Expression, purification, crystallization and preliminary X-ray analysis of the HER3–9E12 Fab complex

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9E12 is a fully human immunoglobulin G₁/κ monoclonal antibody that is specific for the epidermal growth factor receptor 3 (HER3), the overexpression of which has been detected in many tumour types and is associated with poor survival outcomes. To date, knowledge of the molecular mechanism for targeted antibodies that directly inhibit HER3 signalling is limited. Because knowledge of such therapeutic antibodies would help basic immunological therapeutics, structural insights into the HER3–9E12 Fab complex are important. Recombinant human HER3 and Fab fragments of the 9E12 antibody were cloned, expressed and crystallized, and crystallographic data sets were collected. The crystals belonged to space group *P*1, with unit-cell parameters *a* = 74.4, *b* = 98.6, *c* = 99.6 Å, α = 106.0, β = 95.0, γ = 102.5° and diffracted to a resolution of 2.1 Å.

1. Introduction

The HER or ErbB family of proteins (originally named because of their homology to the erythroblastoma viral gene product v-erbB) consists of four receptors (ErbB1–4, also known as HER1–4) and 13 polypeptide extracellular ligands, which contain a conserved epidermal growth factor (EGF) domain. Although the HER family is regarded as the prototypical group of the receptor tyrosine kinase (RTK) family, an important defining feature of the HER network is that two members of the family, ErbB2 (also known as HER2/neu) and ErbB3, are non-autonomous.

Blocking a tumourigenic HER signalling cascade is a key method by which a variety of cancers are treated. To date, the anti-EGFR (EGFR = epidermal growth factor receptor) monoclonal antibody (mAb) cetuximab, the anti-HER2 mAbs trastuzumab and pertuzumab, and the EGFR tyrosine kinase inhibitors erlotinib and gefitinib have played significant roles in clinical practice (Jonker *et al.*, 2007; Meng *et al.*, 2004). The importance of HER3 in cancer has only recently emerged; the upregulation of HER3 is an adverse prognostic factor in many tumour types and is associated with poor survival outcomes (Beji *et al.*, 2012; Lee-Hoeflich *et al.*, 2008; Funayama *et al.*, 1998). Moreover, HER3 has also been implicated in the development of resistance to anti-EGFR or anti-HER2 therapy (Wheeler *et al.*, 2008; Nahta *et al.*, 2006). A shift from HER1 homodimer signalling to HER1/HER3 heterodimer signalling in response to sustained treatment with EGFR inhibitors leads to the reactivation of the PI3K/AKT pathway and bypasses the therapeutic action of these compounds. The upregulation of HER3 seems to be driven by negative feedback from AKT (Sergina *et al.*, 2007). The amplification of the tyrosine kinase receptor MET induces HER3 phosphorylation and PI3K activation, representing another mechanism by which HER3 confers resistance to EGFR-targeting therapies (Engelman *et al.*, 2007). Thus, chemical compounds or biomacromolecules that directly block HER3 signalling are urgently needed.

9E12 is a fully human immunoglobulin G₁/κ monoclonal antibody specific to HER3 that was isolated from a screen of a phage-display library and exhibited superior efficacy in blocking HER2/HER3 heterodimerization and signalling in our ongoing experimental studies (data not shown). Although 9E12 can block HER2–HER3 interaction, the detailed blocking mechanism remains unclear. Mapping the sites on HER3 contacted by the therapeutic antibody

9E12 is an important step toward the understanding and possible manipulation of the mechanism of action of the drug.

Recently, employing crystallographic methods to identify the epitope of an antibody has emerged as a new option to clarify the binding data compared with classical methods such as phage-display peptide library (PDPL) technology (Abdullah *et al.*, 1988; Tao & Maruyama, 2008). Moreover, comparing the epitopes of different antibodies to the same antigen has garnered significant interest in the field of antibody engineering and targeted therapy. For example, the comparison of the different epitopes of adalimumab and infliximab in complex with TNF α using crystallography provided direct evidence of the adalimumab epitope as well as information on a molecular level to further understand the clinical advantages of adalimumab

versus infliximab therapy (Hu *et al.*, 2013). Thus, we launched a crystallographic study to investigate the precise epitope of HER3 recognized by 9E12.

2. Materials and methods

2.1. Macromolecule production

The residues (Ser1–His513) of the ErbB3 extracellular domain (ECD) with C-terminal histidine affinity tags (–GNSHis₆) were prepared as described previously (Schaefer *et al.*, 2011), but we used the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, California, USA) and the FreeStyle 293 expression system (Invitrogen). Briefly, FreeStyle 293 cells were seeded at 5×10^8 cells ml^{–1} in 200 ml medium. PcDNA3.1(+) expression vectors were transfected into cells using FuGene 6 according to the instruction manual. 5 l of HER3-containing medium were loaded into a 10 ml XK26/20 prepacked Ni²⁺–NTA column. The column was washed with 200 ml of buffer consisting of 50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 10 mM imidazole. Elution occurred during a 20 column-volume gradient to 250 mM imidazole. The pooled fractions determined by SDS–PAGE analysis were concentrated to 8 ml and loaded on a 320 ml S200 size-exclusion column equilibrated with 20 mM Tris pH 8, 150 mM NaCl. The fractions containing the pure protein were concentrated to 8 mg ml^{–1}. The purity of the protein was analyzed using SDS–PAGE.

The previously described large non-immunized repertoires of human scFv fragments displayed on phage (Satoh *et al.*, 2000; Samanta *et al.*, 2012) were used for the selection of single-chain antibodies that bound to the human HER3 ECD protein. More than 200 distinct HER3-binding scFv fragments were identified and were ranked based on their affinity for binding to recombinant HER3 ECD protein. A panel of 20 candidate scFv fragments that demonstrated the greatest binding affinity for binding to HER3 were selected for further analysis. These scFv fragments were converted to full IgG molecules (IgG1, κ) and assessed for their ability to inhibit the proliferation of MCF-7 cells in an *in vitro* proliferation assay (data not shown). 9E12 is the antibody with the best inhibition profile.

Antibody 9E12 was cloned, expressed and purified following previously reported procedures. The amino-acid sequence of the antibody 9E12 light-chain V region is QSALTQPASVSGSPGQSI-TISCTGTSSDDLATDVSQYQHPGKAPKLMYDVSFLYSGVS-NRFGSKSGNTASFTLTISGLQAEDEADYYCSTSSPYVFG-GGTKLTVLG. The amino-acid sequence of the heavy-chain V region is EVQLVQSGAELVQPGESLKISCKGSGYSFSGDWIG-WVRQAPGQGLEWMGWISAYNGNTNYADSLQGRVTMTTD-TSTSTAYMELRSLRSDDTAVYYCAREGDGAFDYWGQGLTV-TVSS. The *Xho*I and *Xba*I sites were added to the 5'-end of the heavy-chain variable-region gene (V_H) and an *Nhe*I site was added to the 3'-end. The polymerase chain reaction (PCR) product was cloned into the pGEM-T vector, and the sequence of the product was confirmed using DNA sequencing. V_H was excised *via* *Eco*RV and *Nhe*I digestion and then inserted into the *Eco*RV/*Nhe*I sites of the pAH4604 vector containing the human gamma 1 constant region gene (C_H). The resultant pAH4604–V_H vector was cleaved with *Xba*I and *Bam*HI. The 3.3 kb fragment containing the human antibody heavy-chain gene was cloned into the pcDNA3.1(–) vector (Invitrogen), which was digested with the same restriction enzymes and yielded the heavy-chain expression vector pcDNA3.1(–)V_HC_H. The human kappa-chain constant cDNA (C_L) was obtained as a 0.3 kb PCR product derived from pAG4622. The light-chain variable-region gene (V_L) of 9E12 was fused to the 5'-end of C_L using the overlapping

Figure 1
(a) SDS–PAGE analysis of the purified HER3–9E12 complex. Lane M, molecular-mass marker (labelled in kDa). Lane 1 corresponds to the purified HER3. Lane 2 corresponds to purified 9E12 Fab. Lane 3 corresponds to the protein complex, which is also labelled 3 in the size-exclusion chromatogram (b). Lane 4 corresponds to the excess antibody Fab, which is also labelled 4 in (b). (b) Size-exclusion chromatogram of the HER3–9E12 Fab complex. The plot shows absorbance at 280 nm.

Figure 2
Crystals of a HER3-9E12 complex crystal.

PCR method. The resultant human light-chain gene ($V_L C_L$), which has a *Hind*III site upstream of the start codon and an *Eco*RI site downstream of the stop codon, was cloned into the pGEM-T vector. The sequence of the chain was then verified. $V_L C_L$ was excised *via* *Hind*III and *Eco*RI digestion and ligated into pcDNA3.1 The Zeo(+) vector (Invitrogen) cleaved with the same restriction enzymes yielded the chimeric light-chain expression vector pcDNA3.1 Zeo(+) VLCL. The light-chain and heavy-chain expression vectors were co-transfected into Chinese hamster ovary K1 cells using the Lipofectamine 2000 reagent (Invitrogen). The stable transfectants were isolated *via* limiting dilution in the presence of $600 \mu\text{g ml}^{-1}$ G418 and $300 \mu\text{g ml}^{-1}$ zeocin. The culture supernatants from the individual cell clones were analyzed for antibody production using a sandwich enzyme-linked immunosorbent assay. The assay used goat anti-human IgG Fc (KPL, Gaithersburg, Maryland, USA) as the capture antibodies and goat anti-human kappa-horseradish peroxidase (HRP) Southern

Biotechnology Associates, Birmingham, Alabama, USA) as the detecting antibodies. Purified human IgG1/ κ (Sigma, Saint Louis, Missouri, USA) was used as the standard control. The clones producing the highest amount of recombinant antibodies were selected and grown in a serum-free medium. The recombinant antibodies were purified from the serum-free culture supernatant using protein A affinity chromatography. The antibody concentrations were determined with the absorbance at 280 nm and the purity was confirmed *via* SDS-PAGE analysis (Fig. 1a).

The Fab fragment of the 9E12 for crystallographic investigation was obtained *via* papain digestion of an antibody. The digested protein sample (digested full antibody) was loaded onto a Protein A Sepharose 4 FF column (GE Healthcare). The Fab fragment eluted in the flowthrough was separated from the Fc fragment and was further purified using ion-exchange chromatography with a Q-Sepharose FF column (GE Healthcare). The protein sample was concentrated to 20 mg ml^{-1} and then exchanged into a stock buffer consisting of 30 mM Tris-HCl pH 7.5, 150 mM NaCl. The homogeneity of the protein complex in the crystallographic analysis needs to be carefully examined; for the antibody-antigen complex in our study excess antibody Fab was employed to overcome this problem. Thus, ErbB3-ECD was subsequently mixed with an excess of 9E12 Fab and the complex was purified using gel-filtration chromatography (GE Healthcare). This complex was dialyzed against 20 mM Tris-HCl pH 7.0, 50 mM NaCl and was concentrated to 40 mg ml^{-1} .

2.2. Affinity measurement

To determine binding affinities, surface plasmon resonance (SPR) measurements with a Biacore 2000 were used to determine the monovalent binding affinities of 9E12 as described in Lee *et al.* (2004). 9E12 Fab was directly immobilized on CM5 chips (at $\sim 150 \text{ RU}$), and HER3-ECD (12.5 to 200 nM) was injected into the flow cell. The binding response was corrected by subtracting the RU from a blank flow cell. For kinetic analysis, the 1:1 Langmuir model of

Figure 3
X-ray diffraction pattern of a HER3-9E12 complex crystal obtained using a synchrotron-radiation source. The inset shows a magnification of a sector of the diffraction frame. The numbers give the resolutions of the circles (in Å).

Table 1

Diffraction protocol and data-collection statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.0000
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	74.4, 98.6, 99.6
α , β , γ (°)	106.0, 95.0, 102.5
Resolution range (Å)	50.00–2.10 (2.20–2.10)
Total No. of reflections	487569
No. of unique reflections	77392
Completeness (%)	99.9 (99.3)
Multiplicity	6.3
Average $I/\sigma(I)$	20.56 (5.8)
$R_{\text{merge}}^{\dagger}$ (%)	8.6 (37.3)
Overall <i>B</i> factor from Wilson plot (Å ²)	24.2

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of multiple observations $I_i(hkl)$ of a given reflection.

simultaneous fitting for both dissociation (k_d) and association (k_a) was used. The value for K_d was calculated as k_d/k_a .

2.3. Crystallization

The crystallization conditions for HER3–9F12 Fab were screened using the commercial screens Crystal Screen, Crystal Screen 2, Salt Rx, PEG/Ion and Index (Hampton Research, Laguna Niguel, California, USA). Crystallization assays were carried out using the hanging-drop vapour-diffusion method at 289 K in 16-well plates with two concentrations of HER3–9F12 Fab. For crystallization, each drop consisted of 1 μ l protein solution at a concentration of 15 or 30 mg ml^{−1} in buffer (20 mM Tris–HCl, 50 mM NaCl pH 7.0) and 1 μ l precipitant solution, which were mixed and equilibrated in the 16-well plates. The crystals of the HER3–9F12 Fab complex used in our analyses were obtained using condition No. 33 of Index [1.1 M sodium malonate pH 7.0, 0.1 M HEPES pH 7.0, 0.5% (v/v) Jeffamine ED-2001 pH 7.0]. A subset of the initial crystallization conditions was manually refined in the 24-well format using the hanging-drop vapour-diffusion technique, a final drop size of 2 μ l and a 1:1 mixture of protein and crystallization solutions in addition to screening for additives and streak-seeding. Moreover, different ratios of paraffin oil:silicone oil were also employed for under-oil crystallization (D'Arcy *et al.*, 2003). Finally, addition of glycerol followed by streak-seeding with diluted seed solution (1:100) yielded single, large, rod-shaped crystals (Fig. 2) within 3 or 4 d of setting up crystallization experiments. The initial data were collected using a crystal from a condition consisting of 1.1 M sodium malonate pH 7.0, 0.1 M HEPES pH 7.5, 0.5% (v/v) Jeffamine ED-2001 pH 7.0, 6% glycerol.

2.4. Data collection and processing

The crystals were mounted in a fibre loop, transferred into cryoprotectant solution (13% glycerol) and flash-cooled to 100 K in a nitrogen-gas stream. The preliminary diffraction data were collected on an in-house MAR 165 CCD detector (MAR Research, Hamburg, Germany) at the Department of Biochemistry, Norman Institute for Cancer Research, Canada. The diffraction data were collected from crystals on beamline 11A at the Photon Factory, Japan. The HER3–9F12 Fab crystals showed good-quality diffraction patterns (Fig. 3). All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). A complete data set was collected at a resolution of 2.1 Å on beamline 11A at the Photon Factory, Japan. A summary of the diffraction protocol and data-collection statistics is given in Table 1.

3. Results and discussion

The affinity of HER3 binding to 9E12 is 3.2 nM (K_d) and the purity of the protein complex at the final purification step was at least 95% as monitored by SDS–PAGE loading 0.2 μ g protein (Fig. 1*a*). Under reducing conditions, the antibody Fab yielded two protein bands with molecular masses of ~25 kDa (heavy chain and light chain). Size-exclusion chromatography suggested that HER3–9E12 Fab was in a complexed form because the later peak was the antibody on Superdex 200 using an FPLC purifier (Fig. 1*b*). The protein complex was purified in PBS buffer, exchanged into Tris buffer (20 mM Tris–HCl, 50 mM NaCl pH 7.0) using a 30 kDa cutoff Amicon protein concentrator and further purified. In general, crystals of the protein complex appeared after 3–4 d and continued to grow to maximum dimensions (150 mm) over the following week (Fig. 2). The data were collected using 13% glycerol as the cryoprotectant. The HER3–9E12 complex crystals displayed a good-quality diffraction pattern (Fig. 3). The crystals diffracted to 2.1 Å resolution and belonged to space group *P*1, with unit-cell parameters $a = 74.4$, $b = 98.6$, $c = 99.6$ Å, $\alpha = 106.0$, $\beta = 95.0$, $\gamma = 102.5^\circ$. The molecular-replacement strategy was used to solve the complex structure with *Phaser* (McCoy *et al.*, 2007) using the coordinates of apo HER3 (PDB entry 1m6b; Cho & Leahy, 2002) and GA101 Fab (PDB entry 3pp3; Niederfellner *et al.*, 2011).

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