Different functional sensitivity to mutation at intersubunit interfaces involved in consecutive stages of foot-and-mouth disease virus assembly

Verónica Rincón, Alicia Rodríguez-Huete and Mauricio G. Mateu

Correspondence
Mauricio G. Mateu
mgarcia@cbm.uam.es

Centro de Biología Molecular ‘Severo Ochoa’ (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Small spherical viruses are paradigms of supramolecular self-assembly. Identifying the specific structural determinants for virus assembly provides guidelines to develop new antiviral drugs or engineer modified viral particles for medical or technological applications. However, very few systematic studies have been carried out so far to identify those chemical groups at interfaces between virus capsid subunits that are important for viral assembly and function. Foot-and-mouth disease virus (FMDV) and other picornaviruses are assembled in a stepwise process in which different protein–protein interfaces are formed: 5 protomeric subunits oligomerize to form a pentameric intermediate, and 12 of these stable pentameric building blocks associate to form a labile capsid. In this study, a systematic mutational analysis revealed that very few amino acid side chains involved in substantial interactions between protomers within each pentamer are individually required for virus infectivity. This result contrasts sharply with the previous finding that most amino acid side chains involved in interactions between pentamers during the next assembly step are individually required for infectivity. The dramatic difference in sensitivity to single mutations between the two types of protein–protein interfaces in FMDV is discussed in terms of possible structural strategies for achieving self-assembly and genome uncoating in the face of diverse selective constraints.

INTRODUCTION

Virus morphogenesis entails a cascade of molecular recognition events in which capsid protein subunits and the viral genome are involved (for recent reviews see Agbandje-McKenna & McKenna, 2011; Aksyuk & Rossmann, 2011; Domitrovic et al., 2013; Mateu, 2013a, 2013b; Veessler & Johnson, 2012; Zlotnick & Mukhopadhyay, 2011). Interactions between viral capsid subunits have been identified in atomic or quasi-atomic structural models determined by X-ray crystallography or, in some cases, by high-resolution cryoelectron microscopy or a combination of structural techniques. Theoretical estimates of the energetics of intersubunit interactions have been obtained for many viruses; the VIPER database provides abundant data on both icosahedral virus structures and calculated subunit interaction energies (Carrillo-Tripp et al., 2009). Many structure-based functional studies have provided mechanistic insights into assembly, as well as stability, dynamics, function and evolution, of virus particles (Agbandje-McKenna & McKenna, 2011; Mateu, 2013b). Mutational analyses have addressed the roles of selected capsid residues in these processes. Studies by alanine-scanning mutagenesis have yielded a residue-by-residue functional dissection of protein–protein or protein–nucleic acid interfaces in a few virus particles (del Alamo et al., 2003; Forshey et al., 2002; Ganser-Pornillos et al., 2004; Kim et al., 2011; Mateo et al., 2003; Murray et al., 2007; Pérez et al., 2011; Reguera et al., 2004, 2005; von Schwedler et al., 2003; Wu et al., 2000; Zhang et al., 2010). These studies are also contributing to developing novel applications in biomedicine, biotechnology and nanotechnology, including antiviral drugs (Bocanegra et al., 2012), engineered virus particles for vaccines, gene therapy, drug delivery diagnosis, biomaterials and nanodevices (Bárbara & Blanco, 2013; Bittner et al., 2013; Flenniken et al., 2009; Glasgow & Tullman-Ercek, 2014; Li & Wang, 2014; Mateu, 2011; Smith et al., 2013; Wen et al., 2013). They are also inspiring the design of self-assembling non-biological nanostructures (Lamarre & Ryadnov, 2011).

Foot-and-mouth disease virus (FMDV) (Grubman & Baxt, 2004; Jamal & Belsham, 2013; Mahy, 2005; Sobrino & Domingo, 2004) is both an excellent model for structure–function studies of virus assembly and an economically very important pathogen (Thompson et al., 2002) for which new control strategies are required (Fowler & Barnett, 2012; Paton et al., 2009; Rodríguez & Grubman, 2009). The $P=3$ ($T=1$) icosahedral capsid of FMDV (Acharaya et al., 1989; Curry et al., 1996; Fry et al., 1999; Lea et al., 1994) (Fig. 1a) is formed by 60 copies of each of three proteins (VP1, VP2, VP3) and a small internal polypeptide (VP4), and contains a single-stranded RNA genome. FMDV and other picornaviruses are assembled in the host cell in a stepwise process: First, a protomeric subunit made of one copy of each VP (as a single
polyprotein, P1) is folded; the folded P1 protomer is then proteolytically processed; five processed protomers oligomerize to form a stable pentameric intermediate; and, finally, 12 pentamers associate to form the complete capsid (Fig. 1a). The viral RNA may be co-assembled at this stage to yield the complete virion (Rueckert, 1996). Viral genome uncoating during infection by FMDV involves the acid-induced dissociation of the labile capsid into pentamers (Carrillo et al., 1985; Baxt, 1987). FMD virions are also very sensitive to thermal dissociation, even at neutral pH and low temperatures. As current FMD vaccines are based on the immunogenicity of chemically inactivated but structurally intact virions, FMDV thermostability constitutes a problem for the adequate control of this disease (Hegde et al., 2009). Structural studies and mutational analyses of FMDV are contributing to understanding the molecular determinants of assembly and genome uncoating, and the engineering of viral particles of increased thermostability for the development of improved vaccines (Mateo et al., 2008; Porta et al., 2013; Rincón et al., 2014).

In previous structure-function studies on FMDV assembly and stability, we focused on the interfaces between pentameric subunits which are involved both in the last capsid assembly step and in its acid- or heat-induced disassembly. A systematic mutational analysis by alanine-scanning of the functional role of each of the 42 side chains (per protomer) involved in interpentamer interactions in a foot-and-mouth disease (FMD) virion (Fig. 1b) was carried out (Mateo et al., 2003). The results revealed that the vast majority of amino acid side chains at the interpentamer interfaces, even those that participate only in a few van der Waals (vdW) interprotomer contacts, are critically required for normal completion of the infectious cycle. This observation suggested that the last capsid assembly step during FMDV morphogenesis is very sensitive to single mutations.

In the present study we have investigated the functional role of individual capsid amino acid residues involved in the previous FMDV assembly step, the interaction of capsid protomers to form individual pentamers (Fig. 1b). A thorough functional dissection by alanine-scanning of the interprotomer interfaces in the FMD virion was carried out, and the results were compared with those we had previously obtained for interpentamer interfaces in the same virion. The comparison revealed extreme differences between both interfaces regarding the functional tolerance to mutation of residues in either interface. The structural basis of such differences and the implications for virus function and evolution are discussed.

RESULTS

Analysis of the interprotomer interactions within a pentamer in the FMD virion

Analysis of the intersubunit interactions in the crystal structure of the FMDV C-S8c1 virion (Lea et al., 1994) revealed that each protomer–protomer interface within
the same pentamer involves 93 amino acid residues belonging to either protomer (Fig. 1b). As many as 45 of these residues participate in protomer–protomer association only through a few interactions that involve main chain or $\alpha$-carbon atoms exclusively. In principle, these interactions may be preserved even if these residues are mutated (to alanine or any other residue except glycine), either in nature or in the laboratory. Thus, we focused exclusively on the 48 residues whose side chains (beyond the $\alpha$-carbon) participate in direct interprotomer interactions that would be removed by mutation to alanine (Fig. 1c). Of these, 20 side chains are fundamentally apolar and form a string of buried hydrophobic patches along this narrow but extended protein–protein interface. Two of these side chains correspond to tyrosines (mainly hydrophobic, but involved in hydrogen bonds through their hydroxyl groups). The remaining 28 side chains are fundamentally polar; 10 of them are involved in interprotomer vDW interactions, while 18 others participate also in a total of 17 hydrogen bonds and two salt bridges.

Based on the above analysis, we initially chose alanine-scanning of every interfacial residue whose side chain participates in more than two vDW contacts (including carbon–carbon contacts that bury hydrophobic surface), and/or any hydrogen bond or salt bridge between protomers. Six of these residues participate only in pairwise contacts (N1091–Y3169, K1085–E1175, K2217–E3138); as mutation of only one residue in each pair would be enough to remove the corresponding interactions, only one residue per pair was mutated. Two residues (L1205 and L1206) were excluded because they were located very close to the polyprotein P1 C terminus, outside of the segment in the mutagenized recombinant plasmid that is subcloned in the FMDV infectious clone. As these two residues are involved only in minor interactions between protomers, we considered it unnecessary to include them among the already large and highly representative ensemble of interfacial residues to be tested. To sum up, 19 residues per protomer whose side chains are together involved in nearly all but the presumably weakest interprotomer interactions were selected for mutation (Table 1, Fig. 1c). As representatives of the interfacial residues whose side chains participate only in presumably very weak interactions (one or two vDW contacts), residues M3130 and V3215 were additionally chosen for mutation (Table 1).

**Effect of individual mutations at the interprotomer interfaces in FMDV in host cell infection and production of viral progeny**

Mutation to alanine was used to remove every interaction of the original side chain (beyond C$_\beta$), without introducing any other interaction and with the lowest probability of altering the main chain conformation. The 21 chosen alanine mutations were introduced in the FMDV infectious clone, the corresponding viral RNAs were obtained, and equal amounts of them were used to transfect susceptible cells in parallel with the same amount of non-mutated control RNA. The viral progeny produced at different times after transfection were quantified by titration, and the titres were normalized with respect to the one obtained for the non-mutated control in the same experiment. After the effects of each mutation in FMDV infectivity, the mutants were classified in four groups (Table 2) as follows.

Group I (19 % of the mutants analysed): this group comprised four lethal mutants that yielded no detectable progeny at any time post-transfection (p.t.).

Group II (5 % of the mutants): this group comprised only one mutant that showed a significantly reduced progeny at any time p.t., and comparatively small lysis plaques.

Group III (19 % of the mutants): this group comprised four mutants that showed a significant reduction in titre at short times p.t., but that reached titres similar to that of the non-mutated control at longer times. Sequencing the virus population recovered at the longest times confirmed that the original mutation was still present and that no accompanying mutations had been fixed. All these results indicate that these mutations may decrease the multiplication rate of the virus, but they clearly have no effect on its intrinsic infectivity.

Group IV (57 % of the mutants): this group comprised 12 mutants that showed progeny titres and plaque sizes similar to that of the non-mutated control at any time p.t. Mutants N1060A and N1100A consistently showed somewhat increased (three- to ninefold) titres compared with the control; we were unable to find a likely cause for this moderate increase in infectivity.

In summary, the individual removal of over 80 % of the 21 side chains analysed at each of the five interprotomer interfaces in each of the 12 pentamers in the FMDV capsid had no significant effect in viral progeny production or plaque size (about 60 %), or led only to some delay in particle production (about 20 %). In contrast, fewer than 20 % of the side chains proved to be critical for virus infection.

**Effect of individual mutations at the interprotomer interfaces in steps previous to protomer–protomer association**

Some of the mutated side chains are involved not only in interprotomer interactions, but also in intraprotomer interactions; in addition, some of these side chains are partially exposed on the virion surface and could participate in interactions with other molecules during the viral cycle. Thus, of the few functionally critical residues at the interprotomer interfaces, even fewer could be important for protomer–protomer recognition during virus assembly. This possibility is suggested by the lack of correlation between the observed effect of each mutation on viral production (Table 2), and the reduction in interaction energy between protomers caused by that mutation, based on predictions in the VIPER database (Carrillo-Tripp et al., 2009). As expected,
truncation of most (albeit not all) side chains involved in only a few interprotomer vdW interactions had no significant effect on FMDV infectivity; however, truncation of side chains involved in a larger number of presumably stronger interprotomer interactions led to a wide range of effects, from dramatic (Group I) to none at all (Group IV). This observation suggested that the detrimental effect on virus infectivity of some mutations at these interfaces could be due not to impaired assembly of protomers to yield pentamers, but to the participation of the mutated residues in some other stage of the viral cycle. As some of these residues participate also in intraprotomer and/or intraprotein interactions, impaired protomer folding, a prerequisite for interprotomer association, was a most likely candidate.

To determine if host cells transfected with any of the 21 mutant viral RNAs were able to yield folded capsid protomers, we carried out in situ immunofluorescence assays of cells transfected and analysed a short time p.t. Equal amounts of mutant viral RNA and the non-mutated control RNA were used, and the presence of folded protomers (either free or associated as pentamers or assembled viral particles) was estimated with mAb 5C4. This mAb recognizes a discontinuous epitope that is present in folded protomers but not in unfolded protomers or denatured or native individual capsid proteins. Fig. 2(a) shows results with some representative mutants of each infectivity group, compared with the non-mutated control. The complete results are summarized in Table 2. As expected, the vast majority of the 16 mutations that either had no effect on infectivity or only delayed it (Groups IV and III, respectively) also had no significant effect on the immunofluorescence signal, which probes for folded protomer (the only exceptions were Y1107A, T3016A, I3143A and N2047A, which had a minor effect). In contrast, mutation Y2100A, which led to significantly reduced infectivity (Group II), also reduced the immunofluorescence signal significantly, and lethal mutations (Group I), except R2167A, led to no detectable signal, or a drastically reduced signal.

In another immunofluorescence experiment, mutants with reduced infectivity were probed with mAb SD6, which recognizes a continuous epitope in VP1, irrespective of the state of this capsid protein (unfolded or folded, free or associated in

### Table 1. Residues at the FMDV capsid interprotomer interfaces chosen for mutational analysis

<table>
<thead>
<tr>
<th>Residue*</th>
<th>Hydrogen bonds†</th>
<th>Charge–charge interactions†</th>
<th>VdW contacts†</th>
<th>Exposed side chain</th>
<th>Group according to infectivity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2167</td>
<td>1sc (D3166)</td>
<td>1sc (D3166)</td>
<td>3 (0)</td>
<td>No</td>
<td>I</td>
</tr>
<tr>
<td>K2217</td>
<td>1sc (E3138)</td>
<td></td>
<td>4 (2)</td>
<td>Yes</td>
<td>I</td>
</tr>
<tr>
<td>Q3100</td>
<td>1sc (T1120)</td>
<td></td>
<td>3 (0)</td>
<td>No</td>
<td>I</td>
</tr>
<tr>
<td>Y3169</td>
<td>1sc (N1091)</td>
<td></td>
<td>11 (5)</td>
<td>No</td>
<td>I</td>
</tr>
<tr>
<td>Y2100</td>
<td></td>
<td></td>
<td>16 (10)</td>
<td>No</td>
<td>II</td>
</tr>
<tr>
<td>K1085</td>
<td></td>
<td></td>
<td>3 (0)</td>
<td>Yes</td>
<td>III</td>
</tr>
<tr>
<td>N2047</td>
<td>1sc (2 × S3163)</td>
<td></td>
<td>13 (0)</td>
<td>No</td>
<td>III</td>
</tr>
<tr>
<td>L2051</td>
<td></td>
<td></td>
<td>5 (4)</td>
<td>No</td>
<td>III</td>
</tr>
<tr>
<td>M3014</td>
<td></td>
<td></td>
<td>6 (3)</td>
<td>No</td>
<td>III</td>
</tr>
<tr>
<td>T1050</td>
<td>1mc (A1207)</td>
<td></td>
<td>9 (2)</td>
<td>Yes</td>
<td>IV</td>
</tr>
<tr>
<td>N1060</td>
<td>1sc (T1102)</td>
<td></td>
<td>2 (0)</td>
<td>Yes</td>
<td>IV</td>
</tr>
<tr>
<td>N1100</td>
<td>1mc (R3218)</td>
<td></td>
<td>4 (2)</td>
<td>Yes</td>
<td>IV</td>
</tr>
<tr>
<td>T1102</td>
<td>1sc (N1060)</td>
<td></td>
<td>12 (5)</td>
<td>Yes</td>
<td>IV</td>
</tr>
<tr>
<td>Y1107</td>
<td></td>
<td></td>
<td>9 (8)</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>S2049</td>
<td>1mc (2 × Y3161)</td>
<td></td>
<td>10 (0)</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>N3013</td>
<td>1sc (T1113)</td>
<td></td>
<td>9 (2)</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>T3016</td>
<td>3sc (T1101, N1103, 3 × T1105)</td>
<td></td>
<td>11 (1)</td>
<td>Yes</td>
<td>IV</td>
</tr>
<tr>
<td>T3099</td>
<td>1sc (N1091)</td>
<td></td>
<td>9 (4)</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>M3130</td>
<td></td>
<td></td>
<td>3 (1)</td>
<td>Yes</td>
<td>IV</td>
</tr>
<tr>
<td>I3143</td>
<td></td>
<td></td>
<td>5 (4)</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>V3215</td>
<td></td>
<td></td>
<td>3 (2)</td>
<td>No</td>
<td>IV</td>
</tr>
</tbody>
</table>

*For each residue, the first digit indicates the protein (either VP1, VP2 or VP3) and the last three digits the amino acid position according to Lea et al. (1994).
†For each residue, the interprotomer interactions are indicated. The cut-off distance for hydrogen bonds was 3.5 Å, for charge–charge interactions 3.9 Å, and for VdW contacts 0.5 Å longer than the sum of the VdW radii of the two atoms considered. sc, Side chain; mc, main chain. The total number of VdW contacts and, in parentheses, the number of carbon–carbon contacts are indicated.
‡Residues are assigned to groups according to the effects that truncation of their side chain (by mutation to alanine) had on FMDV infectivity. Group I, no detectable virus titre; Group II, reduced virus titres; Group III, delayed virus production; Group IV, normal virus titres (see text and Table 2).
Table 2. Effects on infectivity and capsid protomer assembly of mutations to alanine at the interprotomer interfaces in the FMDV capsid

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Relative virus titre†</th>
<th>Lysis plaque size‡</th>
<th>Relative fluorescence signal§</th>
<th>Conservation (%)¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45 h p.t.</td>
<td>55 h p.t.</td>
<td>72 h p.t.</td>
<td>Large</td>
</tr>
<tr>
<td>Parental (wt)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Large</td>
</tr>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2167A</td>
<td>Lethal</td>
<td>Lethal</td>
<td>Lethal</td>
<td>No</td>
</tr>
<tr>
<td>K2217A</td>
<td>Lethal</td>
<td>Lethal</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>Q3100A</td>
<td>Lethal</td>
<td>Lethal</td>
<td>Lethal</td>
<td>No</td>
</tr>
<tr>
<td>Y3169A</td>
<td>Lethal</td>
<td>Lethal</td>
<td>Lethal</td>
<td>No</td>
</tr>
<tr>
<td>Consensus or mean</td>
<td>Lethal</td>
<td>Lethal</td>
<td>Lethal</td>
<td>No</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y2100A</td>
<td>0.021 ± 0.008</td>
<td>0.024 ± 0.008</td>
<td>0.083 ± 0.001</td>
<td>Small</td>
</tr>
<tr>
<td>Mean or consensus</td>
<td>0.021</td>
<td>0.024</td>
<td>0.083</td>
<td>Small</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1085A</td>
<td>0.010 ± 0.001</td>
<td>0.128 ± 0.012</td>
<td>2.500 ± 0.141</td>
<td>Large</td>
</tr>
<tr>
<td>N2047A</td>
<td>0.002 ± 0.001</td>
<td>0.009 ± 0.002</td>
<td>0.240 ± 0.007</td>
<td>Small</td>
</tr>
<tr>
<td>L2051A</td>
<td>0.000 ± 0.001</td>
<td>0.007 ± 0.001</td>
<td>0.250 ± 0.007</td>
<td>Large</td>
</tr>
<tr>
<td>M3014A</td>
<td>0.002 ± 0.002</td>
<td>0.016 ± 0.004</td>
<td>0.700 ± 0.03</td>
<td>Small</td>
</tr>
<tr>
<td>Consensus or mean</td>
<td>0.004</td>
<td>0.040</td>
<td>0.925</td>
<td>Small</td>
</tr>
<tr>
<td><strong>Group IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1050A</td>
<td>0.228 ± 0.047</td>
<td>0.600 ± 0.154</td>
<td>1.000 ± 0.141</td>
<td>Large</td>
</tr>
<tr>
<td>N1060A</td>
<td>6.309 ± 0.223</td>
<td>7.692 ± 2.81</td>
<td>7.700 ± 0.494</td>
<td>Large</td>
</tr>
<tr>
<td>N1100A</td>
<td>9.779 ± 0.892</td>
<td>3.077 ± 0.362</td>
<td>ND</td>
<td>Large</td>
</tr>
<tr>
<td>T1102A</td>
<td>0.154 ± 0.018</td>
<td>0.340 ± 0.125</td>
<td>0.400 ± 0.014</td>
<td>Large</td>
</tr>
<tr>
<td>Y1107A</td>
<td>0.379 ± 0.664</td>
<td>0.577 ± 0.123</td>
<td>1.150 ± 0.106</td>
<td>Large</td>
</tr>
<tr>
<td>S2049A</td>
<td>1.067 ± 0.660</td>
<td>0.750 ± 0.353</td>
<td>ND</td>
<td>Large</td>
</tr>
<tr>
<td>N3013A</td>
<td>0.946 ± 0.167</td>
<td>1.769 ± 0.344</td>
<td>ND</td>
<td>Large</td>
</tr>
<tr>
<td>T3016A</td>
<td>2.000 ± 0.098</td>
<td>1.739 ± 0.212</td>
<td>ND</td>
<td>Large</td>
</tr>
<tr>
<td>T3099A</td>
<td>0.268 ± 0.126</td>
<td>4.103 ± 0.025</td>
<td>1.300 ± 0.001</td>
<td>Large</td>
</tr>
<tr>
<td>M3130A</td>
<td>0.857 ± 0.409</td>
<td>2.250 ± 0.106</td>
<td>0.400 ± 0.189</td>
<td>Large</td>
</tr>
<tr>
<td>I3143A</td>
<td>0.763 ± 0.093</td>
<td>1.731 ± 0.74</td>
<td>6.000 ± 0.353</td>
<td>Large</td>
</tr>
<tr>
<td>V3215A</td>
<td>1.500 ± 0.555</td>
<td>2.100 ± 0.883</td>
<td>0.300 ± 0.190</td>
<td>Large</td>
</tr>
<tr>
<td>Mean or consensus</td>
<td>2.0</td>
<td>2.2</td>
<td>2.3</td>
<td>Large</td>
</tr>
</tbody>
</table>

*Virus mutants are classified into groups I, II, III or IV according to the effects of the mutation on infectivity. For each group, the means of the values obtained for the mutants in that group are also indicated.
†Viral titres obtained at different times p.t. have been normalized relative to that obtained for the parental virus (wt). The mean absolute titres obtained for the wt virus were (1.7 ± 0.4) × 10⁷ p.f.u. ml⁻¹ (45 h p.t.),(1.9 ± 0.3) × 10⁷ p.f.u. ml⁻¹ (55 h p.t.) and (2.3 ± 1.4) × 10⁷ p.f.u. ml⁻¹ (72 h p.t.). ND, Not determined.
‡Viruses that yielded plaques with mean sizes similar to or less than half that of the parental virus are respectively classified as large or small. No, no plaques were detected.
§The relative fluorescence signal in in situ experiments (see text and Fig. 2) was determined by dividing the number of cells that were positive with mAb 5C4 by the total number of cells analysed, and dividing those relative values by that obtained for the parental virus (wt). The final normalized value obtained for each mutant gives an indication of the mean relative amount of capsid protomers assembled in the cells. The data were analysed using a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Values that are significantly different (P<0.01) from the wt value are indicated by an asterisk.
¶Percentage conservation is given relative to both the consensus residue and the residue found in CS8c1, except for N1060, for which conservation is indicated relative to consensus (9% relative to CS8c1).

Functional analysis of FMDV capsid interfaces

protemers, pentamers or capsids). We observed a reduction in the total amount of capsid protein that paralleled the reduction observed with antibody 5C4 (results not shown).

The immunofluorescence results suggest that the reduced infectivity observed in a minor fraction of the mutants tested could be due to: (i) impaired RNA replication (leading to a reduction in the amount of template RNA available for translation); (ii) impaired capsid protein translation; or (iii) proteolytic degradation of unfolded protomers that cannot fold properly because of the mutation. We consider it highly unlikely that all of these mutations in widely separate positions in the capsid protein coding regions of the FMDV
Fig. 2. *In situ* immunofluorescence analysis of the presence of FMDV in cells transfected with viral RNA carrying mutations at the interprotomer interfaces: detection of folded protomers (either free or as a part of larger structures). The pictures show representative results with negative (mock) or positive (wt, non-mutated viral RNA) controls, or with examples of Group IV (T1050A, N1100A, T1102A, Y1107A, S2049A, T3099A), Group III (K1085A, L2051A, M3014A), Group II (Y2100A) or Group I (R2167A, Y3169A) mutants. Green and blue fluorescence correspond, respectively, to the signal obtained for folded protomers using mAb 5C4, or to DAPI staining. A summary of the results obtained in these experiments with the 21 mutants tested is included in Table 2.
genome affect RNA replication or translation. Thus, though further study is required, we tentatively favour the possibility that the observed reduced infectivity is due to a defect in protomer folding. Whatever the early step affected in these mutants, the results revealed that protomer–protomer association during FMDV assembly is not substantially affected by (nearly) any of the mutations analysed in this study.

**Effect of individual mutations at the interprotomer interfaces on the stability of infectious FMD virions**

FMDV is easily heat-inactivated, even at physiological temperature. We analysed whether removal of interprotomer interactions in non-lethal mutant virions with reduced (Group II) or delayed (Group III) infectivity could decrease their resistance to heat-induced inactivation. Fig. 3 shows the inactivation kinetics of most Group II and III virions at 42 °C. Fitting to exponential decay reactions revealed only very small or non-significant differences in the inactivation rate constant between any of these mutants and the non-mutated virion. Thus, the observed reduction or delay in progeny virus titres by these mutations is not a consequence of a significant destabilization of the infectious virions obtained as final products.

**Analysis of residue conservation at interprotomer and interpentamer interfaces in FMDV**

The comparative residue-by-residue dissection of interprotomer interfaces (this study), and interpentamer interfaces in the following assembly step (Mateo et al., 2003), in FMDV revealed quite different relationships between structure, interactions and functional effects. To determine whether

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**Fig. 3.** Thermal inactivation kinetics at 42 °C of viruses carrying mutations at the interprotomer interfaces. In each panel, circle symbols correspond to the non-mutated control virion, and square symbols correspond to a Group II or Group III mutant virion as follows: (a) K1085A; (b) N2047A; (c) Y2100A; (d) M3014A. Values given and error bars correspond, respectively, to means ± SD obtained in two or three independent experiments. Error bars smaller than the size of the symbols used are not represented. Fitting of the experimental data to an exponential decay in viral titer is indicated by solid lines.
these substantial differences could be reflected in the way these interfaces had varied during FMDV evolution, we carried out a sequence comparison analysis of 250 strains of the seven FMDV serotypes identified.

Interprotomer interfaces showed a degree of residue conservation that could be qualitatively related overall with the observed effects of individual mutations on infectivity (Table 2). Group I and II residues, whose mutation to alanine proved to be lethal or severely impaired infection, showed a mean conservation of 95–98 %; in contrast, Groups III and IV residues, whose mutation did not prevent normal infectivity (or, in some cases, only delayed virus production), showed a mean conservation of 67–65 %. The global mean conservation of residues at the interprotomer interfaces was 72 %, which is remarkably lower than the mean conservation of capsid regions known to be involved in functional steps of the infectious cycle different from protomer–protomer association.

Residue-by-residue analysis of the interpentamer interfaces had revealed that, contrary to that observed for interprotomer interfaces, individual mutation to alanine of the vast majority (81 %) of the 42 residues tested was lethal or severely impaired infectivity. Accordingly, the global mean conservation of these 42 residues is very high (93 %), much higher than that of the residues at the interprotomer interfaces (72 %). From their effect on infectivity, mutations to alanine at the interpentamer interfaces were classified also into four groups. Interestingly, these groups showed similar mean conservation: 96 % (Group I, lethal mutations), 85 % (Group II, strong reductions in infectivity with partial recovery), 88 % (Group III, significant reductions in infectivity) and 92 % (Group IV, no significant reduction in infectivity). Thus, contrary to what was observed for interprotomer interfaces, for interpentamer interfaces no overall correlation was found between residue conservation and effect on FMDV infectivity. Based on the results of our structure–function analyses, we present in the Discussion a hypothetical comparative model with different selective constraints in the different interfaces in the FMDV capsid.

**DISCUSSION**

**Robust interprotomer interfaces within FMDV pentameric subunits**

The results of this study revealed that most amino acid side chains involved in substantial interactions between protomers within each pentamer in the FMDV capsid are not individually required for virus infectivity. This functional tolerance to disruption of interprotomer interactions was unexpected, as this interface may be considered to be structurally similar to typical protein–protein interfaces. The interprotomer interface is narrow and very elongated rather than roughly circular, but it is still essentially made up of a central, buried hydrophobic region surrounded by a rim of solvent-exposed polar residues (Fig. 1b). In typical protein–protein interfaces, a number of buried hydrophobic side chains provide energetic hotspots that are individually critical for the interaction (Bogan & Thorn, 1998), while no such hotspots were detected in the FMDV interprotomer interfaces.

Two structural differences with typical protein–protein interfaces may contribute to the absence of energetic hotspots in the protomer–protomer interfaces in the FMD virion: (i) the high number of residues and interactions involved in each of these interfaces (about twice the typical number in interfaces between interacting cellular proteins, or in small oligomers), probably leading to high binding affinity; (ii) the high proportion of main chain–main chain interactions (directly insensitive to mutation) that contribute to protomer–protomer binding.

The reduction of viral infectivity caused by individual mutation of a few residues at these interfaces was related mainly to an effect on an early stage of the viral cycle, as the amount of folded protomers was significantly reduced. These few functionally important residues do not form a single discrete patch on the FMDV capsid surface (Fig. 1c), which suggests they may not be involved in binding some unidentified molecule during the viral cycle. A likely, albeit still unproven, explanation is that these residues could impair folding of the unprocessed capsid polyprotein, leading to its early degradation in the cell, before virus assembly begins, leading to reduced amounts of folded protomers (and, thus, pentamers) available for capsid assembly.

**Different tolerance to mutation at interprotomer and interpentamer interfaces in the FMD virion**

Comparison of the results obtained by alanine-scanning of the interprotomer interfaces and interpentamer interfaces in the same FMD virion revealed remarkable functional differences. On the one hand, assembly of protomers into pentamers is essentially insensitive to the loss of interactions between nearly any side chain in one protomer and residues in the neighbouring protomer. On the other hand, assembly of pentamers into complete capsids or virions is critically dependent on the individual interactions between most side chains in one pentamer and residues in the neighbouring pentamer.

Structurally, these different sensitivities to removal of interfacial interactions may be a consequence of the different organization of interprotomer versus interpentamer interfaces. In the interprotomer interfaces, the high number of strong protein–protein interactions, including burial of a large hydrophobic surface area, may make any individual interaction, however strong, of comparatively little relevance for the global interaction. In contrast, the interpentamer interfaces involve fewer residues and are made almost exclusively by polar residues involved in weaker
interactions. In this context, the individual removal of even the weakest interactions can have a significant effect.

From the viewpoint of biological evolution, this quite different structural organization of different interfaces in a virion, and the corresponding functional differences in two consecutive steps of virion assembly, could be the result of different selective pressures. Once assembled, pentamers need not be disassembled into protomers for genome uncoating or any other stage in the infectious cycle. Thus, selection may have favoured energetically strong interfaces between protomers that are quite insensitive to single mutations. In the infected cell, this would ensure the maximum availability of fully formed, assembly-competent pentameric building blocks to reach the critical concentration and amounts needed to form many virions per cell. Interprotomer interfaces that were functionally rather sensitive to mutation, in the face of the very high mutation rate characteristic of RNA viruses, including FMDV (Domingo et al., 2004), would frequently lead to reduced amounts of assembly-competent pentameric building blocks. The relatively low conservation of most residues at the FMDV interpentamer interfaces is also consistent with this scenario. In these interfaces, only those few residues that happen to have a role in other stages of the viral cycle are highly conserved.

In contrast to pentameric subunits, complete FMD virions assembled from them do need to be disassembled for genome uncoating. This, it could be sensibly thought that FMDV could have evolved its essentially polar, energetically weak interpentamer interfaces for facilitating uncoating. Uncoating occurs by mild acidification in the endosomes (Vázquez-Calvo et al., 2012) and is mediated by repulsions between a few histidines that become fully protonated (Acharaya et al., 1989; Curry et al., 1995; Ellard et al., 1999; Maree et al., 2013; van Vlijmen et al., 1998). It could be thought that these repulsions could suffice to unbind the pentamers only if the interactions between the latter are intrinsically weak enough. However, engineered thermostable FMD virions in which the net strength of association between pentamers was increased were as infectious as the natural, thermolabile ones (Mateo et al., 2008; Rincón et al., 2014). Thus, the conserved structural instability of natural FMD virions may be partly due to an unidentified selective pressure unrelated to uncoating (Hegde et al., 2009). Although the selective pressure behind weak interpentamer interfaces in the FMDV virion is unclear, the inevitable tradeoff of such weakness is that loss of a few intersubunit interactions may suffice to tilt the energetic balance and promote dissociation outside the cell, making these interfaces extremely sensitive to single mutations as observed. The high conservation of the vast majority of residues at the interpentamer interfaces in infectious FMDV variants is consistent with this scenario.

Comparison with intersubunit interfaces in other spherical viruses

Very few interfaces between subunits in viral particles have been systematically analysed through mutational analysis so far. However, the structure-based functional differences between different interfaces in the FMD virion may be compared with those found during our analysis of another small spherical virus of another family, the parvovirus minute virus of mice (MVM) (Reguera et al., 2004; Pérez et al., 2011). The results indicate that, in both MVM and FMDV, very stable building blocks (trimers and pentamers of subunits, respectively) are formed in high-affinity association reactions that are, to a remarkable extent, mutation-insensitive. In both viruses, those building blocks self-associate to form the viral capsid in lower-affinity reactions that are, to different extents, mutation-sensitive.

The differences between viruses in sensitivity to mutation at the intersubunit interfaces may be equally revealing. In MVM, a very stable virion that does not require capsid disassembly for genome uncoating (Cotmore & Tattersall, 2012), intertrimer association depends critically only on a few hotspot residues that establish strong interactions, and is relatively insensitive to mutation at the intertrimer interfaces. In FMDV, a labile virion that requires capsid disassembly for uncoating, interpentamer association depends critically on many residues, even those that establish weak interactions, and is much more sensitive to mutation at these interfaces. This high functional sensitivity to mutation at the interpentamer interfaces in FMDV may, however, be not as detrimental to virus evolution as could be expected. Remarkably, the virus manages to accept the introduction of many lethal mutations at these interfaces by fixation of compensatory mutations that frequently occur at a few ‘compensation hotspots’ in defined locations of the viral capsid (Luna et al., 2009). These and other studies have revealed diverse structural adaptations at the interfaces between subunits in viral capsids that allow them to perform the required functions during the viral cycle using different mechanisms and evolutionary strategies.

**METHODS**

**Site-directed mutagenesis and subcloning.** Substitution of amino acid residues in the FMDV capsid (strain C-S8c1) (Sobrino et al., 1983; GenBank sequence accession number AJ133357) was carried out in plasmid p3242/C-S8c1 (Baranowski et al., 1998) using the QuikChange system (Stratagene). The mutant segments were subcloned in the infectious plasmid pO1K/C-S8c1 essentially as previously described (Mateo & Mateu, 2007; Mateo et al., 2003). The presence of the engineered mutations and absence of any other mutation in the subcloned segment were confirmed by DNA sequencing.

**Transcription of viral RNA and transfection of eukaryotic cells.** FMDV RNA was transcribed from linearized non-mutated and mutant pO1K/C-S8c1 plasmids by using the Riboprobe in vitro transcription system (Promega), and used to transfect BHK-21 cells by electroporation as previously described (Mateo & Mateu, 2007; Mateo et al., 2003). The same amounts of every mutant RNA, and of non-mutated RNA as a control, were used in each experiment. Progeny virions were recovered after complete cytopathic effect at different times p.i.
Determination of virus infectivity, amplification of viruses and extraction of viral RNA. Titrations of viruses obtained by transfection of BHK-21 cells were determined at least in duplicate in standard plaque assays. When needed, progeny viruses were amplified by a minimum number of passages in BHK-21 cell monolayers at the highest possible m.o.i., and titrated again. RNA derived from viral populations obtained by transfection or infection was extracted using Trizol LS (Invitrogen) and precipitated with 2-propanol. The RNA was reverse-transcribed to DNA and amplified by PCR as previously described (Mateo & Mateu, 2007; Mateo et al., 2003). The reverse transcription PCR products were purified using centrifugal filter units Amicon Ultra (Millipore). The presence of mutations in the progeny virions was detected by sequencing the capsid region or the entire genome.

Thermal inactivation assays. One millilitre aliquots of virus suspensions adjusted to a titre of $2 \times 10^7$ to $4 \times 10^7$ p.f.u. x ml$^{-1}$ were clarified by centrifugation at 10 000 g for 10 min and subsequently heated at 42 °C for different lengths of time. In general, a mutated virus and the non-mutated control in the same experiment were purified and processed in parallel, to exclude possible differences in the dissociation rate due to slightly differing final conditions between preparations and experiments. The experimental data were fitted to first-order exponential decays by using the program Kaleidagraph (Sinergy Software), which allowed the determination of the dissociation rate constant under the conditions tested (Mateo et al., 2008).

In situ immunofluorescence assays. BHK-21 cells were electroporated with viral RNA as described above, and subsequently cultured on coverslips incubated for 30 h at 37 °C. These were washed with PBS, incubated with 4 % paraformaldehyde, washed again, and incubated with 10 mM glycine–NaOH, pH 8.5. The cell membranes were permeabilized by incubation with 0.2 % Triton X-100 in PBS. The coverslips were washed again, incubated in PBS containing 3 % BSA, and then incubated for 1 h at room temperature with monoclonal antibody 5C4 diluted 1 : 500 in PBS with 3 % BSA. 5C4 recognizes a discontinuous epitope present on the virus particle surface (Lea et al., 1994), and unassembled capsid pentamers and protomers (Szát et al., 1994), but not in isolated capsid proteins. The coverslips were washed thoroughly, and incubated with one or more secondary antibodies diluted 1 : 500 in PBS containing 3 % BSA. Subsequently, the samples were washed and incubated with 1 μg ml$^{-1}$ DAPI (Calbiochem) in PBS for 3 min, washed again, mounted, and visualized in a fluorescence microscope. The percentage of cells that showed fluorescence above detection level with mAb 5C4 relative to total cells (positive for DAPI) was calculated. This method has been repeatedly used by different groups (Ventoso et al., 2010; Poenisch et al., 2015; Lombardo et al., 2000; Martin-Acebes et al., 2009; Popescu et al., 2011) and provides a good estimate of the antigen expressed (folded protomer in this case). Statistical analysis (Table 2) was performed using KaleidaGraph.

Molecular graphics, structural analyses and sequence comparisons. The Protein Data Bank atomic coordinates for the crystal structure of FMDV C-S8c1 (1fmd) (Lea et al., 1994) were inspected graphically using the programs InsightII (Biosym Technologies), RASMOL (Sayle & Milner-White, 1995) and/or Pymol (DeLano, 2002). Contact and solvent accessibility analyses were carried out using the program WHAT IF (Vriend, 1990). Sequence conservation analysis of capsid proteins corresponding to 250 FMDV isolates and deposited in the GenBank (NCBI) was carried out using the alignment program CLUSTAL W (EMBL-EBI).

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