Published in final edited form as:

Biochemistry. 2018 July 03; 57(26): 3556–3559. doi:10.1021/acs.biochem.8b00224.

Co-mobility of GABARAP and Phosphatidylinositol 4-kinase 2A on cytoplasmic vesicles

Yan Chen[1,*], **Hui-Qiao Sun**[2], **John P. Eichorst**[1], **Joseph P. Albanesi**[3], **Helen Yin**[2], and **Joachim D. Mueller**[1]

[1]School of Physics and Astronomy, University of Minnesota, Minneapolis, MN 55455

[2]Departments of Physiology University of Texas Southwestern Medical Center, Dallas, TX 75390

[3]Departments of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75390

Abstract

We previously reported that recruitment of the Type IIA phosphatidylinositol 4-kinase (PI4K2A) to autophagosomes by GABARAP, a member of the Atg8 family of autophagy-related proteins, is important for autophagosome-lysosome fusion. Because both PI4K2A and GABARAP have also been implicated in the intracellular trafficking of plasma membrane receptors in the secretory/endocytic pathway, we characterized their interaction in cells under non-autophagic conditions. Fluorescence fluctuation spectroscopy measurements revealed that GABARAP exists predominantly as a cytosolic monomer in live cells, but is recruited to small cytoplasmic vesicles upon overexpression of PI4K2A. C-terminal lipidation of GABARAP, which is essential for its autophagic activities, is not necessary for its recruitment to these PI4K2A-containing transport vesicles. However, a GABARAP truncation mutant lacking C-terminal residues 103–117 fails to bind to PI4K2A, is not recruited to cytoplasmic vesicles, and does not co-distribute with PI4K2A on subcellular organelles. These observations suggest that that the PI4K2A-GABARAP interaction plays a role in membrane trafficking both under autophagic and non-autophagic conditions.

Keywords

fluorescence correlation spectroscopy; membrane trafficking; Brightness; lipidation; mobility

Autophagy is a multi-step process resulting in the engulfment of bulk cytosol and cytoplasmic inclusions such as aggregated proteins and damaged organelles. It is activated under conditions of nutrient deprivation as a mechanism to provide cells with needed energy sources. Numerous autophagy-related proteins (Atgs), which facilitate the initiation,

[*]**Corresponding** Author: chenx238@umn.edu. Telephone: 612-626-8684. Fax: 612-624-4578.

Author Contributions

Chen, Albanesi, Yin and Mueller designed the experiments and oversaw their execution. Experiments were performed by Sun and Chen.

The authors declare no competing financial interests.

ASSOCIATED CONTENT

Supporting Information

Experimental Procedures and Supplemental Figures.

maturation, and degradation stages of autophagy, have been identified. These include the Atg8 family, ubiquitin-like proteins comprising LC3 (dynein light chain 3) and GABARAP/GATE16 subfamilies [1–3]. Interaction screens[4,5] revealed that GABARAP/GATE16 interact with phosphatidylinositol 4-kinase Type IIα (PI4K2A), one of the four mammalian enzymes that catalyze the phosphorylation of phosphatidylinositol to produce phosphatidylinositol 4-phosphate (PI4P). We found that GABARAP not only binds PI4K2A, but is necessary for PI4P generation on autophagosomes by PI4K2A, and that depletion of either PI4K2A or GABARAP inhibits autophagosome-lysosome (A:L) fusion [6,7]. Furthermore, the catalytic activity of PI4K2A is necessary for A:L fusion, as the PI4K2A depletion defect could be rescued by transfection of wild-type but not kinase-dead PI4K2A or by "shuttling" of PI4P into PI4K2A-depleted cells.

In addition to their well-characterized roles in autophagy, Atg8 proteins have non-autophagic functions in a variety of cellular pathways, including viral replication, cytoskeletal remodeling, and protein trafficking[8]. Indeed, GABARAP was first identified as a binding partner of the GABA(A) receptor[9], mediating the transport of the receptor from the Golgi to the plasma membrane (PM)[10,11]. Consistent with a role in receptor trafficking, GABARAP binds directly to NEM-sensitive factor (NSF)[10] and to microtubules[9]. GABARAP has also been implicated in the PM expression of the angiotensin II receptor[12], the transient receptor potential cation channel subfamily V member 1 (TrpV1, also known as the capsaicin receptor and vanilloid receptor 1)[13], the κ-opioid receptor[14], and HIV-Nef[15]. Thus, GABARAP may function in non-autophagic trafficking by recruiting cargo into Golgi-derived transport vesicles that are destined for the PM[2]. We previously showed that PI4K2A participates in vesicle formation on the Golgi by creating a pool of PI4P that interacts with the adaptors AP-1[16] and GGA1[17]. Other groups have implicated PI4K2A in various aspects of intracellular transport, including the sorting of receptors from endosomes (reviewed in[18–20]).

In this study, we used fluorescence fluctuation spectroscopy (FFS) to characterize GABARAP and its interaction with PI4K2A in living cells. FFS utilizes fluctuations in fluorescence signals from tagged molecules to determine their concentration, mobility, and stoichiometry in a sub-femtoliter optical observation volume within a cell[21–23], see Experimental Procedures). Several unique physical parameters are accessible by analyzing the photon counts emitted from this optical volume. Autocorrelation analysis provides an estimate of the diffusion constant (and, hence, the size) of a fluorescent protein (FP)-tagged particle. Brightness analysis of the fluorescence intensity fluctuations retrieves the normalized brightness b and identifies the average oligomeric state of the labeled protein. The data reveal that cytosolic GABARAP is monomeric at concentrations up to 3 μM, as all GFP-GABARAP brightness values were consistently close to b = 1 (Figure 1A). This result is consistent with prior *in vitro* studies using gel filtration and dynamic light scattering, which indicated that GABARAP is monomeric in solution at concentrations up to 100 μM[24]. We next performed autocorrelation analysis on the same fluctuation data used for brightness analysis to estimate the mobility of GFP-GABARAP. The median residence time, 2.93±1.1 ms (Figure 1B), was independent of concentration. For comparison, the residence time for tandem dimeric GFP (MW ~54 kDa) is approximately 1 ms[25]. Given that GFP-GABARAP has a MW of ~41 kDa, a residence time of 2.9 ms is higher than expected for monomeric

GABARAP, though considerably lower than predicted for a small cytoplasmic vesicle (at least 10 ms)[26,27] Thus, the majority of GABARAP in non-autophagic cells appears to be monomeric, but complexed with soluble (non-membrane-associated) proteins in the cytoplasm. In this respect, it behaves similarly to another Atg8 family member, LC3B[28].

To determine if overexpression of PI4K2A induces the recruitment of cytosolic GABARAP to vesicles, we co-transfected U2OS cells with GFP-GABARAP and mCherry-PI4K2A. All measurements were conducted using a dual-color detection setup. The fluorescence of GFP was separated from the mCherry signal and directed into the green detection channel for brightness analysis of GFP-GABARAP. The red fluorescence signal in the other channel served to identify cells that co-expressed both proteins. As shown in Figure 1A, the brightness of GFP-GABARAP was significantly greater in cells co-expressing PI4K2A. The brightness increased with GABARAP concentration, which we interpret as the recruitment of GFP-GABARAP proteins to cytosolic vesicles in the presence of PI4K2A.

Autocorrelation analysis of the data revealed an increase in the residence time of GFP-GABARAP from about 2 to 100ms in the presence of mCherry-PI4K2A (Figure 1B). The initial increase in the residence time with concentration followed by plateauing is consistent with a concentration-dependent interaction leading to the recruitment of GFP-GABARAP to vesicles. The observed residence time in excess of 10ms is consistent with values previously observed for cytoplasmic vesicles[26,27]. Thus, we conclude that GABARAP has a strong tendency to associate with cytoplasmic vesicles containing PI4K2A.

We turned to another FFS approach, heterospecies partition (HSP) analysis[26,27,29], to probe directly the co-mobility of GABARAP and PI4K2A. HSP analysis allows one to determine if differently colored species move together as a single complex. This method calculates the brightness λ_G of the green channel and its associated brightness λ_R in the red channel. Each HSP-brightness $\lambda = (\lambda_R, \lambda_G)$ corresponds to a point on a two-dimensional brightness plot. The HSP brightness of GFP-labeled protein complexes is localized along the green line of the two-dimensional brightness plot (Figure 2). This is a consequence of the broad emission spectrum of GFP, which results in a split of the fluorescence across both detection channels. The slope of this line represents the brightness ratio of λ_R and λ_G, which equals the intensity ratio of the two channels. The presence of mCherry in a GFP-labeled protein complexes leads to an additional brightness contribution in the red channel, shifting the HSP brightness to the right of the green line (gray zone, Figure 2).

U2OS cells were co-transfected with GFP-PI4K2A and mCherry-GABARAP. Only cells expressing both proteins were subjected to HSP analysis. All measured HSP brightness pairs were to the right of the green line, indicating the presence of diffusing complexes simultaneously carrying GFP-PI4K2A and mCherry-GABARAP (Figure 2A). Similar results were obtained in HeLa cells (Figure 2A), suggesting that co-mobility of the two proteins is not cell line dependent. The HSP data demonstrate the recruitment of GABARAP to PI4K2A-containing vesicles. Interestingly, the measured HSP brightness pairs are distributed along a straight line, indicating the presence of a fixed stoichiometric ratio of GFP- and mCherry-labeled proteins within the diffusing complexes.

We also checked the co-mobility of GFP-PI4K2A with another ATG8 family protein, LC3. The resulting HSP brightness values are plotted in Figure 2B. Although there is slight co-mobility of GFP-PI4K2A and mCherry-LC3, the association was much weaker than that of GFP-PI4K2A and mCherry-GABARAP (Figure 2A).

We next used deletion analysis to identify regions in GABARAP that are necessary for its interaction with PI4K2A. GABARAP contains two structural domains: an N-terminal microtubule-binding domain (residues 1–26) and a C-terminal ubiquitin-like domain (residues 27–117). Like other Atg8s, GABARAP is anchored to the membrane by post-translational attachment of phosphatidylethanolamine to glycine 116, which becomes exposed upon cleavage of the C-terminal leucine. This modification is essential for trafficking of the GABA(A) receptor[30] but not the angiotensin II receptor[31]. To determine if lipidation is necessary for GABARAP binding to PI4K2A, we generated a mutant, GABARAP$^{1–115}$, which lacks the C-terminal glycine and leucine residues. HSP analysis (Figure 3A) revealed that GABARAP$^{1–115}$ was recruited to PI4K2A-containing vesicles essentially identically to full-length GABARAP (compare with Figure 2A; also see Figure S1B). Likewise, full-length GABARAP and GABARAP$^{1–115}$ displayed similar co-localization with PI4K2A in confocal images of non-autophagic HeLa cells (Figure 3B). Thus, whereas lipidation of GABARAP is essential for its role in autophagy[32] and for GABA receptor trafficking[30], this modification is apparently not required for the interaction of GABARAP and PI4K2A.

In contrast to GABARAP$^{1–115}$, a more extensively truncated mutant, GABARAP$^{1–102}$, failed to associate with PI4K2A-containing vesicles (Figure 3A) or to co-localize with PI4K2A-containing organelles (Figure 3B). As expected LC3$^{1–106}$, an analog as GABARAP$^{1–102}$, shows no interaction with GFP-PI4K2A (Figure 2B). Co-immunoprecipitation experiments provided biochemical confirmation that PI4K2A interacts with GABARAP$^{1–115}$ but not GABARAP$^{1–102}$ in cell lysates (Figure 3C). Thus, a segment within residues 103–115 in GABARAP is critical for its interaction with PI4K2A or for proper GABARAP folding.

In summary, our FFS experiments demonstrate that GABARAP exists predominantly as a cytosolic monomer over a broad concentration range. Co-expression of PI4K2A recruits GABARAP to large cytoplasmic structures. Based on their residence times (inversely related to their diffusion constants), and on the co-presence of PI4K2A, which behaves as an integral membrane protein[33], we believe that these structures are small, membrane-bounded vesicles. Both GABARAP and PI4K2A have been localized to the Golgi apparatus. Therefore, these vesicles may be transport carriers that deliver both proteins to the PM. In addition to the increase in residence time, the brightness of GABARAP also increases substantially upon co-transfection of cells with PI4K2A, suggesting that multiple GABARAP proteins are present on each vesicle. The amount of vesicular GABARAP is strongly correlated with the expression level of PI4K2A, and the two proteins co-exist on vesicles at nearly stoichiometric levels. These results indicate that GABARAP and PI4K2A interact tightly. Although lipidation of GABARAP is essential for its role in autophagy, here we show that its lipidation is not required for the interaction of GABARAP and PI4K2A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This research was supported by the National Institutes of Health GM121536 (H.Y., J.P.A, and J.D.M) and GM064589 (Y.C. and J.D.M.).

ABBREVIATIONS

PI4K2A	phosphatidylinositol 4-kinase Type Iiα
FP	fluorescent protein
PI4P	phosphatidylinositol 4-phosphate
FFS	fluorescence fluctuation spectroscopy
A:L	autophagosome-lysosome

REFERENCES

(1). Abdollahzadeh I, Schwarten M, Gensch T, Willbold D, and Weiergräber OH (2017) The Atg8 Family of Proteins—Modulating Shape and Functionality of Autophagic Membranes. Front. Genet 8.

(2). Schaaf MBE, Keulers TG, Vooijs MA, and Rouschop KMA (2016) LC3/GABARAP family proteins: autophagy-(un)related functions. FASEB J. 30, 3961–3978. [PubMed: 27601442]

(3). Slobodkin MR, and Elazar Z (2013) The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy. Essays Biochem. 55, 51–64. [PubMed: 24070471]

(4). Behrends C, Sowa ME, Gygi SP, and Harper JW (2010) Network organization of the human autophagy system. Nature 466, 68–76. [PubMed: 20562859]

(5). Jović M, Kean MJ, Dubankova A, Boura E, Gingras A-C, Brill JA, and Balla T (2014) Endosomal sorting of VAMP3 is regulated by PI4K2A. J. Cell Sci 127, 3745–3756. [PubMed: 25002402]

(6). Wang H, Sun H-Q, Zhu X, Zhang L, Albanesi J, Levine B, and Yin H (2015) GABARAPs regulate PI4P-dependent autophagosome:lysosome fusion. Proc. Natl. Acad. Sci. U. S. A 112, 7015–7020. [PubMed: 26038556]

(7). Albanesi J, Wang H, Sun H-Q, Levine B, and Yin H (2015) GABARAP-mediated targeting of PI4K2A/PI4KIIα to autophagosomes regulates PtdIns4P-dependent autophagosome-lysosome fusion. Autophagy 11, 2127–2129. [PubMed: 26391226]

(8). Subramani S, and Malhotra V (2013) Non-autophagic roles of autophagy-related proteins. EMBO Rep. 14, 143–151. [PubMed: 23337627]

(9). Wang H, Bedford FK, Brandon NJ, Moss SJ, and Olsen RW (1999) $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. Nature 397, 69. [PubMed: 9892355]

(10). Kittler JT, Rostaing P, Schiavo G, Fritschy J-M, Olsen R, Triller A, and Moss SJ (2001) The Subcellular Distribution of GABARAP and Its Ability to Interact with NSF Suggest a Role for This Protein in the Intracellular Transport of GABAA Receptors. Mol. Cell. Neurosci 18, 13–25. [PubMed: 11461150]

(11). Jacob TC, Moss SJ, and Jurd R (2008) GABAA receptor trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci 9, 331–343. [PubMed: 18382465]

(12). Cook JL, Re RN, deHaro DL, Abadie JM, Peters M, and Alam J (2008) The trafficking protein, GABARAP, binds to and enhances plasma membrane expression and function of the angiotensin AT1 receptor. Circ. Res 102, 1539–1547. [PubMed: 18497328]

(13). Laínez S, Valente P, Ontoria-Oviedo I, Estévez-Herrera J, Camprubí-Robles M, Ferrer-Montiel A, and Planells-Cases R (2010) GABAA receptor associated protein (GABARAP) modulates TRPV1 expression and channel function and desensitization. FASEB J. 24, 1958–1970. [PubMed: 20179142]

(14). Chen C, Wang Y, Huang P, and Liu-Chen L-Y (2011) Effects of C-terminal Modifications of GEC1 Protein and γ-Aminobutyric Acid Type A (GABAA) Receptor-associated Protein (GABARAP), Two Microtubule-associated Proteins, on κ Opioid Receptor Expression. J. Biol. Chem 286, 15106–15115. [PubMed: 21388957]

(15). Boeske A, Schwarten M, Ma P, Tusche M, Mötter J, Möller C, Neudecker P, Hoffmann S, and Willbold D (2017) Direct binding to GABARAP family members is essential for HIV-1 Nef plasma membrane localization. Sci. Rep 7.

(16). Wang YJ, Wang J, Sun HQ, Martinez M, Sun YX, Macia E, Kirchhausen T, Albanesi JP, Roth MG, and Yin HL (2003) Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. Cell 114, 299–310. [PubMed: 12914695]

(17). Wang J, Sun H-Q, Macia E, Kirchhausen T, Watson H, Bonifacino JS, and Yin HL (2007) PI4P promotes the recruitment of the GGA adaptor proteins to the trans-Golgi network and regulates their recognition of the ubiquitin sorting signal. Mol. Biol. Cell 18, 2646–2655. [PubMed: 17494868]

(18). Balla T (2013) Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev 93, 1019–1137. [PubMed: 23899561]

(19. Clayton EL, Minogue S, and Waugh MG (2013) Mammalian phosphatidylinositol 4-kinases as modulators of membrane trafficking and lipid signaling networks. Prog. Lipid Res 52, 294–304. [PubMed: 23608234]

(20). Boura E, and Nencka R (2015) Phosphatidylinositol 4-kinases: Function, structure, and inhibition. Exp. Cell Res 337, 136–145. [PubMed: 26183104]

(21). Chen Y, Wei L-N, and Müller JD (2003) Probing protein oligomerization in living cells with fluorescence fluctuation spectroscopy. Proc. Natl. Acad. Sci. U. S. A 100, 15492–15497. [PubMed: 14673112]

(22). Chen Y, and Müller JD (2007) Determining the stoichiometry of protein heterocomplexes in living cells with fluorescence fluctuation spectroscopy. Proc. Natl. Acad. Sci. U. S. A 104, 3147–3152. [PubMed: 17307882]

(23). Macdonald P, Johnson J, Smith E, Chen Y, and Mueller JD (2013) Brightness analysis. Methods Enzymol. 518, 71–98. [PubMed: 23276536]

(24). Coyle JE, Qamar S, Rajashankar KR, and Nikolov DB (2002) Structure of GABARAP in Two Conformations: Implications for GABAA Receptor Localization and Tubulin Binding. Neuron 33, 63–74. [PubMed: 11779480]

(25). Li J, Chen Y, Li M, Carpenter MA, McDougle RM, Luengas EM, Macdonald PJ, Harris RS, and Mueller JD (2014) APOBEC3 multimerization correlates with HIV-1 packaging and restriction activity in living cells. J. Mol. Biol 426, 1296–1307. [PubMed: 24361275]

(26). Li J, Barylko B, Johnson J, Mueller JD, Albanesi JP, and Chen Y (2012) Molecular Brightness Analysis Reveals Phosphatidylinositol 4-Kinase IIβ Association with Clathrin-Coated Vesicles in Living Cells. Biophys. J 103, 1657–1665. [PubMed: 23083708]

(27). Li J, Barylko B, Eichorst JP, Mueller JD, Albanesi JP, and Chen Y (2016) Association of Endophilin B1 with Cytoplasmic Vesicles. Biophys. J 111, 565–576. [PubMed: 27508440]

(28). Kraft LJ, Nguyen TA, Vogel SS, and Kenworthy AK (2014) Size, stoichiometry, and organization of soluble LC3-associated complexes. Autophagy 10, 861–877. [PubMed: 24646892]

(29). Wu B, Chen Y, and Müller JD (2010) Heterospecies partition analysis reveals binding curve and stoichiometry of protein interactions in living cells. Proc. Natl. Acad. Sci. U. S. A 107, 4117–4122. [PubMed: 20142515]

(30). Chen Z-W, Chang C-SS, Leil TA, Olcese R, and Olsen RW (2005) GABAA receptor-associated protein regulates GABAA receptor cell-surface number in Xenopus laevis oocytes. Mol. Pharmacol 68, 152–159. [PubMed: 15814572]

(31). Alam J, Deharo D, Redding KM, Re RN, and Cook JL (2010) C-terminal processing of GABARAP is not required for trafficking of the angiotensin II type 1A receptor. Regul. Pept 159, 78–86. [PubMed: 19766149]

(32). Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, and Yoshimori T (2004) LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J. Cell Sci 117, 2805–2812. [PubMed: 15169837]

(33). Barylko B, Gerber SH, Binns DD, Grichine N, Khvotchev M, Südhof TC, and Albanesi JP (2001) A novel family of phosphatidylinositol 4-kinases conserved from yeast to humans. J. Biol. Chem 276, 7705–7708. [PubMed: 11244087]

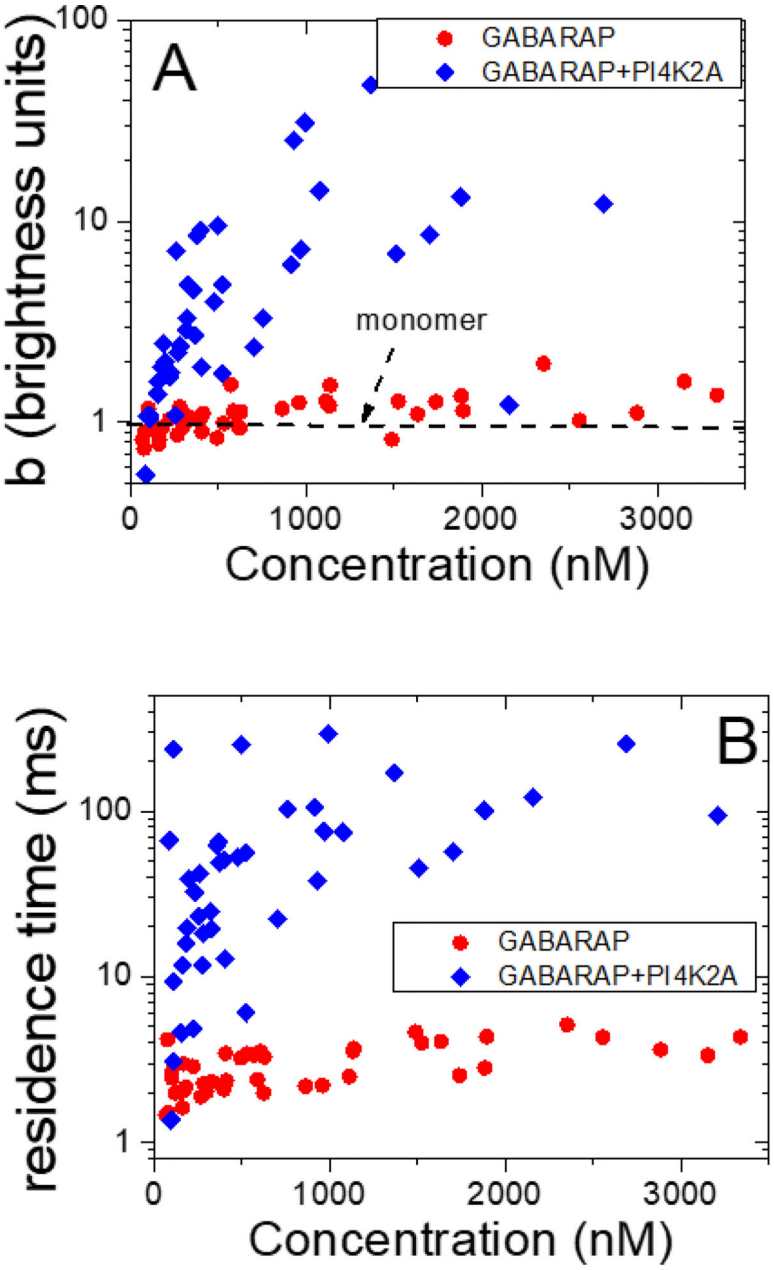

Figure 1. Characterization of GABARAP in the cytosol by FFS.

Molecular brightness (A) and residence time (B) of GFP-GABARAP as a function of protein concentration either in the absence (●) or presence of mCherry-PI4K2A (♦).

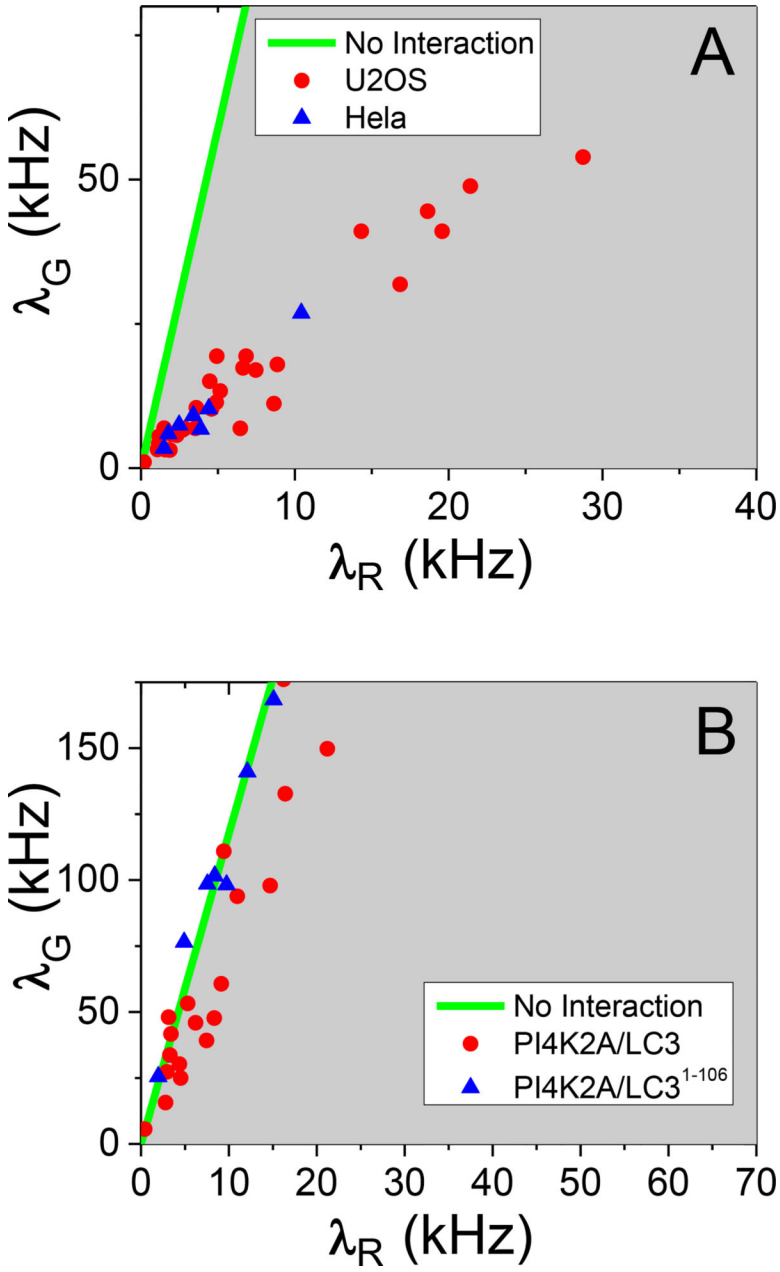

Figure 2. Plots of the HSP brightness from the red and green channel, respectively.

The green line represents HSP brightness values of GFP-labeled proteins that do not co-migrate with mCherry-labeled proteins. The gray shaded zone demarks all possible brightness vectors where GFP-labeled proteins co-migrate with mCherry-labeled proteins. (A) HSP brightness of GFP-PI4K2A and mCherry-GABARAP. HSP brightness vectors for U2OS (●) and Hela (▲) cells coexpressing GFP-PI4K2A and mCherry-GABARAP are shown. All HSP brightnesses are in the shaded area, clearly indicating that GFP-PI4K2A and mCherry-GABARAP are co-mobile. (B) HSP brightness of GFP-PI4K2A and mCherry-LC3. HSP brightness vectors for GFP-PI4K2A coexpressed with wt mCherry-LC3 (●) and with mCherry-LC3 (1–106) (▲).

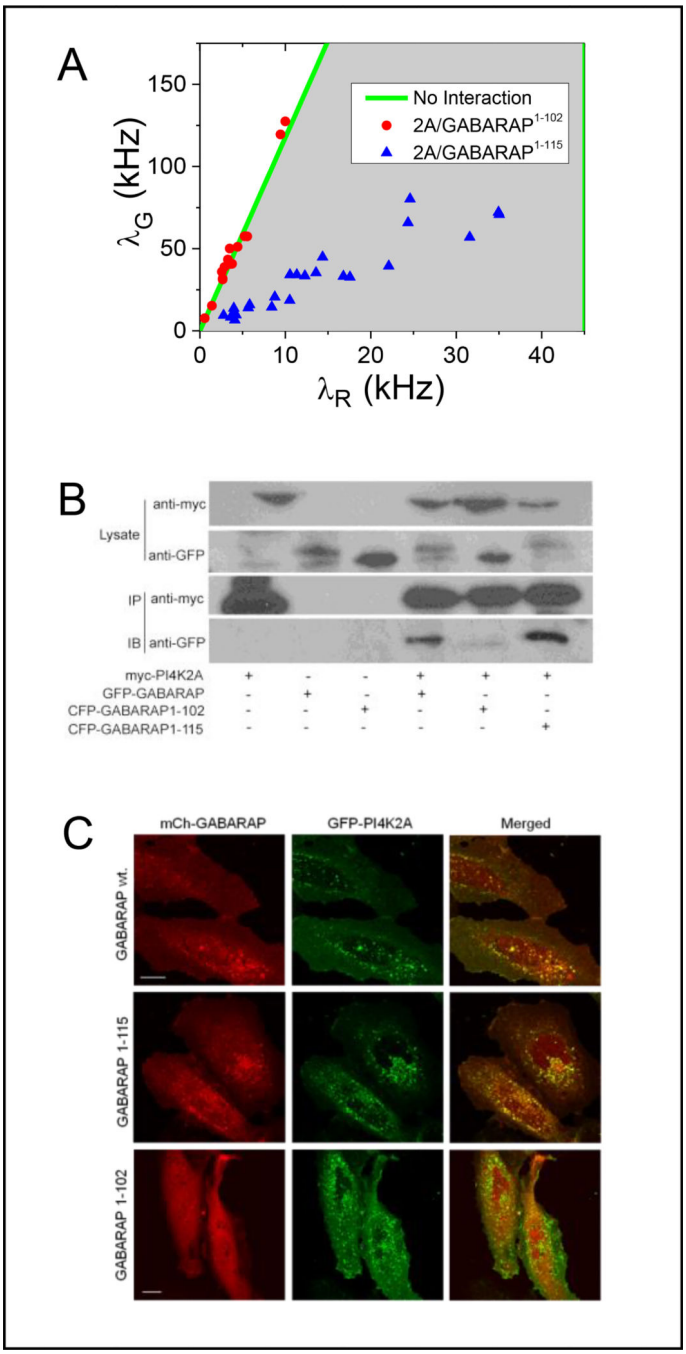

Figure 3. Characterization of GABARAP mutants.

(A) Dual-color HSP Brightness vectors for GFP-PI4K2A and mCherry-GABARAP$^{1–102}$ (●), or mCherry-GABARAP$^{1–115}$ (▲). (B) Myc-PI4K2A coimmunoprecitates with CFP-GABARAP$^{1–115}$ and not GABARAP$^{1–102}$. HEK-293 cells co-transfected with myc-PI4K2A and CFP-GABARAP$^{1–102}$ and CFP-GABARAP$^{1–115}$ were lysed and immunoprecipitated with anti-myc. Coimmunoprecipitated CFP-GABARAPs was detected by Western blotting with anti-GFP. (C) Using deletion analysis to identify regions in GABARAP that are necessary for its interaction with PI4K2A. Hela cells co-transfected with mCh-

GABARAP$^{1–102}$ GABARAP$^{1–115}$ with GFP-PI4K2A. Confocal images show that full-length GABARAP and GABARAP$^{1–115}$ have similar co-localization with PI4K2A and GABARAP$^{1–102}$ co-localization with PI4K2A was impaired. (Scale bar, 10μm).