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Antibody-like proteins that capture and neutralize SARS-CoV-2

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To combat SARS-CoV-2 and any unknown emerging pathogens in the future, the development of a rapid and effective method to generate high-affinity antibodies or antibody-like proteins is of critical importance. We here report a high-speed in vitro selection of multiple high-affinity antibody-like proteins against various targets including the SARS-CoV-2 spike protein. The sequences of monobodies against the SARS-CoV-2 spike protein were successfully procured within only four days. Furthermore, the obtained monobody efficiently captured SARS-CoV-2 particles from the nasal swab samples of patients and exhibited a high neutralizing activity against SARS-CoV-2 infection ($IC_{50} = 0.5$ nM). The high-speed in vitro selection of antibody-like proteins would be useful for the rapid development of a detection method and a neutralizing protein against a virus responsible for an ongoing, and possibly a future, pandemic.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, which was caused by the severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2), has negatively and deeply affected our lives and societies. To control the COVID-19 pandemic in addition to any emerging, heretofore unknown pathogens in the future, it is of paramount importance to rapidly generate multiple high-affinity antibodies or antibody-like proteins against virus proteins, thus developing both a detection method for the virus(1) and a neutralizing protein against it(2).

The identification of a cross-reactive antibody among those developed against SARS-CoV is the fastest way to obtain an antibody(3-7). In principle, however, this approach would yield lower-affinity antibodies with cross-reactivity to the original virus. The isolation of monoclonal antibodies from virus-infected patients is another possible approach(8), but the collection of B cell samples and the identification and large-scale production of effective antibodies represent a long-term research and development project.

In vitro selection is advantageous in terms of isolation speed of antibody-like proteins (ALPs) because time-consuming animal immunization is not necessary to generate ALPs. This method also allows the use of immunoglobulin protein scaffolds, including single-chain variable fragments(9, 10), single-domain antibodies(11), and non-immunoglobulin proteins. Such non-immunoglobulin proteins as a lipocalin(12), a domain of fibronectin(13), the Z-domain of protein A(14), the ankyrin repeat motif(15), and SH3 domain of Fyn(16)

allow a greater chance to obtain high-affinity ALPs.

Phage display is the method that is used most commonly for in vitro selection(17). ALP libraries were expressed in *E. coli* and displayed on the surface protein of phage. The *E. coli* transformation step using phage DNA, the efficiency of which limited the library size practically to 10^9 to 10^{11} , is the main disadvantage of phage display. To generate highly diverse protein libraries, typically 10^{12} to 10^{13} , a cell-free translation system has been used for mRNA display(18-20). However, this display method requires multistep manipulations to synthesize protein/mRNA/puromycin-linker (PuL) complexes; the transcription of the mRNA; the attachment of the PuL to the mRNA; and the translation of library proteins. These multistep manipulations render mRNA display a time-consuming method, with more than weeks typically being necessary to complete six to seven rounds of the procedure (2-3 days per round).

We previously modified the mRNA display method and developed a high-speed in vitro selection method, i.e., the transcription-translation coupled with association of puromycin linker (TRAP) display, for the identification of macrocyclic peptides (Fig. S1A)(21). In the TRAP display, macrocyclic peptide/mRNA/PuL complexes are automatically synthesized via the simple addition of the peptide template DNA, thus skipping two time-consuming steps: the transcription of mRNA and the ligation of PuL. We reported the completion of six rounds of selection in approximately 14 hours using TRAP display, and obtained macrocyclic peptides with nanomolar affinity to their target protein. We also

performed selection against the vascular endothelial growth factor receptor 2 (VEGFR2), and obtained a macrocyclic peptide with VEGF-induced angiogenesis inhibitory activity(22).

Here, we report the development of an improved TRAP display to facilitate the selection of ALPs (Fig. 1A and Fig. S1A). Subsequently, we performed test selections against the human epidermal growth factor receptor 1 (EGFR1) and the epithelial growth factor receptor type 2 (HER2) using a nanobody (the camelid single-domain antibody)(11, 23, 24) and a monobody (the 10th type III domain of human fibronectin) (13) as backbone proteins. We further used this method to rapidly obtain monobodies against the S1 subunit of SARS-CoV-2 spike protein. The selected high-affinity monobodies were used to capture SARS-CoV-2 particles from nasal swab samples of patients. We also increased the affinity of a monobody by the dimerization. The monomeric and dimeric monobodies were used as a neutralizing protein against SARS-CoV-2 infection.

Results

Development of the improved TRAP display for antibody-like protein selection

We started our study by measuring the display efficiencies of the nanobody and monobody on the mRNA, because they determine the diversity of ALP pools and the selection efficiency against a target protein. The monobody DNA template and the DNA-puromycin linker (dPuL, Fig. S1B) were added to the TRAP system, followed by the determination of the display efficiency of the monobody on the mRNA. In contrast with our expectations, less than 3% of the dPuL formed a monobody/mRNA/dPuL complex; moreover, most of the dPuL (87%) did not form an mRNA/dPuL complex (Fig. S1C). As an mRNA sufficient to capture the dPuL (1 μ M) was produced within 10 min in the reaction mixture (Fig. S2A), an unexpected reaction occurred that prohibited the formation of the mRNA/dPuL complex.

We found that the DNA part of the dPuL that was complementary to the 21mers of the 3'-end mRNA sequence (Fig. S1B) was transcribed to RNA in a promoter-independent manner(25), and formed an undesired DNA/RNA duplex; therefore, it inhibited the formation of the mRNA/dPuL complex (Fig. S2B). To prevent promoter-independent transcription, we added a 2'-methoxy (OMe) modification on the ribose because it was reported to prevent T7 promoter-dependent transcription(26). Indeed, the 2'-OMe modification also prevented the promoter-independent transcription, even after an incubation of 30 min (Fig. S2B).

We then used the 2'-methoxy-modified PuL (mPuL) in the TRAP display. As expected, the efficiency of the formation of the mRNA/mPuL complex was increased, from 11% (dPuL) to 60% (mPuL), and the display efficiency was augmented, from 3% (dPuL) to 22% (mPuL) (Fig. S1C). We also analyzed

the display efficiency of the anti-triclocarban nanobody(23, 24) on the mRNA. The efficiency of the formation of the mRNA/mPuL complex was increased, from 9% (dPuL) to 45% (mPuL); moreover, the display efficiency of the nanobody on the mRNA was also increased, from 2% (dPuL) to 15% (mPuL) (Fig. S1C).

The TRAP display was very useful to simplify the selection procedure, but it was not ideal for the first-round selection, because the diversity of the library was limited by the amount of the DNA template added in the translation mixture (see Fig. S2C for the optimization of DNA concentration). To maximize the diversity of libraries, we usually stock an mRNA pool and add it to the translation reaction after the formation of the mRNA/PuL complex in the first-round selection. Therefore, we also analyzed the display efficiency of the nanobody/monobody on the mRNA in the first-round format. We found that the display efficiencies were similar to that of the TRAP display format for both the monobody (19%) and nanobody (15%) (Fig. S1D).

Next, we conducted a test selection using the monobody and nanobody libraries against EGFR1 and HER2 (Fig. S3). High-affinity binders (K_D = 1.3 nM and 3.5 nM for EGFR1, and K_D = 4.4 nM and 13 nM for HER2) were obtained from the monobody library, whereas mid-affinity ones were obtained from the nanobody library (K_D = 47 nM and 32 nM for EGFR1, and K_D = 11 nM and 9.9 nM for HER2).

High-speed selection of monobodies against the SARS-CoV-2 spike protein S1 subunit

According to the results presented above, we focused on the monobody for the subsequent library construction. We introduced 8 or 10 random residues at the BC loop (Pro25-Val29) and 10 or 12 random residues at the FG loop (Gly77-Lys86) of the monobody (Fig. 1B), according to previous reports(13, 20, 27, 28). We used a Tyr-, Ser-, Gly-, and Trp-rich codon mix to construct the random residues(27, 28). The preparation of the mRNA library took 6 months after we ordered the oligonucleotides, because of the oligonucleotide synthesis using trinucleotide phosphoramidites(29) and the large-scale PCR and transcription procedures that were applied to maintain the diversity of the monobody library. However, once the pool of mRNA was prepared, it could be used for multiple in vitro selection studies against various targets because of the large amount of mRNA produced.

With the pool mRNA in our hands, we received the two biotinylated targets, the SARS-CoV-2 spike protein S1 subunit (S1-biotin) and the receptor binding domain (RBD-biotin), and started the TRAP display selection (Fig. 1A). In the first round of the selection, an mRNA template, rather than a DNA one, was used to maximize the diversity of the monobody library (1 μ M mRNA in a 500 μ L scale; monobody display efficiency on the mRNA of about 10%; 3×10^{13} calculated

molecules; Fig. S4). We used mixed targets in the first round, to optimize the effort of the large-scale preparation of the cell-free translation system. From the second round, we used the TRAP display to boost the speed of selection, and simultaneously conducted selection procedures against each target. We observed an enrichment of binder monobodies against both targets in the third round of selection (Fig. 1C). To obtain higher-affinity monobodies, we performed an additional three rounds of selection using a 10-times lower target concentration (2 nM). After the six rounds of selection, the recovered cDNAs of monobodies were sequenced. Notably, because of the high-speed in vitro selection feature of the TRAP display (Fig. 1A), we successfully completed the selection within 3 days, and obtained the monobody sequences on the fourth day.

Receptor binding domain-specific monobodies with low- to sub-nM K_D values

As the same clones were enriched through the selection against both the S1 subunit and the RBD, we selected the nine most-enriched clones in the pool (Table 1) for further study. To confirm the binding activity, we immobilized the purified monobodies (Table S1, Fig. S5) on a microwell plate and added either the S1 subunit (S1-HRP) or the RBD conjugated with streptavidin-horseradish peroxidase (Stv-HRP). The results of the enzyme-linked immunosorbent assay (ELISA) showed that the all monobodies were able to bind to both targets (Fig. 2A). In fact, the wild-type monobody (WT, the control protein with the original loop sequences) did not bind to either of the targets. These results clearly indicated that the selected loop sequences were responsible for the binding to the RBD of the S1 subunit.

To study the stability of monobodies, C-terminally biotinylated monobodies prepared by sortase A reaction (monobody-biotin, Fig. S5) were incubated overnight at various temperatures, and the remaining binding activity was measured by ELISA using an S1 subunit-immobilized microplate and Stv-HRP. We found that all monobodies were stable at 37°C, and that five of them retained half of the binding activity after incubation at 50°C overnight (Fig. S6).

We further assessed the kinetic parameters of all nine monobodies using bio-layer interferometry (BLI). S1-biotin was immobilized on the streptavidin-immobilized sensor and various concentrations of monobodies were added, to determine the kinetic parameters based on global fitting (Fig. 2B, Table 1). The seven clones exhibited a sub-nM to nM-level affinity against the S1 subunit (clone 1, K_D = 2.47 nM; clone 4, K_D = 1.94 nM; clone 6, K_D = 0.76 nM; clone 10, K_D = 2.02 nM; clone 11, K_D = 3.23 nM; clone 12, K_D = 1.37 nM; clone 18, K_D = 0.65 nM). Since mutations were found in the clone 6 (S55W), clone 11 (L62M and S to T in the BC loop), and clone 12 (D67H), we also determined the kinetic parameters of the

back mutants (6b, 11b, and 12b). The affinities were similar to those observed for the parental ones, suggesting that these mutations do not contribute to the binding affinity. It was interesting to obtain high-affinity monobodies, because the in vitro selection of ALPs sometimes required additional affinity maturation after the initial selection to obtain high-affinity monobodies. We believe that this finding is attributable to either the high antigenicity of the S1 subunit or the high diversity/quality of the library prepared using trinucleotide phosphoramidite synthesis(27-29).

Monobodies bound to the SARS-CoV-2 spike protein S1 subunit but not to that of SARS-CoV

Next, we studied the specificity of the monobodies for the SARS-CoV-2 spike protein S1 subunit, because this parameter is important for the development of a future diagnostic assay for this virus. Since the SARS-CoV-2 spike protein sequence exhibits greater similarity with that of SARS CoV than it does with other human coronavirus (30), we decided to test the specificity of the monobodies against the S1 subunit of SARS-CoV-2 and SARS-CoV. The pull-down experiment using monobody-immobilized beads showed that four monobodies (clones 4, 6, 9, and 10) bound to the SARS-CoV-2 S1 subunit, but not to the SARS-CoV S1 subunit (Fig. 3A), suggesting that these monobodies are specific for the SARS-CoV-2 S1 subunit. In turn, four monobodies (clones 1, 11, 12, and 18) bound to both targets, and clone 16 did not bind to either of the S1 subunits because of a low binding affinity (Table 1). Clone 16 pulled down a spike protein trimer (Fig. 3A), probably because of a multivalent effect of the target.

Monobodies inhibited the interaction between ACE2 and the S1 subunit

To determine whether the binding site of the monobodies overlapped with that of the angiotensin-converting enzyme 2 (ACE2), we also tested the inhibition of the S1 subunit/ACE2 interaction (31) afforded by the monobodies. The S1 subunit was immobilized on a microplate and the pre-mixed solution of ACE2-HRP and each monobody was added. The results of ELISA showed that monobody clones 4, 6, 9, and 10 at a concentration of 100 nM inhibited the binding of the S1 subunit to ACE2 (Fig. 3B); moreover, the inhibition was retained for three of the monobodies (clones 4, 6, and 10) at the low concentration of 10 nM (Fig. 3C). Interestingly, these monobodies also exhibited specificity toward the SARS-CoV-2 S1 subunit (Fig. 3A), probably because the ACE2 binding subdomain of the RBD contains more substitutions than other regions of the spike protein (5, 6).

Application of monobodies in sandwich ELISA

The nine monobodies studied above exhibited binding activity toward the S1 subunit. This observation led us to develop

a sandwich ELISA for the detection of the S1 subunit, because it requires a pair of antibodies that recognize distinct epitopes of the S1 subunit. To explore for such monoclonal pairs, we tested all combinations of the cloned monoclonal antibodies in a sandwich ELISA format and found that the monoclonal antibodies recognized three different epitopes (Fig. 3D, Table 1). Monoclonal clones 1, 11, 12, and 18 shared an epitope that was not located at the ACE2-binding surface (Figs. 3B and 3D). The FG loop sequences of these monoclonal antibodies were similar (xLWGYxTxWD; with x representing a non-conserved residue), suggesting that they are the main antigen-binding loop. Monoclonal clones 6 and 10 shared another epitope that was likely located in the ACE2-binding subdomain (Figs. 3B and 3D). These clones exhibited a conserved sequence in the FG loop (xYxGPxxYGxxEx), which will be investigated in a future study. Monoclonal clones 4 and 9 shared another epitope that was also likely located in the ACE2-binding surface (Figs. 3B and 3D). No conserved sequence was detected in the BC loop or the FG loop of these clones. Therefore, we identified 16 pairs that can be used in further sandwich ELISA. Among them, we tested clone 10 as a capturing monoclonal antibody and clone 12 as a detecting monoclonal antibody, because the pair gave one of the highest signal-to-noise ratios in the sandwich ELISA experiment (Fig. 3D). The results of sandwich ELISA showed that a combination of monoclonal antibodies could specifically detect the SARS-CoV-2 spike protein trimer and the S1 subunit (Fig. 3E). Although the sensitivity of this system was not sufficient to detect the target in actual samples (10^3 particles of SARS-CoV-2 in a 100 μ L solution, corresponding to 1 fM spike protein), its combination with other detection methods, such as digital ELISA(32) or loop-mediated isothermal amplification(33), may help to overcome the problem of the detection limit in a future study.

Capture of SARS-CoV-2 virions using monoclonal antibodies from a cultivated sample

Next, we studied the binding of monoclonal antibodies to SARS-CoV-2. Each monoclonal-biotin was incubated with SARS-CoV-2 particles in phosphate-buffered saline (PBS) and the viral particles bound to the monoclonal antibodies were collected on the magnetic beads. An RT-qPCR assay of the SARS-CoV-2 RNA extract in the supernatants and the bead eluents showed that a large proportion of the SARS-CoV-2 particles was detected in the bead eluents for all monoclonal antibodies, with the exception of the WT monoclonal antibody (Fig. 4A), proving the binding activity of all selected monoclonal antibodies toward SARS-CoV-2 particles.

Based on the experiment described above, we realized that this pull-down method also would be useful for enhancing the detection limit of SARS-CoV-2. To test this idea, SARS-CoV-2 particles were suspended in a PBS solution at various concentrations (0.1–10,000 particles/ μ L), and the number of particles was analyzed by pull-down RT-qPCR using

monoclonal clone 4 or by a traditional RT-qPCR. The result showed a wide linear dynamic range of this method (Fig. 4B). The detection limit indeed was increased from 1 to 0.1 particles/ μ L.

Monoclonal antibody capture of SARS-CoV-2 virions from nasal swab samples of patients

We then performed a pull-down of SARS-CoV-2 particles from nasal swab samples of patients (Fig. 4C). Of note, the actual patient samples (sometimes in the form of highly viscous liquids) were very different from cultured samples, primarily because they contain many unknown components. We tested 16 samples, and found that the monoclonal antibody captured SARS-CoV-2 even in these nasal swab samples. To our knowledge, this is the first example of an antibody or an ALP to capture SARS-CoV-2 from patient nasal swab samples. Interestingly, for samples #8, #15, and #16, the capture rates were significantly lower than the remaining cases. We reasoned that this might be because the monoclonal antibody binding site was hidden by neutralizing antibodies developed in the patients.

Finally, we performed a pull-down direct RT-qPCR for nasal swab samples (400 μ L) of patients (Fig. 4D). We tested four samples this way and found that the monoclonal antibody captured SARS-CoV-2 even in higher-volume nasal swab samples. The number of RNA genomes were increased 25–77 times after the pull-down technique was employed. Moreover, RNA genome in the nasal swab sample #4 was detected only by the pull-down direct RT-qPCR but not by traditional direct RT-qPCR, suggesting the increased sensitivity of the pull-down direct RT-qPCR assay.

Binding activity of monoclonal antibody tandem dimers

A dimeric form of a protein binder usually showed higher affinity than the monomeric one(7, 16); therefore, we next synthesized the tandem dimers of a monoclonal antibody. We chose clone 4, 6b, and 12b to synthesize the corresponding tandem dimers (TD4, TD6b, and TD12b) because these clones showed the highest affinity in each group defined by the binding competition assay (Fig. 3D). As expected, the affinities of these tandem dimers were drastically improved (Fig. 5A, Table 1): the K_D displayed less than 1 pM because of the immeasurable k_{off} value (10^{-7} /s).

To understand why the dimerization of monoclonal antibodies drastically improved the affinity, we first immobilized decreased amounts of S1 protein on the sensor chip and analyzed monoclonal antibody dissociation. The results showed that the monoclonal antibody dimers still dissociated very slowly (Fig. S7A). Next, when the monoclonal antibodies were immobilized on the sensor chip and the S1 protein was used as an analyte, we observed a similar K_D value for Clone 6b (Fig. 2 vs. Fig. S7B, Table 1) and no improvement on the K_D values for the tandem dimers (Fig. 2 vs.

Figure 5B, Table 1). These results suggest that the tandem connection of monobodies does not improve the affinity, but bringing up the bridging effect, *i.e.*, an avidity effect.

Monobodies exhibited a high neutralizing activity against SARS-CoV-2 infection

The monobody clone 6b showed high affinity against the S1 subunit of the SARS-CoV-2 spike protein and inhibited the interaction between ACE2 and the S1 subunit (Fig. 3C). These findings led us to analyze the neutralizing activity of monobody clone 6b and TD6b against SARS-CoV-2 infection. We added 4-fold serially diluted monobody solutions to VeroE6/TMPRSS2 cells(34) in 96-well culture plates, followed by infection with SARS-CoV-2 (10^5 TCID₅₀/mL) for 1 hour at 37°C. After 36 hours of incubation with fresh medium, SARS-CoV-2 mRNA amounts in the supernatant were measured by qRT-PCR. The monobody clone 6b showed a very low half maximal inhibitory concentration (IC₅₀ = 0.5 nM, Fig. 5C), even in the monomeric form. Its neutralizing activity was increased in the dimeric form (IC₅₀ = 0.4 nM), although the data obtained for 390 pM TD6b fluctuated, probably because of the high sensitivity of VeroE6/TMPRSS2 cells. More importantly, these IC₅₀ values were comparable to those of the high-affinity human neutralizing antibodies reported recently(35-37), indicating the usefulness of TRAP display for the selection of highly active ALPs.

Discussion

Here, we developed a high-speed in vitro selection method to obtain ALPs against various targets. We demonstrated a constant development of high-affinity binders (K_D values in the low- or sub-nM range) against EGFR1, HER2, and the SARS-CoV-2 spike protein. Furthermore, our selected monobodies recognized a recombinant spike protein as well as SARS-CoV-2 particles, demonstrating applicability for a sandwich ELISA for a rapid antigen test. Using the monobody before performing pull-down RT-qPCR also increased the detection limit of SARS-CoV-2. More importantly, the monobody captured SARS-CoV-2 in nasal swab samples of COVID-19 patients. Actual patient samples are very different from cultured samples because they consist of inhomogeneous liquid and contain many unknown components, including various proteases, mucins, and neutralization antibodies. We believe that the monobodies procured in our study will soon be useful to develop effective diagnostic tools and that such tools will contribute to the worldwide effort to overcome the COVID-19 pandemic. The monobody also had a very low IC₅₀ value against SARS-CoV-2 infection, which was comparable to those of the antibodies that were reported recently as being highly active for neutralizing SARS-CoV-2(35-37). Because the backbone of the monobody was the 10th type III domain of human fibronectin, it might be useful as a neutralizing ALP

against SARS-CoV-2 infection.

We received the SARS-CoV-2 spike protein S1 subunit on April 7, 2020 as a target of the TRAP display selection and, using the methods described herein, obtained high-affinity monobody sequences on April 10, 2020. The high-speed feature of the improved TRAP display is therefore useful for a rapid response to subspecies of SARS-CoV-2(38, 39) in addition to potential new viruses causing future pandemics.

Materials and Methods

Materials

The oligonucleotides were purchased either Fasmac Co., Ltd. (Japan) or Nippon Bio Service (Japan). The primer/probe set for RT-qPCR and positive control RNA for N2 were purchased from Applied Biosystems, Inc. (NY, USA). The synthetic DNAs were acquired from GenScript (NJ, USA). The sequences of the primers and synthetic DNAs are listed in Data file S1. SARS-CoV-2 protein active trimer (SPN-C52H8), human ACE2 protein (AC2-H5257), biotinylated 2019-nCoV S1 subunit-Avitag (S1N-C82E8), biotinylated 2019-nCoV S protein RBD-Avitag (SPD-C82E9), SARS-CoV-2 (COVID-19) S1 subunit (S1N-C52H4), and SARS S1 subunit (S1N-S52H5) were all purchased from ACROBiosystems (DE, USA). EGF receptor/Fc chimera and ErbB2/Fc chimera were obtained from R&D systems (MN, USA). Creatine kinase, creatine phosphate, and *E. coli* tRNAs were purchased from Roche Diagnostics (Japan). The restriction enzymes were obtained from New England Biolabs (MA, USA).

Preparation of monobody mRNA libraries for selection against SARS-CoV-2 targets

To prepare an A-fragment DNA of the monobody library, FN3F0.F83 (1 μ M), FN3F1-2.F29(P) (1 μ M), and the FN3FF1coR8.F73 (0.5 μ M) or FN3FF1coR10.F79 (0.5 μ M) were ligated by T4 DNA ligase (75 μ L in total, 75 pmol for each oligonucleotide) with an assistance of Fn3an1.R20(3NH2) (2 μ M), and Fn3an2-1.R20(3NH2) (2 μ M). As a codons for the randomized residues, we used a codon mix with the following ratios: 20% Tyr, 10% Ser, 15% Gly, 10% Trp, and 3% each of all the other amino acids except for Cys, which is similar to the original cocktail (30% Tyr, 15% Ser, 10% Gly, 5% Phe, 5% Trp, and 2.5% each of all the other amino acids except for Cys)(27, 28). After the ligation, the entire mixture was added to the reaction mixture (10 mM Tris-HCl pH 8.4, 100 mM KCl, 0.1% (v/v) Triton X-100, 2% (v/v) DMSO, 2 mM MgSO₄, 0.2 mM each dNTP, 0.375 μ M T7SD8M2.F44, 0.375 μ M FN3BsaI.R40, and 2 nM of *Pfu-S* DNA polymerase) and the amplified by PCR (15 mL in total, seven cycles of PCR). B-fragment DNA was prepared by the same procedure using FN3FF2co.F72(p), FN3F3coR10.F70(p), FN3F3coR12.F76(p), Fn3an3.R20(3NH2) for ligation, and FN3BsaI.F33, FN3Pri2.R44 for amplification.

The amplified A-fragment DNA and B-fragment DNA were purified by phenol/chloroform extraction and isopropanol precipitation. One end of each DNA product was digested with *Bsa*I (New England Biolabs, MA, USA) as per the manufacturer's protocol, and the DNA products were then purified by phenol/chloroform extraction and isopropanol precipitation. The products were ligated to each other (1 μ M, 200 μ L) to synthesize full-length DNA products, and they were amplified using T7SD8M2.F44, G5S-4Gan21-3.R42, and *Pfu*-S DNA polymerase (60 mL in total, four cycles of PCR). The products were purified through phenol/chloroform extraction and isopropanol precipitation. The DNA template was transcribed by in vitro run-off transcription, and the mRNA was purified by isopropanol precipitation followed by PAGE purification. The mRNA/HEX-mPuL was prepared by a similar procedure described above. The resulting complex was directly used in the first-round of selection.

In vitro selection of monobodies against SARS-CoV-2 Spike protein S1 subunit and RBD of the S1 subunit by the improved TRAP display

For first-round selection, 1 μ M mRNA/ Pu-OMe-linker was added to a reconstituted translation system, and the reaction mixture (500 μ L) was incubated at 37°C for 30 min. After the reaction, 41.7 μ L of 200 mM EDTA (pH 8.0) was added to the translation mixture. A reverse transcription buffer (41.1 μ L of 0.78 M Tris-HCl pH 8.4, 1.16 M KCl, 0.37 M MgCl₂ and 0.08 M DTT), 5 mM dNTPs (66.7 μ L), 100 μ M FN3S.R29 (10 μ L), and 28.7 μ M HMLV (27.5 μ L) were added to the translation mixture, and the resulting solution was incubated at 42°C for 15 min. The buffer was exchanged to HBST buffer using Zeba Spin Desalting Columns. In order to remove the bead binders, the resulting solution was mixed with Dynabeads M-280/M-270 streptavidin (1:1) (Thermo Fisher Scientific) at 25°C for 10 min. The supernatant was mixed with 4.8 μ L of 7.3 μ M biotinylated 2019-nCoV S1 subunit-Avitag and 2.1 μ L of 16.5 μ M biotinylated 2019-nCoV S protein RBD-Avitag, and then incubated at 25°C for 5 min. After recovering the target proteins with Dynabeads M-270 streptavidin, the resulting beads were washed with HBST buffer thrice, and PCR premix (1 mL) was added. The beads were heated at 95°C for 5 min, and the amount of the eluted cDNAs was quantified by SYBR green-based quantitative PCR using T7SD8M2.F44 and FN3Lip.R20 as primers. The eluted cDNAs were PCR-amplified using T7SD8M2.F44, G5S-4Gan21-3.R42, and *Pfu*-S DNA polymerase, and was purified by phenol/chloroform extraction and isopropanol precipitation. The post-translation and post-reverse transcription reaction mixtures were analyzed by using the same procedure as described above.

For the second-round selection, the resulting DNA (about 3–10 nM in final concentration) was added to the TRAP system and the reaction mixture (40 μ L) was incubated at 37°C

for 30 min. After the reaction, 8 μ L of 100 mM EDTA (pH 8.0) was added to the translation mixture. Reverse transcription mixture (24 μ L; 150 mM Tris-HCl pH 8.4, 225 mM KCl, 75 mM MgCl₂ and 16 mM DTT, 1.5 mM dNTPs, 7.5 μ M primer, and 3.4 μ M HMLV) were added to the translation mixture, and the resulting solution was incubated at 42°C for 15 min. The buffer was exchanged to HBST buffer using Zeba Spin Desalting Columns. To remove bead binders, the resulting solution was mixed thrice with M270/M280 magnetic beads (1:1) at 25°C for 20 min. Each target (20 nM final) was added to the half portion of the supernatant. After removing the supernatant, the beads were washed with HBST buffer thrice, and the PCR premix was added to the beads. Quantitation of cDNA and amplification and purification of DNA were performed by the same procedure as described for the first-round selection.

For the third- to sixth-round selection, the procedure was performed similarly as that of the second-round, except for the volume of the reaction mixtures (5 μ L), the target concentration (2 nM in fourth-round to sixth-round), and bead washing conditions (an extensive wash in the sixth-round selection against S1 subunit). After the sixth-round selection, the sequences of the recovered DNAs were analyzed using an Ion Torrent instrument (Thermo Fisher Scientific).

Affinity measurement of selected nanobodies and monobodies

The affinity measurement was performed against the EGF receptor/Fc chimera or the ErbB2/Fc chimera immobilized on an anti-Human IgG Fc Capture biosensor (ForteBio), biotinylated 2019-nCoV S1 subunit-Avitag or biotinylated monobodies immobilized on a streptavidin biosensor (ForteBio), using Octet system (ForteBio, CA, USA), as described in the manufacturer's instructions. The binding assay was performed at 30°C in the buffer D. Each step in the binding assay was as follows: equilibration for 150 s, association for 900 s, and dissociation for 900 s (EGF and HER2); equilibration for 150 s, association for 600 s, and dissociation for 600 s (S1 subunit). Buffer D' [50 mM Hepes-KOH pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween 20, and 1% (w/v) PEG6000] was used when the S1 subunit was an analyte.

Binding assay of monobodies against SARS-CoV-2 S1 or S1 RBD proteins

The ELISA microplate with 96 wells was coated with 100 μ L of 100 nM monobody in HBS2 buffer (25 mM Hepes-K pH 7.5, 150 mM NaCl) overnight. The microplate was washed once with HBST2 buffer [HBS2 with 0.05% (v/v) Tween 20], blocked with 1% (w/v) bovine serum albumin (BSA) in HBS2 buffer at room temperature for one hour, and then was washed once with HBST2 buffer. S1-HRP or RBD-HRP was prepared by mixing biotinylated SARS-CoV-2 spike protein S1

subunit (S1-biotin) or the receptor binding domain (RBD-biotin) with the same amount of streptavidin-HRP (Stv-HRP) in HBST2BP buffer [HBST2 with 0.1% (w/v) BSA and 0.1% (w/v) PEG6000], and was added to the microplate with a final concentration of 10 nM. The microplate was incubated at room temperature for one hour. The microplate was washed five times with HBST2 and 200 μ L of SuperSignal ELISA Pico was added to each well. The chemiluminescence was measured immediately using the SpectraMax M5 plate reader (Molecular Devices).

Pull-down of S proteins with monocbody-immobilized beads

Biotinylated monocbody (1 μ M, 3.3 μ L) in HBST2BP buffer was mixed with Dynabeads M-280 streptavidin (10 mg/mL, 6.6 μ L, pre-washed by HBST buffer) at room temperature for 5 min. After removing the supernatant, the beads were washed twice with HBST buffer, and 2.5 μ L of 100 nM each analyte (SARS-CoV-2 spike protein S1 subunit, SARS-CoV spike protein S1 subunit, SARS-CoV spike protein trimer) in buffer D was added to a one-third fraction of the beads. After mixing at room temperature for 30 min, the supernatant was collected. The beads were washed twice with HBST buffer. The supernatant and heat-elution from the beads were loaded on SDS-PAGE gel and was stained using Sypro Ruby.

Inhibition of ACE2 / CoV-2 S1 subunit interaction by monocbodies

ELISA was performed using a microplate with 96 wells, coated with 100 μ L of 2 nM SARS-CoV-2 Spike protein S1 subunit in HBS2 buffer overnight. The microplate was washed once with HBST2 buffer, blocked with 1% (w/v) BSA in HBS2 buffer at room temperature for one hour, and was then washed once with HBST2 buffer. Biotinylated angiotensin-converting enzyme 2 (ACE2-biotin) complexed with same amount of Stv-HRP in HBST2BP buffer was added to a final concentration of 1 nM together with 100 nM or 10 nM monocbody. Alternatively, a monocbody (10 nM in total) was added to the ACE2-HRP-preincubated S1-immobilized microplate. The microplate was incubated for one hour at 37°C. The plate was then washed five times with HBST2 buffer. After addition of SuperSignal ELISA Pico (200 μ L) to each well, the chemiluminescence was recorded immediately using a SpectraMax M5 plate reader.

Sandwich ELISA using monocbodies

The sandwich ELISA was performed using a microplate with 384 wells, which was coated with 25 μ L of 100 nM of the capturing monocbodies (9 clones plus WT) in HBS2 buffer overnight. The microplate was washed once with HBST2 buffer, blocked with 1% (w/v) BSA in HBS2 buffer at room temperature for one hour, and was then washed once with HBST2

buffer. SARS-CoV-2 S1 subunit (10 nM) in HBST2BP buffer was added. The microplate was incubated at room temperature for one hour, and was subsequently washed once with HBST2 buffer. Biotinylated detecting monocbody (9 clones plus WT) complexed with same amount of Stv-HRP in HBST2BP buffer was added to a final concentration of 10 nM, and the plate was incubated at room temperature for 30 min. The microplate was washed five times with HBST2 buffer, and 50 μ L of SuperSignal ELISA femto (Thermo Fisher Scientific) was added to each well. The chemiluminescence was immediately recorded using the SpectraMax M5 plate reader.

For the other sandwich ELISA, a microplate with 96 wells was coated with 100 μ L of 100 nM monocbody clone 10 in HBS2 buffer overnight. The microplate was washed once with HBST2, blocked with 1% (w/v) BSA in HBS2 buffer at room temperature for one hour, and was washed once with HBST2 buffer. One of the analytes, either the SARS-CoV-2 S1 subunit, the spike protein trimer, or the SARS-CoV S1 subunit was incubated with the detecting monocbody-HRP (clone 12, 1 nM) on ice for one hour. The solution was then added to the capturing monocbody-immobilized microplate and was incubated at room temperature for one hour. The microplate was washed five times with HBST2 buffer and 200 μ L of SuperSignal ELISA femto was added to each well. The chemiluminescence was immediately recorded using the SpectraMax M5 plate reader.

Binding assay of monocbodies toward SARS-CoV2 particles

The SARS-CoV-2 virus was isolated from Vero-E6 cells (ATCC) from a nasal swab sample of a patient in the Nagoya Medical Center, Japan. Virus-infected cells were grown in Dulbecco's modified Eagle medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and penicillin (100 U/mL) and streptomycin (100 μ g/mL) (Thermo Fisher Scientific). Four days after infection, the supernatant was harvested. The supernatant was clarified by centrifugation and filtration through a 0.45- μ m-pore-size syringe filter (Merck Millipore), and used for the virion-binding assay. The virus particle amounts were measured using droplet digital PCR using the One Step RT-ddPCR Advanced Kit for Probes (Bio-Rad) with a primer/probe set for N2 (Data file S1)(40).

SARS-CoV-2 particles (5×10^4 particles/140 μ L) and 10 nM monocbody in HBST buffer were incubated at 25°C for 10 min. This was followed by addition of Dynabeads M-280 streptavidin (10 mg/mL, 10 μ L, pre-washed by PBS buffer), which were incubated with the solution for 10 min. After the incubation, the supernatant was collected for further analysis. The beads were washed three times with HBST, and were suspended with 140 μ L of HBST buffer. Viral RNA was extracted from both the supernatant and the bead suspension using the QIAamp Viral RNA Mini Kit (Qiagen) as described in the

manufacture's protocol. The 5 μ L out of the 60 μ L RNA-containing solution was added to a RT-qPCR reaction mixture (One Step PrimeScript III RT-qPCR Mix, TAKARA-Bio) using a primer/probe set for N2 (40). The target copy numbers were determined using Thermal Cycler Dice Real Time System III (TAKARA-bio), which constantly showed about 3-times higher number of RNA genomes than that of above RT-ddPCR.

Pull-down RT-qPCR for study of the detection limit of SARS-CoV2 particles

SARS-CoV-2 virus particles (0.1–10,000 particles/ μ L based on viral RNA copy number, 700 μ L) were incubated with 2 nM monoclonal clone 4 in PBS (10 mM phosphate pH 7.4, 2.7 mM KCl, 137 mM NaCl) containing 0.05%(v/v) Tween 20 at room temperature for 10 min. The solution was mixed with Dynabeads M-280 streptavidin (10 mg/mL, 8 μ L, pre-washed by PBS buffer) at 25°C for 10 min. The beads were washed with HBST three times and were suspended in HBST. Viral RNA from the bead suspension or the original solution (14 μ L) were extracted using the QIAamp Viral RNA Mini Kit. For RT-qPCR, the One Step PrimeScript III RT-qPCR Mix with the N2 primer/probe set was used. The target copy numbers were determined using Thermal Cycler Dice Real Time System III (TAKARA-bio) as aforementioned.

Pull-down of SARS-CoV-2 in nasal swab specimens from patients

For the pull-down assay using nasal swab specimens from patients, we used residual nasal samples after diagnostic tests. The study was approved by the ethical committee at the Nagoya Medical Center (registration #2019-087) and conducted according to the tenets of the Declaration of Helsinki. Written informed consent for use of the residual samples were obtained from all participants. 35 μ L of each nasal sample was mixed with 2 nM monoclonal clone 4 to a total volume of 100 μ L in HBST buffer and incubated at 25°C for 10 min. The mixture was then incubated for another 10 min with Dynabeads M-280 streptavidin (2.5 mg/mL, 40 μ L, pre-washed by HBST buffer). The RNA copies in both the supernatant and the bead fraction were measured using RT-qPCR, as described above.

Pull-down direct RT-qPCR assay of SARS-CoV-2 in nasal swab specimens from patients

Each residual sample of nasal swab from 4 patients was used for this assay. Briefly, 0.5% Tween-20 (45 μ L) and monoclonal clone 4 (5 μ L of 200 nM) were added into 400 μ L of the swab sample in PBS. The mixture was incubated at 25°C for 10 min, followed by addition of Dynabeads M-280 streptavidin (2.5 mg/mL, 10 μ L, pre-washed by HBST buffer). After 10-min incubation, the beads were washed with 1 mL of HBST three

times and resuspended with 2.5 μ L of HBST containing carrier RNA. The bead suspension or the original swab solution (2.5 μ L) were mixed with a RT-qPCR reaction mixture (47.5 μ L, the PrimeDirect Probe RT-qPCR Mix, TAKARA-bio) with a primer/probe set (Data file S1). The RNA copy numbers were determined using the Thermal Cycler Dice Real Time System III (TAKARA-bio) as aforementioned. The detection level of positive control RNA using the PrimeDirect Probe RT-qPCR Mix was $> \sim 25$ copies/well.

Virus neutralization assay

SARS-CoV-2 neutralization assay was performed using VeroE6/TMPRSS2 cells(34) that were obtained from JCRB cell bank, Ibaraki, Japan. The cells (5×10^3 cells per well, 50 μ L) were seeded in 96-well culture plates and incubated at 37°C for 18 hours before infection. Monoclonal antibodies were four-fold serially diluted (from 400 nM to 24.4 pM) and 20 μ L of the diluted monoclonal antibodies were added into each culture well. Cells were infected with 10 μ L of SARS-CoV-2 (10^5 TCID₅₀/mL) for 1 hour at 37°C. The supernatant was removed, and 80 μ L of Dulbecco's modified Eagle medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and penicillin (100 U/mL) and streptomycin (100 μ g/mL) (Thermo Fisher Scientific). After incubation at 37°C supplied with 5% CO₂ for 36 hours, the culture supernatants were harvested. The SARS-CoV-2 RNA amounts in the supernatants were measured by RT-qPCR using the SARS-CoV-2 Direct Detection RT-qPCR kit (Takara Bio). The half maximal inhibitory concentrations (IC₅₀) were determined using GraphPad Prism version 6.0.

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SUPPLEMENTARY MATERIALS

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Fig. 1. Selection of monobodies against the SARS-CoV-2 spike protein using the TRAP (the transcription-translation coupled with association of PuL) display. **A.** Schematic representation of the TRAP display. Monobody-mRNA complexes were synthesized simply by adding the template DNA to the TRAP system. After RT, selection, and PCR, the amplified DNAs were added to the TRAP system to reproduce a monobody library for the subsequent round of selection. **B.** Structure of the monobody backbone (10th type III domain of fibronectin, 1TTG). The BC and FG loops (labeled in blue) were randomized with the indicated number of residues. **C.** Progress of the TRAP display selection. After each round of selection, the recovered cDNA was quantified by real-time PCR. The recovery of cDNA was calculated by dividing the amount of recovered cDNA by the theoretical amount of mRNA/PuL (1 μ M). At the fourth round, the selection pressure was increased by decreasing the target concentration from 20 nM to 2 nM. The sequences of the monobodies are shown in Table 1. Abbreviations: RT, reverse transcription; Stv, streptavidin; Bio, biotin; PCR, polymerase chain reaction; NTD, N-terminal domain; RBD, receptor binding domain; ACE2, angiotensin-converting enzyme 2; Pu, puromycin; PuL, Pu-linker.

Fig. 2. Binding activity of each clone against the SARS-CoV-2 spike protein S1 subunit and the RBD. A. Determination of the binding domain. A schematic representation of the ELISA is provided on the left. S1 subunit–HRP or RBD–HRP (10 nM) was added to the monobody-immobilized microplate. Error bars, standard deviation of each experiment (in triplicate). **B.** Determination of kinetic parameters by BLI. A schematic representation of the BLI is provided at the top. S1 subunit-biotin was immobilized on a streptavidin-sensor chip, and monobodies (2.5–40 nM) were used in the kinetic analysis. Some mutations were identified in clones 6, 11, and 12. These mutations were reversed in the clones 6b, 11b, and 12b. The data are depicted in blue, and the fit data to a 1:1 binding model is shown in black. The determined kinetic parameters of the monobodies are provided in Table 1. Abbreviations: WT, 10th type III domain of fibronectin; RLU, relative luminescence units; HRP, horseradish peroxidase; Nus, Nus-Tag fused at the C terminus of the monobody; BLI, Bio-layer interferometry. Other abbreviations are as mentioned above.

Fig. 3. Characterization of monobodies and the application for the sandwich ELISA. **A.** A schematic representation of the pull-down assay is provided at the top. The SARS-CoV S1 subunit (100 nM), SARS-CoV-2 S1 subunit (100 nM), or SARS-CoV-2 S protein trimer (100 nM) were pulled down by monobody-immobilized beads. The supernatant and the heat elution from the beads were loaded onto SDS-PAGE followed by stained with Sypro Ruby. **B.** A schematic representation of the ELISA is provided on the left. ACE2-HRP (1 nM) was mixed with each monobody (100 nM) and was added to an S1-immobilized microplate. **C.** ACE2-HRP (1 nM) was mixed with each monobody (10 nM clone 4, 6, 9, or 10), and was added to an S1-immobilized microplate (pre). Alternatively, a monobody was added to an ACE2-HRP-incubated S1-immobilized microplate (post). **D.** A schematic representation of the sandwich ELISA is provided on the left. The S1 subunit (10 nM) was added to the monobody-immobilized microplate. The S1 subunit bound with the capturing monobody was identified by detecting monobody-HRP (10 nM). All possible combinations were tested. **E.** Titration curves of the SARS-CoV-2 S1 subunit, SARS-CoV-2 spike protein trimer, and SARS-CoV S1 subunit. After incubation of the analyte with the detecting monobody-HRP (clone 12, 1 nM), the solution was added to the capturing monobody (clone 10)-immobilized microplate. Error bars, standard deviation of each experiment (in triplicate). Error bars, standard deviation of each experiment (in triplicate). Abbreviations: S1, SARS-CoV or SARS-CoV-2 spike protein S1 subunit, or SARS-CoV-2 spike protein trimer; N.D., not determined. Other abbreviations are as mentioned above.

Fig. 4. Monobody binding to SARS-CoV-2 from culture and patient samples. **A.** Monobody binding activities toward SARS-CoV-2 particles. SARS-CoV-2 particles bound with monobody-biotin were pulled down by M280-streptavidin beads. The RNA genomes in the supernatant and on the beads were quantified by RT-qPCR after RNA extraction. **B.** The detection limits of the traditional RT-qPCR method and the pull-down RT-qPCR method. Various concentrations of SARS-CoV-2 (0.1–10,000 particles/μL) were assayed. SARS-CoV-2 particles were collected from 700 μL of solution by pull-down using monobody clone 4 and viral RNA was quantified by RT-qPCR after RNA extraction. Alternatively, the original solution (14 μL) was subjected to RT-qPCR. **C.** Pull-down of SARS-CoV-2 particles from nasal swab samples (35 μL) of patients. The number of RNA genomes in the supernatant and on the beads were quantified by RT-qPCR after RNA extraction. **D.** Pull-down direct RT-qPCR using nasal swab samples of patients. SARS-CoV-2 particles were collected from 400 μL of nasal swab samples by pull-down and were directly added to a RT-qPCR reaction mixture. Alternatively, an original nasal swab solution (2.5 μL) was added to a RT-ddPCR reaction mixture. Error bars indicate standard deviation of RT-qPCR results (in triplicate). N.D., not detected.

Fig. 5. The affinity of monobody tandem dimers and the neutralization of SARS-CoV-2 infection. **A.** Determination of kinetic parameters of tandem dimers by BLI. S1 subunit-biotin was immobilized on a streptavidin-sensor chip, and the tandem dimers (2.5–40 nM) were used in the kinetic analysis. **B.** The biotinylated tandem dimers of monobody were immobilized on a streptavidin-sensor chip, and the S1 subunit (2.5–40 nM) was used in the kinetic analysis. TD4, TD6b, and TD12b are tandem dimers of the corresponding clones 4, 6b, and 12b connected with a (GGGGS)₃ linker. The data are depicted in blue, and the fit data to a 1:1 binding model is shown in black. The determined kinetic parameters of the monobodies are provided in Table 1. **C.** Monobody-mediated neutralization of SARS-CoV-2 infection in VeroE6/TMPRSS cells. The x-axis value indicates the final concentration of the indicated monobody (6b, TD6b, or WT) for each assay well. The experiment was performed with triplicate samples. The copy number of each RNA in the supernatant is plotted

Table 1. Monobodies obtained by the TRAP display selection against the SARS-CoV-2 spike protein S1 subunit.

The table contains the following information: sequences of the BC and FG loops of the monobodies, the kinetic parameters determined by BLI, the inhibitory activity of the S1 subunit and ACE2 interaction, SARS-CoV-2 S1 subunit specificity, and clones with binding competition. Mutations were identified in the body sequence of clone 6 (S55W), clone 11 (L62M), and clone 12 (D67H). A Ser to Thr mutation in the BC loop of clone 11 was introduced during cloning. These mutations were reversed in the clones 6b, 11b, and 12b. Clones TD4, TD6b, and TD12b are tandem dimers of the corresponding clones. *, monobody was immobilized on sensor chip. N.D., not detected.

Clones	BC loop	FG loop	K_D (nM)	k_{on} (1/Ms) $\times 10^5$	k_{off} (1/s) $\times 10^{-4}$	CoV-2 specificity	S1/ACE2 inhibition	Binding competition
1	GVYDELGH	SLWGYYTMWD	2.47	3.10	7.66	–	–	11, 12, 18
4	PSSRYEHYQF	WTGDVPWYWLVN	1.94	3.48	6.76	+	+	9
6	GGDYVGYY	TYNGPWIYGEEI	0.76	1.47	1.11	+	+	10
9	VYNVYPGT	GSGVKYVVYRRS	17.8	1.83	32.6	+	+	4
10	GHQDYGVS	YYMGPDVYGRSEY	2.02	1.24	2.51	+	+	6
11	TYGSSYGL	ELWGYLTSWD	3.23	2.87	9.25	–	–	1, 12, 18
12	EIYYEIGD	RLWGYYTQWD	1.37	2.89	3.97	–	–	1, 11, 18
16	PRSFDDSQ	GYQFVVYRYGG	24.8	1.22	30.3	N.D.	–	N.D.
18	MYGVVHGV	SLWGYETYWD	0.65	2.19	1.42	–	–	1, 11, 12
6b	GGDYVGYY	TYNGPWIYGEEI	0.42	2.34	0.99			
11b	SYGSSYGL	ELWGYLTSWD	2.53	3.56	9.01			
12b	EIYYEIGD	RLWGYYTQWD	0.62	3.44	2.13			
TD4	PSSRYEHYQF	WTGDVPWYWLVN	<0.001	5.45	<0.001			
TD6b	GGDYVGYY	TYNGPWIYGEEI	<0.001	2.48	<0.001			
TD12b	EIYYEIGD	RLWGYYTQWD	<0.001	6.11	<0.001			
WT	PAVT	GRGDSPASSK	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6b*	GGDYVGYY	TYNGPWIYGEEI	1.18	0.95	1.13			
TD4*	PSSRYEHYQF	WTGDVPWYWLVN	3.13	1.66	5.21			
TD6b*	GGDYVGYY	TYNGPWIYGEEI	2.92	0.54	1.59			
TD12b*	EIYYEIGD	RLWGYYTQWD	1.96	0.82	1.62			