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Short communication

Biochemical characterization of a family of proteins that stabilizes a plant Ran protein in its GTP-bound conformation

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Abstract

Ran-binding proteins (RanBP) are a group of proteins that bind to Ran (Ras-related nuclear small G-protein) and thus control the GTP/GDP-bound states of the Ran and couple the Ran GTPase cycle to cellular processes. In an effort to identify potential downstream effectors for PsRan1-dependent cellular processes, we detected a group of pea Ran (PsRan1)-binding proteins and characterized their biochemical activities. A Ran overlay assay using [^{32}P-GTP]-labeled PsRan1 revealed three PsRan1-binding proteins (33, 45, and 85 kDa in size) from total protein extracts of dark-grown pea plumules. These proteins bound preferentially to the Ran-GTP over Ran-GDP conformation and subsequently stabilized its GTP-bound status. We propose that they are a family of proteins that maintain the Ran protein in the active conformation and are potential downstream mediators for PsRan1-dependent cellular processes. Our report provides the basis for characterizing and dissecting Ran downstream targets and Ran-mediated events, and it thus facilitates our understanding about the roles played by Ran/RanBP signaling pathways during plant growth and development.
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1. Introduction

Ran/TC4 (Ras-related nuclear small GTP-binding protein) is a small GTP-binding protein that binds and subsequently hydrolyzes GTP, so that it acts as a molecular signaling transducer whose active/inactive state depends on the binding of GTP or GDP, respectively. Ran has multiple roles in cellular processes, including nuclear import and export of proteins and RNA, assembly of mitotic spindle and nuclear envelope, and regulation of cell cycle progression [6,11,27]. These complex and variable functions of Ran are achieved in part by its interaction with downstream target proteins, called Ran-binding (interacting) proteins (RanBPs), which bind to either the GTP or GDP-bound form of Ran and thus either control the GTP/GDP-bound states of Ran or help couple the Ran GTPase cycle to a variety of cellular processes.

Several Ran-binding proteins have been identified, and their roles in mediating Ran functions in yeast and mammalian systems are under intensive investigation. These proteins include a family of nucleoporins, karyopherins, Ran GTPase activating factor (RanGAP), RanBP1, RanBP2, RanBP3, Ran guanine nucleotide exchange factor RCC1, NTF2, Mog1p, Dis3 and RanBPM [8,25]. Protein import and export through the nuclear pore complex is mediated by the modulation of Ran status in concert with the actions of other importin/karyopherin and exportin superfamily [8]. Unequally distributed RanBPs across the nuclear membrane (RanGAP and RanBP1 on the cytoplasmic side and RCC1 on the nucleoplasmic side) keep the cytoplasmic Ran mainly in a GDP-bound form and nuclear Ran in a GTP-bound form [14]. This asymmetric distribution of Ran-GTP and -GDP is a major driving force giving directionality to nuclear import/export and circulating importin/

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exportin cargos. Critical roles of Ran-GTP and Ran-GDP were also described in the regulation of mitotic spindle formations in actively dividing somatic cells [2,15,29]. From these studies, it become clear that the Ran-GDP/-GTP switches that are controlled by RanBPs, play a very important role both in regulating the proper function of Ran in nuclear transport in the interphase cells and in directing nuclear assembly and cell cycle progression in dividing cells.

It appears that the functions of Ran signaling in nuclear transport and mitotic progression are well conserved in plants and animals. Tomato Ran protein was functionally homologous with a yeast Ran-like protein in suppressing the effects of a mutation in a yeast homolog of RCC1 (a RanGTP-binding protein) [1]. Nuclear import of Ran relies on a small RanGDP-binding protein, Nuclear Transport Factor 2 (NTF2), and recently identified *Arabidopsis* NTF2 homologues functionally replace the essential *NTF2* gene in yeasts [33]. Rice importin β1 in the presence of importin α1 and Ran-GDP mediates the docking of NLS proteins to the nuclear envelope and their subsequent translocation into the nucleus [13]. The *Arabidopsis* ortholog of exportin 5, HASTY, which controls phase changes and plant morphology, is located at the periphery of the nucleus and interacts with AtRAN1, and presumably regulates nucleocytoplasmic transport of molecules involved in several different morphogenetic pathways [3]. Expression of the *Medicago* RanGAP1 in the *rna1* yeast mutant, rescues its RanGAP deficient phenotypes, such as defects in RNA processing and nucleocytoplasmic mRNA transport [24]. These observations together with the localization patterns of plant RanGAPs in the interphase and mitotic cells led the authors to postulate that functions of these proteins in the regulation of nuclear transport, re- establishment of the nuclear envelope, and in the organization of microtubules are well conserved. Contrary to these similarities, some fundamental differences of plant Ran system from the vertebrate mechanism also exist [21]. For example, *Arabidopsis* RanGAP1 lacks SUMOylated C-terminal domain of vertebrate RanGAP, but instead contains a plant-specific N-terminal WPP domain directing plant-specific mitotic targeting of the protein to the cell plate [12].

Significant progress in animals and yeasts has been made in clarifying the Ran/RanBP signaling pathways, but relatively little is known about them in plants, partly because not many families of plant Ran-binding proteins have been identified or characterized. We previously showed that an *Arabidopsis* PsRan1-binding protein, AtRanBP1c, is a cytoplasmic protein that acts as a coactivator of Ran-GTPase activating protein (RanGAP) [17], and it is critically involved in the regulation of auxin-induced root growth and development [18]. These observations were further supported by a recent report showing that overexpression of a wheat Ran in *Arabidopsis* alters primordial meristem, mitotic progress, and sensitivity to auxin [31]. Nonetheless, to further dissect the Ran/RanBP pathways and systematically understand the roles of Ran signaling in plants, it is necessary to identify more Ran downstream components with which it interacts. Ran targets that are preferentially bound to Ran-GTP, would provide useful information since the GTP-bound status of Ran is known to maintain the signaling switches "on" in many cellular processes. In this study we performed a Ran overlay assay, revealed the existence of a family of three additional Ran-binding proteins, characterized their binding specificity to Ran-GTP and the likely role they play in maintaining Ran-GTP conformation.

2. Materials and methods

2.1. Bacterial expression and purification of PsRan1

A full-length cDNA of *psRan1* gene was obtained from a cDNA library of etiolated pea plumules using an EcoRI/XhoI fragment of an *Arabidopsis* Ran/TC4-like clone, ATTS1902 (ARBC, Ohio), and used as a hybridization probe according to the manufacturer's protocol in λZAPII cDNA library construction kit (Stratagene, USA).

For the expression and purification of the protein, a full-length DNA fragment encoding PsRan1 was amplified by PCR using a forward primer with a NdeI adaptor (5′-CATTCA*TATG*GCCTTGCCTAATCAG) and a reverse primer with a BamHI adapter (5′-CGCGGGATCCACGACTCACTATAGGG) (the restriction sites are underlined, and the start codon is written in italic). The PCR product was digested with NdeI and BamHI, and then subcloned into pET15-b, which was restricted with the same enzymes. In-frame cloning and orientation were confirmed by sequencing across the junction between the pET15-b and the *psRan1*. Bacterial expression in BL21(DE3) and purification of the PsRan1 protein were carried out by following the manual for pET-His-Tag affinity purification system (Novagen, USA).

2.2. Expression of PsRan1 in yeasts and a GTP-binding assay

Yeast strain (*Saccharomyces cerevisiae* Y190) was obtained from ARBC (Ohio). The GAL4 DNA-binding domain vector (pGBT9) was purchased from Clontech (Palo Alto, USA). For a generation of a Y190 strain containing the *psRan1* expression construct fused to pGBT9, a cDNA of *psRan1* containing the full coding region of Ran was amplified by PCR using a forward primer with an EcoRI adapter (5′-ATTCGAATT*CATG*GCCTTGCCTAATC) and a reverse primer with a BamHI adapter (5′-CGGGATCCACGACTCACTATAGGG) (restriction sites are underlined, the start codon is written in italic). The PCR product was digested with EcoRI and BamHI, and then subcloned into pGBT9, which was restricted with the same enzymes. The *psRan1*-pGBT9 construct was then transformed into the Y190 strain by the PEG/LiAc method according to the Clontech's manual for the Matchmaker two-hybrid system (Clontech, USA).

The expression of the fusion protein of PsRan1 and GAL4 DNA-binding domain was confirmed by showing that the total cellular extracts prepared from the transformed yeast, contained a protein that bound to GTP at the molecular weight expected for the fusion protein. In brief, 50 μg of total yeast extracts was obtained by a TCA precipitation method [32],

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