Biochem. J. (2002) **365**, 369–378 (Printed in Great Britain)

GGA proteins associate with Golgi membranes through interaction between their GGAH domains and ADP-ribosylation factors

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ADP-ribosylation factors (ARFs) are a family of small GTPases that are involved in various aspects of membrane trafficking events. These include ARF1–ARF6, which are divided into three classes on the basis of similarity in the primary structure: Class I, ARF1–ARF3; Class II, ARF4 and ARF5; and Class III, ARF6. Previous studies identified a novel family of potential ARF effectors, termed GGA1–GGA3, which interact specifically with GTP-bound ARF1 and ARF3 and are localized to the *trans*-Golgi network (TGN) or its related compartment(s) (GGA is an abbreviation for Golgi-localizing, γ-adaptin ear homology domain, ARF-binding protein). In the present study we have shown that ARF proteins belonging to the three classes, ARF1, ARF5 and ARF6, can interact with all GGA proteins in a yeast two-hybrid assay, *in vitro* and *in vivo*. Segmentation of GGA proteins and isolation of GGA mutants defective in ARF binding have revealed that a limited region within the GGA homology domain, which is conserved in the GGA family, is essential for ARF binding. Expression in cells of GTPase-restricted mutants of ARF1 and ARF5 blocks dissociation of GGA proteins from membranes induced by brefeldin A. However, neither of the ARF mutants recruits GGA mutants defective in ARF binding. On the basis of these observations, we conclude that at least ARF1 (Class I) and ARF5 (Class II) in their GTP-bound state cause recruitment of GGA proteins on to TGN membranes. In contrast, on the basis of similar experiments, ARF6 (Class III) may be involved in recruitment of GGA proteins to other compartments, possibly early endosomes.

Key words: γ-adaptin, brefeldin A, *trans*-Golgi network, yeast two-hybrid system.

INTRODUCTION

In eukaryotic cells, membrane trafficking along the exocytic and endocytic pathways is mediated primarily by vesicular transport. Coated carrier vesicles bud from a donor compartment and fuse with an acceptor compartment to deliver cargo molecules. The vesicle budding is initiated by recruitment of specific coat-protein complexes from cytosol on to donor membranes. Three classes of coated vesicles have been well characterized to date: COPII-, COPI- and clathrin-coated vesicles (for reviews, see [1–3]). COPII-coated vesicles are involved exclusively in anterograde transport from the endoplasmic reticulum (ER) to the ER–Golgi intermediate compartment [4]. COPI-coated vesicles are formed from the ER–Golgi intermediate compartment and the Golgi complex, and may be involved in retrograde transport [5–7]. The major constituents of clathrin-coated vesicles are clathrin and heterotetrameric adaptor protein (AP) complexes [3,8,9]. Four AP or AP-related complexes have been identified. The AP-1 complex is found mainly at the *trans*-Golgi network (TGN) and is responsible for delivery of lysosomal hydrolases. The AP-2 complex is found at the plasma membrane and is involved in internalization of receptors from the cell surface. The AP-3 complex appears to be involved in delivery of some proteins to lysosomes and related compartments. Little is known about the function of the AP-4 complex. Each AP complex is composed of two large subunits ($>$ 100 kDa) often called as adaptins, one medium subunit ($\approx$50 kDa) and one small subunit ($\approx$ 20 kDa): γ- and β1-adaptins, μ1 and σ1 in AP-1; α- and β2-adaptins, μ2 and σ2 in AP-2; δ- and β3-adaptins, μ3 and σ3 in AP-3; and ϵ-and β4-adaptins, μ4 and σ4 in AP-4 [3,8,9].

Recruitment of the AP complexes (except for AP-2) and COPI complex from cytosol on to membranes is triggered by membrane binding of a family of small GTPases, ADP-ribosylation factors (ARFs). In mammals, the ARF family members are divided into three classes based on sequence similarity: Class I (ARF1–ARF3); Class II (ARF4 and ARF5); and Class III (ARF6) (reviewed in [10,11]). Class I ARFs, especially ARF1, have been most extensively studied and shown to regulate the assembly of coat-protein complexes on to vesicle budding sites, including the COPI, AP-1, AP-3 and AP-4 complexes [12–18]. ARF6, the only member of Class III, functions in endosome–plasma membrane recycling system and in remodelling of the actin cytoskeleton [19]. Little is known about the roles of the Class II ARFs. Like other GTPases, ARF cycles between a GDP-bound inactive state and a GTP-bound active state. GDP-bound ARF is primarily cytosolic, although being weakly associated with membranes, whereas the active GTP-bound form binds tightly to membranes, where it encounters effectors. For instance, ARF1-GTP promotes formation of COPI-coated vesicles at the pre-Golgi intermediates and at the Golgi complex by inducing assembly of the COPI complex on to membranes and possibly by activating lipid mediators [10,11,20].

Recently, a family of potential ARF effectors, referred to as GGAs [Golgi-localizing, γ-adaptin ear homology (AGEH)

Abbreviations used: ER, endoplasmic reticulum; AP, adaptor protein; TGN, *trans*-Golgi network; ARF, ADP-ribosylation factor; GGA, Golgi-localizing, AGEH (γ-adaptin ear homology) domain, ARF-binding protein; VHS, Vps27p/Hrs/STAM; GGAH, GGA homology; GST, glutathione S-transferase; HA, haemagglutinin; BFA, brefeldin A; Q71L mutation (etc.), $Gln^{71}\rightarrow Leu$ (etc.); GTP[S], guanosine 5′-[γ-thio]triphosphate; WT, wild-type; EEA1, early endosomal autoantigen 1; Flag, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys.

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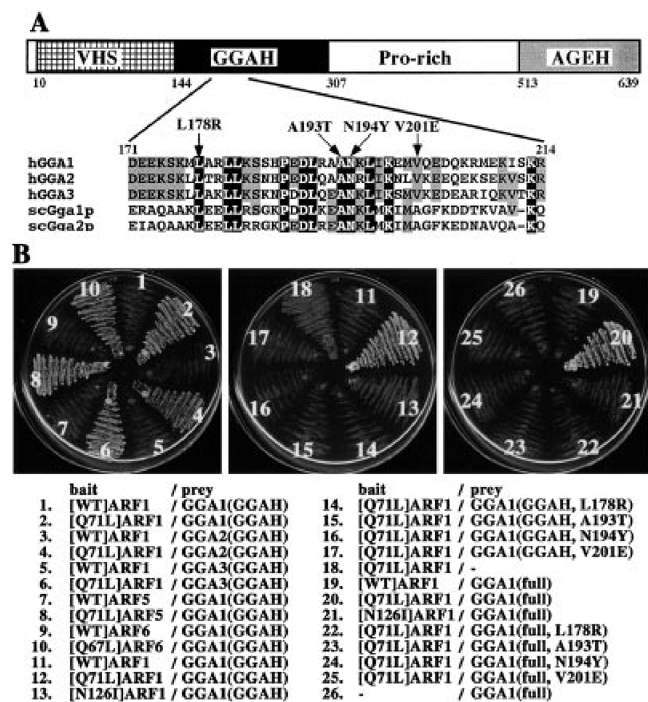

Figure 1 Yeast two-hybrid assays for interactions between ARFs and GGA proteins

(**A**) Schematic representation of the structure of GGA1 and alignment of the GGAH domain sequences of human (h) and *S. cerevisiae* (sc) GGA proteins. Amino acid residues conserved in all the GGA proteins are shown as white letters on a black background, and those conserved in at least three members are shown as black letters on a grey background. Positions of amino acid substitutions that result in defects in ARF binding are indicated. (**B**) Growth of yeast cells carrying an ARF bait vector and a GGA prey vector on histidine-deficient plates. Combinations of the bait and prey vectors are indicated under the growth images.

domain, ARF-binding proteins], have been identified in mammals [21–25] and in yeast [23,24,26]. The three mammalian (GGA1–GGA3) and two yeast (Gga1p and Gga2p) proteins have a common structural organization (see Figure 1A). The N-terminal $\approx$140-amino-acid region resembles the VHS (Vps27p/Hrs/STAM) domain (reviewed in [27]). Immediately downstream of the VHS domain there is a $\approx$160-amino-acid region conserved in the GGA family which we refer to as the GGAH (GGA homology) domain. The domain is responsible for binding to GTP-bound ARFs [22,24]. The C-terminal $\approx$130-amino-acid region is homologous with the ear domain of γ-adaptin, which we therefore refer to as the AGEH domain, and is able to interact with various cytosolic proteins such as γ-synergin and Rabaptin-5 [23,25,28]. The GGAH and AGEH domains are connected by a proline-rich region reminiscent of the hinge regions of adaptins. In mammalian cells, GGA proteins are associated with membranes of the TGN or a compartment apposed to the TGN [21–25]. Deletion of both the *GGA1* and *GGA2* genes in yeast causes defects in sorting of carboxypeptidase Y and Pep12p and in processing of invertase [23,24,26,29]. Furthermore, most recent studies from our [30,31] and other [32–35] laboratories have shown that the VHS domains of mammalian GGA proteins interact with the cytoplasmic domains of sorting receptors, including mannose 6-phosphate receptors, which cycle between the TGN and endosomes to deliver cargo molecules to lysosomes. These data indicate that GGA proteins are involved in membrane trafficking events from the TGN to endosomal/lysosomal compartments under the regulation of ARF.

In the present study we characterize the interaction between ARF and GGA proteins and show that association of GGA proteins with TGN membranes is regulated by ARFs.

EXPERIMENTAL

Plasmid construction

Q71L ($Gln^{71}\rightarrow$Leu) mutations of ARF1 and ARF5, a Q67L mutation of ARF6 and an N126I mutation of ARF1 were introduced into their cDNAs of mouse origin [36] by a PCR-based strategy. cDNA fragments covering the VHS+GGAH (amino acids 1–326), VHS (amino acids 1–147), GGAH (amino acids 141–326) and AGEH (amino acids 515–639) domains of human GGA1, and the GGAH domains of human GGA2 (amino acids 157–342) and GGA3S (amino acids 107–286) were amplified by PCR of their respective full-length cDNA fragments [25]. For two-hybrid analyses, the cDNA fragments for ARFs and GGAs were subcloned into the pGBT9 bait vector and the pGAD10 or pGAD424 prey vector (ClonTech) respectively. For expression in *Escherichia coli* as fusion proteins with glutathione S-transferase (GST), the cDNA fragments for the GGAH domains of GGA1, GGA2 and GGA3 were subcloned into the pGEX-4T-2 vector (Amersham Pharmacia Biotech). For expression in mammalian cells as proteins tagged with a haemagglutinin (HA) or Myc epitope sequence, the cDNA fragments for ARFs and GGAs were subcloned into the pcDNA3-HAC or pcDNA3-MycC [36] and pcDNA3-HAN [37] vectors respectively.

Antibodies

Monoclonal mouse antibodies to γ-adaptin (100.3) was purchased from Sigma. Monoclonal mouse antibodies to GM130 (clone 35) and EEA1 (clone 14) were from BD Transduction Laboratories. Monoclonal rat anti-HA antibody (3F10) and monoclonal mouse anti-Myc antibody (9E10) were from Roche Diagnostics and Santa Cruz Biotechnology respectively. Alexa-488-conjugated anti-mouse IgG was from Molecular Probes. Cy3-conjugated and peroxidase-conjugated anti-rat IgGs were from Jackson ImmunoResearch Laboratories.

Yeast two-hybrid analysis and reverse two-hybrid screening

Yeast two-hybrid analysis was performed as described previously [38–40]. Briefly, yeast Y190 cells were co-transformed with a pGBT9-based bait vector and pGAD-based prey vector, and were grown on synthetic medium lacking tryptophan and leucine. Colonies were picked up and streaked on to the same medium for a filter assay for β-galactosidase activity or on to medium containing 25 mM 3-aminotriazole and lacking tryptophan, leucine and histidine for a growth assay under histidine-deficient conditions.

To screen for mutants of the GGA1 GGAH domain that cannot interact with GTP-bound ARF, the cDNA fragment for the GGAH domain was randomly mutagenized by the error-prone PCR method. The fidelity of PCR was reduced by increasing the concentration of $MnCl_2$ in the reaction mixture as described in [41]. The mutagenized cDNA fragment was subcloned into the pGAD10 vector and transformed into Y190 cells harbouring the pGBT9 vector for [Q71L]ARF1. The transformed cells were plated on medium lacking tryptophan and leucine and

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Table 1 Interaction of ARF with GGA revealed by two-hybrid analysis

+ +, Colonies developing a blue colour within 15 min; +, colonies developing a blue colour within 3 h of incubation; −, colonies not developing a blue colour within 3 h.

			Development of blue colour	
No.	Bait	Prey	Growth on histidine-deficient plate	β-Galactosidase activity
1	[WT]ARF1	GGA1(full)	−	−
2	[Q71L]ARF1	GGA1(full)	+	+ +
3	[N126I]ARF1	GGA1(full)	−	−
4	[Q71L]ARF1	GGA1(VHS + GGAH)	+	+ +
5	[Q71L]ARF1	GGA1(VHS)	−	−
6	[Q71L]ARF1	GGA1(GGAH)	+	+ +
7	[Q71L]ARF1	GGA1(AGEH)	−	−
8	[Q71L]ARF1	GGA2(full)	+	+
9	[Q71L]ARF1	GGA2(GGAH)	+	+
10	[Q71L]ARF1	GGA3(full)	+	+ +
11	[Q71L]ARF1	GGA3(GGAH)	+	+ +
12	[WT]ARF5	GGA1(GGAH)	−	−
13	[Q71L]ARF5	GGA1(GGAH)	+	+ +
14	[WT]ARF6	GGA1(GGAH)	−	−
15	[Q67L]ARF6	GGA1(GGAH)	+	+
16	[Q71L]ARF1	GGA1(full, L178R)	−	−
17	[Q71L]ARF1	GGA1(GGAH, L178R)	−	−
18	[Q71L]ARF1	GGA1(full, A193T)	−	−
19	[Q71L]ARF1	GGA1(GGAH, A193T)	−	−
20	[Q71L]ARF1	GGA1(full, N194Y)	−	−
21	[Q71L]ARF1	GGA1(GGAH, N194Y)	−	−
22	[Q71L]ARF1	GGA1(full, V201E)	−	−
23	[Q71L]ARF1	GGA1(GGAH, V201E)	−	−

subjected to a filter assay for β-galactosidase activity. Colonies that developed a pale-blue colour or did not develop a blue colour after 18 h incubation with the β-galactosidase substrate, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside, were picked up and the selected pGAD10-based plasmid clones were subjected to sequence analysis.

Pull-down assay

The GGAH domain of each GGA protein fused to the C-terminus of GST was expressed in *E. coli* BL21(DE3) cells and purified using GSH–Sepharose 4B beads (Amersham Pharmacia Biotech Ltd.) as described previously [40]. Cell lysates were prepared from HEK-293 cells transfected with an HA-tagged ARF expression vector as follows. HEK-293 cells grown on a 10-cm-diameter plate were transfected with the ARF–HA expression vector using a FuGENE6 transfection reagent (Roche Diagnostics) and incubated for 24 h. The cells were scraped into 0.5 ml of homogenization buffer [25 mM Hepes/KOH (pH 7.2)/1 mM EDTA/1 mM $MgCl_2$/1 mM dithiothreitol/100 mM NaCl/0.1 % Triton X-100] containing a Complete™ protease-inhibitor mixture (Roche) by 90 strokes with a Dounce homogenizer. The homogenate was centrifuged at 800 ***g*** for 10 min at 4 °C in a microcentrifuge to remove unbroken cells. The supernatant was then centrifuged at 120000 ***g*** for 1 h at 4 °C and the supernatant was used for pull-down assays.

Cell lysates containing 60 μg of protein were preincubated with 200 μM GDP or the GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[S]) for 30 min and then with 10 μg of the GST-fusion protein prebound to GSH–Sepharose beads for 30 min at room temperature. The beads were washed three times with homogenization buffer and proteins were eluted from the beads by boiling in SDS/PAGE sample buffer, electrophoresed on an SDS/12.5 %-polyacrylamide gel, and blotted on to an Immobilon P membrane (Millipore). The blot was incubated sequentially with monoclonal rat anti-HA antibody and with peroxidase-conjugated anti-rat IgG, and detected using Renaissance Chemiluminescence Reagent *Plus* (NEN Life Science Products).

[^{35}S]Methionine-labelled wild-type ARF1 ([WT]ARF1) and [Q71L]ARF1 were prepared by *in vitro* translation and transcription of their cDNAs in pcDNA3-HAC using a TNT T7-coupled reticulocyte-lysate system (Promega) and an EXPRE^{35}S^{35}S protein labelling mix (NEN Life Science Products). The labelled proteins were pulled down with GST or GST–GGAH in the presence of GDP or GTP[S] as described above, subjected to SDS/PAGE and analysed using a BAS5000 bioimaging analyser (Fuji).

DNA transfection and immunofluorescence analysis

DNA transfection and immunofluorescence analysis were performed as described previously [25,38,42]. Briefly, HeLa cells grown in wells of eight-well Lab-Tek-II chamber slides (Nunc) were transfected with the expression vector for HA-tagged GGA alone or together with that for Myc-tagged ARF using the FuGENE6 reagent, incubated for 10–16 h, and processed for indirect immunofluorescence analysis. The transfected cells were fixed with 4 % (w/v) paraformaldehyde and permeabilized with 0.1 % Triton X-100 in PBS. Where indicated, the cells were treated with 5 μg/ml brefeldin A (BFA) for 2 min prior to fixation. The cells were then incubated sequentially with a combination of monoclonal rat anti-HA antibody and either monoclonal mouse anti-γ-adaptin or anti-Myc antibody, and with a combination of Cy3-conjugated anti-rat IgG and Alexa-488-conjugated anti-mouse IgG. The stained cells were observed using a confocal laser scanning microscope (TCS-SP2; Leica).

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RESULTS AND DISCUSSION

Interactions between GGAH domains and GTP-bound ARFs

Previous studies revealed an interaction between [Q71L]ARF3 and the GGAH domain of GGA3 [22] and that between [Q71L]ARF1 and the GGA3 GGAH domain [24]; the ARF mutants are defective in their intrinsic GTPase activity and are predominantly bound to GTP [43]. However, those studies did not systematically analyse the interaction between ARFs and GGA proteins. For example, they did not examine whether ARFs of Classes II and III can also interact with the GGAH domain. We therefore addressed this point. To this end, we took two approaches – the yeast two-hybrid assay and the pull-down assay using GST-fusion proteins.

To confirm the previously reported data [22,24], we first examined, using the two-hybrid system, whether only the GTPase-restricted mutant of ARF can interact with GGA proteins and whether the GGAH domain is responsible for the interaction with ARFs. As shown in Figure 1(B), streaks 19–21, and summarized in Table 1, rows 1–3, the full-length GGA1 construct showed an interaction with a GTPase-restricted mutant of ARF1 ([Q71L]ARF1), but not with [WT]ARF1 or its nucleotide-free mutant ([N126I]ARF1). Subsequently, segmentation of full-length GGA1 revealed that only the fragments containing the GGAH domain can interact with [Q71L]ARF1 (Table 1, rows 4–7).

We then tested whether [Q71L]ARF1 can also interact with GGA2 and GGA3. As shown in Figure 1(B), streaks 3–6, and in Table 1, rows 8–11, [Q71L]ARF1, but not [WT]ARF1, interacted with the full-length and GGAH domain constructs of both GGA2 and GGA3. Thus all GGA proteins can interact with the GTPase-restricted form of ARF1 through their GGAH domain.

Previous studies used ARF1 and ARF3, both of which belong to the Class I, for their interaction assays [22,24]. We therefore examined whether ARFs of the other two classes can also interact with GGA1. As shown in Figure 1(B), streaks 7–10, and in Table 1, rows 12–15, [Q71L]ARF5 and [Q67L]ARF6 also interacted with the GGAH domain of GGA1. In contrast, [WT]ARF5 or [WT]ARF6 did not show a significant interaction. Similarly, GTPase-restricted mutants of ARF5 and ARF6, but not their WT, interacted with the GGAH domains from GGA2 and GGA3 (results not shown). Thus interactions were observed in all the examined combinations of GTPase-restricted ARFs and the GGAH domains in the two-hybrid assays.

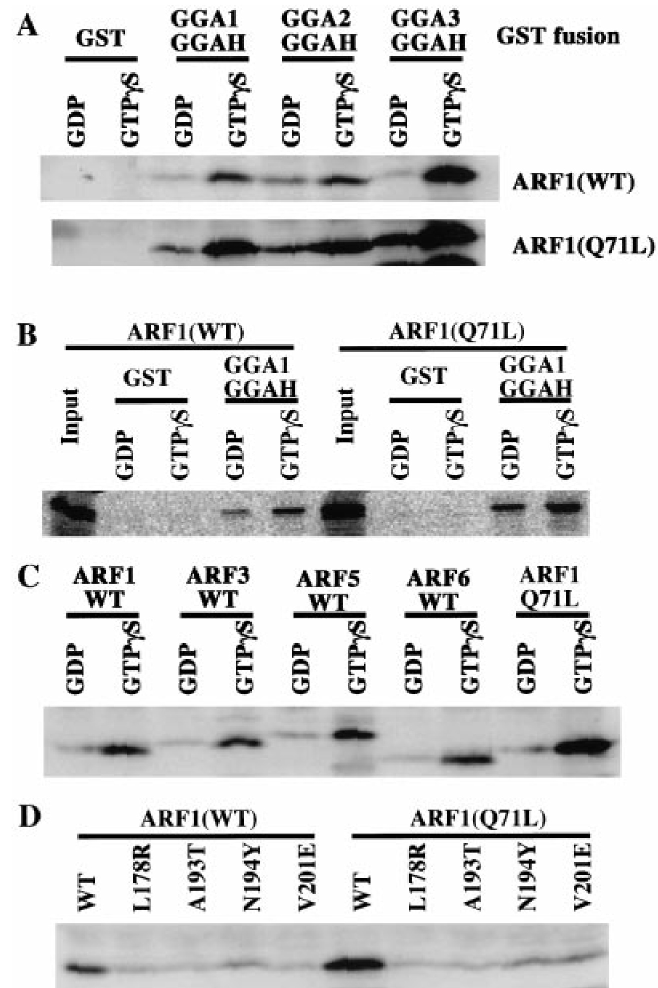

Figure 2 Pull-down assays for interactions between ARFs and the GGAH domains

(**A**) GST or GST fused with the GGAH domain from GGA1–GGA3 was prebound to GSH–Sepharose beads and incubated with lysates from HEK-293 cells transiently expressing HA-tagged [WT]ARF1 ['ARF(WT)', upper panel] or [Q71L]ARF1 ['ARF1(Q71L)', lower panel] in the presence of GDP or GTP[S] ('GTPγS'). The material bound to the beads was subjected to Western-blot analysis using the anti-HA antibody described in the Experimental section. (**B**) [^{35}S]Methionine-labelled [WT]ARF1 or [Q71L]ARF1prepared by *in vitro* transcription and translation was pulled down with the GST–(GGA1 GGAH domain) fusion protein prebound to GSH–Sepharose beads in the presence of GDP or GTP[S] and analysed as described in the Experimental section. (**C**) The GST–(GGA1 GGAH domain) fusion protein prebound to GSH–Sepharose beads was incubated with lysates from HEK-293 cells transiently expressing HA-tagged [WT]ARF1, [WT]ARF3, [WT]ARF5 or [WT]ARF6, or [Q71L]ARF1, in the presence of GDP or GTP[S], and the bound material was subjected to Western-blot analysis. (**D**) GST fused with WT GGA1 GGAH domain or either of its mutants isolated by reverse two-hybrid screening was prebound to GSH–Sepharose beads, incubated with lysates from HEK-293 cells transiently expressing HA-tagged [WT]ARF1 or [Q71L]ARF1 in the presence of GTP[S]. The bound material was subjected to Western-blot analysis.

We then examined the interactions biochemically. The GST-fusion protein of the GGAH domain of GGA1, GGA2 or GGA3 was expressed in *E. coli* and purified with GSH–Sepharose beads. The fusion protein prebound to GSH–Sepharose beads was incubated in the presence of GDP or GTP[S] with lysates from cells transiently expressing [WT]ARF or its GTP-bound mutant tagged with HA. The materials bound to the beads were then subjected to Western-blot analysis using anti-HA antibody. As shown in Figure 2(A), upper panel, [WT]ARF1 was efficiently pulled down with the GGAH domain fusions of GGA1, GGA2 and GGA3 in the presence of GTP[S]. In contrast, the pull-down efficiencies were very low in the presence of GDP, and GST as a negative control did not pull down ARF1, even in the presence of GTP[S]. When lysates from cells expressing [Q71L]ARF1-HA were used, the pull-down efficiencies were significantly increased in the presence of GTP[S] (Figure 2A, lower panel; note that the images shown in the upper and lower panels are derived from the same blot at the same exposure time). Furthermore, the efficiencies in the presence of GDP were also increased. This might be caused by the fact that the GTP molecules associated with the mutant ARF protein in the cell lysates were not hydrolysed to GDP because of its lack of intrinsic GTPase activity.

Because detection of the pulled-down proteins by chemiluminescence–Western blotting is often non-quantitative – although it is an effective procedure to detect interactions qualitatively – radiolabelled ARF1 was prepared by *in vitro* transcription and translation in the presence of [^{35}S]methionine, pulled down with GST–GGA1 GGAH domain in the presence of GDP or GTP[S] and autoradiographically detected after SDS/PAGE (Figure 2B). Estimation of the band densities using a bioimaging analyser

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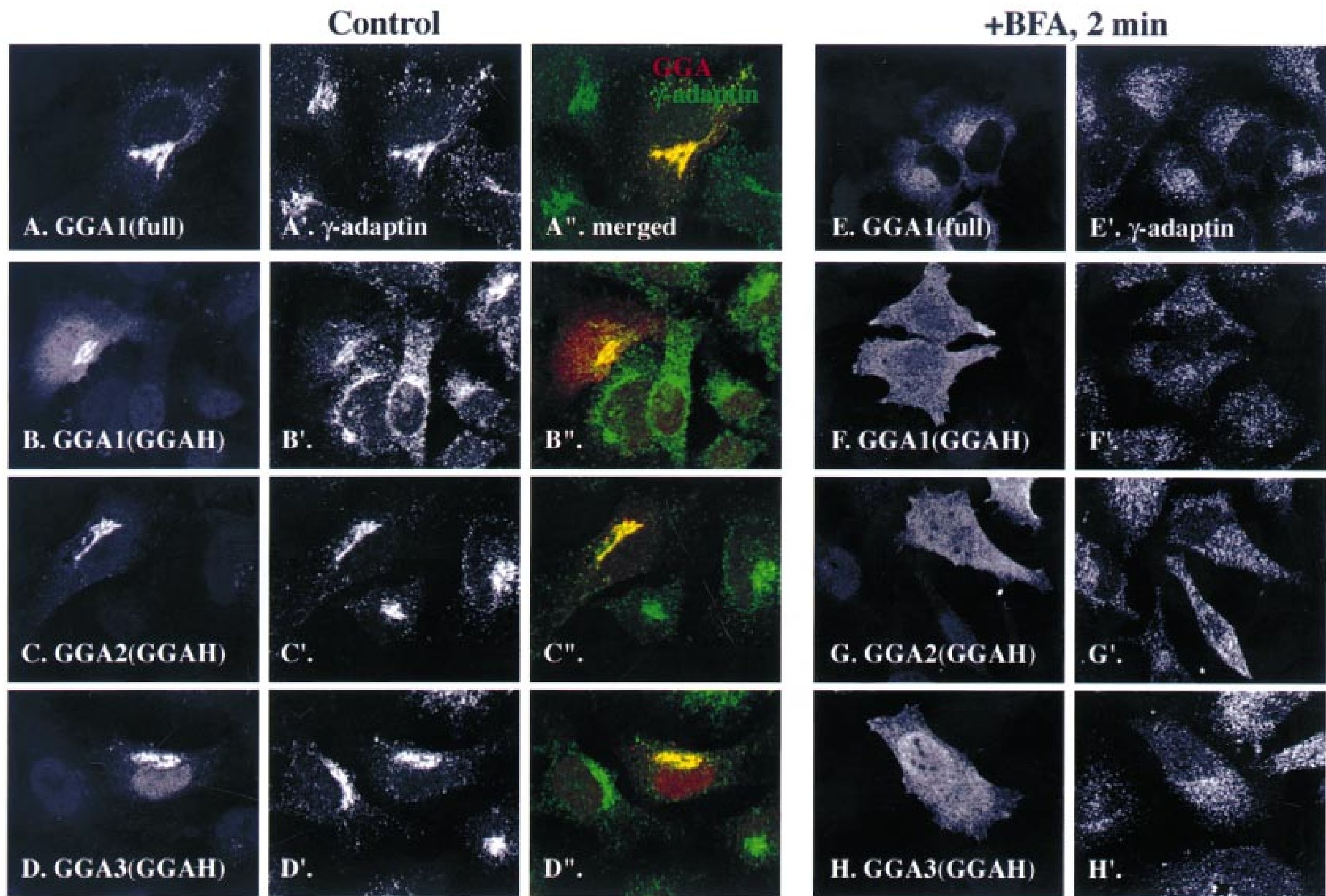

Figure 3 Immunofluorescence analysis of the localizations of GGA1 and GGAH domains and their sensitivity to BFA

HeLa cells transiently transfected with an expression vector for HA-tagged full-length GGA1 (**A** and **E**) or the GGAH domain from GGA1 (**B** and **F**), GGA2 (**C** and **G**) or GGA3 (**D** and **H**) were left untreated (**A**–**D**) or treated with 5 μg/ml BFA for 2 min (**E**–**H**), and double-stained with antibodies to HA (**A**–**H**) and γ-adaptin (**A′**–**H′**) as described in the Experimental section. Merged images are shown in (**A′′**)–(**D′′**). Red, GGA; green, γ-adaptin; yellow shows the overlap.

revealed that, in the presence of GTP[S], [WT]ARF1 was pulled down about 4-fold more efficiently than that in the presence of GDP. In the case of ARF1[Q71L], the pull-down efficiency in the presence of GTP[S] approximately doubled as compared with that in the presence of GDP. The data indicate that the chemiluminescence detection of the pulled-down ARF proteins is, to some extent, quantitative.

Subsequently we examined the specificity of the GGA1 GGAH domain towards the ARF isoforms by the pull-down assay. As shown in Figure 2(C), all ARF isoforms examined, namely ARF1 and ARF3 (Class I), ARF5 (Class II) and ARF6 (Class III), were efficiently pulled down with the GGAH domain fusion in the presence of GTP[S].

Taken together, the two-hybrid and pull-down data indicate: (i) that all GGA proteins are able to interact with GTP-bound ARFs; (ii) that the GGAH domain is sufficient for ARF binding; and (iii) that the domain interacts with all ARF isoforms examined at comparable efficiencies.

GGA is recruited on to the TGN by virtue of the interaction with GTP-bound ARF through its GGAH domain

We then set out to examine whether GGA proteins are recruited on to TGN membranes in an ARF-dependent manner or GGA proteins mediate recruitment of ARFs. Previous studies have shown that the GGAH domain is sufficient for targeting of GGA2 [21] and GGA3 [24] to the TGN. By immunofluorescence analysis of cells transfected with HA-tagged GGA constructs, we reproduced the data and demonstrated that this was also the case with GGA1. As shown in Figure 3, similar to full-length GGA1 (Figure 3A), the GGAH domain of GGA1 (Figure 3B) was localized to perinuclear structures. The localization overlapped with that of γ-adaptin, a subunit of the TGN-associated clathrin adaptor complex, AP-1, in the perinuclear region (Figures 3A–3A′′ and 3B–3B′′), indicating that the GGAH domain contains sufficient information for targeting of GGA1 to TGN membranes. Similarly, the GGAH domains from GGA2 and GGA3 were localized to TGN-like perinuclear structures (Figures 3C–3C′′ and 3D–3D′′ respectively).

To examine the involvement of ARF in the recruitment of the GGAH domains to TGN membranes, the cells expressing the GGAH domain were treated with BFA. Sensitivity to BFA of membrane association of peripheral Golgi proteins has shown to reflect their *in vivo* interaction with activated ARFs, because this fungal metabolite inhibits guanine nucleotide exchange factors for ARFs, thereby interfering with membrane association of ARFs (reviewed in [44]). Furthermore, peripheral membrane proteins are dissociated into the cytoplasm by relatively short

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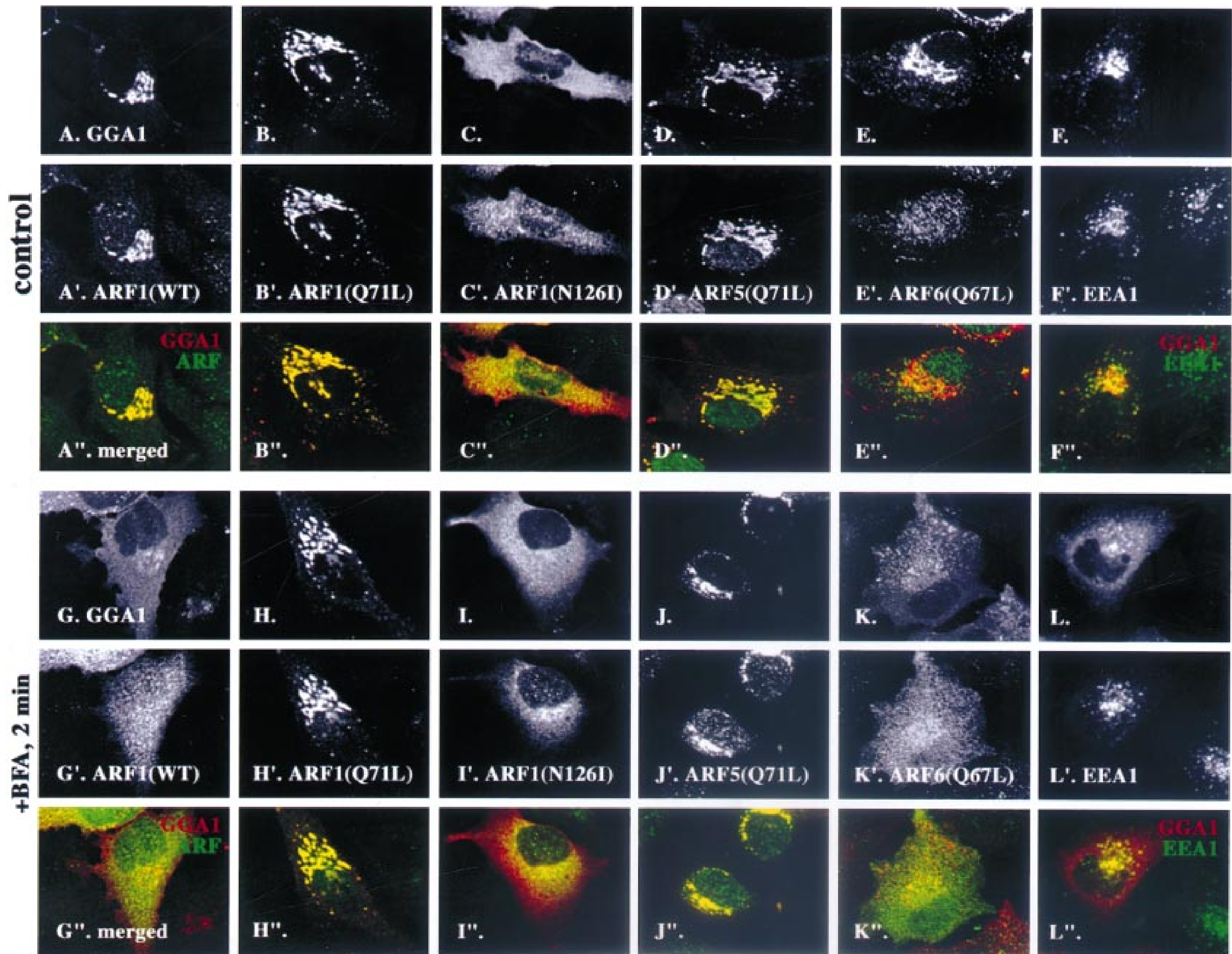

Figure 4 Effects of ARF expression on the localization and BFA-sensitivity of GGA1

HeLa cells transiently transfected with the expression vector for HA–GGA1 along with that for Myc-tagged [WT]ARF1 (**A** and **G**), [Q71L]ARF1 (**B** and **H**), [N126I]ARF1 (**C** and **I**), [Q71L]ARF5 (**D** and **J**) or [Q67L]ARF6 (**E**, **F**, **K** and **L**) were left untreated [**A**–**F**] or treated with 5 μg/ml BFA for 2 min (**G**–**L**) and double-stained with antibodies to HA (**A**–**L**) and either Myc (**A′**–**E′** and **G′**–**K′**) or EEA1 (**F** and **L**). Merged images are shown in (**A″**)–(**L″**). Red, GGA; green, ARF or EEA1; yellow shows the overlap.

BFA treatment (< 30 s), whereas redistribution of integral Golgi membrane proteins to the ER mediated by membrane tubules requires much longer treatment (> 15 min) (reviewed in [5,44,45]). As shown in Figures 3(E)–3(H), all the GGA constructs tested, full-length GGA1 and the GGAH domains from GGA1–GGA3, were redistributed into the cytoplasm within 2 min of treatment with 5 μg/ml BFA. This observation makes it likely that association of GGA proteins with the TGN is dependent on the interaction between the GGAH domain and activated ARF.

To support the above speculation, we performed the following experiments. Cells were first co-transfected with an expression vector for HA–GGA1 and that for either [WT]ARF1 or [Q71L]ARF1 tagged with a Myc epitope, and in turn treated with BFA. Because [Q71L]ARF1 is restricted to a GTP-bound state [43], this mutant expressed in cells was expected to antagonize the effects of BFA [46,47]. As shown in Figure 4, the staining for GGA1 in the [Q71L]ARF1-expressing cells (Figure 4B) was similar to that in cells expressing [WT]ARF1 (Figure 4A), although the Golgi-like structures were rather fragmented in the cells expressing the ARF1 mutant. The ARF1-positive structures represent the Golgi complex, since the ARF1 staining was superimposed on that for GM130 (Figures 5A–A″ and 5B–B″), a Golgi matrix protein [48]. In contrast, when treated with BFA, the GGA1 staining patterns in the cells expressing wild-type and mutant ARF1 were different from each other. In cells expressing [WT]ARF1, both GGA1 and ARF1 were redistributed into the cytoplasm (Figures 4G–G″), whereas in the [Q71L]ARF1-expressing cells, both GGA1 and the ARF1 mutant remained associated with the Golgi-like structures (Figures 4H–4H″). Similar results were obtained with a longer BFA treatment (15 min; results not shown). Thus the association of GGA1 with the TGN appears to be dependent on GTP-bound ARF1. Moreover, the notion of an ARF-dependent association of GGA1 with the TGN was supported by an experiment using cells expressing [N126I]ARF1, which is defective in GTP-binding and dominant-negatively affects coat-protein association [43]. Expression of the dominant negative mutant redistributed not only GGA1 (Figures 4C–4C″), but also GM130 (Figures 5C–5C″)

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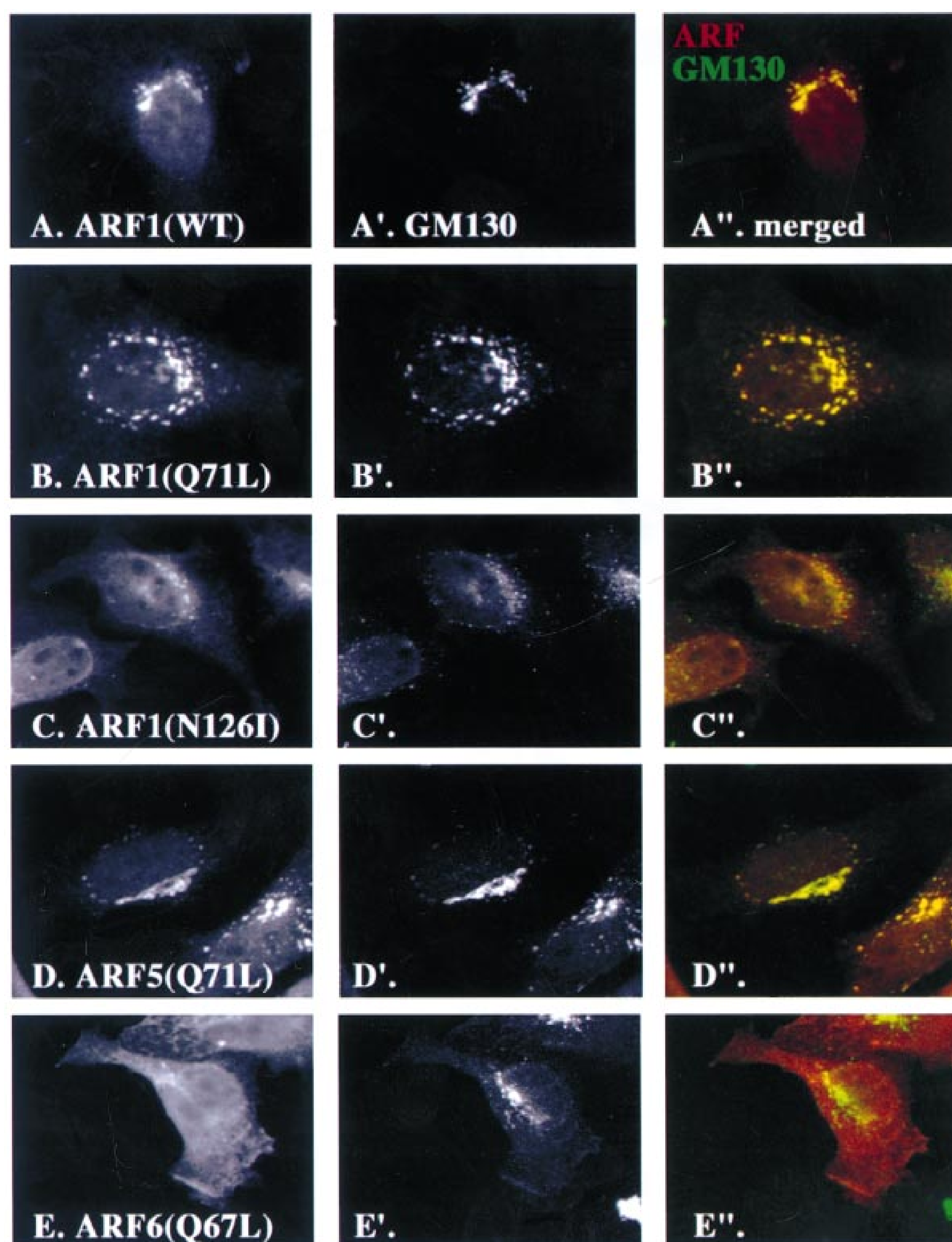

Figure 5 Comparison of ARF localization with that of GM130

HeLa cells transiently transfected with the expression vector for HA-tagged [WT]ARF1 (**A**), [Q71L]ARF1 (**B**), [N126I]ARF1 (**C**), [Q71L]ARF5 (**D**) or [Q67L]ARF6 (**E**) were double-stained with antibodies to HA (**A**–**E**) and GM130 (**A′**–**E′**). Merged images are shown in (**A′′**)–(**E′′**). Red, ARF; green, GM130; yellow shows the overlap.

and γ-adaptin (results not shown). Experiments were also performed using cells co-transfected with a construct for either ARF mutant and a construct for the GGAH domain of either GGA1, GGA2 or GGA3, and essentially the same results were obtained (results not shown). Taken together, these observations are consistent with the notion that GTP-bound ARF1 recruits GGA proteins on to TGN membranes through its interaction with the GGAH domain. Furthermore, this notion is confirmed by experiments using GGAH domain mutants defective in ARF binding (see below).

Next, we examined whether ARFs belonging to the other two classes, ARF5 (Class II) and ARF6 (Class III), were also able to recruit GGA proteins *in vivo*. Expression of [WT]ARF5 or [WT]ARF6 did not alter the GGA1 localization (results not shown). In cells expressing [Q71L]ARF5, the staining for GGA1 was found in the perinuclear region and superimposed on that for the ARF5 mutant (Figures 4D–4D′′). Again, the [Q71L]ARF5 staining overlapped that for GM130 (Figures 5D–5D′′). Treatment of the [Q71L]ARF5-expressing cells with BFA did not significantly affect the localization of the ARF5 mutant or GGA1 (Figures 4J–4J′′). Similar effects of [Q71L]ARF5 expression were observed for GGA2 and GGA3 localization (results not shown). These observations indicate that, like ARF1 (Class I), ARF5 (Class II) in its GTP-bound state is also capable of recruiting GGA proteins *in vivo*. In contrast, the results obtained using [Q67L]ARF6-expressing cells were somewhat different. In

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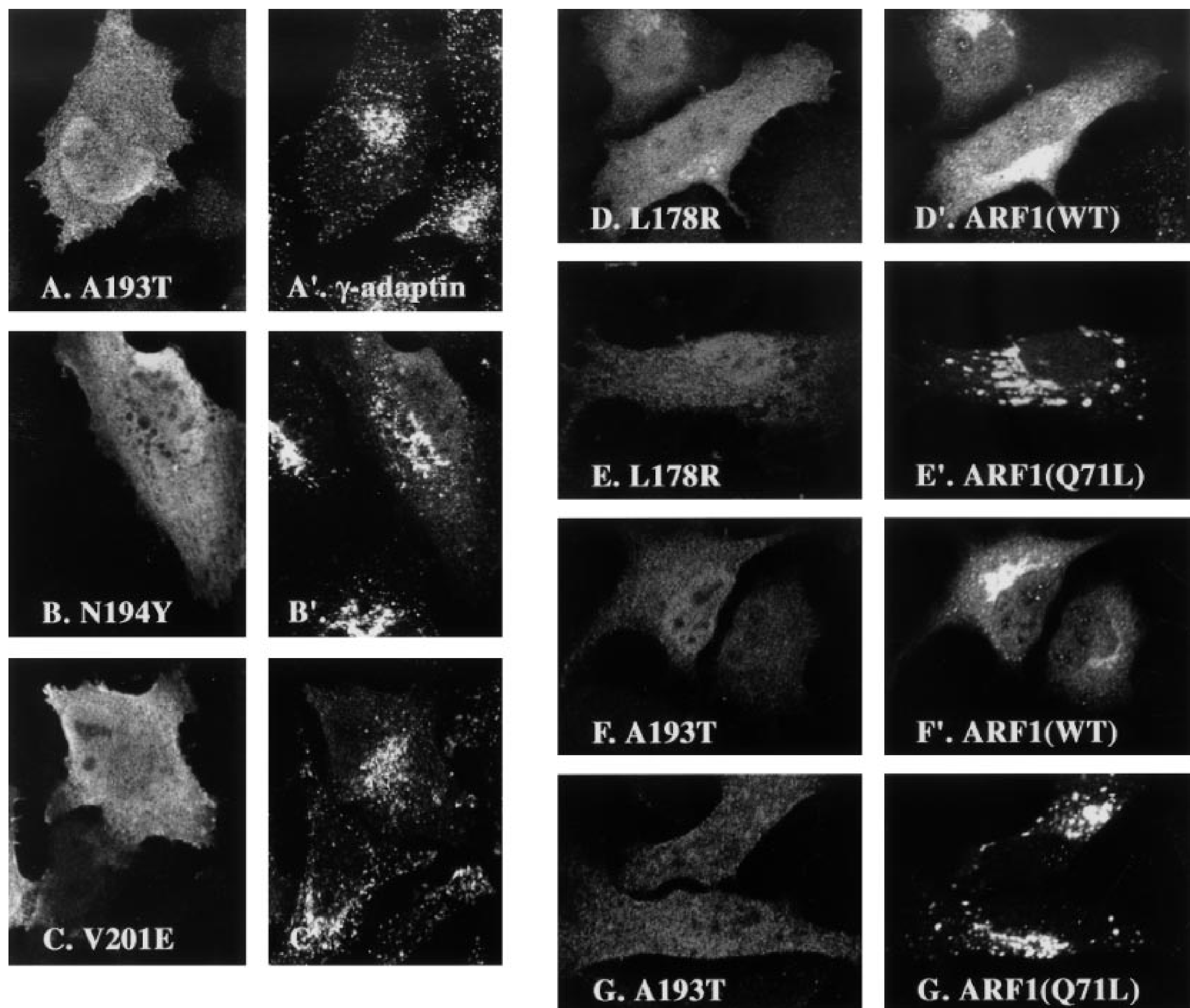

Figure 6 Localization of GGA1-GGAH-domain mutants defective in ARF binding

(**A**–**C**) HeLa cells transiently transfected with the expression vector for the HA-tagged GGA1 GGAH domain with an A193T (**A**), N194Y (**B**) or V201E (**C**) substitution were double-stained with antibodies to HA (**A**–**C**) and γ-adaptin (**A′**–**C′**). (**D**–**G**) HeLa cells transiently transfected with the expression vector for HA-tagged GGA1 GGAH domain with an L178R (**D** and **E**) or A193T (**F** and **G**) substitution in combination with the vector for Myc-tagged [WT]ARF1 (**D** and **F**) or [Q71L]ARF1 (**E** and **G**) were double-stained with antibodies to HA (**D**–**G**) and Myc (**D′**–**G′**).

these cells, in addition to typical Golgi-like structures, punctate structures throughout the cytoplasm and the cell periphery were also stained for GGA1 (Figure 4E). This may be implicated in the functions of ARF6, namely regulation of endocytic recycling, cytoskeletal organization and cell motility. When the [Q67L]ARF6-expressing cells were treated with BFA, GGA1 was no longer associated with Golgi-like structures and it was largely cytosolic (Figure 4K). These observations suggest that ARF6 (Class III) is not involved in recruitment of GGA proteins to Golgi compartments. However, punctate structures containing GGA1, reminiscent of endosomes, were still observed in the [Q67L]ARF6-expressing cells (Figure 4K). The structures may represent early endosomes, since the punctate staining for GGA1 overlapped that for EEA1 (early endosomal autoantigen 1) when the [Q67L]ARF6-expressing cells were treated with BFA (Figures 4L–4L′′). Taken together with the two-hybrid and pull-down data, it is possible that ARF6 could regulate functions of GGA proteins in compartment(s) other than the Golgi, probably early endosomes.

Isolation and characterization of GGAH domain mutants defective in ARF binding

We then set out to isolate GGAH domain mutants that cannot interact with ARFs in order to delineate the specific region and amino acid residues within the domain that are responsible for ARF binding. To this end, a cDNA fragment covering the GGA1 GGAH domain was subjected to error-prone PCR, subcloned into the two-hybrid prey vector and transformed into reporter yeast cells harbouring the bait vector for [Q71L]ARF1. By a filter assay for β-galactosidase activity, clones that developed a pale-blue colour or did not develop a blue colour were selected.

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Sequence analysis revealed that several of the clones thus selected had frameshift mutations, and dozens had single miss-sense mutations or double or triple mutations. To avoid confusion, we thereafter focused on the mutants with frameshift mutations and with single miss-sense mutations. One of the frameshift mutants, which did not develop a blue colour, had one nucleotide deletion at the codon for Phe^{168}, and another, which did develop a pale-blue colour, had a deletion at the codon for Lys^{226} (results not shown), indicating at least the 58-amino-acid region within the GGAH domain is essential for interaction with GTP-bound ARF. Four miss-sense mutants (L178R, A193T, N194Y and V201E) had single nucleotide substitutions that resulted in amino acid substitutions within this region (see Figure 1A). None of the amino acid substitutions were expected to significantly alter the local conformation of the domain, according to the Chou–Fasman secondary-structure prediction program and the Lupas coiled-coil structure prediction program. As shown in Figure 1(B), streaks 14–17, and summarized in Table 1, rows 17, 19, 21 and 23, yeast cells harbouring the prey vector for either of the GGAH domain mutants along with the [Q71L] ARF1 bait vector did not grow on a histidine-deficient plate or exhibit β-galactosidase activity. The same results were obtained with the full-length GGA1 construct containing either of the amino acid substitutions (Figure 1B, streaks 22–25, and Table 1, rows 16, 18, 20 and 22). Among the four mutations, three (L178R, A193T and N194Y) were at residues conserved in all the human and yeast GGA proteins, and the other (V201E) was at a residue conserved in all human GGAs (Figure 1A). While the present study was in progress, Puertollano et al. [49] reported that mutation of Asn^{194} of the GGA3 GGAH domain, which corresponds to Asn^{194} of GGA1, to alanine abolished its interaction with [Q71L]ARF1, this finding being in good agreement with our own. These results suggest that these residues are indispensable for GGA function.

The two-hybrid data were corroborated biochemically. As shown in Figure 2(D), even in the presence of GTP[S], [WT]ARF1 and [Q71L]ARF1 were pulled down at extremely low efficiencies using the GST–GGAH domain mutants as compared with those using the WT GGAH domain.

ARF binding and Golgi association of the GGAH domain correlate with each other

We examined whether binding to ARF was a prerequisite for association of the GGAH domain with the TGN by exploiting the domain mutants. As shown in Figure 6, all the GGAH domain mutants, L178R (Figure 6D), A193T (Figure 6A), N194Y (Figure 6B) and V201E (Figure 6C), were found in the cytoplasm, which is quite different from the localization of the WT GGAH domain (Figure 3B). Furthermore, neither [WT] ARF1 nor [Q71L]ARF1 co-expressed gave rise to recruitment of the GGAH domain mutants to the Golgi region (Figures 6D–6G), indicating that the GGAH domain mutants cannot interact with GTP-bound ARF1 *in vivo*. Thus binding to ARF and localization to the TGN of the GGAH domain correlate with each other.

Conclusions

In the present study we have shown that all GGA proteins can interact with all classes of ARF proteins through their GGAH domains. Furthermore, by immunofluorescence analyses of cells co-expressing either the GGA protein or its mutant defective in ARF binding and either WT or GTPase-restricted mutant of ARF, we conclude that at least Class I and Class II ARFs in their GTP-bound state, but not ARF6 (Class III), recruit GGA proteins on to the TGN by interacting with the GGAH domains. ARF6 might be implicated in recruitment of GGAs on to compartment(s) reminiscent of early endosomes, although the physiological significance of the recruitment is currently unclear.

While the present study was in progress, Puertollano et al. [49] reported that binding of GGA to ARF1 interferes with the activity of GTPase-activating protein, probably due to its sharing the same binding site on ARF1, and transiently stabilizes GTP-bound ARF1 on TGN membranes. Our results suggest that this is also the case for ARF5 and, possibly, for ARF6.

We thank Dr Natsuko Yahara for technical advice. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, from the Japan Society for Promotion of Science, and from the University of Tsukuba Research Projects. H.T. is a recipient of a fellowship from the Japan Society for Promotion of Science for Japanese Junior Scientists.

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Received 18 March 2002; accepted 12 April 2002
Published as BJ Immediate Publication 12 April 2002, DOI 10.1042/BJ20020428

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