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Journal Title: Journal of Virology
Volume: Volume 87, Number 6
Publisher: American Society for Microbiology | 2013-03-01, Pages 3583-3586
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/JVI.03029-12
Permanent URL: https://pid.emory.edu/ark:/25593/s272x

Final published version: http://dx.doi.org/10.1128/JVI.03029-12

JVI Journals.ASM.org

The Receptor-Binding Site of the Measles Virus Hemagglutinin Protein Itself Constitutes a Conserved Neutralizing Epitope

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Here, we provide direct evidence that the receptor-binding site of measles virus (MV) hemagglutinin protein itself forms an effective conserved neutralizing epitope (CNE). Several receptor-interacting residues constitute the CNE. Thus, viral escape from neutralization has to be associated with loss of receptor-binding activity. Since interactions with both the signaling lymphocyte activation molecule (SLAM) and nectin4 are critical for MV pathogenesis, its escape, which results from loss of receptor-binding activity, should not occur in nature.

Measles virus (MV) is an enveloped virus that belongs to the genus *Morbillivirus* of the family *Paramyxoviridae* and possesses two types of surface glycoprotein spikes, the hemagglutinin (H) and fusion (F) proteins. MV infection is initiated by binding of the H protein to cellular receptors on the target host cells. The binding triggers membrane fusion between the virus envelope and the host cell plasma membrane mediated by the F protein. The signaling lymphocyte activation molecule (SLAM) on a subset of immune cells and nectin4 at adherens junctions are the principal receptors for MV (1–4). The H and F proteins are both neutralizing targets, but greater amounts of antibodies (Abs) are elicited against the H protein than the F protein, and the H protein-specific antibodies mainly contribute to neutralization of MV infection *in vivo* (5–8). All the available data suggest that measles eradication is biologically feasible (9, 10), and one of the major factors that would ensure measles eradication is the single-serotype nature of MV. Our previous paper suggested that the receptor-binding site (RBS) on the H protein, which is exposed outside the heavy N-glycan shields, may constitute a major neutralizing epitope that contributes to the single-serotype nature of MV (11). However, our recent paper (12) showing the detailed locations of five epitopes (*I*, *II*, *iv*, *v*, and *vi*) on the H protein provided insufficient evidence that the RBS acts as a neutralizing epitope. Here, we provide direct and concrete evidence that the RBS itself forms an effective conserved neutralizing epitope (CNE) which provides a strong functional constraint against change.

In the present study, we characterized a new mouse monoclonal antibody (MAb), 2F4. MAb 2F4 was produced using a cell line expressing the H protein of the wild-type IC-B strain as an antigen. For competitive binding enzyme-linked immunosorbent assays (cELISAs), MV antigens precoated on cELISA plates (Denka Seiken) were incubated with previously reported MAbs (E81, E128, E185, E39, and E103) (12, 13), 2F4, or phosphate-buffered saline for 1 h and then incubated with MAb 2F4 labeled with biotin using a biotin labeling kit (NH2; Dojindo Laboratories). After three washes, the MV antigens bound by the MAbs were incubated with a streptavidin-horseradish peroxidase (HRP) conjugate (Thermo Scientific) for 1 h before addition of the TMB substrate (3,3′5,5′-tetramethylbenzidine; Denka Seiken). The plates were incubated for 25 min, and the colorimetric reactions were stopped by addition of H_2SO_4. The optical density (OD) values were analyzed using a microplate reader (Bio-Rad). The data revealed that binding of 2F4 was not inhibited by MAbs recognizing epitopes *I*, *iv*, *v*, and *vi* (E81, E185, E39, and E103, respectively) (12) (Table 1; the epitope recognized by 2F4 is tentatively termed epitope *vii*). On the other hand, E128 recognizing epitope *II* (12) showed partial inhibition of 2F4 binding (Table 1). Epitope *II* is shielded by the N-glycan modification at amino acid position 416 (N416-sugar), and viruses with the N416-sugar (genotypes D3, D4, D5, and D9 among the eight genotypes) are not neutralized by E128 (12). Eight distinct recombinant MVs (rMVs) encoding a *Renilla* luciferase reporter gene and an H gene derived from different MV genotypes (A, B3, D3, D4, D5, D8, D9, and H1) were used as neutralization targets for 2F4 (Table 2), as reported previously (12). The data clearly demonstrated that, unlike E128 recognizing epitope *II*, 2F4 showed high neutralizing titers against all eight rMVs (Table 2). These data suggested that epitope *vii* is a CNE and is distinct from all the other five epitopes (*I*, *II*, *iv*, *v*, and *vi*).

When the H protein-SLAM interaction was assessed by surface plasmon resonance assays, as reported previously (12), MAb 2F4 blocked binding of the H protein to SLAM, as did MAbs B5 and E128 recognizing epitopes *I* and *II*, which are located near the RBS (12) (Table 3). These data suggest that epitope *vii* is located at or near the RBS. A panel of rMVs possessing amino acid substitutions at the RBS were analyzed for neutralization by 2F4. On the D3 genetic background (IC323 strain [14]), four rMVs encoding enhanced green fluorescent protein (EGFP) and possessing mutations at a SLAM-interacting residue (R533A) (Fig. 1B) and nectin4-interacting residues (F483A, Y543S, and S544A/Y541S) (Fig.

Received 29 October 2012 **Accepted** 26 December 2012
Published ahead of print 2 January 2013
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doi:10.1128/JVI.03029-12

TABLE 1 Summary of competitive binding ELISAs of anti-H protein MAbs

Unlabeled MAb	Antigenic site	Biotinylated MAb 2F4 (*vii*) inhibition[a]
E81	*I*	−
E128	*II*	+
E185	*iv*	−
E39	*v*	−
E103	*vi*	−
2F4	*vii*	++

[a] ++, complete inhibition; +, partial inhibition; −, no inhibition.

1C) (15–19) were generated and assessed for virus spreading in SLAM-positive (SLAM$^+$) B95a and nectin4$^+$ II-18 cells (20) in the presence or absence of 2F4 (Fig. 2). The rMVs with F483A and Y543S were reported previously (15). As shown in Fig. 2, in the absence of 2F4 [Ab(−)], wild-type MV spread efficiently in both cell types using SLAM or nectin4. On the other hand, rMVs with F483A, Y543S, or S544A/Y541S lost the ability to use nectin4 as a receptor and thus failed to spread in II-18 cells, even in the absence of 2F4. Similarly, rMV with R533A lost the ability to use SLAM as a receptor and did not spread in B95a cells. However, in the presence of 2F4, the mutant MVs showed advantages for spreading. Even in the presence of 2F4, rMVs with F483A, Y543S, or S544A/Y541S were able to spread in SLAM$^+$ B95a cells, and rMV with R533 was able to spread in nectin4$^+$ II-18 cells, whereas the wild-type MV infection was completely neutralized by 2F4. Similar experiments were performed using luciferase-expressing rMVs (21). Two rMVs with mutations at SLAM-interacting residues (D505S and R533A) (17, 19) (Fig. 1B) and two rMVs with mutations at nectin4-interacting residues (F483A and Y543S) (15, 16, 18) (Fig. 1C) were generated (rMVs with F483A and Y543S were reported previously [15]). In part, as reported previously, rMVs with F483A (15) and Y543S (15) were unable to bind to nectin4 (2, 3), and those with R533A (16) and D505S (in the present study) lost the ability to use SLAM (Table 4). Meanwhile, the F483A, Y543S, D505S, and R533A substitutions all resulted in escape from neutralization by 2F4 (Table 4). These data demonstrated that the RBS used for interactions with both SLAM and nectin4 constitutes epitope *vii* recognized by 2F4 (Fig. 1). Consequently, MV escape

TABLE 2 Neutralizing titers against MV strains possessing H genes from different genotypes

Cell line	Genotype, yr isolated	Neutralizing titer[a]	
		2F4 (*vii*)	E128 (*II*)[b]
B95a	A, 1954	22,141	1,968
	B3, 2009	11,070	1,968
	D3, 1984	22,141	15
	D4, 2009	22,141	31
	D5, 2001	11,070	15
	D8, 2009	44,281	1,968
	D9, 2010	11,070	15
	H1, 2009	22,141	1,968
II-18	A, 1954	11,070	62,977
	B3, 2009	11,070	62,977
	D3, 1984	11,070	123
	D4, 2009	11,070	31
	D5, 2001	22,141	<4
	D8, 2009	22,141	62,977
	D9, 2010	11,070	123
	H1, 2009	22,141	62,977

[a] Data represent neutralizing titers for 1 mg/ml of immunoglobulin.
[b] Data for E128 are taken from reference 12.

TABLE 3 Competitive binding of anti-H protein MAbs against SLAM[a]

MAb	Antigenic site	Competitive activity[b]
2F4	*vii*	++
B5	*I*	++
E128	*II*	++
E103	*vi*	−

[a] The detailed procedure has been previously reported (12).
[b] ++, complete inhibition; −, no inhibition.

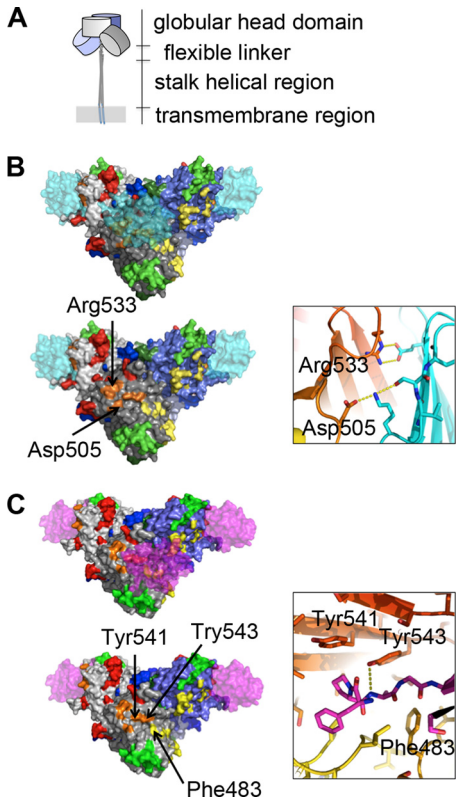

FIG 1 Location of epitope *vii* on the H protein tetrameric structure. (A) Diagram of an H protein tetramer (a dimer of H protein dimers). The four H protein molecules are shown in dark gray, light gray, dark purple, and light purple. (B) Location of SLAM-interacting residues, which constitute epitope *vii* on the tetrameric H protein structure in complex with SLAM (19). SLAM is shown in translucent cyan. SLAM interacting with the gray H protein is deleted in the structure shown at the bottom. A detailed view in a ribbon and stick diagram is shown in the box. SLAM is shown in cyan, and H protein is shown in orange or yellow. (C) Location of nectin4-interacting residues, which constitute epitope *vii*. The tetrameric structure was reconstructed using data by Hashiguchi et al. (19) and Zhang et al. (18). Nectin4 is shown in translucent magenta. Nectin4 interacting with the gray H protein is deleted in the structure shown at the bottom. A detailed view in a ribbon and stick diagram is shown in the box. Nectin4 is shown in magenta, and H protein is shown in orange or yellow. (B and C) The figures were produced using PyMOL (Schrödinger; http://www.pymol.org). The amino acid residues demonstrated or suggested to constitute portions of epitopes are shown in colors as follows: residues on beta-sheets 1, 2, 3, 4, 5, and 6 (19) are shown in blue, dark green, light green, yellow, orange, and red, respectively.

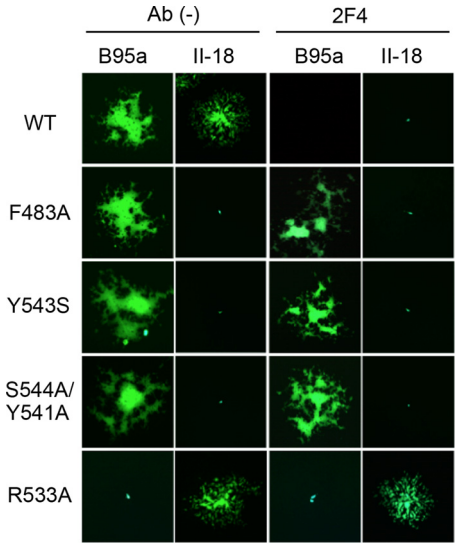

FIG 2 Effects of specific substitutions on virus infectivity and MAb neutralization. EGFP autofluorescence in MV-infected SLAM$^+$ B95a cells and nectin4$^+$ II-18 cells is shown. B95a and II-18 cells were infected with rMVs containing F483A, Y543S, S544A/Y541S, and R533A substitutions in the presence or absence of MAb 2F4. The panels show representative images observed using a fluorescence microscope.

from neutralization by 2F4 is penalized by loss of affinity for one of the principal MV receptors.

Apparently, a previously reported MAb, I-41, also recognizes this *vii* epitope (22), since a substitution at position 552 (the phenylalanine at this position is predicted to interact with SLAM [19]) allowed MV to escape from neutralization. Another previous study implied that MAb 80-II-B2 recognizes a region containing residues 505 and 506 within this epitope (23). The H protein of the CAM-70 MV vaccine strain features a mutation at position 505. Accordingly, the CAM-70 vaccine strain does not react with 80-II-B2 and binds to SLAM inefficiently (23, 24). Previous competitive binding studies revealed that MAb 16-DE6 recognizes the same antigenic site as I-41 (25), and that an 16-DE6 escape mutant contained, among other changes, an arginine-to-glycine substitution at position 533 (R533G) (22). Previously characterized MAbs

TABLE 4 Neutralizing titers against recombinant MVs possessing the IC-H protein with various amino acid substitutions

Cell line	Mutation	Infectivity	Neutralizing titer[a]			
			B5 (*I*)	E81 (*I*)	E103 (*vi*)	2F4 (*vii*)
B95a	Wild type (−)	+	7,536	1,727	10,231	22,141
	F483A	+	3,768	1,727	10,231	<346
	Y543S	+	1,884	1,727	2,558	692
	D505S	−	NA	NA	NA	NA
	R533A	−	NA	NA	NA	NA
II-18	Wild type (−)	+	3,764	27,631	20,462	11,070
	F483A	−	NA	NA	NA	NA
	Y543A	−	NA	NA	NA	NA
	D505S	+	1,884	13,815	20,462	<5
	R533A	+	1,884	27,631	20,462	173

[a] Data represent neutralizing titers for 1 mg/ml of immunoglobulin. NA, not applicable.

B2 and BH26 similarly recognize this epitope (6, 25, 26), which appears to be a premier neutralizing epitope for the H protein. Residues located in this epitope may be involved in SLAM-binding activity (6, 26). Therefore, this region probably corresponds to epitope *vii* identified in our study.

In summary, the present data demonstrate that both the SLAM- and nectin4-interacting residues themselves constitute a CNE. Since these residues are critical for interaction with either SLAM or nectin4, viral escape from neutralization has to be associated with loss of the receptor-binding activity. Since efficient MV spreading mandates interactions of the H protein with these two discrete proteinaceous receptors, SLAM and nectin4 (4), residues within this domain are likely to be under high selective pressure to maintain their molecular identity, and contribute to the sustainability of the single-serotype nature of MV.

ACKNOWLEDGMENTS

We thank T.A. Sato, Y. Yanagi, and T. Seya for providing MAbs, N. Ito and M. Sugiyama for providing BHK/T7-9 cells, and K. Taira for providing an MV isolate. We also thank all the members of Department of Virology 3, NIID, Japan, for technical support.

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