The effect of the D614G substitution on the structure of the spike glycoprotein of SARS-CoV-2


The majority of currently circulating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viruses have mutant spike glycoproteins that contain the D614G substitution. Several studies have suggested that spikes with this substitution are associated with higher virus infectivity. We use cryo-electron microscopy to compare G614 and D614 spikes and show that the G614 mutant spike adopts a range of more open conformations that may facilitate binding to the SARS-CoV-2 receptor, ACE2, and the subsequent structural rearrangements required for viral membrane fusion.

SARS-CoV-2 | Coronavirus | spike | cryo-EM | D614G

The spike glycoproteins of coronaviruses are responsible for receptor binding and membrane fusion during the initial stages of virus infection. Viruses that have spike proteins containing the amino acid substitution D614G are currently predominant in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, and it has recently been shown that G614 viruses have higher infectivity and produce higher viral loads than D614 viruses.

We, and others, have shown that the D614 spike adopts several different conformations, including a “closed” conformation in which the receptor-binding domain (RBD) is mostly closed and cannot bind to the human ACE2 receptor. We have also shown that both furin cleavage and the presence of ACE2 increase the proportion of the spikes that adopt open conformations and suggested that the D614G substitution could also promote the spike’s “opening.” To better understand the impact of the D614G substitution, we have now solved the cryo-electron microscopy (cryo-EM) structure of the G614 spike and compared it to that of the D614 spike recently solved by us and others.

Results

The expressed G614 protein was stable and suitable for high-resolution single-particle cryo-EM reconstruction. Strikingly, the structures we solved (Table 1) show that the G614 spike exists predominantly in the open, receptor-binding-competent conformation, in contrast to the D614 spike (Figs. 1 and 2A; 87% of G614 spikes have either one or two erect RBDs, compared with 17% in our D614 structure (5) and less than 50% in the structures of D614 reported elsewhere (7, 8), none of which showed two erect RBDs). G614 spikes with two erect RBDs account for 20% of observed particles. This conformation has only previously been observed for the furin-cleaved protein on exposure to ACE2 (6), including by tomography on virions.

The minority of G614 spikes (15%) that do not have one or more RBDs erect adopt a partially closed conformation, which is less tightly packed than the closed conformation of the D614 spike, with several constituents of the G614 spike displaying more local flexibility. Comparison of the contact area between each monomer of the trimer with a single neighboring monomer indicates 4,000 Å² for the closed conformation of the G614 spikes, compared to 5,900 Å² for the closed D614 spike (5). This substantial change in contact area is clearly evident in Fig. 2B, where the RBDs are not packed as closely together in the G614 spikes as they are in the D614 spikes. This more dilated packing arrangement in G614 spikes is also accompanied by a clockwise, rigid-body rotation of the N-terminal domains (NTDs) (as viewed in Fig. 2B) by comparison with the D614 spikes (a similar direction of movement to that observed for D614 spikes upon transition from the closed to the open conformation). There are several regions at the virus-membrane-distal top of the G614 spikes in this closed conformation that display more local flexibility, associated with poorer local resolution (SI Appendix, Fig. 1), than the corresponding regions of the D614 spikes, presumably resulting from the substantial decrease in monomer packing surface. Most notably, these regions include the large loop (residues 468 to 491) at the tip of the RBD and to a lesser extent the rest of the surface of the receptor-binding motif of the RBD as well as surface loops of the NTD that are furthest away from the symmetry axis. Consistent with this looser packing of the RBDs in the closed form of the G614 spike is the lack of density for a bound fatty acid (10) that is present in our D614 spikes close to the RBD/RBD binding interface.

Inspection of the closed and open G614 and D614 spike structures reveals the likely mechanistic reason for the differences in domain orientation (Fig. 2C–E). As reported before, the residue D614, located on the NTD-associated subdomain, in the closed conformation of the D614 spike forms a salt bridge.


PNAS 2021 Vol. 118 No. 9 e2022586118
https://doi.org/10.1073/pnas.2022586118 | 1 of 4

Published February 12, 2021.

The spike proteins of most current severe acute respiratory syndrome coronavirus 2 isolates contain a D614G substitution, by comparison with the spike protein of initial isolates. In this study we present high-resolution, single-particle cryo-electron microscopy structures of the G614 spike variant showing that it adopts a predominantly open conformation, unlike the D614 spike that is mostly closed. We conclude that the D614G substitution promotes “opening” of the spike, priming it for binding to the receptor ACE2 and possibly for its subsequent role in membrane fusion. The observed open conformation of the G614 spike may be the reason for the current virus’ reported increased infectivity and its current predominance.

Significance

The spike proteins of most current severe acute respiratory syndrome coronavirus 2 isolates contain a D614G substitution, by comparison with the spike protein of initial isolates. In this study we present high-resolution, single-particle cryo-electron microscopy structures of the G614 spike variant showing that it adopts a predominantly open conformation, unlike the D614 spike that is mostly closed. We conclude that the D614G substitution promotes “opening” of the spike, priming it for binding to the receptor ACE2 and possibly for its subsequent role in membrane fusion. The observed open conformation of the G614 spike may be the reason for the current virus’ reported increased infectivity and its current predominance.

The authors declare no competing interest.

The open access article is distributed under Creative Commons Attribution License 4.0 (CC BY).

1D.J.B. and A.G.W. contributed equally to this work.

2To whom correspondence may be addressed. Email: Donald.Benton@crick.ac.uk, John.Skehel@crick.ac.uk, Antoni.Wrobel@crick.ac.uk, or Steve.Gamblin@crick.ac.uk.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2022586118/-/DCSupplemental.

Published February 12, 2021.

for review October 29, 2020; reviewed by Wah Chiu and Herbert W. Virgin)
with K854 from the S2 component of the neighboring monomer (5). Concomitant with opening of the RBD, and subsequent receptor engagement, the NTD-associated subdomain undergoes structural rearrangements and a rigid-body shift that lead to breaking of the D614–K854 salt bridge (Fig. 2D). Disruption of the salt bridge correlates with unfolding of the 827 to 855 region of S2, which may prime the downstream, putative fusion-peptide-containing sequence (residues 815 to 825) for membrane fusion, as the S1 and S2 chains become more separate (6). With a glycine residue at position 614 a salt bridge cannot be formed and thus the structures of the G614 spike in the open and closed conformation are very similar to each other in this region and, importantly, similar to the open conformation of the D614 spike (Fig. 2D). In contrast, the local structures of the D614 spike in this region are markedly different between the open and closed forms (Fig. 2E).

**Discussion**

A number of biological features have been reported to distinguish the D614G mutant that may correlate with these differences in structure. They include in particular higher virus loads based on estimates of virus RNA in the infected respiratory tract (4), increases in case fatality (11), and up to ninefold increases in virus production in vitro (2, 12). There does not appear to be evidence of increased transmissibility (13), but from genetic analysis increases in distribution of the variant are consistent with a selective advantage (14). Recently, Yurkovetskiy et al. have addressed several aspects of the mutant viruses’ properties, including the structural basis of the open/closed conformation of the spike, that raise two issues pertinent to our work (12). First, conclusions are made about the extent of the open RBD conformations of G614 spikes that are not corroborated by deposited models or density maps. Our analysis of the deposited density map (12) indicates that it is highly anisotropic, restricting reliable interpretation of the RBD location (SI Appendix, Fig. 2A and B). Second, Yurkovetskiy et al. (12) suggest that the effect of the D614G substitution is mediated by the loss of a hydrogen bond to a threonine residue at position 859 of S2. This mechanism is inferred from earlier studies (4, 7).

**Table 1. Cryo-EM data collection, refinement, and validation statistics**

<table>
<thead>
<tr>
<th>Data collection and processing</th>
<th>Closed D614G (EMD-12229) (PDB ID code 7BNM)</th>
<th>One erect RBD D614G (EMD-12230) (PDB ID code 7BNN)</th>
<th>Two erect RBDs D614G (EMD-12231) (PDB ID code 7BNO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage, kV</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Electron exposure, e⁻/Å²</td>
<td>36.8</td>
<td>36.8</td>
<td>36.8</td>
</tr>
<tr>
<td>Defocus range, μm</td>
<td>-1.0 to -3.0</td>
<td>-1.0 to -3.0</td>
<td>-1.0 to -3.0</td>
</tr>
<tr>
<td>Pixel size, Å</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>Symmetry imposed</td>
<td>C3</td>
<td>C1</td>
<td>C1</td>
</tr>
<tr>
<td>No. of final particle images</td>
<td>30,000</td>
<td>150,000</td>
<td>45,000</td>
</tr>
<tr>
<td>Map resolution, Å (FSC threshold = 0.143)</td>
<td>3.6</td>
<td>3.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Map resolution range, Å</td>
<td>3.5–5.5</td>
<td>3.5–5.5</td>
<td>4.0–8.0</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial model used (PDB ID code)</td>
<td>6ZGE</td>
<td>6ZGG</td>
<td>7A93</td>
</tr>
<tr>
<td>Model resolution, Å (FSC threshold = 0.5)</td>
<td>3.7</td>
<td>3.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Model sharpening B factor, Å²</td>
<td>-102.7</td>
<td>-115.3</td>
<td>-96.7</td>
</tr>
<tr>
<td>Model composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonhydrogen atoms</td>
<td>25,971</td>
<td>25,788</td>
<td>25,509</td>
</tr>
<tr>
<td>Protein residues</td>
<td>3,207</td>
<td>3,207</td>
<td>3,197</td>
</tr>
<tr>
<td>Ligands</td>
<td>63</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>Rmsd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths, Å</td>
<td>0.008</td>
<td>0.003</td>
<td>0.007</td>
</tr>
<tr>
<td>Bond angles, °</td>
<td>0.787</td>
<td>0.576</td>
<td>0.989</td>
</tr>
<tr>
<td>Validation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MolProbity score</td>
<td>1.88</td>
<td>2.11</td>
<td>2.15</td>
</tr>
<tr>
<td>Clashscore</td>
<td>9.22</td>
<td>11.10</td>
<td>12.94</td>
</tr>
<tr>
<td>Poor rotamers, %</td>
<td>0.54</td>
<td>0.00</td>
<td>0.75</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favored, %</td>
<td>94.26</td>
<td>93.05</td>
<td>90.67</td>
</tr>
<tr>
<td>Allowed, %</td>
<td>5.74</td>
<td>6.95</td>
<td>9.33</td>
</tr>
<tr>
<td>Disallowed, %</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

![Mutant (G614) vs WT (D614)](https://doi.org/10.1073/pnas.2022586118)

Fig. 1. Space-filling representation of the two predominant open forms of G614 spike and the predominant closed form of D614 spike. The one- and two-RBD-erect forms of G614 spike make up 87% of particles, while the closed form represents 83% of the D614 spike particles in our previous study (5). The three subunits of spike are colored in blue, goldenrod, and rosy brown for G614 and in lighter shades of the same colors for D614. The molecule is viewed with its long axis vertical.
that did not allow unambiguous modeling of the protein structure in the described region. Considering earlier work from our group and others (6, 9, 15) on the closed RBD form of spike (SI Appendix, Fig. 2C), our conclusion is that the evidence strongly supports the role of a salt bridge between D614 and K848, and not T859, in stabilizing the closed spike conformation.

As a consequence of the D614G substitution, a tightly closed structure observed for the D614 spike is not formed and the G614 spike assembly adopts a greater range of more open and flexible conformations than its D614 counterpart. The greater conformational flexibility of the G614 spike explains how it more readily adopts the receptor-binding-competent conformation and suggests how the presently circulating strains of SARS-CoV-2 might have fitness advantage over the D614 variant and display higher infectivity. At the same time, the more open conformation may result in the exposure of epitopes for additional neutralizing antibodies; indeed, recent reports suggest that the D614G substitution increases the susceptibility of SARS-CoV-2 to neutralization (16). As a consequence, as populations become more exposed to SARS-CoV-2, and as more people acquire an immune reaction to the virus, it is likely that the G614 form will have less selective advantage.

**Materials and Methods**

**Protein Production.** The construct coding for the D614G mutant was based on the furin-uncleavable version of the SARS-CoV-2 spike protein ectodomain with a set of stabilizing mutations (R682S, R685S, K986P, and K987P) that we described before (5).

---

*Benton et al.* PNAS | 3 of 4

The effect of the D614G substitution on the structure of the spike glycoprotein of SARS-CoV-2

---

*https://doi.org/10.1073/pnas.2022586118*
The G614 spike protein was produced very similarly to the D614 spike we described before (3). Briefly, it was expressed in E. coli (Gibco) growing in suspension at 37 °C in an 8% CO2 atmosphere transfected with ExpIEm-amine 293 (Gibco) and 1 mg of DNA per liter of culture. The enhancers were added 20 h after the transfection, according to the manufacturer's instructions (2), and the cells were then moved to 32 °C and the supernatant containing the protein was harvested on the fifth day posttransfection. The collected supernatant was clarified, bound overnight to TALON beads (Takara), briefly washed, eluted with 200 mM imidazole, concentrated, and gel-filtered on a Superdex 200 Increase 10/300 GL column (GE Life Sciences) into 150 mM NaCl and 20 mM Tris, pH 8.

Cryo-EM Sample Preparation and Data Collection. The G614 spike glycoprotein was frozen on 200-mesh Quantifoil R/2 grids glow-discharged for 30 s at 25 mA. Four microliters of spike protein at ~0.5 mg/mL in 150 mM NaCl and 20 mM Tris, pH 8, supplemented with 0.1% octyl glycoside was applied on a grid at 4 °C, blotted for 4 to 4.5 s using a Vitrobot MkIII, and plunged-frozen into liquid ethane.

Data were collected using a Titan Krios operating at 300 kV. Images were recorded using a Falcon III detector operating in electron counting mode. Images were recorded as a 40-s exposure, fractionated into 32 frames, with an exposure of 0.5 ms.

Cryo-EM Data Processing. The frames of the collected movies were aligned using MotionCor2 (17) implemented in RELION (18), and the Contrast Transfer Function (CTF) was fitted using CTFfind4 (19). Particles were picked using cryoYLO (20) using a model trained on manually picked micrographs. Picked particles were subjected to two rounds of two-dimensional classification in cryoSPARC (21), retaining classes with clear secondary structure. An ab initio three-dimensional (3D) model was generated using cryoSPARC, which was used as an initial model for 3D classification in RELION, separating into 10 classes. Particles in classes which pertained to the closed form, one eReB RD form, and two eReB RD forms were refined using RELION 3D-Autofine, followed by Yasuhiko polishing (22).

12. L. Yurkovetskiy et al., Structural and functional analysis of the D614G SARS-CoV-2 spike in suspension at 37 °C in an 8% CO2 atmosphere transfected with ExpIEm-amine 293 (Gibco) and 1 mg of DNA per liter of culture. The enhancers were added 20 h after the transfection, according to the manufacturer's instructions (2), and the cells were then moved to 32 °C and the supernatant containing the protein was harvested on the fifth day posttransfection. The collected supernatant was clarified, bound overnight to TALON beads (Takara), briefly washed, eluted with 200 mM imidazole, concentrated, and gel-filtered on a Superdex 200 Increase 10/300 GL column (GE Life Sciences) into 150 mM NaCl and 20 mM Tris, pH 8.