A detailed mutagenesis study of flavivirus cross-reactive epitopes using West Nile virus-like particles

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Human flavivirus infections elicit virus species-specific and cross-reactive immune responses. The flavivirus envelope (E) glycoprotein is the primary antigen inducing protective immunity; however, the presence of cross-reactive antibodies in human sera creates problems for serodiagnosis. Using a West Nile virus-like particle system, we performed mutagenesis across all three E protein functional domains to identify epitope determinants for a panel of monoclonal antibodies (mAbs) raised against different flaviviruses and exhibiting diverse patterns of cross-reactivity. Residues within the highly conserved fusion peptide were the only epitope determinants identified and were important not only for broadly cross-reactive mAbs recognizing all of the medically important flavivirus serocomplexes, but also for less-broad, complex-reactive mAbs. Moreover, different substitutions at specific fusion peptide residues produced highly variable effects on antibody reactivity and virus-like particle secretion. These results support and extend the conclusion that the fusion peptide region constitutes an immunodominant epitope stimulating antibodies with diverse patterns of cross-reactivity.

Flavivirus virions are approximately 500 Å (50 nm) in diameter and contain three structural proteins, capsid (C), premembrane/membrane (prM/M) and envelope (E) proteins (Kuhn et al., 2002; Mukhopadhyay et al., 2003). The flavivirus E glycoprotein is the primary antigen inducing protective immunity and it is essential for receptor binding and membrane fusion (Allison et al., 2001; Crill & Roehrig, 2001; Lindenbach & Rice, 2001). The E protein contains three structural and functional domains. Domain I is an eight-stranded β-barrel, containing two large insertion loops forming the elongated dimerization domain II and the highly conserved internal fusion peptide. Domain III has an Ig-like structure containing the primary receptor-binding motifs (Modis et al., 2003; Rey et al., 1995). Monoclonal antibody (mAb) studies demonstrate that domain I contains predominantly type-specific non-neutralizing (Nt) epitopes, domain II contains cross-reactive epitopes eliciting both Nt and non-Nt antibodies and domain III contains strongly Nt type-specific epitopes (Heinz et al., 1982; Roehrig, 2003; Roehrig et al., 1998).

Human flavivirus infections elicit both virus species-specific and flavivirus cross-reactive antibody responses inducing long-term virus-specific protection (Calisher et al., 1989; Kuno, 2003). However, flavivirus-induced cross-reactive serum antibodies offer only transient cross-protection and actually create a number of difficulties for the public health response to these viruses (Mackenzie et al., 2004; Sabin, 1952; Tesh et al., 2002). Serodiagnosis of secondary flavivirus infections in areas with multiple, co-circulating flaviviruses can be especially problematic, due to the inability to differentiate primary from secondary cross-reactive serum antibodies using currently available, wild-type (wt) viral antigens (Martin et al., 2002). This cross-reactivity problem decreases the efficiency and effectiveness of public health responses to flavivirus epidemics and interferes with important estimates of disease burden (Chang et al., 2004; Kuno, 2003). West Nile virus (WNV) was introduced into New York City in 1999 and has since spread epidemically across North America, creating serodiagnostic difficulty in differentiating WNV-infected patient sera from endemic St. Louis encephalitis virus (SLEV)-infected sera (Lanciotti et al., 1999; Martin et al., 2002). Using a WNV prM/E expression plasmid (Davis et al., 2001; Holmes et al., 2005) as a DNA template both for mutagenesis and for the transient expression of WNV virus-like particles (VLPs), we performed mutagenesis across the E protein to attempt to identify and to ablate cross-reactive E protein epitopes.

We previously published and successfully applied a procedural algorithm identifying flavivirus E protein group-reactive epitopes (epitopes stimulating antibodies
that recognize members of all four medically important flavivirus serocomplexes) using dengue virus serotype 2 (DENV-2) VLPs (Crill & Chang, 2004). With the identification of flavivirus group cross-reactive epitopes (CREs), we extended our focus here to the identification of flavivirus complex CREs in the Japanese encephalitis virus (JEV) serocomplex, which includes the medically important viruses JEV, WNV, SLEV and Murray Valley encephalitis virus. The identification of serocomplex CREs required altering a number of the previously utilized epitope identification criteria. Here we did not limit ourselves to examining residues within structural domain II, since published results indicate that some complex and subcomplex CREs are mapped onto domains I and III in addition to domain II (Roehrig, 2003). Amino acid conservation across the JEV complex was examined and (i) residues conserved across all member viruses of the complex were favoured; (ii) if residues were conserved within, but not across, the entire complex, then residues with shared identities between WNV and SLEV were favoured.

Using this structure-based design approach we identified 33 candidate CRE residues in WNV and selected 17 of these residues as highly probable flavivirus group-, complex- and subcomplex-CRE determinants (Supplementary Table S1, available in JGV Online; Table 1). Stability calculations (ddG) were determined with FOLD-X.

Table 1. mAb reactivity and relative secretion for wt WNV and E protein fusion peptide VLP mutants

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<th>VLP mutant</th>
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<th>4G2</th>
<th>6B6C-1</th>
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*CR, mAb cross-reactivity; poly, polyclonal MHIAF (undetermined cross-reactivity); grp, flavivirus group cross-reactive (all flaviviruses tested); comp +, supercomplex cross-reactive (JEV complex and YFV); comp, JEV complex cross-reactive; type, WNV (Kunjin) type-specific.

†Virus species used to immunize mice for production of mAbs. D2V, Dengue virus serotype-2; MVEV, Murray Valley encephalitis virus; Kun, WNV (Kunjin).

‡Standardized measurements of VLP secretion from transiently transformed CHO cells. Presented as percentage of the wt plasmid VLP secretion (arbitrarily set to 100%).

§We were not able to obtain enough of these VLPs to measure reactivity by these mAbs beyond 1:32 000 [log10(1/32 000)] = 4.5].
Mutations were introduced into the WNV E gene using a QuikChange Multi site-directed mutagenesis kit (Stratagene) and pCBWN as DNA template. The sequences of the mutagenic primers used are listed in Supplementary Table S1 (available in JGV Online). The pmR/E plasmid and transcriptional elements of all purified plasmids were sequenced entirely upon identification of the correct mutation. Automated DNA sequencing was performed using a CEQ 8000 genetic analysis system (Beckman Coulter) and analysed using CEQ 8000 (Beckman Coulter) and Lasergene (DNASTAR) software.

CHO-K1 cells (ATCC CCL 61) were grown at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium/F-12 nutrient mixture (D-MEM/F-12; GIBCO) supplemented with 10% heat-inactivated fetal bovine serum. CHO cells were electroporated with wt and mutated pCBWN using 10% heat-inactivated fetal bovine serum. CHO cells were recovered in 50 ml D-MEM/F-12, seeded into 150 cm² culture flasks for VLP expression and were unable to detect secreted VLPs from any of the Gly104 plasmid-transformed cells. Other constructs that secreted very low VLP levels were K118V (approx. 1% of wt), G106Y and L107G (4%) and L107Y (5%). Mutant plasmids that most dramatically increased VLP secretion relative to wt were L107R (160%), S276D (200%), R166Y (250%), G106R (300%) and the G106R/L107R and G106V/L107R double mutants (200 and 300% of wt respectively).

We did not observe a strong correlation between the predicted ddG of mutant E proteins and VLP secretion from cells transformed with these plasmids (Supplementary Table S1, Table 1). The noticeable exception was that ddG estimates for all possible substitutions at Gly104 were much higher than those for any other residue, and that we were unable to detect secreted VLP Ag from any of the four different Gly104 substitutions examined (Table 1). However, predicted stability and increased mutant VLP secretion did not extend to other E protein residues. The G104N/L107R double mutant was predicted to be more stable than the wt E protein (ddG = −0.16), yet we were unable to detect secreted VLPs from this plasmid. Similarly, the poorly secreting G106Y, L107V and K118V mutants all had ddG values <0.0, predicting increased stability relative to wt, whereas L107R, G106V/L107R and G106R/L107R all had positive ddG values, even though these plasmids secreted VLPs at levels of 200–300% of the wt plasmid (Table 1). We did not observe any correlations between VLP secretion from mutant plasmids and decreases or increases in localized hydrogen bonding by the specific substitutions in the WNV E protein structural model (data not shown). In DENV-2, a G104H E protein substitution also prevented VLP secretion and plasmid-transformed cells exhibited mAb reactivities (detected by immunofluorescence) consistent with the disruption of protein conformation (Crill & Chang, 2004). We have also observed dramatic reductions in VLP secretion from SLEV and JEV pmR/E expression plasmids with Gly104 substitutions (unpublished results). Thus, although we do not know the mechanism preventing the secretion of Gly104 mutant VLPs, it appears to be a consistent phenomenon with substitutions at this highly conserved fusion peptide residue.

We assembled a panel of E protein-specific mAbs of variable cross-reactivity that were raised against different flaviviruses (Table 1). The reactivity patterns of the mAbs included flavivirus group cross-reactive mAbs recognizing viruses from all four pathogenic flavivirus serocomplexes (of JEV, DENV, Yellow fever virus (YFV) and Tick-borne encephalitis virus), a supercomplex cross-reactive mAb recognizing all members in the JEV complex and WNV-specific mAbs. Secreted Ag was concentrated from Ag-ELISA positive tissue-culture fluids by centrifugation overnight in a Beckman type 19 rotor at 19,000 r.p.m. and resuspended in TN buffer (50 mM Tris, 100 mM NaCl, pH 7.5) to 1/100
concentrations producing an mAb end-point determination with MHIAF by selecting Ag the original volume. Concentrated Ag was standardized for mAb end-point determination with MHIAF by selecting Ag concentrations producing an $A_{50\,nm} = 1.0$ using a Synergy HT microplate reader and KC4 microplate data analysis software (both BioTek). Standardized Ag concentrations were then used for mAb end-point determination in Ag-ELISA. This Ag-ELISA protocol is the same as that used to measure VLP secretion, with the exception that the previously determined standardized Ag concentrations and varying mAb concentrations were used instead of polyclonal MHIAF. mAb end-point alterations were only considered significant when they differed by greater than fourfold (two dilutions) from the reactivity of wt pCBWN VLP.

Of the 17 potential CRE residues initially selected for mutagenesis (Supplementary Table S1), only the G106V and L107Y substitutions significantly reduced mAb reactivities (Table 1, Fig. 1). We did not measure mAb reactivity of the non-secreting G104N substitution and the remaining 14 substitutions did not alter the reactivity of any of the mAbs examined (Supplementary Table S1, Table 1). The lack of alterations in mAb reactivity for many of the prospective CRE substitutions suggests potential limitations of our CRE identification algorithm. However, the range of mAb reactivities observed for different individual substitutions introduced at Gly106 and Leu107 (Table 1) suggests that the individual substitutions chosen might not significantly influence the mAb binding kinetics, yet other substitutions could have. It is also possible that there are very few CREs that do not incorporate or are not associated with the fusion peptide region. The G106V substitution ablated the reactivity (reduced to below the limits of detection) of three out of five flavivirus group-reactive mAbs (4G2, 6B6C-1 and 4A1B-9) and of the supercomplex cross-reactive mAb 2B5B-3. The L107Y substitution similarly ablated the reactivity of two group-reactive mAbs (4G2 and 23-1) and of the same supercomplex-reactive mAb, thus the individual Gly106 and Leu107 substitutions examined together dramatically reduced the reactivity of four out of the five flavivirus group-reactive mAbs and one out of three supercomplex- and complex-reactive mAbs (Table 1). Neither of these substitutions altered the reactivity of either the WNV-specific mAbs or the WNV-specific polyclonal MHIAF. Based on the promising nature of these reductions in cross-reactive mAb recognition, and because of the lack of CRE determinants identified outside the fusion peptide region, we chose to examine the effects of multiple substitutions at fusion peptide residues Gly104, Gly106, Leu107 and combinations thereof to assess their effect both on VLP secretion and on mAb reactivity.

We introduced a total of four, four and five individual substitutions at each of fusion peptide residues Gly104, Gly106 and Leu107 respectively, and four double-mutant combinations of these substitutions. We observed dramatic variation both in VLP secretion levels and in mAb reactivity for different substitutions both within and between these three E protein residues (Table 1). None of the substitutions introduced for Gly104 secreted measurable VLPs and even when combined with the better-than-wt-secreting L107R substitution, VLP secretion from the G104N/L107R double mutant was below the limits of detection (Table 1). The four Gly106 substitutions produced plasmids secreting VLPs at levels ranging from 4 to 300 % of wt and the five substitutions examined for Leu107 secreted VLPs at levels ranging from 4 to 160 % of the wt pCBWN expression plasmid (Table 1).

The effect of the different Gly106 and Leu107 substitutions on mAb reactivity was highly diverse. Some mAbs lost
reactivity to mutants containing any substitution at either Gly106 or Leu107. All nine Gly106 and Leu107 substitutions produced VLPs lacking measurable reactivity by the DENV-2-raised group-reactive mAb 4G2 and all except G106A lost recognition by the SLEV-raised supercomplex-reactive mAb 2B5B-3. Conversely, none of the individual Gly106 or Leu107 substitutions altered the recognition of the JEV-raised group-reactive mAb 23-2 (Table 1). Other mAbs exhibited highly variable reactivity patterns dependent upon the specific substitution and position. For example, group-reactive mAb 6B6C-1 reactivity was ablated only by substitutions at Gly106, specifically G106V and G106Y, whereas other Gly106 substitutions and all Leu107 substitutions exhibited only minor reductions. This pattern of both Gly106 and Leu107 acting as epitope determinants of mAb 4G2 and only Gly106 affecting mAb 6B6C-1 was also observed for DENV-2 VLPs (Crill & Chang, 2004). Substitutions at Gly106 or Leu107 alone did not reduce the reactivity of either of the JEV complex-reactive mAbs. The one exception was the reduced reactivity of mAb 16 for G106Y VLP. Conspicuously, many of the Gly106 and Leu107 mutants exhibited increased reactivity for the two JEV complex mAbs (Table 1).

When Gly106 and Leu107 substitutions were combined into single VLPs, not only were mAb reductions from the individual substitutions additively combined, we also observed ‘non-additive effects’. For example, the G106V VLPs were non-reactive with mAb 6B6C-1 but exhibited normal reactivity with mAb 23-1, whereas the L107R VLP exhibited normal reactivity for 6B6C-1 and no measurable reactivity for mAb 23-1. However, the G106V/L107R VLP not only lost all measurable reactivity by both 6B6C-1 and 23-1, but also by mAb 23-2, the reactivity of which was not altered by either substitution alone (Table 1). These non-additive effects on mAb reactivity in the double mutants were even more pronounced with the G106R/L107H and G106R/L107R VLPs. Both of these constructs exhibited complete loss or dramatic reductions of reactivity to all eight cross-reactive mAbs in our panel including flavivirus group-, supercomplex- and JEV complex-reactive mAbs (Table 1).

These results both confirm and extend recent studies identifying flavivirus fusion peptide residues as broadly cross-reactive epitope determinants (Allison et al., 2001; Crill & Chang, 2004; Goncalvez et al., 2004; Stiasny et al., 2006; Zhang et al., 2006; unpublished results from SLEV). It is intriguing, yet not altogether surprising, that substitutions at Gly106 and Leu107 can act as epitope determinants of antibodies with such distinct cross-reactivity profiles. The surface area of conserved fusion peptide residues is much smaller than the typical Ab–Ag interface. Antibodies recognizing the flavivirus fusion peptide region are expected to incorporate less-conserved nearby residues into their binding footprint. Stiasny et al. (2006) recently emphasized this point to explain the observed heterogeneity of the binding patterns of broadly cross-reactive mAbs tested against different flaviviruses. The results presented here are consistent with the interpretation that variation in the virus-specific antigenic landscape surrounding strongly conserved fusion peptide residues can also elicit antibody responses exhibiting highly variable patterns of cross-reactivity.

The results presented above, and those of others (Allison et al., 2001; Crill & Chang, 2004; Goncalvez et al., 2004; Stiasny et al., 2006; Throsby et al., 2006; Zhang et al., 2006), demonstrate that the flavivirus fusion peptide region forms an important immunodominant epitope stimulating a diverse spectrum of cross-reactive antibodies. Insights and application of the knowledge gained from these studies will be critical for improving the efficiency of the public health response to flavivirus epidemics and for improving global estimates of flavivirus disease burden.

Acknowledgements

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References


