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# The role of SARS-CoV-2 genetic background in the emergence and success of spike mutations: the case of the spike A222V mutation

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**Background.** Most SARS-CoV-2 variants of concern present mutations on the spike (S) protein associated with higher infectivity and/or resistance to vaccines and antibody therapies. Many key mutations map to the Receptor Binding Domain (RBD) and are involved in the interaction with the host receptor ACE2 or with antibodies. Although most mutations are well-characterized, these studies often rely on isolated RBDs or in proline-substituted stabilized spikes, which could mask long distance effects of mutations outside the RBD but affecting the degree of exposure of the latter. Our aim was to develop an experimental platform for rapid production and characterization of spike variants "as natural as possible" to better understand the effect of certain mutations in the stability and interaction with ACE2 or antibodies.

**Methods.** 1) Mutagenesis and cloning of S variants in appropriate expression vectors. 2) Production of wild-type and mutated S, RBD and ACE2 proteins using baculovirus/insect and mammalian cultures. 3) Monitor stability of S variants by thermofluor and negative-staining electron microscopy (EM); 4) Analysis of protein conformation in solution by size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) and native-red electrophoresis. 5) Biolayer interferometry protein-protein interaction assays. 6) Structural characterization by cryo-EM. 7) Molecular dynamics (MD) simulations of conformational changes associated to certain mutations.

**Results.** We developed a pipeline for evaluating biophysically and structurally the impact of different mutations on the SARS-CoV-2 spike protein. We produced >30 S protein variants, most of them without stabilizing prolines and few with intact furin site. We also produced 9 RBD variants and >70 variants of monomeric and dimeric ACE2. Combining different techniques, we analyzed the stability of the recombinant proteins and showed that the spike variants are in an equilibrium between at least two conformations, that is influenced by temperature and by certain mutations. We also measured how the mutations affect the binding kinetics of the interaction between the spike or RBD variants with ACE2. Using cryoEM and MD simulations we provide detailed information on the impact of mutation A222V (characteristic of the 20E (EU1) and the Delta subvariant AY.4.2) on the transmissibility of the virus.

**Conclusion.** This collaborative consortium of several CSIC groups resulted in a pipeline for the fast characterization of known and future spike variants, and of protein ancestors. We observed mutation-driven changes in the stability and binding of the S variants, a conformational equilibrium of the spike, and a different behavior with artificially stabilized proteins. The results support our working hypothesis that the use of "as natural as possible" S proteins is important to accurately understand the molecular mechanisms governing SARS-CoV-2 infectivity.

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