Intracellular localization of GGA accessory protein p56 in cell lines and central nervous system neurons

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ABSTRACT

Adaptor protein complex-1 (AP-1) and Golgi associated, γ -adaptin ear containing, Arf binding proteins (GGAs) are clathrin adaptors that regulate membrane trafficking between the *trans*-Golgi network (TGN) and endosomes. p56 is a clathrin adaptor accessory protein that may modulate the function of GGAs in mammalian cell lines. However, the precise relationship between p56 and the three GGAs (GGA1–3), as well as the physiological role of p56 in tissue cells, remain unknown. To this end, we generated an antibody against p56 and determined its cellular localization. In ARPE-19 cells and mouse embryonic fibroblasts, p56 was found to be localized as fine puncta in the TGN. Interestingly, the depletion of each clathrin adaptor by RNAi revealed that this localization was dependent on the expression of GGA1, but not that of GGA2, GGA3, or AP-1. Using immunohistochemistry in the mouse central nervous system (CNS), p56 was clearly detected as scattered cytoplasmic puncta in spinal motor neurons, cerebellar Purkinje cells, and pyramidal neurons of the hippocampus and cerebral cortex. Moreover, double labeling with organelle markers revealed that the majority of these puncta were closely associated with the TGN; however, a small fraction was associated with endosomes or lysosomes in spinal motor neurons. Collectively, these results indicate a functional association of p56 with GGA1, suggesting an important role of p56 in larger CNS neurons.

Cargo sorting coupled with the generation of clathrin-coated vesicles at the *trans*-Golgi network (TGN) and endosomes is regulated by adaptor protein complex-1 (AP-1) and Golgi-localized, γ -adaptin ear-containing, ADP ribosylation factor-binding proteins (GGAs). The AP-1 complex is a heterotetramer composed of γ -adaptin (γ 1 or γ 2 encoded by the *AP1G1* or *AP1G2* gene), β -adaptin (β 1 encoded by the

AP1B1 gene), μ -adaptin (μ 1A or μ 1B encoded by the *AP1M1* or *AP1M2* gene), σ -adaptin (σ 1A, σ 1B, or σ 1C encoded by the *AP1S1*, *AP1S2*, or *AP1S3* gene) (8, 26). On the other hand, there are three known GGAs (GGA1, 2, and 3) in mammals, each of which acts as a monomeric adaptor consisting of VHS, GAT, hinge, and GAE domains (1, 27). Membrane recruitment of these adaptors requires Golgi-localized phosphatidylinositol 4-phosphate (PI4P) and the GTP-bound form of the GTPase, Arf1. In this process, sorting signals are recognized in the cytoplasmic domain of cargoes, such as mannose 6-phosphate receptors (MPRs), sortilin, and β -site amyloid precursor protein cleaving enzyme 1 (BACE1) (1, 5, 9). Recent studies have suggested that the three GGAs are not redundant, but rather play their own roles in

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cargo sorting (5, 7, 37).

Clathrin adaptor functions may be regulated by several accessory proteins that bind the ear domain of γ -adaptin of AP-1 and/or the GAE domains of GGAs. These include γ -synergin (11, 29, 36), aftiphilin (10, 23), γ -Bar/Gadkin (28), enthoprotin/Clint1/epsinR (12, 13, 24, 39), rabaptin-5 (4, 11, 22, 34), GAK (14), and p56 (3, 20, 21). Studies have shown that aftiphilin, γ -Bar/Gadkin, GAK, and p56 regulate cathepsin D sorting (10, 14, 21, 28), and that aftiphilin and γ -synergin regulate the secretion of the von Willebrand factor (19). In addition, p56 is one of the GGA accessory proteins, and it has been suggested that p56 plays a role in the long-range movement of GGA/clathrin-containing transport carriers in certain cell lines (20, 21). However, these studies utilized only cell lines, and a tissue-based study has yet to be performed. Therefore, in this study, we focused on p56 and initially generated an antibody against this accessory protein. We then investigated the GGA-dependency of p56 in its membrane recruitment and the localization of p56 in CNS neurons.

MATERIALS AND METHODS

Antibodies and cells. The p56 rabbit polyclonal antibody was generated by injecting a unique peptide [amino acids 1–15 (MDDDDFGGFEEAETF)] of human p56 into rabbits. The antiserum was then used for Western blot analyses, and the affinity-purified antibody was used for immunostaining in both cell lines and tissues. The other antibodies used in this study were as follows: rabbit antibodies against GGA1 (Santa Cruz Biotechnology; sc-30102), p44/42 MAP kinase (Cell Signaling Technology; 4695); mouse antibodies against GGA2 (BD transduction laboratories; 612612), GGA3 (BD transduction laboratories; 612310), γ -adaptin (BD transduction; 610385), EEA1 (BD transduction laboratories; 610456), LAMP1 (Santa Cruz Biotechnology; sc-19992), GAPDH (Santa Cruz Biotechnology; sc-32233), LBPA (a gift from Dr. Toshihide Kobayashi, RIKEN), a sheep antibody against human TGN46 (Novus Biologicals; NB110-60520), and a goat antibody against mouse TGN38 (Santa Cruz Biotechnology; sc-27681).

ARPE-19 cells were purchased from ATCC. Mouse embryonic fibroblasts (MEFs) were kindly provided by Dr. Masaaki Komatsu from Niigata University.

Knockdown by siRNA. The siRNAs used in the present study were as follows; human GGA1 (5'-GGC AAGUCCGCUUUCUCAAC-3'), human GGA2

(5'-GGAGUUCUGCUGUACAAACAG-3'), human GGA3 (5'-CUGCAGUGCCCAAGUCAUGA-3'), human γ -adaptin [ON-TARGET plus SMARTpool (Dharmacon; L-019183-00-0005)], and human p56 [ON-TARGET plus SMARTpool (Dharmacon; L-018638-01-0005)].

ARPE-19 cells were transfected with the indicated siRNA using Lipofectamine RNAiMAX (Invitrogen). Three days later, cells were processed for immunostaining or Western blotting.

Mice. Two 8-week-old C57 BL/6J mice were purchased from CLEA Japan, Inc. For immunohistofluorescence microscopy, mice were fixed by cardiac perfusion according to the standard protocol using 4% paraformaldehyde (PFA), 4% sucrose, and 0.1 M phosphate buffer (PB; pH 7.4). Tissues were excised and treated with 4–20% sucrose for cryoprotection, and further embedded in OCT compound. Cryosections were prepared and immunostained with the antibodies above. For Western blotting, excised organs were washed with cold phosphate-buffered saline (PBS) and frozen with liquid nitrogen. This study was approved by the Ethics Committee for Animal Research of Fukushima Medical University (approval numbers: 29007, 27016, 25003, 23007, and 21104). All animal experiments were performed according to the guidelines of Fukushima Medical University.

Western blot analyses. Frozen organs were homogenized in lysis buffer (PBS containing 1% Triton X-100 and protease inhibitor [Roche]). ARPE-19 cells were washed three times with cold PBS and lysed using the same lysis buffer. The protein concentrations of the lysates were measured by using the BCA Protein Assay Reagent (Pierce Biotechnology, Inc). For SDS-PAGE, 5–10 μ g/lane of protein was applied and separated, followed by transferring to a PVDF membrane. The membrane was blocked with PBS containing 5% skim milk and 0.1% Tween 20 for more than 30 min, followed by incubation with the primary antibodies detailed above. For the secondary antibodies, HRP-conjugated anti-rabbit and anti-mouse antibodies were used. For signal detection, the ECL prime reagent and LAS4000 mini (GE Healthcare) were used.

Immunofluorescence and immunohistofluorescence microscopy. Cultured cells were fixed with 4% PFA in 0.1 M PB (pH 7.4), and processed for permeabilization and blocking with PBS containing 0.1% Triton X-100 and 0.4% BSA for more than 30 min. The cryosections prepared above were blocked with 5%

Fig. 1 Characterization of the anti-p56 antibody. **(A and B)** ARPE-19 cells were treated with control siRNA (si-Ctrl) or siRNA for p56 (si-p56). Cells were lysed for Western blot analysis using the anti-p56 antibody (**A**) or double immunofluorescence microscopy with anti-p56 and anti-TGN46 antibodies (**B**). In (**A**), the band for p56 is indicated by arrowhead. Molecular weights (kDa) are shown on the right. Signals for p56 (green) and TGN46 (red) were merged and shown in the right column. Note that p56 localizes in the TGN and the cytosol. Bar, 20 μ m. **(C)** MEFs were fixed for double immunofluorescence microscopy with anti-p56 and anti-TGN38 antibodies. Signals for p56 (red) and TGN46 (green) were merged and are shown in the right. Bar, 20 μ m.

normal donkey serum in PBS for 60 min. They were then immunostained with primary antibodies. For secondary antibodies, donkey anti-rabbit, -mouse, or -goat antibodies conjugated with Alexa Fluor fluorescent dye were used. Fluorescent images were then obtained using a confocal microscope FV1000 (Olympus).

RESULTS

To investigate the intracellular localization of the p56 protein in both cell lines and tissues, an antibody against p56 was generated using a synthesized peptide corresponding to amino acids 1–15 of human p56. The sequence of this peptide was identical to mouse p56. The antibody was first examined in ARPE-19 cells, which are derived from human retinal pigment epithelial cells. Western blot analyses showed a strong band for p56 in control cells, which disappeared when p56 expression was suppressed by siRNA (Fig. 1A). Immunofluorescence microscopy revealed that p56 was localized in the TGN46-positive TGN (Fig. 1B), consistent with what has been

reported previously (21). p56 was also detected as a diffuse signal in the cytosol (Fig. 1B). Importantly, the specificities of both the TGN and cytosolic signals were confirmed by their drastic decreases in p56-depleted cells by siRNA (Fig. 1B). A similar pattern of p56 localization was also observed in MEFs (Fig. 1C).

p56 has been shown to interact with GGAs and the γ -adaptin subunit of the AP-1 complex *in vitro* (20). However, the TGN localization of p56 has been shown to be dependent on GGAs, but not on AP-1, in HeLa cells (20, 21). Therefore, we reevaluated this dependency in ARPE-19 cells. Using Western blotting, the suppression of GGA1, GGA2, GGA3, and γ -adaptin by each siRNA did not influence the amount of p56 (Fig. 2). Similarly, the depletion of p56 did not affect the expressions of each clathrin adaptor (Fig. 2). By immunofluorescence, GGA1-depletion caused a disappearance of the p56 signal from the TGN region, whereas GGA2-depletion slightly increased the signal in the TGN (Fig. 3A and B). The depletion of GGA3 or γ -adaptin had no effect on p56 localization (Fig. 3C and D). When

Fig. 2 Western blot analysis of p56 and each clathrin adaptor in the siRNA experiment. ARPE-19 cells were treated with siRNAs as indicated on the top, which were then lysed for Western blot analysis with antibodies (Ab) as indicated on the left. Molecular weights (kDa) are shown on the right. GAPDH was included as a loading control. Note that the depletion of GGA1, GGA2, GGA3, or γ -adaptin did not influence the expression levels of p56.

the siRNA for p56 was applied, the localizations of GGA1, GGA2, GGA3, and γ -adaptin in TGN did not significantly change (Fig. 3E–H). Because the above Western blotting revealed that the amount of p56 was unchanged by GGA1- or GGA2-depletion, we reasoned that GGA1-depletion caused the dissociation of p56 from the TGN, while GGA2-depletion slightly increased the recruitment of p56 onto the TGN. Therefore, these results suggest that the TGN localization of p56 is positively regulated by GGA1 and negatively by GGA2 in ARPE-19 cells.

We next applied the p56 antibody to mouse tissues, as it was shown to detect p56 in MEFs (Fig. 1C). When the lysates of the brain, lung, spleen, or testis were examined by Western blot analysis, p56 was expressed higher in the brain than the other regions (Fig. 4). Therefore, we focused on the CNS in the following analyses. As shown in Fig. 5, p56 was detected as punctate signals primarily in the perikarya and occasionally in the dendrites of cerebral cortex neurons, hippocampal pyramidal cells, cerebellar Purkinje cells, and spinal cord motor neurons. Be-

cause the pattern of the signal was somewhat different from that observed in cultured cells (Fig. 1), detailed localizations were determined in spinal cord motor neurons using markers for the TGN (TGN38), early endosomes (EEA1), late endosomes (LBPA), and late endosomes/lysosomes (LAMP1). As shown in Fig. 6A, a large part of the p56-positive puncta was present in the vicinity of the reticular TGN structure, and the extent of colocalization with the TGN marker appeared lower than that in the cell lines (Fig. 1). This difference might be due to larger TGN in neurons compared with perinuclear compacted TGN in the cell lines, and/or lower occupancy of the clathrin coated pits on the TGN membrane in neurons, which should be clarified in future. In addition, a small fraction of the p56-positive puncta was associated with structures positive for EEA1, LBPA, and LAMP1 (Fig. 6B–D).

DISCUSSION

p56 has the ability to bind to both the GAE domains of GGAs and the ear domain of γ -adaptin *in vitro* (20). However, previous studies have clearly indicated that both the expression and the Golgi localization of p56 are not affected in μ 1A-adaptin-deficient fibroblasts (20) or HeLa cells treated with siRNA for γ -adaptin (21). Similarly, our study using ARPE-19 cells showed no alteration in the expression and localization of p56 by the depletion of γ -adaptin, which strengthens previous findings that p56 functions independently of AP-1. On the other hand, our results of the present study demonstrated that the TGN localization of p56 was affected by the depletion of GGA1, but not of GGA2 or GGA3. Importantly, this GGA1 depletion did not affect the protein expression of p56. These observations are slightly different from those previously reported by Mardones *et al.*, where both the expression and the TGN localization of p56 were partially suppressed by the depletion of each GGA (21). While this discrepancy cannot be fully explained, differences in the cell lines and/or RNAi procedures utilized may have influenced the results. It should be noted that in the brains of GGA1-, GGA2-, GGA3-, or GGA1/3 knockout mice, the protein levels of p56 are unchanged (5), consistent with our results. It would be interesting to know the intracellular distribution of p56 in those mice. In conclusion, our findings confirmed the preference of p56 as an accessory protein for GGAs, and suggest the importance of GGA1 in mediating its function.

So, what is the function of p56? It has been pre-

Fig. 3 Immunofluorescence analysis of p56 and each clathrin adaptor in the siRNA experiment. (**A–D**) Dependency of p56 on clathrin adaptors. ARPE-19 cells were treated with control siRNA (si-Ctrl), or each siRNA against GGA1 (**A**), GGA2 (**B**), GGA3 (**C**), or γ -adaptin (**D**; si- γ -ad), followed by fixation. They were immunostained with the anti-GGA1 or anti-p56 antibody (**A**), or double-immunostained with a combination of the anti-p56 antibody and either anti-GGA2 (**B**), anti-GGA3 (**C**), or anti- γ -adaptin (**D**; anti- γ -ad) antibodies. Thus, only (**A**) shows different fields for the two antibodies. Note that only GGA1-depletion caused the disappearance of the TGN signal for p56 (**A**). Bars, 20 μ m. (**E–H**) Dependency of clathrin adaptors on p56. ARPE-19 cells were treated with control siRNA (si-Ctrl), or siRNAs against p56. They were then immunostained with the anti-GGA1 or the anti-p56 antibody (**E**), or double-immunostained with a combination of the anti-p56 antibody and either, the anti-GGA2 (**F**), anti-GGA3 (**G**), or anti- γ -adaptin (**H**; anti- γ -ad) antibodies. Thus, only (**E**) shows different fields for the two antibodies. p56-depletion did not affect the localizations of four clathrin adaptors. Bars, 20 μ m.

Fig. 4 Western blot analysis of p56 in the mouse brain, lung, spleen, and testis. Lysates of the mouse brain, lung, spleen, and testis were analyzed by Western blotting using the anti-p56 antibody (p56). GAPDH and p44/p42 MAPK were used as loading controls. Molecular weights (kDa) are shown on the right.

Fig. 5 Immunohistological localization of p56 in the mouse CNS. Cryosections of the cerebral cortex (cc, A), hippocampus (hippo, B), cerebellum (cerebel, C), and spinal cord (sc, D) were immunostained using the anti-p56 antibody (anti-p56) or normal rabbit IgG as a control (cont). The boxed regions in the middle column are magnified on the right (enlarged). Note that p56-positive puncta are discerned mainly in the perikarya of large neurons. Bars, 100 μ m (left and middle column) and 20 μ m (right column).

Fig. 6 Intracellular localization of p56 with reference to organelle markers in spinal motor neurons. Spinal cord cryosections were double-immunostained with combinations of the anti-p56 antibody (anti-p56) and anti-TGN38 (A), -EEA1 (B), -LBPA (C), or -LAMP1 antibodies (D). They were then labeled with appropriate secondary antibodies. In the right two columns, the green and red colors indicate the signals for p56 and each marker, respectively. Boxed regions in the merged images are magnified and shown in the enlarged images. Note that the p56-positive puncta are associated not only with the TGN (A), but also with endosomes/lysosomes (arrowheads in B, C, and D). Bars, 10 μ m.

viously shown that RNAi-mediated depletion of p56 in HeLa cells causes the missorting of the lysosomal enzyme, cathepsin D, and decreases the mobility of GGA-containing transport carriers, suggesting that p56 supports the long-range transport of carriers containing lysosomal enzymes (21). This is consistent with our observations that p56-positive puncta were primarily detected in large-sized neurons, such as hippocampal pyramidal cells, cerebellar Purkinje cells, and spinal cord motor neurons. It is also conceivable that p56 contributes to the efficient delivery of lysosomal enzymes in such large neurons.

The TGN localization of p56 in cultured cells indicates the function of this molecule at the TGN membranes. However, we found that a small but significant fraction of p56 was associated with endocytic structures in spinal cord motor neurons, suggesting that it may also function in relation to GGAs at the endosomes *in vivo*. In fact, accumulating evi-

dence indicates that GGAs function at endosomes by regulating the recycling and/or degradation of physiologically and pathologically important cargoes. These include BACE1 (6, 15, 17) and LR11/SorLA (7), which are involved in Alzheimer's disease pathology, TrkA (18), c-Met through the Crk adaptor under HGF stimulation (30), the α 2B-adrenergic receptor (40), Polycystin-1,2 (16), β 1-integrin (33), and EGFR (32, 37). In light of our findings that p56 is specifically recruited by GGA1, GGA1-specific cargoes may prove to be linked to p56. Among the studies above, LR11/SorLA (7) has been reported as a GGA1-specific cargo because the depletion of GGA1, but not GGA2 or GGA3, decreases LR11/SorLA protein levels. Importantly, it has been shown that the expression of GGA1 protein is decreased in the brains of Alzheimer's disease patients (38). Therefore, although future studies are warranted, p56 may have a function in regulating the transport of LR11/

SorLA in neurons and may be implicated in Alzheimer's pathology.

Unexpectedly, we found that a fraction of the p56-positive puncta was associated with LAMP1-positive puncta in spinal motor neurons, which may represent late endosomal structures (31). However, it cannot be excluded that p56 plays unknown roles in the lysosomes of CNS neurons.

Genome-wide association studies have associated the *CCDC91* gene that encodes p56 with several diseases and clinical imaging parameters. To date, *CCDC91* has been identified as one of the susceptible genes for the ossification of the posterior longitudinal ligament of the spine (25) and bipolar disorder (2). Moreover, a SNP in the *CCDC91* gene was associated with fractional anisotropy observed by MRI imaging, which reflects a combination of myelin thickness, fiber coherence and axon integrity (35). The latter two studies support the role of the p56 gene in the CNS. In conclusion, our findings in the present study, together with those previously reported, warrant further investigation of this clathrin accessory protein in the CNS.

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