Cryo-EM structure determination of small proteins by nanobody-binding scaffolds (Legobodies)

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Contributed by Tom A. Rapoport, August 26, 2021 (sent for review August 13, 2021; reviewed by Yifan Cheng and Gabriel C. Lander)

We describe a general method that allows structure determination of small proteins by single-particle cryo-electron microscopy (cryo-EM). The method is based on the availability of a target-binding nanobody, which is then rigidly attached to two scaffolds: 1) a Fab fragment of an antibody directed against the nanobody and 2) a nanobody-binding protein A fragment fused to maltose binding protein and Fab-binding domains. The overall ensemble of \( \sim 120 \) kDa, called Legobody, does not perturb the nanobody–target interaction, is easily recognizable in EM images due to its unique shape, and facilitates particle alignment in cryo-EM image processing. The utility of the method is demonstrated for the KDEL receptor, a 23-kDa membrane protein, resulting in a map at 3.2-Å overall resolution with density sufficient for de novo model building, and for the 22-kDa receptor-binding domain (RBD) of SARS-CoV-2 spike protein, resulting in a map at 3.6-Å resolution that allows analysis of the binding interface to the nanobody. The Legobody approach thus overcomes the current size limitations of cryo-EM analysis.

Significance

Structure determination by cryo-EM is difficult or impossible to apply to proteins smaller than \( \sim 100 \) kDa, excluding many membrane proteins and proteins of pharmaceutical importance from the analysis. Here, we report on a general method that allows structure determination of small proteins. The method is based on the availability of a nanobody to a target protein. The nanobody is then rigidly attached to two scaffolds: 1) a Fab fragment of an antibody directed against the nanobody and 2) a nanobody-binding protein A fragment fused to maltose binding protein and Fab-binding domains. We call the overall ensemble Legobody. The method is demonstrated for two small proteins that have sizes of \( \sim 22 \) kDa.

Author contributions: X.W. conceived the idea; X.W. designed research with input from T.A.R.; X.W. performed research; X.W. analyzed data; X.W. and T.A.R. wrote the paper; and T.A.R. supervised the project.

Reviewers: Y.C., University of California San Francisco Medical Center; G.C.L., The Scripps Research Institute

Competing interest statement: Stanford University has filed a patent application on behalf of the authors regarding the nanobody-binding scaffold protein constructs.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2115001118/-/DCSupplemental.

Published October 7, 2021.

https://doi.org/10.1073/pnas.2115001118
fragments. Nanobodies can be selected from immunized camels or from large in vitro libraries displayed by phages, yeast cells, or on ribosomes (12, 13), and can be produced in large quantities in a fairly short time. They often lock a protein into a fixed conformation, particularly in the case of membrane proteins, and have been used extensively to determine X-ray structures. The small size of nanobodies (~12 to 15 kDa) limits their direct application in cryo-EM, but the problem might be overcome if one could increase their size with the rigid attachment of a large scaffold. One reported approach is to fuse a scaffold into a loop of the nanobody, generating a “megabody” (14). However, the linker consisted of β-strands between the nanobody and scaffold, which caused some flexibility and limited the use of the scaffold for particle alignment in cryo-EM analysis.

Here, we describe a versatile method that allows cryo-EM analysis of even the smallest protein once a tightly binding nanobody is available. The size of the nanobody is increased to ~120 kDa by two rigidly attached scaffolds. The overall design is reminiscent of a Lego construction, so we propose to call the scaffolds/nanobody ensemble “Legobody.” The utility of the Legobody method is demonstrated by structures of two small proteins (22 kDa and 23 kDa) that are asymmetric monomers and have a size well below the estimated limit for direct cryo-EM single-particle analysis (~40 kDa) (15). The Legobody approach can easily be applied to any target protein and should greatly expand the use of cryo-EM single-particle analysis by overcoming the current size limitations.

**Results**

**Generation of a Nanobody-Binding Fab.** Our first nanobody-interacting scaffold is a Fab fragment of an antibody that is directed against a surface present in many nanobodies and not involved in target interaction. To generate such a Fab, we raised monoclonal antibodies in mice against a nanobody (Nb_0) that contains a framework of a His tag attached to the heavy chain. The yield is rather low, the constant regions of the light and heavy chains were mutated based on the crystal structure of a Fab/PrAD complex providing an opportunity to use both scaffolds at the same time.

To increase the stability of the ensemble, we fused two Fab-binding domains to the C terminus of MBP (Nb_MBP) and its antigen MBP (12). After several rounds of selection, hybridoma clone 8D3 was chosen to minimize the immunogenicity of the antigen-interacting complementarity-determining regions (CDRs) of Nb_0 were chosen to minimize the immunogenicity of the antigen-interacting surface (see SI Appendix, Table S1 for sequence). To select for monoclonals that do not perturb nanobody–antigen interaction, hybridoma clones were screened with a complex of a nanobody against MBP (Nb_MBP) and its antigen MBP (12). After several rounds of selection, hybridoma clone 8D3 was obtained, which produced monoclonal antibodies that strongly bind to both Nb_0 and the Nb_MBP/MBP complex.

Although it is possible to directly use the antibodies secreted by clone 8D3 to generate Fab fragments, future applications are greatly facilitated if the Fabs can be made recombinantly. To this end, we first determined the DNA sequences of the regions coding for the variable regions of the light and heavy chains of clone 8D3. These sequences were then combined with the sequences coding for the constant regions of murine IgG1, and both genes were expressed together in HEK293 cells. Because the yield of Fabs was rather low, the constant regions of the light and heavy chains were replaced with those of human IgG. The resulting Fab 8D3 was expressed in HEK293 cells as a secreted protein and purified by nickel-nitrilotriacetic acid (Ni-NTA) chromatography on the basis of a His tag attached to the heavy chain. The yield is ~5 to 8 mg from 1 L of cell culture. Recombinantly purified Fab 8D3 forms a stable complex with nanobody Nb_0, as shown by comigration of the proteins in size-exclusion chromatography (Fig. 1.4).

To identify the exact interaction surface, we determined a crystal structure of the complex of Fab 8D3 and Nb_0. After confirming that the crystals contained both components (SI Appendix, Fig. S1A), a structure of the complex was determined at 1.8-Å resolution (Fig. 1B and SI Appendix, Table S2). As expected, Fab 8D3 binds to a surface of Nb_0 that is distal from the CDRs and contains conserved amino acids present in many nanobodies (Fig. 1C and SI Appendix, Fig. S1B). The Fab-interacting amino acids of Nb_0 are located in loops between β-strands A and B, C and C’, E and F, as well as in segments of the β-strands A and G. It should be noted that four amino acids (LEHH) introduced by the cloning of the His tag are also involved in the interaction (Fig. 1C).

The extensive interactions between the Fab and nanobody generate a rigid interface, a conclusion supported by the B factor profile of the X-ray structure (SI Appendix, Fig. S1C).

**Generation of an MBP-Based Scaffold Interacting with Nanobodies.** The second scaffold was developed on the basis of reports that protein A from *Staphylococcus aureus* can bind to nanobodies (16). Protein A contains five repeats of three-helical bundles (domains A–E). All these domains associate with the constant region of IgG antibodies, but also bind with different affinities to the variable region of the heavy chain of some antibodies (human Vp3 family) (17), a region that is similar in sequence to the common framework of many nanobodies. Consistent with this sequence homology, protein A has been reported to interact with nanobodies in a similar way as with Fabs (18). To identify the strongest binding protein A domain, we fused domain D (PrAD) and the most divergent domains C and E (PrAC and PrAE) through a long, flexible linker to MBP (MBP_L_PrAC, MBP_L_PrAD, and MBP_L_PrAE) and tested these fusions for their interaction with a nanobody. Coelution of the proteins in size-exclusion chromatography showed that all three domains interact with the nanobody, but domain C forms the most stable complex (SI Appendix, Fig. S2A).

Next, we grafted domain C of protein A (PrAC, ~6 kDa) to MBP to generate a larger nanobody-binding partner. MBP is frequently used as an N-terminal fusion partner, as it can increase the solubility of its fusion partners. Although fusions can be designed as helical extensions of the C-terminal helix of MBP and have been extensively used in X-ray crystallography (19), such linkers are not rigid enough for cryo-EM analysis. To generate a more rigid connection between PrAC and MBP, we used the “shared helix” approach (20), applying it to a helix of domain C that is not involved in nanobody interaction (SI Appendix, Fig. S2B). Residues on one side of this helix were mutated to those of MBP’s C-terminal helix that face the core of MBP. The resulting construct MBP_PrAC (Fig. 1D) could be expressed in *Escherichia coli* and purified in large quantities. Like the MBP fusion of PrAC containing a flexible linker (MBP_L_PrAC), MBP_PrAC interacted with the nanobody in pull-down experiments (Fig. 1E).

**Legobody Assembly.** The nanobody surfaces interacting with Fab 8D3 and MBP_PrAC are not overlapping (Fig. 2A and B), providing an opportunity to use both scaffolds at the same time. To increase the stability of the ensemble, we fused two Fab-binding domains to the C terminus of MBP_PrAC. One is the domain D of protein A (PrAD), which has been shown to interact with the variable region of the heavy chain of Fabs (21). The other is protein G (PrG), which is known to strongly interact with the constant region of the heavy chain (22). All domains were connected by short linkers, generating a scaffold designated MBP_PrA/G (Fig. 2A and B). To allow the interaction of PrAD with Fab 8D3, some residues in the variable region of the heavy chain were mutated based on the crystal structure of a Fab/PrAD complex (PDB code 1DEE), generating Fab 8D3.2. Avidity effects increase the binding constants of both the MBP_PrAC and Fab scaffolds for the nanobody, making the overall assembly very stable.

The complex between Fab 8D3.2 and MBP_PrA/G was assembled before adding a nanobody. All three components comigrated in size-exclusion chromatography (Fig. 2C). Negative-stain EM showed strong structural features for the different parts of the Legobody (Fig. 2D), suggesting overall rigidity of the assembly, an assumption confirmed by subsequent cryo-EM analysis (see below).
surface lysine residues were modified with low molecular weight molecules at the water–air interface. To alleviate this problem, surface lysine residues were modified with low molecular weight polyethylene glycol (PEG), a previously introduced method that makes the surface of proteins more hydrophilic and reduces their denaturation on the grids (25). Although the particles still showed some aggregation and preferred orientation (SI Appendix, Fig. S3), the cryo-EM analysis was straightforward, as the size and unique shape of the Legobody greatly facilitated particle picking and two-dimensional (2D) and three-dimensional (3D) classification. The final 3D refinement of the selected particles resulted in a 3D reconstruction with an overall resolution of 3.2 Å and little directional differences in resolution (SI Appendix, Fig. S3 and Table S3). With the exception of the relatively flexible PrAD domain, all parts of the Legobody and KDELR had well-resolved structural features, allowing even the visualization of the maltose molecule bound to MBP (Fig. 4A and SI Appendix, Fig. S4A). The local resolution ranged from 3.0 to ∼4.0 Å and showed that the interactions between the two scaffolds and the nanobody, as well as the connection between PrAC and MBP, are all rigid (Fig. 4B). Because the nanobody is tightly associated with the KDELR, and because the center of alignment of the Legobody is at the position of the nanobody, an excellent map was obtained for the KDELR (Fig. 4C). The local resolution of this part of the map ranged from 3.0 to ∼3.5 Å and all amino acid side chains of the KDELR were clearly visible. In addition, several bound phospholipid molecules could easily be identified. The cryo-EM structure of the KDELR–nanobody complex is almost identical to that obtained by X-ray crystallography (SI Appendix, Fig. S4B) (24).

Case Study II: Receptor-Binding Domain of the SARS-CoV-2 Spike Protein (∼22 kDa). Our second test protein for the Legobody method was the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein.
The RBD allows SARS-CoV-2 to bind to the ACE2 receptor and infect human cells (26). This interaction is of great medical interest, particularly during the current pandemic, and therefore many RBD-neutralizing nanobodies have been generated (27). The RBD has a molecular weight of only $\approx 22$ kDa.

The RBD was expressed in HEK293 cells as a secreted protein and purified by Ni-NTA chromatography on the basis of an attached His tag. The protein was mixed with the preassembled Legobody containing a reported nanobody (Nb_RBD) against the RBD (28). The complex was further purified by size-exclusion chromatography.

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Fig. 2. Assembly and purification of the Legobody. (A) Scheme of the Legobody assembly. Fab_8D3-2, a derivative of Fab_8D3, and MBP_PrAC bind directly to the nanobody (Nb). The Fab-interacting D domain of protein A (PrAD) and protein G (PrG) were fused sequentially to the C terminus of MBP_PrAC via short linkers, generating MBP_PrA/G. Interacting surfaces are colored and highlighted by dashed lines. (B) Model of the assembled Legobody in cartoon representation. The model was assembled from structures of Nb, dFab_8D3 (Fig. 1B), FabPrAD (PDB code 1DEE), and FabPrG (PDB code 1IGC), and by manually building a model for MBP_PrAC from the structures of MBP (PDB code 1ANF) and PrAC (PDB code 4NPD). (C) Legobody assembled with a nanobody was subjected to gel filtration. Fractions were analyzed by SDS-PAGE and Coomassie blue staining. (D) Purified Legobody was analyzed by negative-stain EM. Shown are representative 2D class averages, with density for the nanobody highlighted by an arrow.

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Fig. 3. Purification of a KDELR/Legobody complex. (A) Scheme for the purification of the KDELR/Legobody complex. KDELR solubilized in detergent was immobilized on streptavidin beads. The resin was incubated with purified Legobody. Nanodisc-scaffolding protein MSP1D1 and lipids were added, and the reconstitution of nanodiscs was initiated by the addition of BioBeads. After extensive detergent removal, the complex was eluted from the resin with biotin. (B) The eluted complex of KDELR, Legobody, and nanodisc was subjected to gel filtration and the elution of protein followed by the absorbance at 280 nm (Upper). Fractions between the indicated dashed lines were analyzed by SDS-PAGE and Coomassie blue staining (Lower). Fractions indicated by an asterisk were pooled and used for EM analysis. (C) The purified complex was analyzed by negative-stain EM. Shown are representative 2D class averages, with density for the nanodisc highlighted by an arrow.
and analyzed by cryo-EM. After 2D and 3D classification, followed by 3D refinement, a map with an overall resolution of 3.6 Å was obtained, again with little directional differences in resolution (Fig. S5 and SI Appendix, Fig. S5 A–E and Table S3). The local resolution ranged from ∼3.4 to ∼4.4 Å and showed good density for the central regions of the Legobody and target protein (Fig. 5C). Specifically, the RBD region showed good side-chain density for amino acids at the interface with the nanobody (Fig. 5D). The cryo-EM structure of the RBD/nanobody complex is almost identical to that obtained by X-ray crystallography (SI Appendix, Fig. S5F) (29). Some polypeptide loops distal to the binding interface were invisible in the cryo-EM map (SI Appendix, Fig. S5F). The biased particle orientation suggests that this may be caused by the partial denaturation of the RBD at the water/air interface, i.e., by issues unrelated to the Legobody method. The moderate preferred particle orientation observed with the KDELR and the somewhat stronger bias seen with the RBD are likely not caused by the Legobody itself, as the populated angles were different.

**General Applicability of the Legobody Method.** The Legobody method was developed using nanobodies that have a common framework similar to that used in two in vitro libraries (12, 13). The surfaces interacting with the Fab and MBP_PrAC are essentially identical in these libraries and therefore all selected nanobodies can be used directly for the Legobody method. However, nanobodies generated in alpaca often have a different framework. For example, a nanobody directed against the ALFAtag peptide, which is frequently used to purify or visualize fusion proteins (30), differs significantly in its framework sequence (sequence identity ∼75%) and would therefore not allow interaction with our scaffolds. Specifically, nine amino acid residues in the Fab- and PrAC-interacting regions are different from the ones in the common framework. However, when these residues are mutated, the resulting Nb_ALFA can be assembled into a Legobody (SI Appendix, Fig. S6 A and B). These mutations do not affect antigen binding, as GST-tagged ALFA peptide was able to pull down the preassembled Legobody containing the modified nanobody (SI Appendix, Fig. S6B). We therefore believe that all nanobodies can be used, regardless of whether they are obtained from in vitro libraries or from animal species.

**Discussion**

Here we describe a general method that allows cryo-EM structures to be determined for small proteins. Our Legobody approach thus overcomes current limitations of cryo-EM analysis and greatly expands its use. The method can be applied to any target protein once a tightly binding nanobody is available. The nanobody is assembled into a Legobody by the binding of two scaffolds, a Fab fragment...
and a MBP molecule to which domain C of protein A domain has been grafted (MBP_PrAC). All interactions were designed to be rigid. In addition, Fab-interacting domains were fused to MBP_PrAC to further solidify the complex. The Legobody has a characteristic shape, consisting of two lateral arms, formed by the two scaffolds, and a central lobe, contributed by the nanobody. The overall size (∼120 kDa) and shape of the Legobody, and the center of alignment at the position of the nanobody, greatly facilitate all steps of cryo-EM analysis, from particle picking, classifications, to final refinement. We demonstrate the utility of the Legobody method with two examples of small target proteins (KDELR [23 kDa] and the RBD [22 kDa] of the SARS-CoV-2 spike protein). The membrane protein KDELR poses a particular challenge for cryo-EM analysis, as it is small, has no domains outside membrane, and no symmetry to facilitate particle alignment in EM images. The protein tends to aggregate during purification and on cryo-EM grids at the water–air interface of thin ice. To determine its structure, we not only used the Legobody approach, but also employed two other tricks, which likely are applicable to other challenging membrane proteins. First, we used a purification strategy, in which the KDELR/Legobody complex was incorporated into a nanodisc while bound to beads (Fig. 3A). This strategy reduces aggregation of the receptor and increases its stability in solution. Second, before applying the sample to EM grids, we modified surface lysines with low-molecular-weight PEG. We have used this protocol routinely for other proteins (25, 31, 32), and it often dramatically reduces particle aggregation and preferred particle orientation. Using standard cryo-EM data analysis, we were able to obtain a high-quality map for the KDELR, with density visible for all amino acid side chains. The map would have allowed straightforward de novo model building.

The KDELR is representative of a large group of membrane proteins, which are of small size and pose similar challenges for cryo-EM analysis. Examples include G protein–coupled receptors, solute carrier transporters, and membrane-embedded enzymes, many of which are of great interest for drug development. The Legobody method now makes all these proteins accessible to cryo-EM analysis. Of course, the target proteins might adopt several physiologically relevant conformations and a nanobody might select only one of them.

The RBD of the SARS-CoV-2 spike protein also presents a challenge for cryo-EM analysis, as it is small and consists mainly of β-strands and extended polypeptide segments, which are more difficult to model into a map than α-helices. The map obtained with the Legobody approach was of good quality, especially at the RBD/nanobody interface, with side-chain density for all interacting amino acids. Because this interface is the region of medical interest, our results show that cryo-EM can be used to optimize RBD-neutralizing nanobodies, which may be important for the quick response to future virus pandemics. By comparison with X-ray crystallography, cryo-EM requires only small amounts of protein and can be performed in a significantly shorter time period.
A possible limitation of the Legobody method could arise from steric clashes between the scaffolds and targets. To test whether this is a serious problem, crystal structures of complexes of nanobody with small monomeric target proteins were aligned with the Legobody structure on the basis of the nanobody. Observed steric clashes are listed in SI Appendix, Table S4. For soluble proteins, only three examples were found in which the Fab or MBP_PrAC would clash with the target. For membrane proteins, embedded into detergent micelles, nanodiscs, or lipid cubic phase, clashes would sometimes be caused by the PrAD domain or the MBP_PrAC scaffold. No clashes were observed for the Fab, but the number of available structures is too small to exclude their existence in other cases. Clashes with the PrAD domain can be avoided by deleting this domain and connecting MBP_PrAC directly to PrG via a suitable linker. In fact, this domain bound only weakly to its intended binding site on the Fab and should therefore be dispensable even in the original design. Tests for the compatibility of the scaffolds with the nanobody/target interaction are straightforward (similar to the pull-down experiments in SI Appendix, Fig. S6B), which could also be used to quickly screen for nanobodies that are compatible with both scaffolds, avoiding nanobodies that would cause steric clashes.

Because of the modular design of the Legobody, only one of the two scaffolds can be attached to the nanobody. For example, for certain targets the Fab fragment might be sufficient. If only the MBP_PrAC scaffold is used, we recommend fusing the nanobody to the N-terminus of MBP_PrAC via a flexible linker. However, the use of both scaffolds together not only increases the size of the target but also provides a unique shape that is easily recognizable in EM images. In addition, the both scaffolds may increase the solubility and monodispersity of the target protein. For example, the KDELR tends to aggregate during purification if either the Fab or the MBP_PrA/G scaffold are omitted.

The Legobody used in this study could easily be modified to further increase its molecular mass, stability, and rigidity, which would help to further improve the resolution of the cryo-EM maps. For example, the three-helix bundle of PrAC could be engineered to increase its binding affinity to the nanobody or it could be grafted onto other large proteins. In addition, fusions could be generated with protein L (33) or a modified version of protein M (34), which bind to the Fab at different sites than the fusion partners used in the current Legobody design. We believe that the current Legobodies and their possible variations will make cryo-EM structure determination of small proteins a routine method.

Methods

Purification of Nanobodies. Genes for His-tagged or Strep II-tagged nanobodies were cloned into the pET 26b vector (Novagen). The expression and purification of all His-tagged nanobodies have been described previously (13). For immunization of mice, the His tag was removed by treating purified nanobody Nb_0 with carboxypeptidase A (Sigma) overnight at 4 °C to remove the His tags. The sample was subjected to size-exclusion chromatography on a S200 Increase 10/300 GL column (Cytiva) in 25 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol. The peak fractions of the complex were stored for future use. The mixture was then added dropwise into the medium containing HEK293(-) cells to reach a final cell density of 2 million/mL. The cells were cultured at 37 °C for 12 to 16 h before addition of 10 μM butyrate to induce expression. Medium containing secreted Fabs was harvested ∼48 to 62 h posttransfection. Purification of the Fab was carried out as follows: Harvested medium free of cells was supplemented with 50 mM Tris pH 8.0, 200 mM NaCl, 20 mM imidazole, and 1 μM Nitis. His-tagged Fabs were purified by Ni-NTA chromatography. The beads were washed extensively with 25 mM Hepes pH 7.4, 200 mM NaCl, 20 mM imidazole. Fabs were eluted in 25 mM Hepes pH 7.4, 200 mM NaCl, 200 mM imidazole. Eluted Fabs were concentrated and buffer was exchanged into 25 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol. Aliquots were snap frozen for future use.

Based on reports that Fabs interact preferentially with domain D of protein A (17) and on a crystal structure of a complex of Fab and this domain (PDB code 1DEE), we introduced several mutations in the variable region of the heavy chain of the partially humanized Fab_8D3 (see above), resulting in Fab_8D3 (mutations: G14, N199, K198, K197, H196, S169; the sequence is shown in SI Appendix, Table S1). Fab_8D3-2 was purified in the same way as the original Fab_8D3.

Determination of a Crystal Structure of the Nb_0/Fab_8D3 Complex. Purified His-tagged Fab_8D3 was mixed with purified His-tagged Nb_0 nanobody at a molar ratio of 1:3. The mixture was treated with carboxypeptidase A (Sigma) overnight at 4 °C to remove the His tags. The sample was subjected to size-exclusion chromatography on a S200 increase 10/300 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl. The peak fractions of the complex were pooled and concentrated to 10 mg/mL and used to set up crystal screens.

Purified Nb_0/Fab_8D3 complex (0.2 μL of a 10 mg/mL solution) was mixed with 0.2 μL of mother liquor containing 0.2 M ammonium formate, 20 to 22% wt/vol PEG 3350 using a Mosquito robot (TTP LabTech). Crystals were grown at 4 °C in a drop reservoir method using a reservoir of 0.1 mL mother liquor and reached full size in about 2 wk. Crystals were cryoprotected before harvest in a solution containing mother liquor supplemented with 25 mM Hepes 7.5 and 18% ethylene glycol. X-ray diffraction data were collected on the 24-ID-E beamline at the Advanced Photon Source (APS). Initial phases were obtained by molecular replacement using crystal structures of Fabs and nanobodies with similar amino acid sequences as search models. In both search models, the CDRs were removed.

Purification of MBP Fusions. The sequences of all MBP fusion proteins are given in SI Appendix, Table S1. Based on crystal structures and modeling, we predicted residue Ala405 of MBP_PrA/G-His6 to be close to the Fab and therefore mutated it to histidine to boost the interaction. All variants of MBP were purified by Ni-NTA chromatography on a μL column of NTA-ligand equilibrated to 25 mM Hepes pH 7.4, 200 mM NaCl, 20 mM imidazole. Eluted Fabs were concentrated and buffer was exchanged into 25 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol. The peak fractions were stored for future use.

Purification of GST-Fused ALFA Peptide. GST-fused ALFA peptide was purified as follows: The gene for the ALFA peptide was cloned into the pGEX6p1

_Cit. by guest on October 7, 2021

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https://doi.org/10.1073/pnas.2115001118
vector (Cytiva). The expression was induced by addition of 1 mM IPTG for 5 h at 30 °C. The cells were lysed by sonication in 25 mM Hepes pH 7.4, 400 mM NaCl. The proteins were purified by GST resins using lysis buffer. After elution with reduced glutathione (GSH), proteins were subjected to size-exclusion chromatography on a S200 increase 10/300 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl. Then, the mixture was incubated with a chosen nanobody added at a threefold molar excess over MBP,PrA/Vg with FabBD3 2 at a molar ratio of 1:1.1 in 25 mM Hepes pH 7.4, 150 mM NaCl. Then, the mixture was applied to an amylase resin and the complex was eluted with 25 mM Hepes pH 7.4, 150 mM NaCl, 20 mM maltose. The Legobodies were further purified by size-exclusion chromatography on a S2000 increase 10/300 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 2 mM maltose. The peak fractions of the complexes were concentrated and stored for future use.

**Purification of Legobodies.** Legobodies were assembled by first incubating purified MBP,PrA/Vg with FabBD3 2 at a molar ratio of 1:1.1 in 25 mM Hepes pH 7.4, 150 mM NaCl. Then, the mixture was incubated with a chosen nanobody added at a threefold molar excess over MBP,PrA/Vg. The sample was applied to an amylase resin and the complex was eluted with 25 mM Hepes pH 7.4, 150 mM NaCl, 20 mM maltose. The Legobodies were further purified by size-exclusion chromatography on a S200 increase 10/300 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl, 5% glycerol, 2 mM maltose. The peak fractions of the complexes were concentrated and stored for future use.

**Purification of a Complex of KDELR and Legobody.** The codon-optimized gene for the full-length KDELR with a S8P tag at its C terminus was cloned into the pRS425-Gal1 vector (ATCC 87331) (36). The expression of the receptor and preparations of the membrane fractions were carried out as previously described (37). The receptor were solubilized in 30 mL of 25 mM Hepes pH 7.4, 400 mM NaCl, 1% DMNG (Anatrace) for 1 h. After removing insoluble material by ultracentrifugation, the lysate was incubated with 250 μL streptavidin resin (Thermo Fisher) for 1.5 h. The beads were collected and an excess of purified Legobody was added to the bound KDEL receptor to promote complex formation on the resin. After 1 h of incubation, the resin was washed with eight column volumes of 25 mM Hepes pH 7.4, 150 mM NaCl, 2 mM maltose, 0.02% DMNG. Nanodiscs were assembled on the resin by adding 1.25 mM lipids (POPC/DOPE [Avanti] at a 4:1 ratio in DDM [Anatrace]) and 25 μM nanodisc-scaffolding protein MSP1D1 in 700 μL of washing buffer. After 30 min of incubation, the detergents were removed by the addition of two aliquots of 40 mg of Bio-Beads and overnight incubation. The next day, the streptavidin resins were separated from Bio-Beads SM-2 (Bio-Rad), taking advantage of their different rates of sedimentation by gravity. The streptavidin resins were washed by 25 mM Hepes pH 7.4, 150 mM NaCl, 2 mM maltose, and bound material was eluted with biotin. The KDEL receptor/ Legobody complex in a nanodisc was then purified by size-exclusion chromatography on a Superdex 200 increase 5/150 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl, 2 mM maltose. The peak fractions of the complex were concentrated, snap frozen, and stored for cryo-EM analysis.

**Purification of a Complex of the SARS-CoV-2 Spike RBBD Domain and Legobody.** The codon-optimized gene for the RBBD domain (residues 334 to 526) of SARS-CoV-2 spike protein with an N-terminal Flag tag and a C-terminal His8 tag was cloned into the pCAGEN vector. The RBBD was expressed and purified in the same way as the Fab. After elution from Ni-NTA beads, the protein was further purified by size-exclusion chromatography on a Superdex 5/200 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl. Peak fractions were mixed with Legobody at a molar ratio of 3:1. The mixture was subjected to size-exclusion chromatography on a Superdex 200 increase 5/150 GL column in 25 mM Hepes pH 7.4, 150 mM NaCl, 2 mM maltose. The peak fractions of the complex were concentrated, snap frozen, and stored for cryo-EM analysis.

**Cryo-EM Sample Preparation and Data Acquisition.** The KDELR/Legobody complex at 0.8 mg/mL was PEGylated by incubation with MS(PEG)12 methyl-PEG-NHS-ester (Thermo Fisher) at a 1:40 molar ratio for 2 h on ice to reduce preferred particle charging. The PEGylated sample was then applied to a glow-discharged Quantifoil gold grid (1.2/1.3, 400 mesh). The grids were blotted for 30 s on a filter and plunge frozen in liquid ethane using a Vitrobot Mark IV instrument (Thermo Fisher). The RBBD/Legobody complex at 2.5 mg/mL was incubated with MS(PEG)12 methyl-PEG-N-hydroxysuccinimide (NHS)-ester (Thermo Fisher) at a 1:25 to 128 molar ratio for 2 h on ice. Right before plunge freezing, the PEGylated samples were diluted, using the gel filtration buffers supplemented with detergent IGEPA-L CA-630 (Sigma), so that the final protein and detergent concentrations were 1.2 mg/mL and 0.005%, respectively. The grids were frozen in the same way as described for the KDELR/Legobody sample.

Cryo-EM data for all samples were collected on a Titan Krios electron microscope (FEI) operated at 300 kV and equipped with a K3 direct electron detector (Gatan) at Harvard Cryo-EM Center for Structural Biology. A Gatan Imaging filter with a slit width of 25 eV was used to remove inelastically scattered electrons. All cryo-EM movies were recorded in counting-mode using SerialEM. For the KDELR/Legobody sample, the nominal magnification of 81,000x corresponds to a calibrated pixel size of 1.06 Å on the specimen. The exposure rate was 23.38 electrons/A²/s. The total exposure time was 2.2 s, resulting in a total electron exposure of 51.44 electrons/A², fractionated into 50 frames (44 ms per frame). For the RBBD/Legobody sample, the calibrated pixel size was 1.06 Å. The exposure rate was 23.3 electrons/e/A²/s. The total exposure time was 2.164 s, resulting in a total electron exposure of 50.42 electrons/A², fractionated into 50 frames (44 ms per frame). The defocus range for both samples was between −1.0 and −2.6 μm.

**Cryo-EM Image Processing.** For the KDELR/Legobody complex, dose-fractionated movies were subjected to motion correction using the program MotionCor2 (37) with dose weighting. The program CTfInd4d (38) was used to estimate defocus values from the summed images from all movie frames. During data collection, the particles (close to 1 million) picked by YOLO (39) by “on-the-fly” analysis using an automatic workflow at Harvard Medical School. The particles were then subjected to 2D classification (T2, 80 classes, 30 iterations) in Relion 3.1 (40). For 3D classification, an initial model was generated ab initio in Relion 3.1. After one round of 3D classification (T4, 5 classes, 30 iterations), there was only one class with clear protein secondary structure features. Particles of this class were selected for refinement. After refinement, the initial resolution was determined to be 3.8-Å overall nominal resolution. This initial 3D reconstruction was used as a template to perform autopick in Relion 3.1 on the entire dataset, resulting in 2,532,161 particles. After 2D classifications (T2, 100 classes, 30 iterations), repicked particles were “seeded” with particles used in the previous 3D refinement for 3D classification. After 3D classification (T4, 5 classes, 35 iterations), particles from this class showing the protein secondary structure were selected. The whole complex was selected. After removing duplicates, the particles were subjected to 3D refinement, followed by polishing, Contrast Transfer Function (CTF) refinement, and another round of 3D refinement. Local 3D classification without image alignment (T20, 5 classes, 25 iterations) was performed using a mask including only the nanobody and KDELR. A total of 246,878 particles were finally selected for 3D refinement using a mask excluding the nanodisc and RBBD domain, washed out protein A (Nb₀/Fab BD3).

For the RBBD/Legobody complex, data analysis was performed in a similar way, except that particles from the on-the-fly analysis were not refined. Local resolution calculation and map sharpening were both performed in Relion 3.1. All reported resolutions are based on gold-standard refinement procedures and the Fourier Shell Correlation (FSC) = 0.143 criterion. Histograms of directional FSC curves and sphericity values were calculated with the 3DFSC server (41).

**Model Building.** All model building was done in Coot. For the crystal structure of Nb₀/Fab BD3, the initial phases were obtained by molecular replacement using the Phaser module in Phenix (42). The search models contained a nanobody, as well as the variable and constant regions of a Fab of similar framework sequence. After obtaining an initial density map, the model was refined with rigid bodies and then modified manually. The final model was further validated using the Phenix framework module with the final model with PDB file 6I6J. The flexible D domain of protein A (1DEE) and a nanobody, as well as the variable and constant regions of a Fab of similar framework sequence. After obtaining an initial density map, the model was refined with rigid bodies and then modified manually. The final model was further validated using the Phenix framework module with the final model with PDB file 6I6J. The flexible D domain of protein A (1DEE) and a nanobody, as well as the variable and constant regions of a Fab of similar framework sequence. After obtaining an initial density map, the model was refined with rigid bodies and then modified manually. The final model was further validated using the Phenix framework module with the final model with PDB file 6I6J. The flexible D domain of protein A (1DEE)

**Data Availability.** All study data are included in the article and/or supporting information. The coordinates of the atomic models of Nb₀/Fab BD3, KDELR/Legobody, and RBBD/Legobody have been deposited in the Protein Data Bank with accession codes EM-24728 (46) and EM-24729 (47), respectively. The cryo-EM maps of the KDELR/Legobody and RBBD/Legobody have been de-
conducted at the Northeastern Collaborative Access Team beamlines, which are funded by the National Institute of General Medical Sciences (NIGMS) from the NIH (P30 GM124165). The Eiger 16M detector on 24-ID-E is funded by an NIH-Office of Research Infrastructure Programs High-End Instrumentation grant (S10OD021527). This research used resources from the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract DE-AC02-06CH11357. We thank C. Bahl for initial efforts to use Rosetta for the design of fusions between MBP and Protein A domains, T.-C. Hu for help with the generation of Fab, the SBGrid team for software and workstations support, and Maofu Liao and Susan Shao for comments on the manuscript. This work was supported by a Jane Coffin Child fellowship to X.W. and an NIGMS award to T.A.R. (T01GM052586).