Structural Insights into the Down-regulation of Overexpressed p185<sub>her2/neu</sub> Protein of Transformed Cells by the Antibody chA21<sup>*</sup>

Huihao Zhou<sup>1</sup>, Zhao Zha<sup>1</sup>, Yang Liu<sup>1</sup>, Hongtao Zhang<sup>1</sup>, Juanjuan Zhu<sup>1</sup>, Siyi Hu<sup>1</sup>, Guodong Shen<sup>1</sup>, Liansheng Cheng<sup>1</sup>, Liwen Niu<sup>1</sup>, Mark I. Greene<sup>2</sup>, Maikun Teng<sup>3</sup>, and Jing Liu<sup>4</sup>

From the<sup>4</sup> School of Life Sciences, Hefei National Laboratory for Physical Sciences at Microscale and<sup>6</sup> Key Laboratory of Structural Biology, Chinese Academy of Sciences, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230027, China and the<sup>‡</sup> Department of Pathology and Laboratory Medicine, University of Pennsylvania school of Medicine, Philadelphia, Pennsylvania 19104

p185<sub>her2/neu</sub> belongs to the ErbB receptor tyrosine kinase family, which has been associated with human breast, ovarian, and lung cancers. Targeted therapies employing ectodomain-specific p185<sub>her2/neu</sub> monoclonal antibodies (mAbs) have demonstrated clinical efficacy for breast cancer. Our previous studies have shown that p185<sub>her2/neu</sub> mAbs are able to disable the kinase activity of homomeric and heteromeric kinase complexes and induce the conversion of the malignant to normal phenotype. We previously developed a chimeric antibody chA21 that specifically inhibits the growth of p185<sub>her2/neu</sub>-overexpressing cancer cells <i>in vitro</i> and <i>in vivo</i>. Herein, we report the crystal structure of the single-chain Fv of chA21 in complex with an N-terminal fragment of p185<sub>her2/neu</sub>, which reveals that chA21 binds a region opposite to the dimerization interface, indicating that chA21 does not directly disrupt the dimerization. In contrast, the bivalent chA21 leads to internalization and down-regulation of p185<sub>her2/neu</sub>. We propose a structure-based model in which chA21 cross-links two p185<sub>her2/neu</sub> molecules on separate homo- or heterodimers to form a large oligomer in the cell membrane. This model reveals a mechanism for mAbs to drive the receptors into the internalization/degradation path from the inactive hypophosphorylated tetramers formed dynamically by active dimers during a "physiologic process."

p185<sub>her2/neu</sub> is one of the four receptor tyrosine kinases of the ErbB family. We initially found that both homodimerization and heterodimerization with other ErbB receptors will induce transphosphorylation of the intracellular domains and result in the downstream signaling for cell proliferation and transformation. Moreover our studies have established that heterodimerization leads to increased signaling and transforming activity (1, 2). Significant overexpression of p185<sub>her2/neu</sub> results in abnormalities in cell signaling and can cause cell transformation. Early studies from several laboratories found that <i>her2/neu</i> gene was amplified and overexpressed in 20–30% of breast and ovarian cancers. Breast cancers that have p185<sub>her2/neu</sub> overexpressed have a more aggressive course associated with higher relapse rates (3). p185<sub>her2/neu</sub> represents the first oncoprotein target amenable for drug intervention and immunotherapy in which disabling the kinase reverses the malignant properties of the transformed cell and renders the tumor sensitive to chemotherapy and radiation therapy (4–8).

The p185<sub>her2/neu</sub> protein possesses a similar architecture to the other three ErbB members of this family. These kinases are type 1 transmembrane proteins and comprise an extracellular domain (ECD)<sup>4</sup> with four subdomains (I/L1, II/S1, III/L2, IV/S2), a single transmembrane helix, an intracellular tyrosine kinase domain, and a C-terminal tail (9). Recent crystallographic studies revealed that the subdomains II and IV contribute to dimerization events of the ErbB receptors (10, 11). Monoclonal antibodies that bind the ectodomains of these ErbB proteins have many consequences that can be associated with different epitope regions of the ectodomains. Binding to subdomains II and IV can limit dimerization of p185<sub>her2/neu</sub>. The mechanical disabling of the formation of dimeric complex is the therapeutic purpose of certain anti-p185<sub>her2/neu</sub> monoclonal antibodies (mAbs), such as the humanized mAb Pertuzumab. Pertuzumab binds near the center of subdomain II and appears able to directly interrupt the dimerization of p185<sub>her2/neu</sub> (12). The crystal structure of the Trastuzumab Fab fragment in complex with the p185<sub>her2/neu</sub> ECD revealed that Trastuzumab binds to the juxtamembrane subdomain IV of ECD (13). This interaction may also influence dimerization of the p185<sub>her2/neu</sub> transmembrane region as well as blocking the proteolytic cleavage of p185<sub>her2/neu</sub> ECD, a mechanism that

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* This work was supported by Grants from the National Natural Science Foundation of China (Grants 30570362, 30121001, 30025012, and 30571066), Hi-Tech Research and Development Program ("863" Program) of China (Grant 2006AA02A245), Ministry of Science and Technology of China (Grants 2006CB806500, 2006CB910200, and 2006AA02A318), Chinese Academy of Sciences (Grant KSCX2-YW-R-60), and National Major Special Science and Technology Project of China (Grant 2009ZX09102-223).

† The atomic coordinates and structure factors (code 3H3B) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S6.

§ These authors contributed equally to this work.

1 To whom correspondence may be addressed. Tel.: 86-551-3606314; Fax: 86-551-3606314; E-mail: mkteng@ustc.edu.cn.
2 To whom correspondence may be addressed. Tel.: 86-551-3601437; Fax: 86-551-3601443; E-mail: jliu@ustc.edu.cn.

4 The abbreviations used are: ECD, extracellular domain; scFv, single-chain Fv; CDR, complementarity-determining region; EGFR, epidermal growth factor receptor; r.m.s.d., root mean square deviation; TRITC, tetramethylrhodamine isothiocyanate.
may be relevant to altering p185<sub>her2/neu</sub> functionality in vivo (13).

p185<sub>her2/neu</sub> is expressed at about 40,000 copies/cell in normal tissues, whereas in cancer cells, it can reach 10<sup>6</sup> copies/cell. Down-regulation of p185<sub>her2/neu</sub> has been shown for several inhibitory antibodies as a mechanism to dampen p185<sub>her2/neu</sub>-mediated transformation. Although some studies indicated that 4D5 could down-regulate cell surface p185<sub>her2/neu</sub> receptor, it was reported that by binding to its epitope in the receptor, Trastuzumab prevented p185<sub>her2/neu</sub> ectodomain cleavage mediated by matrix metalloprotease (14). The cleavage produces a shed ectodomain as well as a kinase-active membrane-bound p95 fragment, which can be detected from advanced breast tumors that are insensitive to Trastuzumab treatment (15). However, degradation of both p185<sub>her2/neu</sub> and p95 can be observed in the presence of HSP90 inhibitor (16), suggesting an important role for HSP90 in the prevention of p185<sub>her2/neu</sub> internalization and degradation.

Synergistic down-regulation of p185<sub>her2/neu</sub> levels on her2/neu transformed cells have been observed when combinations of antibodies recognizing different epitopes on p185<sub>her2/neu</sub> ECD are used (17). A mechanism has been proposed in which the combination of antibodies cross-links the receptors, thus forming larger antibody-receptor lattices to enhance internalization (17, 18). A physiologic pathway has been defined in which antibody disables ErbB receptor dimers, leading to the formation of inactivated tetramers and then internalization (19).

Trastuzumab-resistant tumors have been identified after patients achieved an initial response to Trastuzumab-based regimens. Development of new antibody drugs or combinations of ectodomain binding monoclonal antibodies is a reasonable approach to limit the emergence of resistance or even to treat the single antibody-resistant tumor (8, 20). The anti-p185<sub>her2/neu</sub> antibody chA21 mediates specific inhibitory effects on p185<sub>her2/neu</sub>-overexpressed cancer cells (21, 22), as well as human breast and ovarian cancer xenograft (23, 24). Our previous data revealed that chA21 is a potent down-regulator of p185<sub>her2/neu</sub> (22).

Here, we report the x-ray crystal structure of the chA21 single-chain Fv (scFv) in complex with the first 192 residues (designated as EPI) of the N terminus of p185<sub>her2/neu</sub>. The complex structure, scFv-EPI, presents the first detailed atomic level description of a monoclonal antibody binding to this region of the p185<sub>her2/neu</sub> ectodomain. The study documents that chA21 recognizes an epitope located on the opposite surface of the subdomain II of p185<sub>her2/neu</sub> ECD. Our experiments indicate that interactions with this epitope facilitate chA21 cross-linking of receptors and leads to down-regulation of overexpressed ErbB receptors on the cancer cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trastuzumab and Pertuzumab were obtained from Genentech. Preparation of chA21 and its scFv was described previously (25). The p185<sub>her2/neu</sub> antibody Ab-1 (NeoMarkers) contains two monoclonal antibodies, e2-4001 and 3B5, that were raised against the cytoplasmic domain and the C terminus of the receptor, respectively. The polyclonal antibody Ab-1 (NeoMarkers) was generated using an immunogen similar to what was used to obtain 3B5. Polyclonal anti-EGFR antibody (sc-03) and polyclonal anti-ErbB3 antibody (sc-285) were from Santa Cruz Biotechnology. The GAPDH antibody was obtained from KangChen Bio-tech. Horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG and FITC-conjugated goat anti-rabbit IgG were purchased from Pierce.

**Crystallization**—Crystallographic analysis of scFv-EPI complex was described previously (25). The complex structure was solved by the molecular replacement method using the program PHASER (26). The structures of the free chA21 scFv (Protein Data Bank [PDB] code 2GJJ) and residues 1–192 of p185<sub>her2/neu</sub> ECD (PDB code 2A91) were used as the searching models. REFMAC5 (27) and COOT (28) were employed for refinement and iterative adjustments of the model. TLS (29) refinement was performed using PROCHECK (30) for stereochemical restraints. All details for structure refinement and model quality are described in Table 1. The atomic coordinates and structure factors have been deposited in the PDB under the code 3H3B.

**Cell Culture**—The human mammary cancer cell line SK-BR-3, which endogenously expresses epidermal growth factor receptor (EGFR), p185<sub>her2/neu</sub>, and ErbB3, was a generous gift from Prof. Jun Wang (University of Science and Technology of China, Hefei, China). The cells were incubated at 37 °C, 5% CO<sub>2</sub> in RPMI 1640 supplemented with 10% FBS, 10 units/ml penicillin, 10 μg/ml streptomycin (all from Invitrogen).

**Western Blotting**—SK-BR-3 cells were seeded into 6-well plates by 2 × 10<sup>5</sup> cells/well and grown until 80% confluency. After serum starvation for 24 h, the cells were treated with the indicated antibodies at 37 °C, with or without heregulin-β (Sigma) preincubation at 4 °C. Then the cells were harvested and lysed in 1 × sample loading buffer (50 mM Tris-HCl, pH 6.8, 20 mg/ml SDS, 10% glycerol, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF) or radioimmune precipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor mixture (Roche Applied Science). Cell lysates in equal aliquots were subjected to 8% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes. After incubation with 5% bovine serum albumin in TTBS buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for blocking, the membranes were incubated with the indicated primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody. The protein signals were then detected using enhanced ECL chemiluminescence reagent (Pierce).

**RESULTS**

**Overall Structure of scFv-EPI Complex**—The structure of scFv-EPI complex was determined to 2.45 Å resolution with R/R<sub>free</sub> factors = 20.1%/25.5% (Table 1). There are two EPI (Chains A and B) and two scFv (Chains C and D) molecules in the asymmetric unit of the complex crystal (supplemental Fig. 1). The overall electron density for these four molecules was quite good, and only a few residues on the N and C terminus of each molecule, the linkers (residues 113–134) between the
Complex Structure of chA21 and p185

TABLE 1
Statistics of x-ray diffraction data collection and structure refinement
Values in parentheses are for last resolution shell.

| Data collection | | | |
|-----------------|-----------------|-----------------|-----------------|
| Resolution (Å)  | 30.0-2.45 (2.58-2.45) | | |
| Wavelength (Å)  | 1.000 | | |
| Space group     | P2_1_2_1_2_1 | | |
| Unit cell parameters (Å) | a = 82.2, b = 87.2, c = 108.5 | | |
| Unique reflections | 29,113 (4139) | | |
| Redundancy      | 3.6 (3.2) | | |
| Completeness (%)| 99.4 (98.5) | | |
| Average I/σ(I)  | 9.3 (2.3) | | |
| Rmerge (%)      | 7.2 (33.6) | | |

Refinement

| Reflections for refinement/test | 27,646/1461 | | |
| Rwork = Rfree (%) | 20.1/25.5 (25.1/33.5) | | |
| r.m.s. bond (Å) | 0.008 | | |
| Mean B factor (Å²) | 24.5 | | |
| Non-hydrogen protein atom | 6572 | | |
| Ramachandran plot (%) | 89.3 | | |
| Most favored regions | 9.8 | | |
| Additional allowed regions | 0.7 | | |
| Disallowed regions | 0.2 | | |

* Rmerge = Σhkl(|Fobs(h) − ΣFcalc(h)|/ΣFcalc(h)), where Fobs is the observed intensity for reflection h and Fcalc(h) is the calculated structure factor for reflection h.

** Rwork = Rfree = Σhkl(|Fcalc(h)| − |Fobs(h)|)/Σhkl|Fcalc(h)|, where Fcalc(h) and Fobs(h) are the observed and calculated structure factors for reflection h, respectively.

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* Rmerge = Σhkl(|Fcalc(h) − Fcalc(h)|/ΣFcalc(h)), where Fcalc(h) is the weighted average intensity for all observations of reflection h.

** Rwork = Rfree = Σhkl(|Fcalc(h) − Fobs(h)|)/ΣhklFcalc(h), where Fcalc(h) and Fobs(h) are the observed and calculated structure factors for reflection h, respectively.

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One distinction exists at surface-exposed residues 99 –112, which is a p185her2/neu-specific loop not found in other ErbB receptors (supplemental Fig. S3). In p185her2/neu structures reported previously (12, 13, 32) this loop could not be fully traced due to its high flexibility, whereas it is quite ordered in our structural model because of its extensive interactions with the scFv (Fig. 1C).

Overview of the Interface of the scFv-EPI Complex—The complex structure indicates that the CDRs of light chain (L) and heavy chain (H) of chA21 scFv form a pocket able to interact with a large area of the C-terminal part of EPI (Fig. 2A). The antibody-antigen interface buries 953 Å² of the scFv surface and 886 Å² of EPI, which represent about 8.5% of the total scFv accessible surface area (about 11,251 Å²) and 9.7% of the EPI accessible surface area (9168 Å²), respectively.

The CDRs L1, L2, L3, H1, H2, and H3 contribute 220 (23.2%), 33 (0.3%), 93 (9.8%), 71 (7.5%), 263 (27.7%), and 303 Å² (32.0%) of the scFv surface, respectively. The CDRs of chA21 scFv are labeled. The CDRs superposition of chA21 scFv in the free form (PDB code 2GJJ, magenta) and in the complex (colored the same as panel A). Their overall structures are almost identical. C, the structure superposition of EPI in the free form (PDB code 2A91, red; PDB code 1N8Z, cyan; PDB code 1N8Z, gray) and in the complex (colored the same as panel A). Their overall structures are very similar, but the p185her2/neu-specific loop is disordered before the binding of chA21 scFv. The figures were prepared with the program PyMOL (48).

light and heavy chains of both scFv chains, and residues Lys-178, Gly-179, and Ser-180 in Chain B were not observed. Ramachandran plot analysis revealed that only Ala-57 residues in both scFvs fell into disallowed regions. Ala-57 locates at the tip of the complementarity-determining region (CDR) L2 and has been found to participate in forming classic γ-turn structures seen in immunoglobulins (31). The details of refinement statistics and model quality of the final model are summarized in Table 1.

Among the four molecules in the asymmetric unit, Chains A and C form one antigen-antibody complex, whereas Chains B and D form another. These two complexes are disposed in the asymmetric unit through a non-crystallographic two-fold rotation symmetry axis (supplemental Fig. S1), and their structures are almost identical (root mean square deviation (r.m.s.d.) of 0.705 Å for 405 comparable Cα atoms) (supplemental Fig. S2). Therefore, only the complex structure comprising Chains A and C, which has better electron density, is used in the following structural analysis and discussion.

In the complex, the scFv folds into two β-barrel domains formed by the V₄ and V₁ fragments, respectively (Fig. 1A). In comparison with the structure of the free chA21 scFv that was determined previously (22), both their overall structure (r.m.s.d. of 0.527 Å for 218 comparable Cα atoms) and the conformations of the six CDRs are almost identical (Fig. 1B). These data indicate that antibody-antigen interactions do not cause obviously structural changes in the chA21 scFv.

EPI consists of the p185her2/neu ECD subdomain I (residues 1–172) and the first C2 module (residues 173–192) of subdomain II. Subdomain I adopts a β-helical structure, and the N terminus of subdomain II folds closely to it (Fig. 1A). The overall structure of EPI in this complex is very similar to that of the corresponding region of the previously described p185her2/neu ECD (12, 13, 32).

One distinction exists at surface-exposed residues 99 –112, which is a p185her2/neu-specific loop not found in other ErbB receptors (supplemental Fig. S3). In p185her2/neu structures reported previously (12, 13, 32) this loop could not be fully traced due to its high flexibility, whereas it is quite ordered in our structural model because of its extensive interactions with the scFv (Fig. 1C).
Analysis of the complex structure shows that 28 residues of p185$^{her2/neu}$ participate in the antibody-antigen interaction. These residues belong to three discontinuous loops, which are labeled as loop I, II, and III from the N terminus to the C terminus, respectively (Fig. 2A). Loop I (residues 100–105) is the N-terminal part of the p185$^{her2/neu}$ specific loop. It locates nearby the CDRs L3 and H2 of chA21 and contributes 22.9% of the total interface area of the EPI molecule. Loop II (residues 135–144) is behind loop I and III and contributes only about 7.9% of the total interface. The loop III (residues 163–187) contributes 69.3% area of the total interface, which seems to be the major binding region for chA21 scFv.

Interactions between chA21 scFv and p185$^{her2/neu}$ EPI—In total, the interactions between chA21 scFv and EPI consist of 17 hydrogen bonds and 172 van der Waals contacts (<4 Å) (supplemental Tables S1 and S2). Neither salt bridges nor bridging water molecules were found. All of the 17 hydrogen bonds are contributed by L1, L3, H2, and H3 of scFv (supplemental Table S2). As for p185$^{her2/neu}$, the loops I, II, and III participate in forming 4, 3, and 10 hydrogen bonds, respectively (supplemental Table S2).

Asn-103 is key residue on loop I, and it embeds its side chain to a cleft formed by residues Tyr-100 (L3), Trp-102 (L3), and Tyr-239 (H3) of chA21 scFv (Fig. 2B). Asn-103 contributes 16 van der Waals contacts (supplemental Table S1) and forms three hydrogen bonds with Tyr-100 (L3) and Tyr-239 (H3) (Fig. 2C and supplemental Table S2). Another important residue in loop I is Asn-102, which forms a hydrogen bond with Thr-193 (H2) of scFv (Fig. 2C and supplemental Table S2).

Loop II is partially covered by loops I and III and interacts mainly through the protruding side chains of residues Tyr-189 (H2) and Tyr-236 (H3) of chA21 scFv. The main chain nitrogen atom of Asn-136 (II) forms a hydrogen bond with Tyr-189 (H2) (Fig. 2D), and Pro-137 (II) forms the hydrophobic stacks with the phenyl group of Tyr-189 (H2) (Fig. 2B). The Tyr-141 (II), Asp-143 (II), and Thr-144 (II) in combination with Arg-166 (III), Cys-170 (III), and Pro-172 (III) form a big cavity. Tyr-236 (H3), which locates at the tip of the CDR H3, embeds its side chain to this cavity and forms four hydrogen bonds with Tyr-141 (II), Asp-143 (II), Arg-166 (III), and Cys-170 (III) (Fig. 2, B–E, and supplemental Table S2). This structural feature indicates that Tyr-236 (H3) is important for the activity of chA21, a dominant contributory feature of aromatic residues we have described previously (33).

Loop III could be separated into an N-terminal part (residues 163–175) and a C-terminal part (residues 185–187), which are linked by a loop behind these two parts. The N-terminal part of loop III lies on the interface, and the main chain atoms of its residues Arg-166 (III), Cys-170 (III), and Pro-172 (III) contribute six hydrogen bonds altogether (Fig. 2, E and F). The C-terminal part of loop III locates nearby CDR L1, where Glu-185 (III) forms two hydrogen bonds with Tyr-31 (L1) and Asn-33 (L1) of chA21 scFv (Fig. 2F). Additionally, the imidazole ring of His-171 (III) of EPI was sandwiched between the side chains of Tyr-31 (L1) and Tyr-239 (H3) of scFv. The Pro-172 (III) was deeply embedded to the pocket formed by Asn-34 (L1), Lys-36 (L1), Tyr-236 (H3), and Glu-237 (H3) (Fig. 2B). These observations indicate the essential roles of His-171 (III) and Pro-172 tern in many antibody-antigen complexes, which we have described previously (33, 34). Particularly, the L2 loop of the light chain does not protrude from the scFv surface and only forms three van der Waals contacts with EPI (supplemental Table S1). A limited if not minimal role of L2 in antibody-antigen interaction is frequently observed, and we as well as others have noted this feature previously (33–35).
in the interaction affinity and specificity of antibody-antigen interactions and agree with our former mutagenesis results, in which mutations H171A and P172A reduced the affinity 200- and 700-fold as compared with the wild-type p185HER2/NEU (22).

chA21 Binds to the Opposite Surface of the p185HER2/NEU Dimerization Interface—The complex structure indicates that the chA21 epitope is located at the C-terminal part of subdomain I and the N terminus of subdomain II of p185HER2/NEU ECD. This is an epitope distinct from those described for Trastuzumab and Pertuzumab, which are located at subdomains IV and II, respectively. By recognizing this epitope, chA21 binds to the region of p185HER2/NEU located on the opposite side of its dimerization interface (Fig. 3A).

To understand the role of chA21 in dimerization of p185HER2/NEU and other ErbB members, we developed a model for a complex comprising a p185HER2/NEU ECD homodimer and chA21 scFv. The EGFR homodimer structure (PDB code 1IVO) (11) was used as the template to model the p185HER2/NEU homodimer, and the two EGFRs were replaced with two p185HER2/NEU proteins (PDB code 1N8Z) (13) by superimposing residues 232–292 of p185HER2/NEU to residues 226–286 of EGFR. The scFv-EPI complex structure model was next superimposed to one p185HER2/NEU member of the homodimer.

In this modeled complex, the chA21 scFv does not cause steric conflict that is able to disrupt the homodimer directly (Fig. 3B). The heterodimers of p185HER2/NEU with EGFR and ErbB3 were also modeled as described (36), and binding of chA21 would not cause steric conflicts in these heterodimers either (data not shown). These structural observations suggest that chA21 uses a different strategy to inhibit functional tyrosine kinase complexes of p185HER2/NEU-overexpressing cancer cells.

Down-regulation of p185HER2/NEU by chA21 Requires Bivalency and Is Dose-dependent—Previously, we showed in FACS analysis that p185HER2/NEU was internalized from the cell surface by the original murine antibody A21 (22). We investigated receptor down-regulation ability of chA21 in SK-BR-3 cells and compared chA21 with other antibodies. p185HER2/NEU was clearly down-regulated after 24 h of treatment with chA21 and was hardly detectable after 48 h (Fig. 4A). Before the treatment reached 24 h, we noted the accumulation of a fragment of about 130 kDa, which appears to be an intermediate product formed during p185HER2/NEU degradation (Fig. 4A). The overall level of p185HER2/NEU was not significantly affected by Trastuzumab or Pertuzumab (Fig. 4B). Interestingly, the combination of chA21...
The elucidation of targeted therapy has changed cancer treatment (4, 7). We have tried to define the principles by which ectodomain targeting of ErbB receptors can disable kinase complexes and reverse the malignant phenotype of human tumors in vitro and in vivo.

There are shared biological effects of most p185her2/neu ectodomain binding monoclonal antibodies. However, some epitopes to which inhibitory antibodies bind may help explain their anti-tumor activities (38–40). Epitopes for some anti-p185her2/neu antibody drugs are located at ECD subdomains II and IV. Both Trastuzumab and Pertuzumab recognize what may represent dimerization interfaces of p185her2/neu.

In this work, the crystal structure of scFv-EPI complex reveals that chA21 recognizes a novel epitope at the top of p185her2/neu ECD. This unique epitope locates to the opposite side of the dimerization interface. chA21 exploits a related but partially distinct mechanism to mediate p185her2/neu down-modulation. We propose a cross-linking model as an explanation of the molecular mechanism of action of chA21, which also extends our previous observation of receptor inactivated tetrameric forms induced by antibodies (19).

In cancer cells that overexpress p185her2/neu and other ErbB receptors, we first reported that p185her2/neu forms homodimers or heterodimers on the cell surface (2, 41). By binding to its epitope in p185her2/neu, the bivalent chA21 molecule can cross-link two p185her2/neu receptors in different homodimers to form a large complex (Fig. 5). This cross-linked complex would be efficiently internalized and degraded. Such a model explains perfectly the receptor down-regulation data we observed for chA21.

In a preliminary characterization of such chA21-induced oligomeric complexes, we have analyzed chA21 and p185her2/neu ectodomain-human Fc fusion protein (ECD-Fc) on non-reducing SDS-PAGE. In the presence of cross-linking reagent bis(sulfosuccinimidyl)disulphate, several high molecular weight species can be observed in the sample containing chA21 and ECD-Fc (supplemental Fig. S4). Our current data have outlined some biochemical features regarding the process of forming chA21-induced complexes.

**Bivalency**—chA21 requires bivalency to function as a cross-linker. The monovalent scFv form of chA21 cannot simultaneously capture two p185her2/neu molecules. As a result, although the scFv recognizes the same epitope as chA21, it fails to cross-link p185her2/neu molecules to form a large complex. Detectable down-regulation of p185her2/neu cannot be observed for scFv (Fig. 4B). We also observed this bivalency-dependent feature for the original mAb that down-regulates oncogenic p185her2/neu (4).

**Stoichiometry**—A certain stoichiometric ratio between the antibody and the receptor is required. In our experiment, we observed the highest down-regulation when cells were treated with 100 nM chA21. With lower concentration of chA21 (e.g. 10 nM), the large receptor-antibody complex could not efficiently form due to the lack of enough cross-linkers. When the chA21 concentration is too high (e.g. 1000 nM), chA21 levels may lead to self-competition, resulting in binding to single species of p185her2/neu. A cross-linked complex would not efficiently form in this case (supplemental Fig. S5). This explains the bell-

## DISCUSSION

The eludication of targeted therapy has changed cancer treatment (4, 7). We have tried to define the principles by which ectodomain targeting of ErbB receptors can disable kinase complexes and reverse the malignant phenotype of human tumors in vitro and in vivo.

There are shared biological effects of most p185her2/neu ectodomain binding monoclonal antibodies. However, some epitopes to which inhibitory antibodies bind may help explain their anti-tumor activities (38–40). Epitopes for some anti-p185her2/neu antibody drugs are located at ECD subdomains II and IV. Both Trastuzumab and Pertuzumab recognize what may represent dimerization interfaces of p185her2/neu.

In this work, the crystal structure of scFv-EPI complex reveals that chA21 recognizes a novel epitope at the top of p185her2/neu ECD. This unique epitope locates to the opposite side of the dimerization interface. chA21 exploits a related but partially distinct mechanism to mediate p185her2/neu down-modulation. We propose a cross-linking model as an explanation of the molecular mechanism of action of chA21, which also extends our previous observation of receptor inactivated tetrameric forms induced by antibodies (19).

In cancer cells that overexpress p185her2/neu and other ErbB receptors, we first reported that p185her2/neu forms homodimers or heterodimers on the cell surface (2, 41). By binding to its epitope in p185her2/neu, the bivalent chA21 molecule can cross-link two p185her2/neu receptors in different homodimers to form a large complex (Fig. 5). This cross-linked complex would be efficiently internalized and degraded. Such a model explains perfectly the receptor down-regulation data we observed for chA21.

In a preliminary characterization of such chA21-induced oligomeric complexes, we have analyzed chA21 and p185her2/neu ectodomain-human Fc fusion protein (ECD-Fc) on non-reducing SDS-PAGE. In the presence of cross-linking reagent bis(sulfosuccinimidyl)disulphate, several high molecular weight species can be observed in the sample containing chA21 and ECD-Fc (supplemental Fig. S4). Our current data have outlined some biochemical features regarding the process of forming chA21-induced complexes.

**Bivalency**—chA21 requires bivalency to function as a cross-linker. The monovalent scFv form of chA21 cannot simultaneously capture two p185her2/neu molecules. As a result, although the scFv recognizes the same epitope as chA21, it fails to cross-link p185her2/neu molecules to form a large complex. Detectable down-regulation of p185her2/neu cannot be observed for scFv (Fig. 4B). We also observed this bivalency-dependent feature for the original mAb that down-regulates oncogenic p185her2/neu (4).

**Stoichiometry**—A certain stoichiometric ratio between the antibody and the receptor is required. In our experiment, we observed the highest down-regulation when cells were treated with 100 nM chA21. With lower concentration of chA21 (e.g. 10 nM), the large receptor-antibody complex could not efficiently form due to the lack of enough cross-linkers. When the chA21 concentration is too high (e.g. 1000 nM), chA21 levels may lead to self-competition, resulting in binding to single species of p185her2/neu. A cross-linked complex would not efficiently form in this case (supplemental Fig. S5). This explains the bell-

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**Figure 5.** chA21 down-regulates p185her2/neu by cross-linking dimerized receptors to form a large complex in the cell membrane, which causes internalization. A, chA21 cross-links the homodimerized p185her2/neu to facilitate its internalization and down-regulation. B, EGFR and ErbB3 can also be included into the large complex once stimulated by their ligands and heterodimerize with p185her2/neu. HRG, heregulin.
shaped dose-dependent curve (Fig. 4C). To confirm this stoichiometry aspect of cha21-mediated down-regulation, we also studied the effect of cha21 on MCF-7, another cell line with much less expression of p185her2/neu. As predicted, p185her2/neu down-regulation was not observed at 100 nM, the concentration optimized for SK-BR-3 (supplemental Fig. S6A). Consistently, treating the SK-OV-3 cells, which also express high levels of p185her2/neu, with 100 nM cha21 also caused significant down-regulation (supplemental Fig. S6B).

Synergistic Effect with Other Antibodies—Our studies have shown that inactive tetrameric receptors can be readily induced by Trastuzumab (19). We expect that in the presence of cha21, these oligomeric structures can be further cross-linked for degradation (Fig. 4B). This would lead to the synergistic effect of the combination of treatment of cha21 with Trastuzumab or Pertuzumab.

Effect on Heterodimers of ErbB Receptors—ErbB receptors other than EGFR are considered to be resistant to ligand-induced down-regulation (42, 43), and p185her2/neu can also impose an inhibitory effect on EGFR internalization (44, 45). Because the pathogenesis and prognosis of cancers are correlated with abnormal signaling events shared by the activation of members of EGFR family, it seems wise and necessary to disrupt multiple ErbB receptor functions at the same time. However, antibodies such as Trastuzumab and Pertuzumab cannot universally prevent the formation of heterodimers as the dimer interfaces they bind to are only important for certain types of heterodimers (36).

Although EGFR and ErbB3 are not directly recognized by cha21, they can form heterodimers with p185her2/neu. In SK-BR-3 cells, it has been estimated by a mass spectrometry study that about 10–20% of p185her2/neu proteins are associated with EGFR. These heteromers can be cross-linked by cha21 (Fig. 5B), leading to our observation of EGFR and ErbB3 degradation after cha21 treatment (Fig. 4D).

The unique capability of cha21 to down-regulate multiple ErbB receptors may provide a therapeutic opportunity. Acquired resistance to monomeric antibodies has been reported as a result of the up-regulation of active heterodimers of ErbB receptors after anti-EGFR or anti-p185her2/neu antibody treatment (46, 47). It will be of interest to investigate whether this feature of cha21 to target heterodimers of ErbB receptors leads to less acquired resistance in human therapy.

The crystal structure of scFv-EPI complex was determined to 2.45 Å resolution and revealed the detailed antibody-antigen interactions necessary to further improve cha21 for better affinity and specificity. The complex also provides insight into the structural basis for understanding the p185her2/neu down-regulating activity of cha21. Our experiments indicate that cha21, a bivalent antibody, binds to an epitope of p185her2/neu that promotes cross-linking to form larger complexes that are internalized and degraded. These studies add to our efforts to delineate the principles needed to rationally target tumor oncoproteins.

Acknowledgments—We thank Prof. Yuhui Dong and Prof. Peng Liu of Beijing Synchrotron Radiation Facility for assistance in X-ray diffraction data collection.

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