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## Recombinant Sendai viruses with L1618V mutation in their L polymerase protein establish persistent infection, but not temperature sensitivity

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### Abstract

The Sendai virus pi strain (SeVpi) isolated from cells persistently infected with SeV shows mainly two phenotypes: (1) temperature sensitivity and (2) an ability of establishing persistent infection (steady state). Three amino acid substitutions are found in the Lpi protein and are located at aa 1088, 1618, and 1664. Recombinant SeV(Lpi) (rSeV(Lpi)) having all these substitutions is temperature sensitive and is capable of establishing persistent infection (steady state). rSeVs carrying the fragment containing L1618V show both phenotypes. rSeV(L1618V), in which leucine at aa 1618 is replaced with valine, has the ability of establishing persistent infection, but is not a temperature-sensitive mutant, indicating that the ability of a virus to establish persistent infection can be separated from temperature sensitivity. The amino acid change at 1618(L→V) coexisting with aa 1169 threonine is required for acquirement of a temperature-sensitive phenotype. Three amino acid substitutions are also found in the Ppi protein, but rSeV(Ppi) does not show these phenotypes.

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*Keywords:* Sendai virus; Paramyxovirus; Parainfluenza virus; Persistent infection; L protein; Temperature sensitive

### Introduction

Persistent viral infection of cultured cells could be classified into two types, that is, steady-state infection and carrier state infection (Walker and Hinze, 1962). Infection of human conjunctiva cells by mumps virus (MuV), a member of paramyxovirus, easily leads to steady-state infection (Walker et al., 1966). On the other hand, when persistent infection of MuV in murine L929 cells was established, the culture contained two types of virus-infected cells (Ito et al., 1986). The majorities were in “carrier state” and only a few cells were in “steady state” (Ito et al., 1986). Which type of persistent infection is established depends on the combination of virus and cell.

When cells such as HeLa and BHK are infected with Sendai virus (SeV) at a relatively high moi, only a few cells survive. The surviving cells, subcultured for a long time, develop into Sendai virus persistently infected cells (steady-state infection) (Nagata et al., 1972). We previously reported a temperature-sensitive phenomenon of virus maturation in BHK cells persistently infected with Sendai virus (Nagoya strain; BHK/SeV cells) (Nagata et al., 1972). Incidentally, infectious viruses could be isolated from carrier cells by inoculating of the cells into eggs, and the virus (Sendai virus pi strain; SeVpi) was found to be a temperature-sensitive mutant (Kimura et al., 1975). Intriguingly, SeVpi shows almost no cytotoxicity at permissive or nonpermissive temperature and has an ability of establishing virus-persistent infection in various cells without the aid of the DI virus (Yoshida et al., 1982). It has been commonly accepted that these phenotypes of SeVpi are caused by M or HN proteins (Kimura et al., 1975; Kondo et al., 1993; Yoshida et al., 1979). However,

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recombinant SeVs (rSeVs) possessing HNpi or Mpi proteins are not temperature sensitive and are incapable of establishing persistent infection (Nishio et al., 2003). Because none of the virus-specific polypeptides are detected in cells primarily infected with SeVpi and incubated at 38 °C, and none of the virus-specific proteins is pulse labeled at 38 °C (Nishio et al., 2003), it is inferred that the temperature-sensitive step is at an early stage of infection and the polymerase protein(s) is a temperature-sensitive protein. The polymerases of paramyxovirus are composed of L and P proteins. However, which viral component(s) is temperature sensitive and is related to the ability of establishing persistent infection remains to be proved.

In this study, the complete L and P genome sequences of SeV Nagoya strain and SeVpi were determined, and three amino acid substitutions were found in each of the proteins. Recombinant SeV having the Lpi protein showed temperature sensitivity and had an ability of establishing persistent infection, while one possessing the Ppi protein did not show these phenotypes.

## Results

### Comparison of amino acid sequences of the L proteins

We have recently reported that the temperature-sensitive step in SeVpi replication is at an early stage of infection and the L protein was not detected in L929/SeVpi cells incubated at 38 °C (Nishio et al., 2003), suggesting that the temperature-sensitive protein(s) is the L protein. The L gene sequences of all the viruses listed in Table 1, except for the Nagoya parent strain of the SeVpi virus, were reported in our previous study (Nishio et al., 2003). At first, the complete L genome sequences of SeV Nagoya strain were determined. Subsequently, the amino acid sequences deduced from the nucleotide sequences of six different Sendai virus strains [Nagoya (parent wild strain), Z (parent virus of recombinant virus), Fushimi, Hamamatsu, Enders and SeVpi] were compared with each other (Table 1). Three amino acid substitutions unique to the SeVpi L protein were found

and located at aa 1088, 1618, and 1664 (Table 1). The SeVpi L protein differs from that of the Nagoya strain at several additional positions, but these changes are also common to other SeV strains (Table 1). aa 1088 is in the conserved domain IV, and both aa 1618 and 1664 are located between conserved domains V and VI. Furthermore, three amino acid substitutions were also found in the Ppi protein, another component of virus polymerases (data not shown).

### Construction of the recombinant SeV carrying the L protein of SeVpi strain

Consequently, to investigate the role of the L proteins of SeVpi in the phenotypes, we prepared the chimeric recombinant SeV (rSeV) generated by replacing the corresponding regions of L protein of SeV Z strain with fragments including amino acid substitutions of Lpi (Table 2). Fragment *ApaI-NheI* (aa 380–1135), fragment *NheI-XhoI* (aa 1136–1636), and fragment *XhoI-KpnI* (aa 1637–nt 15281; in noncoding region) were named Fr:A1088S, Fr:L1618V, and Fr:I1664V, respectively. The chimeric recombinant viruses were designated as rSeV(Fr:A1088S), rSeV(Fr:L1618V), rSeV(Fr:I1664V), rSeV(Fr:A1088S/L1618V), rSeV(Fr:A1088S/I1664V), and rSeV(Fr:L1618V/I1664V) (Table 2). Furthermore, we constructed rSeV having all the fragments and was named rSeV(Lpi) (Table 2). All of the rSeVs were inoculated intrallantoically into 10-day-old embryonated eggs, which were incubated at 32 °C for 3 days, and then the allantoic fluids were harvested.

### *rSeV(Lpi)* is temperature sensitive and is capable of establishing persistent infection

L929 cells were primarily infected with SeV Nagoya strain, SeVpi, rSeV(PA), or rSeV(Lpi) at an moi of about 10 and were incubated at 32 or 38 °C for 20 h. rSeV(PA) is the parent recombinant SeV (Z strain). Then hemadsorption test and immunostaining were carried out. The cells infected with Nagoya strain or rSeV(PA) exhibited significant hemadsorption at both 32 and 38 °C (Fig. 1A). On the other hand, cells infected with

Table 1  
Comparison of amino acid residues of L protein

Strain	Position of amino acid residue																	
	96	218	322	535	625	626	752	798	852	971	981	1088	1091	1169	1207	1618	1664	2117
Z	H	V	T	E	E	N	M	E	K	G	V	A	R	I	S(C) <sup>a</sup>	L	I	I
Fushimi	Q	V	T	K	K	K	I	E	R	D	V	A	R	T	S	L	I	I
Hamamatsu	Q	V	T	K	K	K	I	E	R	D	V	A	K	T	S	L	I	I
Enders	–	V	T	K	K	K	I	E	R	D	V	A	R	T	S	L	I	I
Nagoya	Q	I	A	K	K	K	I	K	R	D	I	A	K	T	S	L	I	V
SeVpi	Q	I	A	K	K	K	I	K	R	G	V	S	K	T	S	V	V	V

<sup>a</sup> aa1207 of rSeV(Pa) is cysteine.

Table 2  
Temperature sensitive and lower cytopathogenic phenotypes of various rSeVs

Virus	Position of amino acid residue					Temperature sensitivity	Ability of establishing persistent infection
	1088	1169	1207	1618	1664		
SeV Nagoya	A	T	S	L	I	–	–
SeVpi	S	T	S	V	V	+	+
rSeV(PA)	A	I	C	L	I	–	–
new/rSeV(PA)	A	I	S	L	I	–	–
rSeV(Lpi)	S	T	S	V	V	+	+
rSeV(Fr:A1088S)	S	I	C	L	I	–	–
rSeV(Fr:I1664V)	A	I	C	L	V	–	–
rSeV(Fr:A1088S/I1664V)	A	I	C	L	V	–	–
rSeV(Fr:L1618V)	A	T	S	V	I	+	+
rSeV(Fr:A1088S/L1618V)	S	T	S	V	I	+	+
rSeV(Fr:L1618V/I1664V)	A	T	S	V	V	+	+
rSeV(L1618V)	A	I	C	V	I	–	+
new/rSeV(L1618V)	A	I	S	V	I	–	+
new/rSeV(I1169T)	A	T	S	L	I	–	–/+ <sup>a</sup>

<sup>a</sup> new/rSeV(I1169T)-infected cells showed little cpe at 38 °C, and persistent infection could be established at 38 °C.

SeVpi or rSeV(Lpi) showed no hemadsorption at 38 °C, while exhibited significant hemadsorption at 32 °C (Fig. 1A). Subsequently, infective titers of these viruses were measured at 32 and 38 °C. As shown in Fig. 1A, rSeV(PA) is not temperature sensitive, while rSeV(Lpi) is temperature sensitive (Fig. 1A). The virus-specific antigens were detected in cells infected with rSeV(PA) and incubated at 32 or 38 °C. However, none of virus-specific antigens could be detected in cells infected with rSeV(Lpi) and incubated at 38 °C (Fig. 1C). In the next experiment, L929 cells were primarily infected with rSeV(PA) or rSeV(Lpi) at an moi of about 10, incubated at 32 or 38 °C, and the culture fluids were harvested at various periods. HA production was undetected in culture fluids of rSeV(Lpi)-infected L929 cells at 38 °C and was delayed in cells at 32 °C compared with that in cells infected with rSeV(PA) (Fig. 1B), showing that rSeV(Lpi) is a temperature-sensitive virus.

SeVpi was found to show almost no cytotoxicity and to have an ability of establishing persistent infection in various cells (steady state) without the aid of the DI virus even at permissive temperature (Yoshida et al., 1982). Therefore, whether rSeV(Lpi) has an ability of establishing persistent infection was investigated. L929 cells were infected with SeV Nagoya strain, SeVpi, rSeV(PA), or rSeV(Lpi) at an moi of about 100, incubated at 32 °C for 4 days, and then these cells were stained with Gimsa's solution. L929 cells infected with SeVpi or rSeV(Lpi) showed no or little cpe, while SeV Nagoya and rSeV(PA) induced severe cpe (Fig. 2A and data not shown). Furthermore, cells infected with rSeV(Lpi) could be subcultured at 35 °C (Fig. 2B), indicating that rSeV(Lpi) has the ability of establishing persistent infection (steady state). On the other hand, rSeV(Ppi) is not temperature sensitive and is incapable of establishing persistent infection (data not shown).

It is clarified from above findings that replacement of corresponding regions by the Lpi protein of SeVpi can confer the temperature-sensitive phenotype and the lower cytopathogenic property on SeV Z strain (wild strain), showing that these phenotypes are caused by the Lpi protein.

*rSeVs carrying the fragment containing L1618V are temperature sensitive*

The cells primarily infected with rSeV(Fr:A1088S), rSeV(Fr:I1664V), or rSeV(Fr:A1088S/I1664V) exhibited significant hemadsorption at both 32 and 38 °C (Fig. 3A). On the other hand, cells infected with all of rSeVs carrying the fragment containing L1618V showed no hemadsorption at 38 °C, while exhibited significant hemadsorption at 32 °C (Fig. 3A). Subsequently, infective titers of various recombinant SeV(Lpi) were measured at 32 and 38 °C. As shown in Fig. 3A, rSeV(Fr:A1088S), rSeV(Fr:I1664V), and rSeV(Fr:A1088S/I1664V) are not temperature sensitive, while all of rSeVs carrying the fragment containing L1618V are temperature sensitive. Furthermore, no virus-specific polypeptides were detected in cells infected with all of rSeVs carrying the fragment containing L1618V and incubated at 38 °C by immunofluorescent staining (Fig. 3B and data not shown). Subsequently, HA production by L929 cells primarily infected with rSeV(Fr:A1088S), rSeV(Fr:L1618V), rSeV(Fr:I1664V), or rSeV(Fr:A1088S/I1664V) was examined. rSeV(Fr:A1088S), rSeV(Fr:I1664V), and rSeV(Fr:A1088S/I1664V) induced HA production at 38 °C almost as much as at 32 °C (Fig. 3C). However, HA production could not be detected in the culture fluids of cells infected with rSeV(Fr:L1618V) and incubated at 38 °C, and HA production was delayed in these cells at 32 °C (Fig. 3C). These findings point out that the region containing aa 1618 is involved in temperature sensitivity.

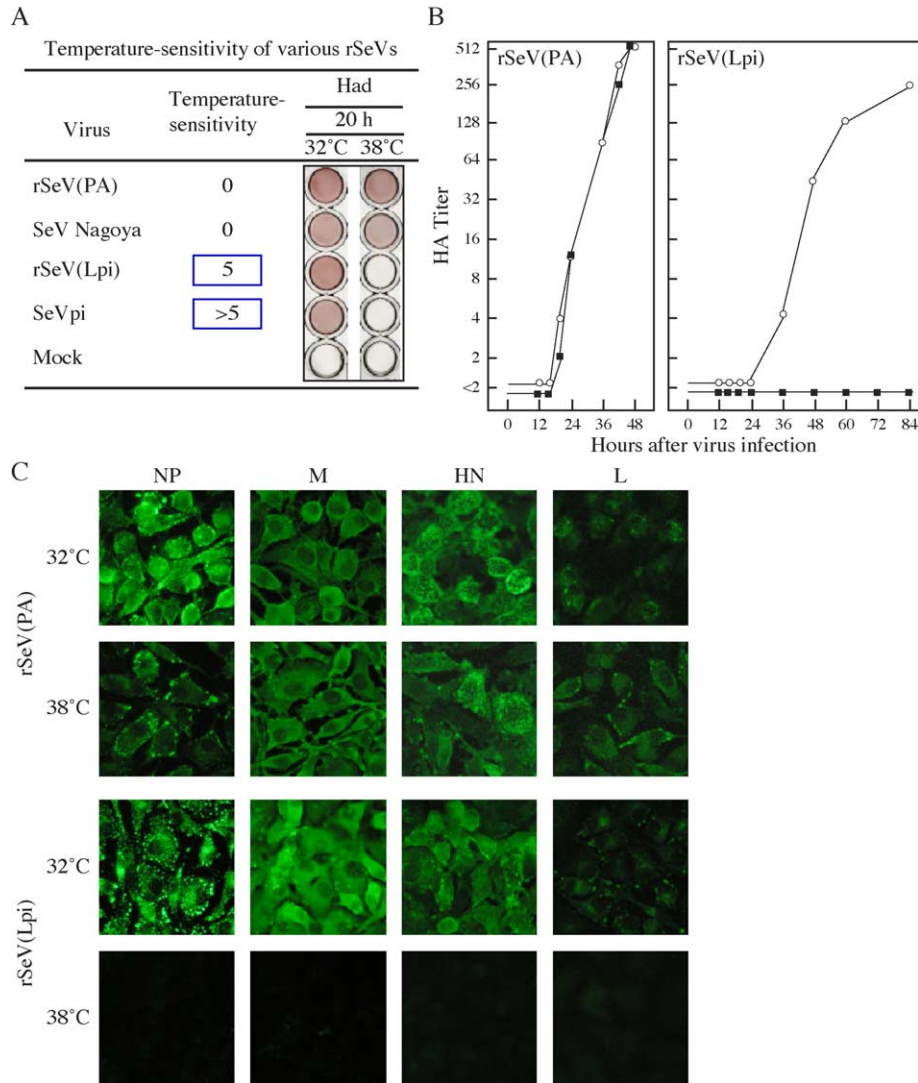


Fig. 1. Temperature sensitivity of rSeV(Lpi). (A) Virus infectivities of SeV Nagoya strain, SeVpi, rSeV(PA), and rSeV(Lpi) were measured at 32 or 38 °C. Decrease (log<sub>10</sub>) in infective titer at 38 °C compared to the titer at 32 °C is shown as “temperature sensitivity”. In next experiment, L929 cells were infected with these viruses at an moi of about 10, incubated at room temperature for 2 h, and then washed three times. Subsequently, the cells were transferred to 32 or 38 °C, were incubated for 20 h, and then hemadsorption test was carried out. (B) L929 cells were primarily infected with rSeV(PA) or rSeV(Lpi) at an moi of about 10, incubated at room temperature for 2 h, and transferred to 32 (○) or 38 °C (■). Subsequently, the culture fluids were harvested at various periods and HA activities of the culture fluids were measured. (C) L929 cells were primarily infected with rSeV(PA) or SeV(Lpi) at an moi of about 10, incubated at room temperature for 2 h, and then were washed three times. Subsequently, the cells were transferred to 32 or 38 °C, incubated for 20 h, and then immunostained with anti-NP, -M, -HN, or -L mAb.

#### Virus-specific polypeptide synthesis in L929 cells infected with various rSeVs

L929 cells infected with rSeV(PA), rSeV(Fr:A1088S), rSeV(Fr:L1618V), or rSeV(Fr:I1664V) were incubated at 32 °C, fixed at various periods, and then virus-specific polypeptides were detected by ELISA using anti-NP or anti-HN antibody. As shown in Fig. 4A, the virus-specific polypeptide syntheses were delayed in cells infected with rSeV(Fr:L1618V) even at permissive temperature compared with the cells infected with rSeV(PA), rSeV(Fr:A1088S), or rSeV(Fr:I1664V).

#### rSeV(Fr:L1618V) shows no cell toxicity and has the ability of establishing virus-persistent infection

L929 cells were infected with various recombinant viruses at an moi of about 100, incubated at 32 °C for 4 days, and then these cells were fixed. L929 cells infected with any rSeVs carrying the fragment containing L1618V showed no or little cpe, but rSeV(Fr:A1088S), rSeV(Fr:I1664V), and rSeV(Fr:A1088S/I1664V) induced severe cpe (Fig. 4B and data not shown). Furthermore, cells infected with any rSeVs carrying the fragment containing L1618V could be cultured to steady-state persistence (Figs. 4C and D, and data not shown),

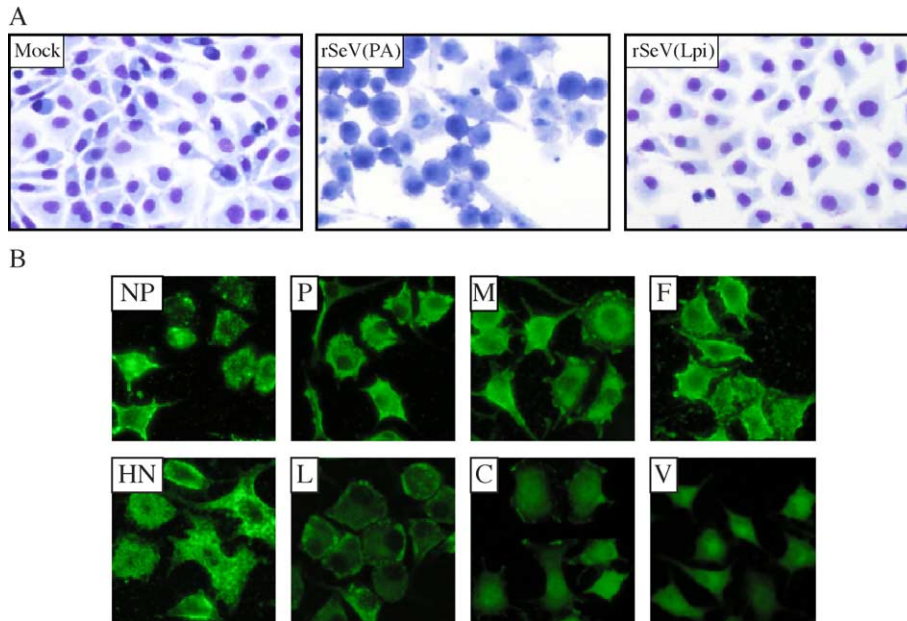


Fig. 2. rSeV(Lpi) is temperature sensitive and is capable of establishing persistent infection. (A) L929 cells were infected with either rSeV(PA) or rSeV(Lpi) at an moi of about 100, incubated at 32 °C for 4 days, and then stained with Giemsa's solution. (B) L929 cells were infected with rSeV(Lpi) at an moi of about 100, incubated at 32 °C for 48 h, and then subcultured three times at 35 °C (permissive temperature). Subsequently, the cells were immunostained with anti-NP, -P, -M, -F, -HN, -L, -C, or -V antibody. Uninfected cells were not stained with any antibody (data not shown).

showing that these viruses have the ability of establishing persistent infection (steady state). These findings show that the region containing aa 1618 is related to an ability of establishing persistent infection.

*The amino acid residue(s) responsible for the ability of establishing persistent infection and temperature sensitivity*

















To exactly identify the amino acid residue(s) responsible for the ability of establishing persistent infection and temperature sensitivity, we constructed the recombinant SeV having the mutant L protein, that is, L1618V in which leucine at aa 1618 was replaced with valine. This rSeV was designated as rSeV(L1618V). Surprisingly, this rSeV(L1618V) was not a temperature-sensitive mutant (Fig. 5), suggesting that amino acid change at 1618(L→V) does not result in a temperature-sensitive phenotype. On the other hand, it is a noteworthy finding that rSeV(L1618V) shows no or little cpe (Fig. 6A) and is capable of establishing persistent infection (Fig. 6B), indicating that the ability of virus to establish persistent infection can be separated from temperature sensitivity.

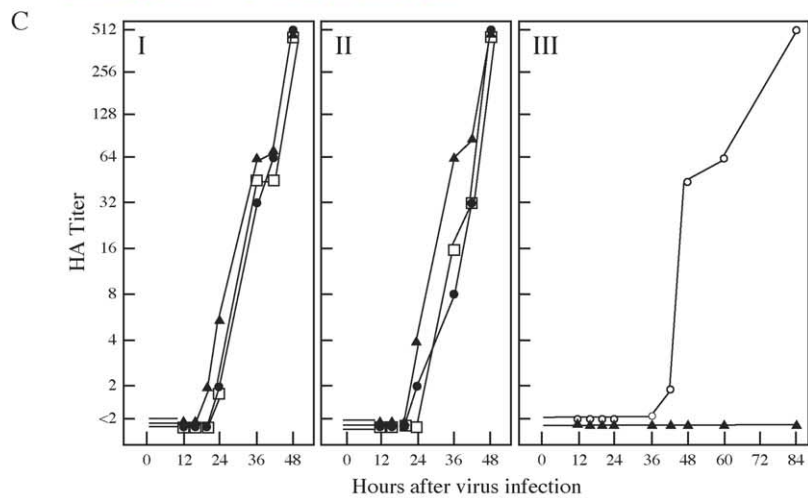
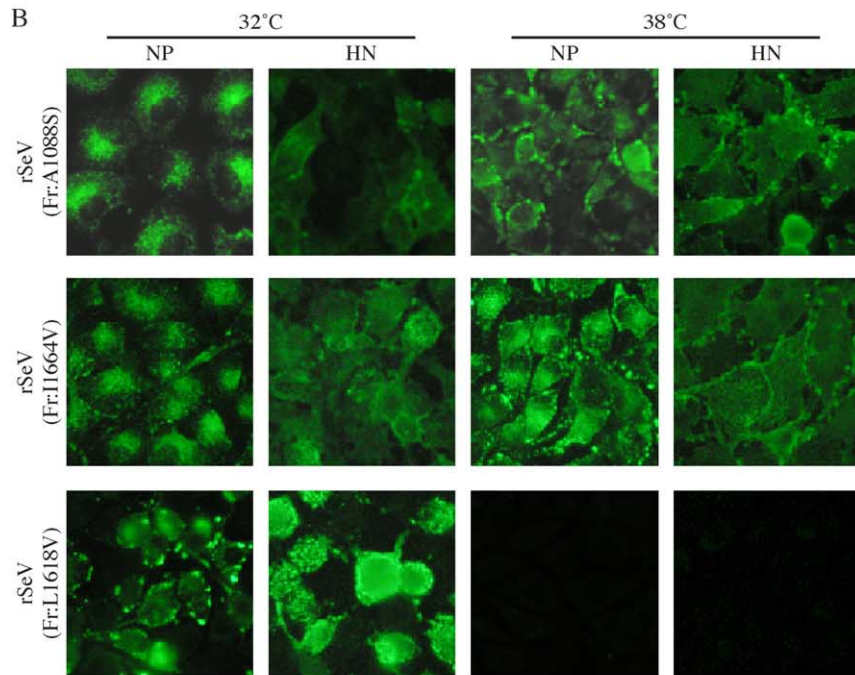
Fr:L1618V contains another amino acid (aa 1169) that is different between L proteins of SeVpi and SeV Z strain (Table 1). Furthermore, aa 1207 of the L protein of rSeV(PA) is found to be cysteine, but that of all the SeVs including Z strain is serine (Table 1). This mutation might be inserted during either construction or isolation of the recombinant virus. Therefore, we further constructed new recombinant viruses, that is, new/rSeV(PA) in which cysteine at aa 1207 was replaced with serine, and new/

rSeV(I1169T) in which isoleucine at aa 1169 and cysteine at aa 1207 were replaced with threonine and serine, respectively (Table 2). In addition, we constructed new recombinant viruses, that is, new/rSeV(L1618V) in which cysteine at aa 1207 and leucine at aa 1618 were replaced with serine and valine, respectively (Table 2). “new/rSeV (I1169T/L1618V)”, in which isoleucine at aa 1169, cysteine at aa 1207, and leucine at aa 1618 are replaced with threonine, serine, and valine, respectively, is completely identical to rSeV(Fr:L1618V) (Table 2). As described above, rSeV(Fr:L1618V) was not only capable of establishing persistent infection, but also showed a temperature-sensitive phenotype. Interestingly, new/rSeV(L1618V) was found not to be temperature sensitive (Fig. 5), although the virus has the ability of establishing persistent infection (Figs. 6B and C). The phenotypes of new/rSeV(L1618V) are almost identical to those of rSeV(L1618V).

new/rSeV(I1169T) was not temperature sensitive (Fig. 5), although HA production at 38 °C was extremely low (Fig. 7). The cells infected with new/rSeV(I1169T) showed rounding at 32 or 35 °C (Fig. 6A), but the virus-infected cells were slow to die. However, because new/rSeV(I1169T)-infected cells hardly multiplied, persistent infected cells could not be established at 32 or 35 °C. Intriguingly, new/rSeV(I1169T)-infected cells showed little CPE at 38 °C (Fig. 6A), and these cells could be subcultured at 38 °C. When these cells were subcultured seven times at 38 °C, these cells were immunostained with anti-NP mAb and hemadsorption test was carried out. Almost all the cells had NP antigens and exhibited positive hemadsorption (Fig. 6D), indicating that persistent infection could be established at 38 °C. These findings show

**A** Temperature-sensitivity of various rSeVs

Virus	Temperature-sensitivity	Had			
		20 h		3 days	
		32°C	38°C	32°C	38°C
rSeV(Fr:A1088S)	0				
rSeV(Fr:I1664V)	0				
rSeV(Fr:A1088S/I1664V)	0.5				
rSeV(Fr:L1618V)	>5				
rSeV(Fr:A1088S/L1618V)	5.25				
rSeV(Fr:L1618V/I1664V)	5.25				
Mock					



that threonine at aa 1169 subtly influences the cytopathogenesis.

Taken together, these findings indicate that only one amino acid substitution at 1618(L→V) changes the cytotoxic virus to the attenuated virus capable of establishing persistent infection, and the amino acid change at 1618(L→V) coexisting with aa 1169 threonine is required for the acquirement of a temperature-sensitive phenotype.

#### HA production by rSeV-infected cells

HA titers of the culture fluids of L929 cells primarily infected with SeVpi, rSeV(L1618V), new/rSeV(PA), new/rSeV(L1618V), or new/rSeV(I11669T) were measured. HA production could not be detected in cells infected with SeVpi and incubated at 38 °C, while was found in cells infected with rSeV(L1618V), although the titers at 38 °C were lower compared with at 32 °C (Fig. 7). Intriguingly, HA production in new/rSeV(PA)-infected cells was significantly lower at 38 °C than at 32 °C, showing aa 1207 (S or C) influences virus production at 38 °C to a certain extent. In addition, HA production was extremely low in cells infected with either new/rSeV(L1618V) or new/rSeV(I1169T) and incubated at 38 °C. At 5 days post-infection, hemadsorption test was carried out. As shown in Fig. 7, new/rSeV(L1618V)-infected cells exhibited detectable hemadsorption at 32 and 38 °C, while SeVpi-infected cells showed no hemadsorption at 38 °C.

#### Discussion

The SeV pi strain used in this study was isolated from a Sendai virus persistently infected cells that had been cultured for a long period (Nagata et al., 1972; Yoshida et al., 1979). Intriguingly, SeVpi can readily establish persistent infection in various cells without the aid of the DI virus (Yoshida et al., 1982). In this study, the complete L genome sequences of SeV Nagoya strain and SeVpi were determined. Subsequently, the amino acid sequences deduced from the nucleotide sequences of six different Sendai viruses were compared with each other. Three amino acid substitutions were found in the Lpi protein and were located at aa 1088, 1618, and 1664. rSeV(Lpi) having these three amino acid substitutions showed a

temperature-sensitive phenotype. Furthermore, L929 cells infected with rSeV(Lpi) showed no or little cpe, and cells infected with rSeV(Lpi) could be subcultured subsequently, indicating that rSeV(Lpi) has the ability of establishing persistent infection (steady state). These findings indicate that the L protein of SeVpi is the determinative viral protein for both temperature sensitive and lower cytopathogenic phenotypes. On the contrary, rSeV having the Ppi protein, another component of virus polymerases, does not show these phenotypes.

None of rSeV(Fr:A1088S), rSeV(Fr:I1664V), or rSeV(Fr:A1088S/I1664V) showed these phenotypes. On the other hand, cells infected with all of rSeVs carrying the fragment containing L1618V were not only capable of establishing persistent infection, but also showed a temperature-sensitive phenotype, indicating that the region around aa 1618 is involved in these phenotypes. To exactly identify the amino acid residue(s) responsible for the ability of establishing persistent infection and temperature sensitivity, we further constructed two recombinant SeVs, rSeV(L1618V) and new/rSeV(L1618V). Expectedly, these rSeVs was capable of establishing persistent infection. However, they were not temperature-sensitive mutants, suggesting that one amino acid change at 1618(L→V) only does not cause a temperature-sensitive phenotype. Fr:L1618V contains another amino acid (aa 1169) that is different between L proteins of SeVpi and SeV Z strain. aa 1169 of the Z strain is isoleucine, while that of the L proteins of other five strains including SeV Nagoya strain and SeVpi is threonine. These findings indicate that the amino acid change at 1618(L→V) and threonine at aa 1169 is necessary for acquirement of a temperature-sensitive phenotype. Intriguingly, the ability of virus to establish persistent infection can be separated from temperature-sensitive phenotypes.

When the amino acid sequences of L proteins from a variety of Mononegaviruses were compared, it has been found that there are six regions of good conservation, designated domains I to VI (Ogawa et al., 1992; Poch et al., 1990; Sidhu et al., 1993). It has been proposed that these conserved domains constitute the enzymatic activities of the L protein, while the more variable regions between the domains contribute to the overall conformation of the protein. Domain I is composed mainly of hydrophobic residues (Poch et al., 1990). In domain II, there is a charged

Fig. 3. rSeV(Fr:L1618V) is temperature sensitive. (A) Virus infectivities of various recombinant Sendai virus were measured at 32 or 38 °C. Decrease (log<sub>10</sub>) in infective titer at 38 °C compared to the titer at 32 °C is shown as “temperature sensitivity”. In the next experiment, L929 cells were infected with various rSeVs at an moi of about 10, incubated at room temperature for 2 h, and then were washed three times. Subsequently, the cells were transferred to 32 or 38 °C, incubated for 20 h, and then hemadsorption test was carried out. The cells infected with rSeV(Fr:L1618V) were also incubated for 3 days. (B) L929 cells were primarily infected with rSeV(Fr:A1088S), rSeV(Fr:I1664V), or rSeV(Fr:L1618V) at an moi of about 10, incubated at room temperature for 2 h, and then washed three times. Then, the cells were transferred to 32 or 38 °C, incubated for 20 h, and then immunostained with anti-NP or -HN mAb. (C; I and II) L929 cells were primarily infected with rSeV(Fr:A1088S) (●), rSeV(Fr:I1664V) (▲), or rSeV(Fr:A1088S/I1664V) (□) at an moi of about 10, and the cells were transferred to 32 (I) or 38 °C (II). The culture fluids were harvested at various periods and HA activities of the culture fluids were measured. (III) L929 cells were primarily infected with rSeV(Fr:L1618V) at an moi of about 10 and the cells were transferred to 32 (○) or 38 °C (▲). Subsequently, the culture fluids were harvested at various periods and HA activities of the culture fluids were measured.

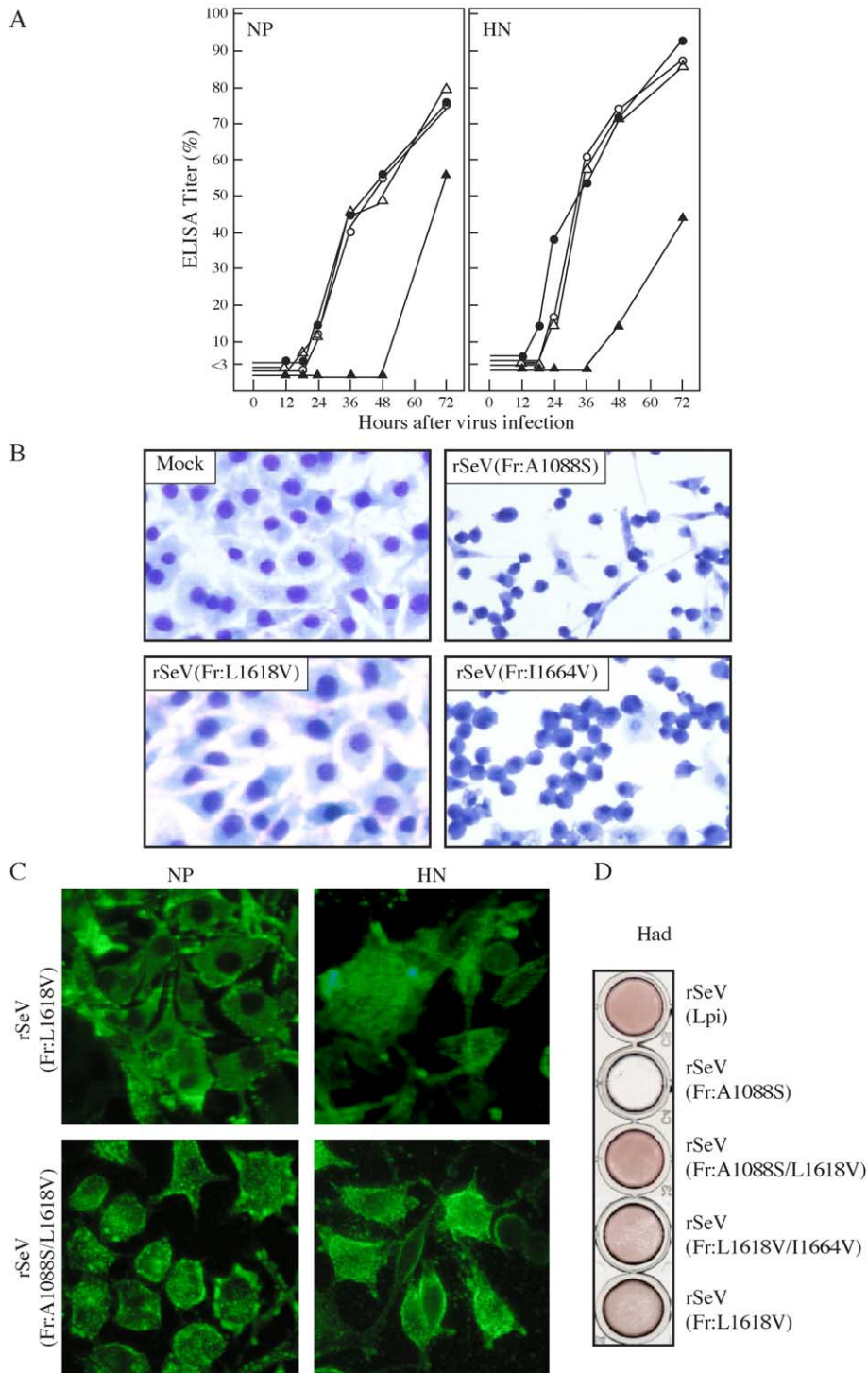


Fig. 4. rSeV(Fr:L1618V) shows no cell toxicity and has the ability of establishing virus persistent infection. (A) L929 cells were primarily infected with rSeV(PA) (●), rSeV(Fr:A1088S) (○), rSeV(Fr:I1664V) (△), or rSeV(Fr:L1618V) (▲) at an moi of about 10 and incubated at 32 °C, and the cells were fixed at various periods. The amount of virus-specific proteins was measured by ELISA using anti-NP and -HN antibodies. ELISA titers are expressed as percentages of the titer at 96 h postinfection. (B) L929 cells were infected with either rSeV(Fr:A1088S), rSeV(Fr:L1618V), or rSeV(Fr:I1664V) at an moi of about 100, incubated at 32 °C for 4 days, and then were stained with Giemsa’s solution. (C) L929 cells were infected with rSeV(Fr:L1618V) or rSeV(Fr:A1088S/L1618V) at an moi of about 100, incubated for 48 h at 32 °C, and then further subcultured 10 times at 35 °C. Subsequently, the cells were immunostained with anti-NP or -HN mAb. (D) L929 cells were infected with rSeV(Lpi), rSeV(Fr:A1088S), rSeV(Fr:A1088S/L1618V), rSeV(Fr:L1618V/I1664V), or rSeV(Fr:L1618V) at an moi of about 100, incubated at 32 °C for 48 h, and then further subcultured five times at 35 °C. Subsequently, the hemadsorption test was carried out. rSeV(Fr:A1088S)-infected cells were scarcely found after five passages.



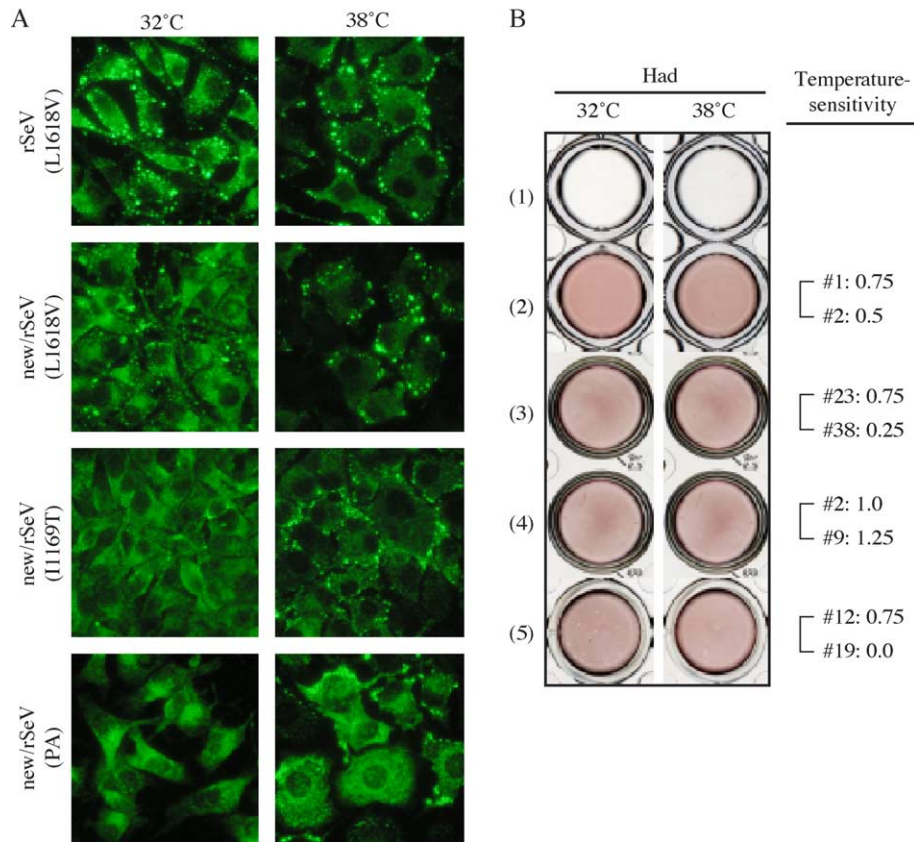


Fig. 5. rSeV(L1618V) and new/rSeV(L1618V) are not temperature sensitive. (A) L929 cells were primarily infected with rSeV(L1618V), new/rSeV(L1618V), new/rSeV(I1169T), or new/rSeV(PA) at an moi of about 10, incubated at room temperature for 2 h, and then washed three times. Then the cells were transferred to 32 or 38 °C, incubated for 20 h, and then immunostained with anti-NP mAb. (B) Hemadsorption test was also carried out at 20 h postinfection. (1) Mock-infected, (2) rSeV(L1618V)-infected, (3) new/rSeV(L1618V)-infected, (4) new/rSeV(I1169T)-infected, and (5) new/rSeV(PA)-infected cells. “Temperature sensitivities” of these viruses (# = clone number) were also measured. Decrease (log<sub>10</sub>) in infective titer at 38 °C compared to the titer at 32 °C is shown as “temperature sensitivity”.

putative RNA binding motif (Smallwood et al., 1999). Domain III has a long continuous stretch of conserved amino acids (VQGDNQ) and is anticipated to represent a RNA polymerase active site and RNA-binding site (Malur et al., 2002; Schnell and Conzelmann, 1995). There are six invariant proline residues and a long continuous stretch of conserved amino acids (RNIGDP) in domain IV, which may be a purine-binding domain. All cysteine and histidine residues are conserved in domain V (Ogawa et al., 1992), which is thought to be a metal binding site. Domain VI possesses a putative purine nucleotide binding element (Canter et al., 1993). In addition, domain VI is predicted to have methyltransferase activity (Bujnicki and Rychlewski, 2002; Ferron et al., 2002; Hercyk et al., 1988).

The L subunit of the RNA-dependent RNA polymerase of negative strand RNA viruses is believed to possess all the enzymatic activities necessary for viral transcription and replication. Mutations in the L proteins of human parainfluenza virus type 3 (PIV3) and vesicular stomatitis virus have been shown to confer temperature sensitivity to the viruses (Feller et al., 2000a; Hunt and Hutchinson, 1993; Skiadopoulos et al., 1998). Skiadopoulos et al. (1998) reported that the L polymerase protein of *cp45* (live

attenuated vaccine candidate of PIV3) contained three amino acid substitutions at positions 942, 992, and 1558, and a recombinant PIV3 virus possessing all three amino acid substitutions was almost as temperature sensitive as *cp45*. Only SeV Y942H L protein was temperature sensitive in both transcription and replication (Feller et al., 2000b). McAuliffe et al. (2004) and Newman et al. (2004) have recently reported that the introduction of the Y942H mutation of the L gene of HPIV3 *cp45* into the homologous position of rHPIV1 yields a virus that possesses the temperature sensitive and attenuated phenotypes. Feller et al. (2000b) isolated Sendai L protein mutated in domains IV and VI that is temperature sensitive for transcription and replication in vitro. One of these, SS3, with changes in amino acids 1004–1006 just downstream of amino acid 992, was temperature sensitive for both transcription and replication. In addition, mutations in SS10 (aa 1041–1043) in domain IV and in SS13 (aa 1798–1801) in domain VI were shown to be temperature sensitive. Two amino acid substitutions, at aa 1197 (N→S) and at aa 1795 (K→E) of the L protein, were found in persistently Sendai virus, but the meaning of these substitution is not clear (Bossow et al., 2000).

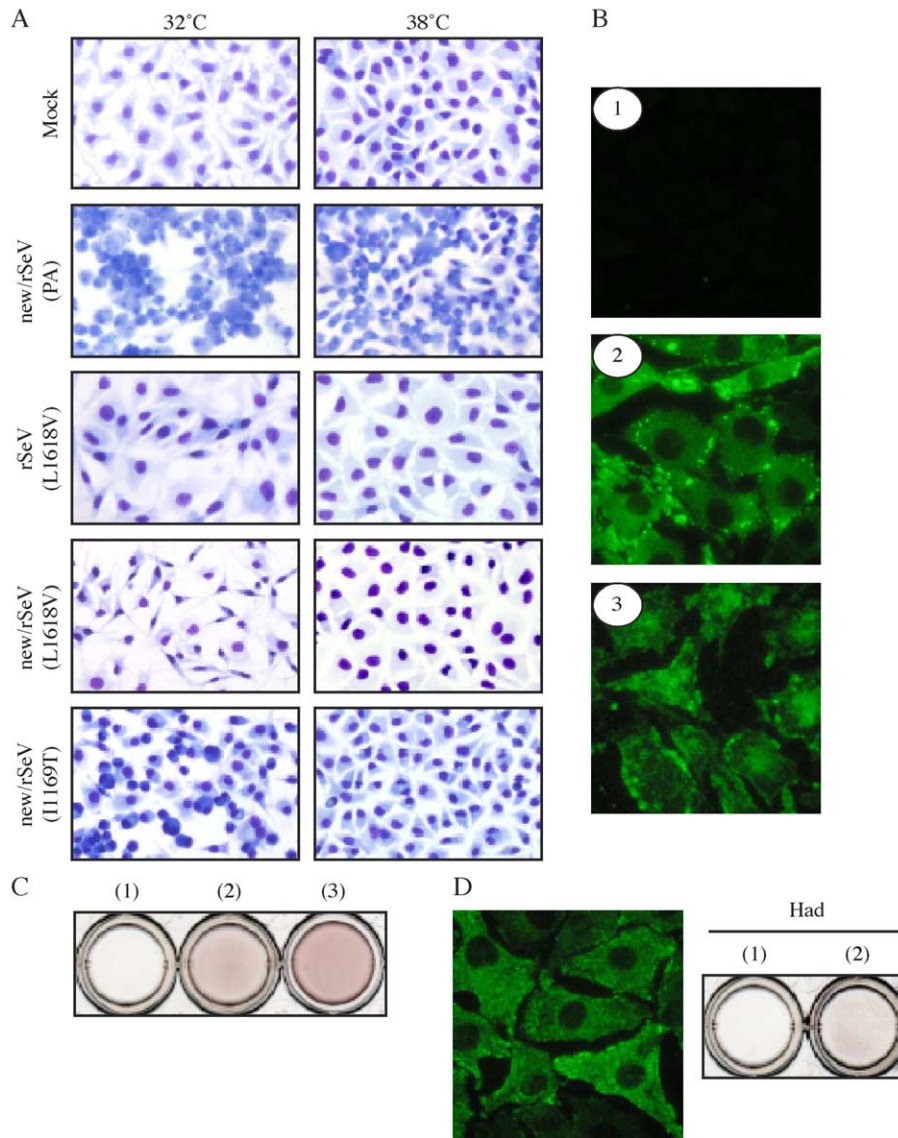


Fig. 6. rSeV(L1618V) and new/rSeV(L1618V) have the ability of establishing virus-persistent infection. (A) L929 cells were infected with either new/rSeV(PA), rSeV(L1618V), new/rSeV(L1618V), or new/rSeV(I1169T) at an moi of about 100, incubated at 32 or 38 °C for 4 days, and then stained with Giemsa's solution. (B and C) L929 cells were infected with rSeV(L1618V) (2) or new/rSeV(L1618V) (3) at an moi of about 100, incubated at 32 °C for 48 h, and then subcultured seven times at 35 °C. Subsequently, the cells were immunostained with anti-NP mAb. Hemadsorption test was also carried out (C). (D) L929 cells were infected with new/rSeV(I1169T) at an moi of about 100, incubated at 38 °C for 48 h, and then subcultured seven times at 38 °C. Subsequently, the cells were immunostained with anti-NP mAb. Hemadsorption test was also carried out. (1) Mock-infected cells; (2) cells infected with new/rSeV(I1169T).

A temperature-sensitive mutation, in general, is thought to act through destabilization of protein folding, which is aggravated by increased temperature. HA production and synthesis of virus-specific polypeptides are delayed in cells infected with temperature-sensitive mutants such as rSeV(Fr:L1618V) even at permissive temperature, suggesting that protein folding of these L proteins is perturbed at permissive temperature. aa 1618 is located between conserved domains V and VI, and the role of this inter-region has not been analyzed. This region is highly variable, which is hypothesized to have specialized function in individual viruses. Skiadopoulou et al. (1999) reported that aa 1558, near aa 1618, in the PIV3 (*cp45*) was related to temperature

sensitive and attenuated phenotypes. In addition, substitution mutations in the SeV L protein at aa 1571, near aa 1558 and 1618, affected multiple polymerase activities (Horikami and Moyer, 1995). Thus, it is inferred that the region around aa 1618 has an important role on transcription and replication.

rSeV(L1618V) and new/rSeV(L1618V) have the ability of establishing persistent infection, but these viruses showed no temperature-sensitive phenotype. Non-ts Sendai virus can establish persistent infection with the aid of the DI virus (Roux and Waldvogel, 1981). In addition, the attenuated mutations in the C and F proteins are non-temperature-sensitive, attenuated mutations (Skiadopoulou et al., 1999). On the contrary, all the attenuated mutations in the PIV3 L

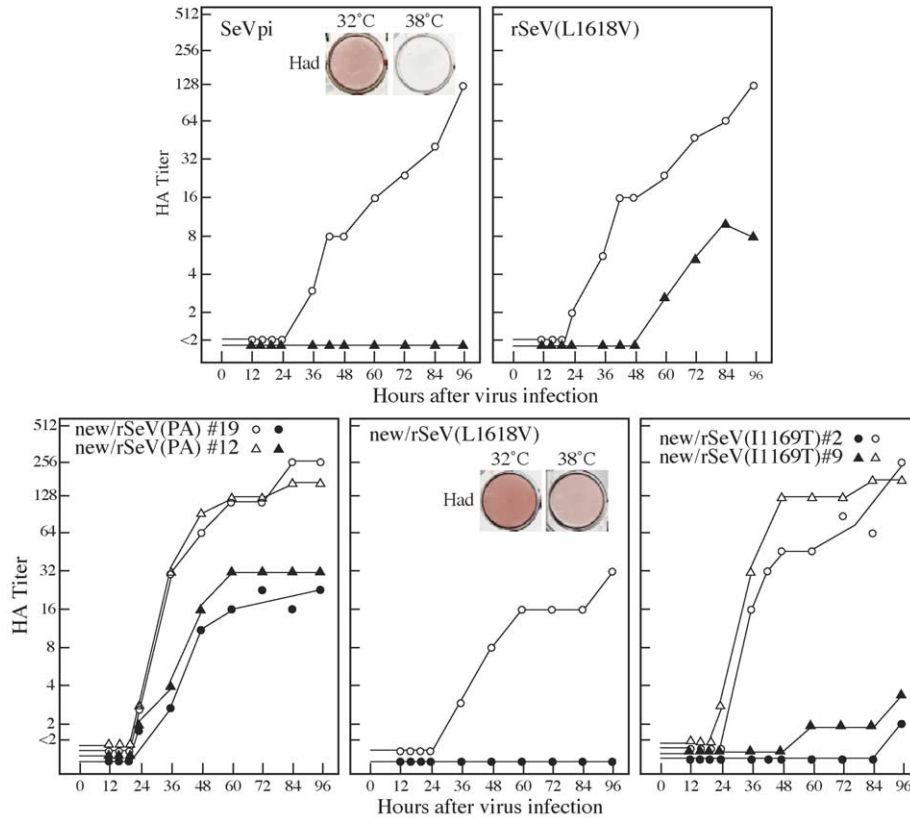


Fig. 7. (A) HA production by rSeV-infected cells. L929 cells were primarily infected with SeVpi, rSeV(L1618V), new/rSeV(PA) (#12,#19), new/rSeV(L1618V), or new/rSeV(I1169T) (#2,#9) at an moi of about 10 and then transferred to 32 (open) or 38 °C (solid). Subsequently, the culture fluids were harvested at various periods and HA activities of the culture fluids were measured. At 5 days postinfection, hemadsorption test was carried out. The different # show different clones of virus.

and SeV L proteins are temperature-sensitive mutations. Thus, the finding that the ability of virus having mutant L protein to establish persistent infection can be separated from the temperature-sensitive phenotypes is interesting. Infective titers at 38 °C of rSeV(L1618V), new/rSeV(L1618V), and new/rSeV(I1169T) are slightly lower than those at 32 °C. When L929 cells infected with new/rSeV(L1618V) or new/rSeV(I1169T) were incubated at 38 °C, HA production was extremely low, showing that virus replication of both the rSeVs was suppressed at 38 °C. In addition, new/rSeV(I1169T) shows no ability of establishing persistent infection at 32 °C, but is capable of establishing persistent infection at 38 °C. Therefore, the phenotypes such as temperature sensitivity and lower cytopathogenic phenotypes of rSeV(Fr:L1618V) are probably due to the synergistic effect of new/rSeV(L1618V) and new/rSeV(I1169T) phenotypes. The elucidation about the molecular mechanism by which L1618V substitution is attenuating mutation without temperature sensitivity is required for further investigation. We are deeply interested in the pathogenicity in mice of a variety of rSeVs constructed in this study, and analysis of the pathogenicity in mice is in progress.

In summary, the temperature-sensitive phenotype and lower pathogenic property of the SeVpi are caused by the L

protein, though it has been accepted for a long time that these phenotypes are caused by HNpi or Mpi (Kimura et al., 1979; Kondo et al., 1993). Amino acid 1618 is related to the ability of establishing persistent infection, and both aa 1169 and 1618 are involved in temperature sensitivity. One amino acid substitution causes dramatic phenotype changes of SeV. Intriguingly, the ability of establishing persistent infection can be separated from a temperature-sensitive phenotype.

## Materials and methods

### Viruses and cells

Wild strain of Sendai virus (SeV) used in this study was Nagoya strain. The SeV pi strain (SeVpi) was isolated from BHK cells persistently infected with SeV (Nagoya strain), which had been maintained for more than 10 years and showed temperature sensitivity. SeVpi was cloned by three successive isolations of plaques formed on LLCMK2 cells incubated at 32 °C (Yoshida et al., 1979). rSeV(PA) was the parent recombinant SeV derived from Z strain (Garcin et al., 1998). The virulence of SeV Nagoya strain is slightly weak compared with that of SeV Z strain. The pi strain of SeV (E-

5 strain) was kindly donated by Dr. T. Yoshida. SeVpi shows almost no cytotoxicity at 32 or 38 °C, and have an ability of establishing virus-persistent infection in various cells without the aid of DI virus at permissive temperature (steady state). Cells used in the present study were mouse L929 cells.

#### *Antibody*

Anti-SeV polyclonal antiserum was described previously (Ito et al., 1987). Anti-NP protein of and anti-HN protein of human parainfluenza type 1 virus (HPIV-1) monoclonal antibodies (mAbs), which are cross-reactive with SeV, were previously reported (Komada et al., 1992). Anti-M and anti-F and anti-L protein mAbs were kindly donated by Dr. C. Orvell and Dr. D. Kolakofsky, respectively. Anti-V and anti-C protein polyclonal rabbit sera were donated by Dr. A. Kato.

#### *Hemadsorption tests*

Tests for the capacity of cells to adsorb erythrocytes to their surface were performed. The culture medium was removed and the monolayer was washed with phosphate-buffered saline (PBS). One milliliter of a 0.4% suspension of guinea pig erythrocytes in PBS was added, and the cultures were at room temperature for 30 min. Unadsorbed erythrocytes were removed by washing with PBS, and the extent of erythrocytes adsorption was observed microscopically.

#### *Viral titration*

Monolayer cultures in 96-well plate were infected with 0.1 ml of 10-fold serial dilutions of SeV-containing fluid and incubated for 4 days. Subsequently, virus-infected cells were washed with MEM, to which was added 100 µl of a 0.4% suspension of guinea pig erythrocytes in MEM, and left at room temperature for 30 min. After removal of unadsorbed erythrocytes by washing with PBS, the hemadsorbed cells were examined microscopically.

#### *Hemagglutination (HA) titration*

Hemagglutination was titrated by doubling dilutions in 50 µl in U type 96-well plates and was detected by the addition of 50 µl of 0.4% guinea pig erythrocytes. The highest dilution showing partial agglutination contained one HA unit.

#### *Immunofluorescent staining*

The cells were fixed with 3% formaldehyde for 15 min at room temperature and rinsed twice with PBS. The cells were permeabilized with 0.05% Tween 20 in PBS (PBS/T) for 30 min and washed twice with PBS. The cells were then incubated for 60 min with primary antibody and washed five

times with PBS. Next, the cells were incubated for 60 min with FITC-labeled secondary antibodies and washed with PBS. Immunofluorescent-stained cells were analyzed using a fluorescent microscope.

#### *ELISA*

The cells were fixed with 3% formaldehyde at room temperature for 20 min, washed with PBS, and then treated with PBS/T for 15 min. The cells were treated with specific mAb for 30 min, washed three times with PBS/T, and then treated with peroxidase-conjugated IgG fraction of goat anti-mouse immunoglobulins serum (Cappel laboratories, Cochranville, PA) for 30 min. Other procedures of ELISA were similar to those described by Hishiyama et al. (1988).

#### *The ability of establishing of virus-persistent infection*

Some of the most common effects of viral infection are morphologic changes such as cell rounding and detachment from the substrate, cell lysis, and syncytium formation. Thus, when we observed virus-infected cells with a microscope, the presences of cell rounding, cell lysis, and syncytium formation were checked at every subcultures. The ability of establishing of persistent infection was judged by the following criteria: (1) when L929 cells are primarily infected with virus at an moi of about 100, no or little cytopathic effect (cpe) is observed; (2) the virus-infected cells are successfully subcultured at least five times without cell damage; and (3) almost all cells infected with the virus and subcultured more than five times exhibit hemadsorption and have the NP and HN antigens.

#### *Construction of recombinant SeV possessing the Lpi and mutant L proteins*

Recombinant SeVs (rSeVs) were prepared according to the modified Kolakofsky's method (Garcin et al., 1998; Nishio et al., 2003). Mutations were introduced into L gene by PCR. Virus recovery was carried out as described previously (Garcin et al., 1998; Nishio et al., 2003), except that BSR T7 cells (expressing cytoplasmic T7 RNA polymerase) were transfected with the full-length cDNA and IRES-containing pTM1 plasmids encoding the N, P, and L proteins for 72 h at 37 °C. All of the rSeVs were inoculated intrallantoically into 10-day-old embryonated eggs, which were incubated at 32 °C for 3 days, and then the allantoic fluids were harvested. The nucleotide sequence of each rSeV was analyzed for confirming isolated rSeVs.

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