Specific depletion of GGA2 causes cathepsin D missorting in HeLa cells*

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Summary. Three mammalian GGAs (Golgi-localized, γ-ear-containing, ARF-binding proteins), GGA1, 2, and 3 have been implicated in the sorting of mannose 6-phosphate receptor (MPR). To investigate the distinct roles of GGA2 in lysosomal enzyme transport, we established two stable cell lines that had a reduced expression of GGA2 by RNA interference. The expression levels of GGA2 were approximately 5% of the control levels, whereas those of non-targeted GGA1 and GGA3 were not apparently reduced. The depletion of GGA2 did not cause changes in the overall distribution of GGA1, GGA3, cation-dependent MPR, or cation-independent MPR. However, the cell lines showed increased secretion of a lysosomal enzyme, cathepsin D. In addition, a moderate expression of the dominant negative VHS-GAT domain of GGA2 had no effect on the trans-Golgi network (TGN) signal of three GGAs, nor was the GGA2 signal affected by the expression of VHS-GAT domain of GGA1 or 3. These results suggest that GGA2 is recruited to the TGN independently of the other GGAs and is required for the efficient sorting of lysosomal enzymes.

Introduction

The Golgi-localized, γ-ear-containing, ADP ribosylation factor (ARF)-binding proteins (GGAs) constitute a family of monomeric clathrin adaptor proteins that are involved in the trans-Golgi network (TGN) to endosome trafficking of the mannose 6-phosphate receptors (MPRs). Three GGAs (GGA1, 2, and 3) exist in humans and share a common structural organization composed of four domains: an N-terminal VHS (VPS27, Hrs and STAM) domain, followed by a GAT (GGA and TOM1) domain, a hinge domain, and a GAE (c-terminal γ-adaptin ear) domain (Bonifacino, 2004; Ghosh and Kornfeld, 2004). The GGAs bind to DXXLL motif of MPRs via the VHS domain (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001). The GAT domain binds to a small GTPase ARF1 that is essential for the association of GGAs to the TGN membrane (Dell’Angelica et al., 2000; Puertollano et al., 2001; Boman et al., 2002; Takatsu et al., 2002). Also, the c-terminus of this domain (C-GAT) can bind ubiquitin, which may be implicated in the sorting of ubiquitinated cargo (Puertollano and Bonifacino, 2004; Shiba et al., 2004). The hinge domain mediates the interaction with clathrin and the γ-adaptin ear of adaptor protein complex-1 (AP1) (Puertollano et al., 2001; Zhu et
al., 2001; Doray et al., 2002b). Finally, the GAE domain interacts with several accessory proteins such as γ-synergin and rabaptin-5 (Hirst et al., 2000; Takatsu et al., 2000; Lui et al., 2003; Mattera et al., 2003; Shibata et al., 2004).

Although the three GGAs in humans appear to behave similarly, they also have some differences: 1) GGA1 and 3, but not GGA2, have an internal DXXLL motif that binds to the self VHS domain, exerting a previously proposed autoinhibition mechanism in binding to the cargo membrane proteins (Doray et al., 2002a; Ghosh and Kornfeld, 2003); 2) the crystal structure of a portion of the VHS domain of GGA2 significantly differs from that of GGA1 or 3 (Zhu et al., 2003); 3) the amino acid sequence in the C-GAT region of GGA2 is different from that of GGA1 or GGA3, and in fact, the binding affinity of GGA2 to ubiquitin is quite low (Perrone and Bonifacino, 2004; Scott et al., 2004; Shibata et al., 2004); 4) the short splicing variant of GGA3, whose VHS domain is unable to bind to the DXXLL motif, is predominantly expressed in human cell lines and tissues except the brain (Wakasugi et al., 2003); and 5) GGA2 and 3 undergo apical growth factor (EGF)-induced phosphorylation (Kamitaka et al., 2005). These results suggest the possibility that each GGA possesses its own distinct roles.

As to whether they act together or function independently, a recent study showed that the three GGAs do interact with each other and that RNA interference (RNAi) of each GGA resulted in decreased levels of the others and their redistribution from the TGN to cytosol, indicating that the threeGGAs act together to sort MPRs (Ghosh et al., 2003). However, a more recent study revealed that this is not the case; depletion of any one GGA does not affect the levels or TGN localization of the other two (Mardones et al., 2007). Thus, further experiments are required for the confirmation or modification of conclusions reached in previous studies. As a complementary study, we examined stable cell lines in which the GGA2 expression was specifically decreased, and further performed a dominant negative experiment using VHS-GAT domain. The results suggest that GGA2 independently functions in the sorting of lysosomal enzymes in HeLa cells.

Materials and Methods

Antibodies

Mouse monoclonal antibodies against the clathrin heavy chain (clone 23), EEA1 (clone 14), GGA2 (clone 27), GGA3 (clone 8), GM130 (clone 35) were purchased from BD Transduction Laboratories (Lexington, KY). Mouse monoclonal antibodies against cation-dependent (CD)-MPR (2D4) and lamp1 (H4A3) were purchased from the Developmental Studies Hybridoma Bank (Iowa, City, IA). Rat polyclonal antibodies against human GGA1 and GGA2 were prepared as described previously (Yogosawa et al., 2006). Other primary antibodies used in this study were as follows: mouse monoclonal antibodies against Transferrin Receptor (H68.4; ZYMED Laboratories Inc., San Francisco, CA), β-COP (Affinity BioReagents, Golden, CO), β-actin (AC-15; Sigma Chemical, Saint Louis, MO), and γ-adaptin (100.3; Sigma Chemical); sheep monoclonal antibody against human TGN46 (Serotec, Oxford, UK); rabbit polyclonal antibodies against rat cation-independent (CI)-MPR (a gift from E. Komnami, Juntendo University School of Medicine, Tokyo) (Mun et al., 1993), and cathepsin D (Calbiochem, San Diego, CA). Secondary antibodies against mouse, rat, rabbit, and sheep IgG coupled with Alexa594 or Alexa488 were purchased from Molecular Probes, Inc. (Eugene, OR). Horseradish peroxidase (HRP)-conjugated rabbit anti-rat antibody was purchased from DAKO (Glostrup, Denmark). HRP-conjugated sheep anti-mouse and donkey anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

DNA construction

The p-SUPER RNAi system (OligoEngine, Seattle, WA) was used to knockdown the GGA2 expression. The siRNA target sequence for human GGA2, 916GAGATCTTCGACAAAC934, was designed using the manufacturer’s protocol. Briefly, forward- and reverse-oriented oligonucleotides for this sequence were synthesized, annealed and then inserted into the HindIII/BglII site of the pSUPER neo vector. The DNA sequence was confirmed using dye-terminator cycle sequencing kit (Applied Biosystems; Foster City, CA) before experiments.

Expression vectors containing HA-tagged VHS-GAT of human GGA1 and 3 were constructed as described previously (Wakasugi et al., 2003). cDNA for GGA2 was provided by K. Nakayama (Kyoto University)/(Takatsu et al., 2000). VHS-GAT of GGA2 (aa 342) was amplified by polymerase chain reaction and subcloned into pcdNA3-HAN (Wakasugi et al., 2003).

Cell culture and RNAi experiment

HeLa cells were grown in an α-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml
streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Transient and stable transfections were performed using FuGENE6 (Roche, Indianapolis, IN) as described in the manufacturer's protocol. To establish cell lines stably expressing the siRNA, cells were trypsinised 48 to 50 h after the transfection, and selected in fresh medium supplemented with 400 μg/ml of G418.

**Western blot analysis**

Control and GGA2-depleted HeLa cells were collected using a cell scraper, pelleted, and lysed in a buffer solution (0.05 M Tris-HCl / pH 7.5, 0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM EDTA) containing protease inhibitors (10 μg/ml leupeptin, 50 μg/ml aprotinin, 2 mM benzamidine, 1 μg/ml pepstatin, 50 μM 4-aminophenyl methanesulfonyl fluoride hydrochloride [p-APMSF]). The cell lysates were subjected to SDS-PAGE, and the proteins were then transferred onto polyvinylidene fluoride membranes (Immobilon, Millipore, Bedford, MA). The blots were immunolabeled with the primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-rat IgG. The chemiluminescent signal produced by ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) were detected using the Amplify ECL system (Amersham Pharmacia Biotech).
Biotech) was detected and measured using LAS-3000 mini (Fujifilm Co., Tokyo). Statistical analysis was carried out by Student’s t-test on more than 5 experiments.

**Immunofluorescence microscopy**

Cells stably or transiently (2–3 days) expressing siRNA for GGA2 were fixed in 3% paraformaldehyde/phosphate-buffered saline (PBS) for 15 min at room temperature. The excess paraformaldehyde was quenched by a 10 min-incubation in 50 mM ammonium chloride/PBS. For single or double immunolabeling procedures, cells were permeabilized with 0.1% Triton X-100/PBS, incubated for 20 min in 10% normal goat serum/PBS, and then with one or two of the primary antibodies diluted with 10% normal goat serum/PBS overnight at 4°C or for 30 min at room temperature. They were further incubated with appropriate secondary antibodies, washed, and mounted on glass slides. They were observed by confocal microscopy, using an LSM5 Pascal (Carl Zeiss, Jena, Germany).

Expression of the VHS-GAT domain of each GGA was performed as described previously (Wakasugi et al., 2003).

**Immunoprecipitation and cathepsin D sorting assay**

Control and GGA2-depleted HeLa cells were incubated in methionine-free Dulbecco MEM (Life Technologies;
Fig. 3. Localization of two MPRs in GGA2-depleted cells. A and B: G2D-1 cells and control HeLa cells were co-cultured overnight and fixed. They were double-stained with rat anti-GGA2 antibody and either rabbit anti-CI-MPR (A) or mouse anti-CD-MPR (B) antibody. Arrowheads indicate G2D-1 cells. C: G2D-1 cells were fixed and double-stained with rabbit anti-CI-MPR and sheep anti-TGN46 antibodies. D and E: HeLa cells were transiently transfected with the siRNA construct for GGA2 and cultured for 50 h. They were fixed and double-stained with rat anti-GGA2 antibody and either rabbit anti-CI-MPR (D) or mouse anti-CD-MPR (E) antibody. Arrowheads indicate GGA2-depleted cells. Bars: 50 μm (A, B, D, and E); 20 μm (C).

Rockville, MD) containing 10% dialyzed FBS for 30 min, followed by incubation with 10 MBq/ml of [35S]methionine/cysteine (Redivue™ PRO-MIX; Amersham Pharmacia Biotech) in the same medium for 30 min. The cells were further chased for 4 hrs with α-MEM supplemented with 10% FBS and 5 mM mannose 6-phosphate. After washing with PBS 3 times, the cells were lysed with the cell lysis buffer (0.05 M Tris-HCl / pH 7.5, 0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The resulting lysates were precleared with Protein A-Sepharose CL4B (Amersham Pharmacia Biotech) for 1 h at 4°C and centrifuged for 15 min. The supernatant was incubated, first with the anti-cathepsin D antibody overnight at 4°C, and then with Protein A-Sepharose CL4B for 1 h at 4°C. After washing the sepharose, the immune-complexes were eluted by boiling in the SDS-gel sampling buffer for 5 min and then subjected to SDS-PAGE. The gels were fluorographed and imaged using BAS5000 (Fuji-film Co.). The signals for procathepsin D in the media, and the intermediate and matured form of cathepsin D in the lysates were measured. Missorted cathepsin D was calculated as a percentage of secreted procathepsin D in the total. Statistical analysis was carried out by Student’s t-test on 5 experiments.
Results

Establishment of GGA2-depleted HeLa cell lines

We stably established two cell lines expressing siRNA for GGA2, which were named as G2D (GGA2-knock down)-1 and G2D-2. First, the expression levels of GGA1, 2, and 3 were examined by quantitative Western blot analysis (Fig. 1A, B). The GGA2 expression in G2D-1 and G2D-2 was decreased to 6.5% and 4.1% of the control, respectively. G2D-1 showed an increased (~150%) expression of both GGA1 and GGA3, while in G2D-2, GGA1 was slightly decreased (67.1%) and GGA3 was increased (188%). We next investigated other marker molecules for the TGN, endosomes, and lysosomes (Fig. 1A). Transferrin receptor, an early/recycling endosomal marker, and lam1p, a lysosomal marker, were increased in G2D-1. A Golgi marker, GM130, was slightly increased in G2D-2. CI-MPR was also slightly increased in both cell lines. Other markers were not significantly altered. Therefore, these cell lines showed a specific depletion of GGA2 and no concomitant loss of non-targeted GGA1/3 as has been shown in the previous study (Ghosh et al., 2003).

Recruitment of GGA1/3 to the TGN is independent of the presence of GGA2

Because it has been previously proposed that the three GGAs interact with each other in cooperating in the MPR transport (Ghosh et al., 2003), it is still possible that the up-regulated GGA1 or GGA3 in our GGA2-knock down cells may be non-functional. We therefore examined by immunofluorescence microscopy whether the GGA1 and GGA3 are associated with the TGN membranes. To directly compare differences, a co-culture of control and G2D-1 cells was analyzed. As shown in Figure 2A and B, GGA1 and GGA3 were localized in the perinuclear area in both the control and G2D-1 cells, and no significant difference was observed between the two cells. Double immunolabeling revealed that the perinuclear signal for GGA1 or GGA3 in G2D-1 cells was colocalized with that for a TGN marker, TGN46. Similar results were obtained in G2D-2 cells (data not shown) and in cells transiently (2 days) expressing siRNA for GGA2 (Fig. 2E, F). These results strongly suggest that the recruitment of GGA1 and GGA3 to the TGN is independent of the presence of GGA2 in HeLa cells.

Fig. 4. Missorting of cathepsin D in GGA2-depleted cells. Wild type HeLa, HeLa transfected with the vector alone, and GGA2-depleted HeLa cells (G2D-1 and G2D-2) were subjected to a pulse-chase experiment to measure the sorting efficiency of a lysosomal protease cathepsin D. After pulse-labeling with [35S] methionine for 30 min, cells were chased for 4 h. Then intracellular and secreted cathepsin D was immunoprecipitated to monitor its maturation and secretion. Procathepsin D (pro), and intermediate (int) and matured (mat) forms of cathepsin D in cell lysate (C) and the medium (M) are shown in A. The percentage of secreted cathepsin D (procathepsin D in the medium) in total was calculated and shown in B. Bars indicate SD of means (n = 5). *p < 0.05 (Student's t-test).

No significant redistribution of two MPRs was observed in GGA2-depleted cells

Depletion of one of GGAs was reported to cause a redistribution of MPR into endosomal compartments (Ghosh et al., 2003; Puertollano and Bonifacino, 2004). We next examined whether similar changes occur in the GGA2-depleted cells. As shown in Figure 3, CI-, and CD-MPR were primarily localized in the perinuclear region in both control and G2D-1 cells. The perinuclear signal for CI-MPR in G2D-1 cells corresponds to the TGN because it was colocalized with the TGN46 signal. MPRs were also distributed in numerous dots in the peripheral
Fig. 5. Overexpression of dominant-negative VHS-GAT domains. A–G: HeLa cells overexpressing HA-tagged VHS-GAT domains (VG) of GGA1 (A and C), GGA3L (B and D), or GGA2 (E, F, and G) were fixed and double-labeled with a combination of anti-HA and anti-GGA1 antibodies (A, B, and F), anti-HA and anti-GGA3 antibodies (C, D, and E), or anti-HA and anti-GGA3 antibodies (G). The left column shows cells expressing each VHS-GAT domain (arrowheads) as indicated. The right column shows an immunofluorescent signal for endogenous GGA1, 2, or 3. Bars: 50 μm. H: Percentage of cells that show disappearance (+), decrease (+/−), or no alteration (−) in the TGN signal of endogenous GGA1, 2, or 3 by the expression of each VHS-GAT domain. The name of each group is shown as a GGA signal followed by a slash and expressed VHS-GAT domain. Staining of each GGA was examined in 20 cells per coverslip, which was repeated three to five times. Bars indicate SD of means.
region in G2D-1 cells, the pattern of which could not be distinguished from that of control HeLa cells. The same results were obtained in G2D-2 cells (data not shown), and in cells transiently (2 days) expressing siRNA for GGA2 (Fig. 3D, E). We therefore, concluded that GGA2 depletion in HeLa cells does not cause any significant alteration in the overall MPR distribution.

\textbf{Sorting of cathepsin D was disturbed in GGA2-depleted cells}

To examine whether GGA2 depletion actually disturbs the sorting of lysosomal enzymes, we performed a cathepsin D sorting assay. Cells were pulse-labeled with \[^{35}\text{S}\] methionine/cysteine for 30 min, and then chased for 4 h in the presence of 5 mM mannose 6-phosphate that inhibits the reuptake of secreted cathepsin D. As shown in Figure 4A, secreted (or missorted) procathepsin D was detected in the media, and properly sorted cathepsin D appeared as processed intermediate and matured forms in the cell lysates. When the percentage of procathepsin D was measured, both GGA2 depleted cells showed significantly higher levels of secreted procathepsin D than control HeLa cells or those containing the vector alone (p < 0.05). This result indicates that GGA2-depletion causes the missorting of lysosomal enzymes.

\textbf{Expression of dominant-negative VHS-GAT domains}

We previously reported that a modest expression of the VHS-GAT domain of GGA1 or GGA3L resulted in the disappearance of the endogenous GGA1 in the TGN, suggesting that GGA1 and GGA3L share the same binding sites on the TGN membrane \textit{in vivo} (Wakasugi et al., 2003). Consequently, we next incorporated GGA2 in this experiment. As shown in Figure 5A and B, expression of GGA1 or GGA3L VHS-GAT led to the dispersion of the endogenous GGA1 in the TGN, confirming our previous results. However, these did not affect the endogenous GGA2 signal (Fig. 5C, D). Moreover, a moderate expression of GGA2 VHS-GAT did not affect the TGN signal of the three GGAs, including GGA2 itself (Fig. 5E, F, G). Together with the above RNAi experiment, these results suggest that GGA2 is independently recruited onto the TGN membrane.

\textbf{Discussion}

Previous studies using a transient expression of siRNA showed that a silencing of GGA1, 2, and/or 3 caused distinct morphological changes in the TGN (Ghosh et al., 2003) and in the distribution of CI-MPR (Ghosh et al., 2003; Puertollano and Bonifacino, 2004) and non-targeted GGAs (Ghosh et al., 2003). Unexpectedly, not any of these was observed in both GGA2-depleted cell lines and cells transiently expressing siRNA for GGA2. These results, however, do not indicate that GGA2 is a redundant molecule because the missorting of a lysosomal enzyme, cathepsin D, was apparently increased in these cell lines.

The present study showed that GGA2 depletion did not cause the MPR redistribution. One may consider that GGA1 and GGA3 compensated for the loss of GGA2, as their expression levels tended to be increased in the two cell lines and they were distinctly localized at the TGN. Although this compensation may function in maintaining the general distribution of MPR, it does not appear to be sufficient to compensate for the loss of the sorting efficiency of lysosomal enzymes. In the case of API-deficient fibroblasts, MPR is redistributed into endosomes and more lysosomal enzymes are secreted into the extracellular space. In these cells, the recycling rate between the plasma membrane and endosomes are increased, indicating important roles of API in retrograde transport from endosomes back to the TGN (Meyer et al., 2000, 2001). In a similar way, GGA3-depleted cells also induced an accumulation of CI-MPR in endosomes together with an internalized epidermal growth factor (Puertollano and Bonifacino, 2004). In this case, however, the loss of interaction of GGA3 with ubiquitin or TSG101, a component of the ubiquitin-dependent sorting machinery, may somehow be implicated in the endosomal sorting of the MPR. Considering that GGA2 is supposed to have lower affinity to ubiquitin than GGA1 or GGA3 (Puertollano and Bonifacino, 2004; Scott et al., 2004; Shiba et al., 2004), it is conceivable that the specific GGA2 depletion has no influence on the endosomal trafficking of MPRs — and thus caused no drastic redistribution of MPRs. Therefore, we speculate that GGA2 may primarily function at the TGN for the efficient sorting of cargos. More detailed analyses, such as the precise localization of MPRs at electron microscopic levels, and the kinetic analyses of intracellular transport of MPRs, are required to understand the mechanisms of the missorting of cathepsin D in GGA2-depleted cells.

Ghosh et al. (2003) have demonstrated that the depletion of any one of the GGAs resulted in the others being
redistributed from the TGN, which apparently contradicts our results. Most recently, however, Mardones et al. (2007) showed that the individual depletion of each GGA had no effect on the levels of the other two GGAs, suggesting that each GGA behaves as an independent species. The latter notion was supported by the present study. In addition, we performed a dominant-negative experiment, which showed that the TGN localization of GGA2 was not disturbed by the expression of GGA1 or GGA3L VIS-GAT domain. It was rather puzzling that VHS-GAT of GGA2 itself had no effect on the endogenous GGA2 localization. Probably, GGA2 binds to the TGN membrane mainly through its GAT domain, which is dependent on the ARF1 function. Thus, a greater expression level is required to disturb GGA2 distribution. In other words, the VIIS domain of GGA2 may be less important for the recognition of cargo proteins than that of GGA1 or GGA3, and may function in other important steps of lysosomal enzyme transport.

In conclusion, we have been first to make HeLa cell lines whose GGA2 expression was stably and specifically depleted. Secretion of a lysosomal enzyme, cathepsin D, was increased in these cell lines, suggesting that they would be suitable tools to explore the distinct functions and properties of GGA2 and possible but as yet undetectable transport steps of the MPR trafficking.

Acknowledgments

We wish to thank Drs. E. Kominami and K. Nakayama for kindly providing us with rabbit anti-C1-MPR antibody and cDNAs for GGA1 and 2, respectively. The monoclonal antibodies against CD-MPR developed by Dr. D. Messner, and that against lamp 1 developed by Drs. J. T. August and J. E. K. Hildreth, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. We are also indebted to members of Uchiyama's lab for helpful discussions.

References


