FEBS Letters 587 (2013) 3656–3660

FEBS Letters

journal homepage: www.FEBSLetters.org

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

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ARTICLE INFO

Article history:
Received 3 September 2013
Accepted 16 September 2013
Available online 25 September 2013

Edited by Veli-Pekka Lehto

Keywords:
ARL6ip1
Conophylline
Topology
Redox-sensitive luciferase assay

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.

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 (ARL6ip1) 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

Abbreviations: ARL6ip1, ADP-ribosylation factor-like 6-interacting protein 1; CNP, conophylline; BCNP, biotinyl-aminoconophylline; ER, endoplasmic reticulum; Gluc, *Gaussia* luciferase; TM, transmembrane

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.09.017

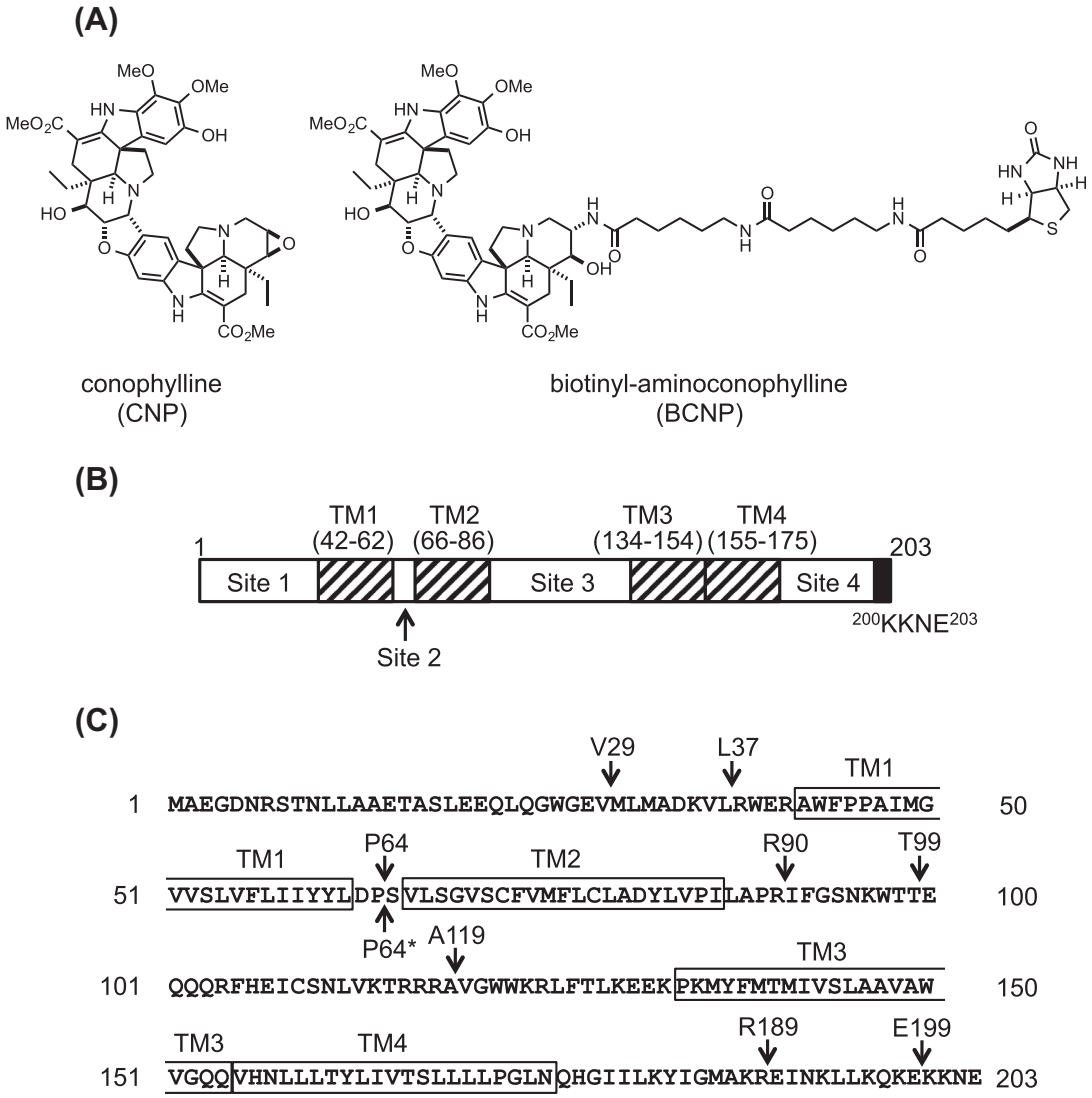

Fig. 1. Structure of CNP and BCNP and primary structure of ARL6ip1. (A) Structure of CNP and BCNP. (B) Putative transmembrane regions (hatched bars; TM 1–4) and non-transmembrane regions (open boxes; Site 1–4) of ARL6ip1. A black area indicates the KKNE sequence. (C) Amino acid sequence of human ARL6ip1. 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-galactosidase 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,