Structural features of interfacial tyrosine residue in ROBO1 fibronectin domain-antibody complex: Crystallographic, thermodynamic, and molecular dynamic analyses

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Abstract: ROBO1, fibronectin Type-III domain (Fn)-containing protein, is a novel immunotherapeutic target for hepatocellular carcinoma in humans. The crystal structure of the antigen-binding fragment (Fab) of B2212A, the monoclonal antibody against the third Fn domain (Fn3) of ROBO1, was determined in pursuit of antibody drug for hepatocellular carcinoma. This effort was conducted in the presence or absence of proteinscience.org

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Abbreviations: CDR, complementarity-determining region; Fab, antigen-binding fragment of antibody; Fn, fibronectin Type-III domain; Fn3, the third Fn domain of human ROBO1; Fv, variable fragment of antibody; IPTG, isopropyl-β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; LY50A, mutant in which Tyr is substituted with Ala at position 50 of the light chain of Fv; mAb, monoclonal antibody; MD, molecular dynamics; scFv, single-chain Fv; sROBO1, soluble ROBO1; TEV, tobacco etch virus.

Additional Supporting Information may be found in the online version of this article.

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of the antigen, with the chemical features being investigated by determining the affinity of the antibody using molecular dynamics (MD) and thermodynamics. The structural comparison of B2212A Fab between the complex and the free form revealed that the interfacial Tyr$^{50}$ (superscripts L, H, and F stand for the residues in the light chain, heavy chain, and Fn3, respectively) played important roles in Fn3 recognition. That is, the aromatic ring of Tyr$^{50}$ pivoted toward Phe$^{68}$, forming a CH/n interaction and a new hydrogen bond with the carbonyl O atom of Phe$^{68}$. MD simulations predicted that the Tyr$^{50}$-Phe$^{68}$ interaction almost entirely dominated Fab-Fn3 binding, and Ala-substitution of Tyr$^{50}$ led to a reduced binding of the resultant complex. On the contrary, isothermal titration calorimetry experiments underscored that Ala-substitution of Tyr$^{50}$ caused an increase of the binding enthalpy between B2212A and Fn3, but importantly, it induced an increase of the binding entropy, resulting in a suppression of loss in the Gibbs free energy in total. These results suggest that mutation analysis considering the binding entropy as well as the binding enthalpy will aid in the development of novel antibody drugs for hepatocellular carcinoma.

Keywords: antigen–antibody interaction; antibody engineering; crystallography; molecular dynamics; thermodynamics

Introduction
Antibody-based cancer therapy has become well established over the past decades and is one of the most important strategies for treating tumor patients.$^{1,2}$ Anticancer antibody drugs have been developed to target cell surface antigens that are overexpressed in human cancer cells relative to normal tissue. Early anticancer antibody drugs utilize immunological mechanisms to kill cancer cells. This strategy requires that many target molecules are present on cancer cells and that multiple antibodies are bound to each cell. Antibodies conjugated with radioisotopes or cytotoxic agents also defeat cancer cells effectively, but there is considerable invasion into normal tissue. Currently, pretargeting strategies are proposed for advanced hepatocellular carcinoma. Actually, a crystal structure of an antigen–antibody complex can indicate which residues should be mutated to upgrade preliminary antibodies to antibody drugs with the desired properties.

The human homologue of the Drosophila roundabout (robo) gene, ROBO1, encodes an axon guidance receptor, which is defined as a novel subfamily of the immunoglobulin superfamily.$^{12}$ As Drosophila robo functions as a gatekeeper controlling midline crossing,$^{12–14}$ ROBO1 is a member of the neural cell adhesion molecule family. Recently, ROBO1 also represents a novel immunotherapeutic target and a sensitive serological marker for hepatocellular carcinoma because the ROBO1 gene expression is upregulated in hepatocellular carcinoma. Actually, ROBO1-positive cells were observed in more than 80% of hepatocellular carcinoma.$^{15}$

ROBO1 contains five repeats of immunoglobulin (Ig) domains, three repeats of fibronectin Type-III (Fn) domains, a transmembrane domain, and an intracellular tail.$^{13}$ Among these domains, the structure of the first Ig domain has been determined by X-ray crystallographic analysis as the complex with the second leucine-rich repeat domain of SLIT2,$^{16}$ a known ligand for ROBO1. ROBO1/SLIT2 signaling has been shown to be involved in cancerous angiogenesis.$^{17}$ The third Fn domain of ROBO1 (Fn3) is located closest to the transmembrane region, but its function remains unknown. Typically, Fn domains are estimated to be present in about 2% of all human proteins.
and found in organisms as evolutionarily distant as bacteriophages. Moreover, Fn domain has a stable framework structure and consequently a high thermostability, which is utilized as a scaffold for the generation of stable proteins in the protein engineering. Therefore, in ROBO1, Fn domains may contribute to stabilizing the extracellular region and the interaction with SLIT2.

In this study, we elucidated the crystal structure of the Fab fragment of B2212A (the specific antibody against human ROBO1 Fn3 domain), in the presence or absence of the antigen at 1.7 and 1.6 Å resolution, respectively, to uncover the structural features of Fn3 as well as to understand the recognition mechanism of B2212A antibody. Consequently, the structural comparison of the complexed and free forms of Fab revealed that the interaction between Fab and Fn3 was almost fully relying on the interfacial Tyr residue of Fab. Furthermore, from the results of molecular dynamics (MD) simulations and isothermal titration calorimetry (ITC), we discussed the thermodynamic features between antigen and antibody (as single-chain variable fragment, scFv) and the structural clues for molecular design of antibodies that display high-affinity binding.

Results

Overall structure of ROBO1 Fn3-B2212A Fab complex

The crystal structures of B2212A Fab were determined by X-ray crystallographic analysis in the presence or absence of Fn3 domain of human ROBO1 at resolutions of 1.7 and 1.6 Å, respectively, (Table I).

The final electron density map is well resolved for most of the complex with the exception of residues 31–35 and 83–85 of the Fn3 domain that are associated with weak electron density (i.e., disordered). However, the crystal structure allowed the interactions between Fn3 and Fab to be examined in atomic detail. A total of 1669 Å² (15.3%) of the solvent-accessible surface was buried in the interface between Fn3 and Fab: 804 Å² on Fn3, 508 Å² on the heavy chain of Fab, and the remaining 357 Å² on the light chain.

The B2212A Fab fragment showed the typical immunoglobulin fold. The overall structure of the framework regions was very similar to that of the structure of anti-gp41 Fab NC-1 (PDB ID code 3OZ9), which was used as a starting model for the
molecular replacement method. The root mean square deviation (RMSD) for 212 equivalent whole C\textalpha atoms of each whole Fab was 1.6 Å, but that for 103 equivalent C\textalpha atoms in the variable region was 0.69 Å as the result of structural refinement. The differences between the crystal structures of the Fab complex and the free form were described.

ROBO1 Fn3 also exhibited the typical Fn domain fold [Fig. 1(A)]. The structure of Fn3 consisted of seven \beta-strands, which form a sandwich of two antiparallel \beta-sheets, one containing three strands and the other four strands. The RMSD for 88 equivalent C\textalpha atoms between ROBO1 Fn3 and the 10th Fn domain of human fibronectin\textsuperscript{20} was 1.2 Å, which was first determined by the crystal
structure of the mammalian Fn domain (PDB ID code 1FNA).

**ROBO1 Fn3-B2212A Fab interface**

The Fn3 epitope for B2212A Fab consisted of residues coming from distant parts of the linear sequence, but these residues were made contiguous by the folding of the protein. Mainly, the β-sheet spanning residues 49–56 of Fn3 bound with the heavy chain and several parts of loop regions spanning residues 17–19 and 68–75 associated with the light chain [Fig. 1(B,C) and Supporting Information Fig. S1]. On the contrary, only four CDRs among six participated in the interaction with Fn3. Namely, the second and third CDRs of the light chain (CDR-L2 and CDR-L3, respectively) and the first and third CDRs of the heavy chain (CDR-H1 and CDR-H3, respectively), made 14 hydrogen bond contacts with Fn3 [Fig. 1(B,C), Table II].

The surface of Fn3 interacting with B2212A Fab was not noticeably concave. However, there was a protrusion of the Phe F68 side chain that penetrated into the small cavity in Fab and formed a CH–π interaction. In detail, the Phe F68 side chain located in hydrophobic cluster spanning residues 64–70 (Val–Val–Ile–Pro–Phe–Leu–Val) was embedded in a hydrophobic surface consisting of Tyr L50, Tyr L32, and Pro H105 [Fig. 1(D)].

### Table II. Residues Forming Hydrogen Bonds Between ROBO1 Fn3 and B2212A Fab in the Complex

|             | B2212A-Fab | Fn3 domain |
|-------------|------------|------------|
| Residue Atom | Residue Atom | Distance (Å) |
| Light chain |            |            |
| Tyr49 OH    | Thr19 N    | 3.0        |
| Tyr50 OH    | Phe68 O    | 2.6        |
| Arg53 NH1   | Asn17 O    | 3.4        |
| Asn92 O     | Arg50 NH2  | 3.1        |
| Asn92 OD1   | Arg50 NH2  | 3.5        |
| Heavy chain |            |            |
| Thr28 OG1   | Thr56 O    | 3.6        |
| Asp31 OD2   | Thr56 N    | 2.7        |
| Asp31 OD1   | Thr56 OG1  | 2.7        |
| Tyr32 OH    | Lys55 NZ   | 3.1        |
| Asn101 ND2  | Thr49 O    | 3.1        |
| Asn101 ND2  | Arg50 O    | 3.2        |
| Asn101 ND2  | His52 O    | 3.0        |
| Tyr104 OH   | Tyr51 O    | 2.6        |
| Tyr104 OH   | Tyr75 OH   | 2.6        |

To investigate the function of Tyr L50 in antigen recognition, we computationally constructed LY50A mutant Fv and performed the MD simulations under the same conditions as those used for the wild-type B2212A Fv. As a result, LY50A mutation led to a decreased binding interaction by +37 kJ/mol (Table III). Although Phe F68 and Arg F50 further reduced the binding interaction (+22 and +6.0 kJ/mol, respectively), Lys F55 and Thr F56 gained binding interaction (−7.5 and −22 kJ/mol, respectively). These results may indicate that the global structural change of the antigen–antibody interface was induced by the LY50A mutation; however, most of the important residue pairs were retained except for the interaction of the replaced Tyr L50 (Table IV) producing the characteristic structural change, as shown earlier.

### MD simulations

To investigate the interactions between the antigen and the antibody in more detail, we conducted MD simulations of the Fn3 complex and the B2212A Fv in physiological saline. The MD simulations led to the calculation that the interaction energy between Fn3 and Fv was −498 kJ/mol, which was considerably smaller than that of a typical example of antigen–antibody complexes (e.g., HEL and HyHEL10 is −745 kJ/mol).

The residue of Fn3 that had the highest interaction energy with B2212A was Phe F68 (−83 kJ/mol), followed by Arg F50 and Thr F56 (−52 and −47 kJ/mol, respectively). The interaction energies of Tyr F51, His F52, Lys F55, and Val F70 in Fn3 exceed −20 kJ/mol (Table III). These results may indicate that both the hydrophobic cluster from Val F64 to Val F70 and the region from Thr F49 to Thr F56 in the third loop play an important role in the epitope. Also, Asn F17 and Thr F19 were observed to have a large interaction with B2212A. Interestingly, Phe F68 interacted most strongly with Tyr L50 (Table IV) producing the characteristic structural change, as shown earlier.

To investigate the structural changes of B2212A Fab on binding of ROBO1 Fn3

To investigate the structural changes of B2212A Fab on binding of Fn3, the crystal structure of the free form of B2212A Fab was superimposed on the Fn3-complexed structure. No major conformational changes occurred in the structure of the B2212A Fab on complex formation [Fig. 2(A)]. Comparison of variable region between the complex and the free form of Fab gave a RMSD of 0.55 Å, corresponding to the Cα atoms. Importantly, the averaged distances between the corresponding Cα carbon atoms in CDR-L1 and L2 were calculated to be 0.8 and 1.3 Å, respectively (Supporting Information Table S1). Significant differences were also found for the side chain. Notably, on binding of Fn3 the aromatic ring of Tyr L50 was rotated by 92° around the Cα–Cβ bond to form a hydrogen bond with the carbonyl O atom of Phe F68, while Tyr F50 made a van der Waals interaction with Phe L32 in the free form [Fig. 2(B)]. In fact, the electron density map corresponding to the side chain of Tyr L50 showed two alternative conformations in the complexed form. Tyr F50 was predominantly associated with Phe F68, while the electron density showed that the OH group of Tyr L50 was directed to Phe L32 in the free form [Fig. 2(C)].
decreased the interaction with ArgL53, and increased the interaction with HisL55 and SerL56. The other was HisF52, which lost interaction with AsnH101 but strengthened interaction with TyrH104.

**Thermodynamic interactions between sROBO1 and B2212A scFvs**

To uncover the role of TyrL50 residue in the antigen recognition, the wild-type and LY50A-mutated B2212A scFvs, and sROBO1 were prepared. The interaction between B2212A scFvs and sROBO1 with ITC was subsequently measured (Fig. 3; Table V).

The binding enthalpy ($\Delta H$) for the interaction between the LY50A mutant scFv and sROBO1 ($-38.9$ kJ/mol) was compared to that between the wild-type scFv and sROBO1 ($-49.3$ kJ/mol). This demonstrated that the increase of $\Delta H$ was $+10.4$ kJ/mol by substituting TyrL50 with AlaL50. In opposition, the entropic term ($T\Delta S$) also increased by $+4.2$ kJ/mol ($+0.84$ to $+5.02$ kJ/mol). As a result, LY50A did not lead to a large difference in Gibbs free energy ($\Delta G$) compared with the wild-type scFv-sROBO1 interaction ($+5.8$ kJ/mol). The net result was that the binding constant for LY50A was as much as one-tenth of that for the wild type.
In this article, we report the crystal structure of the Fn domain of mammalian ROBO1 complexed with its antibody B2212A Fab. Structural comparison of B2212A Fab between the complexed and the free form revealed that the TyrL50 residue rotated by

| Table III. Top 10 Ranking of the ROBO1 Fn3 Interaction Energies on Wild-Type B2212A Fab Binding, and Their Changes With Respect to LY50A Mutant Fab |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Potential energy (kJ/mol) |
| Fn3 residue | Wild type | LY50A |
|            | Electrostatic potential | Lennard-Jones potential | Total | Electrostatic potential | Lennard-Jones potential | Total | ΔTotal |
| Phe68      | -37.7      | -45.3    | -83.0   | -7.99     | -52.9    | -60.8   | -22.2  |
| Arg50      | -22.3      | -29.2    | -51.5   | -25.8     | -19.7    | -45.5   | -6.00  |
| Thr56      | -43.5      | -3.84    | -47.4   | -62.7     | -6.86    | -69.5   | 22.1   |
| Asn17      | -27.1      | -20.0    | -47.0   | -27.1     | -21.3    | -48.4   | 1.40   |
| Lys55      | -35.6      | -8.35    | -43.9   | -41.4     | -10.0    | -51.4   | 7.50   |
| His52      | -28.9      | -7.98    | -36.9   | -35.6     | -12.4    | -48.0   | 11.1   |
| Tyr51      | -17.8      | -19.0    | -36.8   | -1.82     | -22.5    | -24.3   | -12.5  |
| Thr19      | -11.7      | -12.9    | -24.6   | -18.3     | -7.75    | -26.0   | 1.40   |
| Val70      | -0.478     | -20.3    | -20.8   | 1.17      | -10.3    | -9.08   | -11.7  |
| Ile53      | -0.793     | -17.0    | -17.8   | -1.19     | -14.6    | -15.7   | -2.10  |
| whole      | -257       | -241     | -498    | -234      | -227     | -461    | 37     |

| Table IV. Significant Interaction Pairs Between Fn3 and B2212A Fv Complex formed with |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Residues of Wild-type Fv Fv     | Residues of ROBO1 Fn3 |
|                                | Wild-type Fv | LY50A Fv |
| TyrL50*                       | Phe68        | Val70       | Leu69          | Pro71 |
|                               | (-29)        | (-10)       | (-7)           | (-6) |
| Arg53                         | Asn17b       | Thr19       |
|                               | (-16)        | (-7)        |
| LeuL54                        | Asn17b       | Arg50       |
|                               | (-16)        | (-9)        |
| TyrL49                        | Thr19        | Phe68       |
|                               | (-16)        | (-13)       |
| AsnL92                        | Arg50        |
|                               | (-9)         |
| HisL55                        | Asn17b       | Phe68       |
|                               | (-12)        | (-6)        |
| SerL56                        | Asn17b       |
|                               | (-7)         |
| AspH101                       | Thr56        | Lys55       |
|                               | (-41)        | (-34)       |
| AsnH101                       | Arg50        | Thr49       |
|                               | (-26)        | (-8)        |
| TyrH104                       | Tyr51        | Tyr75       | Phe68          | His52b |
|                               | (-21)        | (-13)       | (-12)          | (-8) |
| ValH103                       | Arg50        | Tyr51       |
|                               | (-11)        | (-8)        |
| ProH105                       | Phe68        |
|                               | (-11)        |
| TyrH102                       | Arg50        |
|                               | (-10)        |
| TyrH132                       | Lys55        |
|                               | (-9)         |
| AspH107                       | Phe68        |
|                               | (-7)         |
| ThrH28                        | Thr56        |
|                               | (-6)         |

The interaction pairs that exceed -6 kJ/mol are given.

a Interaction of AlaL50 was calculated for the LY50A mutant.
b AsnF17 and HisF52 were altered their interaction priorities by Ala-substitution of TyrL50.
Parentheses indicate the corresponding interaction energies (kJ/mol).
more than 90° with amino acids in other CDR loops showing no noticeable motion on binding of the ROBO1 Fn3 domain. In other words, there was no significant change in the interface other than TyrL50 residue. Therefore, to derive the structural clue for molecular design to develop antibody drugs, we almost focused on clarifying the role of TyrL50 of B2212A Fab on Fn3 recognition.

During the formation of antigen–antibody complexes, local conformational changes of both molecules have often been observed, leading to high specificity and affinity.\(^{21,22}\) It can be supposed that induced fitting of an antibody to its antigen is critical for high specificity and affinity. Induced fitting can be achieved by small movements of side chains, by structural modifications such as deformation of CDR loops, or by a change in the relative orientation of variable domains. In this case, configuration of the side chain of TyrL50 plays a crucial role in the antigen–antibody interaction, even though the overall RMSD between the complex and the free form of Fab is very small value of 0.55 Å. The hallmark of this reorganization results in the Tyr L50 aromatic ring being rotated around the axis of the Cα–Cβ bond, generating a new hydrogen bond with the carbonyl O atom of Phe F68 on binding of Fn3 (Fig. 2). Meanwhile, the MD simulations suggested that the hydrophobic residues, TyrL49, TyrL50, TyrH104, and ProH105, contribute mainly during complex formation (Tables III and IV). These results suggest that B2212A Fab gained binding energy by rotating the TyrL50 aromatic ring toward the Phe68 residue, thus picking up a hydrogen bonding interaction.

In general, binding interfaces are closely packed\(^{6,23}\) and systematic mutagenesis studies have revealed which “hot spots” tend to be intolerant of mutations. Remarkably, amino acid substitutions outside of the hot spots are typically tolerated.\(^{24}\)

### Table V. Thermodynamic Parameters of the Interactions Between sROBO1 and B2212A scFvs

|                  | WT       | LY50A    |
|------------------|----------|----------|
| \(K_d\) (nM)     | 19 ± 4.0 | 238 ± 109|
| \(K_a\) (× 10^8 M\(^{-1}\)) | 5.6 ± 1.2 | 0.53 ± 0.3 |
| \(\Delta G\) (kJ/mol) | −49.7 ± 0.4 | −43.9 ± 1.3 |
| \(\Delta H\) (kJ/mol) | −49.3 ± 0.8 | −38.9 ± 1.3 |
| \(T\Delta S\) (kJ/mol) | 0.84 ± 1.7 | 5.02 ± 0.4 |

Experimental procedures are described in the text. All data are shown by the mean ± SEM of at least three independent measurements. The abbreviations used are as follows: \(K_d\), dissociating constant; \(K_a\), binding constant; \(\Delta H\), \(\Delta S\), \(\Delta G\), changes in binding enthalpy, entropy, and Gibbs energy, respectively.
significant bias in the amino acid composition has been found among interface residues. For example, these regions are particularly enriched in Tyr, which plays an important role for antigen recognition because the aromatic ring of Tyr is capable of the formation of CH–π and π–π interactions in addition to forming a hydrogen bond. As demonstrated in the case of HEL/\textit{HyHEL}-10, there is an interfacial Tyr dictating the correct ternary structure of variable regions in both the light and heavy chains and the antigen. Interestingly, 50th residue of the light chain of \textit{HyHEL}-10 is Tyr, which is important on binding of HEL. In addition, the Tyr^{50} was comparably conserved (52% frequency) in 55 antiprotein antibody crystal structures (\textit{κ} light chain of IgG2a in particular). Taken together, these results suggest that the Tyr^{50} is significant for the antigen–antibody interaction, not only including antigen recognition, but also for stabilization of the antigen–antibody complex.

To verify the role of the Tyr^{50} residue in the recognition of Fn3, the mutation analysis by using LY50A was performed by ITC to investigate whether \( \Delta G \) and \( \Delta H \) were altered. Consequently, the ITC experiments demonstrated that LY50A led to a drastic increase in the binding enthalpy (\( \Delta \Delta H \); +10.4 kJ/mol; Table \textit{V}). This result was also predicted by the MD simulations as described earlier. Unexpectedly, there was relatively small increase in Gibbs energy (\( \Delta \Delta G \); +5.8 kJ/mol), because the loss of binding enthalpy was compensated by the gain of binding entropy (\( T \Delta \Delta S \); +4.2 kJ/mol). That is, the Ala residue provided a large energy gain for \( T \Delta S \) to compensate the loss of \( \Delta G \). Ordinarily, a drug design directs a drug molecule to bind to its target with high affinity and selectivity. As the binding affinity is a combined function of \( \Delta H \) and \( \Delta S \), extremely high affinity requires that both terms contribute favorably to binding. It is often occurred that the phenomenon of enthalpy/entropy compensation rears its head as a problem. For example, in protease inhibitors, the enthalpy gain achieved by additional hydrogen bonding is compensated completely by an entropy loss, resulting in no affinity change. In this case, there was only a small decrease in the affinity of LY50A for sROBO1, which was compensated by an entropy gain. It seems likely that most of the important residue pairs were retained with the global structural change of Fn3-B2212A interface, even though Ala-substitution disrupted the hydrogen bond and hydrophobic interaction (i.e., π–π and CH–π interactions; Tables \textit{III} and \textit{IV}). Therefore, we propose that Ala-substitution is a useful way for surveying not only hot spots but also potentially important residues contributing favorably to binding.

In this study, ITC experiments were also performed with Fn3 as well as sROBO1 as antigen samples. The properties of Fn3 showed the same tendency with those of sROBO1 but were not significant (Supporting Information Fig. \textit{S2} and Table \textit{S2}). These differences can be attributed to the hydration states of the two Fn3 types. The Fn3 domain of sROBO1 is located appropriately with an accurate conformation, and the accessibility of water molecules is restricted due to the ternary structure of sROBO1. However, the free Fn3 fragment is small and naked, presenting a situation where water molecules can be freely accessed. Consequently, the rate of local hydration of the free Fn3 will be higher than that of Fn3 accompanying sROBO1. An antibody must sacrifice binding energy in displacing water molecules to interact with Fn3, most likely breaking hydrogen bonds in the process. In addition, sROBO1 is closely related to the endogenous molecule and relatively stable, whereas the free Fn3 in aqueous solution also could be aggregated probably by the committing its hydrophobic residues (Fig. 1[D]). Therefore, in discussing the thermodynamics of antigen recognition of B2212A, the data from the ITC experiments with sROBO1 were our main focus.

Herein, the interaction energy of each complex, wild-type Fv-Fn3 and LY50A Fv-Fn3, were calculated as the sum of the electrostatic potential and the Lennard-Jones potential using MD simulations. The difference in the interaction energy between the two complex types (\( \Delta \Delta H \)) was largest with the LY50A. It is difficult to predict the value of \( \Delta \Delta S \) using MD simulations. However, both MD simulations and thermodynamic experiments will be indispensable for molecular design of antibody drugs. With the object of structure-function relationship, the crystal structure analysis of LY50A Fab-Fn3 complex is also in progress. In the near future, according to the accumulated knowledge, experiences, and experimental data using these techniques, the MD simulations need to be able to make accurate predictions of \( \Delta \Delta S \) with respect to the formation of the antigen–antibody complex. This will be aided by the development of improved MD algorithms and supercomputing power.

To develop antibody drugs suitable for pretargeting strategies for cancer, it is necessary to construct a scFv conjugated with immunogenicity-lowered streptavidin. In fact, our group has already succeeded in reducing immunogenicity of streptavidin (patent pending). Therefore, once a high-affinity scFv is designed, the antibody drug project will have to progress to the next step (i.e., pharmacokinetic studies). We expect that the antihuman ROBO1 Fn3 antibody will evolve into the magic bullet against hepatocellular carcinoma, or other tumors involving SLIT2/ROBO1 signaling (e.g., angiogenesis) in the near future.
Materials and Methods

Chemicals
All chemicals were purchased from Wako Pure Chemical Ind. (Osaka, Japan) unless indicated otherwise.

Preparation of ROBO1 Fn3 domain
The cDNA fragment of human ROBO1 Fn3 domain (residues 741–837) was cloned into pTAT6 expression vector (generously gifted by Dr. Marco Hyvönen, University of Cambridge, UK). The Fn3 domain was expressed as a hexahistidine-tagged N-terminal thioredoxin A fusion protein via a linker containing a tobacco etch virus (TEV) protease recognition site. The protein was produced in *Escherichia coli* strain BL21Star (DE3; Life Technology, Rockville, MD). Cells were grown at 37°C up to an OD₆₀₀ value of 0.6, and protein induction was performed by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside at 15°C for 24 h. The cells were harvested by centrifugation, resuspended in a lysis buffer [50 mM sodium phosphate buffer (pH 7.4) and 500 mM NaCl], and disrupted by EmulsiFlex-C3 (Avestin, Ottawa, Canada). The crude extract was centrifuged at 100,000g for 30 min at 4°C and the supernatant was subjected to affinity purification with Ni-NTA superflo (Qiagen, Hilden, Germany) resin. The hexahistidine-tagged protein was eluted with an imidazole gradient, and the eluted sample was diluted to 5 mg/mL or less. TEV protease was added (1/100 wt/wt ratio) to the sample, and the solution was dialyzed against gel filtration buffer [20 mM Tris-HCl (pH 8.0) and 600 mM NaCl] at 4°C. A HisTrap HP (GE Healthcare, Little Chalfont, UK) affinity column was used to remove the hexahistidine-tagged thioredoxin and the uncleaved fusion protein away from free Fn3, which was then concentrated and subjected to gel filtration chromatography using a Superdex 75 (GE Healthcare) column. The purified Fn3 protein was concentrated to 5 mg/mL and stored at −80°C. Protein concentration was determined spectrophotometrically based on absorbance at 280 nm using the calculated molar extinction coefficient of the protein.

Preparation of B2212A Fab fragment and ROBO1 Fn3-Fab complex
The monoclonal antibody (mAb) against human ROBO1 Fn3, clone B2212A, was generated as shown in Supporting Information. The ammonium sulfate fraction of B2212A mAb was adsorbed on HiTrap Protein G (GE Healthcare) column. The antibody was subsequently eluted with 100 mM glycine-HCl (pH 2.7) and then dialyzed against a dialysis buffer [100 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, and 2 mM ethylenediaminetetraacetic acid]. Papain was added (1/100 wt/wt ratio) in the presence of 40 mM freshly prepared l-cysteine and digestion was carried out at 37°C for 3 h. The reaction was quenched by the addition of iodoacetamide at a final concentration of 30 mM. The digestion mixture was dialyzed against 20 mM Tris-HCl (pH 8.0), loaded on HiTrap Q (GE Healthcare) anion exchange column, and eluted with NaCl gradient. The purified Fab fragment was mixed with Fn3 in 1:1.2 molar ratio and incubated for 1 h at 20°C. The Fn3-Fab complex was separated from excess Fn3 by gel filtration chromatography with a Superdex 75 column. The purified Fn3-Fab complex was concentrated to 5 mg/mL and stored at −80°C. Protein concentration of the complex was determined spectrophotometrically.

Crystallization
For the crystallization experiments, the purified Fn3-Fab complex was concentrated to 5 mg/mL. All crystallization screening kits used were purchased from Hampton Research (Aliso Viejo, CA). The clusters of thin plate-like crystals were grown using the sitting-drop vapor diffusion method within 2–3 weeks under the following conditions: equal volumes (0.5 μL) of the Fn3-Fab complex and precipitant, 25.5% (wt/vol) polyethylene glycol (PEG) 4000, 0.085 M sodium citrate tribasic dehydrate (pH 5.6), 0.17 M ammonium acetate, and 15% (vol/vol) glycerol (condition 9, Crystal Screen Cryo) were mixed and equilibrated at 20°C. In the case of a free Fab: 25% (wt/vol) PEG 4000, 0.1 M sodium acetate trihydrate (pH 4.6) and 0.2 M ammonium sulfate (condition 20, Crystal Screen), or 22% (wt/vol) PEG 3,350, 0.1 M sodium citrate tribasic dehydrate (pH 5.5), and 0.10% (wt/vol) n-octyl β-D-glucopyranoside (condition 25, PEGRx 2).

Structure determination and refinement
X-ray diffraction data for free Fab and Fn3-Fab complex crystals were collected at 100 K on the beamlines BL41XU and BL44XU at the SPring-8 (Harima, Japan), respectively, using MX225-HE detector (Rayonix, Evanston, IL). The crystal was flash-cooled with a standard nylon loop and flash-cooled in a nitrogen-gas stream at 100 K. Crystals were cryoprotected in a reservoir solution supplemented with 30% (vol/vol) glycerol. Data processing and reduction were carried out with the HKL-2000 program suite. The structures of B2212A Fab and ROBO1 Fn3 domain were determined by molecular replacement using the program MOLREP in the CCP4 program suite with the structures of the Fab fragment of anti-gp41 Fab NC-1 (PDB ID code 3OZ9) and the third Fn domain of human Neogenin (PDB ID code 1X5H) as search models, respectively. There were two monomers in the crystals of free Fab and the Fn3-Fab complex per asymmetric unit, resulting in a solvent content of 42.4% (V_M = 2.15 Å³/Da) and 49.5% (V_M = 2.46 Å³/Da), respectively.
These values were within the frequently observed ranges for protein crystals. Structural refinements were carried out with the program REFMAC version 5.5.01 and PHENIX version 1.8.4. The structures were visualized and manually modified with the COOT software. The stereochemical configurations of the refined structures were validated with the program PROCHECK, which showed that only 0.8% residues are in disallowed regions of a Ramachandran plot in the both crystal structures. The buried surface area was calculated with the program PISA at the PDBe website. Interactions within the Fn3-Fab interface were assigned with the CONTACT program in the CCP4 suite or the PISA program. Data collection and refinement statistics are summarized in Table I.

**MD simulations**

Prior to MD simulations, the coordinates of the fragment of variable region of antibody (Fv) were extracted from the initial structure of the wild-type complex taken from the crystal structure in this work. We utilized Discovery Studio version 3.1 (Accelrys, San Diego, CA) to generate several missing residue coordinates (Fn3 residues from 31 to 35 and 83 to 85), and to computationally generate the mutant structure by replacing TyrL50 of the wild-type structure with Ala (LY50A). The MD simulations were performed for each of the wild-type Fv-Fn3 complex and LY50A Fv-Fn3 complex. The structure was energetically minimized after adding 20,547 water molecules, 63 Na ions, and 60 Cl ions to reproduce the saline physiological environment. In this work, we used a modified version of the AMBER protein model, and the TIP3P water model for describing the interaction of the solvated protein systems. Although the protein coordinates were restrained to those of the minimized structure, the water molecules and ions were equilibrated for 250 ps using a MD simulation. Subsequently, the positional restraints were removed, and three 400 ns MD trajectories were calculated with randomly generated initial velocities for each of the wild type and LY50A complex, respectively. In all the simulations, the temperature and pressure were adjusted to 298 K and 1 atm with the Nose–Hoover thermostat and Berendsen barostat. For both the thermostat and the barostat, the relaxation time constants were set to 0.1 ps. To address the long-range coulombic interactions, the particle mesh Ewald method was used with a real space cutoff of 1 nm. The simulation time step was 2 fs. All chemical bond lengths were kept constant using the LINCS algorithm. Because the RMSD with respect to the crystal structure gradually increased for the first 100 ns, the latter 300 ns trajectories were used for the interaction energy analysis where the long-range electrostatic term was neglected. For systematic comparison, the same analyses were conducted for the hen egg lysozyme (HEL)-HyHEL10 complex (PDB ID code 2DQJ). In this work, all MD trajectories were calculated using GROMACS version 4.5.5.

**ITC experiments**

Prior to ITC experiments, wild type and LY50A mutant of B2212A scFvs and soluble ROBO1 (sROBO1) were prepared (See Supporting Information). Thermodynamic parameters of the interaction between B2212A scFv antibodies and its antigens were determined using MicroCal iTC200 system (GE Healthcare). The experimental conditions were as follows: in a calorimeter cell, the scFv fragments, at a concentration of 5–10 μM in phosphate-buffered saline [10 mM phosphate buffer (pH 7.4), 150 mM NaCl and 45 mM KCl], were titrated with 55.4–96.2 μM solution of antigens in the same buffer at 25°C. The antigen solution was injected 25 times. Thermograms were analyzed with Origin 7 software (GE Healthcare) after subtraction of the thermogram against a buffer background. The enthalpy change (ΔH) and binding constant (Kₐ) for the antigen–antibody interaction were directly obtained from the experimental titration curve fitted to a one-site binding isotherm. The dissociation constant (K₈) was calculated as 1/Kₐ. The Gibbs free energy change (ΔG = −RT ln Kₐ) and the entropy change (ΔS = (−ΔG + ΔH)/T) for the association were calculated from ΔH and K₈.

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