[課程―２]

審査の結果の要旨

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Enteropathogenic *Escherichia coli* (EPEC), is the most prevalent diarrheal pathogen affecting infants and children, and causes a prolonged watery diarrhea in developing countries. This study identified a novel host defense mechanism against EPEC infection that is mediated by ubiquitin-proteasome system (UPS) proteolytic degradation pathways. It focused on the fate of the Tir effector protein in EPEC-attached host cells and provided scientific results below.

1. After EPEC attaches to host intestinal epithelial cell, the bacterium injects effector protein including Tir into the host cell. The levels of three effectors, Tir, EPEC-secreted protein B (EspB) and Mitochondrial-associated protein (Map) were measured in EPEC-infected host epithelial cells at early stages of infection. The levels of Tir in infected host cells showed a time-dependent decrease as compared to EspB and Map, indicating selective degradation of Tir. The translocated Tir was phosphorylated by FYN tyrosine kinase. Binding of Tir to CBL-C, an E3 ligase, was confirmed by Glutathione S-transferase (GST) pull-down assays in a FYN-mediated phosphorylation-dependent manner. Using CBL-C derivatives, Tyrosine Kinase Binding (TKB) region of CBL-C was identified as a Tir binding region.

2. *In vivo* ubiquitination assays showed that Tir was ubiquitinated by CBL-C, but not by CBL-C CA, a Ub ligase activity-defective mutant. The proteasome inhibitor MG132 or Lactacystin rescued Tir from immediate degradation in transfected cells. Silencing of *CBLC*, but not those of *CBL* or *CBLB*, showed significantly decreased levels of ubiquitination and delayed degradation of intracellular Tir. These results indicate that the levels of Tir in host cells are regulated by CBL-C-dependent ubiquitination and proteasomal degradation.

3. *In vitro* kinase assay and GST pull-down assay confirmed that phosphorylation of Tir-Tyr 454 was required for the CBL-C interaction. This Tyr 454-dependent interaction affected the levels of ubiquitination and degradation of intracellular Tir. The degradation of intracellular WT Tir but not Y454F Tir was observed in EPEC-infected cells. This difference was not seen when cells were treated with *CBLC* siRNA. These results indicated that host cell CBL-C targeted Tyr 454 residue of Tir to down modulate the levels of

intracellular Tir protein in cells infected with EPEC.

4. Pedestal structure and bacterial adherence were quantified by confocal microscopy. These results showed that there was nearly two-thirds less bacterial adherence on cells by EPEC Δ*tir*/*tir* WT, as compared to by EPEC Δ*tir*/*tir* Y454F. However, when cells were treated with *CBLC* siRNA, the number of adherent EPEC Δ*tir*/*tir* WT was almost comparable to that for EPEC Δ*tir*/*tir* Y454F. The levels of pedestal formation followed the same trends as bacterial adherence. These data demonstrated that the efficiency of EPEC adherence to host cells was regulated by the interaction of Tir Tyr 454 and CBL-C.

5. *Citrobacter rodentium* mice infection model was examined using strains WT, Δ*tir*, Δ*tir*/*tir* WT and Δ*tir*/*tir* Y451F (amino acid sequence homology to EPEC Tir Y454F) to investigate *in vivo* effects for bacterial loads in the colon. *C. rodentium* colony forming units (CFUs) were significantly increased by infection with *C. rodintium* Δ*tir*/*tir* Y451F as compared with WT, Δ*tir*, or Δ*tir*/*tir* WT. The data further indicated that Tyr-phosphorylation at EPEC Tir Tyr 454/*C. rodentium* Tir Tyr 451 was critical for host regulation against bacterial colonization.

The above findings together demonstrated that a unique host defense mechanism against EPEC infection that was mediated by ubiquitin-proteasome system (UPS) proteolytic degradation pathways. Since this study shed light on a novel biological function of CBL-C as a host defense mechanism against enteric pathogen like EPEC, this study have been deemed worthy of the conferment of a PhD degree.