**INTRODUCTION**

Zoonotic coronaviruses have crossed over from animal reservoirs multiple times in the past two decades, and it is almost certain that wild animals will continue to be a source of devastating outbreaks. Unlike ubiquitous human coronaviruses responsible for common respiratory illnesses, these zoonotic coronaviruses with pandemic potential cause serious and complex diseases, partly due to their tissue tropisms driven by receptor usage. Severe acute respiratory syndrome coronaviruses 1 (SARS-CoV-1) and 2 (SARS-CoV-2) engage angiotensin-converting enzyme 2 (ACE2) for cell attachment and entry (1–7). ACE2 is a protease responsible for regulating blood volume and pressure that is expressed on the surface of cells in the lung, heart, and gastrointestinal tract, among other tissues (8, 9). The ongoing spread of SARS-CoV-2 and the disease it causes, coronavirus disease 2019 (COVID-19), has had a crippling toll on global healthcare systems and economies, and effective treatments and vaccines are urgently needed.

As SARS-CoV-2 becomes endemic in the human population, it has the potential to mutate and undergo genetic drift and recombination. To what extent this will occur as increasing numbers of people are infected and mount counter immune responses is unknown, but already a variant in the viral spike protein S (D614G) has rapidly emerged from multiple independent events and effects S protein stability and dynamics (10, 11). Another S variant (D839Y) became prevalent in Portugal, possibly due to a founder effect (12). SARS-CoV-2 has a moderate mutation rate estimated at $10^{-3}$ substitutions per site per year (13). However, the virus has undergone rapid mutation and adaptation after infecting mink in Denmark, from which it then crossed back to humans (14), causing Danish authorities to order 17 million farmed mink culled to preemptively prevent the possible emergence of vaccine-resistant variants. In addition, large changes in coronavirus genomes have frequently occurred in nature from recombination events, especially in bats where coinfection levels can be high (15, 16). Recombination of Middle East respiratory syndrome CoVs has been documented in camels (17); there are reported cases of recombination between cocirculating SARS-CoV-2 variants (18), and SARS-CoV-2 itself may have emerged through recombination of coronavirus genomes (19). This will all have profound implications for the current pandemic’s trajectory, the potential for future coronavirus pandemics, and whether drug or vaccine resistance in SARS-CoV-2 emerges and becomes widespread.

The viral spike is a vulnerable target for neutralizing monoclonal antibodies that are progressing through clinical trials, yet in tissue culture, escape mutations in the spike rapidly emerge to all antibodies tested (20). Deep mutagenesis of the isolated receptor-binding domain (RBD) by yeast surface display has easily identified mutations in S that retain high expression and ACE2 affinity but are no longer bound by monoclonal antibodies and confer resistance (21). This has motivated the development of cocktails of noncompeting monoclonals (20, 22), inspired by lessons learned from the treatment of HIV-1 and Ebola, to limit the possibilities for the virus to escape. Notably, drugmaker Eli Lilly has a monoclonal monoclonal (LY-CoV555) in advanced trials (NCT04427501) where the selection of resistant virus variants in patients has occurred. A trial update added an arm with a second monoclonal (LY-CoV016), and the company has not reported putative resistance variants in patients receiving the cocktail thus far. However, even the use of monoclonal cocktails does not address future coronavirus spillovers from wild animals that may be antigenically distinct. Large screening efforts were required to find antibodies from recovered patients with SARS-CoV-1 that cross-react with SARS-CoV-2 (23), indicating that antibodies have confined capacity for interacting with variable epitopes on the spike surface and are unlikely to be broad and pan-specific for all SARS-related viruses.

An alternative protein-based antiviral to monoclonal antibodies is to use soluble ACE2 (sACE2) as a decoy to compete for receptor-binding sites on the viral spike (6, 24–27). In principle, the virus has limited potential to escape sACE2-mediated neutralization without simultaneously decreasing affinity for the native ACE2 receptor, rendering the virus less virulent. Wild-type (WT) sACE2 is currently in a phase 2 clinical trial (28), and multiple groups have now engineered sACE2 to create high-affinity decoys for SARS-CoV-2 that rival matured monoclonal antibodies for potent neutralization of infection (27, 29, 30). In our group, deep mutagenesis was used to identify a large number of mutations in ACE2 that increase affinity for S (27). These mutations were dispersed across the interface and
also at distal sites where they are predicted to enhance folding of the virus-recognized conformation. A combination of three mutations, called sACE2v2.4, increases affinity by 35-fold and binds SARS-CoV-2 S [dissociation constant (K_D), 600 pM] with affinity comparable to the best monoclonal antibodies (27). Even tighter apparent affinities are reached through avid binding to trimeric spike expressed on a membrane. Despite engineering being focused exclusively on SARS-CoV-2 affinity, sACE2v2.4 potently neutralized authentic SARS-CoV-1 and SARS-CoV-2 infection in tissue culture, suggesting that its close resemblance to the WT receptor confers broad activity against ACE2-utilizing betacoronaviruses generally. sACE2v2.4 is dimeric and monodisperse without aggregation, catalytically active, highly soluble, stable after storage at 37°C for days, and well expressed at levels greater than the WT protein. Because of both its high activity and favorable properties for manufacture, sACE2v2.4 is a genuine drug candidate for preclinical development.

Engineered, high-affinity decoy receptors, while very similar to natural ACE2, nonetheless have mutations present at or near the interaction surface. There is therefore an opportunity for viral spike variants to discriminate between an engineered decoy and WT receptors, providing a route toward resistance. Here, we show that the engineered decoy sACE2v2.4 binds broadly and tightly to the RBDs of diverse SARS-associated betacoronaviruses that use ACE2 for entry. We further fail to find mutations within the RBD, which directly contacts ACE2 and is where possible escape mutations will most likely reside, that redirect specificity toward the WT receptor. We conclude that resistance to an engineered decoy receptor will be rare, and sACE2v2.4 targets common attributes for affinity to S in SARS-associated viruses.

RESULTS

An engineered decoy receptor broadly binds RBDs from SARS-associated CoVs with tight affinity

The affinities of the decoy receptor sACE2v2.4 were determined for purified RBDs from the S proteins of five coronaviruses from Rhinolophus bat species (isolates LYRa11, Rs4231, Rsl3727, Rs4084, and RsSHC014) and two human coronaviruses, SARS-CoV-1 and SARS-CoV-2. These viruses fall within a common clade of betacoronaviruses that have been experimentally validated to use human ACE2 as an entry receptor (7). They share close sequence identity within the RBD core, while variation is highest within the functional ACE2-binding site (Fig. 1 and fig. S1), possibly due to a coevolutionary “arms race” with polymorphic ACE2 sequences in ecologically diverse bat species (31). Affinity was measured by biolayer interferometry (BLI), with sACE2 [amino acids (aa) S19 to G732] fused at the C terminus with the Fc moiety of human immunoglobulin G1 (IgG1) immobilized to the sensor surface and monomeric 8his-tagged RBD (fig. S2) used as the soluble analyte. This arrangement excludes avidity effects, which otherwise cause artifically tight (picomolar) apparent affinities whenever dimeric sACE2 in solution is bound to immobilized RBD decorating an interaction surface. WT sACE2 bound all the RBDs with affinities ranging from 16 nM to 91 nM for LYRa11, with a median affinity of 60 nM (Table 1). The measured affinities for the RBDs of SARS-CoV-1 and SARS-CoV-2 are comparable to those of published data (4,27,32–34). Engineered sACE2v2.4 displayed large increases in affinity for all the RBDs, with K_D s ranging from 0.4 nM for SARS-CoV-2 to 3.5 nM for the isolate Rs4231, with a median affinity less than 2 nM (Table 1).

The approximate 35-fold affinity increase in the engineered decoy applies universally to coronaviruses in the test panel, and the molecular basis for affinity enhancement must therefore be grounded in common attributes of RBD/ACE2 recognition.

A deep mutational scan of the RBD in the context of full-length S reveals that residues in the ACE2-binding site are mutationally tolerant

To explore potential sequence diversity in S of SARS-CoV-2 that may act as a “reservoir” for drug resistance, we evaluated the mutational tolerance of the RBD by deep mutagenesis (35). Saturation mutagenesis was focused to the RBD (aa C336 to L517) of full-length S tagged at the extracellular N terminus with a c-myc epitope for detection of surface expression. The spike library, encompassing 3640 single amino acid substitutions, was transfected in human Expi293F cells under conditions where cells typically acquire no more than a single sequence variant (36, 37). The culture was incubated with WT, 8his-tagged, dimeric sACE2 at a subsaturating concentration (2.5 nM). Bound sACE2v2.8h and surface-expressed S were stained with fluorescent antibodies for flow cytometry analysis (Fig. 2A). Compared to cells expressing WT S, the library was poorly expressed, indicating that many mutations are deleterious for folding and expression. A cell population was discernable expressing S variants that bind ACE2 with decreased affinity (Fig. 2B). After gating for c-myc–positive cells expressing S, cells with high and low levels of bound sACE2 were collected by fluorescence-activated cell sorting (FACS), called the ACE2-high and ACE2-low populations, respectively (Fig. 2C). Both the expression and sACE2-binding signals decreased over minutes to hours during sorting, possibly due to shedding of the S1 subunit. Cells were therefore collected and pooled from three separate FACs experiments for a combined 8-hour sort time.

Transcripts in the sorted cells were Illumina sequenced and compared to the naïve plasmid library to determine an enrichment ratio for each amino acid substitution (38). Mutations in S that...
Table 1. BLI kinetics for immobilized sACE2-IgG1 binding to coronavirus RBDs.

| CoV strain* | WT sACE2-IgG1† | sACE2.v2.4-IgG1 | \( k_{on} (M^{-1} s^{-1}) \) | \( k_{off} (s^{-1}) \) | \( K_d (nM) \) | \( \chi^2 \) | \( k_{on} (M^{-1} s^{-1}) \) | \( k_{off} (s^{-1}) \) | \( K_d (nM) \) | \( \chi^2 \) |
|-------------|----------------|-----------------|------------------|-----------------|-----------------|----------------|------------------|----------------|-----------------|----------------|
| LYRa11      | 8.7 × 10^5     | 7.9 × 10^{-2}   | 91               | 0.12            | 1.4 × 10^6      | 2.5 × 10^{-3}  | 1.8              | 0.10            |
| Rs7327      | 6.4 × 10^5     | 4.0 × 10^{-2}   | 63               | 0.25            | 9.8 × 10^5      | 1.8 × 10^{-3}  | 1.9              | 0.11            |
| Rs4231      | 3.2 × 10^5     | 2.2 × 10^{-2}   | 69               | 0.04            | 4.5 × 10^5      | 1.6 × 10^{-3}  | 3.5              | 0.10            |
| Rs4084      | 2.9 × 10^5     | 2.5 × 10^{-2}   | 85               | 0.24            | 4.8 × 10^5      | 1.5 × 10^{-3}  | 3.1              | 0.10            |
| RsSHC014    | 8.8 × 10^5     | 2.6 × 10^{-2}   | 29               | 0.20            | 1.6 × 10^5      | 2.0 × 10^{-3}  | 1.3              | 0.29            |
| SARS-1      | 6.6 × 10^5     | 1.2 × 10^{-4}   | 58               | 0.03            | 3.0 × 10^3      | 5.6 × 10^{-6}  | 2.1              | 0.03            |
| SARS-2      | 1.4 × 10^5     | 8.1 × 10^{-3}   | 16               | 0.25            | 6.6 × 10^5      | 2.8 × 10^{-4}  | 0.4              | 0.09            |
| SARS-2 (Y449K) | 2.0 × 10^5    | 9.0 × 10^{-2}   | 46               | 0.67            | 4.3 × 10^5      | 4.0 × 10^{-3}  | 0.9              | 0.71            |
| SARS-2 (NS01W) | 2.4 × 10^5    | 5.4 × 10^{-3}   | 23               | 0.43            | 3.3 × 10^5      | 2.8 × 10^{-4}  | 0.1              | 0.23            |
| SARS-2 (NS01Y) | 2.2 × 10^5    | 1.8 × 10^{-3}   | 0.8              | 0.15            | 3.6 × 10^5      | 1.1 × 10^{-3}  | <0.1             | 0.24            |

*Purified RBDs at five to seven concentrations were used as the soluble analytes. †IgG1 Fc-fused sACE2 was immobilized to anti-human IgG Fc capture biosensors. \( \chi^2 \) values represent the goodness of curve fitting. Acceptable values were considered to be less than 3.

There are two “hotspot” regions for interactions at the interface that determine receptor affinity and species adaptation, centered around ACE2 residues K31 and K353 (39). These regions are also the locations for substitutions to ACE2 residues T27, L79, and N330 in the engineered sACE2.v2.4 decoy. Mutations to RBD residues at these sites tend to be weakly depleted for high ACE2 binding (fig. S4) but are much more tolerant of mutations than structural positions buried in the RBD core. To highlight a few residues, S-Y505 that packs against the hydrocarbon chain of ACE2-K353 is notably more conserved than most other interfacial residues, with the exceptions of partially buried residues like S-R403 and S-Y453 that likely have additional structural roles; S-Y489 contacting ACE2-T27 and ACE2-K31 has an overall weak preference for aromatic amino acids; and S-G485 on a loop packed against ACE2-L79 has its highest tolerance for polar substitutions, possibly to maintain the loop conformation and solubility (fig. S4). Some mutations are found to be highly enriched for ACE2 binding, including small hydrophobic amino acids for S-Q493 and aromatic amino acids for S-N501 that are both anticipated to increase local hydrophobic or aromatic ring packing. This is consistent with observations from yeast surface display of RBD mutants (40). The S-N501Y mutation, which causes a notable increase in affinity for ACE2 (Table 1), has emerged in a
Tagenesis of the RBD on the yeast surface (mean of $\Delta K_D$ on ACE2 binding from deep mutagenesis of S in human cells) is consistent with mutations at these residues reducing ACE2 interactions through defects in folding and decreased surface expression. Compared to single mutations that destabilize the RBD in the closed-down conformation, more extensive engineering with multiple stabilizing mutations has shown to shift the conformational equilibrium of S subunits to the open-up state.

We used targeted mutagenesis to individually test alanine substitutions to all the cysteines in the RBD (fig. S5). We found that all cysteine-to-alanine mutations severely diminish S surface expression in Expi293F cells, including C391A and C525A on the RBD “back-side” that were neutral in the yeast display scan. These differences demonstrate that there are tighter sequence constraints on the RBD in the context of a full spike expressed at a human cell membrane, yet overall, we consider the yeast display and the human cell datasets to closely agree.

For binding to dimeric sACE2v2.4, we note that interface residues were more tightly conserved in the Starr et al. dataset (Fig. 3D), possibly a consequence of three differences between the deep mutagenesis experiments. First, our selections for ACE2 binding of S variants at the plasma membrane appear to primarily reflect mutational effects on surface expression, which is almost certainly more stringent in human cells. Yeast permits many poorly folded proteins to leak to the cell surface. Second, the yeast selections were conducted at single sACE2 concentrations from which apparent $K_D$ changes were computed; the Starr et al. dataset in this regard is very comprehensive. Because of the long sort times required for our human cell libraries where only a small fraction of cells express spike, we sorted at a single sACE2 concentration that cannot accurately capture a range of different binding affinities quantitatively. Third, dimeric sACE2v2.4 may geometrically complement trimeric S densely packed on a human cell membrane, such that avidity masks the effects of affinity-reducing mutations. Nonetheless, there is overall agreement that ACE2 binding often persists following mutations to the RBD surface, and our data simply suggest that mutational tolerance may be even greater than that already observed by Starr et al.

A screen for S variants that preferentially bind WT ACE2 over the engineered decoy

Having shown that the ACE2-binding site of SARS-CoV-2 protein S tolerates many mutations, we asked whether mutations might therefore be found that confer resistance to the engineered decoy sACE2v2.4. Resistance mutations are anticipated to lose affinity for sACE2v2.4 while maintaining binding to the WT receptor and are most likely to reside in the RBD where physical contacts are made. Similar reasoning formed the foundation of a deep mutagenesis–based selection of the isolated RBD by yeast surface display to find escape mutations to monoclonal antibodies, and the results were predictive of escape mutations in pseudovirus growth selections.

To address whether escape mutations from the engineered decoy might be found in the RBD, we repurposed the S protein library for a specificity selection. Cells expressing the library, encoding all possible substitutions in the RBD, were co-cultivated with WT sACE2v2.4 fused to the Fc region of IgG1 and 8his-tagged sACE2v2.4 at concentrations where both proteins bind competitively. It was

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sACE2 2·v2.4-specific mutations were also mapped to RBD loops 498 to 506, contacting the region where the ACE2-α1 helix packs against a β-hairpin motif (Fig. 5A). By comparison, there were no hotspots in the RBD for sACE2 2(WT)-specific mutations. Only a small number of mutations were selectively enriched for preferential binding to WT receptor (Fig. 5B), and the abundance of these putative WT-specific mutations barely rose above the expected level of noise in the deep mutagenesis data. In this competition assay, S binding to WT sACE2 2 is therefore more sensitive to RBD mutations than S binding to engineered sACE2 2·v2.4.

To determine whether the potential WT ACE2–specific mutations found by deep mutagenesis are real as opposed to false predictions due to data noise, we tested 24 mutants of S selectively enriched in the WT-specific gate by targeted mutagenesis (blue data points in Fig. 5B). Only minor shifts toward binding WT sACE2 2 were observed (fig. S6). Two S mutants were investigated further in sACE2 2 titration experiments, N501W and N501Y, which both retained high receptor binding and displayed small shifts toward WT sACE2 2 in the competition experiment. N501 of S is located in the 498·506 loop, and its substitution to large aromatic side chains might alter the loop conformation to cause steric strain with nearby ACE2 mutation N330Y in sACE2 2·v2.4. After titrating the concentrations of S-expressing cells by flow cytometry, it was found that S-N501W and S-N501Y do show enhanced specificity for WT sACE2 2, but the effect is small and sACE2 2·v2.4 remains to be the stronger binder (Fig. 5C).

Dimeric sACE2 2 binds avidly to S protein on a membrane surface; avid interactions are also observed between sACE2 2 and spikes on authentic SARS-CoV-2 in infection assays (27). We used BLI kinetics measurements, in which immobilized sACE2 2-IgG1 interacts with monomeric RBD, to determine how the observed changes in avid sACE2 2 binding to S-expressing cells translate to changes in affinity. Both N501W and N501Y mutants of SARS-CoV-2 RBD displayed increased affinity for WT ACE2 and engineered ACE2 2·v2.4, with larger affinity gains in favor of the WT receptor (Table 1). This aligns with the flow cytometry data indicating a small shift in specificity toward WT ACE2, but not enough to escape the engineered decoy. By comparison, multiple independent escape mutations are

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**Fig. 4. A competition-based selection to identify RBD mutations within S of SARS-CoV-2 that preferentially bind WT or engineered ACE2 receptors.** (A) Expi293F cells were transfected with WT myc-S and incubated with competing sACE2 2(WT)-IgG1 (25 nM) and sACE2 2·v2.4-8h (20 nM). Bound protein was detected by flow cytometry after immunostaining for the respective epitope tags. (B) As in (A), except cells were transfected with the RBD SSM library. A population of cells expressing S variants with increased specificity toward sACE2 2·v2.4 is apparent (cells shifted to the upper left of the main population). (C) Gates used for FACS of cells expressing the RBD SSM library. After excluding cells without bound protein, the top 20% of cells for bound sACE2 2·v2.4-8h (magenta gate) and for bound sACE2 2(WT)-IgG1 (green gate) were collected. (D and E) Agreement between log 2 enrichment ratios from two independent FACS selections for cells expressing S variants with increased specificity for (D) sACE2 2(WT) or (E) sACE2 2·v2.4. R^2 values are calculated for nonsynonymous mutations (black). Nonsense mutations are red. (F and G) Conservation scores are calculated from the mean of the log 2 enrichment ratios for all nonsynonymous substitutions at a given residue position. Correlation plots show agreement between conservation scores for two independent selections for cells within the (D) sACE2 2(WT)- or (E) sACE2 2·v2.4-specific gates.
affinity may have larger effects on avid binding of the weaker-bound WT receptor.

Overall, validation by targeted mutagenesis confirms that the selection can successfully find mutations in S with altered specificity. The inability to find mutations in the RBD that impart high specificity for the WT receptor means that such mutations are rare or may not even exist, at least within the RBD where direct physical contacts with receptors occur. We cannot exclude mutations elsewhere having long-range conformational effects. Engineered, soluble decoy receptors therefore live up to their promise as broad therapeutic candidates against which a virus cannot easily escape.

**DISCUSSION**

The allure of soluble decoy receptors is that the virus cannot easily mutate to escape neutralization. Mutations that reduce affinity of the soluble decoy will likely also decrease affinity for the WT receptor on host cells, thereby coming at the cost of diminished infectivity and virulence. However, this hypothesis has not been rigorously tested, and since engineered decoy receptors differ from their WT counterparts, even if by just a small number of mutations, it is possible that a virus may evolve to discriminate between the two. Here, we show that an engineered decoy receptor for SARS-CoV-2 broadly binds with low-nanomolar $K_D$ to the spikes of SARS-associated betacoronaviruses that use ACE2 for entry, despite high sequence diversity within the ACE2-binding site. Mutations in S of SARS-CoV-2 that confer high specificity for WT ACE2 were not found in a comprehensive screen of all substitutions within the RBD. The engineered decoy receptor is therefore broad against zoonotic ACE2-utilizing coronaviruses that may spill over from animal reservoirs in the future and against variants of SARS-CoV-2 that may arise as the current COVID-19 pandemic rages on. These findings are highly consistent with research of a high-affinity decoy receptor engineered for a different pathogen, HIV. An IgG1 Fc–fused soluble decoy based on HIV receptors, called eCD4-Ig, broadly neutralizes HIV-1, HIV-2, and related simian viruses, with single mutations in the HIV spike protein unable to achieve full escape (47, 48). Together with the results reported here, these studies collectively demonstrate that engineered decoy receptors can achieve exceptional breadth against virus sequence variants. We argue that it is unlikely that decoy receptors will need to be combined in cocktail formulations, as is required for many monoclonal antibodies and possibly designed miniprotein binders to prevent the rapid emergence of resistance (20, 49), facilitating manufacture and distribution. Our findings give insight into how a potential therapeutic can achieve breadth with a low chance of virus resistance for a family of highly infectious and deadly viruses.

**MATERIALS AND METHODS**

### Plasmids

Residue numbers for constructs begin from the start methionine as a.a. 1. The cloning of human codon-optimized, mature S from SARS-CoV-2 (GenBank accession number YP_009724390.1; a.a. V16 to T1273) into the Nhe I–Xho I sites of pcDNA3.1(+)(Invitrogen) with an N-terminal, extracellular c-myc tag is described elsewhere (27). sACE2 (a.a. 1 to 732 encoding a dimer; WT or engineered variant sACE2_v2.4) fused to an 8his purification tag or to human IgG1 Fc (a.a. D221-K447; nG1m1 isosolotype; GenBank KY432415.1) and cloned into the Nhe I–Xho I sites of pcDNA3.1(+) (Invitrogen) is

| sACE2_v2.4-specific | WT-specific |
|---------------------|------------|
| Y449K/Q/S, L455G/ |
| N501W, N501Y       |

**Fig. 5. Mutations within the RBD that confer specificity toward WT ACE2 are rare.** (A) The SARS-CoV-2 RBD is colored by specificity score [the difference between the conservation scores for cells collected in the sACE2 WT- and sACE2_v2.4-specific gates]. Residues that are hotspots for mutations with increased specificity toward sACE2 WT are blue or toward sACE2_v2.4 are purple. The contacting surface of ACE2 is shown as a green ribbon, with sites of mutations in sACE2_v2.4 labeled and shown as green spheres. (B) Log$_2$ enrichment ratios for mutations in S expressed by cell populations collected in the sACE2 WT-specific (x axis) and sACE2_v2.4-specific (y axis) gates. Data are the mean from two independent sorting experiments. S mutants in blue were predicted to have increased specificity for sACE2 WT and were tested by targeted mutagenesis in fig. S6. S mutants in purple were predicted to have increased specificity for sACE2_v2.4 and were tested by targeted mutagenesis in fig. S7. Other nonsynonymous mutations are black. Nonsense mutations are red. (C) WT myc-S (gray) and three variants, Y449K (purple), N501W (light blue), and N501Y (dark blue), were expressed in Exp293F cells and tested by flow cytometry for binding to sACE2 WT-8h (dashed lines) or sACE2_v2.4-8h (solid lines). MFU, mean fluorescence units.

Last, eight representative mutations to S predicted from the deep mutational scan to increase specificity toward sACE2_v2.4 (purple data points in Fig. 5B) were cloned, and seven were found to have large shifts toward preferential sACE2_v2.4 binding in the competition assay (fig. S7). These S mutations were Y449K/Q/S, L455G/R/Y, and G504K. The basis for why the mutations increase specificity toward engineered sACE2_v2.4 is ambiguous, since RBD residues Y449, L455, and G504 are not in direct contact with engineered sites of the receptor. BLI kinetics between immobilized sACE2 IgG1 and monomeric RBD as the analyte showed reduced affinity of a representative mutant, RBD-Y449K, to both WT and engineered sACE2 (Table 1). However, affinity changes in the picomolar range for sACE2_v2.4 are hidden during avid binding to full-length S-Y449K at the cell surface, whereas avid binding of WT sACE2 to S-Y449K (with affinity measured by BLI in the moderate nanomolar range) is substantially reduced. This finding might explain why the competition selection found many mutations that shift specificity toward engineered sACE2_v2.4, as mutations causing small decreases in readily found in S of SARS-CoV-2 that diminish the efficacy of monoclonal antibodies by many orders of magnitude (20, 21).

Overall, validation by targeted mutagenesis confirms that the selection can successfully find mutations in S with altered specificity. The inability to find mutations in the RBD that impart high specificity for the WT receptor means that such mutations are rare or may not even exist, at least within the RBD where direct physical contacts with receptors occur. We cannot exclude mutations elsewhere having long-range conformational effects. Engineered, soluble decoy receptors therefore live up to their promise as broad therapeutic candidates against which a virus cannot easily escape.

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**Fig. 5. Mutations within the RBD that confer specificity toward WT ACE2 are rare.** (A) The SARS-CoV-2 RBD is colored by specificity score [the difference between the conservation scores for cells collected in the sACE2 WT- and sACE2_v2.4-specific gates]. Residues that are hotspots for mutations with increased specificity toward sACE2 WT are blue or toward sACE2_v2.4 are purple. The contacting surface of ACE2 is shown as a green ribbon, with sites of mutations in sACE2_v2.4 labeled and shown as green spheres. (B) Log$_2$ enrichment ratios for mutations in S expressed by cell populations collected in the sACE2 WT-specific (x axis) and sACE2_v2.4-specific (y axis) gates. Data are the mean from two independent sorting experiments. S mutants in blue were predicted to have increased specificity for sACE2 WT and were tested by targeted mutagenesis in fig. S6. S mutants in purple were predicted to have increased specificity for sACE2_v2.4 and were tested by targeted mutagenesis in fig. S7. Other nonsynonymous mutations are black. Nonsense mutations are red. (C) WT myc-S (gray) and three variants, Y449K (purple), N501W (light blue), and N501Y (dark blue), were expressed in Exp293F cells and tested by flow cytometry for binding to sACE2 WT-8h (dashed lines) or sACE2_v2.4-8h (solid lines). MFU, mean fluorescence units.

Last, eight representative mutations to S predicted from the deep mutational scan to increase specificity toward sACE2_v2.4 (purple data points in Fig. 5B) were cloned, and seven were found to have large shifts toward preferential sACE2_v2.4 binding in the competition assay (fig. S7). These S mutations were Y449K/Q/S, L455G/R/Y, and G504K. The basis for why the mutations increase specificity toward engineered sACE2_v2.4 is ambiguous, since RBD residues Y449, L455, and G504 are not in direct contact with engineered sites of the receptor. BLI kinetics between immobilized sACE2 IgG1 and monomeric RBD as the analyte showed reduced affinity of a representative mutant, RBD-Y449K, to both WT and engineered sACE2 (Table 1). However, affinity changes in the picomolar range for sACE2_v2.4 are hidden during avid binding to full-length S-Y449K at the cell surface, whereas avid binding of WT sACE2 to S-Y449K (with affinity measured by BLI in the moderate nanomolar range) is substantially reduced. This finding might explain why the competition selection found many mutations that shift specificity toward engineered sACE2_v2.4, as mutations causing small decreases in
also previously described (27). The RBDs of SARS-CoV-1 (Urbani isolate; GenBank AAP13441.1; a.a. T320 to D518), SARS-CoV-2 (YP_009724390; a.a. T333 to K529), LYRa11 (AHX37558.1; a.a. T324 to D522), Rs7327 (AT098218.1; a.a. T321 to D519), Rs4231 (AT098157.1; a.a. T320 to D518), Rs4084 (AT098132.1; a.a. T321-D519), and RsSHCo14 (AGZ48806.1; a.a. T321-D519) were cloned with N-terminal influenza HA leader peptides (sequence MKTIHALSYIFCLVFA) and C-terminal 8his tags (sequence GSGHHHHHHHH) into the Nhe I–Xho I sites of pcDNA3.1(+).

These plasmids are deposited with Addgene under accession numbers 145145, 164845, and 161821-161826. Mutations were made by overlap extension polymerase chain reaction (PCR) and verified by Sanger sequencing.

**Tissue culture**

Exp293F cells (Thermo Fisher Scientific) were grown in Exp293 Expression Medium (Thermo Fisher Scientific) at 125 rpm, 8% CO₂, 37°C.

**Recombinant protein production**

Plasmids (500 ng of DNA/ml culture) and polyethylenimine (MW 25,000; Polysciences; 3 μg/ml culture) were mixed with OptiMEM (Millipore) to final concentrations of ~1 mg/ml (RBD-8h proteins), fractions were concentrated using centrifugal ultrafiltration devices 10/300 GL column (GE Healthcare Life Sciences) equilibrated with expression Medium (Thermo Fisher Scientific) at 125 rpm, 8% Expi293F cells (Thermo Fisher Scientific) were grown in Expi293 Expression Medium. Fluorescent signals decreased over time during FACS, and therefore, transfected cultures were prepared on a BD FACSAria II at the Roy J. Carver Biotechnology Center. Dead cells, doublets, and debris were excluded by first gating on the main population by forward/side scattering and then excluding 4',6-diamidino-2-phenylindole–positive cells. From the myc–S–positive (Alexa Fluor 647) population, the 20% of cells with the highest and 20% of cells with the lowest anti–HIS-FITC fluorescence for bound sACE2₂(WT)-8h were collected (Fig. 2C). Collection tubes were coated overnight with fetal bovine serum before sorting and contained an Exp293 Expression Medium. Fluorescent signals decreased over time during FACS, and therefore, transfected cultures were prepared on three separate occasions for a combined total of 8-hour sort time. The total numbers of collected cells were 57,000 and 72,700 for the ACE2-high and ACE2-low gates, respectively. Collected cells were centrifuged (500g, 300 s), and pellets were frozen at −80°C. Samples from the independent sorts were pooled during extraction of total RNA.

The competition selection was performed similarly, with the exception that cells expressing the S library were incubated for 30 min in a mixture of 20 nm sACE2₂(WT)-8h and 25 nm sACE2₂(WT)-IgG1. After washing twice, bound proteins were stained for 30 min with anti-human IgG alloglobinocyanin (APC) (clone HP6017, 1/250 dilution; Cell Signaling Technology) and anti–HIS–fluorescein isothiocyanate (FITC) (chicken polyclonal, 1/100 dilution; Immunology Consultants Laboratory). Cells were washed twice and sorted. After gating for the main population of viable cells as described above, the 20% of cells with the highest FITC relative to APC and highest APC relative to FITC signals were collected (Fig. 4C). The total numbers of collected cells were 53,950 [replicate 1: sACE2₂(WT)-specific gate], 42,860 [replicate 1: sACE2₂(WT)-specific gate], 41,420 [replicate 2: sACE2₂(WT)-specific gate], and 34,730 [replicate 2: sACE2₂(WT)-specific gate]. Total RNA was extracted from the collected cells using a GeneJET RNA purification kit (Thermo Fisher Scientific). First-strand complementary DNA (cDNA) was synthesized with Accuscript (Agilent) primed with a gene-specific oligonucleotide. The region of S scanned by saturation mutagenesis was PCR amplified as three overlapping fragments that together span the full RBD sequence. Following a second round of PCR, primers added adapters for annealing to the Illumina flow cell and sequencing primers, together with barcodes for experiment identification. The PCR products were sequenced on an Illumina NovaSeq 6000 using a 2 × 250 nucleotide paired-end protocol. Data were analyzed using Enrich (38), where the frequencies of S variants in the transcripts of the sorted populations were compared to their frequencies in the naïve plasmid library. Log enrichment ratios for all the individual mutations were calculated and normalized.
Flow cytometry analysis of SARS-CoV-2 S mutants

Exp293F cells at 2.0 × 10^6 cells/ml were transfected with plasmid DNA (300 ng/ml of culture for measuring myc-S surface expression and sACE2 competition binding and 500 ng/ml for titration experiments) encoding myc-S variants using ExpiFectamine (Thermo Fisher Scientific) according to the manufacturer’s directions. At 24 hours after transfection, cells were washed with PBS-BSA. To detect surface-expressed myc-S, cells were incubated with anti-myc Alexa Fluor 647 (clone 9B11, 1/250 dilution; Cell Signaling Technology) on a rocker at 4°C for 30 min. To measure competitive binding of WT and engineered receptors, cells were instead incubated with 25 nM sACE2 WT-IgG1 and 20 nM sACE2 v2.4-8h for 30 min at 4°C, washed twice, and stained with anti-human IgG-APC (clone HP6017, 1/250 dilution; BioLegend) and anti–HIS-FITC (chicken polyclonal, 1/100 dilution). For all experiments, cells were washed twice before analysis on an Accuri C6 Flow Cytometer (BD Biosciences), and data were processed with FCS Express (De Novo Software). Quantification of myc-S surface expression is detailed in fig. S5.

Reagent and data availability

Plasmids for RBD protein expression are deposited with Addgene (numbers 145145, 164845, and 161821-161826). Illumina sequencing data were processed with FCS Express (De Novo Software). Quantification of myc-S surface expression is detailed in fig. S5.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/8/eabb1738/DC1

View/request a protocol for this paper from Bio-protocol.

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All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.