



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

## Minireview

## Virus vaccines: proteins prefer prolines

Rogier W. Sanders<sup>1,2,\*</sup> and John P. Moore<sup>1,\*</sup><sup>1</sup>Department of Microbiology and Immunology, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065, USA<sup>2</sup>Department of Medical Microbiology and Infection Prevention, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, the Netherlands\*Correspondence: [rws2002@med.cornell.edu](mailto:rws2002@med.cornell.edu) (R.W.S.), [jpm2003@med.cornell.edu](mailto:jpm2003@med.cornell.edu) (J.P.M.)<https://doi.org/10.1016/j.chom.2021.02.002>

## SUMMARY

Most viral vaccines are based on inducing neutralizing antibodies (NAbs) against the virus envelope or spike glycoproteins. Many viral surface proteins exist as trimers that transition from a pre-fusion state when key NAb epitopes are exposed to a post-fusion form in which the potential for virus-cell fusion no longer exists. For optimal vaccine performance, these viral proteins are often engineered to enhance stability and presentation of these NAb epitopes. The method involves the structure-guided introduction of proline residues at key positions that maintain the trimer in the pre-fusion configuration. We review how this technique emerged during HIV-1 Env vaccine development and its subsequent wider application to other viral vaccines including SARS-CoV-2.

## INTRODUCTION

Vaccines are a crucial component of humankind's fight against infectious diseases, particularly pathogenic viruses. This has been underscored in the past 12 months, when a new virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has triggered a pandemic. More than 100 million people are now known to have been infected, and well over 2 million have died. But the world's scientific resources have also been mobilized to create, evaluate, and produce multiple vaccines with unprecedented speed and magnitude. There are now substantial indications that these vaccines may significantly curb the pandemic during 2021. The speed of the international response was based on the repurposing of existing vaccine technologies. All the leading candidates rely wholly or in substantial part on the ability of the SARS-CoV-2 spike (S) protein to stimulate the production of virus neutralizing antibodies (NAbs) (Graham et al., 2019; Krammer, 2020; Klasse et al., 2021; Moore and Klasse, 2020). For optimal performance, the S protein is almost always engineered to increase its stability, its yield during production processes, and its presentation of key NAb epitopes. The stabilization method used involves the structure-guided introduction of proline substitutions in specific positions that sustain the S protein in the pre-fusion trimeric form. Here, we review how this technology originated in research on HIV-1 envelope (Env) glycoprotein trimers and was then exploited to create improved versions of Env-protein vaccines against other viruses, including but not limited to respiratory syncytial virus (RSV) and SARS-CoV-2.

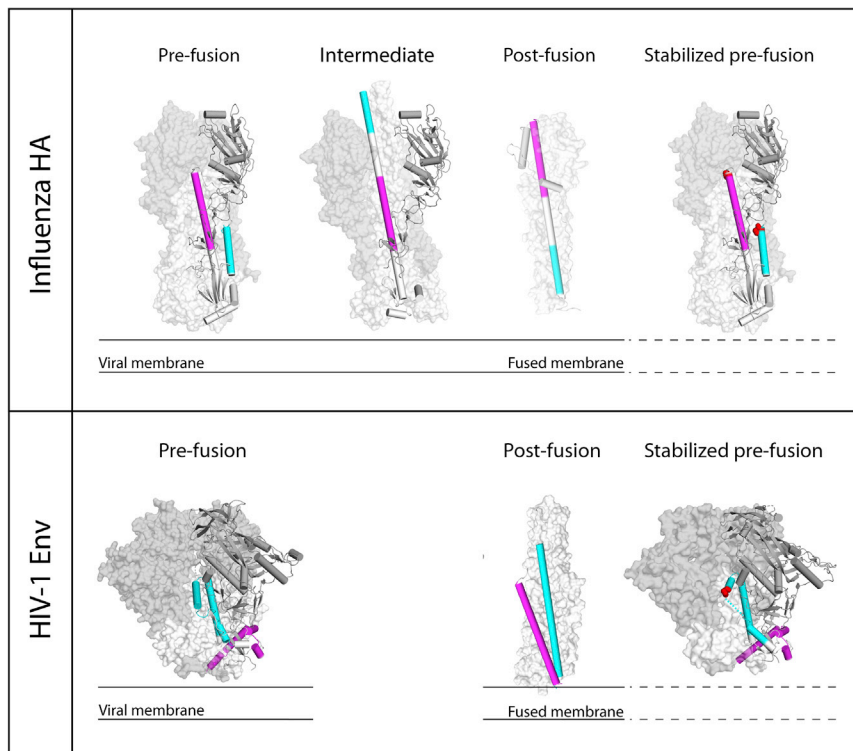
## How class I fusion proteins function

Enveloped viruses such as HIV-1, SARS-CoV-2, RSV, and others fuse at the cell surface or within endosomes in a process that is triggered when their Env (S) proteins, known as class I fusion proteins, bind to one or more cell surface receptors (Kielian, 2014). Although each family of viruses has

evolved its own mechanism of receptor engagement, there are common elements to the events that then drive the fusion of virus and cell membranes and initiate cellular infection. The class I fusion proteins are trimers, each of which comprises a receptor-binding subunit attached to a fusion-mediating subunit, such that the complete entity is a trimer of heterodimers (Kielian, 2014; Murin et al., 2019). The fusion-mediating subunit is anchored to the virus via a membrane-spanning domain. Once assembled and processed by a furin (or sometimes another) protease within the cell, the fusion proteins are maintained in a metastable state known as the pre-fusion conformation (Figure 1). In simplistic terms, the receptor-binding subunit overlays its fusion-mediating counterpart and temporarily locks it into an energetically unfavorable conformation. However, when the receptor-binding subunit engages the appropriate cellular receptor(s), its structure alters in a way that releases the fusion-mediating subunit to itself undergo profound conformational changes (Figure 1). A hydrophobic region at the N terminus of the fusion-mediating subunit now becomes accessible and can insert into the cell membrane, thereby creating a protein linkage between the virus and cell membranes. The release of pent-up energy is sufficient to pull the two membranes together in a way that allows them to fuse. The fusion protein subunits are now in their post-fusion conformations (Kielian, 2014).

These substantial rearrangements within and among the subunits of the class I fusion proteins can only occur if the pre-fusion trimer is metastable. An over-tight association between the subunits would not allow receptor engagement to drive the conformational changes necessary for virus-cell fusion. However, the transition to the post-fusion form can occur spontaneously (i.e., without receptor triggering). When this happens, the ability to drive virus-cell fusion is lost, and the post-fusion proteins trigger antibody responses that are unwanted or worse (see below). Virus evolution creates a "sweet spot," a trimer with just the right degree of stability. Unfortunately, in doing so,





**Figure 1. The influenza HA and HIV-1 and class I fusion proteins**

Upper panels: structural models of the pre-fusion, intermediate, and post-fusion forms of the influenza HA trimer using PDB coordinates 4UNW, 6Y5K, and 1QU1. The prolines that block HA function (Qiao et al., 1998) are indicated in red in the right panel. The prolines are proposed to block the formation of the long helices that are present in the intermediate and post-fusion conformations. Lower panels: structural models of the pre-fusion and post-fusion forms of the full-length HIV-1 Env trimer and the recombinant BG505 SOSIP.664 trimer are shown, as indicated, using PDB coordinates 5FUU, 2EZO, and 6VO1. On one protomer of each trimer, the key helical regions in the gp41 fusion-subunit are highlighted in turquoise (HR1) and magenta (HR2). In the post-fusion form, the previously separated short segments of HR1 and HR2 have been brought together into long helices. On the SOSIP.664 trimer, the position of the I559P substitution is marked in red. It is located in an unstructured region that links two helical elements of HR1, and hinders their transition to the longer helix when the fusion potential of the trimer is activated. Accordingly, the trimer is stabilized in the pre-fusion configuration. The figure was generated by Philip Brouwer.

evolution also creates substantial problems for the designers of vaccines based on the class I fusion proteins.

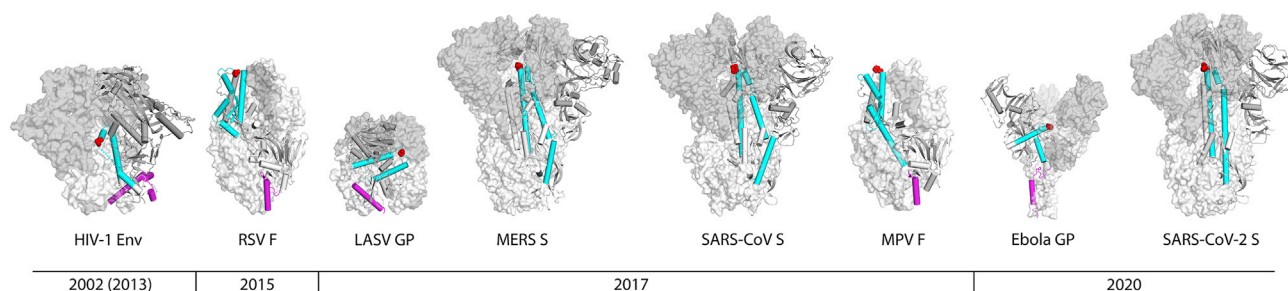
The class I viral fusion proteins are vaccine candidates because they present epitopes for virus-NABs (Murin et al., 2019; Ward and Wilson, 2017). For many viruses, they are in fact the only target for NABs. When used as immunogens, they are intended to induce NABs that, in turn, will bind to the same proteins on the virus surface, impairing their functions and neutralizing infectivity. Most NAB epitopes are located on the receptor-binding subunits, and NABs often, but not always, interfere with receptor binding. In most cases, the relevant NAB epitopes are displayed optimally or only when the trimer is in its pre-fusion conformation. Conversely, post-fusion or other, aberrant protein conformations induce mostly non-neutralizing antibodies (non-NABs) that have no or limited protective capacity. In some cases, non-NABs can even be harmful. Thus, clinical trials of RSV vaccines in the 1960s had to be terminated because vaccinated infants more frequently developed severe disease after subsequent infection compared to controls, and two young children died from RSV infection as a consequence. The proposed mechanism involves the induction of non-NABs resulting in immune complex deposition and complement activation (Browne et al., 2020; Graham, 2020). It follows, then, that RSV vaccines and, by extension, others based on class I fusion proteins are best based on trimers that are in the pre-fusion configuration (Graham, 2016; Graham et al., 2019). But herein lies the problem: the metastable nature of the pre-fusion trimer means that it is very difficult to produce in the vaccine context, particularly when expressed as a recombinant protein. Put simply, the trimer undergoes spontaneous conformational changes and de-

cays into the post-fusion form far too rapidly to be useful. Usually, the various subunits simply dissociate, leaving too few pre-fusion trimers to work with and a considerable quantity of unwanted debris.

Overcoming this obstacle requires the application of protein-engineering methods intended to create stable mimics of the pre-fusion trimer that present key NAB epitopes with appropriate fidelity. A related goal is to minimize the immunogenicity of problematic epitopes for non-NABs.

### The HIV-1 Env SOSIP trimer is the prototype proline protein

The HIV-1 Env trimer was the first to be engineered for increased stability as a recombinant protein. Recombinant Env/S proteins are often produced in secreted form by eliminating the trans-membrane and internal regions that normally link the trimer to the virus (or, in this context) the producer cell membrane. Secreted, soluble proteins are produced in higher yields and are far more straightforward to purify. However, the truncation procedure further destabilizes the pre-fusion HIV-1 Env trimer, which almost immediately disintegrates into its gp120 and gp41 subunits. Eliminating the protease cleavage site between gp120 and gp41 to keep these subunits covalently linked was initially considered to solve this problem, but it became apparent that the modified recombinant trimer did not appropriately mimic the native, virion-associated form (Ringe et al., 2013). Thus, an alternative approach was developed that allowed and embraced furin cleavage while also linking the gp120 and gp41 subunits via an engineered intermolecular disulfide bond, designated SOS (Binley et al., 2000). This strategy was successful in preventing gp120-gp41 dissociation, but the resulting trimers still fell apart, in this case via instabilities within the gp41 subunits. The end product was disulfide-linked gp120-gp41 monomers. It was reasoned that the gp41 subunits were undergoing some form of



**Figure 2. Location of proline substitutions in class I fusion proteins**

The X-ray or cryo-EM structures of the pre-fusion forms of the indicated class I fusion proteins are indicated, together with the years they were obtained. The coloring system used is the same as in Figure 1. PDB coordinates are HIV-1 Env (6VO1), RSV F protein (5C69), LASV GP (5VK2), MERS S protein (5W9J), SARS-CoV S protein (5X58), hMPV F protein (5WB0), Ebola GP (6VKM), and SARS-CoV-2 S protein (6VXX). The figure was generated by Philip Brouwer.

conformational change whereby they transitioned toward the post-fusion form. The analytical techniques available at that time were not adequate for proving this point, and the absence of a structure of the pre-fusion trimer complicated engineering efforts to stabilize the pre-fusion conformation. Using available information on the post-fusion six-helix bundle structure of the gp41 subunits, and the emerging knowledge of how class I fusion proteins function, a substitution was designed that would prevent the formation of the six-helix bundle that is a critical element of the transition to the post-fusion gp41 configuration (Sanders et al., 2002). This approach was based on the hypothesis that disfavoring the post-fusion form would help maintain the gp41 subunit, and as a consequence the entire Env trimer, in its pre-fusion configuration. Studies on influenza hemagglutinin (HA) had shown that appropriately targeted proline substitutions strongly impaired the fusion function of that class I fusion protein (Qiao et al., 1998). Empirical testing of various amino acids and positions identified a proline substitution at isoleucine residue-559 as the most effective (Sanders et al., 2002) (Figure 1). The chemical properties of proline both disfavor helix formation and also confer local rigidity to a polypeptide chain. The resulting I559P change was designated IP. When it was combined with the aforementioned SOS disulfide bond, the SOSIP trimer emerged. Additional refinements over a multi-year period led to the development of the BG505 SOSIP.664 trimer, which could be produced and purified in high yield and thoroughly evaluated in preclinical studies (Sanders et al., 2013, 2015; Sanders and Moore, 2017). High-resolution X-ray and cryoelectron microscopy (cryo-EM) structures of ever-increasing detail were soon obtained, revealing known and previously undiscovered NAb epitopes, as well as spawning additional structure-guided design improvements (Ward and Wilson, 2017). The latter include additional disulfide bonds and cavity-filling mutations that confer additional stability and improved antigenicity (Graham et al., 2019; Sanders and Moore, 2017; Torrents de la Peña and Sanders, 2018). The structures also showed that the I559P substitution did indeed prevent formation of a helix (Ward and Wilson, 2017). The SOSIP design is now the basis of multiple pre-fusion trimer variants that are in or approaching the clinical trial stage of vaccine development (see below). Several of the additional stabilization strategies for which the original SOSIP trimers were the test-beds are also now incorporated into pre-fusion trimers from other viruses (see below).

### Adapting the proline-stabilization method to pneumoviridae

The proline substitution method was next applied to another vaccine-relevant but unstable class I fusion protein: the fusion (F)-protein of the paramyxovirus RSV (Krarup et al., 2015) (Figure 2). RSV causes respiratory infections that can be severe in young children. The absence of an effective vaccine has propelled many efforts to generate an immunogen that elicits robust NABs. Here, the design efforts were guided by prior knowledge of the pre-fusion RSV F structure that was, in turn, aided by the identification of a monoclonal NAb that specifically recognized the protein's pre-fusion conformation (McLellan et al., 2013). The exploration of several stabilization methods included the addition of a C-terminal trimerization domain, altering the exposure of the fusion peptide, eliminating the cleavage site, and adding disulfide bonds and cavity-filling mutations, concepts that were all rooted in strategies tested successfully on the HIV-1 Env trimer (Sanders et al., 2013; Sanders and Moore, 2017). While several of these changes contributed stability to the RSV F pre-fusion trimer, a particularly valuable method focused on proline substitutions of residues E161 and S215 in the hinges linking the alpha-2 helix to alpha-3 and the alpha-4 helix to alpha-5, respectively. The goal was to again prevent formation of a long helix that is an important element of the intermediate and post-fusion forms by stabilizing key protein elements involved in that process. Among multiple substitutions evaluated, the greatest effect was conferred by the S215P change (Krarup et al., 2015). It was noted that S215P is at a structurally equivalent position to the I559P substitution in the HIV-1 Env SOSIP trimer, which in turn suggested there may be mechanistic generality among both the class I fusion proteins and the methods that stabilize them for vaccine purposes. A high-resolution X-ray crystallography structure enabled the impact of the most useful amino acid changes, including S215P, to be better understood (Krarup et al., 2015). The clinical evaluation of the stabilized RSV F trimers is discussed below.

The proline-substitution, helix-disruption method was also integral to the production of stable, pre-fusion trimers from the human metapneumovirus (hMPV) F-glycoprotein ectodomain (Battles et al., 2017) (Figure 2). hMPV is also a pneumovirus that causes respiratory disease and shares some molecular features with RSV. By analogy to the S215P change in RSV F, three residues in the loop linking the alpha-4 and alpha-5 helices were

identified as appropriate positions to assess the impact of proline substitutions: A185, I184, and D186. A comparative study of the three resulting proteins showed that the A185P variant was the most efficiently expressed, and hence it was selected for further studies. Again, a high-resolution crystal structure (2.6 Å) allowed the A185P variant trimer architecture to be defined in detail (Battles et al., 2017).

### ...and to arenaviridae and filoviridae

Viruses in these families cause lethal hemorrhagic fevers. The class I fusion protein on the arenavirus, Lassa virus (LASV), known as the glycoprotein complex (GPC), comprises the GP1 receptor-binding and GP2 fusion-mediating subunits. In this regard, its architecture is similar to HIV-1 Env. Accordingly, strategies that worked for SOSIP trimers were adapted to stabilize the GPC as a fully cleaved, soluble, pre-fusion trimer known as GPCysR4 (Hastie et al., 2017) (Figure 2). The approach taken included an inter-subunit disulfide bond, an improved cleavage site between GP1 and GP2 and the E329P substitution in the metastable region of helical region-1 (HR1) within the GP2 subunit. As with its HIV-1 Env and RSV F counterparts, the modifications allowed the generation of a high-resolution (3.2 Å) X-ray structure that, in turn, revealed where the stabilization changes were located and how they acted (Figure 2).

Producing glycoprotein (GP) trimers from the Ebola (EBOV) and Marburg filoviruses required targeted proline-substitutions as well as additional modifications, such as the removal of the mucin-like domains (Rutten et al., 2020) (Figure 2). Although the overall architecture of the filovirus GPs is quite different from HIV-1 Env and RSV F, all of them are class I fusion proteins. Hence, the proline-based prevention of formation of helices that are involved in the transition from the pre- to post-fusion forms was effective at stabilizing the Ebola GP trimers. Proline substitutions at positions 575, 576, 577, 579, and 581 and the T577P/L579P double change were compared for their impacts on trimer yield and thermal stability in the context of one or two different EBOV sequences, with the T577P variant emerging on top in both cases. Similar helix-disrupting changes were also beneficial to stabilizing the Marburg GP. When combined with an additional stabilization change elsewhere, K588F, a high quality Makona EBOV GP trimer, could be produced for crystallization studies that yielded a 3.5 Å-resolution structure. In turn, the structure provided additional insights into the workings of the T577P and K588F stabilization changes (Rutten et al., 2020).

### Coronavirus S protein vaccines benefit from proline-stabilization

The first foray into the world of CoV S proteins involved the Middle East respiratory syndrome (MERS)-CoV S protein (Pallesen et al., 2017) (Figure 2). MERS-CoV infects camels and horses but is highly dangerous to humans although transmitted with low efficiency between members of our species. Its S protein is another example of a class I fusion protein with two basic subunits: S1 contains the receptor-binding domain (RBD), while S2 contains the fusion machinery. To stabilize the S protein in its pre-fusion form, the by now classic mechanism of disrupting helix-helix interactions within S2 was adopted. Specifically, the V1060P and L1061P substitutions were made within the loop region between HR1 and the central helix (Figure 2). The resulting S-2P mutant

protein was expressed at a 50-fold higher yield than the wild-type construct, allowing its biophysical, receptor-binding, and antigenicity properties to be determined. High-resolution (3.6 Å) cryo-EM structures of the S-2P protein as a Fab-complex were also obtained, revealing valuable information on how the RBD of this type of viral S protein functions to initiate the fusion process. In a comparative immunogenicity study in mice, the S-2P trimer induced NAbs at significantly higher titers than its wild-type counterpart and an S protein monomer. Additional, less detailed studies showed that the S-2P strategy was also effective when applied to the corresponding S proteins from the also lethal SARS-CoV-1 and the HKU1 betacoronavirus that causes common colds (Pallesen et al., 2017; Walls et al., 2019).

The paradigm now established for making stabilized S proteins was of rapid and substantial benefit to immunogen design right at the start of the vaccine response to the COVID-19 pandemic that was triggered by the entry of SARS-CoV-2 into the human population. When the viral sequence became available in January 2020, various research groups rapidly applied the S-2P mutations, in this case K986P and V987P, to the S protein. A 3.5 Å cryo-EM structure of the stabilized S protein was soon determined and its antigenicity and ACE2 receptor-binding properties were explored (Wrapp et al., 2020) (Figure 2). Multiple S protein-based vaccine programs were soon initiated, and some advanced through the various clinical stages within the course of 2020 (Krammer, 2020; Moore and Klasse, 2020; Klasse et al., 2021). The leading vaccines all use the S-2P stabilization method, with the exception of the S protein expressed by the Oxford/AstraZeneca ChAdOx1 nCoV-19 Adenovirus Ad26 vector, which has a wild-type sequence (van Doremalen et al., 2020). Information on the exact design of the S proteins expressed by the CanSinoBio Ad5-nCoV and the Gamalaya Institute's dual Ad26 plus ChAd vectors has not been published, but they are likely to be wild type (Klasse et al., 2021). The immunogenicity benefits of S protein trimer stabilization are summarized below.

Additional stabilization technologies have since been fruitfully applied to the SARS-CoV-2 S protein via structure-guided design (Hsieh et al., 2020; Juraszek et al., 2020). In one study, various disulfide bonds and cavity-filling mutations were evaluated and found to be useful for increasing yield and stabilities. However, the greatest benefits were seen with the HexaPro variant, in which four more proline substitutions (F817P, A892P, A899P, A942P) were added to the original S-2P design to further impede loop-to-helix transitions (Hsieh et al., 2020). In an independent but conceptually similar approach, the most effective additional changes were found to be D614N, A892P, A942P, and V987P. Taken together, these modifications further improved the yield stability and antigenicity of the resulting S protein variant, and their collective impacts were studied at the structural level via cryo-EM (Juraszek et al., 2020). Of note is that two of these substitutions, A892P and A942P, were identified independently in both studies. Data on the immunogenicity of these two new, additionally stabilized constructs are not yet available.

As noted above, the S-2P mutations were clearly beneficial to the immunogenicity of the MERS-CoV S protein (Pallesen et al., 2017). Accordingly, most of the high-level SARS-CoV-2 S protein vaccine programs simply incorporated the twin proline substitutions as a matter of course. There were, however, several immunogenicity experiments in small animals and/or macaques in

which various forms of the S protein were directly compared, including the wild-type and S-2P versions. In the earliest studies, the most commonly used sequence changes in the SARS-CoV-2 S proteins were S-2P and a furin cleavage-site knockout. To assess their impact on immunogenicity, four S proteins were produced that contained neither, one, or both of these changes (Amanat et al., 2020). When mice were immunized twice with each of the four proteins in Addavax adjuvant, antibody and NAb titers were fairly similar after the second dose, with the two cleavage-site mutant proteins inducing the highest titers. However, when the experiment was extended to include a SARS-CoV-2 challenge, the strongest protection was seen in the group given the double mutant (S-2P plus cleavage-site knockout) S protein (Amanat et al., 2020). In a macaque study, seven recombinant Ad26-based vectors, each expressing a different version of the S protein in an adenovirus vector, were given once to groups of 4–6 rhesus macaques before intranasal challenge 6 weeks later with SARS-CoV-2 (Mercado et al., 2020). When antibody responses were measured and protection was assessed by viral load measurements, the virus vector expressing the soluble S.PP (i.e., S-2P) protein outperformed its wild-type counterpart (Mercado et al., 2020). A mouse immunogenicity experiment that also compared the Ad26 virus variants led to a similar conclusion (Bos et al., 2020). Accordingly, Janssen chose the S.PP construct to become its Ad26.COVS.2 clinical vaccine candidate. A comparison of DNA vaccines in macaques showed that the S.dTM.PP construct, a soluble S protein containing the two proline substitutions, a cleavage site knockout, and a trimerization domain, provided stronger protection against SARS-CoV-2 challenge than the corresponding S.dTM wild-type soluble S protein (Yu et al., 2020). Comparative immunogenicity experiments in mice also led to the inclusion of the same double-proline substitution (plus a furin cleavage site knockout) in the S protein expressed by the Sanofi Pasteur clinical mRNA vaccine (initially designated 2P/GSAS but, for clinical trials, renamed as MRT5500). The choice was based on a comparison of four mRNA constructs expressing S proteins that contained either, both, or neither of the above two changes, with an antibody plus NAb titer endpoint. No virus challenge was performed (Kalinin et al., 2020).

In summary, stabilization of the SARS-CoV-2 S protein in its pre-fusion form via the dual-proline method, in some cases combined with other modifications, clearly increases the yield of trimers that can be produced and purified. That factor alone would be beneficial to vaccine development, but there is also compelling evidence that S protein trimer stabilization also improves immunogenicity and protective capacity.

### Clinical trials of Proline-stabilized Class I fusion proteins

Stabilized HIV-1 pre-fusion Env trimers based on the SOSIP design have now been successfully produced and purified under the conditions required for human clinical trials (Table 1). They include the BG505 SOSIP.664 prototype, the DS-SOSIP version that is also based on the BG505 genotype, and a multiply modified variant, BG505 SOSIP.v4.1-GT1.1 that is designed to engage the human germline precursors of broadly active NAbS. SOSIP-based trimers from diverse HIV-1 isolates, consensus sequences, and B cell mosaic sequences are also being evaluated in humans (Table

1). These trials began in late 2019, but their progress has been slowed by the COVID-19 pandemic, and no immunogenicity data are yet publicly available.

As noted earlier, serious side effects, including deaths, occurred in clinical trials of RSV vaccines in the 1960s and were considered to be attributable to the induction of a low ratio of NAbS to non-NAbS (Browne et al., 2020; Graham, 2020). In a much more recent Phase 2b/3 trial, a post-fusion form of recombinant RSV F protein failed to protect older adults and was, again, associated with a poor NAb to non-NAb ratio (Falloon et al., 2017; Graham 2017). However, progress toward a safe and effective vaccine was seen in a Phase 2a trial of the Ad26.RSV.preF vaccine (Sadoff et al., 2020). Here, the stabilized, pre-fusion form of the RSV F protein described above was delivered via an Ad26 vector (Krarup et al., 2015; Sadoff et al., 2020). The outcome was an acceptable safety profile and, of particular relevance, a marked increase in the ratio of the desired NAbS that inhibit virus infection to the undesired non-NAbS that drive infection enhancement (Sadoff et al., 2020).

Hundreds of SARS-CoV-2 vaccine development programs based on S proteins have been initiated during 2020, and dozens are now in human clinical trial stage, including Phase 3 (Krammer, 2020; Klasse et al., 2021) (Table 1). Initial positive data on the efficacy of four of them were reported in press releases from November, 2020 (summarized in Klasse et al., 2021). Two papers on the Phase 3 trials have been published (Voysey et al., 2021; Polack et al., 2020). The Pfizer/BioNTech and Moderna mRNA vaccines are approved for widespread use. The S proteins in the Pfizer/BioNTech, Moderna, and CureVac mRNA vaccines; the Janssen Ad26 vector; and the Novavax recombinant protein vaccines include the two-proline stabilizations, as do most others (Krammer, 2020; Klasse et al., 2021). The Ad26 vector system used in the SARS-CoV-2 S protein vaccine trials is the same as the one that delivered the stabilized RSV F protein (Sadoff et al., 2020; Mercado et al., 2020). The AstraZeneca ChAdOx1 adenovirus vaccine is, in contrast, based on an unmodified S protein (van Doremalen et al., 2020; Voysey et al., 2021). NAb responses to these vaccines vary widely, although cross-study comparisons are compromised by variations in how the titers are determined and presented (Klasse et al., 2021; Krammer, 2020; Moore and Klasse, 2020). As the vaccine design also varies, it is not possible to know whether S protein stabilization has benefitted the human antibody response to S proteins. The animal studies summarized above imply that this is the likely outcome, however, which may be relevant to the apparently reduced efficacy of the non-stabilized AstraZeneca ChAdOx1 adenovirus vaccine compared to the mRNAs (Klasse et al., 2021; Polack et al., 2020; Voysey et al., 2021).

### Conclusion

The stabilization of trimeric class I fusion proteins into their pre-fusion forms has now become a standard vaccine development procedure for which there is a substantial blueprint. The methodology invariably includes introducing one or more proline substitutions into a loop between two helical regions of the fusion-mediating subunit; these changes hinder the transition of the protein toward the post-fusion form and hence stabilize the desired pre-fusion trimer. Inter- or intra-subunit disulfide bonds, cavity-filling substitutions, and other modifications have

**Table 1. Ongoing and completed clinical trials of viral vaccines based on proline-stabilized class I fusion proteins**

Virus	Vaccine	Type	Proline	Phase	n	Location	Trial ID	Sponsor
HIV-1	BG505 SOSIP.664	Protein	I559P	1	60	USA, Kenya	NCT03699241	IAVI
	BG505 SOSIP.664	Protein	I559P	1	105	USA	NCT04177355	NIAID
	ConM SOSIP.v7	Protein	I559P	Exp	30	UK	NCT03816137	ICL
	ConM SOSIP.v7	Protein	I559P	1	24	Netherlands	NCT03961438	AMC
	BG505 DS-SOSIP	Protein	I559P	1	16	USA	NCT03783130	NIAID
	Trivalent MosM SOSIP.v7	Protein	I559P	Exp	32	UK	NCT04046978	ICL
	BG505 GT1.1	Protein	I559P	1	48	USA, Netherlands	NCT04224701	IAVI
RSV	Ad26.RSV.preF	Adenovirus	S215P	1	24	Belgium	NCT03795441	Janssen
	Ad26.RSV.preF	Adenovirus	S215P	1	73	USA	NCT02926430	Janssen
	Ad26.RSV.preF	Adenovirus	S215P	2a	180	USA	NCT03339713	Janssen
	Ad26.RSV.preF	Adenovirus	S215P	1/2a	48	USA, Finland	NCT03303625	Janssen
	Ad26.RSV.preF	Adenovirus	S215P	2a	64	UK	NCT03334695	Janssen
SARS-CoV-2	mRNA-1273	mRNA	K986P V987P	3	30,000	USA	NCT04470427	Moderna
	BNT162b2	mRNA	K986P V987P	3	44,000	USA, Germany, Argentina, Brazil, South-Africa, Turkey	NCT04368728	BioNTech
	Ad26.COVID2.S	Adenovirus	K986P V987P	3	60,000	USA, Argentina, Brazil, Chile, Colombia, Mexico, Peru, South-Africa	NCT04505722	Janssen
	Ad26.COVID2.S	Adenovirus	K986P V987P	3	30,000	USA, Belgium, Colombia, France, Germany, Philippines, South-Africa, Spain, UK	NCT04614948	Janssen
	NVX-CoV2373	Protein	K986P V987P	3	15,000	UK	NCT04583995	Novavax
	NVX-CoV2373	Protein	K986P V987P	3	30,000	USA	NCT04611802	Novavax
	CVnCoV	mRNA	K986P V987P	3	36,500	Germany and others	NCT04652102	Curevac

This table lists all ongoing and completed human studies with proline-stabilized HIV-1 Env and RSV F that we are aware of. Considering the large number of proline stabilized SARS-CoV-2 S-based vaccines that are now in human clinical trials, with new ones starting at regular intervals, we have listed only the vaccines that are furthest along in the evaluation process (e.g., in Phase 3 trials). As even this subset of trials is ever-expanding, our listing is not intended to be definitive, only exemplary. For details of what vaccines are at what stage of the clinical testing programs, it is best to consult routinely updated websites such as <https://www.nytimes.com/interactive/2020/science/coronavirus-vaccine-tracker.html> or <https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines>.

all been applied. High-resolution structures, have guided the positioning of these stabilization changes, usually in an iterative process. Most of these strategies were derived during years of research on the HIV-1 Env trimer and have been successfully extended to multiple counterparts from other viruses. Stabilized pre-fusion trimers from RSV and HIV-1 are now in clinical trials, with promising data emerging on the antibody response to RSV F. The SARS-CoV-2 S protein-based vaccines created during 2020 usually incorporate the dual-proline modification that can be traced back, first to the I559P change made to the HIV-1 Env trimer in 2002 and from there, to stabilization work on RSV F, MERS-CoV S, and other class I fusion proteins.

#### ACKNOWLEDGMENTS

Work in the Moore and Sanders labs is supported by National Institutes of Health grant P01 AI110657, the Bill and Melinda Gates Foundation grants

OPP1132237 and INV-002022, the European Union's Horizon 2020 research and innovation program under grant agreement no. 681137, and a Vici grant from the Netherlands Organization for Scientific Research (NWO). We thank Philip Brouwer for preparing Figures 1 and 2 and Hans Langedijk for inspirational discussions.

#### REFERENCES

- Amanat, F., Strohmaier, S., Rathnasinghe, R., Schotsaert, M., Coughlan, L., García-Sastre, A., and Krammer, F. (2020). Introduction of two prolines and removal of the polybasic cleavage site leads to optimal efficacy of a recombinant spike based SARS-CoV-2 vaccine in the mouse model. *bioRxiv*. <https://doi.org/10.1101/2020.09.16.300970>.
- Battles, M.B., Más, V., Olmedillas, E., Cano, O., Vázquez, M., Rodríguez, L., Melero, J.A., and McLellan, J.S. (2017). Structure and immunogenicity of pre-fusion-stabilized human metapneumovirus F glycoprotein. *Nat. Commun.* 8, 1528.
- Binley, J.M., Sanders, R.W., Clas, B., Schuelke, N., Master, A., Guo, Y., Kajumo, F., Anselma, D.J., Maddon, P.J., Olson, W.C., and Moore, J.P. (2000). A recombinant human immunodeficiency virus type 1 envelope glycoprotein

complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. *J. Virol.* **74**, 627–643.

Bos, R., Rutten, L., van der Lubbe, J.E.M., Bakkens, M.J.G., Hardenberg, G., Wegmann, F., Zuijdgeest, D., de Wilde, A.H., Koornneef, A., Verwilligen, A., et al. (2020). Ad26 vector-based COVID-19 vaccine encoding a prefusion-stabilized SARS-CoV-2 Spike immunogen induces potent humoral and cellular immune responses. *NPJ Vaccines* **5**, 91.

Browne, S.K., Beeler, J.A., and Roberts, J.N. (2020). Summary of the Vaccines and Related Biological Products Advisory Committee meeting held to consider evaluation of vaccine candidates for the prevention of respiratory syncytial virus disease in RSV-naïve infants. *Vaccine* **38**, 101–106.

Falloon, J., Yu, J., Esser, M.T., Villafana, T., Yu, L., Dubovsky, F., Takas, T., Levin, M.J., and Falsey, A.R. (2017). An adjuvanted, postfusion F protein-based vaccine did not prevent respiratory syncytial virus illness in older adults. *J. Infect. Dis.* **216**, 1362–1370.

Graham, B.S. (2016). Vaccines against respiratory syncytial virus: The time has finally come. *Vaccine* **34**, 3535–3541.

Graham, B.S. (2017). Vaccine development for respiratory syncytial virus. *Curr. Opin. Virol.* **23**, 107–112.

Graham, B.S. (2020). Rapid COVID-19 vaccine development. *Science* **368**, 945–946.

Graham, B.S., Gilman, M.S.A., and McLellan, J.S. (2019). Structure-Based Vaccine Antigen Design. *Annu. Rev. Med.* **70**, 91–104.

Hastie, K.M., Zandonatti, M.A., Kleinfelder, L.M., Heinrich, M.L., Rowland, M.M., Chandran, K., Branco, L.M., Robinson, J.E., Garry, R.F., and Saphire, E.O. (2017). Structural basis for antibody-mediated neutralization of Lassa virus. *Science* **356**, 923–928.

Hsieh, C.L., Goldsmith, J.A., Schaub, J.M., DiVenere, A.M., Kuo, H.C., Javanmardi, K., Le, K.C., Wrapp, D., Lee, A.G., Liu, Y., et al. (2020). Structure-based design of prefusion-stabilized SARS-CoV-2 spikes. *Science* **369**, 1501–1505.

Juraszek, J., Rutten, L., Blokland, S., Bouchier, P., Voorzaat, R., Ritschel, T., Bakkens, M.J.G., Renault, L.L.R., and Langedijk, J.P.M. (2020). Stabilizing the closed SARS-CoV-2 spike trimer. *bioRxiv*. <https://doi.org/10.1101/2020.07.10.197814>.

Kalnin, K.V., Plitnik, T., Kishko, M., Zhang, J., Zhang, D., Beauvais, A., Ansova, N.G., Tibbitts, T., DiNapoli, J.M., Huang, P.-W.D., et al. (2020). Immunogenicity of novel mRNA COVID-19 vaccine MRT5500 in mice and non-human primates. *bioRxiv*. <https://doi.org/10.1101/2020.10.14.337535>.

Kielian, M. (2014). Mechanisms of virus membrane fusion proteins. *Annu. Rev. Virol.* **7**, 171–189.

Klasse, P.J., Nixon, D.F., and Moore, J.P. (2021). Immunogenicity of clinically relevant SARS-CoV-2 vaccines in non-human primates and humans. *Sci. Adv.* <https://doi.org/10.1126/sciadv.abe8065>.

Krammer, F. (2020). SARS-CoV-2 vaccines in development. *Nature* **586**, 516–527.

Krupar, A., Truan, D., Furmanova-Hollenstein, P., Bogaert, L., Bouchier, P., Bisschop, I.J.M., Widjojoatmodjo, M.N., Zahn, R., Schuitemaker, H., McLellan, J.S., and Langedijk, J.P.M. (2015). A highly stable prefusion RSV F vaccine derived from structural analysis of the fusion mechanism. *Nat. Commun.* **6**, 8143.

McLellan, J.S., Chen, M., Leung, S., Graepel, K.W., Du, X., Yang, Y., Zhou, T., Baxa, U., Yasuda, E., Beaumont, T., et al. (2013). Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. *Science* **340**, 1113–1117.

Mercado, N.B., Zahn, R., Wegmann, F., Loos, C., Chandrashekar, A., Yu, J., Liu, J., Peter, L., McMahan, K., Tostanoski, L.H., et al. (2020). Single-shot Ad26 vaccine protects against SARS-CoV-2 in rhesus macaques. *Nature* **586**, 583–588.

Moore, J.P., and Klasse, P.J. (2020). COVID-19 Vaccines: “Warp Speed” Needs Mind Melds, Not Warped Minds. *J. Virol.* **94**, e01083-20.

Murin, C.D., Wilson, I.A., and Ward, A.B. (2019). Antibody responses to viral infections: a structural perspective across three different enveloped viruses. *Nat. Microbiol.* **4**, 734–747.

Pallesen, J., Wang, N., Corbett, K.S., Wrapp, D., Kirchdoerfer, R.N., Turner, H.L., Cottrell, C.A., Becker, M.M., Wang, L., Shi, W., et al. (2017). Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. *Proc. Natl. Acad. Sci. USA* **114**, E7348–E7357.

Polack, F.P., Thomas, S.J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J.L., Pérez Marc, G., Moreira, E.D., Zerbini, C., et al.; C4591001 Clinical Trial Group (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* **383**, 2603–2615.

Qiao, H., Pelletier, S.L., Hoffman, L., Hacker, J., Armstrong, R.T., and White, J.M. (1998). Specific single or double proline substitutions in the “spring-loaded” coiled-coil region of the influenza hemagglutinin impair or abolish membrane fusion activity. *J. Cell Biol.* **141**, 1335–1347.

Ringe, R.P., Sanders, R.W., Yasmeen, A., Kim, H.J., Lee, J.H., Cupo, A., Korzun, J., Derking, R., van Montfort, T., Julien, J.P., et al. (2013). Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. *Proc. Natl. Acad. Sci. USA* **110**, 18256–18261.

Rutten, L., Gilman, M.S.A., Blokland, S., Juraszek, J., McLellan, J.S., and Langedijk, J.P.M. (2020). Structure-based design of prefusion-stabilized filovirus glycoprotein trimers. *Cell Rep.* **30**, 4540–4550.e3.

Sadoff, J., De Paepe, E., Haazen, W., Omoruyi, E., Bastian, A.R., Comeaux, C., Heijnen, E., Strout, C., Schuitemaker, H., and Callendret, B. (2020). Safety and immunogenicity of the Ad26.RSV.preF Investigational vaccine coadministered with an influenza vaccine in older adults. *J. Infect. Dis.* <https://doi.org/10.1093/infdis/jiaa409>.

Sanders, R.W., and Moore, J.P. (2017). Native-like Env trimers as a platform for HIV-1 vaccine design. *Immunol. Rev.* **275**, 161–182.

Sanders, R.W., Vesanen, M., Schuelke, N., Master, A., Schiffner, L., Kalyanaraman, R., Paluch, M., Berkhout, B., Maddon, P.J., Olson, W.C., et al. (2002). Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol.* **76**, 8875–8889.

Sanders, R.W., Derking, R., Cupo, A., Julien, J.P., Yasmeen, A., de Val, N., Kim, H.J., Blattner, C., de la Peña, A.T., Korzun, J., et al. (2013). A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog.* **9**, e1003618.

Sanders, R.W., van Gils, M.J., Derking, R., Sok, D., Ketas, T.J., Burger, J.A., Ozorowski, G., Cupo, A., Simonich, C., Goo, L., et al. (2015). HIV-1 VACCINES. HIV-1 neutralizing antibodies induced by native-like envelope trimers. *Science* **349**, aac4223.

Torrents de la Peña, A., and Sanders, R.W. (2018). Stabilizing HIV-1 envelope glycoprotein trimers to induce neutralizing antibodies. *Retrovirology* **15**, 63.

van Doremalen, N., Lambe, T., Spencer, A., Belli-Rammerstorfer, S., Purushotham, J.N., Port, J.R., Avanzato, V., Bushmaker, T., Flaxman, A., Ulaszewska, M., et al. (2020). ChAdOx1 nCoV-19 vaccination prevents SARS-CoV-2 pneumonia in rhesus macaques. *bioRxiv*. <https://doi.org/10.1101/2020.05.13.093195>.

Voysey, M., Clemens, S.A.C., Madhi, S.A., Weckx, L.Y., Folegatti, P.M., Aley, P.K., Angus, B., Baillie, V.L., Barnabas, S.L., Bhorat, Q.E., et al.; Oxford COVID Vaccine Trial Group (2021). Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet* **397**, 99–111.

Walls, A.C., Xiong, X., Park, Y.J., Tortorici, M.A., Snijder, J., Quispe, J., Cameron, E., Gopal, R., Dai, M., Lanzavecchia, A., et al. (2019). Unexpected receptor functional mimicry elucidates activation of coronavirus fusion. *Cell* **176**, 1026–1039.e15.

Ward, A.B., and Wilson, I.A. (2017). The HIV-1 envelope glycoprotein structure: nailing down a moving target. *Immunol. Rev.* **275**, 21–32.

Wrapp, D., Wang, N., Corbett, K.S., Goldsmith, J.A., Hsieh, C.L., Abiona, O., Graham, B.S., and McLellan, J.S. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* **367**, 1260–1263.

Yu, J., Tostanoski, L.H., Peter, L., Mercado, N.B., McMahan, K., Mahrokhian, S.H., Nkolola, J.P., Liu, J., Li, Z., Chandrashekar, A., et al. (2020). DNA vaccine protection against SARS-CoV-2 in rhesus macaques. *Science* **369**, 806–811.