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Synthetic proteins for COVID-19 diagnostics

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ARTICLE INFO

Keywords:
COVID-19 variants
Structure based design
Receptor binding domain
S protein epitopes
ACE2 interaction
Peptide vaccines
Neutralizing antibodies

ABSTRACT

There is an urgent need for inexpensive, rapid and specific antigen-based assays to test for vaccine efficacy and detect infection with SARS-CoV-2 and its variants. We have identified a small, synthetic protein (JS7), representing a region of maximum variability within the receptor binding domain (RBD), which binds antibodies in sera from nine patients with PCR-verified COVID-19 of varying severity. Antibodies binding to either JS7 or the SARS-CoV-2 recombinant RBD, as well as those that disrupt binding between a fragment of the ACE2 receptor and the RBD, are proportional to disease severity and clinical outcome. Binding to JS7 was inhibited by linear peptides from the RBD interface with ACE2. Variants of JS7, such as E484K or N501Y, can be quickly synthesized in pure form in large quantities by automated methods. JS7 and related synthetic antigens can provide a basis for specific diagnostics for SARS-CoV-2 infections.

1. Introduction

Infections with COVID-19, caused by a new coronavirus (SARS-CoV-2) [6,7] have spread throughout the world, causing millions of deaths. The recent drop in Covid-19 hospitalizations in the US can be primarily attributed to rapid roll out of vaccines based on the surface (S) protein of the virus. These vaccines could be rapidly developed because SARS-CoV-2 is closely related, in its sequence and structure [9] to SARS-CoV-1 (SARS), which caused an outbreak with high mortality in 2002–2003 [10–12]. Fortunately, methods developed for studying the structure and antigenicity of the SARS S protein could be adapted to express that of SARS-CoV-2 and its receptor binding domain (RBD), despite the ~20 % diversity in sequence (supplementary Fig. S1) and length (1255/1277 aa for SARS/SARS-CoV2) [4,11]. Both of these viruses enter cells using the human ACE2 receptor [14], distinguishing them from other human pathogenic β-coronaviruses [15]. They also share some epitopes recognized by neutralizing antibodies isolated from survivors [1,16]. At least one SARS-CoV-1 neutralizing monoclonal antibody [17,18] also binds, with less affinity, to the S protein SARS-CoV-2 [19]. Moreover, it was shown recently that a multimeric SARS-CoV-2 RBD nanoparticle adjuvanted with 3M-052/Alum elicited cross-neutralizing antibody responses against SARS-CoV-1, SARS-CoV-2, new SARS-CoV-2 variants and bat CoVs in macaque immunization [20].

There is thus reason to hope that at least initially the current vaccines will protect against the variants that SARS-CoV-2 has continued to accumulate during its path through humanity. However, the ongoing evolution of the virus may affect its phenotype, immune sensitivity and resistance to therapies [5,21–24]. It is thus essential to have sensitive and specific diagnostic tools for the public health community to determine the ability of antibodies in vaccinated individuals to recognize viral variants [13,23,25]. Antigen based assays are also needed to determine the longevity of the immune response in survivors [26–30] or vaccinated individuals [31–33]. As effective treatments are developed, point of care assays will be needed to rapidly discriminate SARS-CoV infections from those with other viruses with similar symptoms [34].

There have been many commercial assays developed for determining antibodies in serum or saliva [35,36] to recombinant versions of the S protein [30], in a trimmer conformation [26], or portions of its RBD [37]. These assays are based on large proteins and it is not clear how variants
will affect their expression or solubility [38].

While small proteins are considered the domain of recombinant techniques [39,41], recent advances allow chemical synthesis of highly purified long peptides and small proteins in large amounts [42–46]. To use this capability to design new diagnostics, and potentially vaccines, against COVID-19, we have thus concentrated on reducing the size of the protein needed to distinguish COVID-19 infections to an area of maximum sequence variation between SARS-CoV-1 and SARS-CoV-2 [8, 47–49]. This area (Fig. 1) also coincides with a region where the epitopes of many COVID-19 neutralizing monoclonal antibodies cluster [50]. As we show here, synthetic proteins representing this region bind antibodies from sera of patients with COVID-19 infections of varying severity. Modifications of this protein could be the basis for detecting variants that may affect treatment protocols.

2. Materials and methods

2.1. Sera

Patient and control sera were collected under separate protocols, are de-identified discarded samples, and are thus considered exempt. Table S1 summarizes the patient characteristics, treatment, and disease outcomes.

Human sera after infection with COVID-19 (10 samples from 9 patients (Table S1)) were negative for residual virus presence. De-identified clinical samples and clinical data were collected from consenting patients under the Observational Protocol for Diseases and Exposures of Public Health Importance (UNMC IRB # 060105), PI, Dr. Mark Kortepeter, U. Nebraska Medical Center; UTMB site-PI, Dr. Susan McEllan, developed by the Special Pathogens Research Network (SPRN) of the National Emerging and Special Pathogens Training and Education Center (NETEC). NETEC and SPRN are funded by the US Department of Health and Human Services Office of the Assistant Secretary for Preparedness and Response (ASPR), CFDA #93.825.

Five control sera (from non-atopic individuals) were collected in the US in February-April of 2019 (Table S2) with informed consent under Colorado Multiple Institution Review Board (COMIRB) 18-0850. All samples are de-identified.

2.2. Proteins and other reagents

Full length recombinant S protein from SARS and SARS-CoV-2 RBD + tags were obtained from Daniel Wrapp (Dartmouth College) and purified as described [51]. Recombinant S protein fragments of the receptor binding domain (RBD), purified from yeast, were received from Wen-Hsiang Chen, Baylor College of Medicine [3,4]. Synthetic proteins (defined as >35 amino acids) and peptides from the RBD were synthesized in the Peptide Core at Los Alamos National Laboratory. Positive control rabbit serum (polyclonal, against SARS/SARS-CoV-2 Coronavirus spike protein subunit 1) was Invtrogen PAS – 81795.

2.3. Peptide and protein synthesis

All reagents and solvents deployed were of peptide synthesis or biotech grade. All amino acids were purchased from P3Bio with the exception of Fmoc His (tBoc), required for high temperature coupling reactions which was obtained from CEM. Dimethylformamide (DMF) and the deployed deprotection reagent, 20 % Pyrrole (prepared as solution in DMF) were obtained from Alfa Aesar. Pyrrole is the suggested Fmoc deprotection reagent by CEM, as elevated reaction temperatures of 105 °C in deprotections using piperidine lead to loss of side chain protecting groups. The peptide coupling reagents, Diisopropylcarbodiimide (DIC) and Oxyma Pure (Ethylylcyanohoxyminoacetaete) were acquired in peptide synthesis grade from AKScientific. General reagents such as N,N-diisopropyl ethyl amine (DIPPEA), trisopropyl silane (isoPr3SiH; TIPS),thioanisole, octaethylenglycol-dithiol and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich and methylene chloride (DCM) was obtained from Fisher Scientific. For HPLC purifications, Acetonitrile was acquired from Alfa Aesar and water was purified in-house (deionized, filtered through a Nanopure to 18.2 MΩ-cm resistivity, and UV-sterilized). Mass spectrometry used highest quality (Optima MS grade) solvents purchased from Fisher Scientific.

2.4. Automated peptide synthesis using the CEM Liberty Prime microwave peptide synthesizer

A CEM Liberty Prime microwave peptide synthesizer was used for solid phase synthesis at high temperature (105 °C). All syntheses were performed at the 0.1 mM scale at the recommended standard instrument chemistry on a Rink amide resin. For the shorter peptides in this publication, single coupling instrument cycles were used, with achieved average coupling yields for cycle of >98.5 %. The synthesis deploys 65 s coupling at 105 °C, direct addition of the pyrrole to the hot resin and deprotection at 105 °C for 45 s, 20 s for the three wash steps for a total single couple cycle time of 2:15 min. For JS7 and JS10 reaction cycles,
double coupling of the amino acids was used and the drain times were increased from 5 to 10 s to accommodate for the resin volume increase over the synthesis cycles. To prevent hydrolysis of acid labile side chain protecting groups during the extended sequence syntheses, 0.1 M DIPEA was added to the Oxyma solution. High average coupling step yields allow even the longest sequences to be obtained in moderate yield.

2.5. Deprotection and removal of the peptides and proteins from the resin

Deprotection used 25 mL of modified “reagent K” mixture: TIPS (1.25 mL/25 mL), thioanisole (0.625 mL/25 mL), octaethyleneglycolithio (1.25 mL/25 mL), a less odorous substitute for EDT (ethylendithiol), and water (1.25 mL/25 mL) in TFA (trifluoroacetic acid). The resin was pretreated with the quencher solution for 5 min, then TFA was added (to final volume of 25 mL). The deprotections were carried out in 50 mL conical tubes of high-density PP, under a blanket of Argon to prevent side reactions from air, for 1.5 h for peptides and 2.5 h for the proteins (>3 KD). The solutions were filtered and the filtrate was concentrated to 10 mL. The peptide was then precipitated into ice cold ether and collected by centrifugation.

2.6. Purification and Analysis of synthetic RBD fragments and peptides

In addition to the longer RBD fragments, JS7 and JS10 (residues 443–505 and 417–505, respectively, of SARS-CoV-2 S protein), for competitive ELISAs, 3 peptides from the JS7 sequence that are most in contact with the ACE2 receptor were synthesized:

- SKVGGNYNYLRFERDISTEIYQAGSTCPNGVEGFNCYFPLQSYGFQPTNGVGY
- SKVGGNYNYLYRLF (443–457)

Purifications (to > 98 % +) were performed on a Waters HPLC preparative workstation with 2545 pump (at 20 mL/min) and using a C18 reverse phase column (Waters BEH 130, 5 μm, 19 × 150) and a gradient from 98 % to 50 % water-acetonitrile with 0.1 % TFA. Peaks were collected based on monitoring at 215 nm using a PDA 2998 detector. Combined product fractions were lyophilized, yielding a white fluffy solid. Peptides were then analyzed for purity by analytical HPLC on a C18 reverse phase column (Waters BEH 130, 5 μm, 4.6 × 150) with a gradient from 98 % to 20 % water-acetonitrile with 0.1 % TFA and by mass spectrometry on Thermo LTQ, Thermo Exacto and ABI 4800 MALDI TOF/TOF mass spectrometers, respectively, in ESI + mode. Additional data on the HPLC conditions and mass spectrometry identification are provided in the supplement.

2.7. Spectral measurements

Circular dichroism (CD) spectrum of JS7 was collected on a Jasco J-815 spectrometer (DIY = 8 s, scan speed 20 mM/min, 10 iterations 178–260 nm) and interpreted with the CDSSTR program [53,54], accessed via the Dichroweb server [55].

Protein/peptide concentrations were determined by direct measurement on a Nanodrop 1000 UV–vis spectrophotometer (Thermo Scientific). JS10 and JS7 (dissolved in water or PBS) were quantified based on calculated extinction coefficients at 280 nm. Peptides 1–3 were dissolved in water to approximately 10 mg/mL and then further dissolved to 5 mg/mL stock solution based on their absorbance at 230 nm.

2.8. Dotspots

Protein dotspots were done as described previously [8]. Proteins (0.25 μg/1 μL spot) were dotted onto nitrocellulose (Millipore, 0.2 μL) and allowed to dry. They were blocked with 5% milk in PBS, washed with PBS and incubated with diluted sera (1:20–1:100 in PBS) for 1 h at RT. The diluted sera were removed, the dotspots washed 3 times with PBS buffer, and then incubated for 1 h in Goat-anti-human-IgG-HRP (Catalog # 2040–05) or Goat-anti-rabbit IgG-HRP (# 4050–05) from Southern Biotech, diluted 1:1000/1:2000 in PBS. After washing 3x with PBS, the spots were developed with 4-Chloronaphthol reagent.

2.9. ELISA and competitive ELISA

Proteins were dissolved to 2 μg/mL in Borate buffer and allowed to bind to 96 well flat bottom plates (Thermofisher) overnight at 4 °C. The peptide or protein solution was removed and plates were blocked with 5% dry milk powder in PBS. The solution was discarded and the plates washed with PBS/0.1 % Tween 20. Sera were assayed alone, or after mixing with the indicated concentrations of proteins or peptides, and incubated 1 h at RT. In each case, the samples were added into the first row and then dilutions were made 1:3 with a multichannel pipettor down the plate. After overnight incubation at 4 °C, the serum dilutions were removed, the plates washed as above, and HRP-labeled secondary antibody (diluted 1:2000 to 1:4000 in PBS) was added. After 1 h, the plates were washed and developed with TMB reagent, using ¼ volume of 2 M Sulfuric acid to stop the reaction.

2.10. Assay for ACE2 binding to S protein

ACE2 receptor binding was measured with a SARS-CoV-2 inhibitor screening kit from Acrobiosystems (Catalog # EP-105), whereby the coating protein used in the assay was 2 μg/mL SARS-CoV-2 RBD (protein 4 in Fig. 3). After coating with the RBD fragment, plates were washed and blocked with 2% BSA in PBS/0.05 % Tween 20. After washing, sera or other samples were applied and the plates incubated overnight at 4 °C. The plates were washed with PBS/0.05 % Tween 20 and a solution of 12 μg/mL biotin conjugated ACE2 fragment was added to all wells. After incubation for 1 h at 37°C, the plates were washed and streptavidin coupled to HRP was added. After another 1 h at 37°C, the plates were washed and developed with TMB reagent and read at 652 nm before and 450 nm after addition of stop solution (¼ volume H₂SO₄).

3. Results

3.1. Characterization of the JS7 antigen

We synthesized two proteins, of about 10 kD and 7 KD (JS10 and JS7, Fig. 1). This area of the RBD structure, in complex with the ACE2 in a cryo-EM structure [56], contains a region of antiparallel β-sheet but is otherwise flexibly structured with 4 tyrosines (Y453, Y489, Y449, Y505) mediating many contacts across the interface. The mutations shown in the figure might even enhance this binding, as it would add yet another tyrosine or positive charge to this region. The CD spectrum of JS7 (Fig. 2) suggests it forms the flexible structure and β-stands that characterize the experimental cryo-EM structure.

3.2. JS7 binds antibodies in sera of COVID-19 patients in a fashion comparable to much larger recombinant proteins

We compared JS7 to four recombinant proteins obtained from other groups, including two versions of the RBD of the SARS-CoV-2. One was expressed with a fusion tag in 293 cells (derived from human embryonic kidney cells) [51] and the other in yeast (proteins 1 and 4, respectively in Fig. 3). Also included were approximately full length S protein [51] of SARS-CoV-1 (JSP-657, protein 2), and its corresponding RBD area (RBD in Fig. 3). The two versions of the SARS-CoV-2 RBD differ in that (1) was purified from a mammalian cell line attached to a linker protein and the other (4) was purified from yeast [4]. An alignment of the RBD sequences (proteins 1,3,4) is included in supplementary materials.

Convalescent sera from 9 hospitalized patients with COVID-19 disease of varying severity, from “mild” to critical, were used for dotspots, ELISAs and an assay for inhibition of association of the RBD with its
cellular receptor, ACE2. As shown in Table 1S, these patients had co-morbidities that have been found, in other studies, to be associated with hospitalization due to COVID-19. Of the 9, 5 were known to be diabetic, and 6 had elevated glucose levels at time of diagnosis. Four of the 6 patients who had severe or critical disease were obese (BMI > 30). Notably, the one patient who succumbed did not have these risk factors but was over 70 years of age.

A dotspot assay showed that all 9 patients’ sera contained antibodies that recognized JS7 (spot #6) and the two SARS-CoV-2 recombinant RBD forms (spots 1 and 4), while recognition of the SARS-CoV-1 recombinant proteins (Spots 2 and 3) varied (Table 1, Table S1). Binding to JS7 was similar, in ELISA assays, to that of the SARS-CoV-2 RBD (Fig. 5). While all patients had antibodies that recognized both SARS-CoV-2 RBD proteins, one patient (16) with mild disease did not recognize the full-length S protein of SARS-CoV-1 (spot 2), in two separate blood draws 5 weeks apart (Table 1, Table 1S). This pattern was similar to 3 of the 5 control sera (Table 2S).

Assays with sera from non-atopic volunteers who participated in a study in early 2019, collected before widespread introductions of SARS-CoV-2 into the US (Table S2), illustrates one of the great advantages to using the small synthetic proteins for diagnostics. Larger recombinant proteins may need to be expressed with protein tags, for solubility and/or ease in purification [76]. As the strong binding of both control and patient sera to protein 1, which still contains such tags, shows, these can cause assay artifacts if left intact (Tables 1, S1, S2). Antibodies in all the control sera showed somewhat less recognition of the RBD fragment of both SARS-CoV-1 and SARS-CoV-2 produced in yeast (proteins 3,4 in Fig. 3; the sequences of all 3 forms of the RBD are compared in Supplementary Material).

3.3. Antibody binding to the RBD or JS7 can be inhibited by peptides from the ACE2/RBD interface

Sera from patients with severe or critical disease had antibodies that interfered with the binding of the SARS-CoV-2 RBD (protein 4 in Fig. 3) to an ACE2 fragment (Fig. 4). Sera from the two patients with mild or moderate disease did not inhibit in this assay. However, both of these patients had antibodies that recognized the RBD and JS7 in dotspots or ELISA (Fig. 5, Table 1, Table 1S). Antibody binding, as measured by ELISA of either the whole RBD (protein 4, Fig. 3) or JS7 was approximately proportional to the severity of the infection (Figs. 5, 6) and inhibited by 3 peptides, underlined in the alignment of Fig. 1, from the JS7 sequence that are most in contact with the ACE2 protein in the cryo-EM structure.

Peptide 1: SKVGGNYNYL
Peptide 2: FERDISTEIYQAGST
Peptide 3: EGFNCYFPLGQFQPTNGVY

The bold residue L in Peptide 1, the most active peptide in preventing binding to JS7 (Fig. 6), is R in the “California” variant of SARS-CoV-2. The bold residues in peptide 3 show two residues that when changed
in SARS-CoV-1 affect ACE2 binding (N479K, T487S) [52]. These residues are most in contact with the ACE2 receptor in structures of the complex (taken from Walls et al. [9]).

As Fig. 5 shows, a mixture of the three peptides prevented binding of the antibodies to the whole RBD, as well as JS7, further emphasizing the importance of this region for immune recognition. In the case of JS7,

### Table 1

| Patient/age | Severity  | Protein dotspot | Binding in ELISA to RBD (4) | Binding in ELISA to JS7 (6) | ACE2 inhibition |
|-------------|-----------|-----------------|-----------------------------|-----------------------------|----------------|
| Female/30–40| mild      | 123456          | $4 \times 10^3$             | $3.7 \times 10^4$            | 0.26           |
| Male/50–60  | severe    |                 | $4 \times 10^3$             | $3.3 \times 10^5$            | -0.98          |
| Male/30–40  | critical  |                 | $3.3 \times 10^5$           | $3.3 \times 10^5$            | -1.28          |
| Female/20–30| Control serum |                | $1.2 \times 10^4$           | $3.6 \times 10^4$            | -0.09          |
only determined IgG binding, a response that normally takes weeks to develop. Additional testing of our antigen with saliva for specific saliva antibodies to the same extent as patients with only mild COVID-19 disease; this was confirmed by ELISA (Figs. 5, 6). This binding could lead to high background and limit the specificity of screening for serum antigens recognizing an area essential for interacting with the cell receptor, ACE2, as it could be competed for with peptides from this region. This suggests it to be an excellent candidate for point of care diagnostics to determine immune system involvement, vaccine efficacy and duration of antibody responses after vaccination or COVID-19 infection.

4. Discussion

Small synthetic proteins and peptides have significant advantages for diagnostic purposes and potentially for use in vaccines. Reagents and linkers needed for assays can be integrated into variants of JS7 during synthesis, while controlling its sequence, stereochemistry and disulfide patterns. As we show here, our synthetic JS7 was recognized by antibodies in convalescent sera to the same extent as the much larger RBD (Table 1 and Figs. 5, 6). This binding was specific for antibodies recognizing an area essential for interacting with the cell receptor, ACE2, as it could be competed for with peptides from this region. This suggests it to be an excellent candidate for point of care diagnostics to determine immune system involvement, vaccine efficacy and duration of antibody responses after vaccination or COVID-19 infection.

4.1. Serum antibodies block the interaction of the RBD with ACE2

Our assays, in keeping with observations of others [34], indicate that very high levels of antibodies against the S-protein are primarily seen after severe infection, a primary limitation on using serum antigen based assays to diagnose ongoing or mild SARS-CoV-2 infection. Two of the control sera contained antibodies that bound three of the four recombinant proteins to the same extent as patients with only mild COVID-19 disease; this was confirmed by ELISA (Figs. 5, 6). This binding could lead to high background and limit the specificity of screening for serum antibodies. This background recognition could be due to previous infections with other coronaviruses [57], or simply reflect germline antibody recognition. In support of the latter, others have found that neutralizing monoclonal antibodies against the SARS-CoV-2 S protein, isolated from many different donors, were from public clonotypes, bound autoantigens and contained relatively few somatic mutations from germline [58]. We note the convalescent serum tests included here only determined IgG binding, a response that normally takes weeks to develop. Additional testing of our antigen with saliva for specific saliva IgA might give earlier and more sensitive detection for less severe illness [35,36].

4.2. Diagnostic and vaccine uses for JS7 and its variants

Diagnostic assays for antibodies to the S protein are needed to determine the efficacy and longevity of response to vaccinations. Further, as more specific treatments are identified, JS7 and variants could prove useful antigens in rapid diagnostic tests to distinguish severely ill patients with COVID-19 from those suffering from other ailments that could have similar symptoms. We also suggest that JS7 or variants thereof could be incorporated into assays previously developed using recombinant RBD, such as haemagglutinin assays [59,60].

The JS7 protein, further modified to display variants and to be serum stable, may also have a future as a vaccine additive or booster. For example, recent methods have been developed to generate one component-synthetic proteins with incorporated adjuvant [61]. JS7 like antigens could also be incorporated into existing vaccine platforms, such as capsid-like particles [62].

The greatest advantage of using small synthetic antigens like JS7 is that the sequences can be rapidly modified to reflect variants now emerging in patients throughout the world [13,63]. Although the N501Y mutant did not alter neutralization by polyclonal antibodies in some test sera [64], other changes in this area at the interface of the RBD and its ACE2 receptor (Fig. 1) may alter the usefulness of treatments, such as convalescent plasma [5], monoclonal antibodies and small molecules designed to disrupt this interaction. While all 9 COVID-19 patients in this study had antibodies that recognized the RBD of SARS-CoV-2, one patient with mild illness did not recognize the whole S protein of SARS-CoV-1 (protein 2), despite the 80 % overall sequence identity (Tables 1, S1).

4.3. Synthetic antigens for screening for CoV infections in animals

Synthetic SARS-CoV-2 antigens could also be useful for assays of antibody responses in animal models, including primates [65], hamsters [66] and mice [67]. While the origins of both SARS viruses are unclear, there is a high probability that similar viruses are circulating, recombining and mutating in some animal reservoir. Cross-over of the human virus into other species could also lead to changes in its infectivity severity, and resistance to therapies [68,70]. Variants or recombinants of these pathogens in animals could result in a virus that combines the high aerosol transmissibility of SARS-CoV-2 [71,72] with the high mortality in humans of SARS-CoV-1 or MERS [15]. Antigen based assays can be particularly useful in estimating the prevalence of COVID-19 infection in animals that may not show obvious illness, such as minks [68] or domestic cats [69].

Several lines of evidence indicate that neutralization by serum antibodies and especially monoclonal antibodies may be limited by
variation in the JS7 area of the S protein. Some variants in this area, which were also identified as escape mutants of monoclonal antibodies [73], can render the virus insensitive to neutralization by convalescent sera [74]. The cross reactive SARS-CoV-1 neutralizing antibody, CR3022, binds the receptor binding domain (RBD) of SARS-CoV-2 with nanomolar affinity [75] but cannot neutralize the newer virus, due to a single amino acid change, P to A in its binding site immediately upstream of JS7 [17,18]. Thus the current sequence of JS7 is just the beginning of those that will be needed in the future to monitor the spread of SARS-CoV-2 variants and their viruses.

In conclusion, we show here that completely synthetic proteins and peptides, which can be produced to high purity in large quantities, can be the basis of specific assays to detect antibodies in sera that are produced in response to infection with SARS-CoV-2. Antibodies in sera of 9 patients who had PCR confirmed, COVID-19 of differing severity recognized the 7.1 kD synthetic fragment, JS7, from the RBD of SARS-CoV-2 with nanomolar affinity [76] but cannot neutralize the newer virus, due to a single amino acid change, P to A in its binding site immediately upstream of JS7 [17,18]. Thus the current sequence of JS7 is just the beginning of those that will be needed in the future to monitor the spread of SARS-CoV-2 variants and their viruses.

Acknowledgements

We thank the reviewers for their helpful comments, which have greatly improved this paper. We thank Wendy S. Baker for expert technical assistance, and Wen-Hsian Chen and others in the group at Baylor College of Medicine and Daniel Wrapp (Dartmouth) and the group of Jason McLellan of the University of Texas at Austin for supplying the recombinant proteins (1-4 in Fig. 3) used throughout this work. Peptide and protein synthesates at LANL were supported by LDRD ER funding; sequence analysis was supported by NIAID R01 AI137332 (to WB/CHS).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.peptides.2021.170583.

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