Epiregulin Recognition Mechanisms by Anti-epiregulin Antibody 9E5

STRUCTURAL, FUNCTIONAL, AND MOLECULAR DYNAMICS SIMULATION ANALYSES

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Epiregulin (EPR) is a ligand of the epidermal growth factor (EGF) family that upon binding to its epidermal growth factor receptor (EGFR) stimulates proliferative signaling, especially in colon cancer cells. Here, we describe the three-dimensional structure of the EPR antibody (the 9E5(Fab) fragment) in the presence and absence of EPR. Among the six complementarity-determining regions (CDRs), CDR1–3 in the light chain and CDR2 in the heavy chain predominantly recognize EPR. In particular, CDR3 in the heavy chain dramatically moves with cis-trans isomerization of Pro103. A molecular dynamics simulation and mutational analyses revealed that Arg40 in EPR is a key residue for the specific binding of 9E5 IgG. From isothermal titration calorimetry analysis, the dissociation constant was determined to be 6.5 nM. Surface plasmon resonance analysis revealed that the dissociation rate of 9E5 IgG is extremely slow. The superimposed structure of 9E5(Fab)-EPR on the known complex structure of EGF-EGFR showed that the 9E5(Fab) paratope overlaps with Domains I and III on the EGFR, which reveals that the 9E5(Fab)-EPR complex could not bind to the EGFR. The 9E5 antibody will also be useful in medicine as a neutralizing antibody specific for colon cancer.

Recently, antibody therapy has been attracting considerable attention as a possible cure for several types of diseases. For instance, trastuzumab is a humanized IgG1κ monoclonal antibody that is targeted for the human epidermal growth factor (EGF) receptor (EGFR)6 (HER2, ErbB-2), which is used in the treatment of metastatic breast cancer (1).

Initially, the EPR precursor protein is expressed as a type I transmembrane protein. A disintegrin and metalloproteinase 17 (ADAM17) catalyzes ectodomain shedding of the EPR precursor protein, which produces mature EPR (2). EPR induces dimerization of EGFR and promotes autophosphorylation in the intracellular kinase domain of EGFR (3). EGFR phosphorylation activates several types of intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and STAT5 pathways (4–6). As a result, proliferation, cell survival, and angiogenesis are induced in the cell.

Although the expression of EPR is suppressed in most adult normal tissues, EPR is overexpressed in human colon, breast, and ovarian cancers (7–10). Therefore, normalization of EGF signaling is expected to cure these cancers. Recently, humanized anti-EPR antibodies with high affinity targeted cytotoxicity have been prepared and characterized (11), and these antibodies have the potential to act as anticancer drugs.

The structure of EPR was first determined by NMR (12). Similar to the other EGF family ligands, EPR (residues Val1–Leu46) is composed of an N-terminal domain (residues Ile3–Glu13) that has a β-hairpin motif called the core region (residues Gly17–Cys32) and a C-terminal domain (residues Val34–Phe45). Three disulfide bridges stabilize the entire EPR structure. For the EGF family antibody ligand, the structures of transforming growth factor β complexed with Fab or single chain Fv of fresolimumab have been reported (13).

The abbreviations used are: EGF, epidermal growth factor receptor; EPR, epiregulin; Fab, fragment, antigen binding; CDR, complementarity-determining region; Fv, variable region of Fab; Vv, variable region of a heavy chain; Vl, variable region of a light chain; Cc, constant region of a light chain; Cm, constant region of a heavy chain; r.m.s.d., root mean square deviation; MD, molecular dynamics; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; hEPR, EPR from H. sapiens; mmEPR, EPR from M. musculus; Vv, association constant; Kd, dissociation constant; ΔH, binding entropy; ΔS, binding entropy; ΔG, Gibbs free energy; m3, triple mutant E27Q/K28N/F29Y.

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Epiregulin Recognition Mechanisms by 9E5(Fab) Antibody

To design an effective humanized antibody, we investigated the antibody recognition mechanism between mature EPR and the 9E5(Fab) fragment by x-ray structural analysis. In this study, we describe the three-dimensional structure of the 9E5(Fab) fragment with and without EPR. Moreover, a molecular dynamics (MD) simulation, isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR) analysis were performed to clarify the structure-function relationship. These findings are expected to aid in the development of future drugs, especially those that target cancers.

Experimental Procedures

Production and Purification of 9E5(Fab)—The 9E5 monoclonal antibody was produced using a method described previously (11). Hybridoma cells were intraperitoneally implanted in BALB/c nude mice (BALB/c-Sc-lu/nu), and ascites were obtained from the mice and examined with a Bio-Scale Mini UNOSphere SUPrA cartridge (Bio-Rad). The peak fractions were injected into a Bio-Scale Mini Bio-Gel P-6 (Bio-Rad).

To prepare 9E5(Fab), the Fc fragments of 9E5 IgG by papain digestion (9E5 IgG-papain, 100:1) were used. The digested samples were loaded onto a Bio-Scale CHT5-1 column (Bio-Rad) and eluted with a linear gradient of 0.5–250 mM sodium phosphate buffer (pH 6.8). The peak fractions were collected and concentrated, and they were then injected onto a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare), which was developed with 20 mM Tris-HCl (pH 7.5) buffer containing 300 mM NaCl. The peak fractions containing 9E5(Fab) were collected and concentrated to 10 mg ml\(^{-1}\) by ultrafiltration with Vivaspin (10-kDa cutoff; GE Healthcare).

Construction of the EPR Expression Plasmids—We constructed EPR from Homo sapiens (hEPR) and Mus musculus (mmEPR) pro-EPR cDNA (residues 1–46), which is elongated by 24 residues toward the N terminus (residues 23 to 46) to improve its fusibility. The EPR gene was cloned into a modified pro-EPR cDNA (residues 1–46), which is elongated by 24 residues toward the N terminus. The molecular replacement was performed with PHASER (16). To determine the 9E5(Fab) crystal structure, the 9E5(Fab) crystal was soaked in cryoprotectant solution (50 mM HEPES-Na (pH 7.3) and 21.5% (v/v) polyethylene glycol (PEG) 4000) and incubated at 20 °C. Crystals of 9E5(Fab) formed within 7 days. For x-ray data collection, a 9E5(Fab) crystal was soaked in cryoprotectant solution (50 mM HEPES-Na (pH 7.3), 24% (v/v) PEG 4000, and 10% (v/v) glycerol) and flash frozen in liquid nitrogen.

Expression and Purification of Recombinant EPRs—Escherichia coli SHuffle T7 cells (New England Biolabs, Ipswich, MA) were transformed with the prepared plasmids. The cells were cultured in lysogeny broth containing 100 μg ml\(^{-1}\) ampicillin at 37 °C until the optical density at 600 nm reached 0.6. The temperature was lowered to 15 °C, and then 0.4 mM isopropyl 1-thio-β-D-galactopyranoside was added to induce protein expression. After 24 h of cultivation, the cells were collected and stored at −80 °C until further use.

The cells were thawed and disrupted with an EmulsiFlex-C3 homogenizer (Avestin Inc., Ottawa, Canada) in 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl, 20 mM imidazole, and 2500 units of Benzonase. After removal of the cell debris by centrifugation, the supernatant was applied to an nickel-nitri-lotriacetic acid Superflow (Qiagen, Hilden, Germany) column and eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl and 500 mM imidazole. HRV3C protease was added to the eluate, and it was dialyzed against dialysis buffer (20 mM Tris-HCl (pH 7.5) containing 600 mM NaCl). To remove the HRV3C protease and uncleaved fusion proteins, the dialyzed sample was applied to GS Trap and His Trap columns (GE Healthcare), and the flow-through fraction was recovered. The sample was concentrated and loaded onto a gel filtration chromatograph with a Hi-Load 16/60 Superdex 75 prep grade column, which was developed with the dialysis buffer. The fractions containing the EPR protein were buffer-exchanged into 20 mM Tris-HCl (pH 7.5) containing 300 mM NaCl and concentrated to 10 mg ml\(^{-1}\).

X-ray Crystallography—The entire crystallization was performed with the sitting drop vapor diffusion method with a VIORAMO 96-well protein crystallization plate (Azone, Edobori, Osaka, Japan). For the crystallization of 9E5(Fab), 0.5 μl of protein solution (10 mg ml\(^{-1}\) 9E5(Fab), 20 mM Tris-HCl (pH 7.5), and 300 mM NaCl) was mixed with 0.5 μl of reservoir solution (50 mM HEPES-Na (pH 7.3) and 21.5% (v/v) polyethylene glycol (PEG) 4000) and incubated at 20 °C. Crystals of 9E5(Fab) formed within 7 days. For x-ray data collection, a 9E5(Fab) crystal was soaked in cryoprotectant solution (50 mM HEPES-Na (pH 7.3), 24% (v/v) PEG 4000, and 10% (v/v) glycerol) and flash frozen in liquid nitrogen.

For crystallization of the 9E5(Fab)-hEPR complex, 0.5 μl of protein solution (10 mg ml\(^{-1}\) 9E5(Fab)-hEPR, 20 mM Tris-HCl (pH 7.5), and 300 mM NaCl) was mixed with 0.5 μl of reservoir solution (100 mM MES monohydrate (pH 6.0) and 14% (v/v) PEG 4000) at 20 °C. The 9E5(Fab)-hEPR complex crystal formed within 7 days. For data collection, a 9E5(Fab)-hEPR crystal was soaked in cryoprotectant solution (100 mM MES monohydrate (pH 6.0), 17% (w/v) PEG 4000, and 20% (v/v) glycerol) and then flash frozen in liquid nitrogen.

The x-ray diffraction data sets for the 9E5(Fab) and 9E5(Fab)-hEPR complex crystals were collected at Photon Factory BL-5A and SPring-8 BL44XU, respectively. The diffraction data were integrated and scaled with HKL2000 (14). The structure of 9E5(Fab) was determined by the molecular replacement method using 82D6A3, which is an antithrombotic antibody (15) (Protein Data Bank code 2ADF), as the starting model with PHASER (16). To determine the 9E5(Fab)-hEPR complex structure, molecular replacement was performed with PHASER.
using the refined 9E5(Fab) structure and the NMR structure of hEPR (Protein Data Bank code 1K37) as the search models (12). Model building was performed using Coot (17), and the structure was refined using REFMAC5 (18) and PHENIX (19); 5% of the reflections were set aside for \( R_{\text{free}} \) calculations (20). The quality of the models was assessed with Ramachandran plots, and model geometry analyses were conducted with Rampage (21). All of the structural figures were drawn with PyMOL (22). The data collection and refinement statistics are summarized in Table 1.

**Molecular Dynamics Simulations**—All of the simulations were performed with the GROMACS 4.6.1 package (23–25) using the Fuji force field (26) for proteins, AMBER force field for ions, and TIP3P water potential. Na\(^+\) and Cl\(^-\) ions were added to produce a neutral solution of 0.15 M. The Nosé-Hoover thermostat (27, 28) with a relaxation time of 1 ps was used to keep the solutions at 298 K. The Parrinello-Rahman scheme (29) was used as a barostat at 1 atm with a relaxation time of 1 ps. The simulation time step was 3 fs, and all of the bond lengths of the proteins were constrained using the LINCS algorithm (30). The leap-frog algorithm was used to integrate the equations of motion, and the particle mesh Ewald method (31) was used to calculate the electrostatic interactions with a real space cutoff of 1.0 nm. The neighbor list cutoff was also set at 1.0 nm. The initial structure was taken from our x-ray crystal structure of the complex. After energy minimization, the heavy atoms of the protein were restrained for 200 ps using a harmonic potential with a force constant of 1000 kJ \( \text{mol}^{-1} \text{nm}^{-2} \) to relax the water molecules. Four NPT (constant number of particles, pressure, and temperature) simulations were then performed for 1 s with initial random velocities that obeyed a Maxwell-Boltzmann distribution at 298 K.

**FIGURE 1. Overall structure of the 9E5(Fab)-hEPR complex.** A, front view of the 9E5(Fab)-hEPR complex. hEPR and the heavy and light chains of 9E5(Fab) are colored pink, green, and cyan, respectively. B, top view of the complex structure. The black dotted squares in A and B show the locations of interaction-1 (enlarged in C), interaction-2 (enlarged in D), and interaction-3 (enlarged in E). C, interaction between CDR-L1, CDR-L3, and CDR-H2 in 9E5(Fab) and the N-terminal domain of hEPR (interaction-1). D, interaction between CDR-H1 and CDR-H2 in 9E5(Fab) and the C-terminal region of hEPR (interaction-2). E, interaction between CDR-L2 and CDR-H3 in 9E5(Fab) and the core region of hEPR (interaction-3). In C, D, and E, oxygen, nitrogen, and sulfur atoms are shown in red, blue, and yellow, respectively. Hydrogen bonds and salt bridges are shown as black dashed lines.
**Epiregulin Recognition Mechanisms by 9E5(Fab) Antibody**

### Table 1

| Data collection and refinement statistics for 9E5(Fab) and the 9E5(Fab)-EPR complex | 9E5(Fab) | 9E5(Fab)/hEPR complex |
|---|---|---|
| Data collection | | |
| X-ray source | Photon Factory BL5a | SPRing-8 BL44XU |
| Detector | ADSC Quantum 210r | Rayonix MX-225HE |
| Wavelength (Å) | 1.0000 | 1.0000 |
| Space group | P2₁ | P2₂ |
| Unit-cell parameters (Å; °) | a = 41.00, b = 79.83, c = 59.98; β = 92.59 | a = 68.60, b = 100.29, c = 187.37 |
| Resolution range (Å) | 50.00–1.60 (1.66–1.60) | 50.00–2.50 (2.59–2.50) |
| Total no. of reflections | 192,615 | 100,371 |
| No. of unique reflections | 48,127 | 22,299 |
| j/σ (I) | 16.2 (2.8) | 28.9 (2.9) |
| Redundancy | 4.0 (3.6) | 4.5 (3.0) |
| Completeness (%) | 95.3 (98.8) | 97.3 (93.4) |
| Rmerge (%) | 6.9 (34.9) | 5.1 (30.9) |

### Refinement statistics

| | | |
|---|---|---|
| Resolution (Å) | 17.27–1.60 | 14.91–2.50 |
| No. of reflections | 46,334 | 22,005 |
| Mean Wilson B value (Å²) | 21.2 | 59.6 |
| r.m.s.d. from ideal values | 3.627 | 3.660 |
| Allowed | 97.2 | 93.5 |
| Favored | 2.8 | 2.8 |
| Resolution range (Å) | 50.00–2.50 | 50.00–1.60 |
| No. of unique reflections | 48,127 | 22,299 |
| Mean Wilson B value (Å²) | 21.2 | 59.6 |
| r.m.s.d. from ideal values | 3.627 | 3.660 |
| Allowed | 97.2 | 93.5 |
| Favored | 2.8 | 2.8 |

### Table 2

**Hydrogen bonds and salt bridges between 9E5(Fab) and hEPR (distance <3.5 Å)**

| Interaction part | hEPR | 9E5(Fab) | Interaction distance A |
|---|---|---|---|
| **Interaction-1** | | | |
| Lys⁴ | O | Tyr⁵² | L1 | Oη | 3.1 |
| Cys⁵ | O | Tyr⁵² | L1 | Oη | 3.5 |
| Ser⁶ | Oγ | Tyr⁵¹ | L3 | O | 2.5 |
| Ser⁶ | Oγ | Asp⁴³ | L3 | O | 3.1 |
| Ser⁶ | N | Asp⁴³ | L3 | O | 3.2 |
| Ser⁶ | Oγ | Asp⁴³ | L3 | Oδ₁ | 3.2 |
| Asp⁷ | N | Asp⁴³ | L3 | O | 3.1 |
| Asp⁷ | Oδ₁ | Lys⁴¹ | L3 | O | 3.2 |
| Asp⁷ | Oδ₁ | Arg⁴⁹ | H2 | η | 3.4 |
| Asp⁷ | Oδ₁ | Arg⁴⁹ | H2 | η | 3.1 |
| Asp⁷ | Oδ₁ | Arg⁴⁹ | H2 | η | 3.4 |
| **Interaction-2** | | | |
| Arg⁹⁸ | Nη | Lys⁴¹ | H1 | O | 3.3 |
| Arg⁹⁸ | Nη | Asp⁴³ | H2 | Oδ₁ | 2.8 |
| Arg⁹⁸ | Nη | Asp⁴³ | H2 | Oδ₁ | 3.5 |
| **Interaction-3** | | | |
| Tyr¹¹ | Oγ | Gly¹⁰¹ | H3 | η | 3.3 |
| Tyr¹¹ | Oγ | Tyr⁵¹ | L3 | Oη | 2.6 |
| Val¹³ | O | His⁴⁹ | L2 | Ne₂ | 2.7 |
| Ser¹⁵ | Oγ | His⁴⁹ | L2 | Nδ₁ | 3.5 |
| Ser¹⁵ | Oγ | His⁴⁹ | L2 | Nδ₁ | 3.1 |
| Gln²⁷ | Ne₂ | Gly¹⁰¹ | H3 | O | 2.4 |
| Gln²⁷ | Ne₂ | Asp⁴³ | H3 | O | 3.4 |
| Gln²⁷ | Ne₂ | Asp⁴³ | H3 | O | 3.0 |
| Asn⁴⁰ | N | Gly¹⁰¹ | H3 | O | 2.8 |

### Results

**Complex Structure of 9E5(Fab)-hEPR**—We determined the structure of the complex of 9E5(Fab) with hEPR at 2.5-Å resolution (Fig. 1 and Table 1). The asymmetric unit contained one 9E5(Fab)-hEPR complex in a rectangular box with approximate dimensions of 35 × 45 × 90 Å. The interaction between 9E5(Fab) and hEPR formed a solvent-accessible surface of ~919 Å², which is in the typical range of interaction surfaces between antibodies and antigens (32).

All six CDRs in 9E5(Fab) (CDR-L1, CDR-L2, and CDR-L3 in the light chain and CDR-H1, CDR-H2, and CDR-H3 in the heavy chain) interacted with hEPR and formed 27 hydrogen bonds or salt bridges as shown in Table 2 with numerous van der Waals contacts.

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**Isothermal Titration Calorimetry**—Thermodynamic analyses of the interaction between EPR and 9E5 IgG were performed with an iTC200 calorimeter (GE Healthcare). In the calorimeter cell experiment, 9E5 IgG was placed in phosphate-buffered saline (10 mM phosphate buffer (pH 7.4), 150 mM NaCl, and 45 mM KCl) at a concentration of 5 μM, and it was titrated with 100–130 μM EPR solution in the same buffer at 25 °C. The EPR solution was injected 25 times. The thermograms were analyzed with Origin 7 software (GE Healthcare) after subtracting a thermogram measured against only the buffer. The enthalpy change (ΔH) and binding constant (Kₐ) for the interaction were directly obtained from the experimental titration curve fitted to a one-site binding isotherm. The dissociation constant (Kₐ) 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 Kₐ.

**SPR Analysis**—SPR was carried out to analyze the interaction between 9E5 IgG and hEPR in a Biacore T100 system. Thioredoxin-fused hEPR was immobilized by an amine coupling method at a level of about 124 resonance units on a CM5 sensor chip (GE Healthcare). The binding of 9E5 IgG to hEPR was accomplished by injecting increasing concentration of 9E5 IgG (3.1–50 μM) into the sensor chip under the buffer condition of HEPES-buffered saline with surfactant P20 (pH 7.4) at a flow rate of 30 μl min⁻¹ at 25 °C. The data were corrected by subtracting the responses from a blank flow cell in which an amine coupling reaction was carried out. The kinetic parameters and the binding affinity were calculated using the bivalent analyte model with Biacore T100 evaluation software (GE Healthcare).
The N-terminal domain of hEPR is recognized by CDR-L1, CDR-L3, and CDR-H2 (Fig. 1C). The C-terminal domain of hEPR is stabilized by CDR-H1 and CDR-H2 (Fig. 1D). The core region of hEPR (GlyE17–CysE32) also superimposed well on the NMR structure (12) (r.m.s.d., 1.0 Å). Although no dynamic movement of the Fv domain and the core region of hEPR occurs, conformational changes occur in the N- and C-terminal domains in hEPR and in CDR-H3 in 9E5(Fab) (Fig. 2 and Table 2).

Movement of CDR-H3 Induced by hEPR Binding—Interaction-3 is composed of the interaction of CDR-L2 and CDR-H3 with the core region of hEPR (TyrE13, TyrE21–ValE23, and SerE26–AsnE28) (Fig. 1F). In interaction-3, little conformational change occurs in hEPR. However, drastic conformational changes occur in CDR-H3 (Fig. 2 and Table 2). The r.m.s.d.

![Diagram of Epiregulin Recognition Mechanisms by 9E5(Fab) Antibody](image)
value of the Cα atoms of CDR-H3 (Arg498–Pro10103; super-
script H refers to the heavy chain) in the presence or absence of
hEPR is 2.4 Å, which is about 2.5 times larger than that of the
variable region of the heavy chain (0.9 Å). Asp102 is originally
hydrogen-bonded to His149 (superscript L refers to the light
chain) in the apo form. However, the hydrogen bond breaks
with the insertion of hEPR, resulting in flipping of Asp102 and
formation of a new salt bridge with Arg108. The Cγ carbon in
the carboxyl group of Asp102 moves more than 10.8 Å, and the
Cα atoms of Gly101 move more than 6.5 Å. A conformational
change from cis-Pro103 to trans-Pro103 also occurs upon
binding with hEPR. Gly100, Gly101, Asp102, and Pro103 in
CDR-H3 form six hydrogen bonds with Tyr13, Gln27, and
Asn28 (Table 2).

Calculation of the Interaction Energy by Molecular
Dynamics—The r.m.s.d. values of hEPR and the Fv part of
9E5(Fab) were compared with the x-ray crystal structure, and
the block average of the total energy was calculated from four
MD simulations (Fig. 3). The block average was calculated
within each 1.5-ns period. Because the system seemed to have
reached equilibrium after about 700–800 ns (Fig. 3), the bind-
ing interactions were analyzed for the trajectories from 900 ns
to 1 μs. The interaction energy is defined here as the sum of the
short range Lennard-Jones (r < 0.9 nm) and coulombic (r < 1.0
nm) interactions between the residue pairs, which are the dom-
inant contributions to the binding of hEPR to 9E5(Fab).

Fig. 4 shows the interaction energies of each hEPR residue,
which are 100-ns time averages of the equilibrated structures in
solution. The solvated structures differed a little from the crys-
tal structure. For example, Table 2 shows that the interaction
distance between the carboxyl oxygen atom of Cys6 of hEPR
and Oη of Tyr32 of 9E5(Fab) is 3.5 Å in the crystal structure.
Although the MD structure provided the shortest O
(Cys6)-OH (Tyr32) distance of 2.6 Å, the longest and time-
averaged distances were calculated to be 4.0 and 6.8 Å, respectively. This explains why the interaction energy of Cys\textsuperscript{66} is small in Fig. 4. Asp\textsuperscript{E9} and Arg\textsuperscript{E40} interact with several atoms as shown in Table 2, and all of the distances are greater than 3.0 Å except for N\textsubscript{η1} (Arg\textsuperscript{E40})–O\textsubscript{82} (Asp\textsuperscript{H152}) (2.8 Å) (Table 2). However, these residues have the strongest and second strongest interaction energies with 9E5(Fab) in Table 3. In the interaction-2 region, Asp\textsuperscript{E9} interacts very strongly not only with Arg\textsuperscript{L95} but also with Arg\textsuperscript{H50}. In the crystal structure, Asp\textsuperscript{E9} forms strong salt bridges. In the interaction-3 region, seven interactions of Asp\textsuperscript{E9} and Arg\textsuperscript{E40} cause large conformational changes of hEPR in the interaction-1 and interaction-2 regions.

Table 3 shows the details of the interaction energies of hEPR residues that are greater than −20 kJ/mol in Fig. 4. In the interaction-1 region, Asp\textsuperscript{E9} interacts very strongly not only with Arg\textsuperscript{H50} but also with Arg\textsuperscript{L95}. In the crystal structure, Asp\textsuperscript{E9} (O\textsubscript{82}) has interaction distances of 3.0 Å with Arg\textsuperscript{H50} (N\textsubscript{η1}) and 3.3 Å with Arg\textsuperscript{L95} (N\textsubscript{η2}). However, in solution, Asp\textsuperscript{E9} has more stable hydrogen bonds and salt bridges with Arg\textsuperscript{H50} than with Arg\textsuperscript{L95}. Thus, Arg\textsuperscript{L95} has higher interaction energies with Asp\textsuperscript{E9} than with Arg\textsuperscript{H50} in Table 3. In the interaction-2 region, both Arg\textsuperscript{E40} and Glu\textsuperscript{E42} interact with CDR-H1 and CDR-H2 and form strong salt bridges. In the interaction-3 region, seven residues of hEPR (Met\textsuperscript{E10}, Tyr\textsuperscript{E13}, Tyr\textsuperscript{E21}, Val\textsuperscript{E23}, Ser\textsuperscript{E26}, Gln\textsuperscript{E27}, and Asn\textsuperscript{E28}) interact with a total of eight residues of EPR (His\textsuperscript{L49} and Tyr\textsuperscript{L60}), CDR-H1 (Asp\textsuperscript{H151} and Tyr\textsuperscript{H133}), and CDR-H3 (Gly\textsuperscript{H100}, Gly\textsuperscript{H110}, Asp\textsuperscript{H102}, and Pro\textsuperscript{H110}) in a relatively weak manner. The total interaction energies in the interaction-1, -2, and -3 regions are 290.0, 214.5, and 195.8 kJ/mol, respectively.

Thermodynamic Analyses—To characterize the binding of the antibody to EPR from a thermodynamic viewpoint, we performed ITC analyses of the interaction of 9E5 IgG with EPR wild type (WT) and hEPR and mmEPR mutants (Table 4 and Fig. 5). The mmEPR triple mutant E27Q/K28N/F29Y (m3) was investigated because of the sequential differences between hEPR and mmEPR (Fig. 6).

hEPR WT showed an exothermic profile, and its binding enthalpy, $\Delta H$, is $−8.6 ± 0.7$ kcal/mol. The interaction has a strong binding affinity ($K_D = 6.5$ nM). The hEPR mutant R40A
showed no heat in the ITC analysis, indicating that ArgE40 is one of the hot spot residues in the interaction between hEPR and 9E5 IgG. The other Ala mutant, hEPR D9A, has a lower binding affinity with a large unfavorable entropy change. The binding energy of hEPR S26R is 1.2 kcal/mol higher than that of hEPR WT. These results indicate that steric hindrance or electrical repulsion reduces the binding affinity.

As expected, no heat was detected for mmEPR WT. In contrast, mmEPR m3 has a similar binding affinity to S26R hEPR. mmEPR R26S exothermically binds to 9E5 IgG, but the dissociation constant could not be determined because of the weak binding.

### Discussion

In this study, we describe the crystal structures of 9E5(Fab) in the presence and absence of its antigen hEPR. To investigate the recognition mechanism of hEPR by 9E5(Fab), we solved the x-ray structure of 9E5(Fab) with and without hEPR. To bind to hEPR, CDR-H3 undergoes the following three characteristic structural changes (Fig. 2). First is the formation of AspH102-ArgH98 salt bridges. AspH102 in 9E5(Fab) without hEPR forms a hydrogen bond with HisL49, thereby contributing to the inter-

### Table 4

| Source | Mutant | $N$ | $K_A$ ($\text{M}^{-1}$) | $K_D$ ($\text{M}^{-1}$) | $\Delta H$ (kcal/mol) | $-T\Delta S$ (kcal/mol) | $\Delta G$ (kcal/mol) |
|--------|--------|-----|------------------------|------------------------|----------------------|------------------------|----------------------|
| hEPR   | WT     | 1.90 ± 0.09 | 16.2 ± 4.9 × 10^7 | 6.5 ± 2.0 | -8.6 ± 0.7 | -2.6 ± 1.0 | -11.2 ± 0.2 |
|        | D9A    | 2.01 ± 0.04 | 6.7 ± 0.2 × 10^7  | 15.0 ± 0.6 | -14.0 ± 0.2 | 3.4 ± 0.2 | 10.7 ± 0.1 |
|        | S26R   | 1.92 ± 0.16 | 2.2 ± 0.8 × 10^7  | 50.6 ± 18.1 | -5.9 ± 0.5 | -4.1 ± 0.7 | -10.0 ± 0.2 |
| mmEPR  | WT     | ND        | ND                 | ND                | ND                  | ND                    | ND                  |
|        | R26S   | ND        | ND                 | ND                | ND                  | ND                    | ND                  |
|        | m3     | 1.87 ± 0.10 | 2.3 ± 0.7 × 10^7  | 46.4 ± 12.0 | -5.0 ± 0.1 | -5.0 ± 0.1 | -10.0 ± 0.2 |

### FIGURE 5. Titration calorimetry of the interaction between 9E5 IgG and EPR. A–G, typical calorimetric titration of 9E5 IgG (5 μM) with 100–130 μM EPR at 25 °C (top) and integration plot of the data calculated from the raw data (bottom). The solid line corresponds to the best fit curve obtained by least square deconvolution. A, hEPR WT; B, hEPR D9A; C, hEPR S26R; D, hEPR R40A; E, mmEPR WT; F, mmEPR R26S; and G, mmEPR m3.
action with CDR-H3 and CDR-L2. The binding of hEPR induces rearrangement of the hydrogen bonds so that AspH102 forms a salt bridge with ArgH98, which was originally exposed to the solvent region, and HisL49 forms a hydrogen bond with SerE26. Second are the conformational changes in the GlyH99–GlyH101 loop. As described above, GlyH101 moves more than 6.5 Å upon binding of hEPR. All of the residues between ArgH98 and AspH102 are glycine, and thus proper contact with hEPR is possible because of the flexibility. The third change is cis-trans ProH103 isomerization. In the structure of 9E5(Fab), ProH103 in CDR-H3 is stabilized by hydrophobic interactions with HisL49 in CDR-L2 and a couple of hydrophobic residues. Although the difference in the energy level between the cis and trans forms is only 2 kJ/mol, the activation energy of cis-trans isomerization is 80–90 kJ/mol (33), meaning that cis-trans isomerization of proline is an energy-requiring reaction.

From the results of the MD simulations, the interaction energy for interaction-3 is relatively small (Fig. 4). However, it is predicted that AspE9 and ArgE40 energetically contribute to interaction-1 and interaction-2, respectively. The ITC analysis clearly indicates that D9A hEPR has a comparable binding affinity with hEPR WT, suggesting loss of entropic energy in D9A and the existence of water molecules around ArgE40 in the counterpart of 9E5(Fab). It also indicates that AspE9 does not contribute to complex formation. Conversely, the R40A mutant of hEPR does not bind to 9E5 IgG, suggesting that ArgE40 is one of the hot spots for 9E5 IgG (Table 4). This interaction energy may contribute to cis-trans isomerization. The formation of a salt bridge between AspH102 and ArgH98 may also contribute to cis-trans isomerization. Once these conformational changes have occurred, it may not be able to return to the structure of CDR-H3, suggesting that the 9E5(Fab)/hEPR complex is difficult to dissociate without some type of energy, such as thermal energy. In fact, SPR analysis indicates that the rate of dissociation is extremely slow (Fig. 7). It is concluded that 9E5(Fab) is an effective antibody against hEPR because 9E5(Fab) strongly binds to hEPR and cannot easily dissociate. 9E5(Fab) can only recognize hEPR, and it can be called a human trap antibody. We will now discuss the specific recognition by 9E5(Fab) from the viewpoint of the amino acid alignment of hEPR (Fig. 6). SerE26–TyrE29 in hEPR interacts with CDR-H3 in 9E5(Fab), corresponding to ArgE26–PheE29 in mmEPR. The results of ITC analysis indicate that the KD value of the S26R mutant of hEPR is about 7 times higher than that of WT (Table 4). In contrast, the binding affinity of mmEPR m3 is on the order of 10^{-8} M. These results suggest that all of the SerE26–PheE29 sequence in hEPR is essential for the specific recognition of 9E5(Fab).
EPR specifically binds to the homodimers of EGFR, ErbB-1, and ErbB-4 (34, 35). To date, three structures of ligands of the EGF family complexed with the EGFR ectodomain have been reported: EGF-ErbB-1 (Protein Data Bank code 1IVO), transforming growth factor/TGF-ErbB-1 (Protein Data Bank code 1MOX), and neuregulin1/EGFR-4 (Protein Data Bank code 3U7U) (36–38). To investigate how to accomplish binding of 9E5(Fab):EPR to EGFR, we superimposed 9E5(Fab):EPR on the EGF-ErbB-1 ectodomain (Fig. 8). hEPR in EPR-9E5(Fab) superimposed well on EGF in EGF-ErbB-1, and the average r.m.s.d. between hEPR and EGF is 0.9 Å. The light chain of 9E5(Fab) does not interact with ErbB-1 and EGF. However, the heavy chain of the N-terminal region (GluH1–GlnH30, CDR-H1 (AsnH28–LysH30 and TyrH33), CDR-H2 (ArgH50–LysH59), and the region from the β-sheet to the α3 310 helix (ThrH71–AsnH77) in 9E5(Fab) interact with Domain I (TyrH88 and AsnH91–SerH92) and Domain III (IleH118–LeuH125, AsnH328, ThrH330, AspH355, and PheH357–ProH358) in ErbB-1. For this reason, the interactions of the heavy chain of 9E5(Fab) prevent binding of the complex of 9E5(Fab):EPR to ErbB-1. This tendency is almost the same as TGF-ErbB-1 and NRG1β-ErbB-4 complexes (37, 38). Therefore, 9E5(Fab)-captured hEPR could not bind to ErbB-1 and ErbB-4.

From the viewpoint of kinetics, the $K_D$ values of 9E5 IgG and hEPR WT are 0.86–6.5 nM, which were observed by ITC and SPR analysis (Fig. 7 and Table 4). hEPR is a much weaker antagonist of the ErbB-1 and ErbB-4 receptors with IC50 values of 2800 nM and >5 μM, respectively (34), indicating that 9E5 IgG binds to hEPR more strongly than ErbB-1 and ErbB-4. According to previous studies, mutational analysis and chemical regeneration suggest that the guanidinium group of ArgH40 in hEPR is essential for binding of the ErbB receptor (40, 41). These results support that 9E5(Fab) acts as not only the simple capturer of EPR but also the competitive neutralization antibody against EGFR with inhibition of the functional residue ArgH40.

In conclusion, 9E5(Fab) binds to only hEPR with rearrangement of the hydrogen bonding network along with cis-trans isomerization of ProH103 and shows high affinity and slow dissociation. MD simulation and ITC analyses uncovered that
Arg⁴¹⁰ acts as a hot spot in the interaction between hEPR and 9E5 IgG. Antibody drugs based on the structure of 9E5 with the conservation of the human trap recognition mechanism are expected, especially for colon cancer.

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