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Amino Acid Substitution(s) in the Stem-Anchor Region of Langat Virus Envelope Protein Attenuates Mouse Neurovirulence

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The identification of variants that are unable to bind membrane receptor preparations (MRPs) has previously been shown to select attenuated yellow fever and Japanese encephalitis viruses. In this study, this methodology has been extended to the tick-borne serocomplex of flaviviruses. Langat (LGT) virus strain TP21 was bound to mouse or human brain MRPs and viruses that escaped binding were isolated and characterized. In addition, variant viruses escaping neutralization by the monoclonal antibody (MAb) 9F9 were also isolated. All of the variant viruses were attenuated for mouse neurovirulence (≥13-fold). Sequence analysis of the prM/E region of the variant viruses identified mutations within the stem-anchor region of the E protein in variants isolated following incubation with mouse or human brain MRPs at a pH ≥ 7.0. The MAb 9F9 variants and MRP variants isolated at pH 5.0, which should induce a conformational shift in the viral E protein, had nearly identical mutations in the prM/M protein immediately N-terminal to the prM/E cleavage site. MAb 9F9 neutralized none of the variant viruses and hemagglutination inhibition assays suggest that the variant virus surface proteins have slightly different conformations compared to the parental virus. These data support previous work indicating that the stem-anchor region of the E protein is important to the surface architecture of the tick-borne flaviviruses. In addition, this study demonstrates that the M protein is at least partially solvent accessible on the virion surface and that the M protein plays a role in maintaining the conformation of the M/E surface complex.

Key Words: stem-anchor region; Langat; membrane receptor; attenuation.

INTRODUCTION

The genus *Flavivirus* in the family *Flaviviridae* consists of small enveloped viruses with a single-stranded, positive-sense RNA genome. Several members of this genus, such as the dengue viruses (DEN1–4), Japanese encephalitis (JE) virus, yellow fever (YF) virus, and the tick-borne encephalitis (TBE) serocomplex of viruses, are arthropod-borne human pathogens. The flavivirus genome is approximately 11 kb in length and encodes three structural proteins [capsid (C), membrane (M), and envelope (E)] and seven nonstructural proteins. The M protein is initially expressed as a pre-M (prM) protein in immature virus particles, but is cleaved by furin (Stadler *et al.,* 1997) during viral assembly to generate the mature virus particle. The flaviviruses are internalized via receptor-mediated endocytosis followed by fusion of the viral membrane with the endosomal membrane (Gollins and Porterfield, 1985). The E protein of flaviviruses is a major determinant of virulence since it plays a critical role in mediation of receptor binding and membrane fusion (McMinn, 1997).

Langat (LGT) virus was originally isolated from a pool of ticks in Malaya and is assigned to the TBE virus serocomplex by serological (Monath and Heinz, 1996) and genetic (Ecker *et al.,* 1999) analyses. Compared with other viruses in the TBE serogroup, LGT virus has a very low virulence in humans (Monath and Heinz, 1996). Langat virus was investigated as an experimental live vaccine against TBE, but a high rate of central nervous system complications was observed among the vaccinees (Mayer, 1975; Pletnev *et al.,* 2000).

The first step of flavivirus infection is the binding of the viral attachment protein to a host cell receptor. This initial step is also a critical determinant of the cell/tissue tropism of the virus (Dimmock and Primrose, 1994; Lentz, 1990; Marsh and Helenius, 1989). Although there is little direct evidence regarding the molecular basis of binding of flaviviruses to host cells, it has previously been shown that JE viruses, selected as mouse brain membrane receptor preparation (MRP) escape variants, were attenuated for mice in both neurovirulence and neuroinvasiveness (Ni and Barrett, 1998; Ni *et al.,* 2000). These data demonstrate that a novel methodology has been developed that can be utilized to isolate and identify specific regions within the viral structural protein that may be involved in binding to the host cell receptor.

In this study, LGT virus was used as a model system for tick-borne flaviviruses to investigate the interaction between the viral attachment protein and the host cell

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TABLE 1

Confirmation That LGT TP21 Virus Mouse and Human Brain MRP^R Variants and MAb^R 9F9 Variants Do Not Bind to MRPs

	Virus strain (variant isolation pH)	Infectivity (log_{10} pfu/ml)		Binding index
		Virus + buffer	Virus + MRP[a]	
Parental	TP21 (pH 7.6)	7.3	4.0	3.3
	TP21 (pH 7.4)	7.3	4.3[b]	3.0
MRP^R variants	MS MRP^R I (pH 7.6)	7.0	6.5	0.5
	MS MRP^R II (pH 7.6)	7.1	6.7	0.4
	MS MRP^R III (pH 5.0)	6.0	5.8	0.2
	MS MRP^R IV (pH 7.0)	6.5[b]	6.1	0.4
	HBG MRP^R (pH 7.4)	6.0	6.0	0.0
MAb 9F9 variants	9F9^R I	6.5 (pH 7.6)	6.5	0.0
	9F9^R II	6.6 (pH 7.6)	6.5	0.1

[a] All variants were tested for MRP binding against mouse brain MRP unless noted.
[b] Variant was tested for MRP binding against human brain grey matter MRP.

receptor. The objectives of this study were to isolate variant LGT viruses that do not bind to mouse (MS) or human brain (HBG) membrane receptor preparations (MRP^R) or do not bind to LGT virus structural protein-specific monoclonal antibodies (MAb^R). These viruses were subsequently evaluated for attenuation of neurovirulence and neuroinvasiveness in mice. The viruses were also screened against a panel of MAbs for hemagglutination inhibition and neutralization activity that varied from the parental virus. Genetic analysis of the structural proteins of viral genome of the variant viruses was carried out to identify mutations that might be responsible for the variant phenotype.

RESULTS

Generation of LGT MRP^R and MAb^R 9F9 variants

Langat virus strain TP21 was passaged once in Vero cells following acquisition from the WHO World Arbovirus Reference Collection to generate a working parental stock of virus. The TP21 virus was incubated with an excess of mouse or human brain MRP to generate MRP^R escape variants. MRP binding was carried out at either pH 5.0, 7.0, or 7.6 to determine the role that pH might play in the ability of virus to bind MRP. Virus that did not bind to the MRP was titrated to determine the total loss of infectivity due to virus binding to MRPs. Initial binding of the parental virus to MRP showed a significant loss in infectivity in the resulting unbound virus supernatants. Langat virus TP21 binding to either mouse or human brain MRP had 3.0 log_{10} and 3.3 log_{10} reductions in infectivity, respectively (Table 1). Similarly, monoclonal antibody neutralization-resistant escape variants (MAb^R) were selected using MAb 9F9. To demonstrate that true variant viruses were produced from the initial binding to MRP or MAb, the variant viruses were again incubated with MRP or MAb and the difference in infectivity determined. The MRP^R and MAb^R 9F9 escape variants had very little reduction in the viral plaque titer following secondary binding and are, thus, true escape variants. Interestingly, the MAb^R variants were also unable to bind MS-MRP[2] (Table 1). The pH at which the initial variants were selected did not seem to affect the ability of the variants to bind to MRP when the secondary binding was performed at pH 7.6.

TABLE 2

Comparison of Monoclonal Antibody Neutralization Tests with LGT TP21 Parent and its MRP^R Variants

MAb	TP21	MS pH 5.0	MS pH 7.0	MS pH 7.6	HBG pH 7.4
13A10	−	−	−	+	+
11H10	+	+	+	+	+
11H12	+	+	+	+	+
9F9	+	−	−	−	−

Note. +, Neutralization. −, No neutralization.

Neutralization analysis of LGT MRP^R variants

The LGT virus MRP^R variants were tested against a panel of MAbs that have been suggested to bind to the prM or E protein of LGT virus (Iacono-Connors *et al.,* 1996). Only three of these MAbs (9F9, 11H10, and 11H12) were found to neutralize the parental TP21 virus (Table 2). Although MAbs 11H10 and 11H12 were able to neutralize all four of the MRP^R pH variants, the MAb 9F9 neutralized the parental virus, but none of the variant viruses. In addition, the MAb 13A10 neutralized the pH

[2] Abbreviations: MS-MRP, mouse brain MRP; HBG-MRP, human brain gray matter MRP; MRP^R, MRP escape variant; MAb^R, monoclonal antibody escape variant; MS-MRP^R I, pH 7.6 variant; MS-MRP^R II, pH 7.6 variant; MS-MRP^R III, pH 5.0 variant; MS-MRP^R IV, pH 7.6 variant.

TABLE 3

Hemagglutination Inhibition Studies Using a Panel of LGT Virus-Specific Monoclonal Antibodies

		LGT virus variants						
Mab	TP21	MRP^R I	MRP^R II	MRP^R III	MRP^R IV	HBG MRP^R	9F9I	9F9II
Group 1								
8H1	<10	None	<10	<10	10	None	<10	<10
13A10	10		<10	<10	10		10	<10
1B10	10		<10	<10	10		10	<10
Group 2								
11H10	10		20	10	10		160	10
11H12	10		80	10	20		160	10
2C7	2560		1280	640	1280		1280	1280
9E3	2560		2560	1280	2560		5120	2560
Group 3								
9E7	2560		2560	640	2560		2560	1280
9F9	320		2560	640	640		1280	1280
10E8	2560		2560	1280	2560		5120	1280
9F12	2560		5120	1280	2560		5120	2560
Titer (pfu/ml)	9×10^6	1×10^8	1.1×10^8	1.6×10^6	4×10^6		1.6×10^6	6×10^6

Note. None, no detectable hemagglutination activity.
All virus titers and HA titers were determined from identical lots off virus harvested 3 dpi.

7.6 MS-MRP^R and HBG-MRP^R (pH 7.4) variants, but not the parental virus, the pH 5.0, or the pH 7.0 MS-MRP^R variant viruses.

Hemagglutination inhibition tests

The flavivirus E protein is believed to be the principal antigen associated with the hemagglutination of these viruses. The panel of MAbs described above has been suggested to bind to the E or M protein of LGT virus (Iacono-Connors *et al.,* 1996). To examine the ability of the MAbs to block hemagglutination of the parental and variant viruses, hemagglutination inhibition (HI) tests were carried out. These data demonstrate that, for this panel of MAbs, there were few differences between the parental and variant viruses. Two of the MAbs, however, had a significant difference in HI tests. The MAbs 11H10 and 11H12 had a HI titer of about 10 against the parental virus, the pH mutant viruses, and one of the 9F9 variants (9F9 II) (Table 3). Against the second 9F9 variant (9F9 I), a 16-fold increase in HI activity was found with MAbs 11H12 and 11H10. When assayed against sucrose-acetone extracted LGT viral antigen, 11H10 and 11H12 MAbs exhibited a 128-fold increase in HI activity compared to the parental virus. MS-MRP^R I (pH 7.6) and HBG-MRP^R (pH 7.4) variants had no HA activity. The viruses do not have homologous mutations (see below) nor do either have mutations within the ectodomain of the E protein that might confer the loss of HA activity. The reason that these two viruses lost HA activity, despite having reasonable viral titers, is unclear, but may be due to conformational changes in the E protein.

Mouse pathogenicity of wild-type and escape variant viruses

The LD_{50} of the parental TP21 virus was initially assayed via the intraperitoneal (ip) route of inoculation to examine viral neuroinvasiveness. This method demonstrated that the ip LD_{50} of the parental virus was greater than 5000 PFU. This result indicated that a study of neuroinvasiveness of the variant viruses was not practical as these viruses were expected to be attenuated in mice. Alternatively, the neurovirulence of the parental and variant viruses was determined by intracerebral (ic) inoculation of 3- to 4-week-old NIH Swiss outbred mice. The parental TP21 virus had an ic LD_{50} of 1.0 PFU, while all of the variant viruses were attenuated (Table 4). The MS MRP^R III (pH 5.0) variant was the least attenuated (13-fold), while the MS MRP^R IV (pH 7.0) was the most attenuated (16000-fold) among the MRP^R variants. The two MAb^R 9F9 variants were both approximately 30-fold attenuated.

Nucleotide and amino acid analysis of variant LGT virus

Nucleotide and amino acid analysis of the M and E genes of parental TP21 and variant LGT viruses was carried out to determine the potential genetic differences responsible for the variant phenotype. Analysis of the MS-MRP^R and HBG-MRP^R variants identified mutations within the stem-anchor region of the E protein but none in the ectodomain of the protein (Table 5). The HBG-MRP^R variant had a mutation at E-416 (L $\rightarrow$ A). The MS-MRP^R IV pH 7.0 variant had an N $\rightarrow$ K change at

TABLE 4

Mouse Neurovirulence Following Intracerebral Inoculation of Parental LGT TP21 Virus, Mouse Brain, and Human Brain Membrane Receptor Preparation Escape (MRP^R) Variants and MAb^R Variants

Virus		Route of Inoculation: Intracerebral (pfu/LD_{50})	Route of Inoculation: Intraperitoneal (pfu/LD_{50})
Parent	TP21	1	>5000
MS variants	MRP^R I (pH 7.6)	1259	ND
	MRP^R II (pH 7.6)	1259	ND
	MRP^R III (pH 5.0)	13	ND
	MRP^R IV (pH 7.0)	16,000	ND
HBG variant	MRP^R (pH 7.4)	160	ND
MAb^R variants	$9F9^R$ I	32	ND
	$9F9^R$ II	30	ND

Note. HBG, human brain gray matter; MAb, monoclonal antibody; ND; not done.

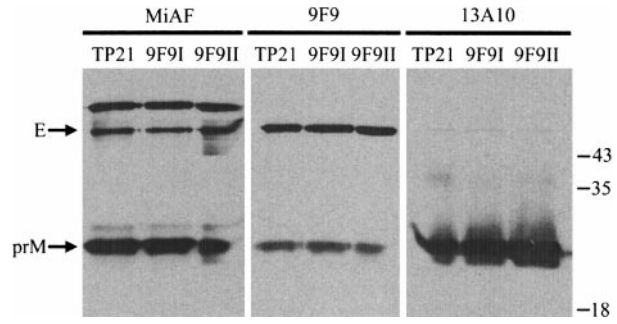

FIG. 1. Western blot analysis of nonreduced Vero cell lysates of LGT virus-infected cells. Cells were infected with wild-type (TP21) virus or 9F9 MAb^R escape variant virus (9F9I and 9F9II). The cell lysates were probed with LGT virus-specific mouse immune ascitic fluid (MIAF) or the MAb 9F9 or 13A10. E and prM proteins are indicated with arrows and the appropriate molecular size standards are also indicated (in kDa).

E-473. This mutation converts an uncharged residue to a charged residue, thus altering the ionic characteristics of that region of the protein. The MS-MRP IV (pH 7.0) variant also had a V → A change at E-440. The MS-MRP^R I and II (pH 7.6) viruses each had an identical mutation at E-438 converting H → Y. Interestingly, the reverse mutation (Y → H) was seen at residue 74 of the M protein (M-74) in the MS-MRP^R III (pH 5.0) virus and both of the 9F9 MAb^R variant viruses. The 9F9 II virus has an additional mutation (V → A) at M-71.

Western blot analysis

Whole-cell lysates from Vero cells infected with LGT virus TP21 and the two 9F9 MAb^R escape mutants were run in nonreducing SDS–PAGE gels and transferred to nitrocellulose for probing in Western blots. Lysates probed with LGT virus-specific mouse immune ascitic fluid (MIAF) detected proteins at the appropriate size for prM, E protein, and NS1 (Fig. 1). When probed with the MAb 9F9, the E-protein band was detected in addition to the prM, confirming previous suggestions by Iacono-Connors *et al.* (1996) that this MAb recognized epitopes on both proteins. Interestingly, there was no apparent difference between the wild-type virus and either of the MAb^R variants, perhaps demonstrating a degree of sequence dependence in the antibody binding epitope. When the lysates were probed with MAb 13A10, only the prM was detected, but with a much higher intensity than MAb 9F9, indicating that the 13A10 epitope was apparently exclusive to prM protein. When run under reducing conditions, the MIAF and MAb 9F9 detected no proteins (data not shown). In addition, under reducing conditions MAb 13A10 bound to a protein of the appropriate size for E protein. Viral protein was not detected in uninfected cell lysates run under either reducing or nonreducing when probed with either MAbs or MIAF (data not shown). These data demonstrate that the epitopes recognized by the MIAF and the two MAbs are conformationally dependent.

DISCUSSION

The principal mechanism utilized by enveloped viruses to facilitate viral entry employs some variation of membrane fusion. In the case of the flaviviruses, this mechanism is thought to proceed via a receptor-medi-

TABLE 5

Comparison of Amino Acid Changes In the M and E Protein Sequence of LGT Virus Strain TP21 and Its MRP^R and MAb^R 9F9 Variant Viruses

AA position	Parental TP21	MS MRP^R variants: pH 7.6	MS MRP^R variants: pH 7.0	MS MRP^R variants: pH 5.0	HBG variant	MAb^R variants: 9F9 I	MAb^R variants: 9F9 II
M-71	V	V	V	V	V	V	A
M-74	Y	Y	Y	H	Y	H	H
E-416	L	L	L	L	A	L	L
E-438	H	Y	H	H	H	H	H
E-440	V	V	A	V	V	V	V
E-473	N	N	K	N	N	N	N

ated event in which the virus interacts with an, as yet unidentified, receptor on the host cell surface. Upon binding, the virus-receptor complex is internalized and packaged into endosomes. The internal pH of the endosome is eventually decreased either by the function of a proton pump or fusion with a lysosome. The decreased pH causes an irreversible conformational shift in the flavivirus E protein which is thought to expose a fusion peptide within the E protein that penetrates the endosomal membrane, resulting in the release of the virus particle into the cytosol. Stiasny *et al.* (1996) reported that the pH change causes the E protein to undergo a two-step process that converts the E protein dimers into trimers. This mechanism requires the stem-anchor region of the E protein. Structural analysis of the stem-anchor region of TBE virus suggests that it contains amphipathic helices and conserved structural elements that are required for proper internalization of the virus (Allison *et al.,* 1999). Previous work examining JE MRP^R escape variants identified mutations in both the E protein ectodomain and the stem-anchor regions that contribute to an attenuation of virulence (Ni and Barrett, 1998). Clearly the role of the stem-anchor region of the E protein is not simply to support the E protein ectodomain, but rather, it plays a vital role in viral entry and maintenance of E protein complexes on the virion surface.

In the current study, mouse (MS) and human brain (HBG)-MRP^R escape variant viruses were identified and sequenced through the prM/E coding region. To examine the role of pH on the ability of LGT virus to bind to MRP, variant viruses were selected at pH 5.0 and 7.6 (HBG pH 7.4) as well as 7.0. All of the variant viruses were attenuated in neurovirulence in mice when compared to the parental virus. In addition, two variant viruses that escaped neutralization by the MAb 9F9 (Iacono-Connors *et al.,* 1996) were also identified and found to be attenuated in mice.

Two of the MS-MRP^R escape variant viruses as well as the HBG-MRP^R variant had mutations in the stem-anchor region of the E protein, while none of the viruses were found to have mutations in the ectodomain of the E protein. Interestingly, these variants were all generated at pH 7.0 or higher. The MS-MRP^R escape variants had mutations in regions previously described as being important to the formation of the heteromeric prM/E complex on the virion surface (Allison *et al.,* 1999). The HBG escape variant had a single change (L → A) at residue E-416, which is immediately adjacent to a putative helical region previously defined as required for the pH-induced conformational shift of the related TBE virus (Allison *et al.,* 1999). Though this mutation could be considered conservative, the increased conformational freedom allowed by the alanine residue compared to the leucine could alter the local conformation in the required helix. The mutations found in the pH 7.0 and 7.6 MS-MRP^R variants were chemically more significant than that of the HBG variant. The pH 7.6 variants had an H → Y mutation at E-438. This mutation not only alters the spatial characteristics of the residue, but may also affect the hydrophilicity and hydrogen-bonding capacity of this position. This mutation could clearly alter the ability of this region to interact with another region of the E protein or with another component of the heteromer. The pH 7.6 variant viruses are also neutralized by the MAb 13A10, suggesting that the H → Y mutation alters the topography of the E protein sufficiently to allow 13A10 to bind to M protein (Fig. 1) and block, directly or indirectly, virus interaction with the host cell receptor. The pH 7.0 variant had two changes, the first of which (E-440 V → A) probably has less influence on protein interactions than the change found at E-473 (N → K), which converts an uncharged residue to a charged residue.

The pH 7.0 and 7.6 variant viruses were attenuated approximately 16,000-fold, suggesting that the changes found in the virus significantly affect the formation of the virus surface complex of prM (M)/E. To more closely examine the mutations within the stem-anchor region of the E protein, wild-type and variant LGT virus amino acid sequences were compared to those of wild-type and attenuated derivative YF and JE viruses with mutations in the stem-anchor region (Fig. 2). These mutations were mapped to previously defined regions of functional significance within the stem-anchor region (Allison *et al.,* 1999). Mutations in the attenuated variants occur within all three of the proposed stem-anchor functional domains. No particular residue appears to have a higher rate of mutation frequency than other residues, although two JE MRP^R escape variants and YF 17D-204 have mutations at adjacent positions in helix 1.

The pH 5.0 MS-MRP^R escape variant and the two 9F9 MAb^R escape variants had mutations that were uniquely different from those found in the other LGT variant viruses. These viruses each had mutations at the C-terminus of the viral M protein immediately adjacent to the cleavage site between the M and E proteins. Each of these viruses had a mutation at M-74 (Y → H), one amino acid N-terminal of the proposed prM/E cleavage site (Fig. 3). The Y → H mutation alters the structural characteristics of the residue and may alter the MAb recognition epitope and protease cleavage efficiency. The finding of the identical mutation in the MS-MRP^R pH 5.0 variant suggests that the Y → H mutation in the M protein limits the conformational shift that normally occurs in the viral E protein, thus facilitating virus entry under conditions that would not normally be suitable for virus:host cell interactions. The low pH condition under which the variant was isolated induces a conformational shift in the E protein that facilitates viral entry and presumably eliminates virus:receptor interaction potential. The escape variant is clearly still able to infect Vero cells, bind the MRP, and thus, presumably, attach to its receptor. These data suggest that M-74 lies within a part of the M protein

	<--Helix 1-->	<----Helix 2 ----->	<------Transmembrane------>
AA residue - - - - >	398 410	431 449	449 474
LGT TP21	REFQKTKKGIERL	SSIGKAVHTVLGGAFNSIF	FGGVGFLPKLLLGVALAWLGLNMRNP
MS-MRP^R pH 7.6	.....	.....Y.....	.....
MS-MRP^R pH 7.0	.....	.....A.....	.....K.
YF Asibi	KL.TQ.M..A...	T.V..GI...F.S..Q.GL	...LNWIT.VIM.AV.I.V.I.T..M
17D 204	.....V...	.....	.....
FNV MS-MRP^R	.....	.....	.....R.....
JE SA14	KA.ST.L..AQR.	N.....Q.F....RTL.	...MSWWITQG.M.AL.L.M.V.A.DR
SA14-14-2	.....	....R.....	.....
P3 MS-MRP^R	.....S.....	.....	.....
P3 MS-MRP^R	.....S.....	...V.....	.....

FIG. 2. Sequence alignment of the stem-anchor region of LGT, YF, and JE viruses. Viruses included in this are LGT virus TP21 (Accession No. M73835), YF Asibi (Accession No. S71025), 17D-204 (Accession No. X15062), FNV MB-MRP^R (Ni *et al.,* 2000), JE SA-14 (Accession No. D90914), JE SA-14-14-2 (Accession No. D90195), and JE MRP^R (Ni and Barrett, 1998). Homologous residues are indicated by (·) and changes are indicated. The YF Asibi and JE SA14 sequences were compared to the LGT TP21 amino acid sequence and any changes noted. The changes in the YF and JE subsets are in comparison to the parental (Asibi or SA14) strain.

that is affected by the pH-induced conformational shift of the E protein. An alternative hypothesis is that the mutation at position M-74 affects the cleavage efficiency between the M and E proteins. However, it is expected that such an effect on virus processing and assembly would be detected in Western blots and in the viral titer. The MAb^R variant and wild-type viruses have similar infectivity titers at 3 dpi (Table 3), indicating that viral propagation is not the obvious consequence of the M-74 mutation. In addition, Western blots of whole-cell lysates of LGT virus-infected Vero cells suggest that the variant viruses do not have differential cleavage from the wild-type virus (Fig. 1).

Initial studies with the MAb 9F9 suggest that the MAb binds to the viral E protein but that the prM is required for

	prM/M	E
LGT	...LVPAYA	∇ SRCTHL...
TBEV	...L**A**P**V**YA	∇ SRCTHL...
MAb^R 9F9 I	...LVPA**H**A	∇ SRCTHL...
MAb^R 9F9 II	...L**A**PA**H**A	∇ SRCTHL...
MRP^R III	...LVPA**H**A	∇ SRCTHL...

FIG. 3. Comparison of the amino acid sequences through the prM/E cleavage site. Viruses included are TBE (Accession No. L40361) and LGT TP21 (Accession No. M73835) in addition to the MAb^R and MS-MRP^R (pH 5.0) variant viruses described in this study. Mutations that vary from the LGT virus are underlined and in bold.

epitope recognition (Iacono-Connors *et al.,* 1996). Western blot analysis of infected cell lysates suggests that the MAb 9F9 recognizes both the E protein and the prM under nonreducing conditions (Fig. 1). MAb 9F9, however, does not recognize M protein in Western blots, suggesting that M protein epitope presentation sufficient for virus neutralization is achieved only after the prM is cleaved and the M and E proteins are complexed. The fact that MAb 9F9 recognizes neither E nor prM under reducing conditions (data not shown) demonstrates that the 9F9 epitope is a conformation-specific epitope rather than a sequence-specific epitope. This being said, it is evident that the mutation at M-74 that is found in the MAb^R escape mutants induces a conformational shift in the prM/E region that affects the ability of MAb 9F9 to bind to intact virus, thus presumably preventing infection. Similarly, the two MAb^R variants do not bind to MS-MRPs (Table 1). The data presented here support the hypothesis by Iacono-Connors *et al.* (1996) that the epitope recognized by MAb 9F9 is one that encompasses both the M and the E proteins, suggesting that they are closely associated in the region of the C-terminus of the M protein.

The data presented in this study reiterate the importance of the E protein stem-anchor region to the viability and infectivity of the flaviviruses. In addition, the identification of the prM/M-E cleavage site as being solvent exposed and accessible to neutralizing antibodies provides clues as to structural organization of the M protein in relation to the E protein. These data contribute to the understanding of the M/E heteromeric complex and

demonstrate the importance of all component parts beyond the E-protein ectodomain.

MATERIALS AND METHODS

All materials were purchased from the Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Viruses and cells

Wild-type LGT virus strain TP21 virus was obtained from the World Arbovirus Reference Center at UTMB. Monkey kidney (Vero and LLC-MK2) cells were grown at 37°C with 5% CO_2 in Eagle's minimal essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2 mM L-glutamine, and antibiotics (0.1 mg/ml neomycin and 0.2 mg/ml streptomycin). For propagation of virus, the cell lines were infected at an m.o.i. of 0.1 for 1 h at 37°C and then grown in EMEM containing 2% HI-FBS. Tissue-culture supernatant containing virus was harvested when approximately 90% CPE was observed. Following clarification by centrifugation, the viral stocks were stored at −70°C in 1-ml aliquots.

Plaque assays

Cells were seeded into six-well plates and grown for at least 24 h. The cells in successive wells were infected with 100 μl of virus in 10-fold serial dilutions. Infection was carried out at 37°C for 30 min. Following infection, the wells were overlaid with EMEM containing 2% HI-FBS and 1% agar. The plates were incubated for 7–8 days and then overlaid as above, but also containing 2% neutral red (v/v) (3.3 mg/ml stock). The plaques were allowed to clear for 2–3 days at 37°C.

Mouse and human brain membrane receptor preparations

Mouse brains were collected from 3- to 4-week-old female BALB/c mice and a human brain from a 76-year-old female. The brain membrane receptor preparations were made based on the method described for neurotransmitter receptor binding assays (Middlemiss and Frozard, 1983). Briefly, the brains were rapidly dissected, weighed, and homogenized in Tris buffer (50 mM Tris, pH 7.6). The homogenate was centrifuged (35,600 *g* for 10 min); the pellet was resuspended in an equal volume of Tris buffer and the process repeated twice more. Between the second and third centrifugation, the homogenate was incubated at 37°C for 10 min. The final pellet was resuspended in a volume of Tris buffer (50 mM pH 7.6) to give a final protein concentration of approximately 20–40 mg wet brain/ml and stored at −70°C.

Generation of virus mouse and human brain MRP escape (MRP^R) variants

Prior to use, aliquots of frozen MRPs and virus stocks were removed from −70°C, thawed quickly at 37°C, and kept on ice. A 100-μl aliquot of neat virus stock was added to 900 μl of mouse or human brain MRPs, such that membrane receptors were in excess, and vortexed to mix. Control virus samples were prepared in the same way, but mixed with 900 μl of Tris buffer instead of MRPs. Samples were incubated at 37°C for 30 min on a rotating stand. Following incubation, the virus-MRP suspension was centrifuged at 13,000 rpm for 10 min to remove the membrane material and bound virus. Residual virus infectivity in the supernatant was determined by plaque assay. Viruses that escaped binding to MRPs were isolated by plaque picking and resuspended in EMEM containing 2% HI-FBS. The viruses were expanded by a single passage through Vero cells. The individual MRP^R variants were assayed for binding to fresh mouse or human brain MRPs. A lack of binding of the virus variants (i.e., no reduction in residual viral titer) to fresh mouse or human brain MRP confirmed that they were true MRP^R escape variants.

Generation of MAb 9F9 escape variants

To generate MAb^R escape variants, 900 μl of LGT virus (about 5×10^5 PFU) was mixed with 100 μl neat MAb 9F9. The mixture was incubated at 37°C for 30 min, and then the mixture was added to monolayers of Vero cells seeded into six-well plates after 10-fold serial dilution with PBS. The cells were infected and overlaid as described above (Plaque Assays). Plaques that appeared at low dilutions of MAb (e.g., neat, 1:10 dilution) were picked after 6–8 days and amplified in Vero cells. The variants were tested against 9F9 antibody a second time and were identified as MAb^R escape variants only if the antibody did not neutralize them.

Plaque-reduction neutralization assays

Assays were performed by mixing dilutions of MAb mouse ascitic fluids (MAbs) (Iacono-Connors *et al.,* 1996), diluted in Hanks' balanced salt solution (HBSS) containing 2% HI-FBS, with approximately 100 PFU of LGT virus. The mixture was incubated at 37°C for 30 min and then added to monolayers of Vero cells that had previously been seeded into six-well plates. After incubation for 30 min at 37°C, the monolayers were overlaid as described above (Plaque Assays).

Hemagglutination inhibition

Hemagglutination (HA) assays were carried out as previously described (Beaty *et al.,* 1995). Briefly, virus supernatants were titrated in twofold serial dilutions and assayed for the ability to agglutinate goose red blood

cells (RBCs) at a range of pH (6.0–7.0). The optimum pH and the appropriate virus dilution representing 4 HA units were determined (1 HA unit being equal to the highest dilution of virus that was able to completely agglutinate the RBCs). Hemagglutination inhibition (HI) assays were carried out using twofold serial dilutions of acetone-extracted LGT virus MAb (Iacono-Connors *et al.,* 1996), beginning with a dilution of 1:10. The endpoint of inhibition was determined as the highest dilution at which hemagglutination was blocked. All viruses were harvested at 3 dpi and assayed immediately for HA activity. Viral titers were determined after a single freeze–thaw cycle.

Mouse pathogenicity

Three- to four-week-old female NIH-Swiss outbred mice (Harlan Bioproducts, Indianapolis, IN) were inoculated with either 20 μl of virus diluted in PBS via the intracerebral route or 100 μl by the intraperitoneal route. Virulence studies utilized five mice per dose group and the LD_{50} of each virus strain was determined.

Sequencing and analysis of the LGT virus structural genes

The RT-PCR, cloning, and sequencing methods for flavivirus RNA have been previously described (Ni *et al.,* 1994). Computer analyses of nucleic acid data and deduced amino acid sequences were performed using the Wisconsin Package (Genetics Computer Group, Madison, WI) data analysis software.

Western blot analysis

Whole-cell lysates from virus-infected Vero cells were run in SDS–PAGE gels under nonreducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with TBST–milk (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 3% dry milk powder) and then probed with primary antibody diluted in TBST–milk. Secondary antibody was HRP-conjugated anti-mouse IgG (H+L) (Sigma, St. Louis, MO). Westerns were visualized using ECL chemiluminescence (Amersham/Pharmacia, Piscataway, NJ).

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