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| メタデータ | 言語: eng 出版者: 公開日: 2017-10-04 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属: |
| URL | http://hdl.handle.net/2297/17279 |

Characterization of ArfGAP1 and FinGER7 /FinGER8 interaction by quantitative yeast two-hybrid analysis

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Abstract: By using a quantitative method for yeast two-hybrid analysis, we tried to identify interacting regions of ArfGAP1, a GTPase activating protein for ARF, and FinGER7 and FinGER8, family members of five span transmembrane proteins localizing in the Golgi apparatus and the ER. The N-terminal region (1-152), which contains a GTPase activation domain, and the C-terminal region (256-406) of ArfGAP1 was dispensable and the central region (153-257) reported to function for sensing membrane curvature and also for targeting to the Golgi apparatus was necessary and enough for the interaction with FinGER7 or FinGER8. A mutation of 211th tryptophan of ArfGAP1 to alanine that disrupts the Golgi targeting and lipid curvature sensing abolished the interaction with FinGER7 or FinGER8 suggesting the role of FinGER7 and FinGER8 for the targeting of ArfGAP1 to the Golgi apparatus. The severe reduction of the interaction was observed by deletion of the N-terminal 31st to 41st residues of FinGER7 showing the importance of this region (31-41) for the interaction. Deletion of any parts of the C-terminal putative transmembrane segments completely abolished the interaction suggesting that the deletion of the transmembrane segments caused some defects for proper insertion on the membrane, proper conformation or topology, or proper subcellular localization for the interaction with ArfGAP1. Replacement of the putative cytoplasmic region or the transmembrane region of FinGER7 by FinGER8 did not recreate the original higher-level interaction suggesting that the cytoplasmic region and the transmembrane region cooperate for the interaction with ArfGAP1.

Key words ARF, Golgi apparatus, GTPase activating protein, Yeast two-hybrid analysis

Introduction

The Golgi apparatus is situated at the center of the vesicular transport pathway. It receives secretory and transmembrane proteins from the ER, processes, sorts and sends out to the final destinations¹⁾. Great details have been described about the molecular mechanism of the vesicular transport machinery. For example, transport vesicles are produced by COPII, COPI and clathrin coat at the ER, the Golgi apparatus and plasma membrane, respectively. The transport vesicles are un-coated and fuse with target membranes mediated by SNAREs and associated factors^{2)~4)}. Ypt/rab family small GTPases are known to regulate vesicle budding and/or fusion at the various steps of vesicular transport pathway^{5)~7)}.

FinGER proteins are a family of five-span transmembrane proteins localizing in the ER and the Golgi apparatus⁸⁾. Eight family members were found in human and they are all expressed widely in various tissues. The Golgi apparatus was fragmented by the overexpression of some of the family members suggesting that the FinGER proteins function in the maintenance of the Golgi structure and/or the transport between the ER and the Golgi apparatus. Yip1p is a putative yeast orthologue of FinGER4 and FinGER5. Yif1p is that of FinGER3, FinGER7, FinGER8. Yip1p and Yif1p are essential yeast gene products localized in the Golgi apparatus⁹⁾¹⁰⁾. The loss of function of these proteins causes a block of the ER to Golgi transport and accumulation of ER membranes. These proteins interact with Ypt1p and Ypt31p, which are

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Abbreviations: ER; endoplasmic reticulum, GAP; GTPase activating protein, SD; standard deviation, TM; transmembrane segment

Ypt/rab family small GTPases shown to function at the Golgi apparatus, suggesting their involvement in the vesicle budding and/or fusion at the Golgi apparatus¹¹⁾¹²⁾. Supporting this possibility, it was reported that Yip1p and Yif1p were necessary to form vesicles competent for fusion to the Golgi apparatus¹³⁾. On the contrary, it was also reported that Yip1p function in the vesicle budding from the ER but not in the fusion to the Golgi apparatus¹⁴⁾. Therefore, it remains obscure whether Yip1p and Yif1p function in the vesicle budding and/or fusion steps.

To explore the function of FinGER proteins, we sought to identify interacting partner(s) of FinGER proteins. Formerly, we were able to find that FinGER1 and FinGER2 interact with FinGER6, FinGER4 and FinGER5 interact with FinGER7 by using yeast two-hybrid analysis⁸⁾. Therefore, we extended the assay to find the interaction with other proteins that are known to function in the vesicular transport between the ER and the Golgi apparatus. Interestingly, we have found that ArfGAP1 showed reproducible interaction signal with FinGER7 and FinGER8. ArfGAP1 is localized on Golgi membrane and catalyzes GTP hydrolysis on Arf1, which is a small GTPase that promote COPI vesicle formation¹⁵⁾. Through GTPase activation of Arf1, ArfGAP1 was shown to control the dynamics of COPI vesicle formation and to be involved in the selection of cargo molecules into a COPI vesicle^{16)~18)}. In addition, it was recently shown that ArfGAP1 function as a sensor of lipid membrane curvature that is only activated on a highly curved transport vesicle membrane but not on a flat cisternal membrane¹⁹⁾²⁰⁾. Therefore, the interaction of FinGER7 and FinGER8 with ArfGAP1 imply that FinGER7 and FinGER8 are involved in the regulation of COPI vesicle formation or cargo sorting into a COPI vesicle.

Here, we developed a highly quantitative method for yeast two-hybrid analysis and tried to substantiate the interaction of FinGER7/FinGER8 and ArfGAP1 and define the interaction regions for both proteins.

Materials and Methods

Quantitative yeast two-hybrid analysis

This is based on MATCHMAKER Two-Hybrid System (Clontech, Takara Bio Inc., Ohtsu, Japan). pGBT9 and pGAD424 were used for the introduction of sample protein coding sequences and Y190 for the host strain. Y190 cells were transformed with test-pair plasmids by a standard method. Transformants were grown in an agar plate containing synthetic complete medium dropped out with leucine and tryptophan (SC-LW)²¹⁾. To circumvent the clonal variation, 10 colonies were mixed and grown on a SC-LW plate for three days. One loop-full of the cells was further grown in 5 ml of SC-LW liquid medium for 15 hours. 2 ml of the culture solution was taken and added to 8 ml of fresh SC-LW liquid medium and incubated for 3

hours. 1ml of the culture solution was taken and absorbance (OD₆₀₀) was measured. The sample was rejected when OD₆₀₀ was far more than 0.8 or far less than 0.5 (use only growing cells at mid-log phase). The sample was then centrifuged at 14,000 g for 30 seconds and the supernatant was removed. The resultant cell pellet was washed with 500 μ l of Z-buffer and suspended in appropriate amount of Z-buffer to be OD₆₀₀ = 2.5 (typically \sim 500 μ l; the measured absorbance of the cells culture suspension was used for the estimation). 100 μ l of the cell suspension was diluted with 900 μ l of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, [pH7.0]) and the absorbance was measured (OD_{600c}). 100 μ l of the cell suspension was rapidly frozen in liquid nitrogen and thawed on a water bath at 25 $^{\circ}$ C and the freeze-thaw cycle was repeated for two more times. 700 μ l of a reaction buffer (Z-buffer containing 0.27%(v/v) 2-mercaotoethanol) and 160 μ l of Z-buffer containing 4mg/ml o-nitrophenyl- β -D-galactopyranoside were added and incubated for appropriate time (typically, 4 hours; the reaction was linear for up to 16 hours). Reaction was stopped by adding 400 μ l of sodium carbonate. The samples were centrifuged for 10 minutes at 14,000 g, 4 $^{\circ}$ C and the supernatant was collected. The absorbance (OD₄₂₀) of the supernatant was measured. We used following parameters for our equipments to calculate the enzymatic activity. The ratio of yeast cell concentration to absorbance is: 4.3×10^7 cells/ml/OD₆₀₀. The ratio of o-nitrophenol concentration to absorbance is: 1.9×10^2 μ M/OD₄₂₀. Therefore, the β -galactosidase activity was calculated by the following formula: $6.1 \times 10^3 \times \text{OD}_{420} / t / \text{OD}_{600c}$ (Units; amol/min/cells). Where t is an incubation time (minutes). An experiment was done in triplicate.

Plasmid construction

The pGBT9 plasmid containing human ArfGAP1 coding sequence was kindly donated by Dr. Nakayama (Kyoto Univ.). FinGER7 and its mutants were cloned into pGAD424. Deletion mutants of ArfGAP1 and FinGER7 were produced by PCR with appropriate pairs of synthesized oligo-DNA. A point mutation was introduced by using an appropriate synthesized oligo-DNA and a single strand DNA containing a target sequence produced from pBluescript II KS+ or pcDNA3 as a vector²²⁾. All the mutant fragments were subsequently cloned and the sequences were checked.

Results and Discussions

Development of a quantitative yeast two-hybrid analysis

We encountered a fluctuation in the β -galactosidase activity for the same combination of test-plasmids among experiments and also among clones when the assay was performed using the original method using o-nitrophenyl- β -D-galactoside recommended by manufacturer

(Clontech). We found that (a) the selection of transformants with a dropout synthetic dextrose (SD) medium, which only contains minimum essential nutrients, and subsequent incubation of cells with a non-selective (YPD) medium resulted in a fluctuation in the induction level of the β -galactosidase activity among pairs of test-plasmids and also among experiments, (b) there was a clonal variation of the β -galactosidase activity for the same pair of test-plasmids and (c) the activity was higher for fresh transformants and became lower for older transformants. To circumvent these problems, we (a) used a synthetic complete (SC) medium, which are supplemented with non-essential nutrients²¹⁾, that induces a higher β -galactosidase activity without the incubation with non-selective (YPD) medium prior to the assay, (b) mixed about ten clones for averaging the clonal variation and (c) always used fresh (\sim 1 week old) transformants for the assay. As a result, we have been able to develop a more reproducible and quantitative assay (experimental details were described in Materials and Methods).

Specific interaction of ArfGAP1 and FinGER7/FinGER8.

We tried to find the interaction of rab1, rab6, GBF1 and ArfGAP1 with all the FinGER proteins (FinGER1–FinGER8) in two-way combinations (either fusion with an activator domain or a binding domain) using a yeast two-hybrid analysis and found that only ArfGAP1 showed significant signals with FinGER7 and FinGER8 (Fig. 1 and unpublished observation). Using the above-developed quantitative assay, we tried to substantiate

the interaction of ArfGAP1 and FinGER7/FinGER8. As is frequently observed in the yeast two-hybrid analysis, the activity was only observed for one way, i. e., only when ArfGAP1 fused with a binding domain (pGBT9) and FinGER proteins fused with an activation domain (pGAD424) (Fig. 1). This may be caused by a difference of the expression levels of proteins (a higher level expression was usually obtained for a binding domain fusion; unpublished observation) or structural limitations of fusion proteins. Although we could not detect significant signals in opposite combinations, the interactions of ArfGAP1 with FinGER7 and FinGER8 appeared to be specific because no significant activity was observed with other FinGER proteins (Fig. 1). No activity was also observed for ArfGAP2 or ArfGAP3 with any of the FinGER proteins confirming the specificity of the interaction (unpublished observation).

Analysis of the interacting region(s) of ArfGAP1 for FinGER7 and FinGER8.

We then tried to map the interacting region(s) of ArfGAP1 with FinGER7 and FinGER8. As shown schematically in Fig. 2 A, we produced series of N-terminal and C-terminal deletion mutants of ArfGAP1. Unexpectedly, some of the constructs gave a higher background activity with an empty activation domain vector (pGAD424) (Fig. 2B). Especially, a central region of the ArfGAP1 (153-257) showed a highest background activity. A C-terminal region (305-406) also gave a higher background activity. It was possible that these regions directly activate the transcription without the interaction with the activation domain. Nevertheless, it was possible to show specific interaction of the deletion mutants of ArfGAP1 with FinGER7 or FinGER8 by subtracting these background activities because the assay was highly quantitative and reproducible. As shown in Fig. 2 C, the pattern of the difference of activities among mutants was similar for FinGER7 and FinGER8 suggesting the mode and the interaction region(s) of ArfGAP1 were similar for FinGER7 and FinGER8. The removal of the 1st to 136th residues containing a GTPase activating domain did not affect the activity suggesting that this region is not necessary for the interaction (compare Full and Δ N137). Conversely, a fragment only containing the 1st to 148th residues (Δ C148) did not show a significant activity suggesting this region did not interact with FinGER7 or FinGER8. Similarly, deletion of the 258th to 406th residues did only slightly reduced the activity again suggesting that the deleted region is not necessary for the interaction (Δ C257). Conversely, a fragment only containing the 254th to 406th residues (Δ N254) did not show a significant activity suggesting this region did not interact with FinGER7 or FinGER8. On the other hand, fragments only containing the 137th to or the 153rd to 257th residues showed activities comparable to the full

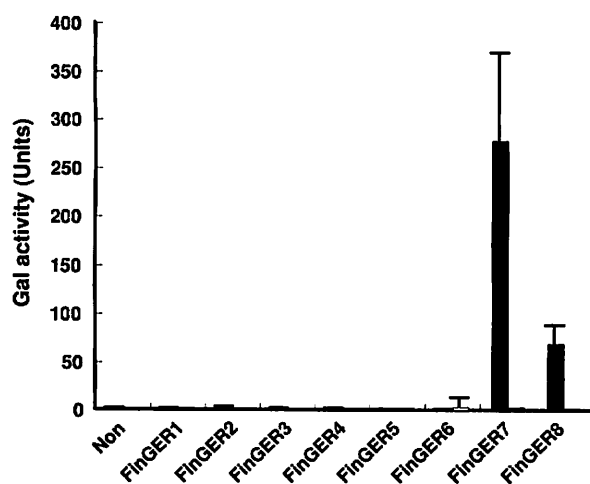


Fig. 1. Specific interaction of ArfGAP1 and FinGER7/FinGER8. Quantitative yeast two-hybrid analysis was performed as described in Materials and Methods. Triplicate assays were performed more than three times and the mean activities were shown (bars) with SD (error bars). ArfGAP1 was fused with an activation domain (open bars) or binding domain (solid bars). FinGER proteins tested were indicated at the bottom. Non: no-insert control.

length, Δ N137 or Δ C257 mutants of ArfGAP1, strongly suggesting that the region containing the 153rd to 257th residues is necessary and enough for the interaction of ArfGAP1 with FinGER7 or FinGER8. It was thought that this region most probably promoted the interaction directly. Further N-terminal or C-terminal deletion abrogated the interaction (Δ N177- Δ C257, Δ N137- Δ C231) suggesting that this region (153-257) is the minimum interaction domain.

Interestingly, the 153-257 region contains an ALPS domain (the 192nd to 231st residues), which was shown to form an amphipathic helix and proposed to sense the curvature of the membrane¹⁹. It was also reported that the same region is important for the Golgi localization of ArfGAP1^{23,24}. The residues between the 203rd and 215th are the core of the ALPS domain and also critical for the Golgi localization of ArfGAP1. Especially, the 211th tryptophan is shown to be critical for the Golgi targeting²⁴ and also for sensing curved membranes¹⁹. Therefore, we tried to analyze the effect of the mutation of the 211th tryptophan to an alanine (W211A) for FinGER7/FinGER8 interaction. Interestingly, W211A completely abolished the interaction (compare Δ N137- Δ C257 and Δ N137- Δ C257/W211A or Δ N153- Δ C257 and Δ N153- Δ C257/W211A). These results clearly indicated that the W211 is necessary for the interaction of ArfGAP1 and FinGER7/FinGER8.

Analysis of the interacting region(s) of FinGER7 for ArfGAP1

We next tried to analyze the region(s) of FinGER7 that is important for the interaction with ArfGAP1. Various deletion mutants of FinGER7 were produced (Fig. 3 A) and the interaction with a full length ArfGAP1 was analyzed. As shown in Fig. 3 B, the activity was preserved by deletion of N-terminal 30 residues (Δ N31). Then, sudden decrease was observed by further deletion of 11 residues (Δ N42) suggesting that a region from the 31st to the 41st residue is important for the interaction. We tried to delete the transmembrane region from the C-terminus. Removal of any of the putative transmembrane segments completely abolished the activity suggesting the transmembrane region is quite important for the interaction. It is possible that deletion of any parts of the transmembrane region hampers the protein to be properly inserted on the membrane, to take a proper conformation or topology, or to be transported to a proper subcellular destination for the interaction. Therefore, we tried to replace the whole transmembrane region of FinGER7 with that of other FinGER proteins to alleviate those effects. We first chose FinGER8, which is most similar to FinGER7 and showed a weaker interaction with ArfGAP1. As shown in Fig. 4, replacement of the transmembrane region or the cytoplasmic region of FinGER7 by those of FinGER8 (F7cyt-F8tm or F8cyt-F7tm) caused the reduction of activities near to the native FinGER8 level.

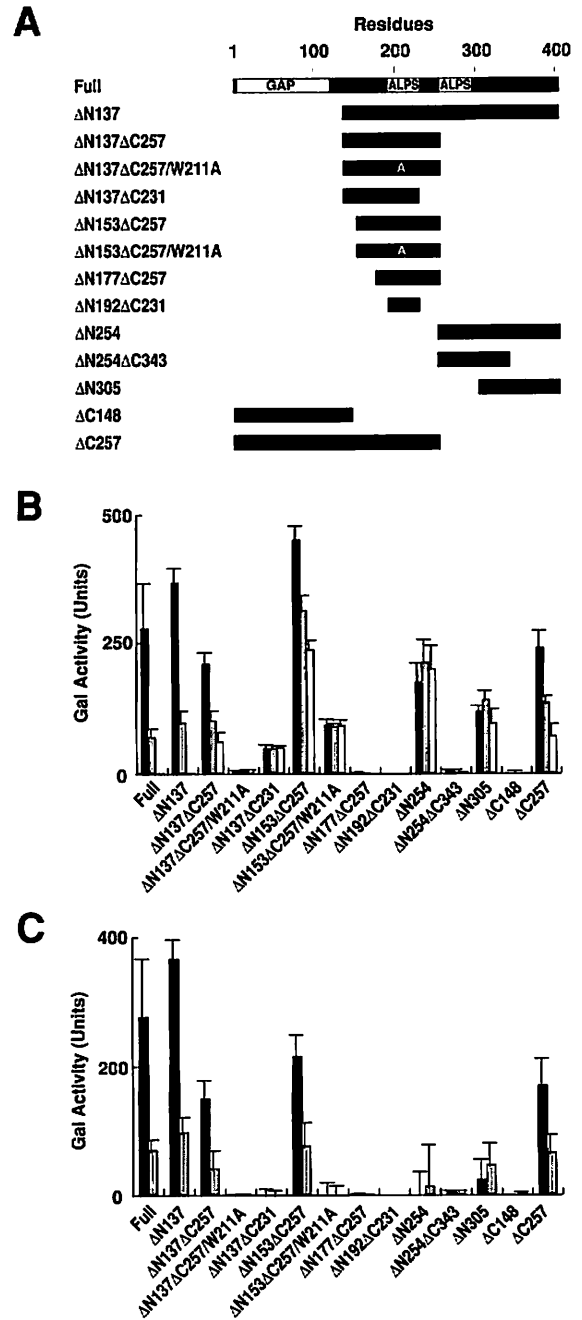


Fig. 2. Interaction of ArfGAP1 mutants with FinGER7/FinGER8 (A) Schematic representation of ArfGAP1 mutants. A GTPase activation domain (GAP) and ALPS domains were shown on the top full length ArfGAP1. The position of the W211A mutation was indicated on a bar. (B) Quantitative yeast two-hybrid analysis was performed as above and the raw activity was shown. Triplicate assays were performed more than two times and the mean activities were shown (bars) with SD (error bars). FinGER7 (solid bars), FinGER8 (gray bars) and control no-insert (open bars). Tested ArfGAP1 mutants as described in (A) were indicated at the bottom. (C) The results subtracted with the background activity were shown. The propagation of error (SD) was calculated and shown (error bars). FinGER7 (solid bars) and FinGER8 (gray bars). Tested ArfGAP1 mutants as described in (A) were indicated at the bottom.

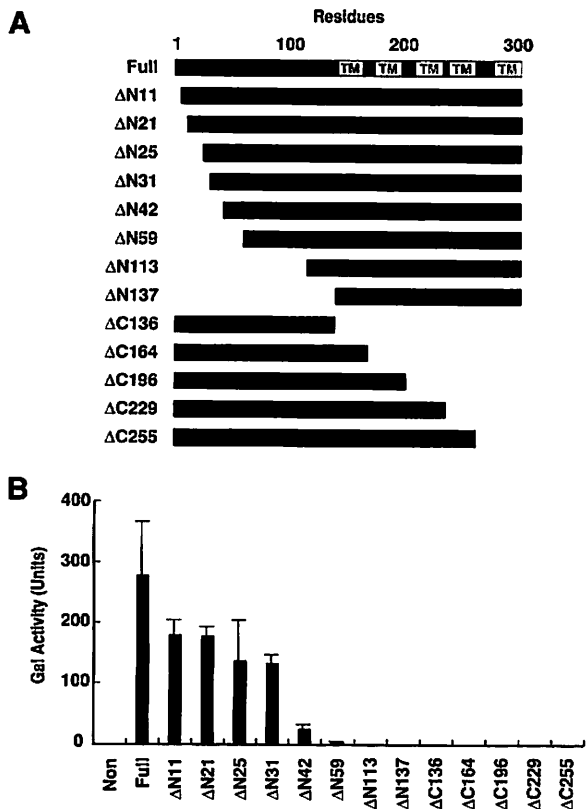


Fig. 3. Interaction of FinGER7 deletion mutants with ArfGAP1 (A) Schematic representation of FinGER7 mutants. Putative transmembrane segments were shown on the top full length FinGER7. (B) Quantitative yeast two-hybrid analysis was performed as above. Triplicate assays were performed more than three times and the mean activities were shown (bars) with SD (error bars). Tested FinGER7 mutants as described in (A) were indicated at the bottom. Non: no-insert control.

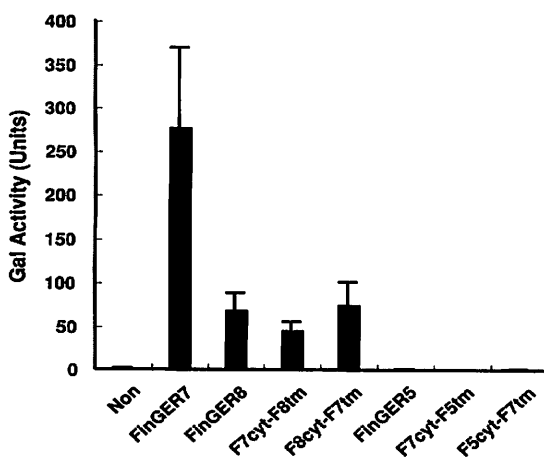


Fig. 4. Interaction of chimeric mutants of FinGER7 and FinGER8 or FinGER5 with ArfGAP1 Putative cytoplasmic region (F7cyt: 1-111) or transmembrane region (F7tm: 112-293) of FinGER7 was swapped with homologous regions of FinGER8 (F8cyt: 1-126, F8tm: 127-311) or FinGER5 (F5cyt: 1-112, F5tm: 113-257). Quantitative yeast two-hybrid analysis was performed as above. Triplicate assays were performed more than two times and the mean activities were shown (bars) with SD (error bars). Non: no-insert control.

We also tried to replace by FinGER5, which did not show interactions with ArfGAP1. FinGER5 interacts with FinGER7 and colocalizes in the same cellular compartment⁸⁾²⁵⁾ and probable to recreate the proper subcellular localization. As a result, no significant activity was observed for these mutants (F7cyt-F5tm or F5cyt-F7tm). The primary sequence of FinGER7 and FinGER8 was 54% identical (68 % similar) and is similarly divergent in both the putative cytoplasmic and transmembrane regions. On the other hand, the primary sequence of FinGER7 and FinGER5 was quite diverged (12% identical, 26% similar). Taken together these results, it was suggested that the cytoplasmic region and the transmembrane region cooperate for promoting the interaction with ArfGAP1 and the cytoplasmic region or the transmembrane region cannot recreate the higher interaction of FinGER7 and ArfGAP1 individually.

Concluding remarks

ArfGAP1 is proposed to function as a subunit of COPI coat and promote the disassembly of COPI coatomer. A detailed mode of functions is still under a debate but available evidences strongly suggest that it is involved in cargo selection or membrane deformation²⁶⁾²⁷⁾. Our finding that ArfGAP1 specifically interacts with FinGER7 and FinGER8 implies that FinGER7 and FinGER8 are also involved in the regulation of the COPI vesicle formation and/or cargo sorting into a COPI vesicle. In this aspect, the fact that FinGER7 interact with ArfGAP1 at the lipid curvature sensing domain overlapping with the Golgi targeting domain appeals for its significance. It is possible that FinGER7 and FinGER8 control the activity of ArfGAP1 to modulate the activation of Arf1 leading to the stimulation or inhibition of the COPI coat assembly on Golgi membranes. Elucidation of the function of FinGER proteins will provide a new insight for the regulation of vesicle formation and cargo selection.

Acknowledgements

We would like to thank Dr. Martin Lowe (University Manchester) for critically reading the manuscript, Mr. Masashi Kitamura, Mr. Gourou Fujii and Dr. Shin-ichiro Yoshimura (Max-Planck-Institute of Biochemistry) for experimental help and discussions, Drs. Akina Saito, Hye-Won Shin and Kazuhisa Nakayama for reagents and helpful discussions and all the member of Nakamura group for comments, discussions and encouragements. This work was supported by a Grant-in-Aid for Scientific Research (18570173), a Grant-in-Aid for Scientific Research on Priority Areas (16044218, 17028016) from MEXT of Japan and research grants from The Naito Foundation (2006). H. A. is a student of International Special Course of Graduate School of Natural Science and Technology, Kanazawa University and supported by the Scholarship for Research Student from MEXT (2004).

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