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学位論文題名

Identification of ZAPS as a positive regulator of RIG-I-mediated antiviral response

(パターン認識受容体RIG-Iを介した抗ウイルス応答を増強する、新規調節因子ZAPSの同定)

学位論文内容の要旨

【Background and purpose】 Pathogen invasion is sensed by pattern recognition receptors (PRRs) of the innate immune system via its recognition of pathogen-associated molecular patterns (PAMPs). Viral RNAs can serve as a PAMP, and the retinoic acid-inducible gene I (RIG-I) is a key PRR for the detection of positive- and negative-stranded RNA viruses in the cytoplasm of cells. RNA carrying 5'-triphosphate modification (3pRNA) is shown to be an essential determinant for viral RNA recognition by RIG-I, which results in the activation of the downstream both interferon (IFN)-regulatory factor (IRF) 3 and nuclear factor-κB (NF-κB) pathways for the production of type I IFNs and inflammatory cytokines. Accumulating evidence has been shown that RIG-I-mediated pathways are important for the activation of innate immune responses to viral infection.

Poly(ADP-ribose) polymerases (PARPs) are known to regulate not only cell survival and cell death, but also other diverse biological processes and pathogeneses of diseases. It has been reported that some PARPs, including PARP-1 and PARP-13 (also termed ZAP; zinc-finger CCCH-type antiviral protein 1), interact with viral molecules and are likely to show a direct regulatory effect of certain viral subsets. However, no interaction of the PARPs with host innate immune responses has been defined. In this study, we clarify the role of PARPs in innate immunity, particularly in terms of its possible involvement in pattern recognition receptor-mediated signaling.

【Methods and Results】 To investigate the role of the PARPs in nucleic acid-induced innate immune responses, we exogenously expressed PARP-1, PARP-2, PARP-7, PARP-9, PARP-12 and PARP-13, all of which were reported to be involved in microbial infection, inflammation and immunity. We performed quantitative RT-PCR (qRT-PCR) analysis, and found that PARP-13/ZAP uniquely showed a marked enhancing effect on the expression of IFN-β mRNA upon transfection with 3pRNA in HEK293T human embryonic kidney cells. Next, we examined whether there is any difference between the two isoforms of PARP-13/ZAP: qRT-PCR analysis also revealed that the shorter isoform (we hereafter call this isoform ZAPS (zinc-finger CCCH-type antiviral protein 1, short form)) rather than the full-form ZAP, was selectively induced by IFN-α or 3pRNA and had a stronger effect on IFN-β mRNA induction by stimulation with 3pRNA.

By conducting qRT-PCR experiments, we found that exogenous expression of ZAPS in HEK293T cells remarkably enhanced the induction of type I IFN genes with 3pRNA stimulation. Consistent with this result, luciferase reporter analyses showed that 3pRNA stimulation resulted in activation of the *IFNB* promoter in a manner dependent on the levels of ZAPS expression. In addition, we conducted RNA interference-based knockdown experiments with small interfering

RNA (siRNA) targeting ZAPS mRNA (siZAPS). qRT-PCR analyses showed that the induction of IFN-β mRNA was severely abrogated in siZAPS-treated $CD14^+$ monocytes as well as similarly treated other cell lines (HEK293T cells and A549 human lung epithelial adenocarcinoma cells). The production of IFN-β protein was also verified by enzyme-linked immunosorbent assay (ELISA). We also found that ZAPS overexpression in HEK293T cells strongly enhanced the 3pRNA-induced activation of NF-κB-driven luciferase reporter gene. Consistently, qRT-PCR analyses showed that the induction of genes encoding other cytokines, such as tumor necrosis factor (TNF), interleukin 6 (IL-6) and CXCL10, in response to 3pRNA stimulation, was diminished in A549 cells, in which ZAPS mRNA was knocked down. These data indicated that ZAPS strongly potentiates the activation of not only type I IFN but also NF-κB in the RIG-I-mediated pathway.

Next, we investigated how ZAPS functions as a potentiator of RIG-I pathway. The IFN-β mRNA expression induced by the exogenous expression of ZAPS in HEK293T cells was abolished by RNAi-mediated downregulation of not only several molecules situated downstream of RIG-I but also RIG-I itself. Therefore, we speculated that ZAPS plays a possible role as a novel regulator in the proximal process of RIG-I-mediated signaling. Confocal analysis and immunoprecipitation assay revealed that ZAPS is in association with RIG-I after 3pRNA stimulation. Moreover, native page and immunoprecipitation assay showed that the formation of RIG-I oligomers induced by Newcastle disease virus (NDV) infection or stimulation with 3pRNA was suppressed in siZAPS-treated HEK293T cells. These findings suggest that ZAPS associates with RIG-I to promote the RIG-I oligomerization.

To evaluate the function of ZAPS in RIG-I-mediated antiviral responses, we infected A549 cells with influenza virus, which are known to activate RIG-I-mediated signaling. Knockdown of ZAPS expression by siRNA impaired influenza virus-induced mRNA expression of type I IFN, IL-6, TNF and CXCL10 and the production of IFN-β protein, which were assessed by qRT-PCR and ELISA, respectively. By qRT-PCR analyses, we also detected higher expression of a viral nucleoprotein gene in siZAPS-treated A549 cells than control siRNA-treated A549 cells following influenza virus infection. Moreover, qRT-PCR analysis showed that exogenous expression of ZAPS in HEK293T cells resulted in much more induction of IFN-β mRNA after influenza virus infection, and consistent with this result, a notable suppression of viral replication was observed by plaque-forming assay. A similar observation was also obtained for NDV infection. These suggest that ZAPS exerts its antiviral activity through the RIG-I-mediated pathways.

【Discussion】 In this study, we demonstrated that PARP-13 is a regulator of RIG-I-mediated antiviral signaling in human cells. We also found that ZAPS, the shorter isoform of PARP-13, is selectively upregulated by stimulation with 3pRNA, possibly through type I IFN signaling. It has been speculated that ZAPS acts as a positive feedback regulator for RIG-I-mediated type I IFN pathway. In this regard, it is the next issue to clarify how each isoform is differently regulated.

Activation of RIG-I after ligand binding is thought to be a multistep process that includes the activation of its ATPase activity, conformational changes and oligomerization. The present data have demonstrated that ZAPS interacts with RIG-I after 3pRNA stimulation, and oligomerization of RIG-I, possibly by stabilizing the RNA-RIG-I complex, although the underlying mechanism needs to be investigated further. The results also indicated that ZAPS expression alone slightly enhances the expression of IFN-β mRNA and the activation of the *IFNB* promoter independently of any ligands. Consistent with these data, we detected the ZAPS-RIG-I interaction before stimulation, albeit very weakly. This disconnection of ligand dependence may suggest that this interaction is sufficient to promote small amounts of signaling but that the overall signaling program is most efficiently triggered by the binding of ligand to RIG-I in addition to its activation by ZAPS.

The result showing that ZAPS is critically involved in antiviral innate immune responses to

influenza virus and NDV raises the possibility that ZAPS have an important role against other viruses which activate RIG-I signaling. It is also needed to investigate the role of ZAPS in other PRR-activated innate signaling. Previous reports show that ZAP has a role in the decay of mRNAs, which are derived from certain viral subsets. Together with this observation, our present data indicate that ZAPS might exert a "dual-mode" defense activity against viral infection. How these activities are regulated remains to be clarified.

【Conclusion】 This study indicates that ZAPS has a key role as a positive regulator of RIG-I signaling during innate antiviral immune responses, suggesting that ZAPS may be an important target for therapeutic intervention in the control of viral diseases.

学位論文審査の要旨

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(パターン認識受容体RIG-Iを介した抗ウイルス応答を増強する、新規調節因子ZAPSの同定)

本研究は、細胞質内ウイルスRNA認識受容体RIG-Iによる抗ウイルス応答を調節する分子を新たに探索し、RIG-I シグナルを増強する分子として、poly(ADP-ribose) polymerase (PARP)-13 shorter isoform (ZAPS)を同定した。ZAPSはtype I interferon (IFN)により発現誘導され、ウイルスRNA存在下でRIG-Iと相互作用し、RIG-Iのオリゴマー化を増強することでtype I IFN、炎症性サイトカイン産生を増加させることが示された。更に、インフルエンザ感染においてZAPSをノックダウンすると、type I IFN産生が低下しウイルス複製が増加すること、逆にZAPSの過剰発現ではNewcastle disease virus 感染でのIFN-β産生が上昇し、ウイルスタイターが低下することから、RIG-Iを介したtype I IFN産生による抗ウイルス応答にZAPSが重要な役割を果たすことが明らかとなった。

審査会において、申請者の発表後、副査の畠山教授から、申請者の実験の進め方について、また全長のZAPとZAPSの種々の組織での発現量の違い、RIG-IとZAPSの結合におけるZnの関与などについて質問があり、申請者は自ら行った実験内容・手技について詳しく説明し、ZAPSとZAPの発現誘導の違いについてもスライドで示しながら答えた。今回同定したshort formのZAPSは、全長のZAP遺伝子からalternative splicingにより生成されると考えられており、siRNAによるノックダウンではZAP, ZAPSの両者がノックダウンされること、強制発現系においてZAPとZAPSの機能の違いが見られることを説明した。副査の佐邊教授から、ZAPSのN末に存在するZnフィンガーに関連した実験上の注意点、および学位論文における序章の書き方についてコメントがあった。また、NF-κBが活性化された細胞においてZAPSによるRIG-Iシグナル増強がどのような効果をもたらすか質問があり、申請者は、想定されることを現時点での実験データから説明し、今後取り組む重要な課題であるとの認識を示し

た。副査の志田教授からは、RIG-I のアダプター分子 MAVS からの IRF-7 活性化について質問があり、適切に答えた。また、ウイルスによる宿主免疫系、特に IFN 産生系の撹乱戦略について討論がなされた。副査の今村教授（指導教員）から、臨床において申請者の研究をどう発展させるか、また炎症疾患との関連について質問があり、申請者は、ZAPS が RIG-I を介した type I IFN と NF-κB の両方のシグナルに関与することから、炎症を増強させる可能性などについて述べ、今後、炎症疾患とウイルス感染との関連を基礎的および臨床的に明らかにすることが重要であるとの見解を示した。最後に主査の松本准教授から、RIG-I 活性化に関与する蛋白群のなかでの ZAPS の位置についての質問、および ZAP, ZAPS によるウイルス RNA 分解系と IFN 産生誘導系への振り分けがどのように行われているかという質問がなされた。申請者は、RIG-I は ZAPS によりオリゴマー化された後、ATPase 活性が起こること、RIG-I のユビキチン化は ZAPS の下流であることを示した。RNA 分解系と IFN 誘導系への分岐については、まだ不明であることをスライドで示して返答した。

申請者はすべての質問に対してその主旨を理解し、自らの研究内容と文献的考察を交えて適切に回答した。

この論文は、自然免疫による抗ウイルス防御において重要な役割を果たす RIG-I の活性化に ZAPS という新規分子が関与することを明らかにした点で高く評価され、今後のウイルス RNA 認識機構の解明および抗ウイルス剤の開発に寄与することが期待される。

審査員一同は、これらの成果を評価し、大学院過程における研鑽や取得単位なども併せ申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。