様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　２ 年　 ７ 月 １５ 日現在

機関番号：２１６０１
研究種目：研究活動スタート支援
研究期間：2018～2019
課題番号：１８Ｈ０６１８３・１９Ｋ２１２８８

研究課題名（和文）Establishing a framework for EGFR attenuation through structure-guided targeting of GGA2

研究課題名（英文）Establishing a framework for EGFR attenuation through structure-guided targeting of GGA2

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交付決定額（研究期間全体）：（直接経費）　　2,300,000円

研究成果の概要（和文）：本研究の目的は、GGA2-EGFR-jxt複合体の構造解析から抗がん剤を設計することである。しかし多くの労力を費やしたがその相互作用を確証することが出来なかった。蛍光顕微鏡を用いた共鳴エネルギー移行による相互作用の検証も行なったが、GGA2-jxt複合体は検出されなかった。GGA2抗体はEGFR依存性の腫瘍細胞を検出する有用なツールになるため、モノクローナル抗体作製用のGGA2を作製し、培養液から抗体を精製した。次にEGFR再利用に関わるAPmuの構造解析を目指した。構造解析に利用できる良質なタンパク質を得られず、哺乳類細胞での発現も乏しいことから、Apmuは細胞毒性を有すると示唆された。

研究成果の学術的意義や社会的意義
I could not achieve my goal. I wanted to develop anticancer drugs to interfere with GGA2-EGFR by structure-based design. No suitable protein complex was made.
I could make protein for GGA2 antibody generation and purification. This antibody could be a tool towards personalised cancer treatment.

研究成果の概要（英文）：The goal is to obtain a GGA2-EGFR-jxt complex to design anticancer drugs. I made many constructs to study the interaction for structural studies. I could not corroborate previous experiments. Therefore,I made fluorescent resonance energy transfer pairs for fluorescence microscopy. Many constructs were screened for interactions, but I was unable to obtain a complex.
A GGA2 monoclonal would be an invaluable tool in detection of GGA2 in tumours of patients with EGFR-dependent cancers. I produced GGA2hinge, which was used to generate monoclonal antibodies. I purified the antibody from culture medium, which is now used to analyse patient materials.
I shifted my project to the mu adaptor protein (APmu) involved in EGFR recycling. Many (un)fused constructs were made for expression in bacterial cells. I was unable to obtain protein for structure studies. Expression in mammalian cells also failed, suggesting that APmu is toxic. More experiments are needed, but that is outside the time limit.

研究分野：Structural biology

キーワード：GGA2　EGFR　Crystallography　Signal-disruption　Protein complex　FLIM　Confocal microscopy　Adaptor protein complex

1．研究開始当初の背景

① The Golgi-localised, γ-adaptin ear-containing, ADP ribosylation factor-binding proteins (**GGAs**) are clathrin adaptor proteins that are involved in correct intracellular compartment delivery of substrate proteins (see Bonifacino. *Nat. Rev. Mol. Cell Biol.*, 2004 for review). Humans have three GGAs (**GGA1-3**) that share 30% sequence identity. GGAs arrange transport of clathrin-coated vesicles between the trans-golgi network (TGN), endosomes and the lysosome. GGAs have four functional regions, a substrate-binding VHS, an adaptor protein binding GAT, an unstructured region that binds clathrin and a C-terminal accessory protein binding domain called GAE (Fig. 1). This project focuses mostly on the cargo-binding VHS domain of GGA2 because we are interested how GGA2 is involved in trafficking and recycling of molecules in certain cancer cells. The VHS domain recognises a specific motif in the cargo molecule called the acid cluster dileucine (ACLL). A well-know substrate of GGAs are the mannose-6-phosphate receptors (MPR) that bind enzymes destined for the lysosome by means of their mannose-6-phosphate targeting signal. GGAs are in charge of delivering MPR and substrate to the lysosome and recycling MPR back to the TGN (Fig. 1). The GGAs are particularly fascinating because they combine several functionalities into only one polypeptide chain unlike the adaptor proteins (APs) complexes.

The epidermal growth factor receptor (**EGFR**) is an essential oncogene for signal transduction in dividing cells to activate or deactivate cell proliferation, thereby EGFR is involved in cancer development. EGFR has an extracellular domain that bind EGF, a transmembrane helix, a juxtamembrane region (jxt) and a C-terminal tyrosine kinase involved in signal transduction (Fig. 1). Upon binding of the EGF signalling molecule EGFR molecules dimerise partly through the jxt region and undergo cross phosphorylation which transfers the signal downstream (Jura et al., *Cell*, 2009). EGFR is of vital importance for proper cell cycle maintenance, which is exemplified by the fact that EGFR is upregulated in certain types of cancer. An important mechanism for attenuation of the EGFR signal is endocytosis (Fig. 1), which is achieved through AP recruitment and clathrin-coating of endocytotic vesicles after EGF activation (Tomas et al., *Trends in Cell Biol.*, 2014 for review). EGFR vesicles are then transported to early endosomes followed by recycling or degradation via late endosomes/multivesicular bodies (Fig. 1).

Knockout of GGA2, but not GGA1/3, is neonatally lethal (Govero et al., *PloS one*, 2012). Dr. Uemura at the Fukushima Medical University showed that siRNA knockdown of GGA2 results in decreased EGFR expression because of enhanced lysosomal degradation. Interestingly, knockdown of GGA1/3 has the opposite effect (Uemura et al., *Sci. Rep.*, 2018). EGFR levels are increased under the influence of GGA2 by a mechanism which has not yet been explored at the atomic level. Uemura et al. proposed that GGA2 recognises the jxt-region of EGFR (Fig. 1), which raises an important question that is at the heart of this project: **1) How does GGA2 recognise EGFR?** Because jxt lacks the necessary acid cluster dileucine, suggesting that GGA2 recognises EGFR through a different mechanism. Mutagenesis of GGA2 N108, essential for cargo ACLL recognition (Misra et al., and Shiba et al., *Nature*, 2002), abolishes jxt binding (Uemura et al., *Sci. Rep.*, 2018), suggesting that the jxt-recognition region is near the ACLL-peptide binding groove. Another question we want to answer in this project is: **2) Why is GGA2 involved in EGFR trafficking and recycling and not GGA1/3?** We know that GGA1, 2 and 3 recognise jxt (Uemura et al., *Sci. Rep.*, 2018), but only knockdown of GGA2 promotes lysosomal targeting. The jxt domain of EGFR has been implicated in dimerisation-

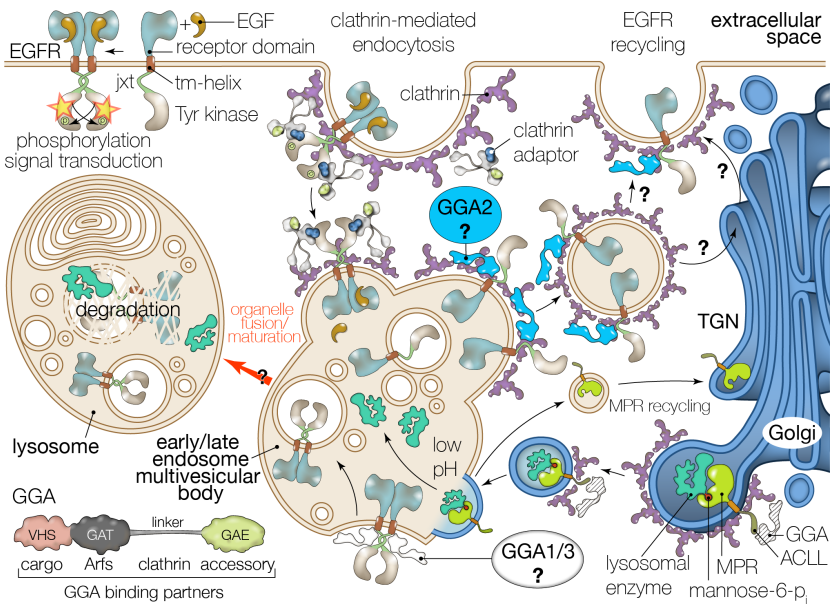

Fig. 1: Simplified vesicle transport model with highlighted GGA and EGFR.

driven activation of the dimeric EGFR signalling complex (Jura et. al. *Cell*, 2009). Non-destructive plasma membrane trafficking of EGFR could be enhanced by GGA2-driven monomerisation (Fig. 1) - through binding of jxt and disruption of the dimer state - since it has been suggested that the monomeric form is prone to recycling (Tanaka et al., *J. Biol. Chem.*, 2018). The answers to why GGA1/3 do the opposite to GGA2 (Fig. 1) must lie in the atomic details of the recognition of the jxt-region and that interaction is the subject of our proposal.

② To study the involvement of GGA2 in tumour development, it would be advantageous to be **able to detect GGA2 levels in patient cancer tissues**. Among the GGA proteins the hinge region is the most dissimilar (~30% sequence identity) while the domains are more conserved (~50-60% sequence identity). Therefore, the hinge region provides the best opportunity against which to generate a GGA2-specific monoclonal antibody that does not interact with GGA1 and 3. Such an approach to generate a GGA2-specific antibody has been used successfully before (Mardones et al., *Mol. Biol. of the Cell*, 2007), however the published hybridoma cell line is not available to us. Therefore, we decided to generate our own monoclonal antibodies using a GGA2hinge-derived peptide.

③ Like GGA2, **other adaptor proteins** (Fig. 1) **are involved in the recycling of EGFR** and as a side project I have been interested in studying APμ adaptor proteins. Some APμ protein members have been extensively studied, but some have unknown cargo and are of interest in the trafficking and recycling of many different proteins involved in cell signalling and cancer.

２．研究の目的

① **Our main research questions is how does GGA2 recognise EGFR jxt and what is the main difference compared to GGA1/3**, we can establish a methodology - through the design of high-affinity molecules - to specifically disrupt GGA2-mediated plasma membrane recycling of EGFR. This will cause attenuation of the EGFR signal by promoting its lysosomal degradation. This route is of particular interest for cancers where tumours show increased presence of GGA2 and EGFR (Uemura et al., *Sci. Rep.*, 2018). This study of the relationship between GGA2 and EGFR takes us into hitherto unexplored avenues of anti-cancer drug development, since the link between EGFR and GGA2 has only recently been discovered (Uemura et al., *Sci. Rep.*, 2018).

② Specific **monoclonal antibodies will be more and more valuable in personalised medicine**. In the future, a GGA2 antibody could be used to identify whether a patient tumour has abnormal levels of GGA2 and treatment strategies could be adapted.

③ We need to **find suitable expression vectors** for the production of the μ adaptor protein. I would want to explore fusions with e.g. maltose binding protein (MBP) in either *E. coli* or mammalian cells. Once we have obtained large quantities of pure APμ we can start crystallisation trials. The structure of APμ will reveal its cargo specificity and we will be able to perform structure-based drug design to develop small molecules to interfere with cargo-recognition and thereby inhibit tumour growth similar to ①.

３．研究の方法

① Previously co-immunoprecipitation experiments were used to probe the interaction between jxt and GGA2 (VHS/VHS-GAT). However, for structural studies those experiments are insufficient, we need large quantities of complexed proteins at high purity. Milligram quantities are required for crystallisation trials. Following an initial hit the crystals are optimised and diffraction data can be collected. At Fukushima medical university we have access to a crystallisation robot as well as synchrotron access through our collaborators.

The main focus of the project is obtaining a crystal structure of a jxt-GGA2 complex, however, it would valuable to probe their interaction both *in vivo* and *in vitro*. A technique that can be used to measure the interactions between two molecules inside the cell is fluorescence lifetime imaging microscopy (FLIM). Two molecules are expressed labeled with Förster resonance energy transfer (FRET) donor acceptor pairs and if they interact the fluorescence lifetime of the donor molecule decreases. These experiments can be performed through a collaboration at Fukushima medical university. Once we have a crystal structure available different methods can be used to probe the interaction between GGA2 and a

drug lead compound. Furthermore, small incremental changes in the optimisation of such a lead can be evaluated using for example co-immunoprecipitation, gel filtration, FLIM or thermal shift experiments.

② Because the hinge region of the GGA proteins is the most variable among GGA1, 2 and 3 it would be best to generate monoclonal antibodies from the hinge alone. Since the hinge is a peptide, fusion to GST would increase expression levels, stability and ease of handling. I decided on a double-tagged system with GST, a cleavable linker, GGA2 peptide and a 6his tag. In that way the peptide can easily be captured on glutathione-agarose beads, cleaved from the GST tag on the column followed by 6his-tag affinity chromatography and gel filtration. After that the hinge peptide would be sent to a company that generates monoclonal hybridoma cell lines after which the antibody can easily be purified from the medium.

③ The APμ adaptor is a two-domain protein with an N-terminal domain and a C-terminal cargo-binding domain. For structural studies I would want to produce both unfused or fused to a carrier or the C-terminal region by itself or fused to a carrier, all constructs would have a 6his-tag for purification.

4．研究成果

① I have produced around 30 different constructs of VHS/VHS-GAT and jxt. These constructs were subjected to different experiments to show binding interaction including gel filtration chromatography, co-immunoprecipitation and FLIM. Gel filtration chromatography is the most import because in the case of successful complex formation the material can immediately be used for crystallisation experiments. Unfortunately, **I was unable to show complex formation** between GGA2 (VHS/VHS-GAT) and jxt. In the case of formation of such a complex VHS/VHS-GAT and jxt would co-migrate through the column, which did not occur (Fig. 2A/B). Because we were not able to obtain a complex structure, we were not able to find out **how GGA2 and jxt interact and why GGA1 and 3 behave differently than GGA2**.

For the FLIM experiments we co-expressed FRET pairs of donor VHS/VHS-GAT (fused to mTurquoise) with acceptor jxt (fused to mCherry) (Fig. 2C). As positive control we used the ACLL C-terminal region of a mannose-6-phosphate receptor (mCherry-ACLL). While co-expression of mTurq-VHS/VHS-GAT with mCherry-ACLL results in a significant decrease of the fluorescence life time from approximately 3.6 ns to 3.2 ns (Fig. 2C/D/E), co-expression with mCherry-jxt does not show such a decrease (Fig. 2D/E). These experiments indicate that while VHS/VHS-GAT are in close proximity to ACLL inside the native-like environment of the cell, jxt does not bind. Furthermore, it could be shown that the decrease in fluorescence lifetime in VHS/VHS-GAT-ACLL was a specific FRET effect because the non-FRET lifetime could partly be recovered upon acceptor photobleaching, which did not happen in the case of jxt (data not shown).

Although these experiments **do not prove or disprove the work that has been done** previously on GGA2 and jxt, they do indicate that the VHS/VHS-GAT-jxt interaction could rather weak or transient. Therefore the GGA2-jxt pair was difficult to observe using my experiments and **may not be an ideal candidate for drug design** as initially anticipated in our project. This is because structure-based drug design relies on tight interactions between drug and drug target. Therefore a different approach would need to be explored to design drugs that interfere with EGFR trafficking.

② GGA2hinge was fused to GST via a protease-sensitive linker followed by a C-terminal 6his-tag (Fig. 2F). This construct expressed well and bound tightly to the glutathione agarose (Fig. 2F lanes lysate and flow through). After that the GGA2hinge-6his could be released from the column by addition of 3C protease (Fig. 2F lane 3C:Release). Following proteolytic release the protein was subjected to 6his-tag affinity chromatography and eluted by imidazole (Fig. 2F lane E1-E3). Subsequently the eluted hinge was further purified by gel filtration (Fig. 2F chromatogram), which resulted in highly pure GGA2hinge (Fig. 2G) that could be used for antibody generation by a company. The antibody from the best clone was purified by Protein G affinity chromatography followed by desalting (Fig. 2H). Currently **the antibody's efficacy** in histological screening for tumours with abnormal GGA2 levels **is being evaluated**.

③ I have generated approximately 20 constructs to express the μ adaptor protein. However, **I was unable to obtain soluble material for structural studies**. Surprisingly, expression in mammalian cells also failed (Fig. 2I), which suggests that there is an intrinsic problem with this protein. Either it can only be expressed at low levels, or the protein is toxic to the cell lines used for protein production. Further experiments are needed to investigate these issues, however this falls outside the project's time limit.

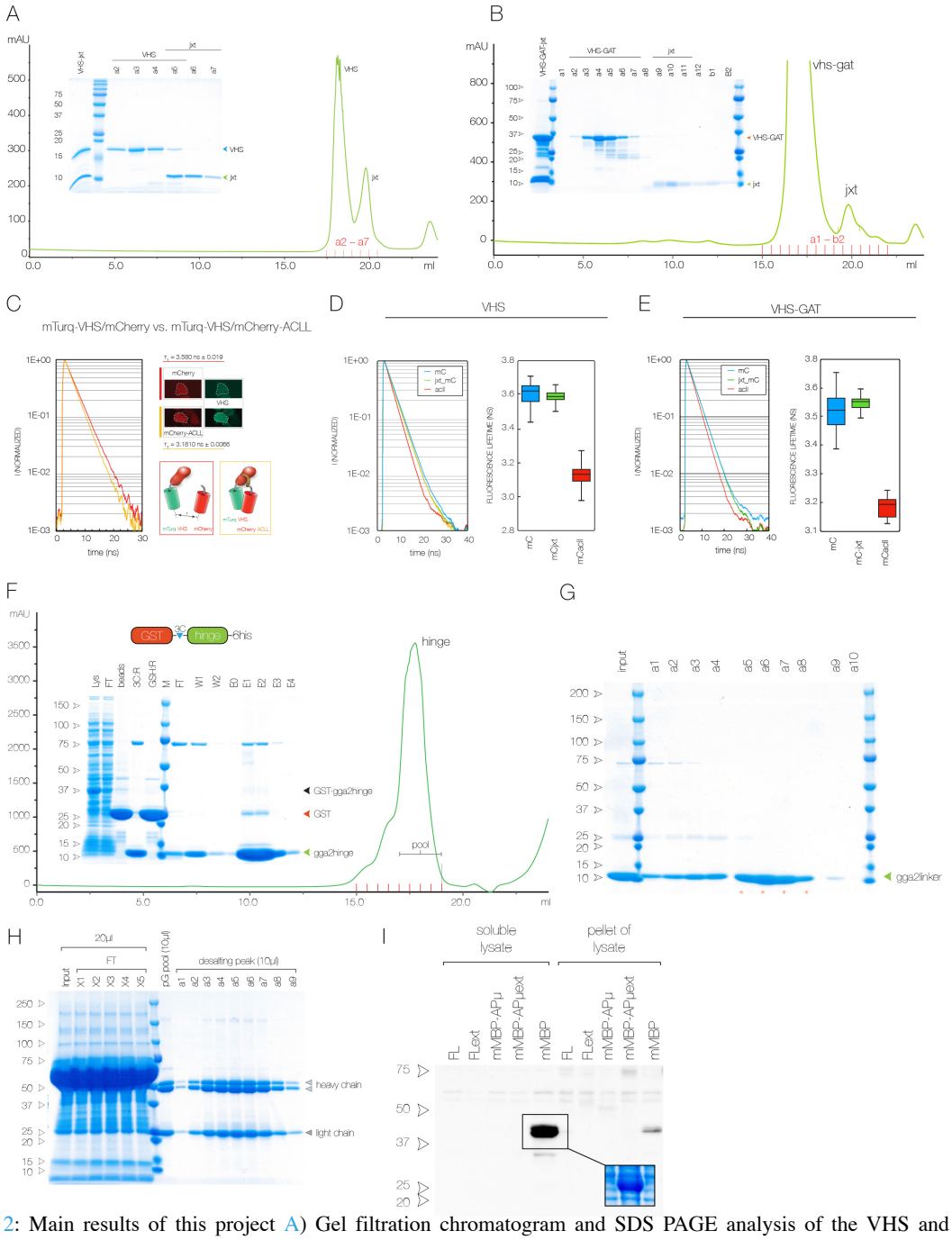

Fig. 2: Main results of this project A) Gel filtration chromatogram and SDS PAGE analysis of the VHS and jxt interaction. B) same as A, but with VHS-GAT. C) In-cell FLIM analysis. Schematic representation of co-expression of mTurquoise-fused VHS and negative control mCherry (bottom right red box) or mTurquoise-VHS and mCherry-ACLL (bottom right, yellow box). Representative example of cells co-expressing these constructs are shown top right. Fluorescence lifetime decay profiles of positive and negative control are shown on the left. D) Decay profile of co-expression experiments of mTurq-VHS with mCherry alone, jxt-mCherry and mCherry-ACLL (blue, green and red respectively) are shown on the left with their averaged fluorescence lifetimes represented with error margins as box plots on the right with the same colours. E) Same as D, but with VHS-GAT. F) Purification of GGA2hinge using glutathione-agarose, nickel-agarose and gel filtration. G) SDS PAGE gel of the chromatogram in F. H) Protein G affinity purification of hybridoma cell medium followed by desalting. I) Expression of APμ constructs in HEK293 cells analysed by anti-his western blot; only mMBP was expressed. The inset shows the mMBP band stained with coomassie, indicating high expression level of mMBP positive control in the cells.

References

Bonifacino. *Nat. Rev. Mol. Cell Biol.*, **5**, 2004 23-32.
Jura et al., *Cell*, **137**, 2009, 1293-1207.
Tomas et al., *Trends in Cell Biol.*, **24**, 2014, 26-34.
Govero et al., PloS one, **7**, 2012.
Uemura et al., *Sci. Rep.*, **8**, 2018.
Misra et al., and Shiba et al., Nature, **415**, 2002, 933-937.
Tanaka et al., J. Biol. Chem., **293**, 2018, 2288-2301
Mardones et al., *Mol. Biol. of the Cell*, **18**, 2007, 3486-3501.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計1件（うち招待講演　1件／うち国際学会　1件）

１．発表者名 Marcel Bokhove
２．発表標題 An embrace: Structural basis of the human endoglin-BMP9 interaction
３．学会等名 International Symposium on Diffraction Structural Biology 2019（招待講演）（国際学会）
４．発表年 2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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