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学位論文名	**ラット E 型肝炎ウイルスの感染細胞からの放出に関与する宿主因子の同定とその機能解析**
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論文内容の要旨

1　研究目的

Recent reports have shown that rat hepatitis E virus (HEV) is capable of infecting humans. Rat HEV is also successfully propagated into human PLC/PRF/5 cells, raising the possibility of a similar mechanism shared by human HEV and rat HEV. It has previously been reported that human HEV recruited tumor susceptibility gene 101 (Tsg101) via its PSAP motif in open reading frame 3 (ORF3) protein and that it required multivesicular body (MVB) sorting for virion egress. However, rat HEV ORF3 has the PxYPMP motif instead of the PSAP motif. This proline-rich sequence is indispensable for rat HEV release, although the release mechanism and the host factors involved remain unclear.

2　研究方法

Morphological analysis of rat HEV particles was performed by transmission electron microscopy (TEM), followed by immune electron microscopy (IEM). To study the utilization of MVB sorting for rat HEV release, I used dominant-negative (DN) mutants of vacuolar protein sorting (Vps)4A or Vps4B and small interfering RNA (siRNA) targeting either Tsg101 or apoptosis-linked gene 2-interacting protein X (Alix) or neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4), in comparison with human HEV. In addition, I used DN mutant of Nedd4. To study the utilization of exosomal pathway for rat HEV egress, I used siRNA targeting Ras-associated binding 27A (Rab27A) or hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs); or the accelerator of exosome release [Bafilomycin A1 (Baf-A1)] or inhibitor of exosome release (GW4869). I performed immunofluorescence assays (IFA) to detect the co-localization of rat HEV ORF3 protein with Tsg101, Alix, Rab27A or Hrs; or rat HEV ORF2 and ORF3 with CD63, an MVB and exosome marker protein. Immunoprecipitation (IP) assays were performed to

study the direct interaction of rat HEV ORF3 protein with host factors Tsg101, Alix or Nedd4. To examine other host proteins that bind to rat HEV ORF3, I constructed FLAG (N-terminal)- or Myc (C-terminal)-tagged rat HEV ORF3 wild-type or its mutant with three proline to leucine substitutions (PQYPMP to LQYLML) in the proline-rich region of ORF3, then subjected them to co-IP assay with anti-FLAG or anti-Myc mouse mAb, followed by gel electrophoresis and silver staining.

3 研究成果

TEM images and IEM showed that rat HEV particles in the exosome fraction of culture supernatant are membrane-associated. Following treatment with detergent and protease, the particles lost their membranes and became membrane-unassociated, suggesting that the capsids of rat HEV particles are individually covered by lipid membrane. The overexpression of DN mutant of Vps4A or Vps4B decreased rat HEV release to 23.9% and 18.0%, respectively, indicating that the enzymatic activities of Vps4A and Vps4B are necessary for rat HEV egress. The release of rat HEV was decreased to 8.3% in Tsg101-depleted cells and to 31.5% in Alix-depleted cells. Similarly, specific depletion of Tsg101 or Alix significantly decreased the human HEV release to 12.8% and 30.9%, respectively. These results indicated that Tsg101 is important for rat HEV release; and that Alix is necessary for the release of both rat HEV and human HEV. In addition, rat HEV ORF3 protein was co-localized with either Tsg101 or Alix. However, the release of both rat HEV and human HEV in Nedd4-depleted cells increased to 507.9% and 436.3%, respectively; while the RNA levels of rat or human HEV in cells transfected with DN mutant of Nedd4 did not differ markedly from that of empty vector, indicating that Nedd4 is not essential for the release of both rat HEV and human HEV.

Rat HEV release was also decreased in Ras-associated binding 27A (Rab27A)- or hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)-depleted cells (to 20.1% and 18.5%, respectively). Furthermore, rat HEV ORF3 was co-localized with either Rab27A or Hrs. The extracellular rat HEV levels in the infected PLC/PRF/5 cells were increased after treatment with Baf-A1and decreased after treatment with GW4869. These results indicated that rat HEV utilizes MVB sorting for its release and that the exosomal pathway is required for rat HEV egress. Furthermore, triple staining showed that the rat HEV ORF2 and ORF3 proteins are co-localized in the cytoplasm, suggesting that membrane-associated rat HEV particles are present within the MVB.

Unlike human HEV ORF3 protein, rat HEV ORF3 protein did not interact with Tsg101, and neither rat HEV ORF3 nor human HEV ORF3 protein interacted with Alix nor

Nedd4 as indicated by co-IP assays. A host cellular protein at an approximate size of 90 kDa was seen to bind to either FLAG- or Myc-tagged wild-type rat HEV ORF3 but not to the ORF3 mutant (LQYLML). This host protein could be an alternative to Tsg101 that can bind to rat HEV ORF3.

4 考察

The present morphological analysis by TEM revealed that rat HEV particles are membrane-associated in culture supernatants and became membrane-unassociated particles of a smaller size after treatment with detergent and protease to remove the membranes. This is in agreement with the findings in human HEV, and suggests that the capsids of rat HEV particles are individually covered by a lipid membrane.

Many enveloped viruses acquire their membrane by hijacking the host cells' endosomal sorting complexes required for transport (ESCRT) machinery to facilitate the final stages of virion release such as for HIV, Ebola virus, or quasi-enveloped virus such as hepatitis A virus (HAV). In human HEV and avian HEV, the ESCRT components or ESCRT-related components involved in this event are Tsg101 and Vps4A/B. In the present study, rat HEV release was revealed to be ESCRT-dependent as the overexpression of DN mutants of Vps4A/B inhibited the rat HEV release, and the depletion of endogenous Tsg101 reduced the release efficiency of rat HEV. I also found that depletion of endogenous Alix decreased the virus release of not only rat HEV but human HEV as well. These results support the notion that the release of rat HEV is ESCRT-dependent with components such as Tsg101, Alix and Vps4A/B.

The rat HEV release was indeed inhibited by the depletion of endogenous Rab27A or Hrs, components required for exosomal secretion. In addition, rat HEV ORF3 protein was co-localized with either Rab27A or Hrs. I also showed that treatment of rat HEV-infected cells with Baf-A1 significantly increased the extracellular rat HEV RNA in a dose-dependent manner, while treatment with GW4869 decreased the extracellular rat HEV RNA, further supporting the notion that rat HEV utilizes the exosomal pathway for its egress, similar to other viruses, such as hepatitis C virus (HCV) and human herpes virus 6 (HHV-6). Taken together, my present findings indicated that rat HEV utilizes MVB sorting and the exosomal pathway to support its egress from infected cells.

As described above, the results from siRNA assays and IFA indicated that both Tsg101 and Alix are associated with rat HEV egress. However, rat HEV ORF3 did not bind to either protein. PxYPMP motif in rat HEV ORF3 protein was suggested to be associated with its egress in a previous study where the substitution of leucine for proline (PQYPMP into

LQYLML) was associated with disrupted membrane formation, and thus decreased virus release. The present analysis suggested that one host cellular protein at an approximate size of 90 kDa was bound to both FLAG- and Myc-tagged rat HEV ORF3 wild-type but not to the LQYLML mutant, therefore, it might be a candidate alternative to Tsg101, which is known to bind to the ESCRT-related motif in human and avian HEV ORF3, PSAP.

5　結論

The present study showed that rat HEV utilizes MVB sorting and the exosomal pathway to support its egress from infected cells. While the results from siRNA assays and IFA indicated that both Tsg101 and Alix are associated with rat HEV release, rat HEV ORF3 did not bind to either protein. I detected one host cellular protein at an approximate size of 90 kDa that bound to rat HEV ORF3 and might be a candidate alternative to Tsg101, associated with virion egress (or with other ORF3-related functions). The exploration of host cellular factors that bind to rat HEV ORF3 to support virion egress warrants further study.

論文審査の結果の要旨

学位論文表題：ラット E 型肝炎ウイルスの感染細胞からの放出に関与する宿主因子の同定とその機能解析

本論文は、ラット E 型肝炎ウイルス（HEV）の感染細胞からの放出機構を解明し、それに関与する宿主因子の同定を試みた研究である。

この研究で申請者は、1）Vps4A, Vps4B の dominant negative (DN) mutant 過剰発現系を用いて、ラット HEV の放出に小胞の細胞内膜輸送系（MVB sorting）が関与すること、2）siRNA による遺伝子ノックダウン実験系および DN mutant 過剰発現系を用いて、宿主細胞因子である Tsg101, Alix, Nedd4 がラット HEV の放出に重要であること、3）exosome の分泌に重要な Rab27A, Hrs のノックダウン実験系や exosome の分泌を亢進・阻害する試薬を用いて、exosome 経路がラット HEV の放出に重要であること、4）ヒト HEV は Tsg101 と結合することで細胞外への放出が促進されるが、ラット HEV は Tsg101 とは結合せず、90 kDa の宿主由来タンパクと結合することがウイルス放出に重要であること、などを明らかにした。

本研究は多岐にわたる研究手法を駆使して行われており、得られた結果は明快であった。本研究を通して得られた結果はウイルス学的に重要な研究成果であると言え、博士の学位を授与するに相応しいものと高く評価できる。この論文はすでに国際誌に掲載が決定しており、加えて申請者は、これに関連する研究について筆頭著者としてすでに 2 報の原著論文と 1 報の総説を国際誌に発表していることを特記したい。

以上のことから、本申請論文は学位論文として合格であると判定された。

なお、審査員からいくつかの軽微な修正点が指摘されたが、それらは全て適切に修正されたことが確認された。

最終試験の結果の要旨

申請者は学位論文に沿った研究内容の発表を行った。発表は大変分かりやすいもので、プレゼンテーションの構成も理解しやすいように作られていた。発表時間は約 30 分間と予定通りであった。

申請者の発表の後に、この研究で見出したウイルスと結合する宿主因子について、siRNA 実験によって得られたウイルスの放出亢進の結果の解釈について、ラットからヒトへの感染の防御について、ラット HEV 感染の治療について、エンベロープの有無による宿主の酵素や免疫に対するウイルスの耐性の違いについて、ラット HEV のラット株化細胞への感染について等、約 30 分間にわたって活発な質疑応答が行われた。申請者は各質問に対して適切に答えており、研究結果の的確な解釈に加えて、同分野の研究背景や研究の進展について多くの知識を得てきていることが確認された。時間を通して有意義な質疑応答が行われたと考える。

論文審査および最終試験から、申請者が研究者として充分な知識と研究遂行能力を有すると評価し、審査員全員一致で最終試験に合格と判断した。