Determination of topological structure of ARL6ip1 in cells: Identification of the essential binding region of ARL6ip1 for conophylline

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Masahiro Kuroda [a], Shintaro Funasaki [a], Tsuyoshi Saitoh [b], Yukiko Sasazawa [a], Shigeru Nishiyama [b], Kazuo Umezawa [a,c,*], Siro Simizu [a,*]

[a] *Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan*
[b] *Department of Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan*
[c] *Department of Molecular Target Medicine Screening, Aichi Medical University School of Medicine, Nagakute, Japan*

ARTICLE INFO

ABSTRACT

Conophylline (CNP) has various biological activities, such as insulin production. A recent study identified ADP-ribosylation factor-like 6-interacting protein 1 (ARL6ip1) as a direct target protein of CNP. In this study, we revealed that ARL6ip1 is a three-spanning transmembrane protein and determined the CNP-binding domain of ARL6ip1 by deletion mutation analysis of ARL6ip1 with biotinyl-amino-CNP. These results suggest that CNP is expected to be useful for future investigation of ARL6ip1 function in cells. Because of the anti-apoptotic function of ARL6ip1, CNP may be an effective therapeutic drug and/or a novel chemosensitizer for human cancers and other diseases.

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1. Introduction

Conophylline (CNP; Fig. 1A), a *Vinca* alkaloid, was isolated from leaves of *Tavertaemontana divaricate* [1]. CNP was later isolated again from *Ervatamia microphylla* as a Ras function inhibitor that induced normal morphology in K-*ras*-transformed NRK cells [2]. CNP showed anti-tumor activities, such as inhibition of cellular chemotactic invasion, inducing normal morphology and growth inhibition in K-*ras*-transformed cells and cancer cells [3]. Moreover, CNP was found to induce pancreatic β-cell differentiation from pancreatic exocrine carcinoma cells [4], and it lowered the blood level of glucose in type-2 diabetes model mice [5]. Therefore, CNP is expected to be applied to β-cell regeneration chemotherapy. In the course of in vivo study, CNP was also found to exert anti-fibrotic actions in pancreatic islets in type-2 diabetes Goto-Kakizaki rats [6].

A recent study identified ADP-ribosylation factor-like protein 6-interacting protein 1 (ARL6ip) as a direct target protein of CNP by

using CNP-linked latex nano-beads [7]. ARL6ip1 was first identified as an interacting molecule of ARL6, a member of the ARL subfamily of small GTPases, by use of the yeast two-hybrid system [8]. ARL6ip1 is an integral transmembrane protein with four predicted transmembrane regions. It consists of 203 amino acids and localizes to the endoplasmic reticulum (ER) membrane [9]. The C-terminal sequence of ARL6ip1, KKNE, corresponds to the KKXX sequence commonly found in the C-terminal region of ER membrane proteins, which might function as an ER retention motif [9,10].

It was reported that overexpression of ARL6ip1 in HT1080 cells exhibits anti-apoptotic activity from multiple apoptotic inducers, caused by inhibition of caspase-9 activity [9]. It was also demonstrated that ARL6ip1 interacts with ARL6ip5 and promotes EAAC1-mediated glutamate transport activity [11]. Since ARL6, with which ARL6ip1 interacts, is likely to be engaged in intracellular trafficking, ARL6ip1 could also regulate intracellular trafficking pathways in the ER membrane, but its cellular functions are not fully defined.

In this study, we determined the topological structure of ARL6ip1 in cells by redox-sensitive luciferase assay, a rapid and recently reported conventional topological assay, using *Gaussia* luciferase (Gluc) [12]. In addition, we identified the important region of ARL6ip1 for interacting with CNP. Taken together, it is suggested that CNP is expected to aid in the further clarification of the

(A)

conophylline
(CNP)

biotinyl-aminoconophylline
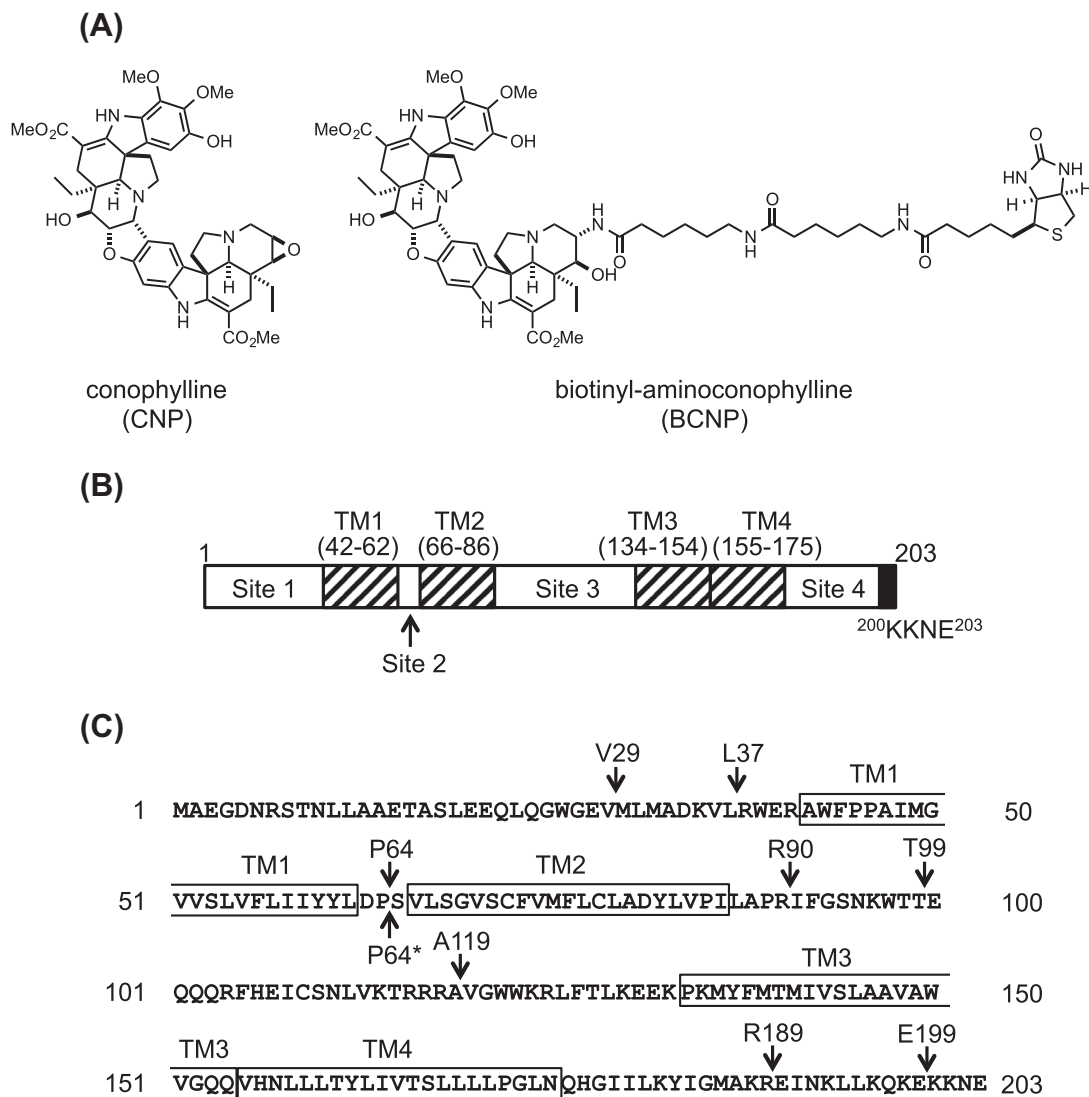(BCNP)

(B)

(C)

Fig. 1. Structure of CNP and BCNP and primary structure of ARL6ip1. (A) Structure of CNP and BCNP. (B) Putative transmembrane regions (hatched bars; TM 1–4) and non-transmembrane regions (open boxes; Site 1–4) of ARL6ip1. A black area indicates the KKNE sequence. (C) Amino acid sequence of human ARL6ip1. Enclosed areas indicate putative transmembrane regions. The residues pointed by arrows indicate the C-terminally Gluc-attached position. For examples, V29 and P64 mean that Gluc was inserted between Val29 and Met30 and Pro64 and Ser65 of human ARL6ip1, respectively. P64* means (M1-P64)-fused Gluc.

role of the cytoplasmic region in ARL6ip1 function. Since ARL6ip1 negatively regulates apoptosis induced by various stimuli [9], our results might provide a rationale to combine CNP and antitumor drugs in cancer therapy.

2. Materials and methods

2.1. Materials

CNP was isolated from the leaves of *E. microphylla* as reported previously [2]. Biotinyl-amino-CNP (BCNP; Fig. 1A) was synthesized from isolated CNP according to our previous report [7]. Synthesized BCNP was confirmed by NMR and ESI-MS.

2.2. Construction of plasmid vectors, cell culture and transfection, redox-sensitive luciferase assay and β-galactosidase assay, in vitro binding assay, and Western blotting

The protocols used for the construction of plasmid vectors, cell culture, transfection, redox-sensitive luciferase assay and β-galac-

tosidase assay, in vitro binding assay, and Western blotting are indicated in Supplementary materials and methods [12–18].

3. Results

3.1. Determination of ARL6ip1 topology using redox-sensitive assay

The predicted transmembrane regions (TMs) of ARL6ip1 obtained in the UniProt database (http://www.uniprot.org/) were shown in Fig. 1B. Although it has been suggested that ARL6ip1 has four predicted transmembrane regions (TM 1–4) and four non-transmembrane regions (Site 1–4), its topology is still unknown. To determine the topology of ARL6ip1, we performed a redox-sensitive luciferase assay [12]. Gluc functions as a redox reporter that requires an oxidative environment for its activity but does not work under reducing conditions.

To evaluate Gluc activities for the identification of ARL6ip1 topology, we constructed a series of Gluc-fused ARL6ip1 proteins (Fig. 1C). HT1080 cells were transfected with these constructs along with a control vector encoding β-galactosidase and lysed,

and Gluc-catalyzed bioluminescence and β-galactosidase activity were measured. The ratio of bioluminescence to β-galactosidase activity of R189 and E199 was much higher than the others, suggesting that the V29 (Site 1), L37 (Site 1), R90 (Site 3), T99 (Site 3), and A119 (Site 3) fusions are localized in the cytoplasm, while the R189 (Site 4) and E199 (Site 4) fusions are in the ER lumen (Fig. 2A). To clarify whether Site 2 of ARL6ip1 is localized in the cytoplasm or ER, we attached Gluc to the C-terminus of P64 of ARL6ip1 (P64) and the C-terminus of a.a. 1–64 of ARL6ip1 (P64*). The results suggests that Site 2 is localized in the cytoplasm and ER lumen data from P64 and P64*, respectively, probably because Site 2 consists of only three amino acids and P64 fails to attain proper conformation caused by steric hindrance of Gluc. These results indicate that Site 1 and Site 3 are localized in the cytoplasm, and Site 2 and Site 4 are localized in the ER lumen. It also suggests that ARL6ip1 is a three-spanning transmembrane protein (Fig. 2C).

3.2. Identification of important region of ARL6ip1 for binding to CNP

To determine which site in ARL6ip1 is essential for interaction with CNP, we constructed GFP-fused ARL6ip1 mutants, as shown in Fig. 3A. HEK 293T cells that were transfected with these constructs were lysed and incubated with NeutrAvidin bead-bound BCNP. GFP-ARL6ip1 (Δ1–41), as well as GFP-ARL6ip1 (WT), was co-precipitated with NeutrAvidin bead-bound BCNP; however, GFP-ARL6ip1 (Δ1–86) and (Δ1–133) were not (Fig. 3B), suggesting that the a.a. 42–86 region of ARL6ip1 is essential for the interaction with CNP.

For further analysis, we constructed a GFP-tagged ARL6ip1 deletion mutant lacking a.a. 42–86 (Δ42–86) and a GFP-tagged truncated a.a. 42–133 ARL6ip1 fragment with a KKNE sequence at its C-terminus (42–133 + KKNE) (Fig. 3A). Fig. 3C shows that GFP-ARL6ip1 (Δ42–86) exhibits very weak binding ability to BCNP as compared to WT. Meanwhile, GFP-ARL6ip1 (42–133 + KKNE) binds to BCNP, but its binding ability is weaker than that of WT. These results indicate that TM 1 and TM 2 are essential for the interaction of ARL6ip1 with CNP, but they are not sufficient for CNP binding. From the results of the topological analysis of ARL6ip1 and the in vitro binding assay, we assume that CNP binds to Site 3, a.a. 87–133, of ARL6ip1, because it is generally considered that compounds bind to non-transmembrane regions of target proteins. Since the binding capacity of GFP-ARL6ip1 (42-133 + KKNE) with
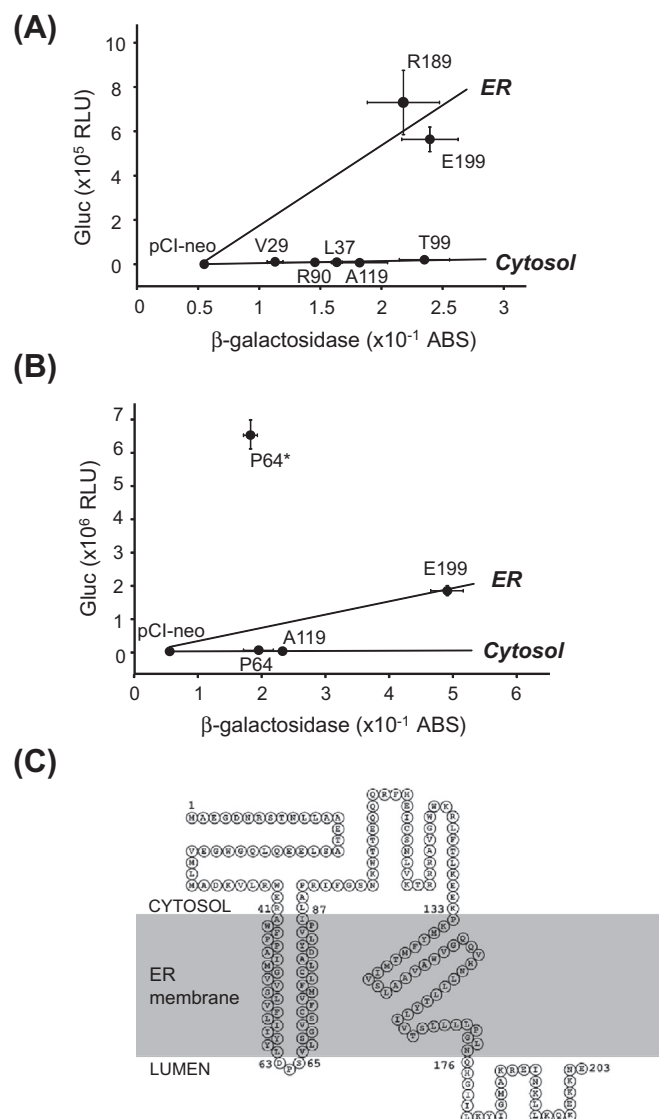

Fig. 2. Determination of topology of human ARL6ip1. (A and B) The bioluminescence/absorbance plot of Gluc-fused ARL6ip1. In the plot, the proteins localized in the ER or cytosol are displaced separately in two groups, as indicated with two straight lines. Data are represented as mean ± S.D. (*n* = 3). (C) Putative topology of human ARL6ip1. The gray area indicates ER membrane.

(A)

GFP

GFP-ARL6ip1(WT)

GFP-ARL6ip1(Δ1-41)

GFP-ARL6ip1(Δ1-86)

GFP-ARL6ip1(Δ1-133)

GFP-ARL6ip1(Δ42-86)

GFP-ARL6ip1(42-133+KKNE)

(B)

Input
IB: Anti-GFP Ab

Pull-down: Neutravidin-beads
IB: Anti-GFP Ab

(C)

Input
IB: Anti-GFP Ab

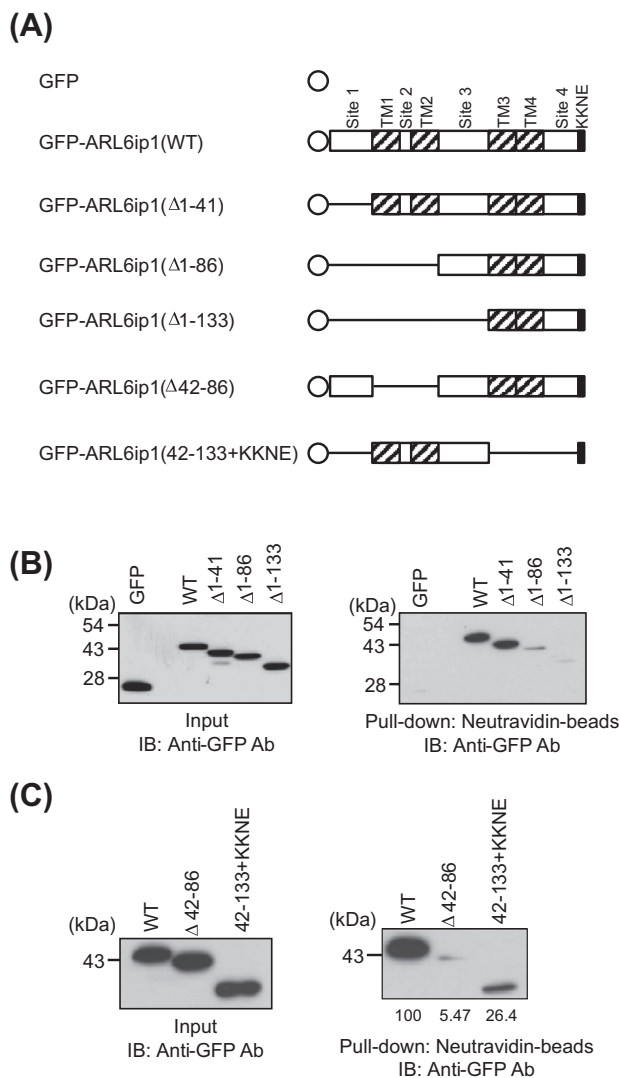Pull-down: Neutravidin-beads
IB: Anti-GFP Ab

Fig. 3. Determination of important region of ARL6ip1 for binding to CNP. (A) Schematic illustrations of GFP-fused ARL6ip1 deletion mutants. (B and C) Cells were transfected with the vectors encoding GFP-ARL6ip1 or deletion form of ARL6ip1 for 30 h. The total cell lysates were extracted, precleared with equilibrated NeutrAvidin beads for 2 h, and mixed with NeutrAvidin bead-bound BCNP for 2 h at 4 °C. A portion of equilibrated lysates was collected as "Input". After washing, the proteins that interacted with NeutrAvidin bead-bound BCNP were eluted with the sample buffer. BCNP-interacted ARL6ip1 was detected by Western blotting using anti-GFP antibody.

CNP is approximately five times higher than that of GFP-ARL6ip1 (Δ42–86), as shown in Fig. 3C, transmembrane regions would be important for forming Site 3 with a stable loop structure that allows it to interact with CNP.

4. Discussion

In this study, we have characterized the topological structure of ARL6ip1 and the CNP-binding region of ARL6ip1. Although ARL6ip1 had been proposed to be a four-spanning transmembrane protein, we showed that ARL6ip1 is a three-spanning transmembrane protein with the N-terminus and C-terminus facing the cytosol and ER lumen, respectively (Fig. 1 and 2). In addition, to identify the region that is critical for the interaction of ARL6ip1 with CNP, we synthesized BCNP and performed an in vitro binding assay. Deletion mutation analysis of GFP-ARL6ip1 revealed that the a.a. 42–86 region of ARL6ip1 is essential for the interaction of CNP with ARL6ip1 (Fig. 3). Further analyses revealed that the only the a.a. 42–86 region is not sufficient for CNP binding (Fig. 3). From the topological analysis of ARL6ip1, we assume that the direct CNP binding site is Site 3 (87–113 a.a.), while the TM regions, such as TM 1 and TM 2,

would be important for forming Site 3 with a stable loop structure that allows it to interact with CNP.

Although the a.a. 42–86 region (TM 1, Site 2, and TM 2) of ARL6ip1 is important for the interaction of ARL6ip1 with CNP, it contains only a 3-a.a. non-TM region: Asp63-Pro64-Ser65. To investigate whether Site 2 is required for the interaction with CNP, we constructed three Site 2 mutant expression vectors (DPA, NPA, and SSS). All mutants were bound to NeutrAvidin bead-bound BCNP, as well as wild-type (DPS) (data not shown). These results suggest that the sequence of Site 2 is not important for the interaction with CNP, while TM 1 and TM 2 would be required for the interaction.

ARL6ip1 consists of the KKNE sequence at its C-terminus, which conforms to the KKXX motif commonly found in ER membrane proteins, mediating their retention in the ER. To investigate whether the C-terminal KKNE sequence also functions as an ER retention signal, we constructed Gluc mutants that were localized to various organelles (Fig. S1). Gluc (−SP), in which the signal peptide of Gluc was deleted, was localized in the cytosol, while full-length Gluc was localized in the ER lumen and extracellular space, and Gluc+KDEL, known as the ER retention a.a. sequence, was

localized in the ER. We also constructed Gluc mutants, including Gluc+KKNE and Gluc (−SP)+KKNE (Fig. S1). The assay shows that Gluc+KKNE is localized in the ER lumen and extracellular space, and Gluc (−SP)+KKNE is localized in the cytosol, suggesting that the C-terminal KKNE sequence does not function for retention in the ER lumen. It has been reported that the cytoplasmic C-terminus di-lysine motifs are important for Golgi-to-ER retrieval and capture by coatomers and packaged into vesicles in their ER–Golgi trafficking [19,20]; however, the KKNE motif in ARL6ip1 may not function as an ER retrieval motif.

In summary, this is the first report of the topological structure of human ARL6ip1. Our results suggest that, contrary to the putative structure with four-transmembrane-spanning domains, ARL6ip1 is three-spanning transmembrane protein. Moreover, we demonstrated that Site 3 of ARL6ip1 is an essential region for interaction with CNP; besides, TM regions would be also important for the stabilization of Site 3. The results provide us to develop a novel screening system for the identification of small-molecule inhibitors and/or peptides that interact with the specific region of ARL6ip1. The role of each site and TM for ARL6ip1 function might be revealed by the obtained inhibitors. Moreover, because CNP showed anti-tumor, anti-diabetes, and anti-fibrotic actions, our findings might provide new clues for understanding the role of ARL6ip1 for these diseases. Furthermore, CNP augmented apoptosis in human cancer cell lines that was induced by treatment with drugs, such as brefeldin A (data not shown); thus, CNP may be a novel chemosensitizer for human cancers.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.09.017.

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