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Rab1a rescues the toxicity of PRAF3

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ABSTRACT

The PRA1-superfamily member PRAF3 plays pivotal roles in membrane traffic as a GDI displacement factor via physical interaction with a variety of Rab proteins, as well as in the modulation of antioxidant glutathione through its interaction with EAAC1 (SLC1A1). Overproduction of PRAF3 is known to be toxic to the host cells, although the factors capable of cancelling the toxicity remained unknown. We here show that Rab1a can rescue the cytotoxicity caused by PRAF3 possibly by "positively" regulating ER-Golgi trafficking, cancelling the "negative" modulation by PRAF3. Our results illuminate the close physiological relationship between PRAF3 and Rab proteins.

1. Introduction

In human, more than 60 members of Rab GTPase family have been found to date, functioning as key molecular switches in membrane trafficking which collectively regulate important biological events comprising secretion, biosynthesis, endocytosis and autophagy in concert with associated factors including GDI (GDP dissociation inhibitor), GEF (guanine exchange factor), GAP (GTPase-activating proteins), REP (Rab escort protein), and GDF (GDI displacement factor) (extensively reviewed in [1]). Each Rab is known to localise at a specific intracellular membrane and distinctively engaging in the modulation of respective cellular homeostasis, which has promoted extensive researches on the molecular mechanism of Rabs including their localisations.

Generally, newly synthesised Rabs are escorted by REP to GGT (geranylgeranyl transferase), where one or two conserved cysteine residues at the very C-termini of Rabs are prenylated. The prenylated Rabs in GDP form (Rab-GDP) are then bound to GDI and stayed in cytosol as inactive form. Although precise mechanism for specific localisation of Rabs has not been fully understood, however, GDFs are found to be involved in the Rab localisation [1,2], in which GDFs expedite the Rab-GDP to localise at the specific membrane with their hydrophobic C-terminus buried on the membrane by repelling the bound GDIs [1]. The membrane-docked Rabs then interact with GEF and are activated to form GTP-bound Rabs (Rab-GTP), thereby resulting in the promotion of the membrane trafficking in concert with Rab effectors [3].

PRA1 (prenylated Rab acceptor 1) proteins are known to function as the GDFs, predicted to be four-membrane spanning proteins, highly conserved amongst vertebrates, and are predominantly localised at the endoplasmic reticulum (ER) or Golgi apparatus. In human, there are three isoforms belonging to PRA1 super family (named PRAF1, PRAF2 and PRAF3), while only one isoform has been found in yeast (PRAF1/Yip3p). In human, PRA1 proteins strongly interacts with broad range of prenylated Ras family small GTPases including Rab proteins [4–6] and Ha-Ras, RhoA, TC21 and Rap1a [7].

It is of note that PRAF3 (alias: GTRAP3–18, ARL6IP5, HSPC127, DERP11, JWA, addicsin, hp22, jmx, Yip6b) participate not only in membrane trafficking through physical interactions with Rab proteins [8] but also in the modulation of antioxidant glutathione through interactions with EAAC1 [9–11]. In addition, yeast two-hybrid screening has identified an interaction between Rab1 and ADP ribosylation factor-like protein 6 / Bardet-Biedl syndrome type 3 (ARL6/BBS3) [12], a component of BBSome which belongs to the ADP ribosylation factor (ARF) GTPase family, and which is required for the normal formation of primary cilia [13–15]. The C-terminal prenylation of Rabs is known to be prerequisite for substantial binding to PRA1 proteins [8], but it remains unknown whether and how PRAF3 can physically interact with ARL6.

In the accompanying paper [16], we report a recombinant protein overexpression method that avoids the cytotoxicity of the expressed protein in the yeast expression system. Overexpression of PRAF1/Yip3p or human PRAF3 (hPRAF3) in itself has been shown to be toxic to the host cells. We postulated that the cytotoxicity could be avoided by application of an EGFP conjugation system to the membrane protein. In this system, the PRAF1/Yip3p and hPRAF3 proteins conjugated with

Abbreviations used: (E)GFP, (enhanced) green fluorescent protein; SD medium, synthetic defined medium

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Table 1
List of expression vectors used in this study.

Inserted gene	Host vector	Selective marker	Promoter	Tag
For yeast expression				
hPRAF3	pYES-DEST52	*URA3*	*GAL1*	–
hRab1a	pAG424GAL-ccdB	*TRP1*	*GAL1*	–
hRab3a	pAG424GAL-ccdB	*TRP1*	*GAL1*	–
hRab8a	pAG424GAL-ccdB	*TRP1*	*GAL1*	–
hARL6	pAG424GAL-ccdB	*TRP1*	*GAL1*	–
EGFP	pYES-DEST52	*URA3*	*GAL1*	–
For human expression				
hPRAF3	pDsRed-Express-N1			DsRed at the C-terminus
hPRAF3	pcDNA-DEST47			GFP at the C-terminus
hRab1a	pcDNA-DEST47			EGFP at the N-terminus*

* For EGFP-tagging to hRab1a, since conserved di-cysteine motif usually occurred at the very C-terminus in Rab species is to be essentially prenylated so that Rabs can be anchored to the target membrane, N-terminally EGFP-fused hRab1a was employed in this study so as not to hinder the Rab1a's original function.

Table 2
Combination of yeast expression vectors used for the growth test.

Vector 1		Vector 2	
Inserted gene	Host vector	Inserted gene	Host vector
hPRAF3	pYES-DEST52	–	pAG424GAL-ccdB
hPRAF3	pYES-DEST52	hRab1a	pAG424GAL-ccdB
hPRAF3	pYES-DEST52	hRab3a	pAG424GAL-ccdB
hPRAF3	pYES-DEST52	hRab8a	pAG424GAL-ccdB
hPRAF3	pYES-DEST52	hARL6	pAG424GAL-ccdB
EGFP*	pYES-DEST52	–	pAG424GAL-ccdB

* Negative control.

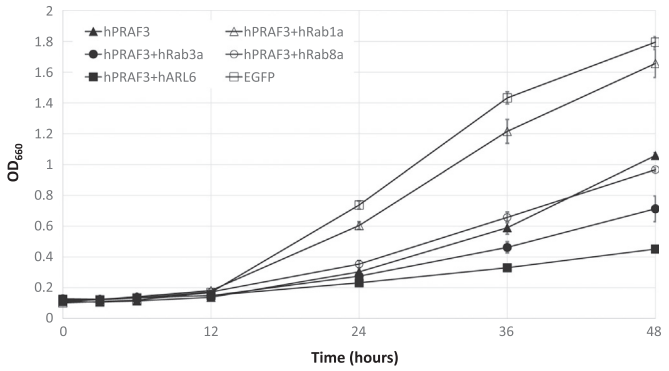

Fig. 1. Co-expression assay of the yeast cells transformed with expression vectors harbouring hPRAF3, hRab1a, hRab3a, hRab8a, hARL6 (and EGFP as a control) genes as listed in Table 2, showing that only hRab1a can relieve the growth level compatible to the control level despite the PRAF3's toxicity (n = 3; statistically significant after 24 h culture (to 48 h culture); $P < 0.01$).

EGFP at either (both) ends of termini exhibit normal growth and are obtained at a level sufficient for functional and structural analysis.

We report here that Rab1a can relieve the cytotoxicity of PRAF3 both in yeast and a human cell expression system. The ability of Rab1a to cancel the toxicity could further imply that PRAF3 and Rabs are closely related to each other physiologically and genetically.

2. Materials and methods

2.1. Preparation of expression vectors

The constructs used in this study are illustrated in Table 1. In brief, the cDNA for hPRAF3 was prepared as described previously [16] and sub-cloned into pDONR221 (Thermo Fisher Scientific, Waltham, MA). The nucleotides coding hRab1a, hRab3a, hRab8a and hARL6 were manufactured by gBlocks® (Integrated DNA Technologies, Coralville, IA) and sub-cloned into pDONR221 (Thermo Fisher Scientific, MA). For the yeast growth test, an entry vector harbouring the hPRAF3 gene was subjected to LR recombination with the pYES-DEST52 vector (Thermo Fisher Scientific, MA), whereas entry vectors harbouring Rabs and ARL6 were also subjected to LR recombination with pAG424-ccdB (Addgene #14151) to produce yeast expression vectors. For microscopic analysis in a human expression system, the nucleotide encoding EGFP was introduced at the N-terminus of the sub-cloned hRab1a constructs by a restriction enzyme-based routine technique and subjected to LR recombination with a pcDNA-DEST47 vector (Thermo Fisher Scientific). For the hPRAF3-DsRed construct, PCR fragment for hPRAF3 was directly cloned into pDsRed-Express-N1 vector (Clontech Laboratories, CA) as previously mentioned [17]. hPRAF3-GFP expression construct was manufactured from the sub-cloned vector in pDONR221 as described above through LR recombination with pcDNA-DEST47 (Thermo Fisher Scientific, MA).

2.2. Yeast growth test

The combinations of expression vectors used for the growth test are summarised in Table 2. Each of the expression-vector combinations were transformed into the INVSc1 strain (Invitrogen, Carlsbad, CA), which has the genotype *MATa his3D1 leu2 trp1–289 ura3–52 MAT his3D1 leu2 trp1–289 ura3–52*, and streaked onto SD lacking uracil and L-tryptophan (abbreviated as SD – URA – TRP hereinafter) on an agar plate supplemented with 2% D-glucose, then allowed to grow at 30 °C for three days. The colonies were inoculated in 5 mL SD – URA – TRP liquid media supplemented with 2% D-glucose and cultured overnight at 30 °C with shaking. Aliquots of the cultured media were thoroughly washed by distilled water and added to freshly prepared SD – URA – TRP liquid media with 2% DL-lactate (Nacalai Tesque, Kyoto, Japan) and 2% D-galactose so as to adjust the OD_{660} to 0.1 (0 h), then cultured at 30 °C with shaking at 120 rpm, and allowed to grow so as to induce the recombinant protein (co-)expression via the *GAL1* promoter system. The OD_{660} was monitored at the time points of 0, 3, 6, 12, 24, 36, and 48 h(s). This yeast growth test was repeated three times. Data are expressed as means ± S.E. Statistical significance was assessed with two-way ANOVA followed by a Bonferroni multiple comparison test using GraphPad Prism 5. Differences were considered significant at $P < 0.05$.

2.3. Viability test in SH-SY5Y cells and immunofluorescence microscopy

hPRAF3 in the pDsRed-Express-N1 vector and hRab1a in the pcDNA-DEST47 vector were co-introduced into SH-SY5Y cells, and their overexpression was permitted for 48 h. The culture condition and reagents used for the (co-)expression were previously described [17]. Immunostaining for monitoring the apoptotic feature of the cells was performed according to those described in [17] except for the