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# Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein

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**Abstract**

Envelope viruses maturate by macromolecule assembly and budding. To investigate these steps, we generated virus-like particles (VLPs) by co-expression of structural proteins of Sendai virus (SeV), a prototype of the family Paramyxoviridae. Simultaneous expression of matrix (M), nucleo- (N), fusion (F), and hemagglutinin-neuraminidase (HN) proteins resulted in the generation of VLPs that had morphology and density similar to those of authentic virus particles, although the efficiency of release from cells was significantly lower than that of the virus. By using this VLP formation as a model of virus budding, roles of individual proteins in budding were investigated. The M protein was a driving force of budding, and the F protein facilitated and the HN protein suppressed VLP release. Either of the glycoproteins, F or HN, as well as the N protein, significantly shifted density of VLPs to that of virus particles, suggesting that viral proteins bring about integrity of VLPs by protein–protein interactions. We further found that co-expression of a nonstructural protein, C, but not V, enhanced VLP release to a level comparable to that of virus particles, demonstrating that the C protein plays a role in virus budding.


*Keywords:* Sendai virus; Virus-like particle; Morphology; Density; Nucleocapsid; C protein

## Introduction

Sendai virus (SeV), a prototype of the family Paramyxoviridae, is an enveloped virus with a nonsegmented negative-strand RNA genome. The SeV virion has two spike glycoproteins on the lipid-containing envelope, the hemagglutinin-neuraminidase (HN) protein and the fusion (F) protein. Lying underneath the envelope is the matrix (M) protein, which bridges the viral glycoproteins and the internal nucleocapsid. The nucleocapsid has a helical structure consisting of genomic RNA enwrapped with the nucleocapsid (N) protein, associated with RNA polymerase subunits, the large (L) protein, and the phosphoprotein (P). Besides these structural proteins, the P gene encodes the C proteins and the V protein. The C proteins are a generic name of four proteins, C′, C, Y1, and Y2, formed by the various start codons and the common stop codon in a reading frame shifted from that of the P protein. The V protein is synthesized from an accessory mRNA generated by RNA editing during transcription and containing an insertion of a pseudotemplated G residue at a specific editing site. These proteins are not basically incorporated into virions and are termed nonstructural proteins (Lamb and Kolakofsky, 2001; Nagai, 1999). SeV replicates in the cytoplasm and is released from infected cells by assembly and budding at the plasma membrane. Although the current model for assembly of the paramyxovirus implicates the M protein as the principal component promoting the budding of the virus particle (Peeples, 1991; Yoshida et al., 1976, 1979), the underlying mechanism of virion formation is still poorly understood.

Previous studies on retroviruses using the virus-like particle (VLP) system have made great progress in identification of functional domains for budding in the gag protein and in elucidation of their budding mechanisms (Accola et al., 2000; reviewed in Freed, 2002). In the VLP system, the

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gag protein is released into the culture medium when expressed in the absence of other viral proteins, forming VLPs that resemble virus particles (Freed, 2002). In negative-strand RNA viruses, M protein-deficient viruses have been generated by using the virus recovery system from cDNA in rabies virus (Mebatsion et al., 1996), measles virus (Cathomen et al., 1998), and SeV (Inoue et al., 2003a). These viruses are invariably defective in virus budding. Furthermore, expression of the M protein or its analogs in the absence of other viral proteins leads to its release into the medium in a lipid-containing vesicle, as was observed for the M protein of vesicular stomatitis virus (VSV) (Justice et al., 1995; Li et al., 1993; Sakaguchi et al., 1999), M1 of influenza virus (Gomez-Puertas et al., 2000; Latham and Galarza, 2001), and VP40 of Ebola virus (Harty et al., 2000; Timmins et al., 2001). In VSV and Ebola virus, ca. 20–40% of the M protein synthesized in cells was released into the medium (Justice et al., 1995; Sakaguchi et al., 1999; Timmins et al., 2001). The M protein of parainfluenzavirus type 1 (PIV1), a paramyxovirus belonging to the same genus Respirovirus as SeV, is also released into the culture medium when expressed alone (Coronel et al., 1999). Co-expression of M and N proteins causes release of vesicles enclosing nucleocapsid-like structures (Coronel et al., 1999). It has also been shown that expression of the SeV M protein results in its release from cells (Sakaguchi et al., 1999; Takimoto et al., 2001). Moreover, the F protein as well as the M protein has been shown to form vesicles to be released when expressed alone, and protein motifs necessary for the release, including putative actin-binding motifs, have been identified (Takimoto et al., 2001). However, a quantitative study on the release has not been performed.

Schmitt et al. (2002) generated VLPs that resembled authentic virus particles by expressing proteins of simian virus 5 (SV5), a member of the genus Rubulavirus of the family Paramyxoviridae, and demonstrated that expression of the M protein alone was not sufficient for its release from cells but that simultaneous expression of the M protein, N protein, and one of the viral glycoproteins, F and HN proteins, was necessary for efficient VLP release from cells. The results of that study show that interactions among multiple viral proteins, not the M protein alone, confer budding competence on VLP, suggesting a different mechanism of paramyxovirus budding.

To further clarify the mechanism of paramyxovirus assembly and budding by using a VLP system, it is important to investigate the effects of other viral proteins, in addition to the M protein, on VLP formation. In the present study, we generated VLPs by co-expressing SeV proteins in various combinations, and we evaluated the contribution of each viral protein to budding by quantifying VLP release from cells. Furthermore, roles of each viral protein in VLP formation towards an authentic virus particle were also investigated by estimating density of the VLPs and by electron microscopy. The results showed that the M protein was indeed a major driving force of SeV budding and that the F and HN proteins had enhancing and suppressive effects on budding, respectively. Furthermore, we show that the four viral proteins M, N, F, and HN, are required for formation of VLPs with density and morphology similar to those of authentic SeV particles and that the C protein enhances VLP release.

## Results

### *VLP formation by expression of SeV M, N, F, and HN proteins*

SeV M, N, F, and HN proteins were simultaneously expressed in 293T cells and metabolically labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine. The culture medium was then collected and directly fractionated by sucrose floatation ultracentrifugation. Viral proteins in the floated fraction showed that these four proteins were released in the form of lipid-containing vesicles (Fig. 1A). Viral proteins in the cells were also analyzed, and the fraction of the released M protein was calculated as described in Materials and methods. The released fraction was 3.6%, which was ca. 1/5 of that of virus particles from infected cells (18.3%) (Fig. 1A).

Sucrose density equilibrium ultracentrifugation of the culture supernatant demonstrated that the M, N, F, and HN proteins were concentrated to fraction 14 (density = 1.17–1.20 g/ml), which was identical to that of authentic virus particles (Fig. 1B). Electron microscopy with negative staining showed the existence of virus-like particles (VLPs) that appeared to be similar to authentic virus particles (Fig. 1C). Herring bone-like, nucleocapsid-like structures were also found beside the VLPs (Fig. 1C). This finding is consistent with a previously reported finding that expression of the SeV N protein in cells resulted in generation of nucleocapsid-like structures (Buchholz et al., 1993). The VLPs seem to contain RNA actually because radioactivity was found at fractions 14–16 in [$^{3}$H]uridine labeling (data not shown). These results indicate that simultaneous expression of the M, N, F, and HN proteins yielded VLPs with morphology and density similar to those of actual virus particles, suggesting that VLP formation can be a model for virus budding. The release efficiency of the VLPs was, however, significantly lower than that of SeV from infected cells.

Amounts of the N protein in the VLPs and virus particles were compared with those of other viral proteins after sucrose density equilibrium ultracentrifugation. Ratios of N protein to M protein in fraction 14 in Fig. 1B were 1.6 for the VLP and 0.7 for the SeV particle. The VLP was shown to contain more N proteins than the amount in the SeV particle. The ratios of other proteins in VLPs and SeV particles appeared to be similar.

We added an exogenous neuraminidase to the medium to prevent VLPs from being trapped on the cell surface by the

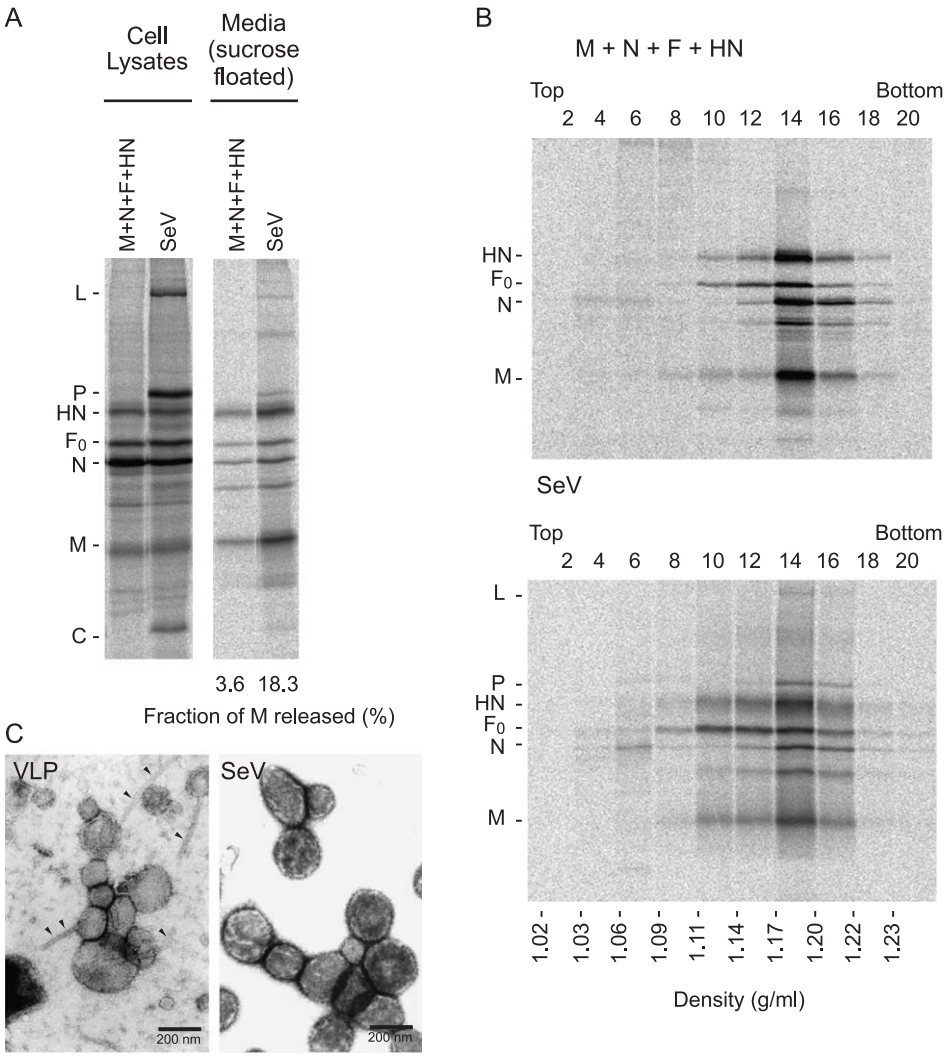


Fig. 1. Comparison of VLPs by co-expression of M, N, F, and HN proteins and SeV. (A) 293T cells were transfected with plasmids for expression of M, N, F, and HN proteins or infected with SeV at an m.o.i. of 10 and labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine from 24 to 48 h post-transfection. The medium was then separated by sucrose floatation ultracentrifugation, and proteins in the floated fraction were analyzed by immunoprecipitation and SDS-PAGE. Proteins in the cells were also analyzed, and the released fraction of the M protein is shown in the figure. $F_0$: precursor of the F protein before proteolytic processing. Proteins from 1/15 of the cell lysates and those from half of the medium were run in a lane. (B) The medium from the labeled cells was then separated by sucrose density equilibrium ultracentrifugation in a 20–50% continuous gradient. Fractions were collected, and SeV proteins in each fraction were analyzed by immunoprecipitation and SDS-PAGE. Density of fractions was measured by weighing each fraction. (C) The medium from transfected 293T cells was concentrated by ultracentrifugation at 24 000 rpm for 1 h through a 7% (w/w) sucrose cushion. The pellet was then suspended in PBS, processed for negative staining, and observed by electron microscopy. Arrowheads indicate nucleocapsid-like structures.

hemagglutinating activity. The addition improved VLP release by ca. 2-fold in some experiments, although it was not always reproducible. The medium was supplemented with neuraminidase in all of the experiments in this study.

*Contribution of each SeV protein to VLP release from cells*

We investigated the role of each SeV protein in VLP formation. Expression of the M protein alone led to an efficient release of M proteins into the medium (Fig. 2A). The efficiency of the M protein release was calculated to be 14.5%. The F protein was also released but with less efficiency (0.7%) (Fig. 2A). Only a trace amount of proteins was released when either the N or HN protein was expressed (Fig. 2A).

Expression of the F protein with the M protein increased the released fractions of both proteins, especially that of the F protein significantly (from 0.7% to 6.5%) (Fig. 2B), suggesting a synergic effect of protein release. In contrast, expression of the HN protein with the M protein resulted in suppression of M protein release (from 14.5% to 0.6%), although the released fraction of the HN protein itself was slightly increased from 0.1% to 0.4%. Expression of the N protein with the M protein also decreased the released fraction of the M protein to 3.2%, while it increased the released fraction of the N protein from 0.03% to 0.4% (Fig.

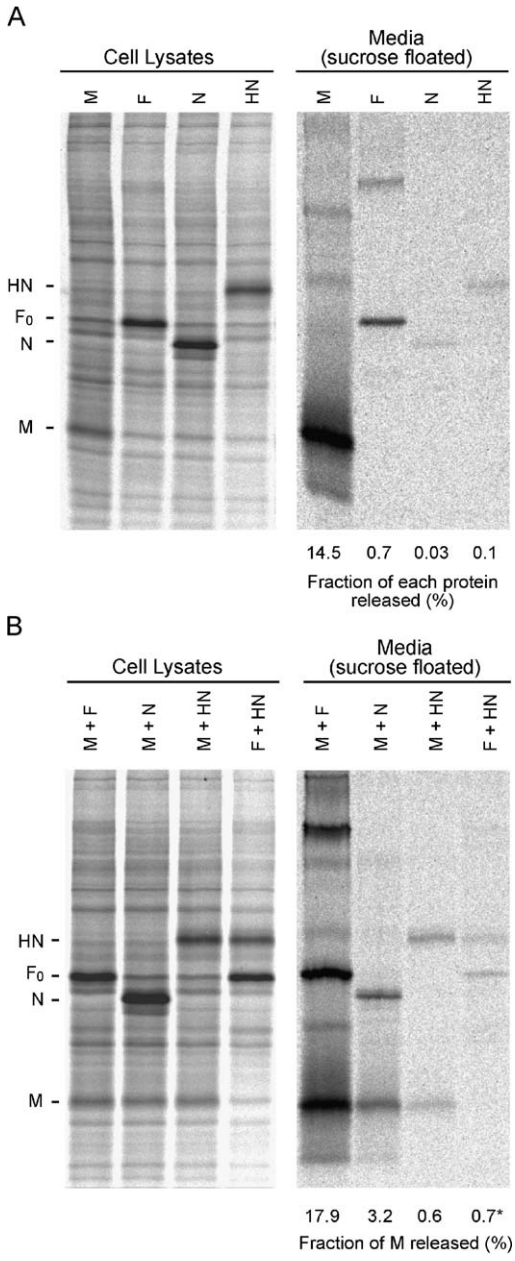


Fig. 2. Protein release by expression of single or double SeV proteins. A single plasmid (A) or two plasmids (B) as indicated in the figure were introduced into 293T cells and labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine from 24 to 48 h post-transfection. The vesicles released were concentrated by sucrose floatation ultracentrifugation, and proteins were analyzed by immunoprecipitation and SDS-PAGE. Proteins in the cells were also analyzed, and protein release was calculated by each protein (A) and by the M protein (B). Asterisk: protein release was calculated by the HN protein. Proteins from 1/15 of the cell lysates and those from half of the medium were run in a lane in these gels.

2B). HN and N proteins could be a burden for M-driven vesicle release. Co-expression of the two glycoproteins, F and HN, did not result in efficient release of these proteins (Fig. 2B).

An apparent constant intracellular level of the M protein in Fig. 2B does not seem to accord with the fact that the released fraction of M protein varied from 0.6% to 17.9%; intracellular M protein level should vary in accordance with the variation in the released fraction. This is due to the sample amount loaded on the gel as described in Materials and methods (1/15 of the cell lysates and half of the medium). The difference between the amounts of M proteins in cells was therefore compressed to 1/7.5, making it difficult to detect the difference between levels of intracellular M protein.

Expression of the M, N, F, and HN proteins resulted in formation of a VLP as described above. Removal of the M protein from this combination resulted in complete suppression of VLP release, supporting the notion that the M protein is a major driving force for release (Fig. 3A). Removal of the F protein also reduced the released proteins to 0.5%, indicating that the F protein has a release-promoting effect (Fig. 3A). Removal of the N protein did not significantly affect the efficiency of release in this case (Fig. 3A). It is notable that without the HN protein, release of the remaining proteins, M, F, and N proteins, was enhanced (8.3%; Fig. 3A). An increase in amounts of transfected HN plasmids resulted in greater suppression of VLP release (Fig. 3B), confirming that the HN protein has an inhibitory effect on release. The inhibitory effect may not be due to cell toxicity of the HN protein because viral protein synthesis appeared to be constant in the transfected cells (Fig. 3B) and no apparent CPE was observed (data not shown). For experiments in this study, 0.5 μg of the HN plasmid was used to adjust the intracellular expression level of HN protein to that in SeV-infected cells.

### *Acceleration of VLP release by co-expression of the C protein*

Effects of nonstructural proteins, C and V proteins, on the VLP release were further examined. Expression of the C protein together with M, N, F, and HN proteins enhanced VLP release by more than 2-fold (Fig. 4). The enhancement was highly reproducible (Fig. 4), showing that the C protein increased the efficiency of VLP release. In contrast, the V protein did not have such an enhancing activity (data not shown). The fraction of M released into VLPs formed by M, N, F, HN, and C was 9.8%, almost half of the M fraction released into SeV particles (Fig. 4).

### *Density and morphology of structures released by expression of viral proteins in various combinations*

We performed sucrose density equilibrium ultracentrifugation to determine the density of released proteins. M protein released by single expression showed a wide range of densities, indicating heterogeneity of M protein-containing structures (Fig. 5). The M protein was found in abundance in fractions 8 to 16 (1.09–1.22 g/ml). The M protein was considered to be released in association with

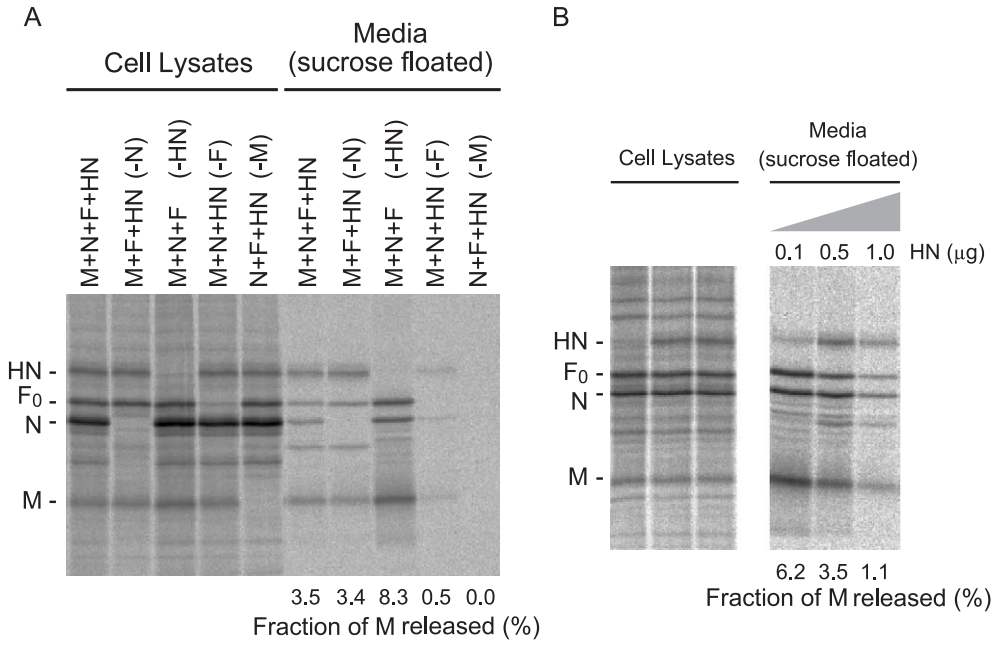


Fig. 3. (A) Contribution of SeV proteins to VLP release from cells. 293T cells were transfected with multiple plasmids as indicated in the figure and labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine from 24 to 48 h post-transfection. The medium was then separated by sucrose floatation ultracentrifugation, and proteins in the floated fraction were analyzed by immunoprecipitation and SDS-PAGE. Proteins in the cells were also analyzed, and protein release was calculated by the M protein. (B) Effect of the HN protein on VLP release. The M, N, and F plasmids together with increasing amounts of the HN plasmid were introduced into 293T cells, and proteins were analyzed. Proteins from 1/15 of the cell lysates and those from half of the medium were run in a lane in these gels.

lipids because treatment with 1% Triton X-100 before ultracentrifugation shifted the distribution (data not shown). The singly expressed F protein was distributed in a narrow range around fraction 14 (1.17–1.20 g/ml) (Fig. 5), and released N protein was slightly detected in a heavier fraction, fraction 16 (1.20–1.22 g/ml) (Fig. 5).

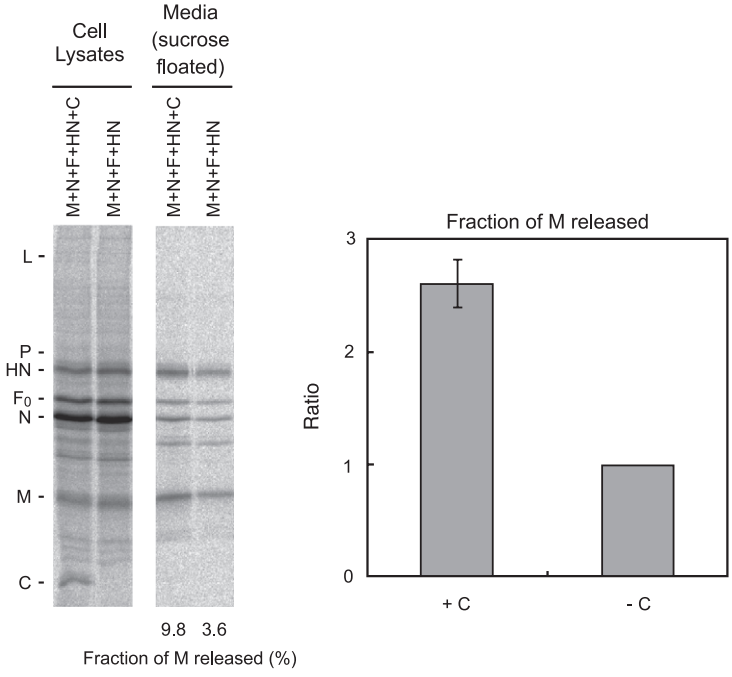


Fig. 4. Effect of C protein on VLP release from cells. 293T cells were transfected with multiple plasmids with or without the C plasmid as indicated in the figure and labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine from 24 to 48 h post-transfection. The medium was then separated by sucrose floatation ultracentrifugation, and proteins in the floated fraction were analyzed by immunoprecipitation and SDS-PAGE. Proteins in the cells were also analyzed similarly, and protein release was calculated by the M protein. Proteins from 1/15 of the cell lysates and those from half of the medium were run in a lane. The graph indicates the ratio of fractions of M released into VLPs from four independent experiments. Error bar indicates standard deviation.

When the F protein was expressed with the M protein, these two proteins co-existed mainly in fractions 12 and 14 (Fig. 5, M + F). The distribution of the M protein was shifted into these fractions (Fig. 5, M + F). A band between the M and $F_0$ proteins was considered to be β-actin because it reacted with anti-β-actin antibody in immunoblotting (data not shown) as described previously (Takimoto et al., 2001). Expression of the N protein with the M protein resulted in co-existence of N and M proteins in a fraction similar to that when the N protein was singly expressed (Fig. 5, M + N). On the other hand, a significant amount of the M protein was found in fractions 8–12 (Fig. 5, M + N), probably having been released independently of the release of the N protein. The changes in distribution and amounts of released proteins by co-expression suggest interactions between F and M proteins and between M and N proteins. In contrast, simultaneous expression of F and N proteins caused no change in protein release or in density distribution compared with those in the case of single expressions (data not shown).

Triple expression of M, N, and F proteins caused co-localization of these three proteins in fraction 14, suggesting that a significant amount of these proteins interacted and formed VLPs with density similar to that of authentic virus particles (Fig. 5, M + N + F). On the other hand, some N proteins were found in a heavier fraction, fraction 18, and some $F_0$ proteins were found in the lighter fractions, fractions 10 and 12, suggesting that some proteins were independently released probably in heterogeneous VLPs. Triple expression of M, N, and HN proteins caused less VLP release in total, but the proteins were fairly concentrated to fraction 14 (Fig. 5, M + N + HN). The glycoproteins F and HN appeared to guide M and N proteins to fraction 14.

Simultaneous expression of M, N, F, and HN proteins resulted in concentration almost all of these proteins to fraction 14, suggesting formation of homogenous VLPs with density similar to that of virus particles (Fig. 1B). Additional expression of the C protein with the four proteins did not alter the distribution of the proteins, although amounts of the proteins were increased overall (data not shown). Convergence by co-expression of viral proteins to fraction 14, which had the same density as SeV particles, was apparent when the percent distribution of the M protein was plotted in a graph (Fig. 5).

Negative staining and electron microscopy demonstrated that the supernatant from the cells expressing only the M

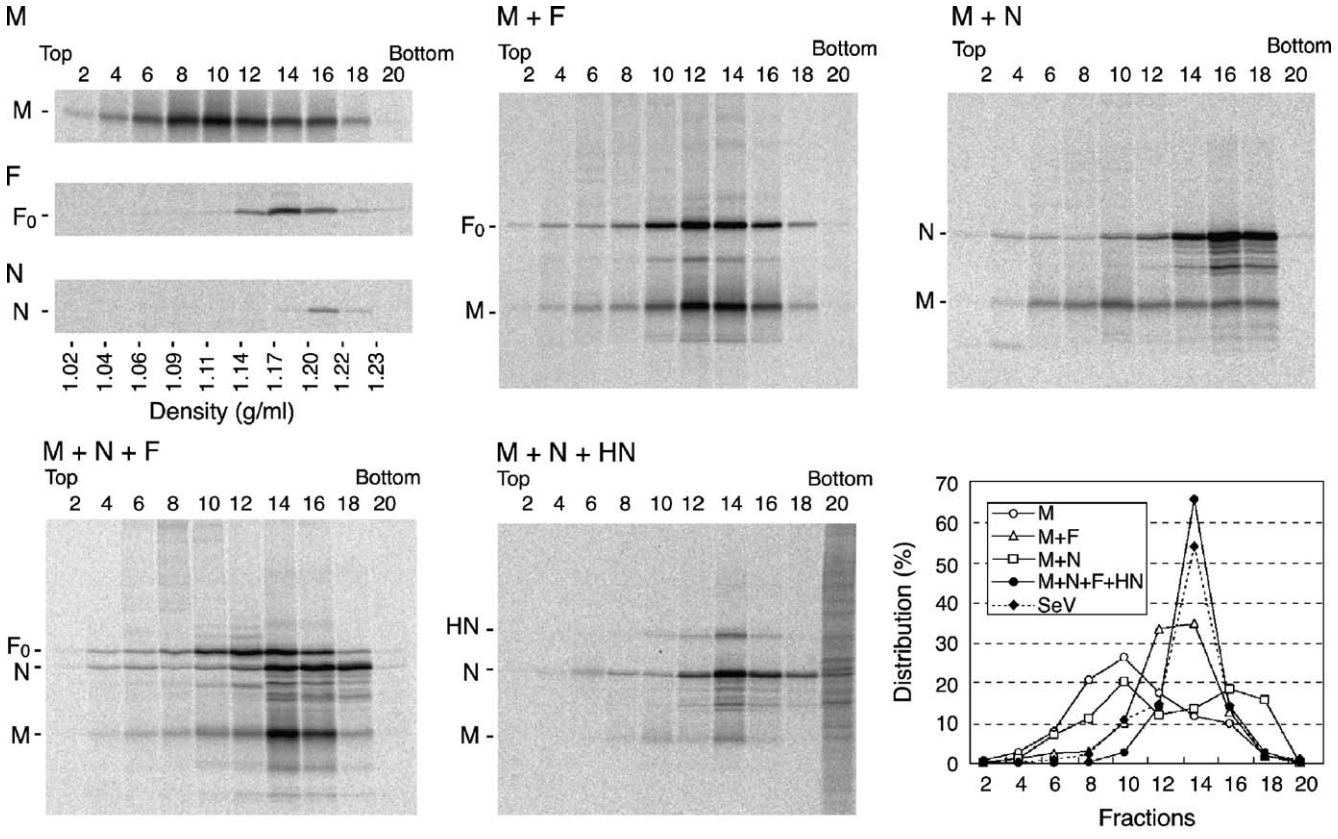


Fig. 5. Distributions of densities of VLPs with an increasing number of plasmids. Plasmids for expression of a single viral protein (M, F, or N) or those for expression of multiple viral proteins (M + F, M + N, M + N + F, or M + N + HN) were introduced into 293T cells and labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine from 24 to 48 h post-transfection. The medium was then separated by sucrose density equilibrium ultracentrifugation in a 20– 50% continuous gradient. Fractions were taken and SeV proteins in each fraction were analyzed by immunoprecipitation and SDS-PAGE. (Graph) Percent distributions of the M protein in the results from each combination of plasmids in Fig. 1B (M + N + F + HN, SeV) and this figure (M, M + F, M + N) are plotted in a graph.

protein had vesicles with smooth surfaces (Fig. 6A). The vesicles from the cells expressing M and F proteins had rough surfaces with some geometric patterns, indicating the presence of abundant spikes on the surfaces (Fig. 6B). Furthermore, the vesicles formed by M, F, and N proteins had nucleocapsid-like structures inside (Fig. 6C). There were also some structures outside the particles (Fig. 6C). Co-expression of M, N, F, and HN proteins generated VLPs similar to virus particles (Fig. 1C), although small vesicles were also seen in the VLPs (data not shown). There was no difference between the morphology of VLPs generated by co-expression of M, N, F, and HN proteins with the C protein and that without the C protein (data not shown).

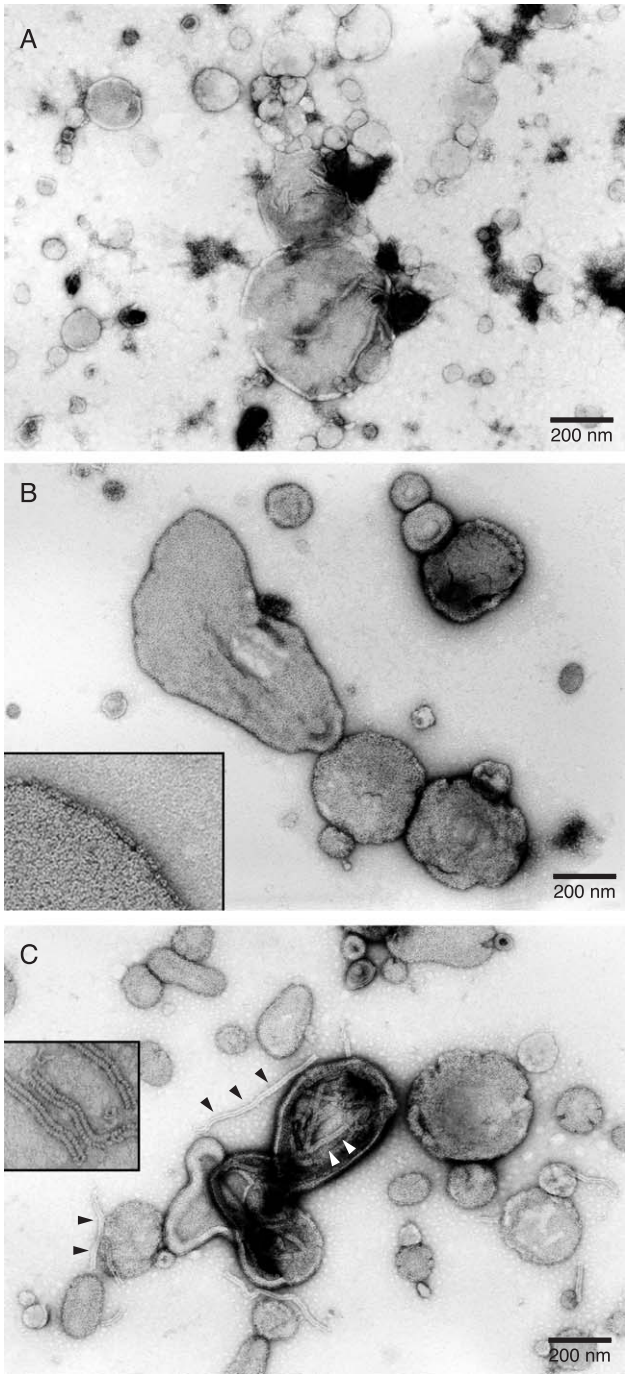


Fig. 6. Electron microscopy of structures released from transfected cells. The medium from transfected 293T cells was clarified by low-speed centrifugation, passed through a 5-μm filter, and centrifuged at 24 000 rpm for 1 h through a 7% (w/w) sucrose cushion. The pellet was resuspended in PBS, processed for negative staining, and observed by electron microscopy. (A) Single expression of M. (B) Double expression of M and F. (Inlet) Magnification of the surface of the VLP. (C) Triple expression of M, N, and F. Arrowheads indicate nucleocapsid-like structures. (Inlet) Magnification of the nucleocapsid-like structures.

## Discussion

In the present study, we expressed SeV proteins and produced VLPs. Co-expression of M, N, F, and HN proteins and sucrose density equilibrium ultracentrifugation concentrated these proteins into a common fraction, to which the authentic virus particles migrated, indicating that these proteins autonomously assembled into VLPs that are comparable to the authentic virus particles in terms of density. The similar densities seem to reflect the common compositions of the virus particles and the VLPs. The VLP formation can thus be used as a model for virus assembly and budding. In this condition, however, protein release was only one-fifth of that of SeV. A higher level of release, almost half of the efficiency of SeV, was obtained by co-expression of C protein together with M, N, F, and HN proteins. To get closer the efficiency of VLP release to that of virus release, some other viral proteins or the nucleocapsid containing the full-length genomic RNA might be necessary.

Schmitt et al. (2002) expressed multiple proteins of SV5, a paramyxovirus classified to the genus distinct from that of SeV, in 293T cells to generate VLPs (Schmitt et al., 2002). The study shows that the M protein alone does not result in generation of VLPs from cells, but co-expression of the N protein and one of the glycoproteins, F or HN protein, together with the M protein is necessary for efficient VLP budding. This situation is different from that of SeV. Furthermore, the glycoproteins, F and HN proteins, have similar budding-promoting effects in SV5, whereas in SeV, the F protein was promoting and the HN protein was inhibitory for VLP budding. The differences revealed by VLP formation may indicate differences of mechanism of virus budding between SV5 and SeV.

An intracellular nucleocapsid structure formed by single expression of the N protein has been reported in paramyxoviruses: SeV (Buchholz et al., 1993), measles virus (Spehner et al., 1991), and Newcastle disease virus (Errington and Emmerson, 1997). Once formed in cells, the nucleocapsid-like structure could be a substitute for the nucleocapsid in VLP budding. We found that the ratio of the N protein to other viral proteins in VLPs was different to that in the virus particles; the ratios of the N protein to the M protein were 1.6:1 in VLPs and 0.7:1 in the virus particles.

The N protein in VLPs appeared to be more abundant than that in the virus, as had been observed in VLP formation in SV5 (Schmitt et al., 2002). The VLPs may contain nucleocapsid-like structures of various lengths, almost all of which are probably shorter than the viral nucleocapsid (15 384 bases). Such differences in length may affect the N protein ratio because difference in length affects the nucleocapsid incorporation and budding as shown by using defective interfering particles (Mottet and Roux, 1989).

Interaction between the M protein and viral glycoproteins has been shown by using biochemical and cell biological methods (Yoshida et al., 1976, 1979, 1986) and by the use of live viruses whose cytoplasmic tails of the glycoproteins were deleted (Fouillot-Coriou and Roux, 2000). Recently, Ali and Nayak (2000) showed the interaction of M and glycoproteins in a raft domain by solubilizing cells with a nonionic detergent and a subsequent sucrose floatation. The shift of fractions observed in the sucrose density ultracentrifugation of VLPs suggested the interaction. On the other hand, Takimoto et al. (2001) did not suggest the interaction, based on the observation that the co-expressed and released M and F proteins were in distinct fractions after sucrose density equilibrium ultracentrifugation, although they suggested a functional interaction by release acceleration of both of the proteins. The reason for the contradiction with ours is unknown. The present study also suggested protein–protein interaction between M and N. This is consistent with the previous reports (Coronel et al., 1999, 2001; Yoshida et al., 1976).

Fouillot-Coriou and Roux (2000) showed by using recombinant SeVs possessing cytoplasmic tail-deleted glycoproteins that a partial deletion of the F tail caused the virus to bud inefficiently, while the rates of incorporation of the F protein to other viral proteins were almost constant. It seems that the F protein is essential for virus budding, as if it is a part of the budding machinery. On the other hand, the tail-less HN basically had no effect on virus budding, and HN mutants are not incorporated in some cases in a cytoplasmic motif-dependent fashion (Fouillot-Coriou and Roux, 2000; Takimoto et al., 1998). Furthermore, efficient budding of an HN-deficient SeV has been reported (Inoue et al., 2003b; Leyrer et al., 1998; Stricker and Roux, 1991). These results suggest that SeV F and HN proteins have different roles in virus budding. Our study showed that the F protein had a promoting effect on VLP budding, while the HN protein had an inhibitory effect. Although Takimoto et al. (2001) demonstrated that the HN protein did not have such an inhibitory effect on M-driven vesicle release, the reason of the discrepancy is not known. The HN protein increased VLPs of uniform density (Fig. 5) and may have enhanced the integrity of VLPs.

Hasan et al. (2000) reported that SeV lacking all of the four C proteins yielded less virus particles and that the particles were irregular in shape, suggesting a role of the C protein in virus assembly and budding. In the present study, addition of the C protein to the VLP system enhanced VLP release from cells. This is strong evidence that a paramyxovirus accessory protein affects virus budding. In infected cells, the exogenous C protein has a suppressive effect on RNA synthesis (Curran et al., 1992; Horikami et al., 1997). Thus, the function of the C protein in virus budding had been difficult to analyze in virus-infected cells. The present VLP system could illustrate the action of the C protein on virus budding. However, the mechanism of acceleration of VLP release by the C protein is unknown and to be resolved. The C protein is known to co-localize with the M and HN proteins in infected cells (Hasan et al., 2000), and it is thought that the C protein acts as a molecular chaperon to facilitate assembly of viral proteins by interacting with M protein. Although the C protein has been shown to interact with the L protein (Horikami et al., 1997), the interaction may not be involved in VLP release because the VLP-forming system in the present study lacked the L protein.

The acceleration of VLP release by the C protein is limited to only 2- to 3-fold, while a C-knockout virus suppressed virus formation by ca. 100-fold (Hasan et al., 2000). Because the C protein has various functions, including anti-interferon activity (Garcin et al., 1999; Gotoh et al., 1999) and inhibition of viral RNA synthesis (Curran et al., 1992; Horikami et al., 1997), the extremely low yield of C-knockout virus may be due to the summation of impediments of these functions in addition to that of the budding-enhancing activity identified in this work.

In summary, we have established a VLP production system of SeV. The generated VLPs had density similar to that of actual virus particles, and the released VLPs were comparable to the virus particles. A novel finding by using the VLP system was that the C protein directly facilitated virus budding. The current VLP system will be useful in further research on virus budding, including research to develop antiviral drugs targeting virus assembly and vaccine development by using VLPs as an immunogen.

## Materials and methods

### *Cells and a virus*

293T cells, human renal epithelial cells expressing the SV40 large T antigen, were propagated in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with glutamine and 10% fetal calf serum. Recombinant SeV derived from the Z strain (Kato et al., 1996) was propagated in embryonated chicken eggs as described previously (Kiyotani et al., 1990).

### *Plasmids*

The plasmids, pCAGGS-M, pCAGGS-N, pCAGGS-F and pCAGGS-HN, for mammalian expression of SeV proteins under the chicken β-actin promoter (Niwa et al., 1991) were described previously (Sakaguchi et al., 1999).

The expression plasmid for the C protein, pCAGGS-C, was constructed in this study.

*Transfection, infection, and metabolic labeling*

Subconfluent 293T cells were transfected with plasmids by using the FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN). For a 60-mm dish, 3.5 μg of plasmids were used in cotransfection experiments: pCAGGS-M, 1 μg; pCAGGS-F, 1 μg; pCAGGS-N, 0.5 μg; pCAGGS-HN, 0.5 μg; pCAGGS-C, 0.5 μg. The plasmid amounts were determined so that intracellular levels of protein expression were similar to those in virus-infected cells. In the case of omission of an expression plasmid, a vector plasmid, pCAGGS, was added to keep the total amount of transfected DNA constant. For a 100-mm dish, a 3-fold larger amount of the plasmids was used. Alternatively, 293T cells were infected with SeV at an m.o.i. of 10.

For metabolic labeling of viral proteins, the medium of transfected or infected 293T cells was replaced with 1.5 ml of DMEM containing one-tenth of the normal amounts of cysteine and methionine and 2.5 MBq/ml of [$^{35}$S]cysteine and [$^{35}$S]methionine mixture ([$^{35}$S]Pro-mix, Amersham Biosciences, Piscataway, NJ), together with 10 mU of bacterial neuraminidase (Roche Diagnostics) after 24 h. After 48 h, the medium and cells were collected separately.

*Sucrose floatation and sucrose density equilibrium ultracentrifugation*

For a sucrose floatation assay, the medium was clarified by centrifugation at 7000 rpm for 5 min, and 1.4 ml of the supernatant was mixed with 2.1 g sucrose to adjust its concentration to 60% (w/w). The supernatant was then transferred to a centrifuge tube, overlaid with 7 ml of 50% (w/w) sucrose/phosphate-buffered saline (PBS) and 0.5 ml of 10% (w/w) sucrose/PBS, and centrifuged at 35 000 rpm for 18 h in a RPS40T rotor (Hitachi, Tokyo, Japan). The top 1.5-ml fraction including the 10% and 50% sucrose boundary was collected and solubilized in a radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 150 mM NaCl] containing 50 mM iodoacetamide (IAA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were solubilized in a RIPA buffer containing IAA and PMSF and then centrifuged at 15 000 rpm for 20 min to remove nuclei and cell debris.

For sucrose density equilibrium ultracentrifugation, the medium was clarified at 7000 rpm for 5 min and was layered onto the top of a 20–50% sucrose/PBS linear density gradient and centrifuged at 35 000 rpm for 18 h in a RPS40T rotor. Fractions were obtained by using an Auto Densi-Flow fractionator (Labconco, Kansas City, MO). Density of fractions was measured by weighing each fraction. Proteins in the fractions were solubilized by adding concentrated RIPA buffer containing IAA and PMSF.

*Immunoprecipitation and SDS-PAGE*

Solubilized proteins in the RIPA buffer were mixed with 2 μl of anti-SeV serum, 1 μl of anti-C serum (kindly provided by A. Kato), and 30 μl of Protein A Sepharose beads, followed by agitation for 18 h at 4 °C. Immune complexes were washed three times with the RIPA buffer, suspended in SDS sample buffer, and boiled for 3 min as described previously (Sakaguchi et al., 1996). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using an 11% gel, and protein bands were visualized and quantitated by using a BAS2000 image analyzer (Fuji Film, Tokyo, Japan). The fraction of viral proteins released into the medium was calculated basically as the ratio of the M protein detected in the medium to the total M protein both in the medium and the cells. Proteins from 1/15 of the cell lysates and those from half of the medium were run in a lane in these gels.

*Electron microscopy*

For electron microscopy, subconfluent 293T cells in 100-mm dishes were transfected with plasmids, and after 48 h, the media were clarified by low-speed centrifugation and passed through a Millex-SV filter with a pore size of 5.0 μm (Millipore, Bedford, MA) to completely remove cells and then pelleted through a 7% (w/w) sucrose/PBS cushion by ultracentrifugation at 24 000 rpm for 1 h. The pellet was resuspended in PBS, placed on Formvar-carbon-coated nickel grids, stained with 4% uranyl acetate, and examined under a JEOL JEM-1200EX II transmission electron microscope as described previously (Uchiyama and Uchida, 1988).

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