ARTICLE P 59-62

Crystal structure of the Fab fragment of burosumab, the first-in-class anti-FGF23 medicine for treating X-linked hypophosphatemia and tumor-induced osteomalacia

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Burosumab is a first-in-class therapy approved to treat X-linked hypophosphatemia and tumor-induced osteomalacia, a condition associated with excessive fibroblast growth factor 23 (FGF23) production. This antibody drug directly targets the excessive FGF23 in patients and inhibits its regulation of phosphate homeostasis and vitamin D metabolism. Here, the Fab fragment of burosumab was expressed, purified, and crystallized. The crystals belonged to the space group $P2_1$, with unit cell parameters $a = 49.51$, $b = 66.22$, $c = 62.05$ Å, and $\beta = 105.99^\circ$. An asymmetric unit of the crystal contains one Fab fragment with a Matthews coefficient of $1.93 \text{ Å}^3 \text{ Da}^{-1}$. The structure was determined at a resolution of 1.45 Å , with $R_{\text{work}}/R_{\text{free}} = 0.169/0.196$. The complementarity determining regions of burosumab construct a negatively charged patch, a putative hot spot for FGF23 binding. This high-resolution structure could be used for predicting the binding mode between burosumab and FGF23.

INTRODUCTION

Fibroblast growth factors mediate signal transduction involved in autocrine, paracrine, or hormonal activities. Human fibroblast growth factor 23 (FGF23) is a 32 kDa glycoprotein mainly produced in bone and plays an important role as a hormonal regulator of phosphate homeostasis (Saito and Fukumoto, 2009). All endocrine FGFs, including FGF19, FGF21, and FGF23, require co-receptor Klotho (α - or β -Klotho) for effective binding to the FGF receptors. The co-receptor α -Klotho facilitates the binding of FGF23 to FGFR1c, which is the most critical receptor for FGF23 signaling under physiological conditions. Excessive FGF23 reduces serum levels of phosphorus by regulating phosphate excretion and vitamin D activation in the kidney. For a long time, blocking the excessive FGF23 has been recognized as a promising therapeutic strategy to treat hypophosphatemic rickets (ADHR Consortium, 2000).

In 2018, the U.S. Food and Drug Administration (FDA) approved the first-in-class anti-FGF23 drug, burosumab (Crysvita®), for treating X-linked hypophosphatemia (XLH), which causes impaired bone growth and development in children (Lamb, 2018). In 2020, the FDA expanded the indications of this drug to include FGF23-related hypophosphatemia in tumor-induced osteomalacia (Whyte, 2021). Burosumab is a fully human monoclonal IgG1κ antibody targeting FGF23. By directly neutralizing excess activities of FGF23 in patients, burosumab

restores phosphate reabsorption by the kidney and increases the activation of vitamin D, which increases gastrointestinal phosphate absorption. Thus, hypophosphatemia and the risk of bone diseases by bone mineralization defects can be significantly reduced.

The structures of human FGF23 and its ternary complex with FGFR1c and α -Klotho have established the molecular basis of the interaction of FGF23 with its receptors, thereby enhancing the understanding of the detailed mechanism of FGF23 signaling underlying phosphate homeostasis (Goetz et al., 2007; Chen et al., 2018).

Despite the clinical success of burosumab, no structural information for the antibody and its interaction with FGF23 has been reported to date. To elucidate the mechanism of action and epitope of burosumab, we expressed, purified, and crystallized the Fab fragments of burosumab. Besides, we determined its crystal structure at the resolution of 1.45 Å . The detailed understanding of the mechanism of action of burosumab from further structural studies can facilitate the design of improved biologics against FGF23.

RESULTS AND DISCUSSION

The Fab fragment of burosumab was overexpressed in the periplasmic region of *E.coli* and purified to homogeneity by the Ni-affinity and size exclusion chromatography (Figure 1A).

The crystals of the burosumab Fab fragments were generated by a hanging-drop vapor diffusion method with a well solution containing 0.03 M NaF, 0.03 M NaBr, 0.03 M NaI, 20% (v/v) PEG500 MME, 10% (w/v) PEG20000, 0.1 M Sodium HEPES, pH 7.5 at 4°C (Figure 1B). X-ray diffraction data were collected to 1.45 Å resolution and the crystals belonged to space group P2₁, with unit cell parameters *a* = 49.51, *b* = 66.22, *c* = 62.05 Å, and β = 105.99° (Table 1). The Matthews coefficient was calculated to be 1.93 Å³ Da⁻¹ with a solvent content of 36.36% (Matthews, 1968), assuming one Fab molecule in an asymmetric unit. After phasing by molecular replacement, the crystal structure was refined to a resolution of 1.45 Å, with $R_{\text{work}}/R_{\text{free}}$ = 0.169/0.196. The asymmetric unit of the crystal contained one molecule of the burosumab Fab with clear electron density on all residues except the residues 132–137 of the heavy chain (Figure 2). The six complementarity determining regions (CDRs) of burosumab would contain the putative paratope for FGF23 binding. Notably, a negatively charged patch is formed by the residues of the CDR regions, including D99, D102, D105 within HCDR3, and D50 of LCDR2, implying its major contribution to FGF23 binding by electrostatic interactions (Figure 3). The high-resolution of the structure would be helpful for predicting the binding mode between burosumab and FGF23. Interestingly, inspection of the surface of FGF23 reveals a highly positively charged spot, which is the heparin-binding site (Figure 4). To obtain further insights on the antigen-antibody interaction, a molecular docking between FGF23 and burosumab was tried using the HADDOCK server with restraints that the acidic and basic residues on the charged spots of each protein participate in interactions (van Zundert et al., 2016). However, the docking result was not reliable, probably due to the conformational flexibility of FGF23.

Further studies to determine the structure of burosumab in complex with FGF23 would elucidate the precise epitope and mechanism of action underlying the blockade of the interaction of FGF23 with FGFR1c or α -Klotho. In addition, the structural study can also provide helpful information for the design of improved anti-FGF23 biologics for treating X-linked hypophosphatemia and tumor-induced osteomalacia.

FIGURE 1 | Purification and crystallization of the burosumab Fab. (A) Reducing SDS-PAGE analysis of the fractions of the burosumab Fab after size exclusion chromatography. Lane M and C contain molecular-weight marker and the sample before SEC, respectively. Due to the similar molecular weight, the heavy and light chains are shown as a single band. (B) Crystals of the burosumab Fab grown in 0.03 M NaF, 0.03 M NaBr, 0.03 M NaI, 20% PEG500 MME, 10% PEG20000 0.1 M Sodium HEPES, pH 7.5 at 4°C.

METHODS

Expression and purification of the burosumab Fab

The gene encoding the burosumab Fab was synthesized after codon-optimization for *E. coli* expression. The synthesized gene was cloned into a modified pBAD vector using *Nhe*I and *Ap*I restriction sites (Table 2). The construct contains the heavy and light chain with the STII signal sequence in each chain for periplasmic secretion and a 6xHis-tag at the C-terminus of the heavy chain (Lee et al., 2016). The plasmid pBAD-burosumab Fab was transformed into *E. coli* Top10F (Invitrogen). The cells were grown at 37°C in LB medium supplemented with 50 µg ml⁻¹ ampicillin. When the OD600 of the culture reached 1.2, the protein expression was induced with 0.2% arabinose, and cells were further grown at 25°C for 15 h. The cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl), and lysed by sonication on ice. After removing cell debris by centrifugation, the burosumab Fab in

TABLE 1 | Data collection and refinement statistics

Data Collection	
X-ray source	PLS 5C
Wavelength (Å)	1.0000
Space group	P2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	49.51, 66.22, 62.05
α , β , γ (°)	90, 105.99, 90
Resolution (Å)	1.45 (1.49–1.45)*
<i>R</i> _{sym} (%)	10.1 (49.5)
<i>I</i> / σ <i>I</i>	6.31 (1.81)
Completeness (%)	99.7 (85.3)
Redundancy	3.4 (2.5)
CC _{1/2}	0.985 (0.845)
Refinement	
Resolution (Å)	1.45
No. reflections	68056
$R_{\text{work}}/R_{\text{free}}$ (%)	16.9/19.6
No. atoms	
Protein	3211
Water	673
Average B-factor (Å ²)	22.7
Protein	21.4
Water	26.9
R.m.s. deviation	
Bond lengths (Å)	0.007
Bond angles (°)	1.239
Ramachandran	
Favored (%)	97.61
Allowed (%)	2.39
Outlier (%)	0.00
PDB code	7VEN

*Values in parentheses are for the outer resolution shell.

the supernatant was purified using HisTrap HP column (Cytiva) and HiLoad 26/60 Superdex 200 pg column (Cytiva). The elution profile of the protein showed a single major peak in the size exclusion chromatography, and the protein quality was evaluated by SDS-PAGE.

Crystallization, data collection, and structure determination

For crystallization, the purified burosumab Fab was concentrated to 20 mg ml⁻¹ in 20 mM Tris, pH 8.0, and 200 mM NaCl. The crystallization was carried out by a vapor-diffusion method with 300 µl of the reservoir solution and the hanging drop of 0.8 µl of protein solution mixed with 0.8 µl of the reservoir solution at 4°C. Clusters of crystals were grown with a reservoir solution containing 0.03 M NaF, 0.03 M NaBr, 0.03 M NaI, 20% (v/v) PEG500 MME, 10% (w/v) PEG20000, 0.1 M Sodium HEPES, pH 7.5 within a week. For cryoprotection, a cluster of crystals was broken with a needle and flash-frozen in liquid nitrogen.

FIGURE 2 | Structure of the Fab fragment of burosumab. (A) Overall structure of the burosumab Fab. The heavy and light chains are colored cyan and pink, respectively. The CDR regions of the heavy and light chains (HCDR1-3 and LCDR1-3) are colored blue and red, respectively. (B) Stereoscopic view of the 2fofc map (1.5 σ contour level) calculated at 1.45 Å resolution on the CDR regions.

FIGURE 3 | Negatively charged patch on the putative paratope of burosumab. (A) Surface representation of the burosumab Fab. The heavy and light chains are colored cyan and pink, respectively. The CDR regions of the heavy and light chains are colored blue and red, respectively. (B) Electrostatic potential generated by PyMOL on the burosumab surface. (C) The residues within the CDR regions. The acidic residues comprising the negatively charged patch are labeled. The green ellipses indicate the putative region of the negatively charged patch for FGF23 binding.

FIGURE 4 | Positively charged spot on the surface of FGF23. Surface representation of the FGF23 with electrostatic potential generated by PyMOL shows a highly positively charged spot on the heparin-binding site (PDB entry 2p93). The bound heparin analogue is represented as a stick model.

TABLE 2 | Macromolecule production

Protein	Burosumab Fab
Source organism	Burosumab
DNA source	Gene synthesis (Bioneer, Inc)
Restriction enzyme	<i>NheI</i> and <i>Apal</i>
Cloning vector	pBAD
Expression vector	pBAD
Expression host	<i>E. coli</i> Top10F
Complete amino-acid sequence of the construct produced	<Heavy chain> QVQLVQSGAEVKKPGASVKVSKCASGYFTTNHYMHWVRQA PGQGLEWMGIINPISGSTSNAQKFQGRVTMTTRDTSTSTVY MELSSLRSEDTAVYYCARDIVDAFDWGGQTMVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTQYIC NVNHKPSNTKVDKKVEPKSCDKTHHHHHH <Light chain> AIQLTQSPSSLASVGDRTITCRASQGISSALVWYQQKP GKAPKLLIYDASSLESGLVPSRFGSGSGTDFTLTISSLQP EDFATYYCQQFNDFYTFGPGTKVDIKRTVAAPSVFIFPPS DEQLKSGTASVCLLNNFYPREAKVQWVDNALQSGNSQE SVTEQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC

X-ray diffraction data for a single crystal were collected at 100 K on beamline 5C of the Pohang Light Source (PLS-II) (Park et al., 2017) to a resolution of 1.45 Å, integrated and scaled using XDS (Kabsch, 2010). The structure was solved by molecular replacement using Phaser software with the structure of the pembrolizumab Fab (PDB code 5ggs, chains A and B) as a search model (McCoy et al., 2007). The variable and constant regions of the search model were separated during molecular replacement due to the elbow flexibility within the Fab moiety. Model building and structure refinement were carried out using PHENIX and COOT software (Emsley and Cowtan, 2004; Adams et al., 2010). Statistics for data collection and refinement are presented in Table 1. All structural figures were generated using PyMol (Schrödinger, Inc.). The atomic coordinates and structure factors for the crystal structure of the burosumab Fab have been deposited in Protein Data Bank (<http://www.rcsb.org>), under the entry 7VEN.

ACKNOWLEDGEMENTS

We are grateful to the staff of beamline 5C at Pohang Accelerator Laboratory for help with the X-ray diffraction experiments. This work was supported by a grant from the National Research Foundation of Korea (NRF-2020R1A2C2008865). This paper was written as part of Konkuk University's research support program for its faculty on sabbatical leave in 2020.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR INFORMATION

U.B.P., H.T.L., Y.K., T.J.J., and N.G. performed the experiments. Y.-S.H. wrote the paper. All authors discussed the results and commented on the manuscript.

Original Submission: Sep 10, 2021

Revised Version Received: Sep 19, 2021

Accepted: Sep 27, 2021

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