Structure of PI5P4Kβ-Nucleotide Complexes Uncover GTP-Sensing Function in Mammalian Cells

Structural biology is not only to provide a structural explanation for known cellular functions but also to unveil unknown cellular functions and identifying the underlying molecular mechanism. Especially, the combination of atomic resolution structures of proteins with reverse genetic approaches, which we named the *structural reverse genetics* approach, can directly connect a biochemical activity of proteins to biological function(s). Utilizing this approach, we have revealed the presence and significance of the GTP-sensing function in mammalian cells. Structure determination of the PI5P4Kβ-nucleotide complexes and the structure-based development of a GTP-insensitive mutant was the key to demonstrating that PI5P4Kβ is a GTP sensor that is critical for tumorigenesis.

In order to maintain and control concentrations of energy molecules, such as ATP and GTP, in response to cellular status, cells have energy sensor proteins that monitor the concentration of specific energy molecule(s) and evoke concentration-dependent signaling for cellular responses [1, 2]. Such energy sensors have been found for ATP and other metabolites, but not for GTP in spite of its importance in protein synthesis and cell signaling. Accumulated lines of evidence have indicated the presence of a GTP bioenergetic system that actively responds to GTP depletion in mammalian cells [3], however, the GTP sensor that receives the GTP concentration as a biological *cue* and converts it into cellular signaling has not been identified.

Recently, we discovered that the lipid kinase PI5P4Kβ is the unknown energy sensor for cellular GTP concentration [4]. Our cell biological and biochemical analyses have revealed that PI5P4Kβ is a unique GTP-utilizing kinase with K_M value (88 μM) for GTP that is suitable to be regulated by physiological changes of the cellular GTP concentration (100–500 μM). These biochemical features, however, only imply that PI5P4Kβ is a candidate GTP sensor and do not necessarily mean that the GTP-dependent activity of PI5P4Kβ has *in vivo* significance. Since PI5P4Kβ has both ATP- and GTP-dependent kinase activities (**Fig. 1A**), conventional reverse genetic approaches, such as knockout or knockdown, cannot be exploited to solve this issue (**Fig. 1B**). To overcome this problem and reveal the *in vivo* significance of the GTP-dependent activity of PI5P4Kβ as a GTP sensor, we decided to solve the structures of the PI5P4Kβ-nucleotide complexes in order to rationally develop a GTP-insensitive mutant that lacks the GTP-dependent kinase activity, while having the intact ATP-dependent activity (**Fig. 1C**).

Although the structure of *apo* PI5P4Kβ has already been solved at 3.0 Å resolution [5], we need higher resolution structures of nucleotide complexes to distinguish the GTP and ATP binding modes. The previously reported crystallization method was insufficient to obtain high resolution diffraction data from PI5P4Kβ-nucleotide complexes, thus we applied our newly developed multi-step soaking method to improve the resolution [6]. The sequential soaking in less invasive polyvinylpyrrolidone

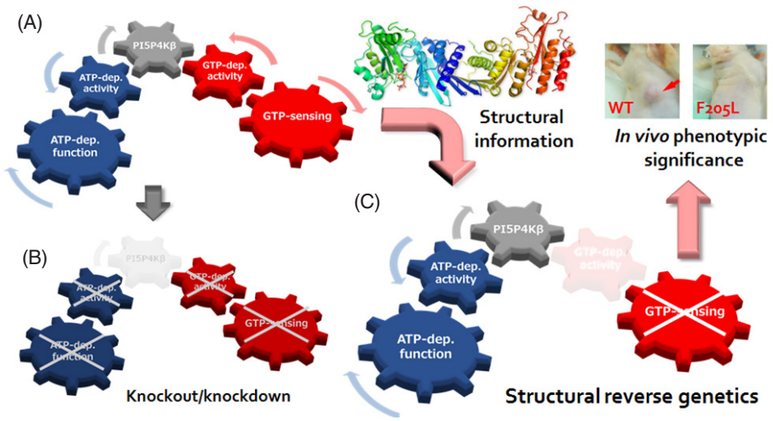

Figure 1: Connecting the GTP-dependent activity of PI5P4Kβ to the GTP-sensing function by the *structural reverse genetics* approach. **(A)** Schematic representation showing that two biochemical activities of PI5P4Kβ drive distinct associated biological functions. **(B)** The conventional reverse genetics strategy affects the whole biochemical activities of PI5P4Kβ and thus all functions associated with PI5P4Kβ are lost. **(C)** With structural information, the *structural reverse genetics* approach removes only the GTP-dependent activity of PI5P4Kβ and thus the *in vivo* phenotypic significance of the associated GTP-sensing function is revealed. Top middle: the whole structure of PI5P4Kβ in complex with GTP analog, GMPPNP (PDB ID: 3X04). Top right: the GTP-insensitive mutation (F205L) disrupted the tumorigenic phenotype associated with WT PI5P4Kβ.

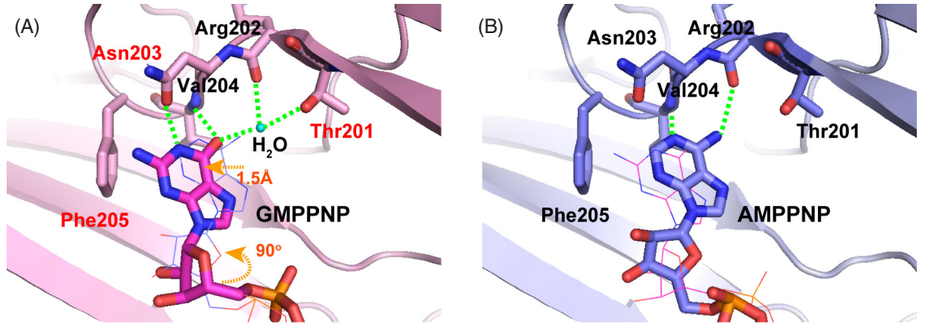

Figure 2: Difference in **(A)** GTP and **(B)** ATP recognition by PI5P4Kβ. In each panel, the positions of counterpart nucleotides are shown by line representation for comparison. Both nucleotide analogs form hydrogen bonds with PI5P4Kβ (green dotted lines) but in distinct connecting patterns. In addition, Phe-205 is in close proximity only for the GTP analog GMPPNP. The residues that are specialized for GTP recognition are highlighted in red in (A).

K15 and then ethylene glycol for better diffraction significantly improved the average resolution of the PI5P4Kβ crystals. The resolutions of the final structures reached 2.60 Å, 2.15 Å, 2.45 Å, 2.70 Å, and 2.60 Å for the apo, AMP, GMP, AMPPNP, GMPPNP complexes, respectively, from the diffraction data obtained at AR-NE3A . These structures demonstrated clear differences in the GTP and ATP binding modes of PI5P4Kβ.

The crystal structures suggested that Thr-201, Asn-203, and Phe-205 are specialized for GTP recognition in PI5P4Kβ (**Fig. 2**), which enabled us to introduce rational mutations to these residues. After confirming that the individual structures of the PI5P4Kβ mutants were almost identical to that of the wild-type (WT) PI5P4Kβ, NMR as well as biochemical experiments were carried out to show the effect of mutation on the GTP- and ATP-dependent activities. The analyses revealed that the PI5P4Kβ^{F205L} mutant showed a decrease in the GTP-dependent kinase activity, without having any perturbation of the ATP-dependent activity, thus we used the mutant to further reveal the *in vivo* significance of the GTP-dependent activity of PI5P4Kβ.

Isogenic cells lines that express the PI5P4Kβ^{F205L} mutant and the wild-type (WT) PI5P4Kβ were established by introducing the mutant and WT PI5P4Kβ to the PI5P4Kβ knockout cell line and the GTP-sensing functions of these two cell lines were compared. The comparison demonstrated that PI5P4Kβ's lack of GTP-sensing activity disrupted the GTP-dependent change of the lipid PI(5)P levels. In addition, two thirds of the metabolites could not faithfully change their level in response to a GTP depletion. These results clearly indicate that PI5P4Kβ is a GTP sensor and provide, for the first time, clear evidence of the presence of the GTP-sensing function in mammalian cells. Furthermore, the cells expressing the PI5P4Kβ^{F205L} mutant abolished the tumorigenic activity observed for the mouse embryonic fibroblast cells expressing WT PI5P4Kβ in mouse xenograft models. It has been shown that the cellular GTP concentration is relatively high in certain cancer cells [7]. The data imply that some cancer cells are taking advantage of the GTP-sensing function for their tumorigenic activity. This knowledge may lead to a new approach to cancer therapy.

As we demonstrated here, the combination of atomic resolution structures of proteins with reverse genetic approaches, which we termed the *structural reverse genetics* approach, can directly connect a biochemical activity of proteins to biological function(s) [8]. With the recent advances in structural biological methods and genome editing technologies, the *structural reverse genetics* approach has come of age.

REFERENCES

[1] D. G. Hardie, F. A. Ross and S. A. Hawley, *Nat. Rev. Mol. Cell Biol.* **13**, 251 (2012).
[2] P. B. Dennis, A. Jaeschke, M. Saitoh, B. Fowler, S. C. Kozma and G. Thomas, *Science* **294**, 1102 (2001).
[3] F. M. Rosenbloom, J. F. Henderson, I. C. Caldwell, W. N. Kelley and J. E. Seegmiller, *J. Biol. Chem.* **243**, 1166 (1968).
[4] K. Sumita, Y. H. Lo, K. Takeuchi, M. Senda, S. Kofuji, Y. Ikeda, J. Terakawa, M. Sasaki, H. Yoshino, N. Majd, Y. Zheng, E. R. Kahoud, T. Yokota, B. M. Emerling, J.M. Asara, T. Ishida, J. W. Locasale, T. Daikoku, D. Anastasiou, T. Senda and A. T. Sasaki, *Mol. Cell* **61**, 187 (2016).
[5] V. D. Rao, S. Misra, I. V. Boronenkov, R. A. Anderson and J. H. Hurley, *Cell* **94**, 829 (1998).
[6] M. Senda, T. Hayashi, M. Hatakeyama, K. Takeuchi, *A. T. Sasaki* and T. Senda, *Cryst. Growth Des.* **16**, 1565 (2016).
[7] T. W. Traut, *Mol. Cell. Biochem.* **140**, 1 (1994).
[8] K. Takeuchi, M. Senda, Y. H. Lo, S. Kofuji, Y. Ikeda, A. T. Sasaki and T. Senda, *FEBS J.* (2016). doi:10.1111/febs.13739

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K. Takeuchi[1,2], M. Senda[3], Y.-H. Lo[3], S. Kofuji[4], Y. Ikeda[4], A. T. Sasaki[4] and T. Senda[3,5] ([1]AIST [2]JST, PRESTO, [3]KEK-IMSS-PF/SBRC, [4]Univ. of Cincinnati College of Medicine, [5]The Graduate Univ. of Advanced Studies (SOKENDAI))