Flaviviruses form a genus in the family *Flaviviridae* (52) and comprise a number of important human pathogens such as yellow fever, dengue, Japanese encephalitis, West Nile, and tick-borne encephalitis (TBE) viruses (30). They are small, enveloped viruses with only three structural proteins, designated C (capsid), M (membrane), and E (envelope). The E protein is oriented parallel to the viral membrane and forms a head-to-tail homodimeric complex (Fig. 1A and B). The structure of the E ectodomain (soluble E [sE])—consisting of about 400 amino acids and lacking the 100 C-terminal amino acids (including the so-called stem and two transmembrane helices)—has been determined by X-ray crystallography for several flaviviruses (Fig. 1A) (25, 34, 36, 38, 44, 55). Both of the essential entry functions—receptor-binding and membrane fusion—after uptake by receptor-mediated endocytosis—are mediated by E, which is therefore the primary target for virus-neutralizing antibodies (11, 42, 43, 45).

As revealed by cryo-electron microscopy (cryo-EM), mature infectious virions have smooth surfaces, comparable to a golf ball (27, 37). Their envelopes are icosahedrally symmetric and consist of a closed shell of 180 E monomers that are arranged in a herringbone-like pattern of 30 rafts of three dimers each (Fig. 1C) (27). On the other hand, capsid-lacking subviral particles, which can be produced in recombinant form by the coexpression of prM and E, have a different symmetry, with 30 particles, which can be produced in recombinant form by the coexpression of prM and E, have a different symmetry, with 30

The peculiar organization of E in virions is reminiscent of the tight packing of capsid proteins in nonenveloped viruses, for which it was shown that the native antigenic structure is strongly dependent on the intact capsid structure and not completely represented by isolated forms of capsid proteins (1, 41, 53). Such modulations of antigenic structure may be due to conformational changes in the course of packaging the capsid proteins into virions and/or to the fact that antibody binding sites at the virion surface are composed of residues that come together only through the juxtaposition of capsid proteins or neighboring protein subunits. Even in the case of spiky viral envelope proteins, the dependence of certain epitopes on the quaternary organization of the envelope glycoproteins has been described (8, 47).

For flaviviruses, structural studies provide evidence for the considerable flexibility of E, especially at the junctions between the individual domains I, II, and III (DI, DII, and DIII) (7, 35, 55), suggesting that soluble forms may display differences in antigenic structure compared to those fixed in the closed envelope shell of whole virions. Furthermore, because of the tight packing of E at the virion surface, certain epitopes may be cryptic in the context of whole virus particles but accessible in soluble forms of E (40, 51).

Studies on the antigenic structure of flaviviruses have used different antigen preparations including virions, recombinant subviral particles (RSPs), and soluble forms and subunits of E (10, 15–17, 32, 39, 40, 46, 49, 51), but so far no systematic comparative analysis of E in different physical forms and quaternary arrangements has been conducted. It was therefore the objective of our study, using TBE virus as a model, to investigate possible structural and/or antigenic differences between (i) soluble dimeric forms of E, including C-terminally truncated sE and detergent-solubilized full-length E (Fig. 1A and
B); (ii) E in the context of whole virions (Fig. 1C); and (iii) E in the context of RSPs (Fig. 1D). For this purpose we used, and further characterized, a set of monoclonal antibodies (MAbs) directed to each of the three domains of E. All of these MAbs have neutralizing activity (17, 24) and therefore, by definition, react with infectious virions.

Through these analyses, we demonstrate that the reactivity of several MAbs is significantly dependent on the quaternary arrangement of E and differs between virions, RSPs, and/or sE dimers. We thus provide evidence for previously unrecognized structural factors that have an impact on the antigenicity of the flavivirus E protein.

MATERIALS AND METHODS

Production of TBE virus and sE dimers. TBE virus strain Neudoerfl (33) was grown in primary chicken embryo cells, harvested 48 h after infection, concentrated by ultracentrifugation, and purified by rate-zonal sucrose density gradient centrifugation and equilibrium sucrose density gradient centrifugation, as described previously (19). This procedure led to the complete separation of virions from subviral particles. sE dimers were produced by limited digestion of purified TBE virions with trypsin at 0°C and subsequent purification by anion exchange chromatography (21).

Mutagenesis and production of subviral particles (RSPs). For the generation of wild-type and mutant RSPs, we used the cDNA clone SV-PEwt (2) of TBE virus strain Neudoerfl, containing the prM and E genes flanked by parts of the C and NS1 genes, under the control of the simian virus 40 early promoter. Single-site mutations in E were introduced using a GeneTailor Site-Directed Mutagenesis System (Invitrogen). RSPs were produced by transfecting COS-1 cells (ATCC CRL 1650) with recombinant plasmids by electroporation (49). After 48 h, the RSPs in clarified cell culture supernatants were pelleted by ultracentrifugation. The pellet was resuspended in TAN buffer (50 mM triethanolamine, 100 mM NaCl, pH 8.0), and the concentration of E protein was determined by enzyme-linked immunosorbent assay (ELISA) after incubation for 30 min at 65°C in the presence of 0.4% sodium dodecyl sulfate (SDS) (22). The maturation state of RSPs (extent of prM cleavage) was analyzed by quantitative Western blotting as described previously (14).

Standardization of E protein contents in different antigen preparations. The protein concentration of highly purified virus preparations (purity verified by SDS-polyacrylamide gel electrophoresis) was determined according to Schaffner and Weissmann (48). E accounts for approximately 70% of the total proteins in virions. The E protein concentration in RSPs was determined by SDS-ELISA as described in Heinz et al. (22) using purified virions as a standard. Under the conditions of this assay, all E molecules are solubilized into monomers, and the result is therefore not influenced by possible differences in antigen recognition of the native E protein in virus and RSPs. Purified preparations of sE (purity verified by SDS-PAGE) were the same as used for crystallographic analysis (21, 44), and the concentration of sE was identical to the

FIG. 1. Structures and schematic representations of the TBE virus E protein, virions, and RSPs. In all panels, DI, DII, and DIII of the E protein are shown in red, yellow, and blue, respectively, and the fusion peptide (FP) is in orange. (A) Ribbon diagram of the sE dimer (top view). (B) Schematic of the full-length E dimer in a top view (upper panel) and side view (lower panel). The position of the two transmembrane helices of the membrane anchor and the two helices of the stem are based on Zhang et al. (54) and are shown in green and purple, respectively. (C) Pseudo-atomic structure of the virion based on cryo-EM reconstructions of dengue and West Nile viruses (27, 37, 54). One of the 30 rafts, each consisting of three parallel dimers, is highlighted. DIIIs of three monomers belonging to one icosahedral asymmetric unit are labeled by white stars. (D) Pseudo-atomic structure of RSP based on cryo-EM reconstructions (12).
total protein concentration of the preparation, as determined according to Schaffner and Weissmann (48).

**MAbs.** A panel of previously described TBE virus E protein-specific MAbs (17, 24) was used. Antibodies were purified from murine ascitic fluids using protein A-Sepharose High Performance columns (GE Healthcare Life Sciences) according to the manufacturer's recommendations. Fab fragments were generated from purified MAbs by papain cleavage as described previously (20) and purified by affinity chromatography (isotype immunoglobulin G2a [IgG2a]) or ion exchange chromatography (isotype IgG1).

**Epitope mapping by four-layer ELISA.** A four-layer ELISA was carried out as described previously (49). Briefly, wild-type and mutant RSPs at a concentration of 0.5 μg/mL E protein were captured by guinea pig anti-TBE virus Ig-coated plates and incubated with predetermined dilutions of each of 22 neutralizing and nonneutralizing E protein-specific MAbs. For the detection of bound antibodies, we used peroxidase-labeled rabbit anti-mouse IgG. Connecting the absorbance values obtained with each of the MAbs yields characteristic reactivity profiles that distinguish wild-type and mutant RSPs.

**Neutralization assay.** TBE virus neutralization assays were performed in baby hamster kidney cells (BHK-21) as described previously (51). Serial dilutions of MAbs were mixed with 10 times the 50% tissue culture infective dose of virus and incubated for 1 hour at 37°C. BHK-21 cells were added, and incubation was continued for three days. The presence of virus in the supernatant was determined by a virology approach employing RSPs and exchanged surface domains.

**Statistical analysis.** Data were analyzed with GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA). Two-tailed t tests were used to compare ELISA avidities, which were considered significantly different when the P value was less than 0.05.

**RESULTS**

Epitope mapping of neutralizing MAbs by mutagenesis of RSPs. In this study, we used a set of 11 TBE virus E protein-specific and neutralizing antibodies, as shown in Table 1. In the case of 8 of the 11 antibodies, information on the location of the corresponding binding sites in E had been obtained previously employing sequence analysis of neutralization escape mutants (23, 32). In order to obtain a more complete picture of these eight epitopes and also of the three additional epitopes defined by the antibodies B2, IU3, and IM3, we used a mutagenesis approach employing RSPs and exchanged surface-exposed residues all over the external surface of E. A total of 51 individual mutations were introduced (Table 2) that affected neither secretion nor maturation of RSPs (see Materials and Methods). The reactivity of the RSP mutants with each of the 11 antibodies was tested in a four-layer ELISA (see Ma-
Two examples of the reactivity profiles obtained are shown in Fig. 2, and all of the results are summarized graphically in Fig. 3. Overall, most of the mutations affecting MAb binding clustered at sites compatible with the size of individual Fab footprints (6, 29). The mutagenesis approach thus provided additional valuable information on the location and, in some cases, the extension of the epitopes. However, in three instances (MAbs A3, IE3, and IU3), mutations affecting the binding of the MAb were too distant to be part of the same Fab footprint. MAb IU3 presents an extreme example of this category as mutations affecting its binding form a continuous patch along DII and also extend to adjacent regions in DI and DIII. Therefore, indirect conformational effects caused by these mutations have to be postulated, and the nature and precise location of this specific epitope remain undefined.

**Antigenic reactivity of MAbs with virions, RSPs, and sE in blocking ELISA.** To assess possible antigenic differences between E in soluble and in particulate forms, the reactivities of each of the MAbs with serial dilutions of purified preparations...
of virions, RSPs, and sE were standardized to equimolar concentrations of E, as described in Materials and Methods. The antigenic reactivities of these standardized preparations were compared in a blocking ELISA format in the absence of detergent to maintain the structural integrity of virions and RSPs (see Materials and Methods) (Fig. 4). The information contained in the blocking curves displayed in Fig. 4 was also used to calculate the relative ELISA avidity of each MAb with respect to its binding to the virus, RSP, and sE (see Materials and Methods) (Table 3).
of the binding characteristics of the whole MAb B4 with that of its Fab fragment. As can be seen from Fig. 4B, the Fab dis-
played no irregularities in the sigmoidal binding curve and thus allowed the estimation of Fab affinity, which was significantly higher with sE than with whole virus (Table 3). In contrast to MAb A3 and Fab B4, MAbs B1 and B2 had lower and similar avidities for sE compared to virions, respectively, but nevertheless were blocked more efficiently by sE than by virions at low antigen concentrations. This comparison suggests a sub-
maximal occupancy of potential binding sites for these two antibodies on virions.

(i) Comparison of the MAb reactivities with virions and RSPs. With 8 of the 11 MAbs (A5, B1, B2, and B4 and, to a lesser extent, IE3, IC3, i2, and IM3) the blocking capacity of RSPs was higher than that of the virus despite the presence of equimolar concentrations of E. Virus and RSPs yielded identical or nearly identical results (for MAbs A4, A3, and IU3) only in a few instances. For MAbs B2 and i2, the higher blocking activity of RSPs might be explained by the higher ELISA avidity of these MAbs with RSPs than with virions (Table 3). This explanation, however, cannot apply to the other MAbs because they have identical ELISA avidities with both particulate forms (Table 3). It is therefore suggested that the reactivity differences observed in the blocking ELISA are due to the fact that the occupancy of E molecules in virions was lower than in RSPs in these cases.

(ii) Comparison of the MAb reactivities with virions and sE.

Two distinct patterns were obtained: MAbs A4 (including its Fab fragment), A5, IE3, IU3, i2, and IM3 exhibited both lower reactivities and lower avidities to sE than to virions (Fig. 4 and Table 3), thus providing evidence for differences in the structure of these epitopes. However, for the remaining MAbs (MAbs A3, IC3, B4, B1, and B2) sE had stronger blocking activity than virions, especially at low concentrations of E (Fig. 4A). In the specific case of A3, this was consistent with an approximately eightfold higher avidity to sE than to virions (Table 3). Avidity calculations were not possible for MAbs IC3 and B4 due to bisigmoidally shaped dose-response curves of sE, indicating differences in avidity at different E/IgG ratios (Fig. 4A). Such a pattern might be caused by antibody-mediated cross-linking of different sE dimers at high concentra-
tions. To assess this hypothesis, a comparison was undertaken of the binding characteristics of the whole MAb B4 with that of its Fab fragment. As can be seen from Fig. 4B, the Fab dis-

### TABLE 3. Neutralizing activities of MAbs and their ELISA avidities to different forms of the TBE virus E protein

<table>
<thead>
<tr>
<th>MAb or MAb-Fab</th>
<th>E domain</th>
<th>NT&lt;sub&gt;50&lt;/sub&gt; (µg/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ELISA reactivity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Virus</th>
<th>RSP</th>
<th>sE dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td>MAb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC3</td>
<td>DI</td>
<td>3.5</td>
<td>1.0</td>
<td>0.6–1.5</td>
<td>1.6</td>
<td>0.9–2.2</td>
</tr>
<tr>
<td>i2</td>
<td>DI</td>
<td>10.1</td>
<td>11.6</td>
<td>10.0–13.2</td>
<td>6.6</td>
<td>4.7–8.5</td>
</tr>
<tr>
<td>A3</td>
<td>DII</td>
<td>23.3</td>
<td>41.0</td>
<td>39.3–42.6</td>
<td>50.1</td>
<td>36.0–64.2</td>
</tr>
<tr>
<td>A4</td>
<td>DII</td>
<td>1.6</td>
<td>4.4</td>
<td>3.2–5.6</td>
<td>4.6</td>
<td>4.2–5.1</td>
</tr>
<tr>
<td>A5</td>
<td>DII</td>
<td>41.1</td>
<td>4.1</td>
<td>2.5</td>
<td>2.0–3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>IE3</td>
<td>DII</td>
<td>273.1</td>
<td>40.0</td>
<td>23.4–55.9</td>
<td>34.0</td>
<td>25.8–62.9</td>
</tr>
<tr>
<td>B4</td>
<td>DII</td>
<td>70.9</td>
<td>1.4</td>
<td>0.9–1.9</td>
<td>1.5</td>
<td>1.2–1.8</td>
</tr>
<tr>
<td>B1</td>
<td>DIII</td>
<td>21.9</td>
<td>3.0</td>
<td>0.3–6.5</td>
<td>2.8</td>
<td>2.0–3.6</td>
</tr>
<tr>
<td>B2</td>
<td>DIII</td>
<td>9.6</td>
<td>4.9</td>
<td>3.0–6.8</td>
<td>4.1</td>
<td>1.8–2.3</td>
</tr>
<tr>
<td>IM3</td>
<td>DII</td>
<td>77.9</td>
<td>3.0</td>
<td>2.3–3.6</td>
<td>2.9</td>
<td>2.2–3.7</td>
</tr>
<tr>
<td>IU3</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15.3</td>
<td>15.8</td>
<td>13.4–18.2</td>
<td>15.3</td>
<td>13.4–18.2</td>
</tr>
<tr>
<td>MAb-Fab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4-Fab</td>
<td></td>
<td>2.1</td>
<td>1.3–2.8</td>
<td></td>
<td>1.4</td>
<td>1.3–1.5</td>
</tr>
<tr>
<td>B4-Fab</td>
<td></td>
<td>5.2</td>
<td>3.9–6.5</td>
<td></td>
<td>4.5</td>
<td>1.2–7.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of antibodies at which 50% neutralization was achieved.
<sup>b</sup> KD and 95% confidence interval (CI) values are the means of at least three independent experiments. NC, not calculable due to an irregular binding curve.
<sup>c</sup> KD values were compared by t tests. P values of <0.05 were rated as significant and are highlighted in bold. NS, not significant.
<sup>d</sup> NA, not assignable to a single domain.
<sup>e</sup> Data are the means of three independent experiments.
<sup>f</sup> Data are the means of two independent experiments.
<sup>g</sup> MAb-Fab, Fab fragment of a specific MAb.
The differences observed between soluble and particulate forms of E dimers through their tight packing in virions. The best evidence for such structural differences was obtained with MAb A4 and its derived Fab fragment, both of which have an at least 10-fold higher affinity for E as part of virions or RSPs than as sE (Table 3). This epitope has been mapped to the edge of DII by both mutagenesis and neutralization escape approaches (Fig. 3 and Table 1), and one could argue that the full epitope in virions is composed of amino acid residues from different E subunits, brought together through their specific quaternary arrangement in virions. Such “overlapping” contributions would of course be absent in sE and could provide an alternative explanation for the avidity/affinity differences observed with the two forms. If this were true, the reactivity of virions and RSPs should also be different because the arrangement of E dimers, and therefore the E dimer contacts in these two particulate structures, are unrelated (herringbone-like in virions and T = 1 icosahedral in RSPs) (Fig. 1C and D). However, the binding curves of MAB A4 and Fab A4 and their deduced avidities/affinities with virions and RSPs are virtually identical. These data therefore suggest true structural differences between sE and E in particles, probably due to the flexibility of E at its domain interfaces (7, 35, 55).

The higher avidity of some MAbs/Fabs for RSPs than virions may indeed be related to the different icosahedral structures and chemical environments of E dimers in these particles. Examples are MAB B2 and Fab B4 (both directed to the lateral side of DIII) and also MAB i2 that has been mapped to the side of DI (Fig. 3). Cryo-EM has demonstrated an extremely tight packing of E dimers at the surface of virions (27, 37), whereas a somewhat looser arrangement has been revealed for RSPs (12). We therefore propose that, even though the E dimer building blocks are the same in virions and RSPs, the differences in quaternary arrangement account for the variations in antigenic reactivity observed between the two forms. Alternatively, the dynamic nature of the flavivirus surface, as first demonstrated for mature dengue virus particles (31), could also account for some of the differences observed.

All of the atomic structures of flavivirus E proteins determined so far consist of the ectodomain only and lack the so-called stem and the double membrane anchor (Fig. 1A). Although cryo-EM revealed that the stem-anchor of the pro-

### TABLE 4. Average number of IgG molecules bound per TBE virion under saturation conditions (occupancy)

<table>
<thead>
<tr>
<th>MAb or Mab-Fab</th>
<th>E domain</th>
<th>No. of bound IgG molecules per virion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC3</td>
<td>DI</td>
<td>34.3 ± 4.0</td>
</tr>
<tr>
<td>i2</td>
<td>DI</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>DII</td>
<td>57.5 ± 0.7</td>
</tr>
<tr>
<td>A4</td>
<td>DII</td>
<td>88.2 ± 4.5</td>
</tr>
<tr>
<td>A5</td>
<td>DII</td>
<td>26.3 ± 4.6</td>
</tr>
<tr>
<td>IE3</td>
<td>DII</td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>DIII</td>
<td>44.7 ± 5.3</td>
</tr>
<tr>
<td>B1</td>
<td>DIII</td>
<td>34.8 ± 5.2</td>
</tr>
<tr>
<td>B2</td>
<td>DIII</td>
<td>37.8 ± 2.9</td>
</tr>
<tr>
<td>IM3</td>
<td>DIII</td>
<td>35.6 ± 4.4</td>
</tr>
<tr>
<td>IU3</td>
<td>NA</td>
<td>37.0 ± 11.3</td>
</tr>
<tr>
<td>MAb-Fab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4-Fab</td>
<td></td>
<td>150.0 ± 38.6</td>
</tr>
</tbody>
</table>

* MAb-Fab, Fab fragment of a specific MAb.

* NA, not assignable to a single domain.

* Data shown in this table are the results from at least four independent experiments (mean ± standard deviation). —, no conclusive results obtained.

**Comparison of full-length and truncated E dimers.** The differences observed between virus and sE might be related not only to the quaternary arrangement of E at the virion surface and/or differences in the accessibility of epitopes but also to the lack of the stem-anchor region in sE. Therefore, a comparative analysis of full-length and sE dimers in a blocking assay format was undertaken, similar to that used for the experiments depicted in Fig. 4A except for the addition of the detergent Tween 20, necessary for keeping full-length E in solution (Fig. 5). Although 6 of the 11 MAbs reacted identically with both forms of E, significant differences in reactivity and avidity were observed with MAbs A3, IM3, and i2. MAbs IU3 and IE3 did not yield conclusive results because of their low reactivity. Two patterns were observed with the MAbs that differentiated between the two forms of E. A3 had a much higher blocking activity and ELISA avidity for the truncated protein (K<sub>D</sub> of 3.4 ± 0.3 nM) than for the full-length E (K<sub>D</sub> of 24.4 ± 4.3 nM), and the opposite was true for the MAbs IM3 (K<sub>D</sub> of 46.3 ± 12.3 nM for sE and 2.7 ± 0.1 nM for full-length E) and i2 (K<sub>D</sub> of >100 nM for sE and 52.3 ± 3.2 nM for full-length E). These data indicate that the presence of the stem-anchor region has a significant impact on the antigenic structure of the E protein ectodomain.

**DISCUSSION**

In this work, we have investigated the dependence of the antigenic structure of the TBE virus E protein on its quaternary arrangement. Using 11 E-specific MAbs as probes, we found significant differences in the antigenicity of soluble and particulate forms of E. The phenomena observed could be related to several factors including epitope accessibility, structural flexibility of E, and different degrees of occupancy by antibodies.

It is an important finding of our study that some of the antibodies displayed a higher avidity for sE than for E in the context of virions (and to a certain extent also RSPs). This was especially striking in the case of MAb A3, which reacts with an epitope at the edge of DII, and for Fab B4, which reacts with the lateral side of DIII (Fig. 3). In both cases, the results obtained can best be explained by a limited accessibility of these epitopes in the context of particles due to lateral contacts between E dimers that characterize the specific icosahedral arrangements of particulate forms (Fig. 1C and D). Such a partially cryptic nature has been described for other epitopes of the flavivirus E protein, including those involving the highly conserved fusion peptide loop at the tip of DII (40, 51). Avidity, however, would not be influenced in a situation in which a subset of a given MAb-defined epitope is totally cryptic while the rest is fully accessible for antibody binding, as described for DIII-specific epitopes at the fivefold symmetry axis of virions (26).

The differences in avidity observed between soluble and particulate forms could also reflect true structural differences of E that relate to its flexibility at the junctions between the three domains and the constraints imposed on individual E dimers through their tight packing in virions. The best evidence for such structural differences was obtained with MAbs A4 and its derived Fab fragment, both of which have an at least 10-fold higher affinity for E as part of virions or RSPs than as sE (Table 3). This epitope has been mapped to the edge of DII by both mutagenesis and neutralization escape approaches (Fig. 3 and Table 1), and one could argue that the full epitope in virions is composed of amino acid residues from different E subunits, brought together through their specific quaternary arrangement in virions. Such “overlapping” contributions would of course be absent in sE and could provide an alternative explanation for the avidity/affinity differences observed with the two forms. If this were true, the reactivity of virions and RSPs should also be different because the arrangement of E dimers, and therefore the E dimer contacts in these two particulate structures, are unrelated (herringbone-like in virions and T = 1 icosahedral in RSPs) (Fig. 1C and D). However, the binding curves of MAB A4 and Fab A4 and their deduced avidities/affinities with virions and RSPs are virtually identical. These data therefore suggest true structural differences between sE and E in particles, probably due to the flexibility of E at its domain interfaces (7, 35, 55).

The higher avidity of some MAbs/Fabs for RSPs than virions may indeed be related to the different icosahedral structures and chemical environments of E dimers in these particles. Examples are MAB B2 and Fab B4 (both directed to the lateral side of DIII) and also MAB i2 that has been mapped to the side of DI (Fig. 3). Cryo-EM has demonstrated an extremely tight packing of E dimers at the surface of virions (27, 37), whereas a somewhat looser arrangement has been revealed for RSPs (12). We therefore propose that, even though the E dimer building blocks are the same in virions and RSPs, the differences in quaternary arrangement account for the variations in antigenic reactivity observed between the two forms. Alternatively, the dynamic nature of the flavivirus surface, as first demonstrated for mature dengue virus particles (31), could also account for some of the differences observed.

All of the atomic structures of flavivirus E proteins determined so far consist of the ectodomain only and lack the so-called stem and the double membrane anchor (Fig. 1A). Although cryo-EM revealed that the stem-anchor of the pro-
tein is not accessible at the virion surface (54), our data provide evidence for a contribution of these sequences to the reactivity of some neutralizing MAbs. Specifically, the DIII-specific MAb IM3 and the DII-specific MAb i2 had significantly stronger reactions with the full-length protein, whereas, in contrast, the DII-specific MAb A3 had much higher avidity for the truncated form. The structural basis of these observations is not clear at present and requires the structural determination of the full-length flavivirus E protein. Given the flexibility of E at its domain junctions, it is quite possible, however, that interactions of the stem-anchor at the bottom of the molecule can have subtle effects on the hinges between the domains and thus influence epitopes recognized by antibodies at the surface.

Blocking ELISAs as performed in this study offer the advantage that the antigen-antibody reaction takes place in solution and is therefore not influenced by denaturation artifacts through the coating of antigens to solid phases. However, it is important to note that the extent of blocking not only is a function of antibody avidity but can also reflect differences in the antibody occupancy of the antigens compared. This was clearly shown through the results obtained with some MAbs that, although they had a higher avidity for the virus, were blocked significantly better by equimolar concentrations of sE. Indeed, a careful analysis of the number of antibody molecules bound to virions under different antibody/virus ratios revealed that only between 30 and 90 molecules, depending on the specific antibody, could be bound to the available 180 epitopes per virion at saturation. One explanation for this limitation is certainly steric hindrance between the bulky antibody molecules. Consistent with this interpretation is the observation that this number could be significantly increased through the use of the corresponding Fab fragment (MAb A4).
to such steric hindrance factors, however, the inaccessibility of a subfraction of the potential 180 epitopes at the virion surface (e.g., at the fivefold symmetry axis) (26, 31) could also contribute to the differences observed. In any case, comparative antigenic analyses of the same protein at equimolar concentrations, with the protein presented in different quaternary arrangements and/or physical forms, have to be treated with great caution in order to avoid misleading interpretations.

RSPs have been valuable tools for studying the properties of flavivirus glycoproteins, especially with respect to particle assembly (4), membrane fusion (3, 9, 14, 49), and antigenic structure (3, 49). As part of the present work, we have also carried out an extensive mutagenesis study with TBE virus RSPs to delineate the epitopes of the 11 neutralizing MAbs used. While in most cases the results were consistent with the possible sizes of Fab-covered areas on protein antigens, some antibodies were affected by mutations at quite distant locations on the E protein surface. In such instances, indirect effects of the mutations on the corresponding epitope have to be postulated. The most extreme example in this context was that of MAb IU3 (Fig. 3). Its reactivity was abolished or impaired by mutations in all three E protein domains, making a location of the actual binding site by the use of this technology virtually impossible. In the absence of other information (e.g., from neutralization escape mutations and/or structures of antibody-antigen complexes), the data obtained in such mutagenesis approaches can therefore lead to erroneous interpretations.

In conclusion, our data provide experimental evidence for several factors that modulate the antigenic structure of the flavivirus E protein. These are most likely related to the flexibility of E at its domain junctions, influences of the stem-anchor, and constraints imposed on E through its assembly into particles. The differences in antigenic reactivity observed between particulate and soluble forms of E are especially relevant with respect to the standardization of antigenic contents and immunogenicity of vaccines that contain different physical forms of flavivirus antigens.

ACKNOWLEDGMENTS

We thank Walter Holzer for help with virus production and the generation of sE dimers and Gabriel O’Riordain for critical reading of the manuscript.

This work was supported by the Austrian Science Fund (Fonds zur Foerderung der wissenschaftlichen Forschung), FWF project number P17035-B09.

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