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研究課題名（和文）Studies on influenza A virus M2-host interactome and its role in virus replication

研究課題名（英文）Studies on influenza A virus M2-host interactome and its role in virus replication

研究代表者

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研究成果の概要（和文）：ウイルス複製中のIAV-M2の多様な役割にもかかわらず、これらの多様な機能の分子基盤についてはほとんど知られていません。 細胞膜に向かう途中で、M2外部ドメインと細胞質尾部は、小胞/オルガネラと細胞質環境にさらされ、そこでさまざまな細胞タンパク質と相互作用する可能性があります。 この研究中に、IAV M2タンパク質と相互作用してウイルス複製に影響を与えるホストタンパク質を特定しました。 M2と原形質膜の相互作用を支配する2つのIAVサブタイプ間で一意に異なる2つのアミノ酸残基（54および57位）が見つかり、ウイルスの出芽/放出に役割を果たしています。

研究成果の学術的意義や社会的意義
Our findings add significant information to the existing scientific knowledge on influenza A virus M2 protein roles in virus infected cells, plausibly providing the basic information that can be used for the development of strategies for virus control and therapeutic measures.

研究成果の概要（英文）：Despite the diverse roles of IAV-M2 during virus replication, little is known about the molecular basis of these diverse functions. While en route to the cell membrane, the M2-ectodomain and cytoplasmic tail are exposed to vesicular/organelle and cytoplasmic environments, where they may interact with various cellular proteins. During this study we identified host-proteins which interact with IAV M2 protein and affect virus replication. We found two amino acid residues (position 54 and 57) uniquely different between two IAV subtypes that governed the interaction of M2 with plasma membrane thereby playing role in virus budding/release. The M2 protein, besides increasing the transmembrane domain (TMD) length and having two inherent lipid raft targeting features, did not associate with lipid rafts suggesting that the M2-TMD length is not the only non-lipid micro-domain localization determinant. These results also suggested that virus release/budding requires optimum length of M2-TMD.

研究分野：Veterinary Science

キーワード：influenza A virus　M2 protein

Formula S-19, F-19－１, Z-19 (common)

1. 研究開始当初の背景

The influenza A virus (IAV) M2 is among the smallest *bona fide* ion channel proteins. Each M2 monomer consisting of 97 amino acid residues is divisible into the N-terminal ectodomain (ED, residues 1–24), middle transmembrane domain (TMD, residues 25–43), and C-terminal domain (residues 44–97). The ED is required for its incorporation in the influenza virion. The M2-TMD acts as proton channel and is involved in virion uncoating and viral ribonucleoprotein (vRNP) release, and the M2-CT is involved in virus assembly, morphogenesis and budding. While *en route* to the cell membrane, the M2-ED and -CT are exposed to vesicular/organellar and cytoplasmic environments, where they interact with various cellular proteins and might interfere/affect the host cellular functions. In contrast to the IAV hemagglutinin (HA) and neuraminidase (NA), M2 is located in the peri-lipid raft regions of cell membrane, despite it has lipid-raft targeting regions. It is speculated, but no scientific evidence, that the smaller length of M2-TMD (19 aa residues) prevents the association of M2 with lipid-rafts.

2. 研究の目的

Despite the diverse roles of IAV-M2 during virus replication, little is known about the molecular basis of these diverse functions in infected cells. The M2 protein from the ingress site to the egress point interacts with various host factors; however very little is known about the M2-host interactome and their role in virus replication. In the present study, we systematically investigated and shortlist the sixty-two host factors, we had identified. The interaction of M2-CT region with plasma membrane affects the process of virus budding and morphogenesis. We, using a monoclonal antibody against M2-ED, identified unique amino acid residues and their role in virus budding. We also focused on the M2-TMD propensity to localize in the non-lipid raft regions of plasma membrane despite having potential lipid-raft targeting regions.

3. 研究の方法

(1) Host cellular proteins were co-immunoprecipitation (Co-IP) from cells transfected with M2-expression plasmid (pCAGGS-M2) using a chemical cross linker (DSS), and anti-M2 ectodomain mAb (rM2ss23R) conjugated to gel. The co-immunoprecipitated proteins were identified by mass spectrometry. Then, we selected three siRNAs for each identified target gene. After optimizing the virus dose, siRNA dose, cell lines and plaque assay for quantifying the virus in siRNA treated cells, screening was conducted. The effect of gene knock-down on virus replication was considered positive or negative if at least 2/3 siRNAs produced similar effect. In this way. The shortlisted genes were further evaluated using various virological and molecular biological techniques.

(2) In order to determine the relation between M2-TMD length and lipid raft association and virus replication, TMD-length mutants were constructed by introducing insertion mutations. Insertion mutations were introduced so that TMDs of mutant proteins were three, six or eight amino acid longer than that of wtM2-TMD. The mutant proteins were characterized by comparing their cell surface expression and cytotoxic potential, an ion channel dependent activity, with that of wtM2 protein. Lipid raft localization of wtM2 and M2-TMD mutants was assessed by TritonX-100 (TX100) solubility assay and immunofluorescence (IF) microscopy. The viruses with M2-TMD mutations were generated by reverse genetics and examined by transmission electron

microscopy (TEM).

(3) The reassortant viruses between A/Aichi/2/1968 (H3N2) (Aichi) and A/ PR/8/1934 (H1N1) (PR8) were constructed using reverse genetics. The swap mutations were introduced at amino acid (aa) residues 54, 57 and 89 between Aichi and PR8 and mutant viruses were generated by reverse genetics. The role of the amino acid residues in virus replication and virus budding was assessed by modifies plaque reduction assay, proximity ligation assay (PLA), confocal microscopy, real-time PCR and other virological techniques.

4. 研究成果

(1) The information regarding the subcellular localization of identified host proteins was retrieved from databases such as DAVID, Locale, STRING, and UniProt. The subcellular localization analysis revealed that many of the host proteins were localized in those subcellular compartments where M2 protein is localized and its ED or CT are exposed such as ER, Golgi network, and plasma membrane (Fig

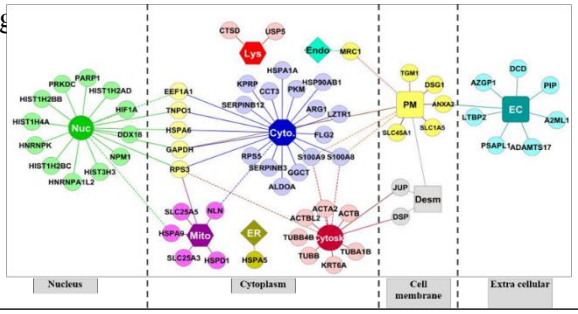

Fig. 1: The nodes in each compartment show the presence of unstimulated host proteins. The host proteins connected with lines can move between subcellular compartments. Abbreviations: Nuc: Nucleus; Cyto: Cytoplasm; Mito: Mitochondria; Lys: Lysosome; Endo: Endosome; ER: Endoplasmic reticulum; Cytosk: Cytoskeleton; PM: Plasma membrane; Desm: Desmosome; EC: Extracellular.

siRNA screening helped to shortlist five host genes whose knock-down caused more than seventy percent reduction (SLC1A5, EEAF1 , FLG2, SLC25A3 and SLC25A5) and three Genes caused increase (TNPO1, PARP1, HSPD1) in virus infectivity. We further focused on a shortlisted host protein, SLC1A5. We found that knockdown of SLC1A5 caused significant reduction in viral replication (Fig. 2) despite increase in virus internalization (Fig. 3). We also confirmed that M2 and SLC1A5 co-localization by IF.

Fig. 2

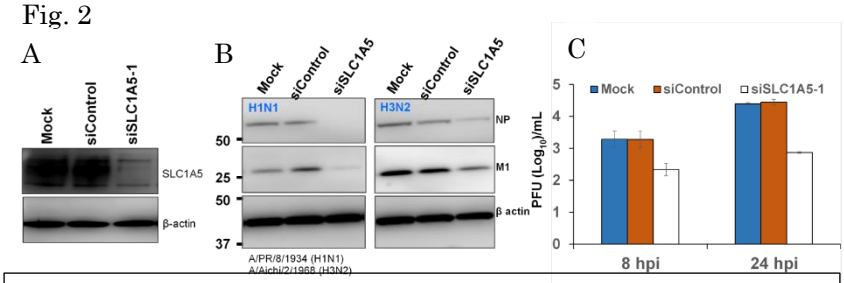

Fig. 2: **A.** Effect of control and gene-specific siRNAs on SLC1A5 expression. **B**. Effect of SLC1A5 on viral proteins in cells infected with different influenza A viruses. **C.** Effect of SLC1A5 knock-down on virus release. Cell supernatant were collected at indicated time points and virus titers were measure by plaque assay.

Fig. 3

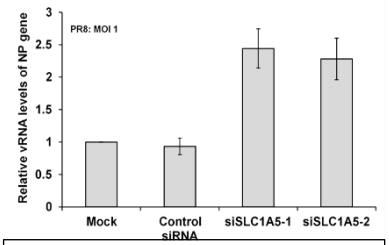

Fig. 3: Effect of SLC1A5 knock-down on virus internalization determined by real-time RT-PCR.

(2) The M2-TMD insertion mutants were constructed (Fig. 4). The immunofluorescence microscopy showed that cell surface expression of M2-TMD mutants was comparable to that of wt-M2 (Fig. 5). The M2 is cytotoxic for cells in ion channel activity dependent manner. So, we compared the cytotoxicity potential of TMD-

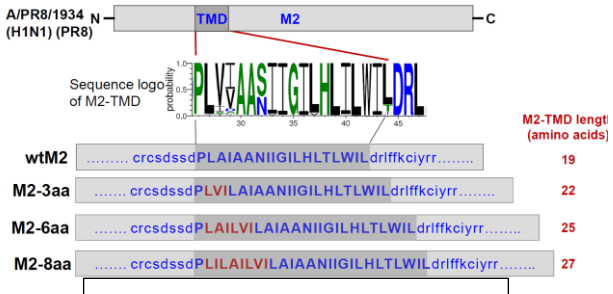

Fig. 4. Schematic diagram showing site of insertion mutations in M2-TMD.

mutants with that of wtM2 using green fluorescence assay (Fig, 6), and found that mutant proteins retained cytotoxicity potential similar to that of WTM2. Then, we evaluated the effect of M2-TMD insertion mutations on lipid raft association. TritonX-100 solubility assay showed that M2-TMD mutants, like wtM2, were largely found in TX100-soluble fractions, the non-raft microdomains; whereas HA remained associated with Lipid rafts (Fig.7). IF staining of lipid rafts and M2 protein, and colocalization analysis also confirmed that increasing the TMD length did not affect their non-lipid raft association character. Then attempts were made to rescue the viruses with M2-TMD mutations using reverse genetics approach. Interestingly, only virus with three amino acid insertions could be rescued, although viral proteins and viral RNA were detected from the supernatants of cells transfected for rescue of wt- and M2-TMD mutant viruses. Electron microscopy of rgPR8/wtM2 and rgPR8/M2-3aa viruses revealed no morphology or size differences (Fig. 8). However, virus could not be rescued using M2-6aa and M2-8aa. The M2 protein, besides increasing the TMD length and having two inherent lipid raft targeting features, did not associate with lipid rafts suggesting that the M2-TMD length is not the only non-lipid microdomain localization determinant. Furthermore, increasing the M2-TMD length did not seem to alter the M2 function, but somehow prevented virus rescue either by destabilizing the virus particles or by preventing the successful virus budding.

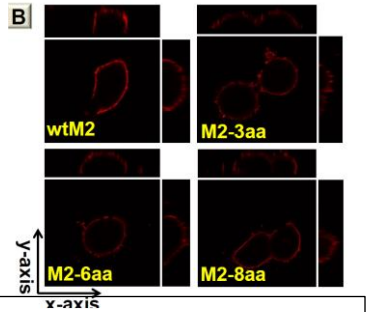

Fig. 5: Expression of wtM2 and M2-TMD mutant proteins on the surface of transfected cells.

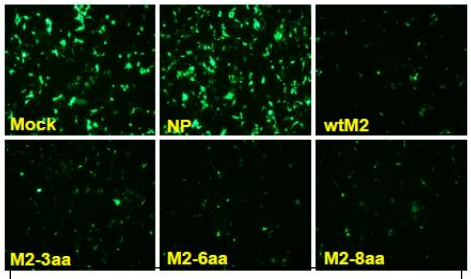

Fig. 6. Cell viability assay determined by green fluorescence assay.

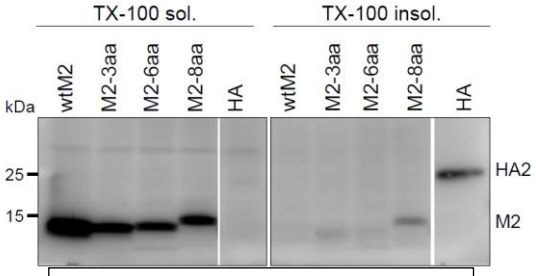

Fig.7. Triton-X solubility assay to determine M2 association with lipid rafts.

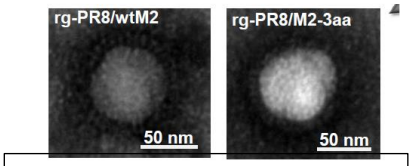

Fig. 8. Transmission electron microscopy of released virus particles.

(3) The anti-M2-ED specific antibody (rM2ss23) showed broad reactivity against M2 proteins of tested influenza A viruses (Fig. 9A). rM2ss23 did not neutralize the A/Aichi/2/1968 (H3N2)

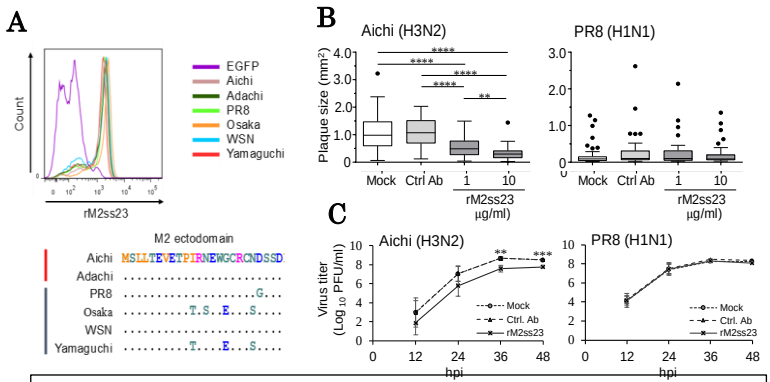

Fig. 9: **A**. Comparison of M2 ectodomain amino acid sequences and binding activity of rM2ss23 with the M2 of A/Aichi/2/1968 (H3N2) (Aichi), A/PR/8/1934 (H1N1) (PR8), and A/chicken/Yamaguchi/7/2004 (H5N1) IAVs. **B.** inhibitory effect of rM2ss23 on the Aichi and PR8 plaque sizes in MDCK cells. Boxplots of plaque sizes. **C.** Growth curves of Aichi and PR8 IAVs in the presence and absence of rM2ss23.

(Aichi) and A/PR/8/1934 (H1N1) (PR8) viruses, and also did not inhibit the ion channel activity of their M2 proteins. However, it inhibited the and also reduced the release of progeny viruses (Fig. 9B and C). Amino acid comparison of the rM2ss23 resistant and sensitive strains revealed that sensitive and resistant viruses differed at aa positions 54, 57 and 89 (Fig. 10A). Swapping aa residues between PR8 and Aichi showed that PR8 became sensitive to the inhibitory effect of rM2ss23 (Fig. 10 B). These findings suggested that amino acid residues at position 54 and 57 influenced the interaction of M2-CT with plasma membrane. Bioinformatics analysis also supported this assumption, since changing the aa residues at positions 54 and 57 changed the net charge and hydrophobicity of the amphipathic helix region. The colocalization of M2 and HA was also studied since HA-M2 are known to interact at virus budding sites. IF study also showed that rM2ss23 caused distinct clustering of Aichi-M2 into patches whereas PR8-M2 showed uniform cell surface distribution. However, replacing the PR8-M2 with Aichi M2 changed the M2 cell surface distribution of PR8/Aichi-M2 reassortant virus (Fig. 11). These findings suggested that amino acid residues in M2 CT affect the process of virus budding/ release. Therefore, it is highly plausible that process of virus budding and release might be different between different IAV strains, and this process is governed by nature of amino acids in M2-CT.

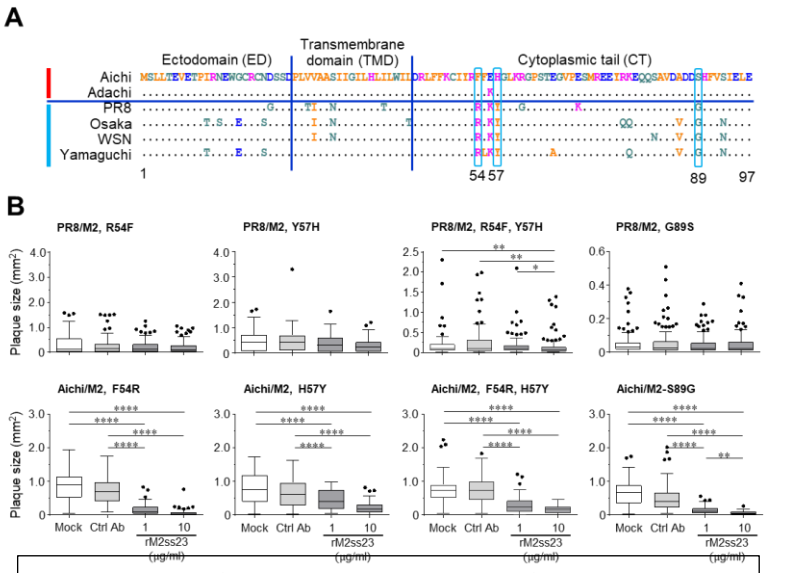

Fig. 10: **A.** Comparison of M2 ectodomain amino acid sequences of rM2ss23 resistant and sensitive strains. **B.** inhibitory effect of rM2ss23 on the mutant Aichi and PR8 viruses. Plaque sizes were measured using modified plaque assay in MDCK cells. Boxplots of plaque sizes.

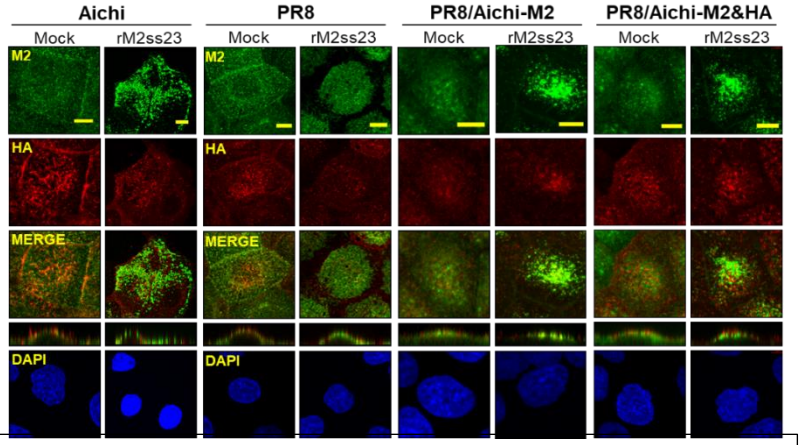

Fig. 11: Cell surface distribution of M2 of indicated viruses in infected MDCK cells. MDCK cells were infected with indicated IAVs at MOI 3 for eight hours. Then, cells were fixed, and cell surfaces were stained for viral M2 and HA proteins using anti-HA and anti-M2 mAbs followed by goat anti-mouse Alexa Fluor 488 for M2, and isotype specific goat anti-mouse IgG2a Alexa Fluor® 594 or goat anti-mouse IgG3 Alexa Fluor® 594 secondary antibodies for HA as explained in materials and methods. Images were taken

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５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　3件／うちオープンアクセス　3件）

１．著者名 Masahiro Sato, Junki Maruyama, Tatsunari Kondoh, Naganori Nao, Hiroko Miyamoto, Yoshihiro Takadate, Wakako Furuyama, Masahiro Kajihara, Hirohito Ogawa, Rashid Manzoor, Reiko Yoshida, Manabu Igarashi and Ayato Takada	４．巻 4;9(1)
２．論文標題 Generation of bat-derived influenza viruses and their reassortants	５．発行年 2019年
３．雑誌名 Scientific Reports	６．最初と最後の頁 1158
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41598-018-37830-x.	査読の有無 有
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３．雑誌名 International Journal of Molecular Sciences	６．最初と最後の頁 2649～2649
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms18122649	査読の有無 有
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１．著者名 Naganori Nao, Junya Yamagishi, Hiroko Miyamoto, Manabu Igarashi, Rashid Manzoor, Aiko Ohnuma, Yoshimi Tsuda, Wakako Furuyama, Asako Shigeno, Masahiro Kajihara, Noriko Kishida, Reiko Yoshida, Ayato Takada	４．巻 8
２．論文標題 Genetic Predisposition To Acquire a Polybasic Cleavage Site for Highly Pathogenic Avian Influenza Virus Hemagglutinin	５．発行年 2017年
３．雑誌名 mBio	６．最初と最後の頁 1-15
掲載論文のDOI（デジタルオブジェクト識別子） 10.1128/mBio.02298-16	査読の有無 有
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１．発表者名 Manzoor Rashid
２．発表標題 Mechanism of antiviral activity of an M2 ectodomain-specific monoclonal antibody against influenza A virus
３．学会等名 17th NSV (Negative Strand Viruses)（国際学会）
４．発表年 2018年

１．発表者名
Manzoor Rashid

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〔図書〕　計0件

〔産業財産権〕

〔その他〕
北海道大学人獣共通感染症リサーチセンター国際疫学部門
http://www.czc.hokudai.ac.jp/en/research/epidemiology/
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http://www.czc.hokudai.ac.jp/research/epidemiology/

６．研究組織

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