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Virology 428 (2012) 48–57

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The N-terminus of the Montano virus nucleocapsid protein possesses broadly cross-reactive conformation-dependent epitopes conserved in rodent-borne hantaviruses

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ARTICLE INFO

Article history:
Received 21 December 2011
Returned to author for revisions
19 January 2012
Accepted 13 March 2012
Available online 19 April 2012

Keywords:
Hantavirus
Montano virus
Nucleocapsid protein
Monoclonal antibody
Conformational epitope
Cross-reactivity

ABSTRACT

The hantavirus nucleocapsid (N) protein is an important immunogen that stimulates a strong and cross-reactive immune response in humans and rodents. A large proportion of the response to N protein has been found to target its N-terminus. However, the exact nature of this bias towards the N-terminus is not yet fully understood. We characterized six monoclonal antibodies (mAbs) against the N protein of Montano virus (MTNV), a Mexican hantavirus. Five of these mAbs recognized eight American hantaviruses and six European and Asian hantaviruses, but not the Soricomorpha-borne Thottapalayam hantavirus. The N protein-reactive binding regions of the five mAbs were mapped to discontinuous epitopes within the N-terminal 13–51 amino acid residues, while a single serotype-specific mAb was mapped to residues 1–25 and 49–75. Our findings suggest that discontinuous epitopes at the N-terminus are conserved, at least in rodent-borne hantaviruses, and that they contribute considerably to N protein cross-reactivity.

Introduction

The hantavirus (family *Bunyaviridae*) genome consists of three RNA segments of negative-sense polarity, Large (L), Medium (M), and Small (S), encoding the viral polymerase, envelope glycoproteins, and nucleocapsid (N) protein, respectively (Schmaljohn et al., 1986). Hantaviruses are normally carried by persistently infected rodents, from which humans are believed to acquire the infection primarily through the inhalation of virus-contaminated excreta; however, human-to-human transmission has also been reported (Martinez et al., 2005). Infection with American hantaviruses causes hantavirus pulmonary syndrome (Hjelle et al., 1994), whereas the viruses originating from Europe and Asia cause hemorrhagic fever with renal syndrome (Avsic-Zupanc et al., 1992).

European and Asian (i.e., Old World) hantaviruses known to cause illness in humans include Hantaan virus (HTNV), Dobrava virus (DOBV), Puumala virus (PUUV), Seoul virus (SEOV), and Amur virus (AMRV). Others, such as Tula virus (TULV) and Hokkaido virus (HOKV), have not been associated with human disease. American (i.e., New World) hantaviruses, such as Sin Nombre virus (SNV), New York virus (NYV), Black Creek Canal virus (BCCV), and Bayou virus (BAYV), are known human pathogens. In South America, viruses so far linked with human illness include Andes virus (ANDV), Laguna Negra virus (LANV), and Lechiguanas virus (LECV). Maporal virus (MAPV), Rio Mamore virus (RIOMV), have not been associated with human disease (Jonsson et al., 2010).

Hantaviruses detected in Mexico include EL Moro Canyon virus (ELMCV) (Torrez-Martinez et al., 1995) and, more recently, Playa de Oro virus (OROV) (Chu et al., 2008). In our recent wild rodent epidemiological survey in Guererro and Morelos States, Mexico, we identified virus RNA of three distinct hantaviruses, currently designated as Montano virus (MTNV), Carrizal virus (CARV), and Huitzilac virus (HUIV), the pathogenicity of which to humans is still unknown (Kariwa et al., 2012).

The hantavirus N protein, in addition to being the most abundant viral protein, plays key roles in hantavirus infection, including encapsidation, binding of the viral RNA genome (Severson et al., 2001; Xu et al., 2002), and initiation of the transcription and

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0042-6822/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.03.001

translation of viral mRNA (Cheng et al., 2011; Severson et al., 2005). The N-terminus of the N protein has been found to be necessary for other viral processes, including oligomerization and N–N interactions (Alminaite et al., 2006; Kaukinen et al., 2001). The N protein is a frequently used antigen for detecting hantavirus antibodies, because it is highly conserved and provokes a strong immune response in humans and mice (Bharadwaj et al., 2000; de Carvalho Nicacio et al., 2002). Although antigenic determinants have been detected over the entire length of the N protein, the strongest response is directed toward the N-terminus (Alfadhli et al., 2002; Boudko et al., 2007; Elgh et al., 1996; Jenison et al., 1994; Lundkvist et al., 1996), which contributes substantially to the overall cross-reactive immune response (Lindkvist et al., 2007; Tischler et al., 2008). As such, deletion of the N-terminus from the protein permits the generation of antigens with serotype-specific applications (Koma et al., 2010; Li et al., 2006; Morii et al., 1998; Tischler et al., 2008).

Monoclonal antibodies (mAbs) are useful tools for studying hantavirus antigenic characteristics due to their specificity. So far, several studies have attempted to generate mAbs with desired specificities. For example, the immunization of mice with more than one antigen or the use of virus core particle delivery systems have generated mAbs with relatively broad cross-reactivity (Geldmacher et al., 2004; Kucinskaite-Kodze et al., 2011). A large proportion of this response was shown to arise from the N-terminus. Evidence gathered through crystal structural analysis and nuclear magnetic resonance (NMR) spectroscopic predictions have revealed that the N-terminus assumes a coiled-coil α-helix conformation (Boudko et al., 2007; Wang et al., 2008). However, the role of this domain in inducing strong and cross-reactive responses remains to be thoroughly investigated.

To broaden our understanding of the antigenic properties of the N protein of Mexican hantavirus, we generated and characterized six mAbs against MTNV N protein expressed in bacteria. Five of the six mAbs showed a strong and broad spectrum of reactivity to both American and Eurasian rodent-borne hantaviruses (reactivity to the Soricomorpha-borne Thottapalayam virus [TPMV] was not observed). The binding regions of the five cross-reactive mAbs were mapped to the N-terminal 13–51 amino acids (aas) of the N protein, with their activity apparently dependent on the preservation of the integrity of the N-terminal domain. Our findings suggest that the N protein coiled-coil α-helix domain possesses conformational epitopes that contribute significantly to cross-reactivity, at least in rodent-borne hantaviruses.

Results

mAb production and isotyping

Six hybridoma clones (2H10, 9E5, 10G5, 2F11, 7E6, and G8B4) that had a strong and specific reaction to MTNV N protein on enzyme-linked immunosorbent assay (ELISA) were established. All six mAbs recognized the hantavirus N protein by Western blotting. All clones were of the IgG1 Kappa subclass except for 10G5, which belonged to IgG2a (Supplementary Table 1).

Reactivity of the mAbs to bacteria- and baculovirus-expressed hantavirus N protein

To determine the extent of mAb reactivity to different hantaviruses, several N proteins expressed in bacteria or a baculovirus system were tested by ELISA. The mAbs recognized bacterially-expressed whole N proteins of homologous MTNV and two other Mexican hantaviruses (HUIV and CARV), as well as SNV (Fig. 1A). The reactivity of the mAbs to the European and Asian hantavirus N antigens was varied. For example, mAb 2H10, 10G5 and 7E6 recognized and reacted with nearly all of the antigens, including ANDV, LANV, MAPV, HTNV, SEOV, AMRV, PUUV, TULV, and HOKV [AB010731]. All of the mAbs except G8B4 also reacted with the N proteins of PUUV, TULV and HOKV whereas 2F11 did not recognize the HTNV, AMRV and SEOV antigens and 9E5 did not react with AMRV and SEOV. G8B4 showed specificity to North American hantaviruses, reacting only with SNV and the three

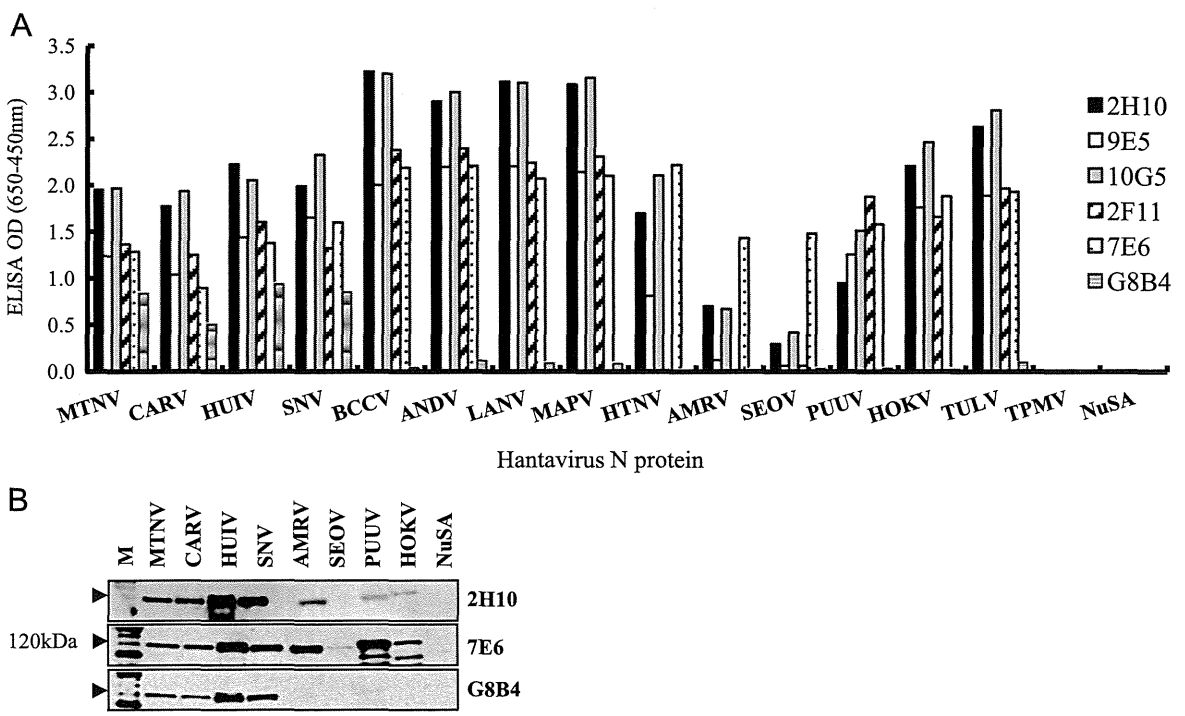

Fig. 1. Reactivity of mAbs to whole hantavirus N protein fused with NuSA (~120 kDa). (A) ELISA reactivity of mAbs to 15 hantavirus N proteins and NuSA as a negative control antigen and (B) reactivity of 2H10, 7E6 and G8B4 to the hantavirus N proteins on Western blotting.

Table 1
Reactivities of mAbs to N proteins of hantaviruses expressed in baculovirus in ELISA.

mAb	North America					South America			Europe and Asia				
	MTNV	CARV	HUIV	SNV	BCCV	ANDV	LANV	MAPV	HTNV	SEOV	PUUV	TULV	TPMV
2H10	+[a]	+	+	+	+	+	+	+	+	+	+	+	−[b]
9E5	+	+	+	+	+	+	+	+	+	+	+	+	−
10G5	+	+	+	+	+	+	+	+	+	+	+	+	−
2F11	+	+	+	+	+	+	+	+	−	−	+	+	−
7E6	+	+	+	+	+	+	+	+	+	+	+	+	−
G8B4	+	−	−	−	−	−	−	−	−	−	−	−	−

[a] ELISA OD: ≥0.2.
[b] ELISA OD: <0.2.

Table 2
Reactivities of mAbs to N proteins of hantaviruses expressed in Vero E6 cells on IFA.

Region	North America				South America			Europe and Asia				
N protein	MTNV	CARV	HUIV	SNV	ANDV	LANV	MAPV	HTNV	SEOV	PUUV	TULV	TPMV
2H10	+[a]	+	+	+	+	+	+	+	+	+	+	−[b]
9E5	+	+	+	+	+	+	+	+	+	+	+	−
10G5	+	+	+	+	+	+	+	+	+	+	+	−
2F11	+	+	+	+	+	+	+	−	−	+	+	−
7E6	+	+	+	+	+	+	+	+	+	+	+	−
G8B4	+	+	+	+	−	+	+	−	−	−	+	−
E5G6[c]	+	−	+	+	+	+	+	+	+	+	+	+[d]

[a] IFA titer: ≥1:100.
[b] IFA titer: <1:100.
[c] Hybridoma culture supernatant.
[d] TMPV N was manipulated to add binding ability to E5G6 (Okumura et al., 2007).

Mexican hantaviruses (MTNV, CARV, and HUIV) although not with BCCV antigen. The reactivity of representative mAbs to bacterially-expressed hantavirus N proteins was confirmed by a strong response on Western blotting. None of the mAbs reacted with NuSA, a negative control antigen (Fig. 1B).

We also examined the reactivity to baculovirus-derived N proteins of various hantaviruses (Table 1). Four of the mAbs (2H10, 9E5, 10G5, and 7E6) recognized baculovirus-derived antigens of North (MTNV, CARV, HUIV, SNV, and BCCV) and South American hantaviruses (ANDV, LANV, and MAPV) as well as the European and Asian viruses (HTNV, SEOV, PUUV, and TULV) but not TPMV. The mAb 2F11 did not react with HTNV or SEOV antigen. G8B4 reacted with homologous MTNV N antigen but did not react with other N proteins examined, including the closely related CARV, HUIV or SNV antigen.

Reactivity of the mAbs to native hantavirus N proteins expressed in Vero E6 cells by indirect immunofluorescence antibody assay (IFA)

The reactivity of the mAbs to native antigens was further assessed by IFA in transfected and infected Vero E6 cells. The mAb reactivity pattern was consistent between the N proteins in both transfected and infected cells. As summarized in Table 2, the five cross-reactive mAbs strongly recognized nearly all American, European, and Asian hantavirus N proteins tested. However, 2F11 did not react with the HTNV or SEOV antigens, while G8B4 only reacted with the N proteins of MTNV, CARV, HUIV, SNV, and weakly to LANV, MAPV and TULV. A manipulated TPMV N protein to add the binding ability to E5G6 was used to confirm expression (Okumura et al., 2007).

The reactivity on IFA was verified by applying the mAbs to authentic hantavirus N protein in virus-infected cells, in which a response similar to that in transfected cells was observed (Table 3). None of the mAbs reacted with TPMV N protein (Tables 2 and 3).

Table 3
Reactivities of mAbs to authentic N proteins of European and Asian hantaviruses in IFA.

mAb	Rodent-borne				Soricomorpha-borne
	HTNV	SEOV	PUUV	TULV	TPMV
2H10	+[a]	+	+	+	−[b]
9E5	+	+	+	+	−
10G5	+	+	+	+	−
2F11	−	+	+	+	−
7E6	+	+	+	+	−
G8B4	−	−	−	+	−
E5G6[c]	+	+	+	+	+[d]

[a] IFA titer: ≥1:100.
[b] IFA titer: <1:100.
[c] Hybridoma culture supernatant.
[d] Anti-TPMV serum.

Determination of the binding regions using truncated MTNV N protein

Given the observed propensity of the mAbs for cross-reactivity to different hantavirus N proteins, we sought to define the regions of the N protein targeted by each mAb. Initially, we applied the mAbs to truncated MTNV N proteins (Fig. 2A). The mAbs recognized the whole N protein (N1–428), N1–80, and N1–150, but not N46–125, N125–275, or N250–428 (Table 4), suggesting that all of the epitopes were located within the first 80 aa residues at the N-terminus.

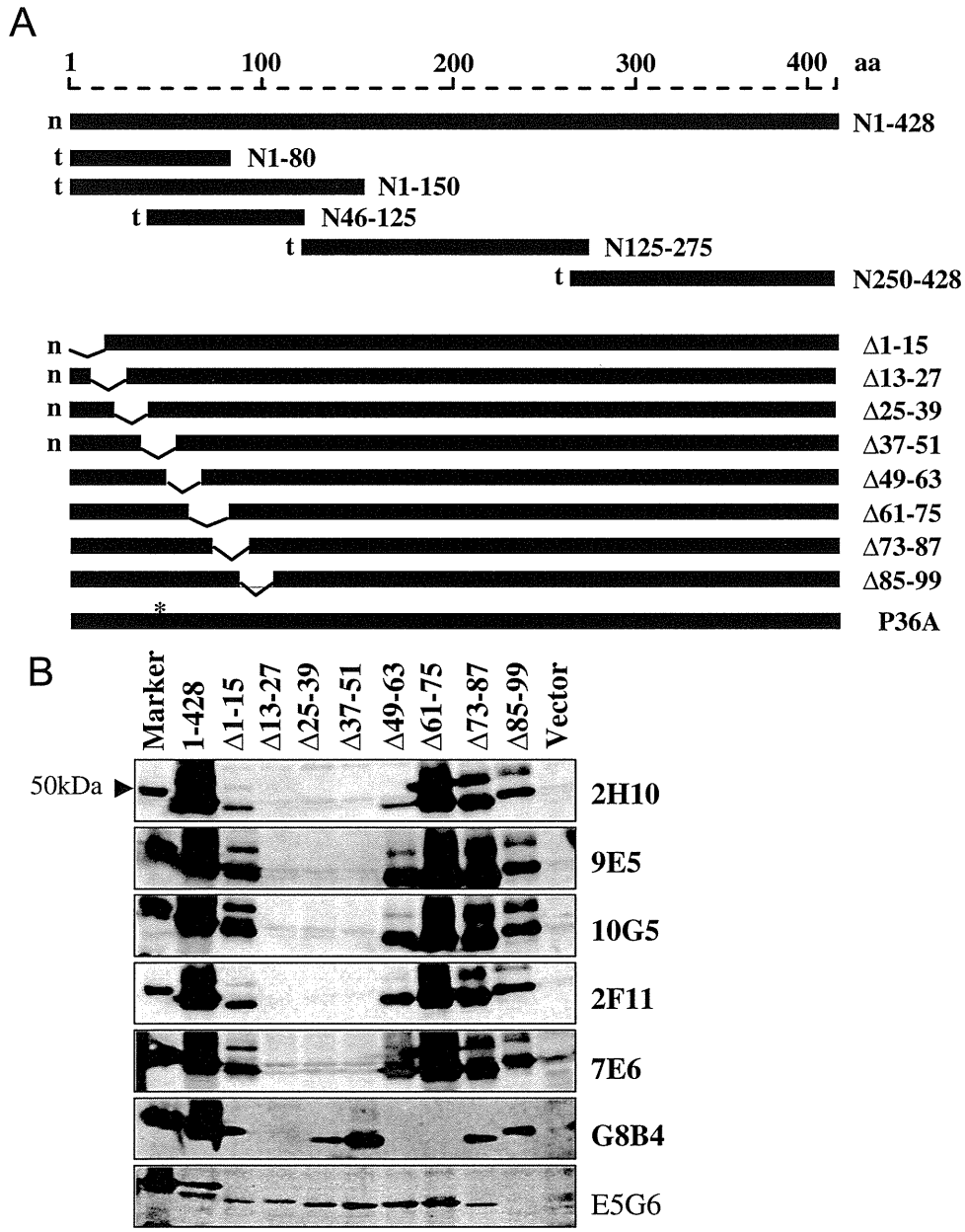

Fig. 2. Diagrammatic representation of MTNV N protein for mapping the epitopes recognized by the mAbs. (A) Full-length N protein and the truncated mutants, N protein mutants with 15aa deletions within the first 100 aas at the N-terminus. Substitution of alanine for proline at residue 36 (P36A) in full-length MTNV N protein. The n and t represent the N fusion protein for antigens expressed in bacteria. The asterisk (*) indicates the position of the alanine substitution in the N-terminus. (B) Assessment of the expression levels of the various N terminus deletion mutant constructs to the six mAbs compared to control mAb E5G6 with a binding region located outside the N-terminus.

Table 4
Reactivities of mAbs to bacterially-expressed full-length and truncated N proteins of MTNV.

mAb	MTNV N protein					
	N1–428	N1–80	N1–150	N46–125	N125–275	N250–428
2H10	+[a]	+	+	−[b]	−	−
9E5	+	+	+	−	−	−
10G5	+	+	+	−	−	−
2F11	+	+	+	−	−	−
7E5	+	+	+	−	−	−
G8B4	+	+	+	−	−	−

[a] ELISA OD: ≥ 0.2.
[b] ELISA OD: < 0.2.

Determination of the binding regions using MTNV N protein deletion fragments

To refine the binding regions of the mAbs within the N-terminus, we constructed and expressed 15 aa-deleted fragments of MTNV N protein covering residues 1–99 with three overlapping residues (Fig. 2A) and analyzed their reactivity by IFA (Table 5). Two major mAb patterns of reactivity were observed. Five mAbs (2H10, 9E5, 10G5, 2F11, and 7E6) recognized Δ1–15-deleted N but lost reactivity to Δ25–39 and Δ37–51 N, although 2F11 and 10G5 reacted weakly with Δ13–27 N. On the other hand, G8B4 showed a different response pattern: recognition was abolished by the Δ1–15 and Δ13–27 deletions, but restored by Δ25–39 and Δ37–51 N successive deletions, while Δ49–63 and Δ61–75 N

Table 5
Mapping of mAbs-binding regions to N protein of MTNV mutant N lacking 15 amino acids were expressed in Vero E6 cells and used as antigen for IFA test.

mAb	MTNV N Protein									Binding site (aa)
	N1–428	Δ1–15	Δ13–27	Δ25–39	Δ37–51	Δ49–63	Δ61–75	Δ73–87	Δ85–99	
2H10	+[a]	+	−[b]	−	−	+	+	+	+	13–51
9E5	+	+	−	−	−	+	+	+	+	13–51
10G5	+	+	+	−	−	+	+	+	+	25–51
2F11	+	+	+	−	−	+	+	+	+	25–51
7E6	+	+	−	−	−	+	+	+	+	13–51
G8B4	+	−	−	+	+	−	−	+	+	1–27, 49–75
E5G6[c]	+	+	+	+	+	+	+	+	+	166–175

[a] IFA titer: ≥1:100.
[b] IFA titer: <1:100.
[c] Hybridoma culture supernatant.

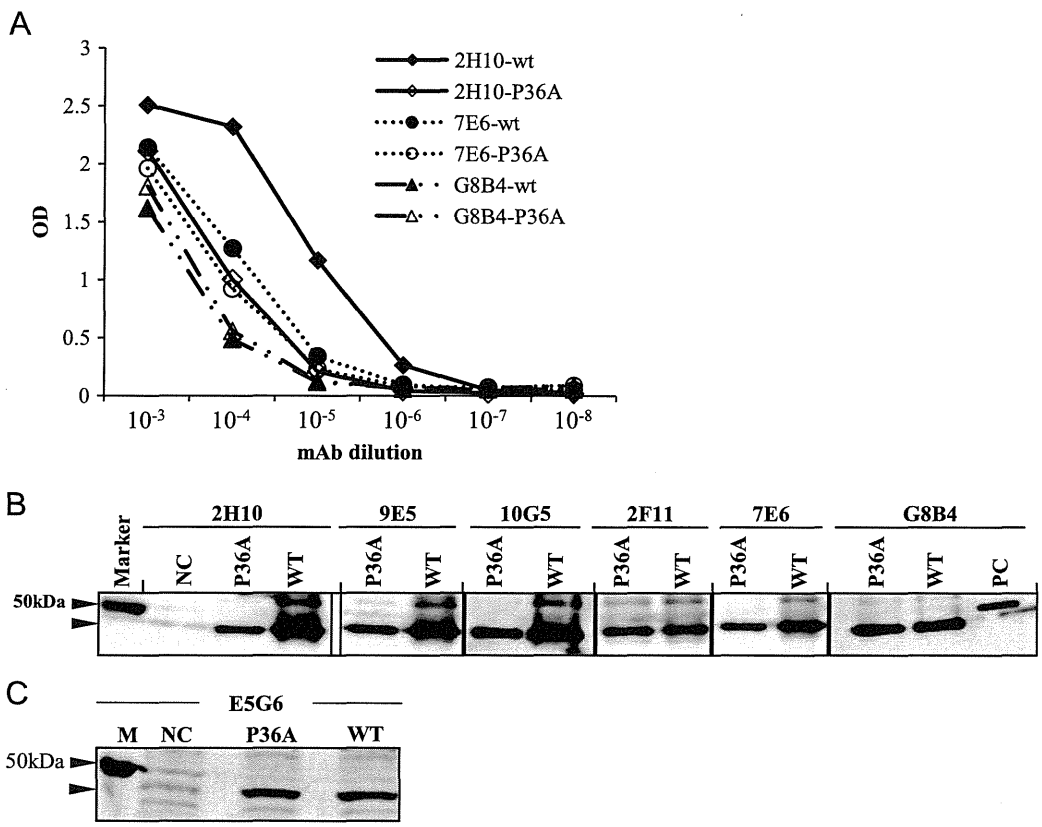

Fig. 3. Evaluation of mAb reactivity to wild-type MTNV N protein (wt) and P36A N protein as a 293T cell antigen lysate. (A) Indirect capture ELISA of biotinylated 2H10 (0.690 mg/mL), 7E6 (0.166 mg/mL), and G8B4 (0.138 mg/mL). (B) Assessment of the reactivity of the six mAbs to P36A N protein mutant and wild-type MTNV N protein (wt) and SNV N protein as a positive control (PC) on Western blotting. (C) Assessing the expression levels of the mutant N protein compared to the wt form using mAb E5G6 with a binding region located outside the N-terminus, empty vector as a negative control (NC).

were not recognized. The reactivity of G8B4 was restored at Δ73–87 and Δ85–99 N. Evaluation of these deleted constructs of MTNV N protein in Western blotting using mAb E5G6 showed comparable levels of expression (Fig. 2B).

Reactivity of the mAbs to MTNV N proline-36 (Pro36) substituted with alanine by IFA, ELISA, and Western blotting

To assess the role of the N-terminal coiled-coil α-helix conformation in mAb reactivity, Pro36 in the full-length N protein was replaced with alanine (P36A). When examined by IFA in transfected cells, all of the mAbs reacted with the P36A mutant; compared with wild type, the intensity varied but was weaker overall, especially for mAb 2H10 (data not shown). A similar pattern was observed on capture ELISA and Western blotting using a 293T cell antigen lysate (Fig. 3A and B). Differential expression of the wild-type and P36A mutant proteins as the cause of the disparity was assessed using mAb E5G6, which had an epitope located outside the N-terminal domain that showed equal band sizes on Western blotting, suggesting comparable levels of expression of the two constructs (Fig. 3C).

Mapping of the topological relationships by a competitive binding assay

We performed a competitive binding capture ELISA to determine the topological relationships of the epitopes within the N-terminus using full-length MTNV N protein (Fig. 4). The assay

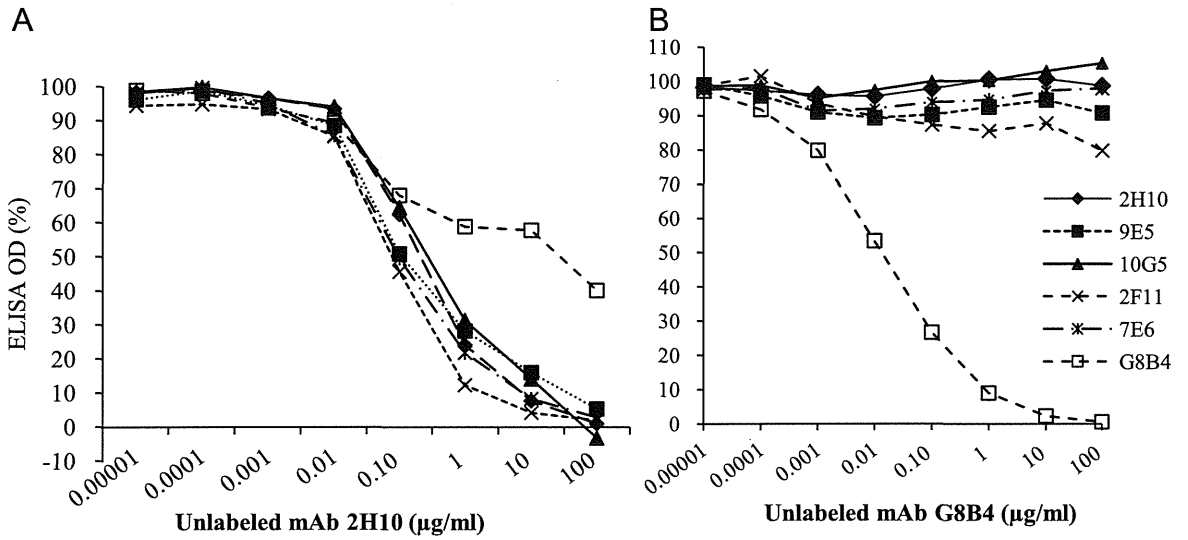

Fig. 4. Competitive ELISA of the six mAbs showing the inhibition of biotin-labeled mAbs on MTNV N protein using 10-fold serial dilutions of unlabeled mAbs. (A) Example of the complete inhibition pattern of mAb 2H10 to the other four mAbs (9E5, 10G5, 7E6, and 2F11), except for G8B4, which exhibited partial inhibition. (B) Reverse set up of G8B4 competitor (unlabeled) showing the complete inhibition to homologous labeled G8B4 and the absence of inhibition of the other five mAbs.

identified two prominent and partially overlapping regions. Five mAbs (2H10, 9E5, 10G5, 2F11, and 7E6) showed complete two-way inhibition, and, as anticipated, G8B4 showed partial inhibition (50–60%) when the five other mAbs were used as competitors (Fig. 4A). However, when the format was reversed (i.e., with G8B4 as the competitor [unlabeled]), no inhibition was observed in the other five mAbs (Fig. 4B), consistent with our deleted mutant epitope mapping results that showed partial overlap between G8B4 and the other mAbs (Table 5).

Discussion

The generation of mAbs for detecting hantaviruses is an important approach to obtain information on the antigenic properties of hantaviruses, especially in Mexico, because few hantavirus-related studies have been conducted so far. In this study, we generated and characterized six mAbs directed against the MTNV N protein that had extended cross-reactivity to a wide range of rodent-borne hantavirus N proteins expressed in bacteria, baculovirus, and mammalian systems. The region of the N protein responsible for eliciting this strong cross-reactivity was mapped to discontinuous epitopes within the N-terminus.

As is common with hantaviruses, we observed that all of the mAbs targeted the N-terminus, a region known to be immunodominant. A preliminary assessment of mAb reactivity on ELISA indicated a relatively wide spectrum of cross-reactivity to other hantavirus antigens. Additionally, we observed that all of the mAbs recognized SDS-treated antigens on Western blotting (Fig. 1B). Because the MTNV N protein used for mouse immunization was expressed in *Escherichia coli*, our initial concern was that these cross-reactive antigenic determinants could be bacteria-specific and absent from other systems or authentic virus N protein. Thus, we examined the reactivity of the mAbs to hantavirus N proteins expressed in other systems.

Although a comprehensive comparison of the antigens could not be made between the bacterial and baculovirus expression systems, the results of the five mAbs (2H10, 9E5, 10G5, 2F11, and 7E6) were consistent. While G8B4 reactivity was restricted mainly to the North American hantaviruses, in bacterial expression, G8B4 reacted with MTNV, CARV, HUIV, and SNV but in baculovirus expression, only homologous MTNV was positive. The reason why the reactivity of G8B4 was lost in baculovirus system is not clear. It is likely that the bacterial expression and other expression systems preserve the epitope conformation. Moreover, mAb 7E6 recognized all of the European and Asian hantaviruses examined, at least to some degree. Furthermore, these differences appeared to have a greater effect on the ability of the mAbs to react with heterologous antigens, such as the European and Asian viruses. As noted, when mAb reactivity to native N protein was examined in mammalian cells by IFA, four mAbs strongly recognized antigens of all the hantaviruses considered (Table 3). This recognition of N protein in virus-infected cells confirmed that the epitopes eliciting the cross-reactive B-cell response to bacterially-expressed antigen were authentic. Additionally, the failure of the mAbs to recognize authentic TPMV antigen showed that the target epitopes may assume a different conformation in Soricomorpha-borne hantavirus N protein or be absent altogether (Yadav et al., 2007).

The reactivity to truncated MTNV N fragments highlights the immunodominant nature of the protein's N-terminus (Elgh et al., 1996; Lundkvist et al., 1996). Moreover, the failure to react with the fragment N46–125 is worth noting because it suggests the need for the complete stretch of N-terminal residues. These results are consistent with previous findings showing that the first 100 aas comprise the immunodominant region of hantavirus N protein (Geldmacher et al., 2004; Gott et al., 1997). Further examination of mAb reactivity using N-terminus-deleted mutants shed more light on the binding properties. We found that those mAbs with the highest level of cross-reactivity (2H10, 9E5, 2F11, 10G5 and 7E6) bound to residues 13–51, a region previously shown to induce a cross-reactive immune response in hantavirus-infected humans (Jenison et al., 1994). Our initial speculation was that the mAbs were targeting linear epitopes because they recognized SDS-treated antigens strongly on Western blotting. However, an evaluation of reactivity using the deleted mutants suggested that the mAbs could be targeting non-linear or conformational epitopes (Table 5).

To further explore this observation, given that the N-terminus forms a coiled-coil α-helix conformation, we hypothesized that if attachment of the mAbs involved straddling between the N protein helices, substituting the residue Pro36 would interfere with reactivity. A previous study demonstrated that the substitution of Pro36 destabilized the coiled–coiled conformation but

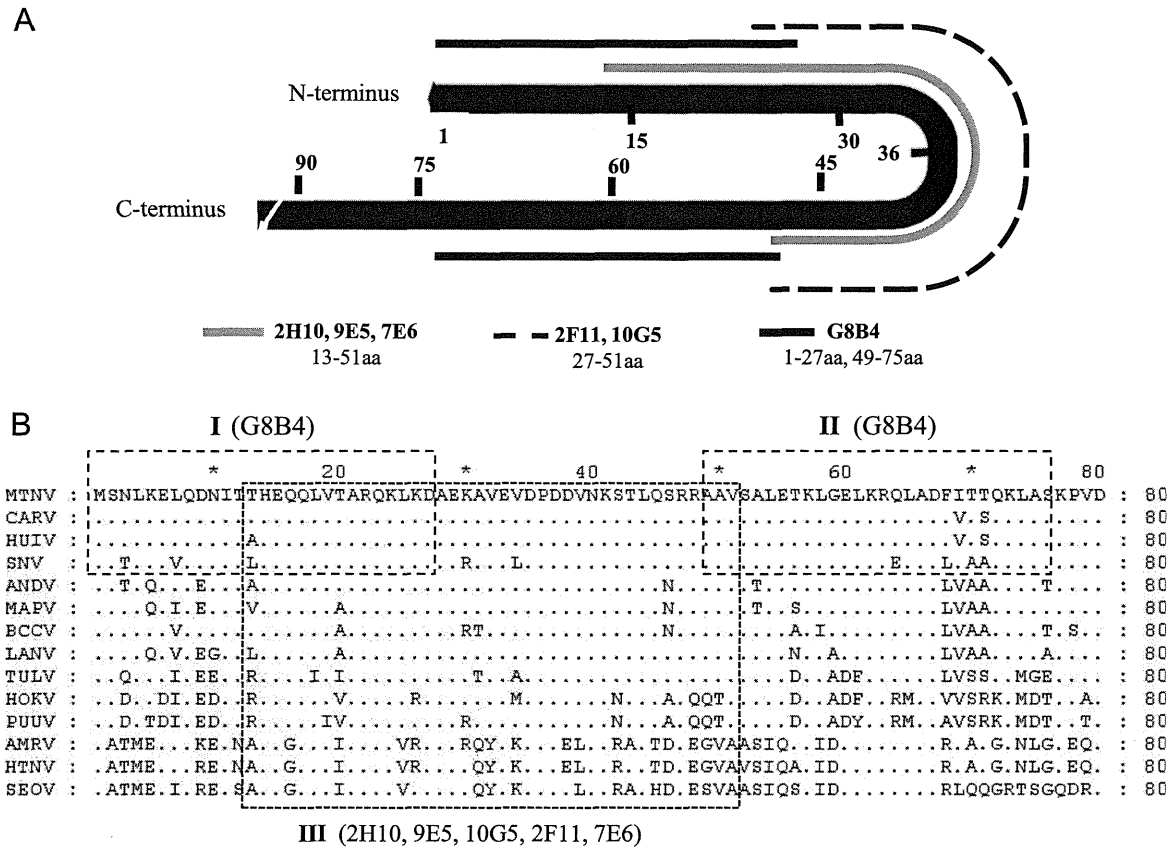

Fig. 5. Binding regions of the mAbs, as determined using truncated and deleted N protein mutants. (A) Schematic view of the residues (aas 1–100) at the MTNV N protein N-terminus recognized by the six mAbs. The mAb and residues necessary for reactivity included: G8B4, (aas 1–27 and 49–75) open bar; 2H10, 9E5, and 7E6 (aas 13–51), gray bar; and 2F11 and 10G5 (aas 25–51), broken lines. (B) Alignment of the N-terminal residues (aas 1–80) from the hantaviruses used in the analysis, showing the binding regions of G8B4 (I and II) and 2H10, 9E5, 10G5, 2F11, and 7E6 (III).

preserved the secondary α-helix structure (Wang et al., 2008). For this experiment, Pro36 was substituted with alanine to uncouple the helix–helix interaction, while, as much as possible, maintaining the secondary structure of the helix. The reactivity of the mAbs to the mutant was lower than that to wild type, suggesting that the substitution destabilized the conformation of the N-terminal domain. Although we changed a single residue, Lindkvist et al. (2008) found that by substituting four residues (aas 34–38) instead, nephropathia epidemica patient polyclonal sera reactivity to a heterologous antigen was significantly affected. This indicates that these residues are involved in inducing cross-reactivity, directly as part of the epitope or indirectly, by conferring conformational stability to the protein.

A competitive ELISA confirmed that five of the six mAbs recognized similar regions. A comparison of the specific reactivity of G8B4 (aas 1–27 and 49–75) to the five other mAbs suggested the presence of broadly cross-reactive conformation-dependent epitopes within aas 13–51 at the N-terminus (Fig. 5A).

Although the exact nature of the effect of the deletions remains undetermined, the complete loss of reactivity of the mAbs to the mutant N protein due to deletions between aas 13 and 51 at the N-terminus suggests that conformational integrity of this region is necessary for antibody recognition. The residues necessary for cross-reactivity could not be defined due to the sequence diversity across the hantaviruses examined (Fig. 5B). Therefore, these cross-reactive mAbs may be useful probes for structure–function relationship studies.

In this study, we showed that within the N-terminus there are conformation-dependent epitopes responsible for inducing extensive cross-reactivity within rodent-borne hantaviruses. These data suggest that although the primary structure of the hantavirus N protein may vary, there are highly conserved antigenic determinants apparently common to rodent-borne hantaviruses, thus pointing to similar structure and function mechanisms.

Methods

Cloning of the full-length genes and truncated and deleted mutants

For the expression of full-length N protein in *E. coli* (strain AD494 pLysS), cDNAs for the entire N protein open reading frame from MTNV, CARV, and HUIV (Kariwa et al., 2012) and SNV, kindly supplied by Dr. C. J. Peters (University of Texas Medical Branch, Galveston, TX, USA), BCCV (Rollin et al., 1995), ANDV (Reynolds et al., 2007), LANV (Johnson et al., 1997), MAPV (Fulhorst et al., 2004), HTNV strain 76–118 (Lee et al., 1982), AMRV (Lokugamage et al., 2004), SEOV (Kitamura et al., 1983), PUUV strain Sotkamo (Vapalahti et al., 1992), HOKV (Kariwa et al., 1995), TULV (Tegshduuren et al., 2010) and TPMV (Carey et al., 1971) were cloned into pET43.1b(+) (Novagen) for expression as NuSA-tagged proteins. The MTNV deletion mutants were similarly prepared (Fig. 1A). Truncated MTNV N protein cDNAs encoding N1–80, N1–150, N46–125, N125–275, and N250–428 were cloned into pET32.1b (Novagen) for expression as thioredoxin (TrxA) fusion proteins (Fig. 1A). For the expression of N protein in mammalian (Vero E6 and 293T) cells, cDNAs encoding full-length or deleted MTNV N protein were cloned into the expression vector pCAGGS/MCS (Niwa et al., 1991).

For baculovirus expression, MTNV, CARV, HUIV, SNV 77734 (Botten et al., 2000), BCCV, ANDV, LANV strain 510B, and MAPV, HTNV, SEOV, PUUV, TULV, TPMV genes encoding full-length N protein were cloned into pFastBac1 (Invitrogen) and expressed in High Five cells, as described previously (Koma et al., 2010).

Preparation of recombinant N protein in bacteria

N protein was expressed as a fusion with NuSA or TrxA in *E. coli* (strain AD494 pLysS) (Fig. 1). Briefly, transformed cells were grown in Luria-Bertani medium containing 100 μg/mL ampicillin to an optical density of $A_{600}=0.6$, and expression was induced for 4 h with isopropyl-β-D-thiogalacto-pyranoside to a final concentration of 0.5 mM. The cells were recovered by centrifugation (6000*g*, 10 min), followed by His-tag affinity purification using ProBond Nickel-chelating resin (Invitrogen) and collection with Elute Buffer (50 mM Tris, pH 8.0, 0.5 M NaCl, and 250 mM imidazole) followed by desalting on PD-10 columns (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The recombinant N proteins were subjected to SDS-PAGE and Western blotting with the hantavirus N-specific mAb E5G6 (Yoshimatsu et al., 1996) to verify correct expression.

Recombinant N protein expression in mammalian systems

For hantavirus N protein expression in mammalian cells, the entire open reading frame of the S segment or a series of truncated or deleted constructs encoding the desired regions were amplified and cloned into pCAGGS/MCS vector. For IFA, Vero E6 cells were seeded on 24-well slides and transfected using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA). Cells infected with the following viruses were used: HTNV 76–118, SEOV SR-11, PUUV Sotkamo strain (Vapalahti et al., 1992), TULV strain Morabia, and TPMV strain VRC-66412.

The transfected or infected cells were fixed with acetone and analyzed for reactivity to the mAbs. To prepare an N protein cell lysate for ELISA and competitive binding assay, 293T cells were grown in six-well plates and transfected. Expression was carried out for 48 h. The cells were then harvested and lysed in Lysis buffer (0.01 M Tris HCl, pH 7.8, 5 mM EDTA, 0.15 M NaCl, 0.6 M KCl, and 2% Triton X-100).

Mouse immunization

Four-week-old BALB/c mice were inoculated with four doses of 0.2 mL of MTNV N-NuSA (750 μg/mL) in incomplete Freund's adjuvant (Rockland, Gilbertsville, PA, USA) at 0-, 14-, 21-, and 48-days intervals. Pre- and post-immune serum was collected and examined by ELISA for adequate specific antibody responses. Four days after the final immunization, the mice were sacrificed and their spleens harvested for the preparation of hybridomas.

All experiments involving animals in this study were approved by the Committee of Animal Experimentation of Hokkaido University.

Production of mAbs

SP2/0-Ag14 myeloma cells were mixed with the spleen cells and fused with polyethylene glycol 1500 (Roche, Indianapolis, IN, USA). The hybridomas were cultured in GIT medium (Nippon Seiyaku, Osaka, Japan) containing 2× HAT supplement (Gibco-Invitrogen, Grand Island, USA) and BriClone (NICB, Dublin, Ireland). MTNV N antibody-secreting hybridomas were cloned at least four times by limiting dilution. ELISA was used to select hybridomas producing anti-N protein-specific antibodies using MTNV N protein and NuSA (negative control antigen). Next, eight-week-old BALB/c mice were inoculated intraperitoneally with antibody-producing hybridomas (10×10^7 cells/mouse) and ascitic fluid was collected within 2 weeks. The supernatant of the ascitic fluid was purified using an Affi-Gel Protein A MAPS II Kit (BioRad, Hercules, CA, USA) and PD-10 desalting columns. The mAb IgG subclasses were determined using IsoQuick Strips (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

ELISA

ELISA microtiter plates (NUNC) were coated with recombinant N protein or NuSA-tagged protein at 2 μg/mL in carbonate-bicarbonate buffer and left at 37 °C for 1 h or 4 °C overnight. Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) was used for blocking. The plates were then incubated with hybridoma culture supernatant or purified mAbs. After washing, 1:4000-diluted peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) was added to the plate (50 μL/well). *o*-phenylenediamine (Sigma-Aldrich) substrate was applied to the plate (100 μL/well) and left at room temperature for 30 min. The optical densities were measured at 450–650 nm using a Labsystem Multiscan MS (Helsinki, Finland). The specific reaction was calculated by subtracting the absorbance of the NuSA tag from the N protein value. All incubations were carried out for 1 h at 37 °C with three between-step washes using phosphate-buffered saline containing 0.05% Tween-20 (PBST).

Western blotting

For Western blotting, the N proteins were separated by SDS-PAGE then transferred to a PVDF membrane (Millipore, Cork, Ireland). Membrane blocking was performed using Block Ace. Next, dilute ascitic fluid or purified mAb (in PBST) was applied, followed by peroxidase-conjugated IgG (Jackson ImmunoResearch). Signals were detected using chemiluminescence ECL detection reagents (GE Healthcare) according to the manufacturer's protocol.

IFA

Vero E6 cells were seeded on 24-well slides and transfected with plasmid constructs encoding full-length, truncated, or deleted N proteins (Fig. 2). Fixed cells were incubated with 10-fold dilutions of purified mAbs, followed by Alexa-488-conjugated anti-mouse IgG antibody (Invitrogen).

Biotinylation and competitive binding assay

The competitive binding assay was performed by a capture ELISA using a streptavidin-biotin labeled system. Purified IgG (2 mg) was biotinylated using a Biotin-AC5-OSu Kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol. Next, 96-well plates were coated with 2 μg/mL mAb (2H10) diluted in PBS, incubated overnight at 4 °C, and blocked with 3% BSA-PBS buffer. A MTNV N protein lysate prepared from 293T cells (1:20) was captured for 1 h, followed by the addition of a 10-fold excess of unlabeled mAb (from 0.1 mg/mL) in 0.5% BSA (in PBST). Biotin-labeled mAbs were added without washing, and then the plate was incubated with streptavidin-peroxidase (Invitrogen) for 30 min at room temperature. The colorimetric reaction was carried out followed by three washes with PBST. The absorbance values (450–650 nm) were categorized as positive ($>75\%$), intermediate (25–75%), or negative ($<25\%$) inhibition in relation to the reference sample without unlabeled/competing mAbs.

For the capture ELISA for the detection of baculovirus N protein in High five cell lysate, reactivity to mAbs detection was carried out using biotinylated mAb E5G6.

Acknowledgments

This work was supported financially by Grants-in-Aid for Scientific Research (16405034, 17255009, and 21405035) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; and by a Health and Labor Sciences Research Grant on Emerging and Re-Emerging Infectious Diseases from the Japanese Ministry of Health, Labor, and Welfare. This work was also supported by the Global COE Program for Zoonosis Control (Hokkaido University).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2012.03.001.

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Journal of General Virology (2012), **93**, 27–38 DOI 10.1099/vir.0.035964-0

A conserved region in the prM protein is a critical determinant in the assembly of flavivirus particles

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Received 3 July 2011
Accepted 21 September 2011

Flaviviruses are assembled to bud into the lumen of the endoplasmic reticulum (ER) and are secreted through the vesicle transport pathway, but the details of the molecular mechanism of virion assembly remain largely unknown. In this study, a highly conserved region in the prM protein was identified among flaviviruses. In the subviral particle (SP) system of tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus, secretion of SPs was impaired by a mutation in the conserved region in the prM protein. Viral proteins were sparse in the Golgi complex and accumulated in the ER. Ultrastructural analysis revealed that long filamentous structures, rather than spherical SPs, were observed in the lumen of the ER as a result of the mutation. The production of infectious virions derived from infectious cDNA of TBEV was also reduced by mutations in the conserved region. Molecular modelling analysis suggested that the conserved region is important for the association of prM–envelope protein heterodimers in the formation of a spike of immature virion. These results are the first demonstration that the conserved region in the prM protein is a molecular determinant for the flavivirus assembly process.

INTRODUCTION

Enveloped viruses bud through cellular membranes, such as the plasma membrane, the membrane of the endoplasmic reticulum (ER) and the Golgi complex. Flaviviruses are generally thought to bud into the ER of virus-infected cells (Lindenbach *et al.*, 2007). Individual particles are subsequently transported to the Golgi complex in transport vesicle and released by exocytosis via the *trans*-Golgi network (Mackenzie & Westaway, 2001). However, intermediate budding structures have not been observed and the details of the mechanism of assembly and secretion are still largely unknown.

Flaviviruses, belonging to the family *Flaviviridae*, include many clinically important human pathogens, such as dengue (1–4) serotype viruses, West Nile virus, Japanese encephalitis virus (JEV), yellow fever virus and tick-borne encephalitis virus (TBEV) (Lindenbach *et al.*, 2007). The flavivirus genome consists of a positive-polarity ssRNA of approximately 11 kb, which encodes three structural proteins: the core (C), pre-membrane (prM)/membrane (M) and envelope (E) proteins. It also encodes seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, within a single long ORF (Chambers *et al.*, 1990). The ORF is translated into a large polyprotein, which is co- or post-translationally cleaved by cellular and viral proteases.

Flavivirus virions are 40–50 nm in diameter, spherical in shape and contain a nucleocapsid and an envelope. The envelope has two viral proteins: the major envelope protein E and the small membrane protein prM/M. Cryo-electron microscopy analysis of the immature dengue virus revealed 60 spikes, each comprising three prM/E heterodimers, that are organized icosahedrally on the surface of the particles (Zhang *et al.*, 2003), in contrast to the smooth surface of mature virions reported previously (Kuhn *et al.*, 2002).

The M protein is synthesized as a precursor protein, prM, in the ER and contains one to three *N*-linked glycosylation sites (Chambers *et al.*, 1990). Newly synthesized E and prM proteins associate to form heterodimers that are incorporated into immature virions (Allison *et al.*, 1995b; Wengler & Wengler, 1989). One of the major functions of the prM protein is a chaperone-like activity aiding folding and maturation of E proteins (Konishi & Mason, 1993; Lorenz *et al.*, 2002). During transport of the virions

A supplementary figure and table are available with the online version of this paper.

through the ER and Golgi complex to the cell surface, the prM proteins of the heterodimers protect the E proteins from premature fusion by the low-pH conditions in the transport vesicles (Guirakhoo *et al.*, 1991, 1992; Heinz *et al.*, 1994; Zhang *et al.*, 2003). Prior to release from the cell, the cellular furin protease cleaves the pr portion from prM, resulting in rearrangement of the E proteins into head-to-tail homodimers on the surface of the mature virus particles (Elshuber *et al.*, 2003; Stadler *et al.*, 1997). Other roles of the prM protein are not well understood.

In flaviviruses, it has been demonstrated that subviral particles (SPs) are assembled and secreted from cells expressing the viral prM and E genes (Allison *et al.*, 1995b; Fonseca *et al.*, 1994; Mason *et al.*, 1991; Op De Beeck *et al.*, 2003; Yamshchikov & Compans, 1993). These SPs are similar to the slowly sedimenting haemagglutinin particles that are released from flavivirus-infected cells in addition to infectious virions (Gritsun *et al.*, 1989; Heinz & Kunz, 1977). SPs contain viral E proteins but lack the nucleocapsid protein and viral RNA. Because the characteristics of the envelope proteins in SPs are structurally and functionally similar to those of authentic virions, SPs have been used in research of the viral E proteins (Allison *et al.*, 1995a, 1999, 2001; Lorenz *et al.*, 2003).

Despite recent advances in our understanding of flaviviruses, the functional residues of the prM ectodomain that are important for assembly and secretion of the virions remain almost unknown. In our previous study, a single point mutation in the prM protein was found to reduce the secretion of TBEV particles, suggesting that prM plays a crucial role in the virus budding process (Yoshii *et al.*, 2004). In the current study, a highly conserved region in the prM protein was identified among flaviviruses. Several amino acid substitutions were introduced into this conserved region by site-directed mutagenesis in the SP systems of TBEV and JEV to examine their effects on the assembly and secretion of SPs. Some of these mutations were then introduced into an infectious cDNA clone of TBEV to confirm the role of the conserved region in the virus replication cycle.

RESULTS

Effect of point mutations in the conserved region of prM proteins on SP secretion

From a multiple-sequence analysis of the flavivirus prM protein, a highly conserved region was identified among flaviviruses (analysis of >4000 strains using Pfam: http://pfam.sanger.ac.uk/; Finn *et al.*, 2010). Table 1 shows the sequence of the conserved region among representative flaviviruses. To investigate the importance of this region in virus replication, alanine substitutions were engineered in this region of the pCAG-TBEME plasmid, which expresses the prM and E proteins of TBEV (Table 2). Alanine substitutions were also engineered at cysteines 68 and 70, but no expression of viral proteins was observed in cells transfected with these plasmids (data not shown). This may have been due to a critical conformational change in the prM protein, as both cysteine residues stabilize the prM structure by disulfide bonds (Li *et al.*, 2008).

pCAG-TBEME plasmids containing the different mutations were transfected into 293T cells. After incubation for 24 h, extracellular E proteins were detected by ELISA. As presented in Fig. 1(a), the secretion of E protein was reduced considerably by prM mutations at proline-63, aspartate-65, valine-66, aspartate-67 and phenylalanine-69. An alanine substitution of glutamate-62 did not affect the kinetics of the E protein. Interestingly, the conservative valine-to-alanine substitution at residue 66 reduced the secretion of the E protein. At position 66, a branched-chain amino acid (valine, isoleucine or leucine) is conserved, and a

Table 1. A conserved sequence in flavivirus prM protein

The non-conserved amino acid in the sequence is shown in non-bold.

Virus*	GenBank accession no.	Amino acid sequence									Amino acid range†
TBEV	AB062063	**E**	**P**	V	**D**	V	**D**	**C**	**F**	**C**	62–70
POWV	NC_003687	**E**	**P**	V	**D**	V	**D**	**C**	**F**	**C**	62–70
LANV	AF253419	**E**	**P**	V	**D**	V	**D**	**C**	**F**	**C**	58–66
JEV	AF069076	**D**	**P**	E	**D**	V	**D**	**C**	**W**	**C**	61–69
WNV	AB185914	**D**	**P**	E	**D**	I	**D**	**C**	**W**	**C**	61–69
DENV	U87411	**E**	**P**	E	**D**	I	**D**	**C**	**W**	**C**	60–68
YFV	X03700	**E**	**P**	D	**D**	I	**D**	**C**	**W**	**C**	57–65
APOIV	NC_003676	**E**	**P**	V	**D**	L	**D**	**C**	**F**	**C**	57–65
Consensus		**E/D**	**P**	–	**D**	**V/I/L**	**D**	**C**	**F/W**	**C**	

*POWV, Powassan virus; LANV, Langat virus; DENV, dengue virus; YFV, yellow fever virus; APOIV, Apoi virus.
†Numbers indicate the amino acid position in the prM protein.

Table 2. The amino acid difference in prM proteins between the parental virus and the plasmids used in this study

The non-conserved amino acid in the sequence is shown in non-bold.

Virus/ mutant	Position*								
	62	63	64	65	66	67	68	69	70
TBEV (pCAG-TBEME and Oshima-IC)									
Wild type	**E**	**P**	V	**D**	**V**	**D**	**C**	**F**	**C**
prE62A	A	–	–	–	–	–	–	–	–
prP63A	–	A	–	–	–	–	–	–	–
prD65A	–	–	–	A	–	–	–	–	–
prV66A	–	–	–	–	A	–	–	–	–
prD67A	–	–	–	–	–	A	–	–	–
prF69A	–	–	–	–	–	–	–	A	–
prV66I	–	–	–	–	I	–	–	–	–
prF69W	–	–	–	–	–	–	–	W	–
	Position†								
	61	62	63	64	65	66	67	68	69
JEV (pcJEME)									
Wild type	**D**	**P**	E	**D**	**V**	**D**	**C**	**W**	**C**
prP62A	–	A	–	–	–	–	–	–	–
prD64A	–	–	–	A	–	–	–	–	–
prV65A	–	–	–	–	A	–	–	–	–
prD66A	–	–	–	–	–	A	–	–	–
prW68A	–	–	–	–	–	–	–	A	–

*Based on TBEV strain Oshima 5-10 (GenBank accession no. AB062063).
†Based on JEV strain Nakayama (GenBank accession no. EF571853).

valine-to-isoleucine substitution was engineered in pCAG-TBEME. Unlike the alanine substitution, the isoleucine substitution did not affect secretion of the E protein, indicating the importance of the branched-chain amino acid at this residue. At position 69, the aromatic amino acid phenylalanine is conserved in tick-borne and no-known-vector flaviviruses, whilst tryptophan is conserved here in mosquito-borne flaviviruses. A phenylalanine-to-tryptophan substitution was engineered at this residue in pCAG-TBEME and this substitution reduced secretion of the E protein.

To confirm the importance of the conserved region in other flaviviruses, similar alanine substitutions were engineered in the pcJEME plasmid expressing the prM and E proteins of JEV. pcJEME plasmids containing the different mutations were transfected into 293T cells. After incubation for 24 h, intracellular and extracellular E proteins were detected by Western blotting. The substitutions of proline-62, aspartate-64, valine-65, aspartate-66 and tryptophan-68 in the prM protein resulted in reduced secretion of the E protein, as observed in TBEV (Fig. 1b). These data indicated that the conserved region in the prM protein of flaviviruses has an important role in the assembly and secretion of the virus particles.

Effect of mutations in the conserved region on prM–E interaction

Interaction between the prM and E proteins is necessary for assembly of the virus particle. To investigate whether the mutations in the conserved region affected the prM–E association, 293T cells transfected with the pCAG-TBEME plasmids were lysed, and co-immunoprecipitation of the prM and E proteins was carried out. As shown in Fig. 2, prM and E proteins were detected in eluates from co-immunoprecipitation samples from cells transfected with pCAG-TBEME carrying the prP63A, prD65A, prV66A or prF69A mutation and in the wild-type plasmid. These data demonstrated that heterodimerization between prM and E was not disrupted by mutations at positions 63, 65, 66 or 69. Following alanine substitution at position 67 (aspartate to alanine), prM and E proteins were not co-immunoprecipitated, indicating that this mutation critically affected the prM–E interaction.

Effect of mutations in the conserved region on intracellular localization

To evaluate whether the mutations in the conserved region affected the intracellular distribution of viral proteins, cells

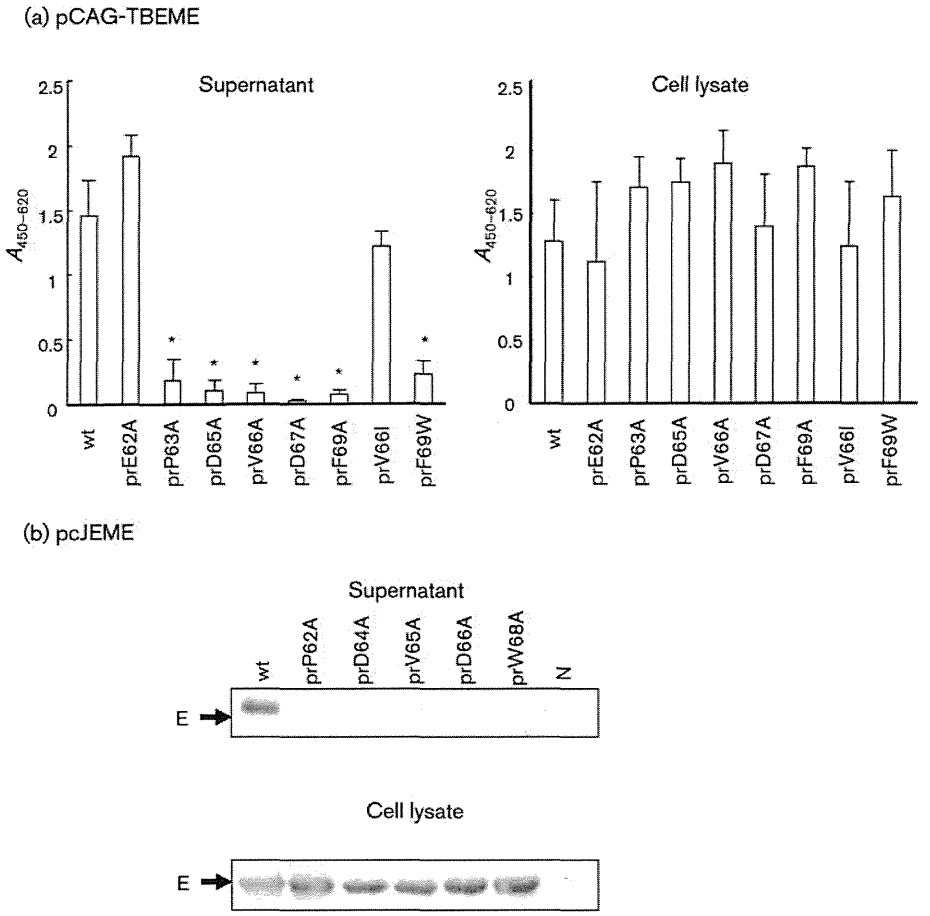

Fig. 1. Mutations in the conserved region of prM reduce the secretion of SPs. PrM and E proteins were expressed in 293T cells by transfection of wild-type (wt) pCAG-TBEME (a) or pcJEME (b) with or without a mutation in the conserved region in prM. After a 24 h incubation, E protein in the solubilized sample was detected by ELISA using TBEV E-specific mAb (a) or Western blotting using JEV E-specific mAb (b). Mean absorbance values (±SD) were calculated from triplicate experiments. Asterisks indicate statistically significant differences compared with the wild-type (wt) plasmid (Student's *t*-test, $P<0.05$). N, Non-transfected control.

transfected with each pCAG-TBEME plasmid were double-stained for TBEV proteins and cellular marker antigens. The distribution of viral proteins in the ER was not affected by the mutations in prM (Fig. 3a). However, whilst the distribution of viral proteins in the Golgi complex was observed in the wild-type-transfected cells, co-localization of viral proteins and Golgi markers was low with the prM protein containing mutations (Fig. 3b). Co-localization of prM and E proteins was similar in all pCAG-TBEME-transfected cells (see Supplementary Fig. S1, available in JGV Online). These data suggested that the mutation in the conserved region of prM caused a defect in the transport of the viral protein to the Golgi complex.

Ultrastructural analysis of particle assembly by prM mutations

To examine whether the mutations in prM affected the assembly of SPs, electron microscope analysis of pCAG-TBEME-transfected cells was performed. As shown in Fig. 4, spherical SPs were observed in the lumen of membrane-bound vesicles in wild-type-transfected cells (Fig. 4a, b). In contrast, filamentous structures were observed in the lumen of membrane-bound vesicles in cells transfected with pCAG-TBEME containing the prP63A, prD65A, prV66A or prF69A mutation (Fig. 4c–f). The filamentous structures were 30–40 nm in width and 0.1 to >1.0 μm in length and had constrictions every 50–100 nm. In prD67A-transfected cells, no SPs or filamentous structures were detected (Fig. 4g). These results indicated that the conserved region of prM has important roles in virion assembly.

Effect of mutations in the conserved region on viral multiplication

To confirm the effect of mutations in prM on viral multiplication, alanine substitutions affecting the secretion

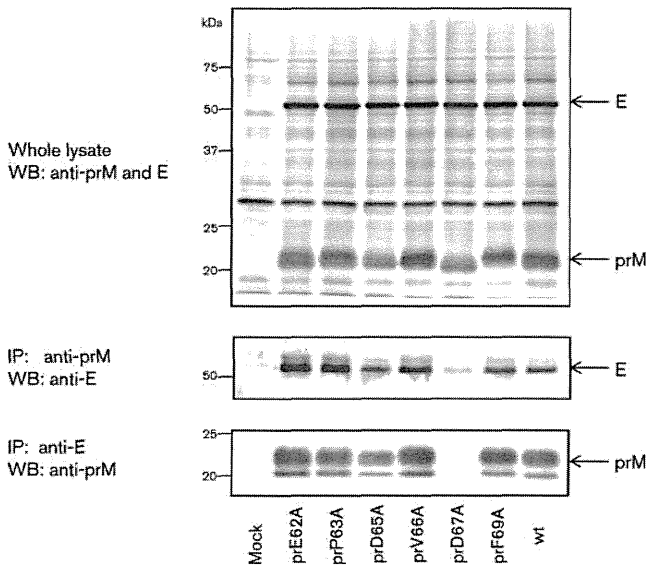

Fig. 2. Effect of mutations in the conserved region of prM on prM–E interaction. The prM protein, with or without (wt) a mutation in the conserved region, was expressed in 293T cells by transfection of the pCAG-TBEME plasmid containing various mutations. After 24 h incubation, cells were harvested, and whole-cell lysate and post-nuclear supernatant immunoprecipitated (IP) with mouse anti-E or anti-prM antibodies were analysed by Western blotting (WB). Protein bands were detected by anti-E and anti-prM rabbit polyclonal antibodies. The positions of the individual proteins are marked and molecular size is indicated (kDa).

and assembly of SPs were engineered in the full-length infectious cDNA of TBEV (Oshima-IC) (Table 2). Baby hamster kidney (BHK-21) cells were transfected with *in vitro*-transcribed mRNA from Oshima-IC, and the culture supernatant was harvested at 8–120 h post-transfection. As presented in Fig. 5(a), cells transfected with Oshima-IC containing prM mutations produced fewer infectious virus particles than cells transfected with wild-type Oshima-IC. The secreted E protein was quantified by ELISA and compared with the infectious virus titre to examine whether the lower level of infectious virus resulting from the presence of prM mutations was due to a reduction in virion secretion or to loss of infectivity of the secreted virion (Fig. 5b). Virus titres were reduced by the prM mutations at a rate similar to that of viral protein secretion, indicating that the infectivity of secreted virions was unaffected by the prM mutations. These results were consistent with the low level of secretion of SPs by prM mutations observed in Fig. 1. Cells transfected with Oshima-IC prD67A produced fewer virus particles than those transfected with Oshima-IC containing the other prM mutations, indicating that this mutation affects the virus multiplication associated with the loss of the prM–E interaction and particle assembly, as observed in the SP experiment.

To investigate the possibility of the appearance of reversion or second-site mutations, each virus was passaged ten times (over a period of 30 days) and the nucleotide sequences of their prM and E genes were determined. Reversion to wild-type sequence was observed in Oshima-IC prP63A at passage 9 and in prD67A at passage 2. No reversion or second-site mutation appeared in Oshima-IC prD65A, prV66A or prF69A, and their growth rates were still slower than wild-type virus. These data confirmed the importance of the conserved region in prM in viral multiplication.

Molecular modelling of the conserved region on trimeric spikes of immature virions

To identify potential amino acid residues that could interact with the conserved regions, the crystal structure of the pr peptide and the E protein was superimposed on the pseudo-atomic structure of the trimeric spikes of three prM–E heterodimers in immature virions. This structure was then refined by simulated annealing with molecular dynamics calculation (Fig. 6 and Supplementary Table S1, available in JGV Online). This highlighted amino acid residues that could potentially interact with the conserved regions of the prM proteins (prM-1, prM-2 and prM-3) within the trimeric spike of the prM–E heterodimers: (i) the fusion peptide of E-2 with the conserved region of prM-1; (ii) arginine-16, lysine-19, methionine-39 and threonine-81 of prM-3 with the conserved region of prM-2; and (iii) arginine-16, lysine-19, methionine-39 and threonine-81 of prM-2 with the conserved region of prM-3. As shown in Fig. 6, whilst the conserved region of prM-1 is located between the fusion loop of E-1 and E-2, the conserved regions of prM-2 and prM-3 were suggested to interact with similar regions of each other, indicating the dimeric association between prM-2 and prM-3. These results suggest that the conserved region in prM could serve as an important domain for the association between heterodimers in the formation of a spike.

DISCUSSION

In flaviviruses, the prM and E proteins play important roles in the assembly and secretion of virions. Many studies have demonstrated that the co-expression of prM and E proteins in cells leads to the formation of SPs that are structurally and functionally similar to native virions (Allison *et al.*, 1995b; Fonseca *et al.*, 1994; Mason *et al.*, 1991; Op De Beeck *et al.*, 2003; Yamshchikov & Compans, 1993). These studies indicated that critical determinants for the assembly and secretion of the virions were present in the prM and/or E proteins. During the assembly process, prM proteins are assumed to coat E proteins. It has been shown that the furin cleavage sequence between the pr region and the M protein is important for maturation of infectious virions prior to their release from cells (Elshuber *et al.*, 2003; Stadler *et al.*, 1997) and that the transmembrane domain of the prM protein serves as a retention signal for the membrane of the ER (Op De Beeck *et al.*, 2003, 2004). In several reports, single point mutations in prM were found

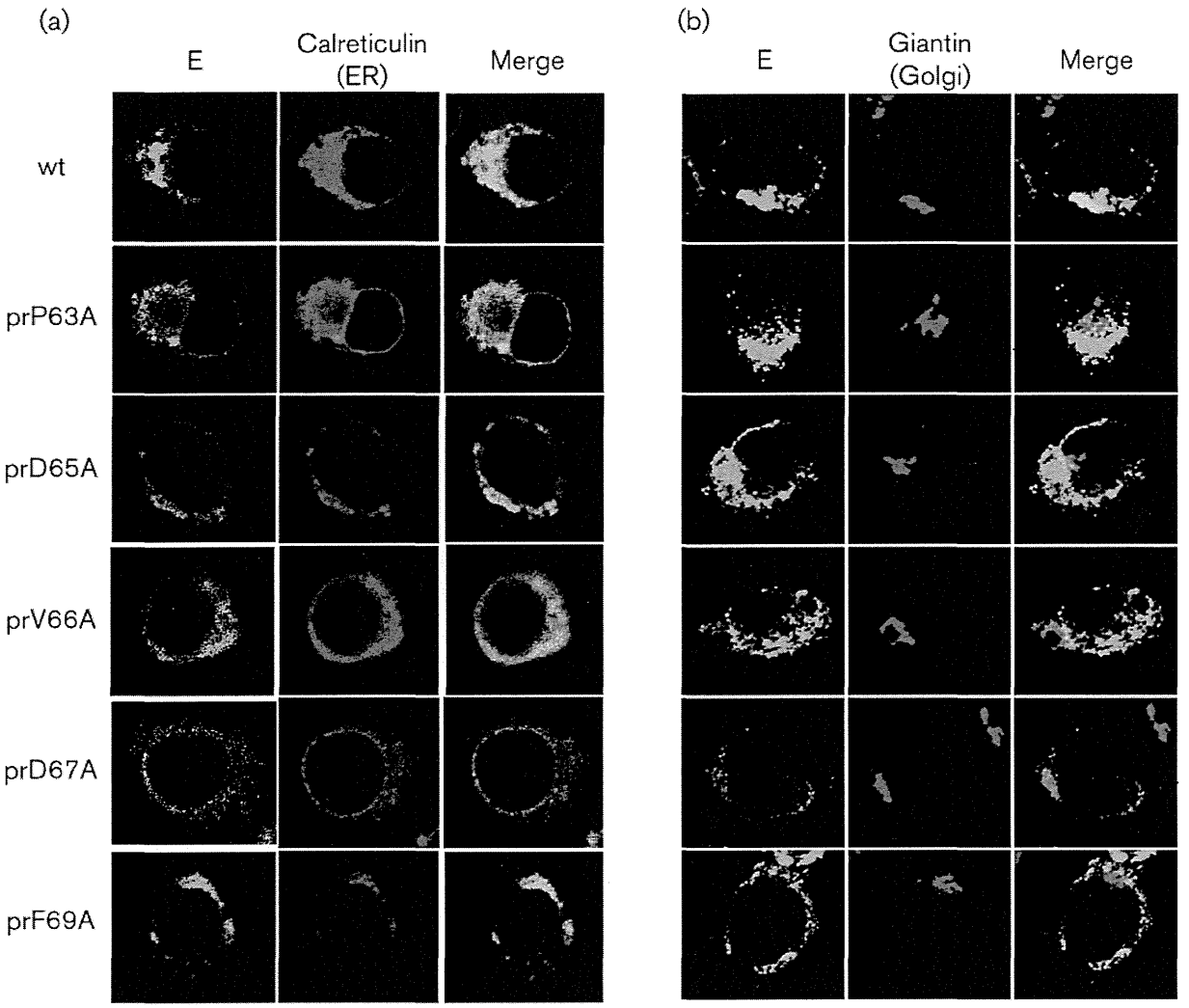

Fig. 3. Intracellular localization of expressed E proteins. 293T cells transfected with pCAG-TBEME wild-type (wt) or various mutant plasmids were fixed and subjected to dual staining with antibodies against calreticulin (an ER marker) (a) or giantin (a Golgi marker) (b) and anti-E antibodies. Co-localization of the E protein with the ER or Golgi marker proteins is depicted in the merged images.

to affect viral particle formation and secretion without preventing prM–E heterodimerization (Pryor *et al.*, 2004; Tan *et al.*, 2009). Another study showed that coordinated cleavage between the C protein and prM promoted the uptake of nucleocapsids into budding flavivirus membranes (Lobigs & Lee, 2004). However, little is known about the functional residues of the prM protein involved in the assembly and secretion process. In this study, we demonstrated for the first time that the conserved region of the prM ectodomain in flaviviruses has critical functions in the assembly and secretion process.

Multiple-sequence analysis revealed that the conserved region in prM is present in a large number of flaviviruses, including tick-borne, mosquito-borne and no-known-vector flaviviruses (Table 1). As shown in our previous study, a proline-to-serine mutation at position 63 of the prM of TBEV reduces the secretion of virus particles (Yoshii *et al.*, 2004) and we considered that the conserved region plays a crucial role in the assembly and secretion of virus particles. It was revealed that the secretion of SPs was impaired by a single mutation in the conserved region of both TBEV and JEV (Fig. 1). This indicated that the conserved region in the prM protein is a critical and common molecular determinant for the assembly and secretion of flaviviruses.

The interaction between the prM and E proteins is important during the early events of virus particle maturation and assembly. Previous studies have shown that the E protein requires co-synthesis of prM to achieve structural conformation, whilst prM folds independently of other viral components (Guirakhoo *et al.*, 1992; Lorenz *et al.*, 2002). PrM–E heterodimerization is a crucial process

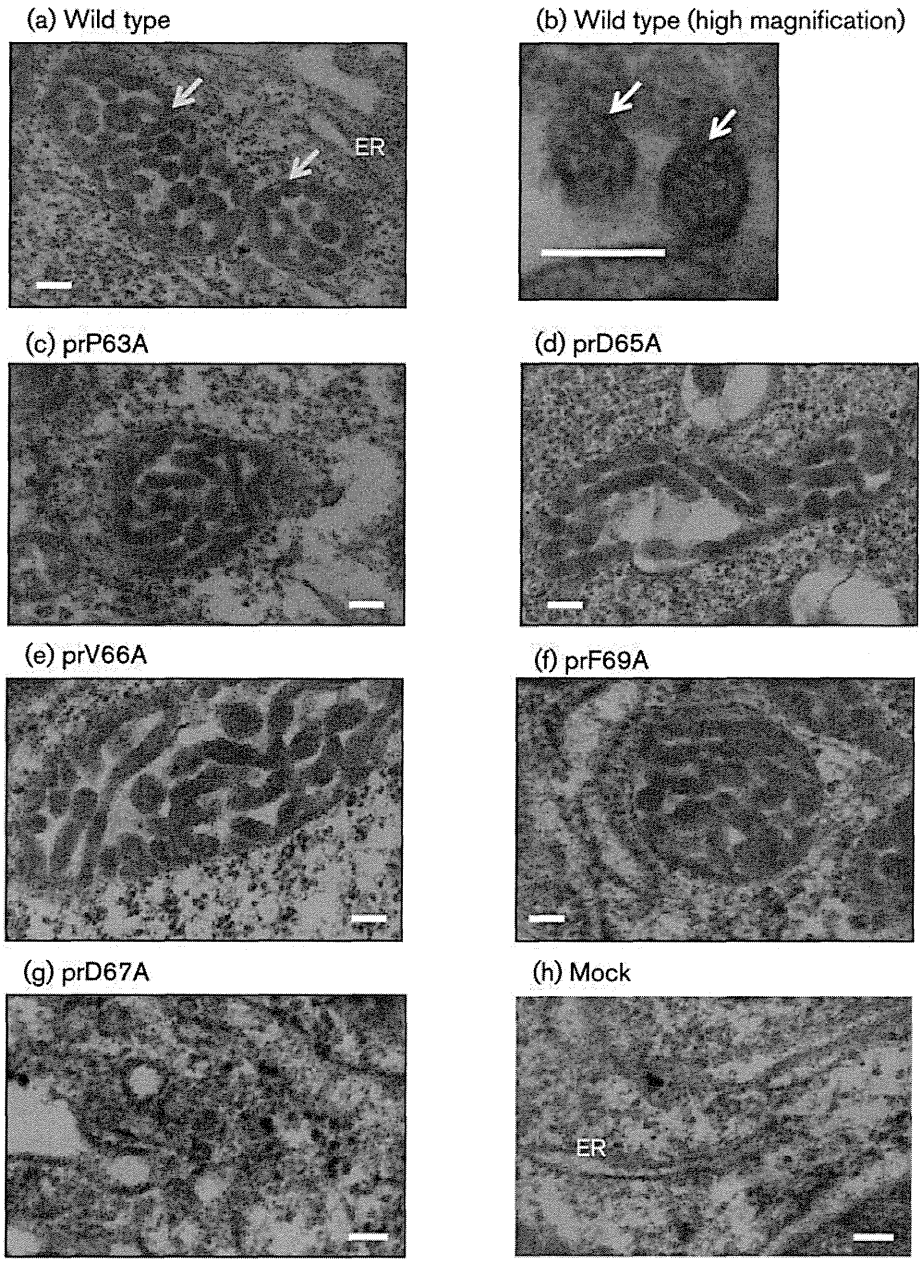

Fig. 4. Electron micrographs of 293T cells transfected with pCAG-TBEME containing various mutations. (a, b) Spherical SPs (arrows; diameter 20–30 nm) were observed in the lumen of membrane-bound vesicles in wild-type-transfected cells. (c–f) Filamentous structures were observed in the lumen of membrane-bound vesicles in cells transfected with pCAG-TBEME containing the prP63A (c), prD65A (d), prV66A (e) or prF69A (f) mutation. (g, h) No particulate and filamentous structures were observed in prD67A-transfected cells (g), similar to mock-transfected control cells (h). Bars, 50 nm.

for sequential virus particle formation. In this study, co-immunoprecipitation experiments demonstrated that prM–E heterodimerization was not impaired by the mutations in the conserved region of the prM protein except for the mutation at position 67 (Fig. 2). This mutation impaired the interaction between the prM and E protein, leading to the failure of further steps in the viral particle assembly process. These data suggested that the reduction in SP secretion by the prM mutations, except for position 67, was due to later steps in the viral particle assembly process and secretion, and not to disruption of prM–E heterodimerization in the early events of virus particle assembly.

Previous reports have shown that flavivirus particles bud into the ER lumen, followed by transport through the

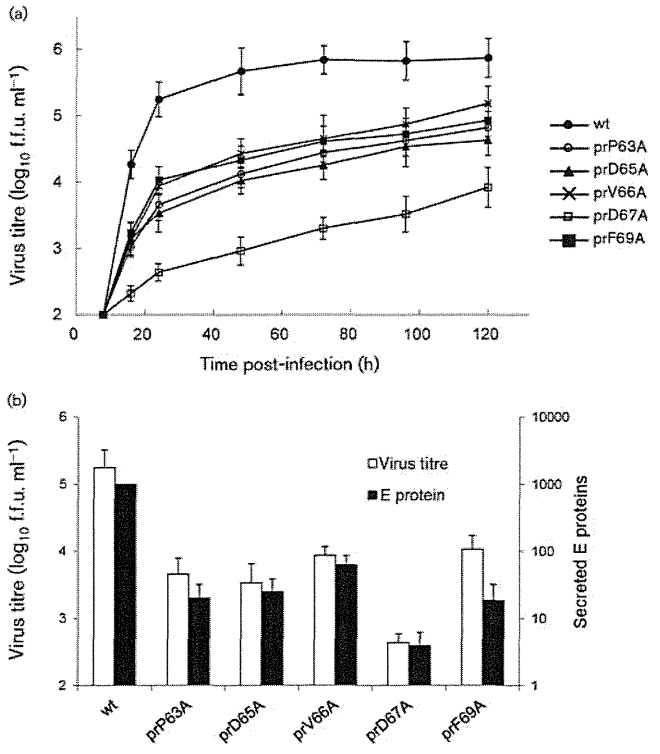

Fig. 5. Effect of mutations in the conserved region of prM on virus particle secretion. (a) Growth curve of the Oshima-IC virus wild-type (wt) and prP63A, prD65A, prV66A, prD67A and prF69A mutants. A monolayer of BHK-21 cells was infected with the individual viruses at an m.o.i. of 0.01. At each time point, the culture medium was harvested and virus titres were determined by focus-forming assay in BHK cells. (b) Comparison of the titre of infectious virus and secreted E proteins. After 24 h incubation, the relative amount of secreted E protein was quantified by ELISA and compared with the virus titre. The amount of E protein from the Oshima-IC wild-type virus was set at 1000. Data are means ± SD from triplicate experiments.

secretory pathway (Mackenzie & Westaway, 2001). In this study, the expressed viral proteins with the prM mutations were not transported to the Golgi complex, and accumulated in the ER (Fig. 3). Furthermore, ultrastructural analysis revealed long filamentous structures, rather than spherical SPs, in the lumen of the ER resulting from the prM mutations (Fig. 4). The width of the filamentous structures was approximately the same as the diameter of the SPs, and constrictions were observed every 50–100 nm. It was suggested that they were derived from abnormal budding in which a particle failed to pinch off, resulting in the bud growing into a tubular structure. Filamentous structures may not undergo transport through the secretory pathway due to their abnormal budding. It is also possible that they are derived from cellular membrane components induced by prM mutations. In any case, the mutations in the conserved region of prM clearly affected the budding process of the virus particle, which must have occurred after the prM–E heterodimerization. No SPs or filamentous structures were observed with the mutation at position 67. This could be attributed to the disruption or the prM–E heterodimerization in the early events of virus particle assembly, as described above. In a study of dengue virus, this negatively charged residue was shown to interact with the positively charged surface of domain II of the E protein (Li *et al.*, 2008).

The budding mechanism of flaviviruses is considered to be distinct from that of other enveloped viruses. In many enveloped viruses, important cytoplasmic domains directing virus budding have been identified, such as late-domain motifs (Bieniasz, 2006; Kail *et al.*, 1991; Morita & Sundquist, 2004; Whitt *et al.*, 1989; Zhao *et al.*, 1994). These domains interact with cellular factors resulting in efficient budding. However, the cytoplasmic loops of the flavivirus prM and E proteins consist of only a few amino acid residues between their two transmembrane regions. Thus, it is considered that the ER-luminal regions of the prM and/or E proteins play critical roles in the assembly of flaviviruses. The ER membrane does not normally form vesicles that bud into the lumen. It is possible that prM–E heterodimers, alone or with cellular factors in the ER lumen, assemble laterally and induce membrane curvature into an isometric lattice, as reported in the budding of membrane transport vesicles (Keen *et al.*, 1979; Wieland & Harter, 1999). The conserved region in prM may have important roles in this process, which were impaired by the mutations, leading to the abnormal budding.

The conserved region in prM could be involved in the oligomerization of the prM–E heterodimers. Immature virions of flaviviruses contain 60 trimeric spikes and each spike consists of three prM–E heterodimers (Zhang *et al.*, 2003). The asymmetrical spike has been considered a single assembly unit because the association between heterodimers within a spike is sufficiently strong, compared with the association between spikes. The dominant contacts between the prM–E heterodimers within a spike are between pre-peptides at the extremities of the spikes. Based on the crystal structure of the pr peptide of dengue virus 2 (Li *et al.*, 2008), the conserved region of prM covers the fusion peptide of the E protein in each heterodimer and aspartate-63 and -65 were involved in the complementary electrostatic patches. Our structural model for an asymmetrical trimer of prM–E heterodimers indicates that the conserved region in prM could serve as an important domain for the association between heterodimers in the formation of a spike (Fig. 6 and Supplementary Table S1). The mutations in the region might affect the association between heterodimers and cause perturbation of oligomerization of prM–E heterodimers, resulting in the inefficient induction of membrane curvature and/or pinch-off.

In summary, we demonstrated that the conserved region in prM is a critical determinant in flavivirus assembly and secretion. Mutations in this region, except for aspartate-67,

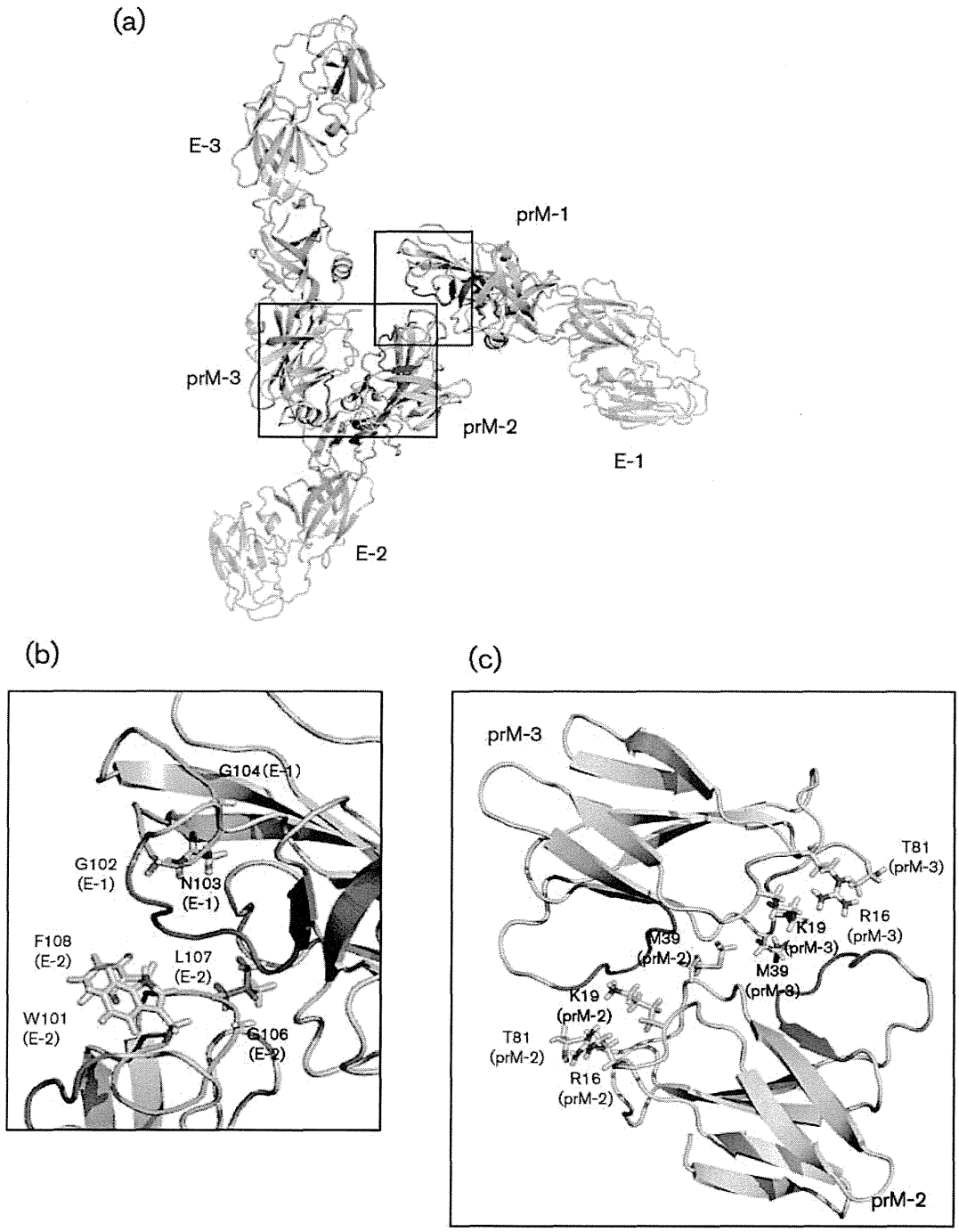

Fig. 6. Structural model for an asymmetrical trimer of prM–E heterodimers. (a) Overall structure of an asymmetrical trimer of prM–E heterodimers. (b) Close-up view of the binding interface between prM-1 and E-1 or E-2. (c) Close-up view of the binding interface between prM-2 and prM-3. E and prM molecules are coloured blue-white and yellow, respectively. The fusion peptide of the E protein and the conserved region of prM protein are shown in green and magenta, respectively. Residues represented by stick models show the amino acid position within 4.0 Å of the conserved region of prM. This figure was prepared using PyMOL (DeLano Scientific; DeLano, 2002).

did not affect the interaction between the prM and E proteins but impaired the budding step, which is independent of previously known functions of the prM protein. These results contribute to the further understanding of prM function, revealing the molecular mechanism of flavivirus assembly and secretion.