Biochemical and Biophysical Research Communications 468 (2015) 580–586

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mammalian farnesyltransferase α subunit regulates vacuolar protein sorting-associated protein 4A (Vps4A) – dependent intracellular trafficking through recycling endosomes

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ARTICLE INFO

Article history:
Received 26 October 2015
Accepted 27 October 2015
Available online 10 November 2015

Keywords:
Farnesyltransferase
Oligomerization
Isoprenylation
Trafficking

ABSTRACT

The protein farnesyltransferase (FTase) mediates posttranslational modification of proteins with isoprenoid lipids. FTase is a heterodimer and although the β subunit harbors the active site, it requires the α subunit for its activity. Here we explore the other functions of the FTase α subunit in addition to its established role in protein prenylation. We found that in the absence of the β subunit, the α subunit of FTase forms a stable autonomous dimeric structure in solution. We identify interactors of FTase α using mass spectrometry, followed by rapid *in vitro* analysis using the *Leishmania tarentolae* cell – free system. Vps4A was validated for direct binding to the FTase α subunit both *in vitro* and *in vivo*. Analysis of the interaction with Vps4A in Hek 293 cells demonstrated that FTase α controls trafficking of transferrin receptor upstream of this protein. These results point to the existence of previously undetected biological functions of the FTase α subunit that includes control of intracellular membrane trafficking.

1. Introduction

Activating mutations of *ras* proto-oncogenes are one of the most common features of human cancers [1]. Targeting mutated Ras by blocking its functionally critical farnesylation has been considered a promising strategy for downregulating its activity [2]. This post-translational modification is carried out by FTase, that catalyzes the attachment of an isoprenoid farnesyl to its substrates [3]. Although farnesyltransferase inhibitors (FTIs) are undergoing clinical trials, their antitumor effect has been independent of Ras mutations. The α subunits of prenyltransferases belong to the tetratricopeptide repeat (TPR) superfamily [4,5]. The β subunit of prenyltransferases encompasses the active site, however, it is inactive in the absence of the α subunit [6]. Despite the wealth of structural data, the role of FTase α subunit in prenylation is unclear. Only two FTase α residues (Lys164 and Gln167) directly interact with lipid and peptide substrates respectively [7–9]. Interestingly, several reports show that the α subunits of prenyltransferases interact with other proteins like β-2 adrenergic receptor and histone deacetylase HDAC6 [10,11]. Understanding prenyltransferase regulation could shed light on the mechanism of action of FTIs. Here we focus on the role of the FTase α subunit within the heterodimer and on its yet unclear function involving interactions with other proteins. We find that FTase α dimerizes in solution. We identify a putative interacting partner of FTase α subunit, Vps4A, and confirm their interaction *in vitro*. Further we demonstrate that the FTase α subunit controls membrane trafficking of the transferrin receptor upstream of Vps4A.

2. Materials and methods

2.1. Protein expression and purification

Recombinant proteins, *R. norvegicus* FTase α and *S. cerevisiae* FTase α were purified using standard protocols. Details are provided in supporting information S1 File Methods.

2.2. Multi-angle laser light scattering (MALLS)

Proteins were injected onto a Superdex 200 HR 10/30 column (GE Healthcare). Data were collected every 0.5 s at a flow rate of 0.5 mL/min and analyzed by ASTRA software.

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http://dx.doi.org/10.1016/j.bbrc.2015.10.148
0006-291X/

2.3. Crosslinking

FTase α subunit was dialyzed against a buffer 25 mM HEPES (pH 8.0) and 150 mM NaCl. The crosslinker Bis [sulfosuccinimidyl] suberate (Pierce) dissolved in 30 mM acetic acid pH 3.0 was added to 20 μM and 10 μM protein solutions at a 3 mM final concentration. The reaction was stopped with a 50 mM of $(NH_4)_2CO_3$.

2.4. Small angle X-ray scattering (SAXS)

Protein solutions were characterized using the SAXS/WAXS beamline at the Australian Synchrotron according to standard procedures. Detailed description can be found in S1 File Methods.

2.5. Protein complex immunoprecipitation (co-IP)

Hek 293 cells were harvested 18 h post transfection and lysed for 30 min at 8 °C with NETN buffer. After removing cell debris, supernatants were incubated with unconjugated sepharose beads. Later, supernatants were incubated overnight at 8 °C with sepharose beads coated with an antibody against GFP [12] – GFP-nanobody, which binds Citrine with a similar affinity as GFP [12]. Captured proteins were eluted as previously described [13] and subjected to trypsin digestion. More detailed description can be found in S1 File Methods.

2.6. LC-MS/MS analysis

The eluate was subjected to in-solution digestion following standard protocols. The extracts were analyzed by LC-MS/MS on a Shimadzu Nano HPLC (Japan) coupled to a QStar Elite mass spectrometer (ABSCIEX, Canada) equipped with a nano-electrospray ion source. The identity of proteins was confirmed using the following criteria: (i) if there were two or above peptides identified with a 95% confidence and a 1% global false discovery rate (FDR), (ii) if one peptide was identified with a 95% confidence and a 1% global FDR but the mass spectrometry data was of good quality. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002458. Further detail is provided in S1 File Methods.

2.7. Leishmania tarentolae cell free system (LTE) template preparation

Open reading frames (ORFs) of prey proteins were obtained from the RIKEN mouse cDNA Library (Facility for Life Science Automation, IMB, UQ, Australia). Full-length RIKEN mouse cDNA clones were originally established by the Genome Exploration Group, RIKEN GSC [14,15]. A mCherry-His or His tag was fused with the ORFs at the C-terminus using a short linker region TCCGGTTCGGGCTCCGGTGGA. Poly(U) species independent translation enhancers (SITS) were used as a translation initiator [16] and combined with ORF-coding fragments together with the mCherry-His-coding fragment by overlap-extension PCR (OE-PCR) [13]. Templates for the expression of N-terminally labeled bait proteins were assembled by combining amplified poly(U)SITS-EGFP DNA fragment and a protein coding fragment. ORFs were also commercially cloned (Diamantina Institute, Australia) into vectors combined with the Gateway® cloning platform (Invitrogen) [17]. Translation reactions primed with 20 nM template were performed at 27 °C for 2.5 h, in the presence of 0.2% of FluoroTect™, a modified charged lysine transfer RNA labeled with the fluorophore BODIPY®-FL (PROMEGA). Proteins were resolved by SDS-PAGE and scanned on a Typhoon Trio instrument with 488 nm excitation and 520 nm emission.

2.8. Pull-down assay

EGFP-bait and prey-mCherry-His or -His tag fused proteins were translated separately for 1 h in 50 μL LTE in the presence of FluoroTect™ (PROMEGA) and subsequently mixed and co-translated for a further 90 min at 27 °C. 10 μL of a 30% GFP-nanobody coated beads slurry was added and incubated for 20 min. Beads were washed 3× with PBS-0.1% Triton X-100 and heated with SDS-PAGE loading buffer, resolved by SDS-PAGE. For the reverse pull-down experiments Co^{2+} affinity resin (Talon, Clontech) was used and the wash buffer contained 20 mM imidazole.

2.9. Co-localization

ORFs were fused at the C-terminus with a mCherry tag by OE-PCR and cloned into the pQCXP vector (Addgene no. 17393) [18]. Hek 293 cells were co-transfected with constructs and subjected to live imaging using a confocal microscope (Zeiss). Further detail is provided in S1 File Methods.

2.10. Reagents

Mouse anti-GFP (G6795 Sigma–Aldrich); rabbit monoclonal anti-mCherry (5993-100 BioVision); IRDye 680 goat anti-mouse 827-11080 and 800 goat anti-rabbit 926-32211 (Li-Cor Biosciences); goat anti-Vps4 (Santa Cruz, sc-21463); IRDye 680 donkey anti-goat (926-68024, Li-Cor Biosciences); mouse monoclonal anti-GAPDH (Sigma, G8795); IRDye 680 goat anti-mouse (827-11080, Li-Cor Biosciences). Vps4A knock down was performed with ON-Target plus SMART pool human Vps4A siRNA (Thermo Scientific) following the manufacturer's protocol.

2.11. Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed on an LSM 710 meta Zeiss confocal microscope as described previously [19]. Further detail is provided in S1 File Methods.

3. Results

3.1. Recombinant FTase α subunit dimerizes in solution

Although FTase α subunit is obligatory for the enzymatic activity [6], it is unclear why the heterodimeric architecture is necessary and evolutionarily conserved. We conjectured that this might be related to functions of the FTase α subunit independent of the role they play in a heterodimer. We generated recombinant mammalian and yeast FTase α subunits for structural and biophysical characterization. Multi-angle laser light scattering (MALLS) revealed that the proteins did not form aggregates. However, the ΔN29 truncation of *R. norvegicus* α subunit calculated molecular weight was 78 kDa, almost twice the theoretical mass of 41 kDa. Similarly, the calculated molecular weight of 62 kDa for the yeast FTase α subunit was significantly higher than the calculated theoretical value of 37.3 kDa (Table 1). To assess the oligomerization state of the FTase α subunit, protein was exposed to the bifunctional, amine-reactive crosslinker BS3. SDS-PAGE analysis (Fig. 1A) revealed rapid formation of an approximately 80 kDa band corresponding to a dimer. The bands corresponding to higher order oligomers were present at longer incubation times. Significantly, bands corresponding to a trimer (approximately 120 kDa) were not observed, suggesting that higher order structures emerge through crosslinking of FTase α homodimers.