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Intracellular *Porphyromonas gingivalis* exploits recycling pathway to exit from infected cells

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Introduction

Human epithelial and mucosal surfaces are a host defense barrier to prevent intrusion by harmful materials and bacteria. In case of gingival sulcus, multilayered squamous gingival epithelium functions a central role in innate immunity against periodontal pathogens, including *Porphyromonas gingivalis*, capable of entering gingival epithelial cells using various host cellular deficiencies. Our previous findings showed that *P. gingivalis* organisms were first encapsulated by early endosomes at the entry process to cells[1]. Followed by localization in early endosomes, some bacteria were sorted to late endosomes/lysosomes, which likely led to degradation of bacteria, and others became co-localized with Rab11A and RalA, host recycling regulator proteins. We also showed that knockdown of Rab11A or RalA disturbed bacterial exit from the infected gingival epithelial cells to the culture media. However, the molecular machineries utilized by *P. gingivalis* for its exit from infected host cells remains unknown.

Basically endosomes consist of three different compartments: early endosomes, late endosomes/lysosomes, and recycling endosomes. Early endosomes receive molecules delivered form the plasma membrane and functions as a sorting station of the endocytic and recycling pathways. It was previously reported that the endocytic and recycling pathways in mammalian cells are regulated by specific small Rab GTPases[2], regulators of many steps of membrane traffic. Approximately 40 members of Rab proteins have been identified in humans. Some endocytic Rab proteins are also shown to be involved in intracellular trafficking of various pathogens. However, no Rab GTPases have been shown to be involved in bacterial exit from infected host cells.

Specific membrane fusions are mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins[3], consisting of at least 38 members in mammalian cells. A SNARE protein contains one SNARE domain, approximately 60-70 amino acids in its N-terminus, except that SNAP23, SNAP25, or SNAP29 contains two SNARE domains. On membrane fusions, SNARE complexes comprise four parallel helix bundles. Because each combination of SNARE complexes has a high specificity, it is crucial for functional membrane fusions that each SNARE protein appropriately localize on its target membrane of organelles. As for the bacterial exit from infected cells, however, the endosomal SNARE remains to be identified.

In this short review, we illustrate the molecular process involved in the exit of intracellular *Porphyromonas gingivalis* from gingival epithelial cells, focused on Rab GTPases and SNARE proteins.

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VAMP2 is involved in *P. gingivalis* exit

To identify the molecular machineries involved in the exit of intracellular *P. gingivalis* from infected host cells, we examined its association with SNARE proteins in human immortalized gingival epithelial (HIGE) cells[4] using confocal laser microscopy. At 1 h after infection, we found that monomeric Cherry (mCherry)-VAMP2-positive puncta structures were co-localized with enhanced green fluorescent protein (EGFP)-FYVE[5] (early endosome marker) and recruited to intracellular *P. gingivalis* ATCC 33277 in gingival epithelial cells (Fig. 1A). Thereafter, its co-localization with the VAMP2 and FYVE was decreased at 5 h after infection in a time-dependent manner (Fig. 1B).

To analyze the involvement of VAMP2 in the bacterial exit from host cells, we performed RNAi knockdown against VAMP2 in HIGE cells. Knockdown of VAMP2 caused the increased co-localization of intracellular bacteria with EGFP-2xFYVE even at 5 h after infection (Fig. 2A-2C).

Moreover, VAMP2 knockdown increased the bacterial accumulation in HIGE cells (Fig. 3A-3C). These results indicate that VAMP2 is involved in the exit of *P. gingivalis* from infected cells.

Involvement of Rab4A in *P. gingivalis* exit

Rab4A is necessary for recycling of the transferrin receptor (TfR) from early endosomes back to the plasma membrane via a fast recycling[6]. We found that mCherry-Rab4A co-localized with intracellular *P. gingivalis* for up to 5 h after infection (Fig. 4A and 4B). RUN and FYVE domain containing 1 (RUFY1) is an Rab4A effector and involved in early endosomal trafficking[7]. Thus, we examined whether RUFY1 translocates to Rab4A-positive endosomes containing *P. gingivalis*. As expected, we found that Myc-RUFY1 was co-localized with EGFP-Rab4A-positive puncta containing *P. gingivalis* (Fig. 4C).

Next we examined the involvement of Rab4A in intracellular traffic with RNAi knockdown. Compared with control siRNA-treated cells, we showed the increased number of bacterial co-localization with EGFP-2xFYVE (Fig. 5A-5C).

In addition, Rab4A-knockdown increased the viable bacterial organisms in HIGE cells, and reduced

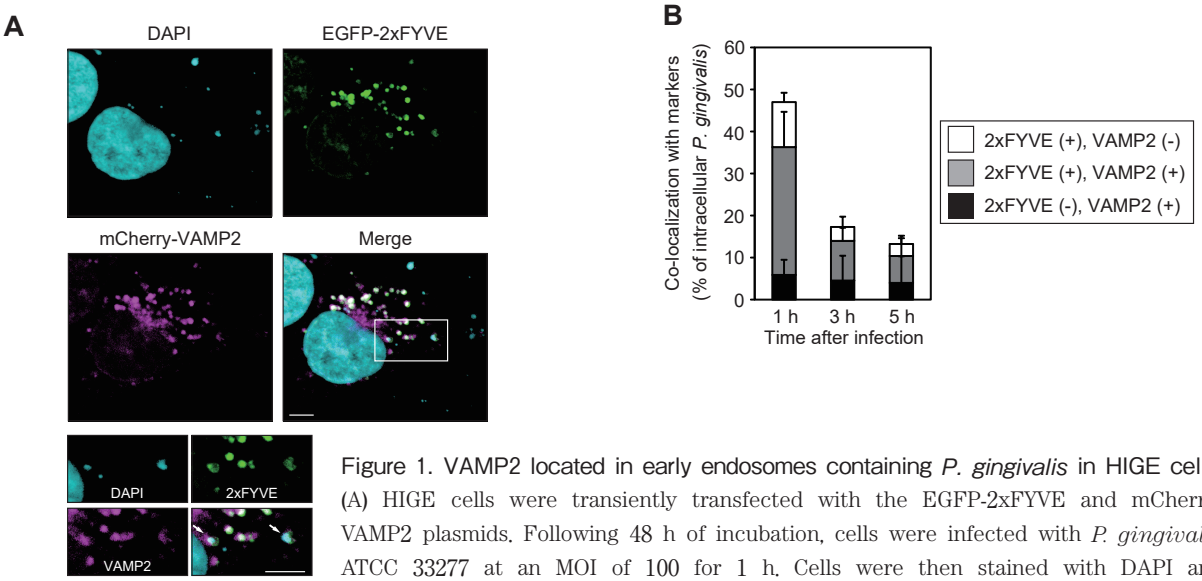

Figure 1. VAMP2 located in early endosomes containing *P. gingivalis* in HIGE cells. (A) HIGE cells were transiently transfected with the EGFP-2xFYVE and mCherry-VAMP2 plasmids. Following 48 h of incubation, cells were infected with *P. gingivalis* ATCC 33277 at an MOI of 100 for 1 h. Cells were then stained with DAPI and analyzed by confocal microscopy. Arrows indicate co-localization. Scale bars, 5 μm. **(B)** Quantification of intracellular *P. gingivalis* associated with markers at the indicated times after infection; white: FYVE (+)/VAMP2 (-), gray: FYVE (+)/VAMP2 (+), black: FYVE(-)/VAMP2 (+). Data shown represent the mean ± SD of 3 biological replicates. At least 20 HIGE cells and 100 bacteria were analyzed per test.

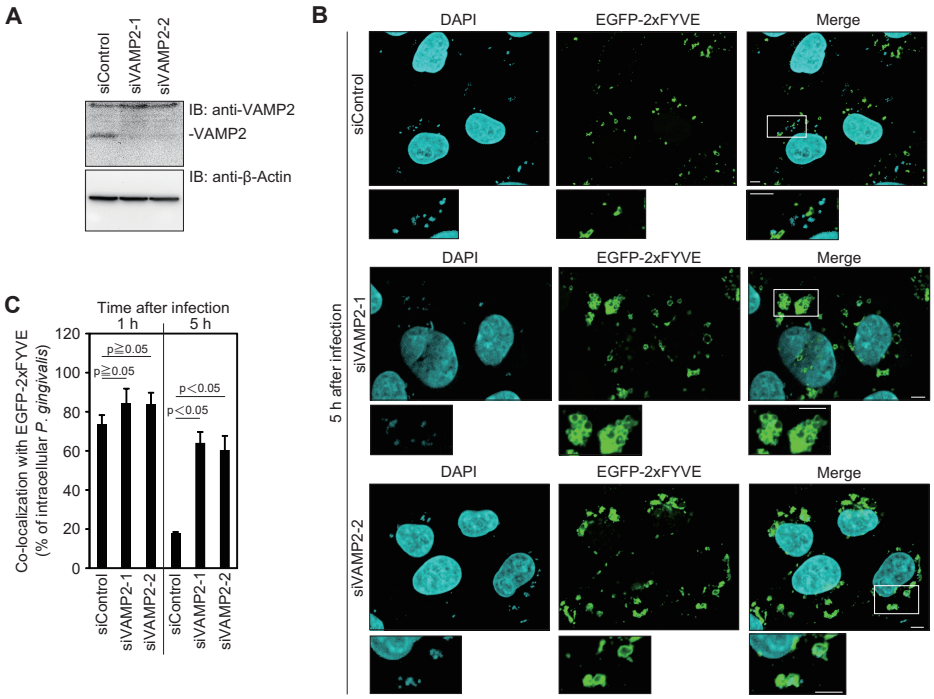

Figure 2. Effects of VAMP2-knockdown on localization of *P. gingivalis* in early endosomes.

(A) HIGE cells were treated with control siRNA or siRNA oligonucleotides against VAMP2. Following 48 h of incubation, cells were analyzed by immunoblotting using the indicated antibodies. **(B)** HIGE cells stably expressing EGFP-2xFYVE were treated with control siRNA or VAMP2 siRNA oligonucleotides. Following 48 h of incubation, cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, then incubated for 1 h with antibiotics and further for 3 h without antibiotics (5 h after infection). They were then stained with DAPI and analyzed by confocal microscopy. Scale bars, 5 μm. **(C)** Quantification of intracellular *P. gingivalis* associated with EGFP-2xFYVE at the indicated time periods after infection. Data shown represent the mean ± SD of 3 biological replicates. At least 20 IHGE cells and 100 bacteria were analyzed per test. *$p < 0.05$, Dunnett's test.

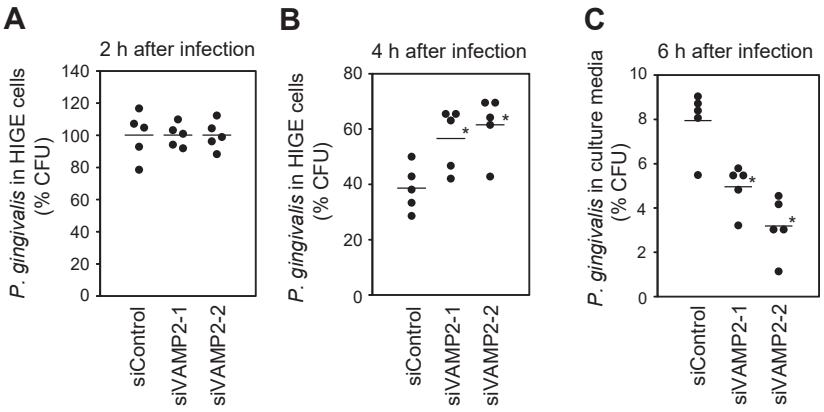

Figure 3. Effects of VAMP2-knockdown on viability of *P. gingivalis* in HIGE cells or culture media.

(A-C) HIGE cells were treated with control siRNA or VAMP2 siRNA oligonucleotides. Following 48 h of incubation, cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, incubated for 1 h with antibiotics and then for the indicated time periods without antibiotics. Quantification of numbers of *P. gingivalis* organisms in HIGE cells **(A, B)** and in culture media **(C)** were determined using a CFU assay, as described in Experimental procedures. Data shown represent 2 biological replicates, with mean represented by lines ($n = 5$). *$p < 0.05$, Dunnett's test.

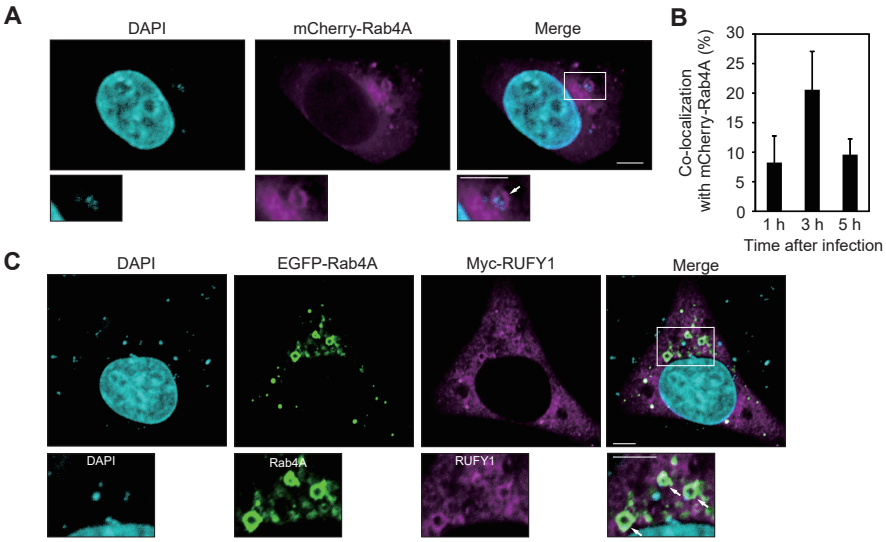

Figure 4. Characterization of Rab4A in HIGE cells.

(A) HIGE cells were transiently transfected with the mCherry-Rab4A plasmid. Following 48 h of incubation, cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, incubated for 1 h with antibiotics, and then for 1 h without antibiotics (3 h after infection). They were then stained with DAPI and analyzed by immunofluorescence microscopy. An arrow indicates co-localization. Scale bars, 5 μm. **(B)** Quantification of intracellular *P. gingivalis* associated with mCherry-Rab4A was performed at the indicated times after infection. Data shown represent the mean ± SD of 3 biological replicates. At least 20 IHGE cells and 100 bacteria were analyzed per test. **(C)** HIGE cells were transiently transfected with the Myc-RUFY1 and EGFP-Rab4A plasmids. Following 48 h of incubation, cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, incubated for 1 h with antibiotics, and then for 1 h without antibiotics (3 h after infection). They were then stained with DAPI and analyzed by confocal microscopy. An arrow indicates co-localization. Scale bars, 5 μm.

the number of viable bacteria at 6 h after infection (Fig. 6A-6C). These results indicate that Rab4A is involved in the exit of intracellular *P. gingivalis* from host cells.

Conclusion and Future Directions

Based on our findings, we propose the following model (Fig. 7). The entering *P. gingivalis* is first sorted to early endosomes, some bacteria are transported to late endosomes/lysosomes or autophagosomes likely for degradation, while others exploit fast recycling to bacterial exit from host cells by recruitment of VAMP2 and Rab4A.

Our results strongly suggest that this pathogen positively utilizes Rab GTPases and SNARE proteins for intracellular trafficking and bacterial survive, further molecular analyses will be needed to answer the following questions: 1) Which proteins(s) is a host receptor in early endosomes sensing for *P. gingivalis* to lead extraordinary fast recycling; 2) How does this pathogen evade intracellular degradation as a whole; and 3) What is the physiological significance for this bacteria in periodontitis.

Because microorganisms have abilities to adapt to various environment changes, their habitat ranges are basically found in many different environments. *P. gingivalis* has a unique habitat in periodontal sites including subgingival dental biofilm and gingival cells[10]. It will be interesting to discover whether the exit of intracellular *P. gingivalis* or its pathogens from gingival epithelial cells is features of pathogens, particularly keystone pathogens in mixed bacterial communities.

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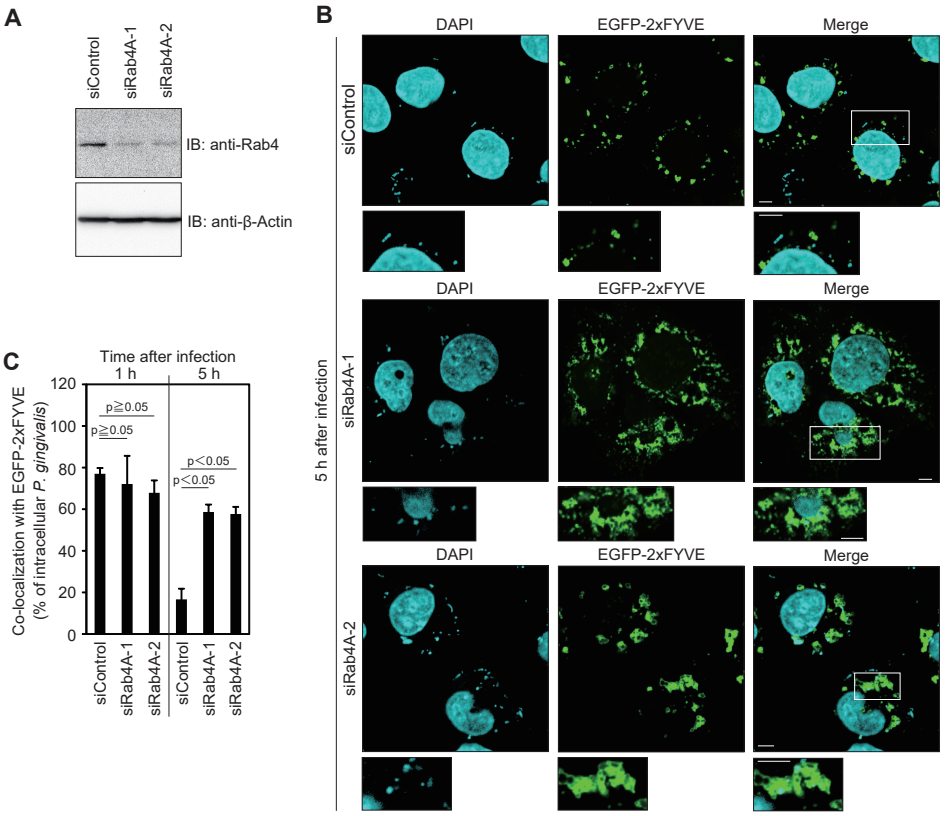

Figure 5. Effects of Rab4A-knockdown on localization of *P. gingivalis* in early endosomes.

(A) HIGE cells were treated with control siRNA or siRNA oligonucleotides against Rab4A. Following 48 h of incubation, cells were analyzed by immunoblotting using the indicated antibodies. **(B)** HIGE cells stably expressing EGFP-2xFYVE were treated with control siRNA or Rab4A siRNA oligonucleotides. Following 48 h of incubation, cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, incubated for 1 h with antibiotics, and then for 3 h without antibiotics (5 h after infection). They were then stained with DAPI and analyzed by confocal microscopy. Scale bars, 5 μm. **(C)** Quantification of intracellular *P. gingivalis* associated with EGFP-2xFYVE was performed at the indicated times after infection. Data shown represent the mean ± SD of 3 biological replicates. At least 20 IHGE cells and 100 bacteria were analyzed per test. *$p < 0.05$, Dunnett's test.

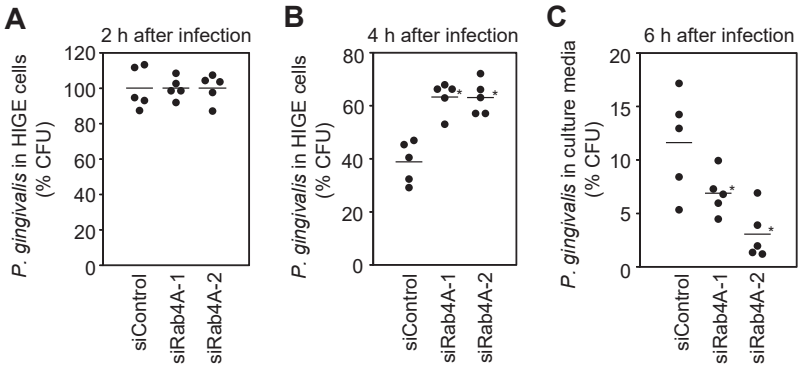

Figure 6. Effects of Rab4A-knockdown on viability of *P. gingivalis* in HIGE cells or culture media.

(A-C) HIGE cells were treated with control siRNA or Rab4A siRNA oligonucleotides. Following 48 h of incubation, cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, incubated for 1 h with antibiotics, and then for the indicated time periods without antibiotics. Quantification of numbers of viable *P. gingivalis* organisms in HIGE cells (A, B) and in culture media (C) was performed with a CFU assay, as described in Experimental procedures. Data shown represent 2 biological replicates, with mean presented by lines ($n = 5$). *$p < 0.05$, Dunnett's test.

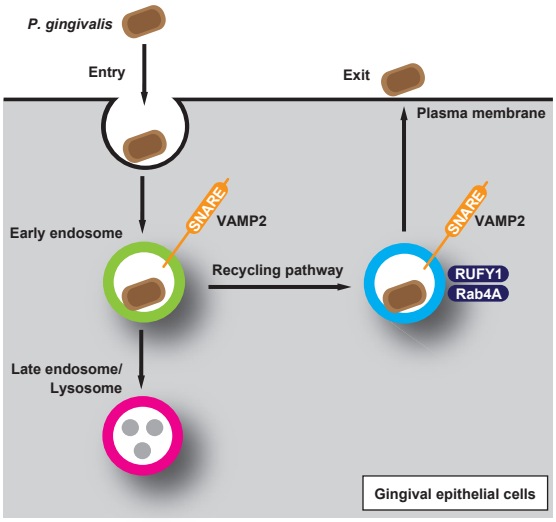

Figure 7. Proposed model of *P. gingivalis* trafficking in human gingival epithelial cells.

P. gingivalis enters gingival epithelial cells and becomes localized in early endosomes. Subsequently, some bacteria are sorted to lytic compartments (late endosomes/lysosomes) likely for degradation, whereas others recruit VAMP2 and Rab4A onto early endosomes. RUFY1 is then recruited and interacts with Rab4A. RUFY1 is phosphorylated by *P. gingivalis* infection and translocated onto endosomes containing bacteria. Finally, the endosomes fuse with the plasma membrane, which allows *P. gingivalis* to escape via the fast recycling pathway for further penetration of gingival tissues.

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