Crystallization and preliminary X-ray crystallographic analysis of the sclerostin-neutralizing Fab AbD09097

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The secreted cystine-knot protein sclerostin was first identified from genetic screening of patients suffering from the rare bone-overgrowth diseases sclerosteosis and van Buchem disease. Sclerostin acts a negative regulator of bone growth through inhibiting the canonical Wnt signalling cascade by binding to and blocking the Wnt co-receptor LRP5/6. Its function in blocking osteoblastogenesis makes it an important target for osteoanabolic therapy approaches to treat osteoporosis, which is characterized by a progressive decrease in bone mass and density. In this work, the production, crystallization and preliminary X-ray diffraction data analysis of a sclerostin-neutralizing human Fab antibody fragment, AbD09097, obtained from a naive antibody library are reported. Crystals of the Fab AbD09097 belonged to space group $P2_1$, with unit-cell parameters $a = 45.19$, $b = 78.49$, $c = 59.20$ Å, $\beta = 95.71^\circ$ and diffracted X-rays to a resolution of 1.8 Å.

1. Introduction

The secreted Wnt factors play an important role in the regulation of bone formation and remodelling during embryonic development as well as during adult life (for reviews, see Baron & Kneissel, 2013; Monroe *et al.*, 2012). Through the activation of different signalling cascades, for example the canonical β -catenin or the noncanonical JNK/planner cell polarity and calcium-releasing pathways, Wnt factors control chondrogenesis (Rudnicki & Brown, 1997), osteoblastogenesis (Bennett *et al.*, 2005) and osteoclastogenesis (Glass *et al.*, 2005), and hence dysregulation of Wnt signalling is often associated with pathological conditions, leading either to reduced or elevated bone mass (for a review, see Krishnan *et al.*, 2006). The canonical pathway is initiated upon binding of Wnt to a heteromeric complex comprising a Frizzled receptor and a coreceptor of the low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) subfamily. This leads to phosphorylation of the cytoplasmic dishevelled, which then inhibits a large complex composed of the glycogen synthase kinase 3, axin and adenomatous polyposis coli, which would otherwise phosphorylate β -catenin and thereby target the latter for proteasomal degradation. When hypophosphorylated β -catenin accumulates in the cytoplasm it is subsequently translocated into the nucleus, where it mediates the transcription of Wnt target genes together with transcription factors of the TCF/LEF family (for a review, see Moon, 2005). Wnt signalling is tightly regulated by a multitude of different extracellular Wnt antagonists, among them members of the dickkopf family (Dkk1–3), Wnt inhibitory factor-1, cerberus

and the related proteins sclerostin and Wise (for a review, see Elston & Clifton-Bligh, 2010). Sclerostin was initially identified from genetic screens of two rare diseases, sclerosteosis and van Buchem syndrome, which are characterized by excessive bone overgrowth, indicating that sclerostin is a strong negative regulator of bone mass (Balemans *et al.*, 2001, 2002). Recent structural and functional analyses have shown that by binding to LRP5/6 sclerostin blocks the interaction of Wnt factors with their coreceptor and hence antagonizes the bone-forming functions of Wnts (Li *et al.*, 2005; Ellies *et al.*, 2006; Weidauer *et al.*, 2009; Veverka *et al.*, 2009; Bourhis *et al.*, 2011; Holdsworth *et al.*, 2012; Boschert *et al.*, 2013). The distinct expression profile of sclerostin in bone and the observation that its genetic deletion in mice did not result in a phenotype other than an increased bone density make sclerostin a highly interesting target for 'bone-anabolic' therapies for bone-destructing diseases such as osteoporosis (for a review, see Papapoulos, 2011). Indeed, antibodies neutralizing sclerostin have been used in several studies, and clinical phase trials with sclerostin-neutralizing antibodies are currently taking place (Li *et al.*, 2009; Ominsky *et al.*, 2010; McColm *et al.*, 2014; Lewiecki, 2014). Here, we present the crystallization and preliminary X-ray data analysis of a new sclerostin-neutralizing Fab antibody fragment obtained by a selection employing phage display.

2. Materials and methods

2.1. Macromolecule production

Sclerostin protein for the selection of Fab antibodies utilizing phage display was produced *via* baculovirus-driven insect-cell expression (Weidauer *et al.*, 2009). Briefly, the cDNA for the mature region of murine sclerostin (residues Gln24–Tyr211; SWISS-PROT entry Q99P68) was cloned into a modified version of the transfer vector pBAC3 (Novagen) to encode a protein with a gp64 signal peptide and an N-terminal hexahistidine tag followed by a thrombin cleavage site. Sf9 and TriEx Sf9 (Novagen) insect cells grown in suspension in serum-free IPL41 medium (Genaxxon) were infected with recombinant baculovirus [multiplicity of infection (MOI) of 5] obtained from co-transfection of the transfer vector with triple-cut virus DNA BacVector3000 (Novagen). The proteins were purified from the supernatant by step-gradient purification using Ni²⁺–NTA metal-affinity chromatography and subsequent cation-exchange chromatography using HiTrap CM Sepharose (GE Healthcare) employing a linear gradient of 0–1 M NaCl in 10 mM HEPES pH 7.5.

Fab antibodies against murine sclerostin were selected from a panning using the HuCAL GOLD phage-display library (Rothe *et al.*, 2008; Knappik *et al.*, 2000). The DNA encoding Fabs of selected phage clones were cloned into an *Escherichia coli* expression vector allowing periplasmic expression of a monovalent Fab equipped with an Myc and hexahistidine tag at the C-terminus of the Fab heavy chain. Preparative production of the sclerostin-neutralizing Fab antibody AbD09097 was performed as described by Jarutat *et al.* (2006).

Table 1

Macromolecule-production information for the Fab AbD09097.

The recognition sequence for proteolytic processing by the endopeptidase thrombin in the heavy chain of the Fab AbD09097 is underlined, and the Myc and hexahistidine sequences are indicated by *italic* and lower-case letters, respectively.

Expression host	<i>E. coli</i> strain TG1F [−]
Complete amino-acid sequence of AbD09097	
Light chain	DIVLTQSPATLSLSPGERATLSCRASQSISSNEL- AWYQQKPGQAPRLLIYDTSNRTATGVPARFSGS- GSGTDFLTITISLEPEDFAVYCCQYYSPIT- FGQGTKEIKRTVAAPSVFIFPPSDEQLKSGT- ASVVCLLNNFYPREAKVQWVDNALQSGNSQE- SVTEQDSKSTYSLSSTLTLSKADYEKHKVYA- CEVTHQGLSSPVTKSFNRGEA
Heavy chain	QVQLVESGGGLVQPGGSLRLSCAASGFTFTNYM- HWVRQAPGKGLVWSTIAYDGSSTYYADSVKG- RFTISRDNKNTLYLQMNSLRADTAIVYCAR- WFTDDIWGGQTLVTVSSASTKGPSVFPLAPSS- KSTSGGTAALGCLVKDYFPEPVTVSWNSGALT- SGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ- TYICNVNHKPSNTKVDKVEPKSEFLVPRGSG- APEQKLISEEDLMDAPHhhhhh

Briefly, transformed bacteria were grown at 30°C until the optical density at 600 nm reached 0.5; protein expression was then induced by the addition of 1 mM IPTG and expression proceeded for 16 h. The cells were chemically lysed (BugBuster, Novagen) and the crude supernatant was applied to metal-affinity chromatography using Ni–NTA resin (Ni–NTA Superflow, Qiagen). The resin was washed with 30 mM imidazole in 20 mM NaH₂PO₄, 500 mM NaCl pH 7.4 and the Fab was eluted with 250 mM imidazole in 20 mM NaH₂PO₄, 500 mM NaCl pH 7.4. The buffer was changed to PBS and the yield and purity of the antibody were determined by UV spectroscopy and SDS–PAGE. To set up crystallization trials, the Fab protein AbD09097 was further purified *via* cation-exchange chromatography employing a 0.05–1 M NaCl gradient in 50 mM sodium acetate pH 5.0 using a high-resolution Tricorn 5/10 Source 15S column (GE Healthcare). The purity and homogeneity were checked by SDS–PAGE. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Fractions from the high-resolution cation-exchange chromatography that contained pure Fab AbD09097 protein were pooled and dialyzed against 50 mM NaCl, 10 mM Tris–HCl pH 7.6 directly before setting up crystallization experiments. The protein was concentrated to 10 mg ml^{−1} using ultrafiltration (Millipore Centricon ultrafiltration devices, 3.5 kDa cutoff). Initial crystallization screening was performed using commercially available sparse-matrix screens such as the PACT, PEGs and PEGs II suites (Qiagen/NeXtal). Crystallization trials were performed using a sitting-drop vapour-diffusion setup in 96-well Greiner CrystalQuick plates. In all initial trial setups 1 µl protein solution in the droplet was mixed with 1 µl reservoir solution and was placed over 100 µl reservoir solution. Optimization and growth of crystals for data acquisition was performed using a hanging-drop vapour-

Table 2
Crystallization of AbD09097.

Method	Vapour diffusion
Plate type	Greiner CrystalQuick plate (screening), NeXtal EasyXtal (production)
Temperature (K)	294
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	50 mM NaCl, 10 mM Tris-HCl pH 7.5
Composition of reservoir solution	20% (w/v) PEG 3350, 0.1 M HEPES pH 7.5, 10 mM ZnCl ₂
Volume and ratio of drop	1:1 ratio protein:reservoir, 2 µl final volume
Volume of reservoir	100 µl (screening), 1 ml (production)

Table 3
Data-collection and processing statistics for the Fab AbD09097.

Values in parentheses are for the outer shell.

Diffraction source	Rigaku MicroMax-007 HF, Cu anode
Wavelength (Å)	1.5418
Temperature (K)	100
Detector	Rigaku R-Axis HTC image plate
Crystal-to-detector distance (mm)	140
Rotation range per image (°)	0.5
Total rotation range (°)	188
Exposure time per image (s)	150
Space group	<i>P</i> 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	45.19, 78.50, 59.20
α , β , γ (°)	90.0, 95.7, 90.0
Mosaicity (°)	0.658
Resolution range (Å)	20.8–1.85 (1.92–1.85)
Total No. of reflections	126045
No. of unique reflections	34408 (2600)
Completeness (%)	97.9 (80.1)
Multiplicity	3.7 (2.9)
$\langle I/\sigma(I) \rangle$	10.5 (3.7)
$R_{\text{r.i.m.}}^{\dagger}$	0.079 (0.371)
$R_{\text{p.i.m.}}^{\ddagger}$	0.054 (0.416)
Overall <i>B</i> factor from Wilson plot (Å ²)	22.1

[†] The redundancy-independent merging *R* factor $R_{\text{r.i.m.}}$ was estimated from R_{merge} by multiplication of the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity. [‡] $R_{\text{p.i.m.}} = \sum_{hkl} \{1/[N(hkl)-1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

diffusion setup employing NeXtal EasyXtal 24-well plates (Qiagen/NeXtal). All crystallization experiments were performed at 294 K in a temperature-controlled incubator. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Crystals of the Fab AbD09097 grew as rod-shaped single crystals with dimensions of about 200 × 50 × 50 µm (Fig. 1). Crystals harvested from the drop were briefly soaked in reservoir solution supplemented with 10% (w/v) glucose to achieve cryoprotection. The crystals were then directly flash-cooled in liquid nitrogen. A complete data set for the Fab AbD09097 was acquired at 100 K from a single crystal using an X-ray home source (Rigaku MicroMax-007 HF X-ray generator, VariMax HF mirror optics and a Rigaku R-Axis HTC image-plate detector). The crystal-to-detector distance was set to 140 mm and data were collected at 100 K by rotating the crystal through 188° (0.5° oscillations) with 150 s exposure per frame. The data were indexed, integrated and scaled using *CrystalClear* v.1.3.6 SP1 (Rigaku) and *iMosflm*

MOSFLM and *SCALA* from the *CCP4* package (Winn *et al.*, 2011; Leslie & Powell, 2007; Evans, 2006; Battye *et al.*, 2011). Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

The secreted Wnt-modulator protein sclerostin has raised great interest since its discovery owing to its potential as a target for the treatment of bone-related diseases (Papapoulos, 2011). Owing to its negative regulation of bone growth through blocking co-receptor binding of different Wnt factors (Bourhis *et al.*, 2011; Gong *et al.*, 2010), inhibition of the ‘inhibitor’ sclerostin restores bone growth, and thus neutralizing antibodies against sclerostin are promising drug candidates for the pharmaceutical industry (for a review, see Papapoulos, 2011). We have attempted to produce antibodies against murine full-length sclerostin in order to use these antibodies as tools to map the functional sites in sclerostin and to analyse its function in cellular assays. The target protein

Figure 1
(a) Rod-shaped crystals of the Fab AbD09097. The crystals grew to maximal dimensions of 200 × 50 × 50 µm within 7 d. (b) SDS-PAGE and subsequent Western blot analysis of crystals as shown in (a) using an anti-His₆ antibody confirmed the presence of full-length Fab protein with the Myc and His₆ tag attached to the C-terminus of the Fab heavy chain.

sclerostin was produced in baculovirus-transfected insect cells, for example TriEx Sf9 cells, as the eukaryotic system ensures correct and homogenous folding as well as glycosylation of the two *N*-glycosylation sites (Weidauer *et al.*, 2009). The presence of the carbohydrate significantly attenuates the 'stickiness' of the sclerostin protein, which might facilitate the panning process of the phage display (see below). For the generation of Fab antibodies, sclerostin was first biotinylated using EZ-link Sulfo-NHS-activated LC-Biotin (Pierce) and an equimolar ratio of biotin to sclerostin. The proteins were subsequently purified by RP-HPLC using a C8 column and a gradient from water to 100% acetonitrile in 0.1% (v/v) trifluoroacetic acid. The biotinylated sclerostin was then incubated in solution with phages from the HuCAL GOLD antibody phage library (Rothe *et al.*, 2008; Knappik *et al.*, 2000). Sclerostin–phage complexes were captured by streptavidin-coated magnetic beads (Invitrogen) and the phages were eluted with DTT.

Fab proteins from crude *E. coli* lysates were tested for specific binding to sclerostin using an ELISA employing immobilized sclerostin protein and control proteins. Among five different Fabs obtained against murine sclerostin, one antibody, AbD09097, showed dose-dependent neutralization of the sclerostin-mediated inhibition of Wnt1 activity, as measured in a Wnt reporter gene assay, and binds to murine and human sclerostin with high affinity (Boschert *et al.*, 2013; van Dinther *et al.*, 2013). To obtain insight into how this antibody recognizes and neutralizes sclerostin, the Fab AbD09097 was produced in *E. coli* on a preparative scale. For purification and immunodetection, the heavy chain of the Fab contained a Myc tag (EQKLSEEDLN) and a hexahistidine sequence at the C-terminus. The sequence tags could be

removed by proteolytic cleavage at a preceding recognition sequence (LVPRGS) using thrombin. After initial capture using metal-ion affinity chromatography (Ni^{2+} -NTA, Qiagen), the Fab AbD09097 protein was further purified employing high-resolution cation-exchange chromatography. As the 29-amino-acid C-terminal peptide extension containing the Myc and His₆ tag was thought to potentially interfere with the production of high-quality crystals, we also prepared Fab AbD09097 protein with the C-terminus removed by thrombin cleavage before purification by cation-exchange chromatography. The homogeneity and the purity of the Fab protein were confirmed by SDS-PAGE. Fab AbD09097 protein with and without the C-terminal sequence tags was subjected to crystallization trials using different commercial sparse-matrix crystallization screens (Qiagen/NeXtal). Needle-shaped crystals with single-crystal morphology could be obtained from the PACT Suite conditions B3 [0.1 M MIB (sodium malonate, imidazole and boric acid in a 2:3:3 ratio) buffer pH 6.0, 25% (w/v) PEG 1500] and B4 [0.1 M MIB buffer pH 7.0, 25% (w/v) PEG 1500] and the PEGs II screen conditions F4 [0.2 M ammonium sulfate, 0.1 M sodium acetate, 22% (w/v) PEG 4000] and F11 [0.2 M ammonium sulfate, 0.1 M sodium acetate pH 5.6, 30% (w/v) PEG 4000]. Single cube-shaped crystals could be obtained from the PACT screen conditions B12 [0.01 M zinc chloride, 0.1 M MES [2-(*N*-morpholino)-ethanesulfonic acid] pH 6.0, 20% (w/v) PEG 6000] and C12 [0.01 M zinc chloride, 0.1 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] pH 7.0, 20% (w/v) PEG 6000], the latter of which was used as a starting condition for optimization screening. Both truncated and full-length versions of the Fab protein yielded similarly shaped crystals under the same conditions, indicating that the long C-terminal peptide sequence did not disturb or alter crystal formation. After optimization screening, diffraction data-quality crystals were finally grown at 294 K from 20% (w/v) PEG 3350, 0.1 M HEPES pH 8.5, 10 mM zinc chloride; the protein concentration was 10 mg ml⁻¹ (Fig. 1). Diffraction data analysis then revealed that crystals of AbD09097 with the Myc and His₆ tag attached to the heavy-chain C-terminus consistently diffracted X-rays to significantly higher resolution (≤ 2 Å) compared with crystals of AbD09097 which had been treated with thrombin to remove these tags (≥ 7 –8 Å) (Fig. 2). Thus, in contrast to the usual assumption that removal of the terminal extension of proteins improves crystallizability and crystal quality, the C-terminal Myc and His₆ tag on the heavy chain of AbD09097 clearly improved the diffraction of these crystals and was required to obtain high-resolution data.

A full data set was then acquired from a single crystal of AbD09097 at 100 K at the home X-ray source. The data consisted of 376 0.5° frames, yielding 126 045 reflections. Indexing and scaling of the data using *CrystalClear* (v1.3.6 SP1; Rigaku) or *iMosflm* (Battye *et al.*, 2011; Leslie & Powell, 2007) and *SCALA* from the *CCP4* package (Winn *et al.*, 2011; Evans, 2006) suggested that the crystal belonged to the monoclinic space group *P*2 or *P*2₁, with unit-cell parameters $a = 45.19$, $b = 75.50$, $c = 59.20$ Å, $\beta = 95.7^\circ$. The redundancy-independent overall $R_{\text{p.i.m.}}$ (precision-indicating merging R

Figure 2
Diffraction image of a crystal of AbD09097 (as shown in Fig. 1). The crystals diffracted to 1.9 Å resolution (exposure time 150 s) as seen from the enlargement in the upper left inset.

factor; Diederichs & Karplus, 1997) was 5.4% for the resolution range 20.8–1.85 Å and the completeness was 97.9%. To determine the packing of the Fab molecule in the asymmetric unit of the crystal, we calculated the Matthews coefficient (Matthews, 1968), which yielded a V_M of $2.01 \text{ Å}^3 \text{ Da}^{-1}$ corresponding to a solvent content of 38.8%. The low solvent content when only a single Fab molecule was present in the asymmetric unit strongly indicated the absence of noncrystallographic symmetry. To solve the structure, we employed molecular replacement using *Phaser* (McCoy *et al.*, 2007) with the crystal structure of the Fab AbD1556 bound to the extracellular domain of the BMP type IA receptor (BR1A) as a template (Harth *et al.*, 2010). Initial attempts to employ the full Fab structure as a search template for calculation of the rotation and translation functions failed, possibly owing to differences in the antibody architecture, namely the hinge angle between the variable and constant domains of the Fab. We then produced combinations of coordinate sets separating the light and the heavy chains, partitioning the variable domain from the constant domain or using each variable and constant region of the light and the heavy chain separately. Only rotation and translation functions employing search templates comprising the constant and variable domains separately or using all four Fab domains individually yielded reasonable results in the molecular-replacement analysis and also confirmed $P2_1$ as the correct space group (see Supplementary Table S1). A subsequent short refinement of the oriented template derived from the search using the four individual Fab domains with *REFMAC* yielded an R_{cryst} of 33.9% and an R_{free} of 38.1%. The sequence of the search-template Fab was exchanged for the sequence of the Fab AbD09097 and the conformational differences in the CDR loops were modelled. It is interesting to note that an initial preliminary visual inspection of the electron-density map after molecular replacement reveals additional density protruding from the C-terminus of the Fab heavy chain, which harbours the Myc and His₆ tag, into a symmetry-related Fab molecule, possibly explaining why removal of the tag resulted in crystals that diffracted X-rays to only low resolution. Refinement of the structure is currently in progress. The final structure of the Fab AbD09097 will provide the first insight into the epitope of a sclerostin-neutralizing antibody and might thus facilitate the future development of other inhibitors targeting sclerostin.

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