High-throughput structure determination of an intrinsically disordered protein

Intrinsically disordered proteins (IDPs) are a class of protein with multiple conformations that play an important role in signal transduction *in vivo*. The conformation of IDPs is significantly influenced by their external environment, and exhibits changes through interactions with binding partners. Identifying the determinants that stabilize the atomic-level structure of IDPs is crucial for understanding their biological functions. One promising method for determining the structures of IDPs is crystallizing IDPs fused with a binding partner. However, the versatility of this approach is limited by the challenges in designing robust scaffold crystals that maintain high diffraction quality.

To address these limitations, proteins that spontaneously crystallize in the cells of specific expression hosts were identified. In-cell protein crystallization ensures rapid, small-scale synthesis of crystals with high diffraction quality, which is particularly useful for crystallization screening to optimize crystal design. In this context, the crystallization of the target protein incorporated into in-cell protein crystals is proposed as a scaffold to immobilize the target protein in well-defined pores within the crystal. We have attempted X-ray structure analysis of target proteins using polyhedra crystals (PhCs), known for their high diffraction quality, produced in insect cells following infection with the cytoplasmic polyhedral virus [1]. Previously, we have successfully determined the structure of a ten-amino acid miniprotein, CLN025, fused to a polyhedrin monomer (PhM), resulting in the formation of PhCs in insect cells using SPring-8 **BL32XU** [2]. These findings highlight the potential of PhCs as versatile scaffolds for various proteins. Additionally, a cell-free expression system from wheat germ extract was used to express PhM. The structure of PhC was determined at a high resolution of 1.80 Å from sub-micron crystals obtained at a reaction scale of 100 μL within 24 h (Fig. 1(a)) [3]. This method contrasts with the conventional *in vitro* crystallization systems, which requires 1 L or more cell culture and several months for crystallization trials and optimization.

By utilizing the versatility of PhCs and the rapid structural analysis enabled by cell-free protein crystallization (CFPC), a pipeline for structure determination optimized for IDPs was established by integrating CFPC with the protein structure prediction software Foldit Standalone (Fig. 1(c)). Our goal was to elucidate the structural regulatory mechanism of the IDP region of c-Myc, a transcription factor known to bind to MAX (Fig. 1(b)) [4]. To clarify the structure-fixation factors of the 11-residue fragment within c-Myc (Y402–K412), which serves as a drug-binding site, we conducted X-ray crystal structure analysis of PhC mutants containing the domain swapped with this c-Myc fragment through several cycles of the CFPC screening method [5].

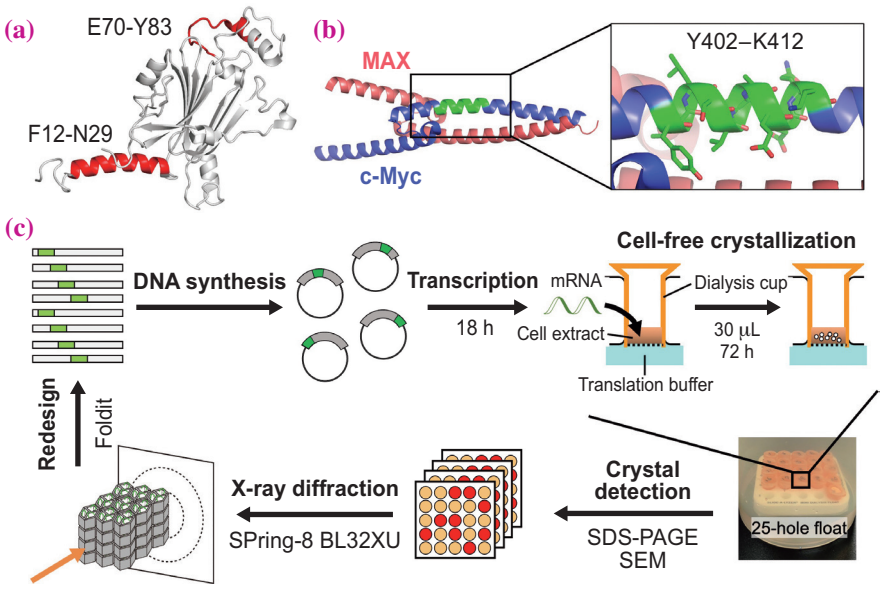

Fig. 1. **(a)** Crystal structure of PhM (PDB ID: 7XWS) and two sites in PhM for swapping with the c-Myc fragment [3]. **(b)** Crystal structure of the c-Myc/MAX complex (PDB ID: 1NKP) [4]. The black box shows the position of Y402–K412, colored green. **(c)** Scheme of high-throughput screening of X-ray crystallography for proteins.

First, six mutant PhCs, in which F12–N29 or E70–Y83 were partially swapped with the c-Myc fragment, predicted to be stably crystallized by Foldit Standalone (Fig. 1(a)). These mutants were rapidly synthesized on a small scale (30 μL) through the CFPC method. The obtained crystals were then subjected to high-resolution structural determination of the IDP region using diffraction data acquisition at SPring-8 BL32XU. In one of the six candidate mutants, the electron density corresponding to the entire length of the c-Myc fragment was successfully observed in the PhC (Fig. 2(a)). The c-Myc fragment in PhC forms a helical structure that is structurally similar to that of the c-Myc/MAX complex (Fig. 2(b)) and the structure predicted by Foldit. Two fragments of c-Myc, directed oppositely in the middle of the N-terminal helical region, interact via hydrophobic interactions involving I403 and V406 and hydrogen bonds at E410 (Fig. 2(c)). This structure was used in further redesigns to identify the key determinants fixing the c-Myc structure. Two additional crystal structures were obtained in which the c-Myc fragments were stepwise replaced with the original PhC sequence. B-factor analysis of these mutants indicated that the lack of the hydrophobic interaction at I403 destabilized the α-helical structure of the c-Myc fragment. In contrast, the lack of hydrogen bonds at E410 did not disrupt the structure.

Finally, this method allowed for the determination of eight crystal structures out of 22 mutants, revealing that interactions of residues I403 and V406 in c-Myc significantly stabilize the α-helical structure. This comprehensive approach enabled the successful determination of the IDP structure and provided insights into the specific residues involved in stabilizing the secondary structure of proteins. These findings underscore the power of our CFPC screening method as a valuable tool for elucidating essential molecular interactions that govern the stability of target proteins.

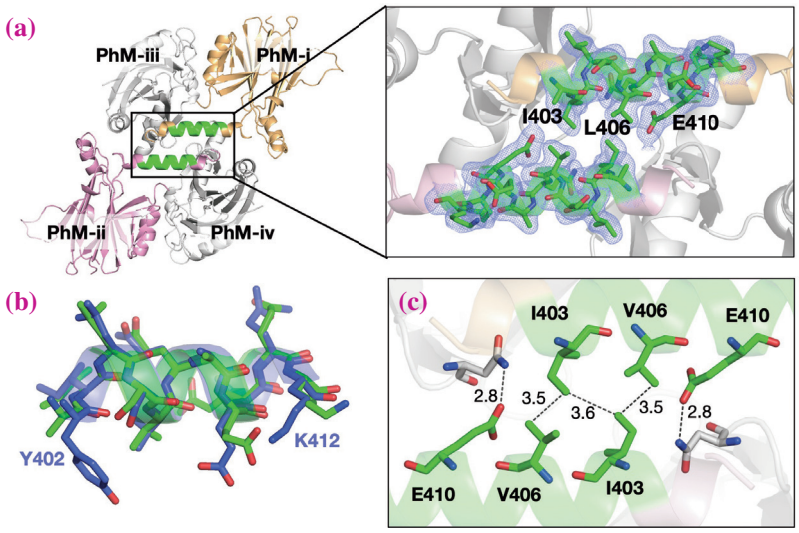

Fig. 2. Crystal structure of the c-Myc fragment fused to PhC. **(a)** Structure of the c-Myc fragment fused PhC tetramer and a close-up view of the c-Myc fragment in PhC. Each monomer in the tetramer is designated PhM-i, PhM-ii, PhM-iii, or PhM-iv. **(b)** Superposed structure of the c-Myc fragment in PhC (green) and the one in c-Myc/MAX (blue) (PDB ID: 1NKP). **(c)** Noncovalent interactions between the c-Myc fragment and surrounding residues in PhC. All fragments of c-Myc and c-Myc/MAX are colored green. The selected $2|F_o|-|F_c|$ electron density maps at 1.0σ are shown in blue. Nitrogen and oxygen atoms are colored blue and red, respectively. The cut-off distances of noncovalent interactions are 3.5 Å and 5.75 Å for hydrogen bonds and hydrophobic interactions, respectively. Hydrophobic interactions of the shortest distance between two residues are shown (c).

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