF3 mAb. These proteins were visualized by chemiluminescence by using Clean-Blot IP Detection Reagent (HRP) (Thermo Scientific).

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**REFERENCES**


