



Rab1a rescues the toxicity of PRAF3

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ARTICLE INFO

Keywords:

Rab
Toxicity
Protein expression
Membrane protein
Endoplasmic reticulum (ER)

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, addicisin, 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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<https://doi.org/10.1016/j.bbrep.2018.03.002>

Received 5 January 2018; Received in revised form 7 March 2018; Accepted 16 March 2018

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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.

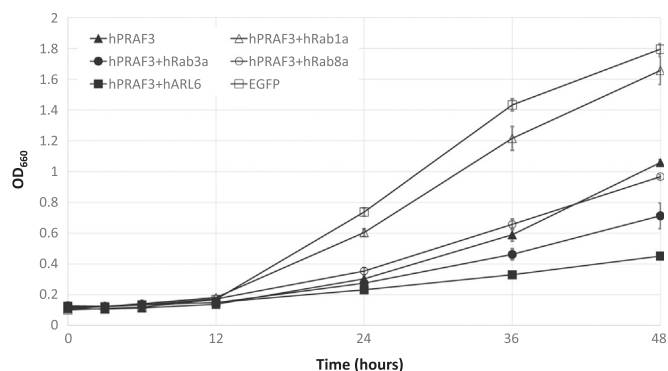


Fig. 1. Co-expression assay of the yeast cells transformed with expression vectors harbouring hPRAF3, hRab1a, hRab3a, hRab8a, hARL6 (and EGFP as a control) genes as listed in Table 2, showing that only hRab1a can relieve the growth level compatible to the control level despite the PRAF3's toxicity ($n = 3$; 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 OD₆₆₀ 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 OD₆₆₀ 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

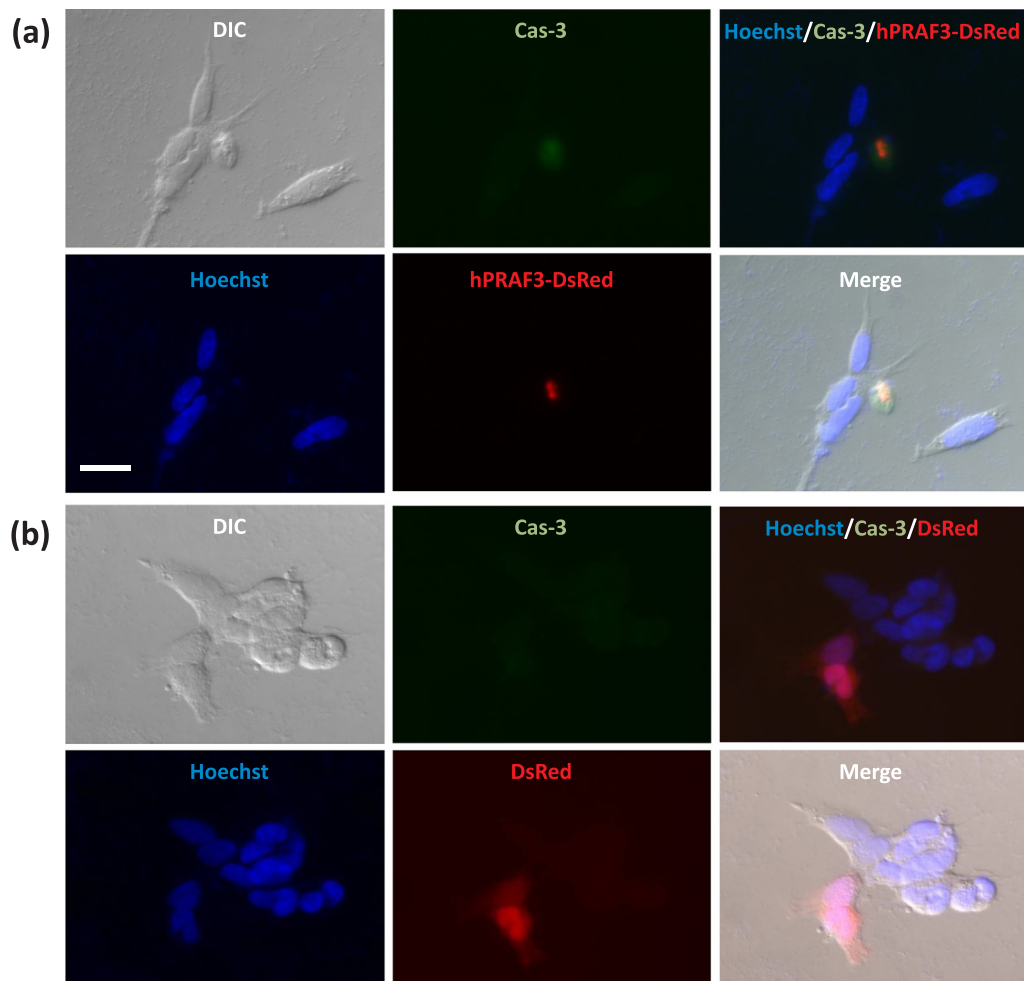


Fig. 2. Apoptotic feature induced by the overexpression of hPRAF3 (*red*) (monitored by anti-cleaved caspase-3 (*green*)). Cells overexpressed with hPRAF3 (**a**) and with DsRed (**b**) as a negative control, confirming that only the hPRAF3 overexpression induces the expression of cleaved caspase-3, implying that hPRAF3 overexpression leads to apoptotic cell death. Scale bar: 20 μm .

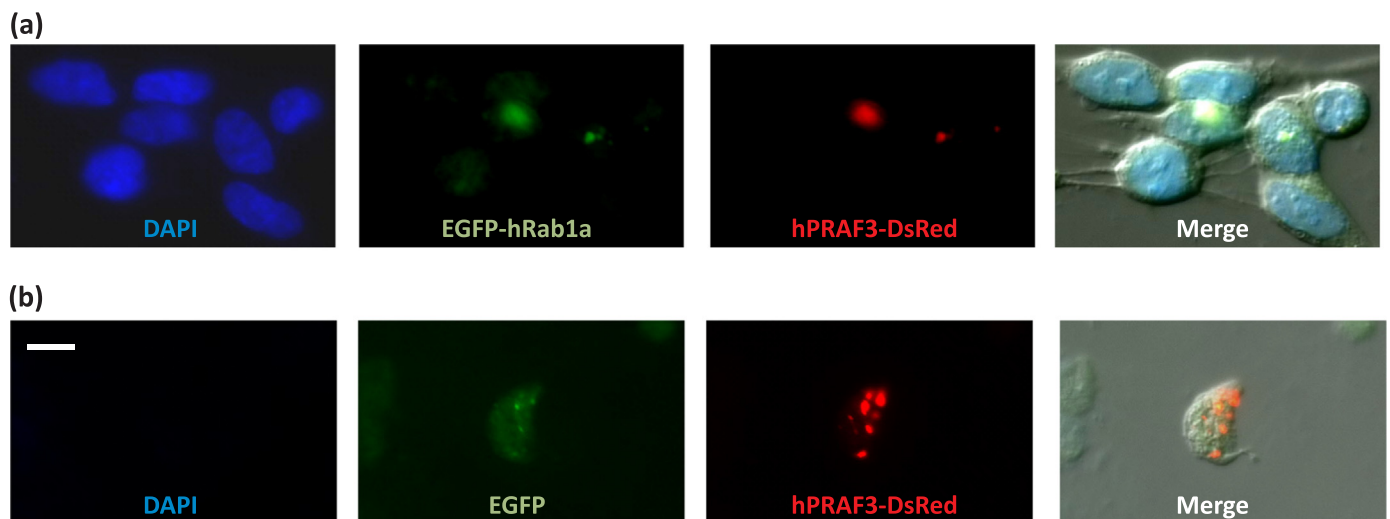


Fig. 3. Difference in cell viability between the cells (**a**) co-expressing hPRAF3 (*red*) and hRab1a (*green*); and (**b**) hPRAF3 (*red*) and EGFP (*green*). Scale bar: 10 μm .

antibodies (primary antibody, anti-cleaved caspase-3 (Asp175) (Cell Signaling Technology, MA); and secondary antibody, goat anti-rabbit IgG Alexa Fluor® conjugated (Thermo Fischer Scientific, MA)). All the microscopic analyses in this study were performed using Axio Imager M1 (Zeiss, Oberkochen, Germany).

3. Results & discussion

As a first step in our effort to identify a factor that can cancel the cytotoxicity of PRAF3, a growth test was attempted using a yeast expression system for the initial screening. In the presence of

heterogeneous expression of hPRAF3, the expression of the rescuing factor was expected to raise the impaired growth rate to the normal level. Because interactions of PRAF3 with Rab proteins [6,8] and with ARL6 [12] have been reported, hRab1a, hRab3a, hRab8a and hARL6 were chosen as screening partners. As we documented earlier [16], the cells that overexpressed hPRAF3 on their own exhibited a low growth rate due to its toxicity (Fig. 1). Among the co-expressions with Rabs and ARL6, only hRab1a yielded a substantial retrieval of the growth rate to a level comparable to the control (EGFP only), indicating that hRab1a can actually function as a rescuing factor.

We further performed a co-expression test of hPRAF3 and hRab1a in human cells in order not only to confirm the cell viability in the human co-expression system, but also to examine the intracellular localisation of both proteins. In preparation for this experiment, the viability of the SH-SY5Y cells with DsRed-fused hPRAF3 overexpression (i.e., without any co-expression partner) was tested. Although apoptotic phenotype induced by PRAF3 has been reported earlier [18], even the DsRed-fused hPRAF3 overexpression was found to lead to the formation of aggregates within the host cells, and triggered apoptotic cell death (Fig. 2, Supplemental Data 1), possibly because DsRed (or GFP)-tagging undermines the stability of hPRAF3—at least in SH-SY5Y cells—sufficiently to cancel the toxicity of hPRAF3, as discussed earlier [16]. We next performed a co-expression test using hPRAF3 and hRab1a co-expressed cells. In comparison with the negative control (hPRAF3 with EGFP; Fig. 3(b), Supplemental Data 2), the hRab1a co-expressed cells exhibited normal cell shape and rigid cell growth, confirming that Rab1a can also cancel the toxicity of PRAF3 in the human cell system (Fig. 3(a)).

This apoptotic propensity caused by hPRAF3 overexpression and relieving effect benefited by hRab1a co-expression might postulate a harmonised gene expression of hPRAF3 with hRab1a. In support of this idea, in *Arabidopsis thaliana*, most AtPRA1 genes are reported to be co-expressed with Rab genes [19], and it may be through this co-expression that eukaryotic cells spontaneously avoid the cellular toxicity in the event where PRA1 proteins are overexpressed.

Rab1 is also known to function as a rescuing factor for the neuronal loss in animal models of Parkinson's disease, possibly by “positively” regulating the ER-Golgi transport to overcome the trafficking block caused by α -Synuclein [20], whose misfolding is closely associated with neurodegenerative disorders including Parkinson's disease [21]. On the other hand, Geng *et al.* reported the cytotoxicity of PRAF1/Yip3p, the only protein belonging to the PRA1 family in yeast, observing abnormal changes in the ER structure when PRAF1/Yip3p was overexpressed, presumably due to inhibition of the ER-Golgi transport [22]; their results implied the negative regulatory function of the PRA1 family in ER-Golgi transport. In support of this notion, PRAF3 is known to serve as a negative regulator of Rab1 [23], inhibiting the Rab1-dependent trafficking. Together, these findings indicate that PRA1 protein overproduction in the host cells should “negatively” regulate ER-Golgi trafficking to an excessive level that might induce ER stress in host cells, leading to a low growth level or apoptosis, whereas Rab1a can serve “positively” in ER-Golgi trafficking, potentially cancelling the negative regulation of the trafficking by PRA1 proteins, and thereby “rescuing” the viability of the host cells.

In conclusion, we postulate here that Rab1a can rescue the cytotoxicity caused by PRAF3, possibly by promoting on the ER-Golgi trafficking. Together with our previous paper [16], these findings suggest that there are at least two reasons for the cytotoxicity of PRA1 proteins. Namely, the cytotoxicity of PRAF3 could be due to (1) the tendency of PRA1 proteins to irreversibly aggregate in the host cells; and (2) the negative regulatory effect of the PRA1 proteins on ER-Golgi trafficking.

Recently, PRA1 and α -synuclein have been shown to interact physically [24], suggesting the importance of the molecular functions of the PRA1 family not only as Rab acceptors but also as direct interactors with α -synuclein, an improved understanding of which could lead to a

more accurate portrait of synucleopathy. We hope that the findings described herein will contribute to the future research on membrane trafficking and neurodegenerative diseases.

Acknowledgements

We would like to thank Drs. Fumio Arisaka (Nihon University, Japan) and Yohei Ohashi (Medical Research Council, Laboratory of Molecular Biology, UK) for their helpful discussions and comments. Part of this work was supported by JSPS KAKENHI Grant Number 15580458 (to T.N., H.O.) and JSPS KAKENHI Grant Number 17K08962 (to M.W.).

Author contributions

H.O. performed all of the experiments and drafted the manuscript, M.W. did microscopic work, K.K.U. performed statistical analysis and T.N. critically reviewed the manuscript.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2018.03.002>.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2018.03.002>.

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