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The yeast Arf-GAP Glo3p is required for the endocytic recycling of cell surface proteins

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ABSTRACT

Small GTP-binding proteins of the Ras superfamily play diverse roles in intracellular trafficking. Among them, the Rab, Arf, and Rho families function in successive steps of vesicle transport, in forming vesicles from donor membranes, directing vesicle trafficking toward target membranes and docking vesicles onto target membranes. These proteins act as molecular switches that are controlled by a cycle of GTP binding and hydrolysis regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In this study we explored the role of GAPs in the regulation of the endocytic pathway using fluorescently labeled yeast mating pheromone α-factor. Among 25 non-essential GAP mutants, we found that deletion of the *GLO3* gene, encoding Arf-GAP protein, caused defective internalization of fluorescently labeled α-factor. Quantitative analysis revealed that *glo3*Δ cells show defective α-factor binding to the cell surface. Interestingly, Ste2p, the α-factor receptor, was mis-localized from the plasma membrane to the vacuole in *glo3*Δ cells. Domain deletion mutants of Glo3p revealed that a GAP-independent function, as well as the GAP activity, of Glo3p is important for both α-factor binding and Ste2p localization at the cell surface. Additionally, we found that deletion of the *GLO3* gene affects the size and number of Arf1p-residing Golgi compartments and causes a defect in transport from the TGN to the plasma membrane. Furthermore, we demonstrated that *glo3*Δ cells were defective in the late endosome-to-TGN transport pathway, but not in the early endosome-to-TGN transport pathway. These findings suggest novel roles for Arf-GAP Glo3p in endocytic recycling of cell surface proteins.

1. Introduction

Endocytosis of membrane proteins and lipids is a process critical for a wide variety of functions as well as the survival of eukaryotic cells. Upon internalization from the plasma membrane, cargo proteins such as cell surface receptors are usually delivered to the early endosome. Subsequently they can be sorted to recycling endosomes that bring the cargo back to the plasma membrane, or to late endosomes/multivesicular bodies en route to the lysosome/vacuole for degradation. Studies of yeast and mammalian cells have shown that these successive steps of vesicular trafficking are strictly regulated by various small GTPase proteins, including Rho, Rab, and Arf/Sar, and that the fundamental roles of these proteins in vesicular trafficking are conserved throughout eukaryotes [1]. These small GTPase proteins act as molecular switches that cycle between two conformational states: an inactive, GDP-bound state and an active, GTP-bound state. Guanine nucleotide exchange factors (GEFs) activate the switch by catalyzing the exchange of GDP for GTP, whereas GTPase-activating proteins (GAPs) increase the intrinsic GTPase activity and inactivate the switch [2].

Arf proteins undergo a cycle of GTP binding and hydrolysis to regulate vesicle formation on a variety of donor membranes, such as those of the Golgi, endosomes, and plasma membrane [3–5]. In mammals, Arf proteins can be divided into three classes based on their amino acid sequence similarity: class I (ARF1 and ARF3), class II (ARF4 and ARF5), and class III (ARF6) [6]. Class I and II Arfs localize primarily to the Golgi apparatus and function there and in compartments along the

Abbreviations: GAP, GTPase-activating protein; GFP, green fluorescent protein; mCherry, monomeric Cherry; GEF, guanine nucleotide exchange factor; VPS, vacuolar protein sorting; TGN, *trans*-Golgi network; ER, endoplasmic reticulum

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secretory pathway. The Class III Arf6 functions mainly at the plasma membrane and has roles in endocytic trafficking [7].

Budding yeast has three *ARF* genes: *ARF1*, *ARF2*, and *ARF3*. Arf1p and Arf2p, are 96% identical and serve as functional homologues, although the level of protein produced from *ARF1* is ~10-fold higher than that from *ARF2* [8]. Through their roles in coat assembly and disassembly, Arf1p and Arf2p are known to facilitate COPI and clathrin coat dynamics on the Golgi apparatus and function in many different steps of intracellular trafficking, such as retrograde transport from the Golgi to the ER and anterograde transport to the lysosome/vacuole [7,9,10]. Previous studies demonstrated that deletion of the *ARF1* gene creates a defect in the secretory pathway, as evidenced by altered glycosylation of secreted proteins [11]. Additionally, recent studies have revealed a role for Arf1p in the exomer-mediated trafficking of cargo proteins from the *trans*-Golgi network (TGN) to the plasma membrane [12–15].

Yeast has six structurally related proteins with the potential to provide GAP activity for the Arf1 and Arf2 proteins [16–18]. Genetic studies have shown that the four known yeast Arf-GAPs have overlapping functions [19]. Two of these Arf-GAPs, Gcs1p and Glo3p, are essential for retrograde transport from the Golgi to the ER [17], however, Gcs1p makes only a small contribution, because *glo3*Δ cells exhibit severely defective retrograde transport, whereas *gcs1*Δ cells show only a minor defect [17]. Glo3p has been identified as a component of COPI vesicles, and a mutant version of Glo3p (Glo3-R59K) prevents the generation of COPI-coated transport vesicles in vitro [20]. It has also been reported that Gcs1p and Age2p mediate the formation and function of TGN-derived transport vesicles destined for the vacuole via both the vacuolar protein sorting (VPS) and AP-3-dependent pathways [21]. However, the role of Arf-GAPs in regulating transport from the TGN to the plasma membrane in the secretory pathway has not been clarified.

In a previous study, we synthesized a fluorescently labeled mating pheromone derivative, which binds specifically to the Ste2p G protein-coupled receptor and is used as a marker for receptor-mediated endocytosis [22,23]. In the present study, using this fluorescent marker, we show that cells lacking Glo3p exhibit defects in α-factor internalization. Quantitative analysis demonstrates that the *glo3*Δ mutant has a defect in α-factor binding to the cell surface. We also show that *glo3*Δ cells have defects in the late endosome-to-TGN transport pathway, and that therefore, endocytosed Ste2p is sorted to the vacuole, not to the plasma membrane via the recycling pathway. We extend these findings to Kex2p and Gap1p, demonstrating that Glo3 seems to play a general role in recycling cell surface and TGN resident proteins from the late endosome and thus preventing them from being turned over in the vacuole.

2. Material and methods

2.1. Yeast strains, growth conditions, and plasmids

The yeast strains used in this study are listed in Table 1. Cells depleted of Arf-GAPs, Rab-GAPs, or Rho-GAPs were purchased from Open Biosystems and strain identities were confirmed by PCR using primers specific to the gene and the selection marker (Supplementary Fig. 1). All strains were grown in standard rich medium (YPD) or synthetic medium supplemented with 2% glucose (SD) and appropriate amino acids. The N-terminal GFP tag was integrated at the endogenous locus of the *GLO3* gene as follows: The GFP (S65T) fragment whose stop codon was replaced with a BglII site was subcloned into BamHI- and NotI-digested pBlueScript II SK (pBS-GFP), and the NotI-SacII fragment, which contains the *Saccharomyces cerevisiae ADH1* terminator and the *His3MX6* module, was amplified by PCR using pFA6a-GFP (S65T)-HIS3MX6 as a template, and inserted into NotI- and SacII-digested pBS-GFP (pBS-GFP-HIS3). To create an integration plasmid, 300-bp 5′ UTR of the *GLO3* gene and the N-terminal fragment of the *GLO3* ORF (nt 1-470) were generated by PCR and cloned into the BamHI or BglII site of pBS-GFP-HIS3. To integrate GFP at the N terminus of the *GLO3* gene, the integration plasmid was linearized by KpnI and transformed into yeast. C-terminal GFP or mCherry tagging of proteins was performed by PCR-based homologous recombination using pFA6a-GFP(S65T) or pFA6a-mCherry, respectively, as a template [24].

Glo3p and its domain deletion mutants were expressed from pRS316 plasmids in *glo3*Δ cells. The full-length *GLO3* gene was amplified by PCR from yeast genomic DNA using primers 5′-CGGGATCCGCTGTTCC CCTGTTACTC-3′ and 5′-CGCTCGAGCAATTAGAAACAGAGATG-3′, and cloned into BamHI- and XhoI-digested pRS316. DNA fragments encoding GLO3ΔGAP (Glo3Δ2-145) or GLO3ΔBoCCS (Glo3Δ214-375) were created by PCR amplification from full-length *GLO3* gene using primers 5′-CACATAGCGACAATGAACGGTCAAGATTCTTCG GAC-3′ and 5′-AGAATCTTGACCGTTCATTGTCGCTATGTGTATAC-3′ or 5′-ACTGTCAC TAAAACGGGCAGAATCGCGGAAAGGTAC-3′ and 5′-TTCCGCGATTCTGC CCGTTTTAGTGACAGTGGTCTTG-3′, respectively, and cloned into pRS316.

2.2. Fluorescence microscopy

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a 100×/NA 1.40 (Olympus) objective and an Orca-AG cooled CCD camera (Hamamatsu), using Metamorph software (Universal Imaging). To assess colocalization with high precision, each image pair was acquired simultaneously using dual-channel 2D imaging system. Simultaneous imaging of red and green fluorescence was performed using an Olympus IX81 microscope, described above, and an image splitter (Dual-View; Optical Insights) that divided the red and green components of the images with a 565-nm dichroic mirror and passed the red component through a 630/50 nm filter and the green component through a 530/30 nm filter.

2.3. Fluorescence labeling of α-factor and endocytosis assays

Fluorescence labeling of α-factor was performed as described previously [22]. For endocytosis assays, cells were grown to an OD600 of ~0.5 in 0.5 ml YPD, briefly centrifuged, and resuspended in 20 μl SM with 5 μM Alexa Fluor 594-α-factor. After incubation on ice for 2 h, the cells were washed with ice-cold SM. Internalization was initiated by the addition of SM containing 4% glucose and amino acids at 25 °C.

2.4. ^{35}S-labeled α-factor internalization assay

Preparation and internalization of ^{35}S-labeled α-factor were performed as described previously [25]. Briefly, the cells were grown to an OD600 of ~0.3 in 50 ml YPD, briefly centrifuged and resuspended in 4 ml YPD containing 1% (w/v) BSA, 50 mM KH_2PO_4, pH 6, and 20 μg/ml of uracil, adenine, and histidine. After adding ^{35}S-labeled α-factor, cell aliquots were withdrawn at various time points and subjected to a wash in pH 1.1 buffer to remove surface-bound α-factor so that internalized α-factor could be measured, or in pH 6 buffer to determine the total amount (internalized and bound) of α-factor. The amount of cell-associated radioactivity after each wash was determined by scintillation counting. Each experiment was performed at least three times.

2.5. CPY colony blot assay

The CPY colony blot assay was performed as described previously [26]. After overnight growth in YPD medium, cells were diluted to 0.2 OD600, and 2 μl of each dilution was spotted on a YPD plate. After incubation at 30 °C for 2 days, a nitrocellulose filter was placed on the plate and then incubated at 30 °C for 1 day. The nitrocellulose filter was washed several times with standard phosphate-buffered saline. The membranes were then subjected to immunoblotting with mouse anti-CPY (Molecular Probes, Eugene, OR). The mouse anti-CPY was used at