WHITE PAPER

De novo inactive GPCR cryo-EM structure and epitope mapping

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Introduction

G protein-coupled receptors (GPCRs), the largest superfamily of cell surface receptor proteins, have an exceptional track record as drug targets, with over 300 approved drugs targeting around 100 GPCRs. Despite this, over 70% of the therapeutically relevant GPCRs remain undrugged. GPCRs can be activated, inactivated, or finely modulated on a seemingly endless spectrum of approaches, meaning GPCR drug development is far from exhausted[1]. To realize the full therapeutic potential of GPCRs, significant challenges need to be overcome. A big obstacle to obtaining sufficiently potent and specific GPCR drugs is the intractability of GPCRs for structural studies by X-ray crystallography, traditionally a workhorse in structure-based drug design (SBDD).

Cryo-electron microscopy (cryo-EM) does not rely on the crystallization of proteins and has been transformative for the structural determination of active GPCRs. Since the first cryo-EM GPCR structure in 2017, there has been an explosion in the number of active GPCR structures being determined, with cryo-EM rapidly becoming a routine technique used for industrial drug discovery[2]. However, cryo-EM structures of inactive GPCRs are more scarce than active complexes, owing to their smaller size (50 kDa versus 150 kDa). To advance the application of cryo-EM for inactive GPCR complexes in SBDD, we determined a *de novo* structure of a class A GPCR target in complex with an antigen-binding fragment (Fab) that is under active pre-clinical development at a small biotech customer (see Figure 1).

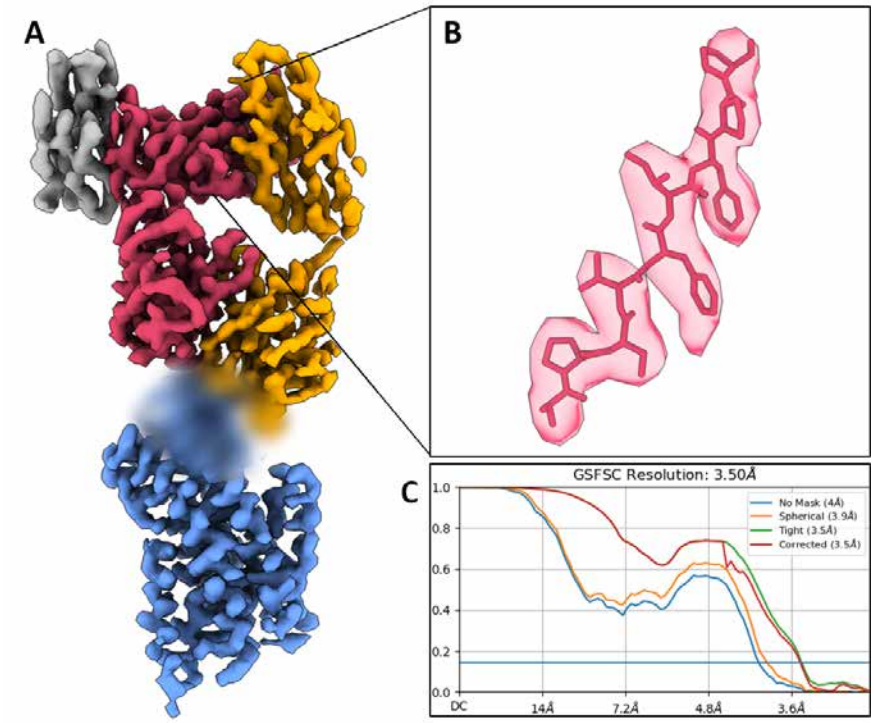

Figure 1. Cryo-EM reconstruction of the GPCR/Fab/VHH complex. (**A**) Cryo-EM density of the ternary complex. Blue: GPCR, Pink: Fab light chain, Yellow: Fab heavy chain, Grey: anti-Fab VHH. The epitope region is blurred to ensure confidentiality. (**B**) Representative density with atomic model from the Fab light chain constant region. (**C**) Gold-standard Fourier shell correlation curves. The 0.143 cutoff is indicated by a horizontal blue line.

Cryo-EM structure determination

We apply a fail-fast approach working with new proteins that comprises three stages: inspection, screening and data collection.

Inspection

Inspection ensures that the protein provided is high enough quality for structural characterization by cryo-EM. During this step, the quality of the vitrified sample is assessed on several parameters such as homogeneity, particle distribution, and density, and in the case of membrane proteins, vitrified ice formation in a given detergent buffer. Here, the inspection stage

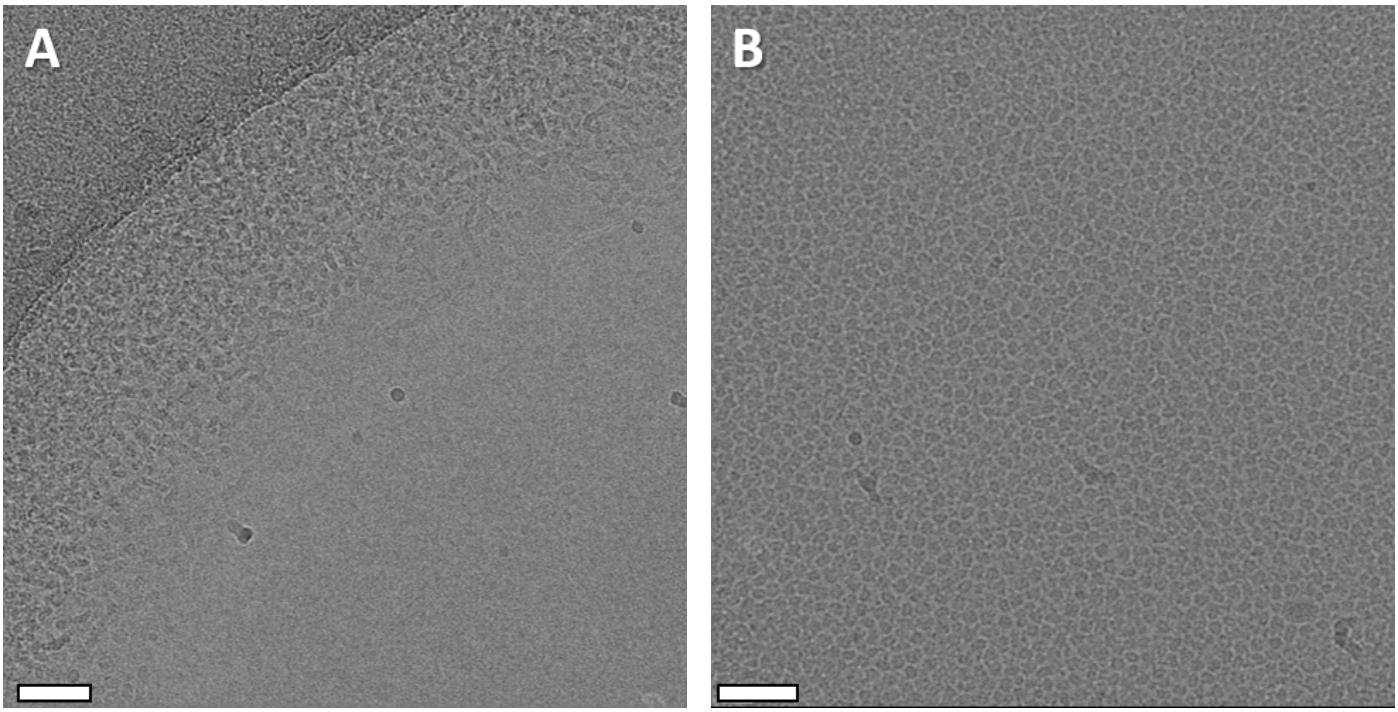

Figure 2. Sample improvement following the preliminary inspection. (**A**) Results from the first sample inspection showing particle clumping near the edge of the hole. (**B**) Results obtained after exchanging the detergent and concentrating the protein complex. *Scale bar = 50 nm.*

revealed that the ice in the middle of the holes was too thin and therefore the particles were clumping and overlapping near the carbon edge (Figure 2A). During optimization only two parameters were varied: detergent used during purification and protein concentration. Changing the detergent from n-Dodecyl-B-D-maltoside (DDM) to glyco-diosgenin (GDN) and concentrating the sample to ~15 mg/ml improved the vitrification and thin particle monolayers were achieved (Figure 2B).

Screening

Screening involves short data collections to determine whether further optimization is needed before acquiring a large dataset. Thermo Scientific™ EPU Software's Multigrid feature enables automated data collection for multiple grids while the cryoSPARC Live™ on-the-fly processing workflow by Structura is ideally suited to rapidly process cryo-EM datasets and to obtain a near real-time view of samples[3].

For this sample, screening datasets failed to produce an intermediate resolution structure of the GPCR/Fab complex with the sample exhibiting preferred orientation and the reconstruction algorithm struggling to align the transmembrane region of the receptor. We reasoned that rigidifying the Fab using a single-domain antibody (VHH) would sufficiently reduce the flexibility and increase the mass of the complex, leading to improved particle alignment and increased chances for successful structural determination.

Data collection

Data collection was performed on a ternary complex comprising the GPCR target, therapeutic Fab, and an anti-Fab VHH. As hypothesized, adding the VHH solved the problems observed during screening and we obtained a 3.5 Å reconstruction of this novel complex after a single data collection (Figure 1). The Thermo Scientific Krios™ G4 Cryo-TEM (Cryo-Transmission Electron Microscope) equipped with a Thermo Scientific Selectris™ X Imaging Filter and Thermo Scientific Falcon™ 4 Direct Electron Detector was used for the data collection while data processing was performed in the cryoSPARC™ single-particle analysis suite[4]. The complete protein-to-structure workflow took a total of five days. Data acquisition and analysis parameters can be found in Table 1.

Data acquisition and processing parameters	
Grid	UltraAuFoil® R1.2/1.3
Camera	Falcon 4 Detector with Selectris X Filter
Slit width (eV)	10
Nominal magnification	130,000x
Pixel size (Å)	0.96
Dose rate (e^-/pix/sec)	6.26
Exposure time (sec)	7.27
Total dose (e^-/Å^2)	53.8
Fractionation	EER
Autofocus	After centering
Hole centering	AFIS (6 µm image shift)
Delay after stage shift (s)	5
Number of images	4,336
Total number of particle picked	1,459,697
Particles in final reconstruction	192,647

Table 1. Parameters used for cryo-EM data acquisition and analysis.

Epitope mapping of a class A GPCR targeting Fab

An atomic model of the GPCR-Fab complex was built and refined using ISOLDE[5]. The analysis of epitope-paratope interface revealed that the interaction between the receptor and the Fab is primarily driven by hydrophobic interactions, six stabilizing hydrogen bonds, and a salt bridge. This analysis provides invaluable information for guiding mutagenesis studies to improve the selectivity and specificity of the anti-GPCR Fab.

Discussion

We were able to determine a *de novo* cryo-EM structure of an inactive class A GPCR in complex with a Fab derived from a pre-clinical antibody. The structure reveals the GPCR-Fab binding interface in molecular detail, which will further inform the design and guide the selection of antibodies that are under development for this target. For instance, cryo-EM can reveal which antibodies display the most potent mechanism of action. The maturation of an antibody can also be carried with greater precision by knowing which exact amino acid residues to focus on. In other words, better antibody therapeutics can be created in less time. Detailed structural information about the epitope may also be used for intellectual property purposes. It is therefore highly desirable to obtain the structures of antibody complexes in the early phases of a discovery program. The present example highlights how aggressive timelines to achieve cryo-EM structures can be maintained by applying a fail-fast approach. The increased speed provided by the latest Falcon 4 Detector and the cryoSPARC™ image processing software support fast decision-making needed to advance a project rapidly.

The result also showcases that cryo-EM is amenable to inactive GPCRs complexed with a Fab, which is paramount for the study of therapeutic antibodies. For small molecule GPCR antagonists, BRIL-fusion constructs in conjunction with anti-BRIL Fabs can also be used (Figure 3)[6]. In both cases, the use of a single-domain antibody stabilizing the Fab appears to be helpful. In addition, our data illustrate that cryo-EM can be used for *de novo* structural determination of inactive GPCRs without the need for stabilizing mutations of the receptor construct.

Conclusion

In summary, we describe a three-step fail-fast approach that we utilized to determine the novel structure of a class A GPCR in complex with a therapeutic antibody, enabling SBDD for this target. We show that the protein-to-structure pipeline can be executed in less than a week, allowing cryo-EM to go hand in hand with the drug development cycles. This work paves the way for routine structural determination of antibody-bound or small molecule antagonist-bound GPCR complexes within the pharmaceutical and biotechnology industry.

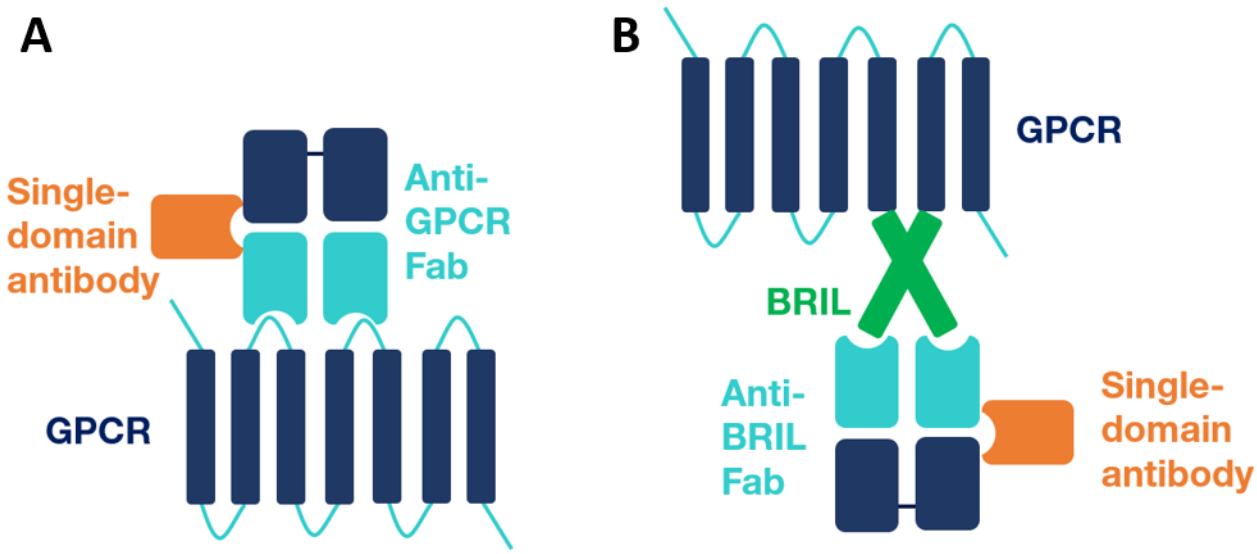

Figure 3. Schematic representation of strategies that could be used for stabilizing small molecule GPCR antagonists. (**A**) Complex stabilization using an anti-GPCR Fab and anti-Fab VHH. (**B**) BRIL-fusion constructs in conjunction with anti-BRIL Fab and anti-Fab VHH.

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