

## ***Chlamydia pneumoniae* CPj0783 interaction with Huntingtin-protein14**

Izumi Yanatori,<sup>1</sup> Yumiko Yasui,<sup>1</sup> Kazunobu Ouchi,<sup>2</sup> Fumio Kishi<sup>1\*</sup>

<sup>1</sup>Department of Molecular Genetics, Kawasaki Medical School, Kurashiki, Okayama, Japan. <sup>2</sup>Department of Pediatrics, Kawasaki Medical School, Kurashiki, Okayama, Japan

Received 8 September 2015 · Accepted 1 December 2015

**Summary.** *Chlamydia pneumoniae* is a Gram-negative, obligate intracellular pathogen that causes community-acquired respiratory infections. After *C. pneumoniae* invades host cells, it disturbs the vesicle transport system to escape host lysosomal or autophagosomal degradation. By using a yeast mis-sorting assay, we found 10 *C. pneumoniae* candidate genes involved in aberrant vesicular trafficking in host cells. One of the candidate genes, CPj0783, was recognized by antibodies from *C. pneumoniae*-infected patients. The expression of CPj0783 was detected at mid to late-cycle time points and increased during the inclusion maturation. Two-hybrid screening in yeast cells revealed that CPj0783 interacted with Huntingtin-interacting protein 14 (HIP14). The specific interaction between CPj0783 and HIP14 could be demonstrated by an in vivo co-immunoprecipitation assay and an in vitro GST pull-down assay. It was also demonstrated that HIP14 was localized in the Golgi apparatus and colocalized with CPj0783. HIP14 has a palmitoyl transferase activity that is involved in the palmitoylation-dependent vesicular trafficking of several acylated proteins. These findings suggest that CPj0783 might cause abnormal vesicle-mediated transport by interacting with HIP14. [Int Microbiol 18(4):225-233 (2015)]

**Keywords:** *Chlamydia pneumoniae* · intracellular pathogens · yeast two-hybrid screening CPj0783–HIP14 · protein mis-sorting · vesicle transport

### **Introduction**

*Chlamydia pneumoniae* is an obligate intracellular pathogen that causes acute and chronic respiratory infections in humans. Almost all humans face the possibility of contracting *C. pneumoniae* infections at least once in their lifetime [9]. *Chlamydia* species have a unique biphasic life cycle initiated by the attachment of the elementary body (EB) to the host

cell. Then, the EB is endocytosed by the cell and is contained within a plasma membrane-derived vesicle, which is quickly modified by the pathogen to establish a replicative form, termed the inclusion [9]. The pathogen remains within the inclusion for the duration of its intracellular development and is transformed into the reticulate body (RB) [11]. While *Chlamydia* grows in the inclusion, the pathogen should escape from phagosomal degradation in host cells. It is thus currently thought to achieve such purposes by expressing certain molecules that inhibit phagosomal maturation and modulate the host-vesicle trafficking pathways. Previously, we reported the functional high-throughput screening system in yeast cells [20]. It has been found that the carboxypeptidase Y-invertase (CPY-Inv) reporter system is a powerful tool to identify the molecules that alter eukaryotic vesicle trafficking pathways. By using a similar expression screening system,

\*Corresponding author: Fumio Kishi  
Department of Molecular Genetics  
Kawasaki Medical School  
577 Matsushima, Kurashiki  
Okayama 701-0192, Japan  
Tel. +81-864621111. Fax +81-86462-1199  
E-mail: fkishi-ygc@umin.ac.jp

it has been reported that several molecules expressed by intracellular pathogens such as *Legionella pneumoniae* inhibit the maturation of the phagosome and/or exit the endocytic pathway [4,17].

We have determined the sequence of the whole genome of *C. pneumoniae* J138 strain [16] and found that this strain features a putative protein coded by its 1069 open reading frames (ORFs). A comprehensive bioinformatics approach has been applied for annotation taxonomy, and approximately half of the predicted ORFs have been found to encode proteins without any known functions. To identify novel *C. pneumoniae* molecules that determine virulence and pathogenicity, we screened 455 ORFs without any known functions in a yeast expression system. Here, we address the newly identified *C. pneumoniae* CPj0783 gene that causes mis-sorting in yeast cells. CPj0783 was found to interact with Huntingtin-interacting protein 14 (HIP14) in vivo and in vitro. HIP14 is classified in both the palmitoyl acyl transferase protein family (PAT family) and ankyrin repeat superfamily (ANK superfamily) [15,18]. HIP14 is most notably involved in the palmitoylation and trafficking of multiple proteins and has been localized to the Golgi apparatus [12,18]. The expression of HIP14 has been detected ubiquitously in human tissues and one of the most essential target proteins is huntingtin (HTT) [15]. Our study suggests that a novel *C. pneumoniae* molecule, CPj0783, would interact with HIP14 and could cause aberrant vesicle trafficking.

## Materials and methods

**Cell culture and infection.** *Chlamydia pneumoniae* J138 was propagated in HEp-2 cells maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum.

**Mis-sorting assay in yeast cells.** We previously developed a carboxypeptidase Y (CPY)-invertase (Inv) reporter system in yeast cells [20]. The mis-sorting assay was performed using this system, as previously described [20]. Yeast cultures were spotted on SC-Ura/fructose with and without 20 µg/ml of doxycycline and grown for 2 days at 30°C. In the invertase overlay assay, the plates were overlaid with a 0.75% agarose solution containing 125 mM sucrose, 100 mM sodium acetate buffer (pH 5.5), 0.5 mM *N*-ethylmaleimide, 10 µg/ml of horseradish peroxidase, 8 units/ml of glucose oxidase and 2 mM *O*-dianisidine. After 5–15 min, photographs were taken with a Nikon D70 digital single-lens reflex camera. Image contrast was adjusted using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

**Protein expression and purification.** For the purification of the glutathione S-transferase (GST)-tagged CPj0783 (25–262 amino acids) fusion protein, *E. coli* JM109 cells transformed with protein-expression plasmids were grown in Luria broth with 100 µg/ml ampicillin at 37°C to an OD<sub>600</sub> of 0.8; then, recombinant GST gene expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration

of 0.3 mM at 37°C for 3 h. Cells were pelleted, resuspended in TNE buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 2% NP-40 and 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication. Lysates were cleared by centrifugation at 20,000 ×g for 15 min at 4°C, and GST-tagged proteins were purified from the supernatant using glutathione-Sepharose 4B beads. After incubation with Sepharose beads for 2 h, the beads were washed five times with TNE buffer, and then the recombinant fusion proteins were eluted with 10 mM reduced glutathione and 50 mM Tris-HCl at pH 9.0.

**Antibodies.** Antibody generation was performed as previously described [19]. Briefly, maltose-binding protein-tagged CPj0783 was purified on amylose resin and used to immunize Japanese white rabbits. The affinity-purified anti-CPj0783 polyclonal antibody (pAb) was purified from antiserum on a HiTrap *N*-hydroxysuccinimide activated column (GE Healthcare Japan) coupled with GST-tagged CPj0783. HRP-conjugated goat anti-human IgA (Monosan, Netherland), anti-human IgG and anti-human IgM (Invitrogen, CA) immunoglobulins were used as the secondary antibodies. HRP-conjugated anti-mouse and anti-rabbit IgGs were purchased from Cell Signaling technology (Danvers, MA, USA). Most other general reagents were purchased from Wako Chemicals, Nacalai Tesque and Sigma.

**Immunofluorescence microscopy.** Cells grown on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 in PBS. Cells were incubated with primary antibodies overnight at 4°C. Secondary antibodies coupled to Alexa 594 were incubated at room temperature on cells for 60 min. The coverslips were washed and mounted on slides with VECTASHIELD (Vector Laboratories). Images were obtained using a Zeiss LSM 700 confocal laser-scanning microscope system or an Olympus BX53 fluorescence microscope.

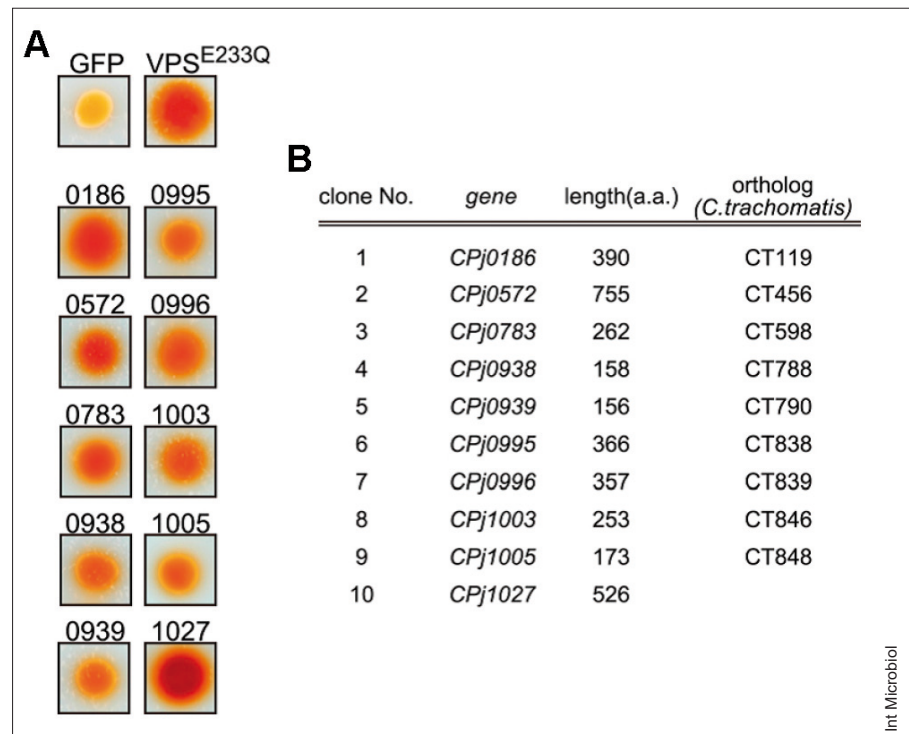
**Yeast two-hybrid assays and α-galactosidase activation assays.** The yeast two-hybrid assay was performed as described in the Matchmaker protocol (Takara Bio Co., Japan). As a bait plasmid, the amino acids 25–262 of CPj0783 were cloned into the GAL4 DNA-binding domain expression vector. As a prey, the Mate & Plate Library-HeLa S3 (normalized) cDNA library was used. To assess α-galactosidase activity in isolated yeast transformants, colorimetric assays were performed as described in the manuscript provided by Takara Bio Co.

**GST pull-down assay.** GST-CPj0783 was incubated with the protein extract from GFP-HIP14 expressing HEp-2 cells at 4°C for 2 h. The suspension of glutathione-Sepharose 4B beads was added, and the incubation was continued for an additional 30 min. The beads were washed five times, and extracted proteins were analyzed by immunoblotting.

## Results

***Chlamydia pneumoniae* molecules and protein mis-sorting in yeast cells.** It was found that the CPY-Inv reporter system is a powerful tool to identify molecules that alter eukaryotic vesicle trafficking pathways [17]. Vacuole protein sorting gene 4 (Vps4) is one of the core proteins which form multivesicular bodies. When Vps4E233Q was expressed in yeast cells, normal endosomal function was perturbed and the CPY-Inv fusion protein was exported out of the cells to the cell surface [17,20]. Then, the media color changed to brown

**Fig. 1.** *Chlamydia pneumoniae* molecular screening using a yeast mis-sorting assay. (A) 455 function-unknown *C. pneumoniae* genes were cloned into a CEN  $P_{Tet-off}$  vector and then expressed in yeast cells. The yeast was spotted on the induction media. The detail method of mis-sorting assay is described in the section of materials and methods. Ten *C. pneumoniae* molecules that caused mis-sorting in yeast cells were obtained from this screening. The candidate genes were analyzed in three independent experiments. (B) Ten *C. pneumoniae* molecules. Nine of these molecules have *C. trachomatis* orthologs, and CPj1027 was specific to *C. pneumoniae*.



as invertase reacted with its substrate in the overlaid agarose and subsequently formed brown precipitates. By using this system, 455 *C. pneumoniae* ORFs were screened and we consequently obtained 10 positive clones (Fig. 1). When each one of the CPj0186, 0572 and 1027 was expressed in yeast cells, they showed strong colorimetric changes in the overlay by the mis-sorting of CPY-Inv. When one of the other seven *C. pneumoniae* ORFs, CPj0783, 0938, 0939, 0995, 0996, 1003 and 1005, was expressed in yeast cells, it showed moderate colorimetric change in the overlay. We summarized the ORF names, predicted amino acid length, and their orthologs in the *C. trachomatis* genome in Fig. 1B. CPj1027 did not have any counterpart in the *C. trachomatis* genome and was one of the *C. pneumoniae* specific ORFs.

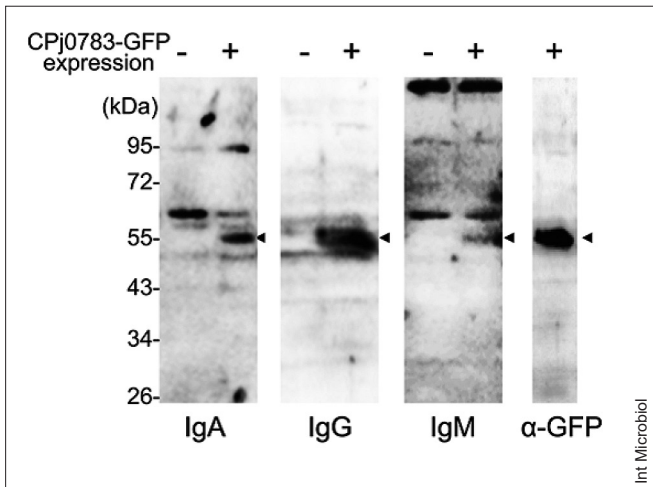
### ***Chlamydia pneumoniae* molecule expression in infected patients.**

We made a genomic screening system for *C. pneumoniae*-specific antigen molecules in the previous study [21] and adapted it to these candidate ORFs in the present study. GFP-tagged ORFs encoded by the ten candidates were expressed in yeast cells as described [21]. Serum samples were collected from eight patients (age range 4–11 years) who had been clinically diagnosed with primary acute *C. pneumoniae* infection. These samples were applied to immunoblot analyses as primary antibodies. Three

different classes of immunoglobulins (HRP-conjugated anti-human IgA, IgG and IgM) were used as the secondary antibodies. The serum samples from these patients did not contain significant anti-*Saccharomyces cerevisiae* antibodies, which would have produced a high level background on the immunoblots (Fig. 2). Therefore, we were able to specifically detect the *C. pneumoniae* antigens under conditions of low-level background. Then, it was revealed that CPj0783 was recognized by all classes of immunoglobulins examined in this study (Fig. 2). When antisera obtained from babies without *C. pneumoniae* infection who were diagnosed by commercially available serologic ELISA test kits were used for immunoblot analysis, we could not obtain any specific signals on the blots (data not shown). These results provide the evidence that CPj0783 had been definitely produced in patients with primary *C. pneumoniae*-infection and was recognized by the host immune systems. CPj0783 was no longer the candidate ORF but a gene undoubtedly working in its genome. Thus, we investigated CPj0783 in details.

### **Expression of *Chlamydia pneumoniae* CPj0783 during inclusion maturation.**

*Chlamydia pneumoniae* has a unique life cycle and two developmental stages, the elementary body (EB) and the reticulate body (RB). EB is the infectious form of *Chlamydia*. After invading the host



**Fig. 2.** CPj0783 expression in *C. pneumoniae*-infected patients. Serum samples were collected from eight patients (age range 4–11 years) who had been clinically diagnosed with a primary acute *C. pneumoniae* infection. Yeast cells were transformed with the amino acids 25–262 of CPj0783 C-terminally fused to GFP (CPj0783-GFP), and then the protein was extracted. The predicted molecular mass of CPj0783-GFP was 56 kDa (arrowhead). Immunoblot analysis was performed using serum samples as the primary antibodies and three different classes of immunoglobulins (HRP-conjugated anti-human IgA, IgG, IgM) as the secondary antibodies. The arrowheads indicate the specific band of CPj0783-GFP that was detected by patient sera or anti-GFP pAb ( $\alpha$ -GFP).

cells (infection time: 0–2 h), *C. pneumoniae* makes inclusion bodies and changes its morphology from EB to RB (2–18 h). RBs are the non-infectious intracellular form, yet they are the metabolically active replicating form. They can then replicate themselves through binary fission (18–48 h). After division, the RBs become EBs (48–72 h). We investigated at which stage of the chlamydial developmental cycle CPj0783 was expressed in *C. pneumoniae*-infected cells. mRNA and protein expression were examined in *C. pneumoniae*-infected cells. Figure 3A shows that the mRNAs of *C. pneumoniae* major outer membrane protein (MOMP) and CPj0783 were detected by RT-PCR in *C. pneumoniae*-infected cells at 48 h post-infection. Then we investigated at which stage of the chlamydial developmental cycle CPj0783 was expressed in *C. pneumoniae*-infected cells. The protein expression of MOMP was detected at 12 h post-infection, and its expression level increased during the developmental cycle (Fig. 3B). According to the MOMP expression levels, bacteria proliferated with the longer incubation period, whereas the protein expression of CPj0783 was detected at 24 h to 66 h post-infection, and a rapid decrease in the CPj0783 expression after 60 h post-infection was observed.

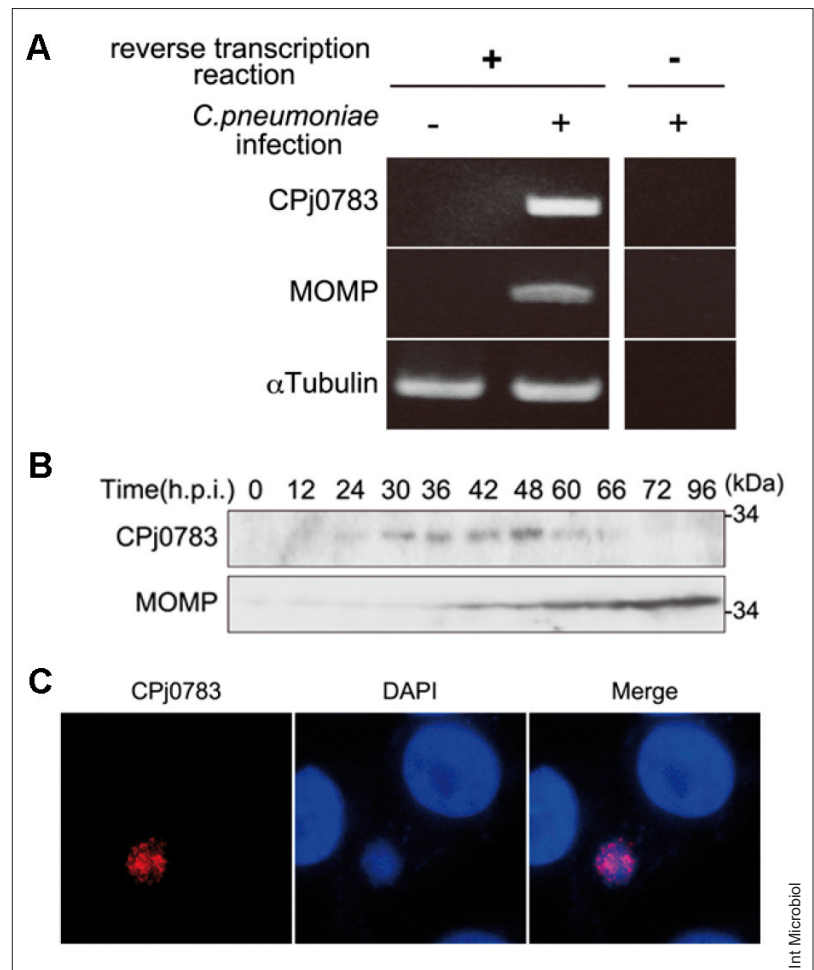
We investigated CPj0783 expression by immunostaining at 48 h post-infection (Fig. 3C). *Chlamydia pneumoniae*-infected cells were analyzed by staining with DAPI and an antibody against CPj0783. DAPI staining clearly showed host cell nuclei and a chlamydial inclusion in the host-cell cytoplasm. Some immunoreactive punctate patterns of CPj0783 expression on a chlamydial inclusion were visualized by staining with the antibody.

**CPj0783-HIP14 interaction in yeast two-hybrid screening.** To identify protein(s) that interact with CPj0783, a two-hybrid screening system in yeast cells was used in the present study. According to the predicted structure of CPj0783, CPj0783 might contain a putative transmembrane region in its N-terminus (Fig. 4A). Therefore, we used amino acids 25–262 of CPj0783 as a bait protein for yeast two-hybrid screening. Two of the positive clones were sequenced, and these clones were identified as part of HIP14 and encompassed amino acids 8–182 which contained the ankyrin repeat domain (Fig. 4A). To confirm the results of a yeast two-hybrid screening, CPj0783 and the cDNA in the bait and prey in the initial screening were exchanged with each other (Fig. 4B), and then an interaction between the bait amino acids 8–182 of HIP14 and the prey amino acids 25–262 of CPj0783 was detected as well (Fig. 4B). HIP14 has five ankyrin repeats in its N-terminal half and six transmembrane domains in its C-terminal half [15]. The clones obtained from the initial screening contained three ankyrin repeats, and therefore we used amino acids 1–303 of HIP14 that corresponded to the entire cytoplasmic region as prey. The resulting plasmid DNAs were transformed into haploid yeast to detect protein-protein interactions. An interaction between CPj0783 and amino acids 1–303 of HIP14, as well as an interaction between CPj0783 and amino acids 1–182 of HIP14, was detected (Fig. 4C). These results suggest that an interaction between CPj0783 and HIP14 in yeast cells could be demonstrated in this way. It was therefore important to clarify the interaction between CPj0783 and HIP14 in other ways, as described below.

**CPj0783 interaction with HIP14 in vivo.** To reveal the interaction between CPj0783 and HIP14 in mammalian cells, we made mCherry-tagged CPj0783 and GFP-tagged HIP14 and co-transfected them into HEp-2 cells. The cell lysates were precipitated with anti-mCherry pAb-protein G beads and analyzed by immunoblotting with anti-GFP pAb. HIP14-GFP was specifically detected in the immunoprecipitate from HEp-2 cells expressing mCherry-CPj0783 (Fig. 5A).



**Fig. 3.** CPj0783 mRNA and protein expression in *C. pneumoniae*-infected cells. (A) HEp-2 cells were infected with *C. pneumoniae* and incubated at 37°C for 48 h. Cells were lysed in Sepasol-RNA I Super for the extraction of total RNA. RNA was treated with DNase I to remove DNA contamination. RNA was reverse-transcribed in three independent experiments. cDNA was amplified using primers specific for CPj0783, MOMP or  $\alpha$ -tubulin. (B) HEp-2 cells were infected with *C. pneumoniae* and incubated at 37°C for the indicated time. Cells were lysed in TNE buffer for the extraction of proteins. CPj0783 or MOMP protein expression was analyzed by immunoblotting with specific antibodies. The experiments were performed in triplicate. (C) HEp-2 cells grown on glass coverslips were infected with *C. pneumoniae* and incubated at 37°C for 48 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 in PBS. Cells were incubated with  $\alpha$ -CPj0783 pAb overnight at 4°C. A secondary antibody coupled to Alexa 594 was incubated on cells for 60 min at room temperature. Images were obtained using an Olympus BX53 fluorescence microscope.



We observed the multiple mCherry-CPj0783 bands on the immunoblot. These multiple bands were detected not only by the anti-mCherry pAb but also by the anti-CPj0783 pAb. mCherry-CPj0783 might be an unstable protein and was cleaved by an unknown mechanism.

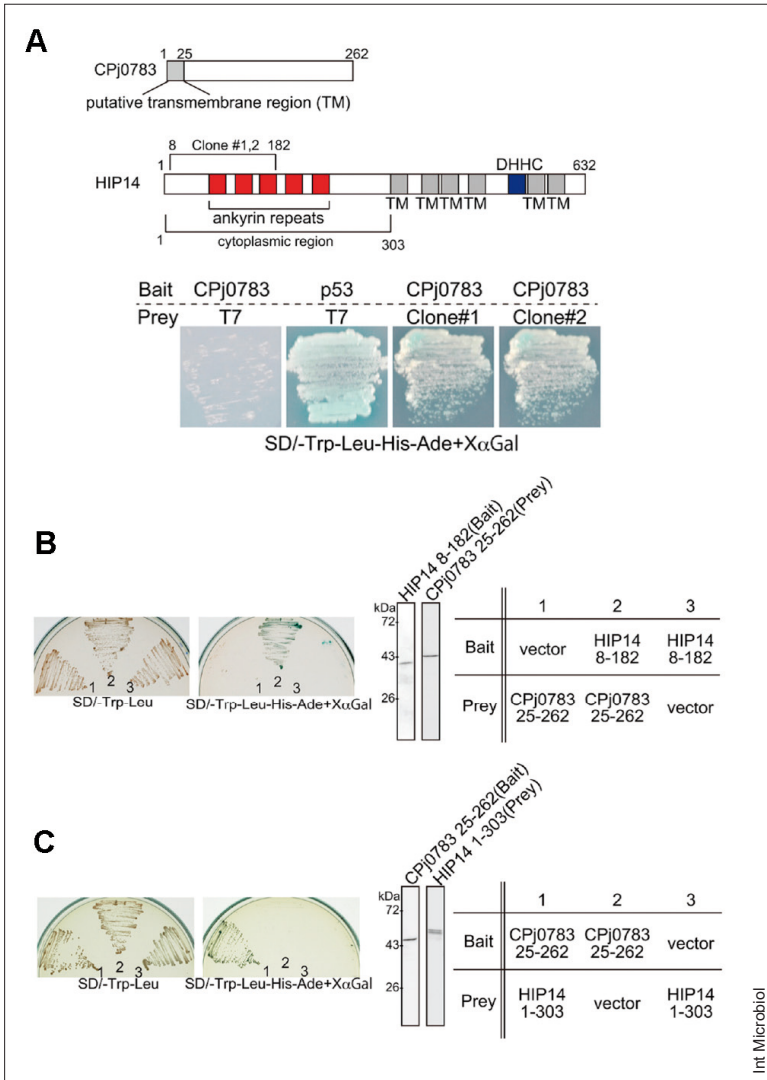
**CPj0783 colocalization with HIP14 in the Golgi apparatus.** We analyzed the subcellular localization of CPj0783 in HEp-2 cells. mCherry-CPj0783 and HIP14-GFP were co-transfected in HEp-2 cells. HIP14-GFP completely colocalized with a Golgi marker (Fig. 5B), as previously reported [7]. The location of CPj0783 clearly corresponded with that of HIP14-GFP. These results suggested that CPj0783 and HIP14 might interact with each other in the Golgi apparatus of mammalian cells.

**CPj0783 interaction with HIP14 in vitro.** To confirm the results of the co-immunoprecipitation assay (Fig. 5A) and the immunostaining study (Fig. 5B), a GST pull-

down assay was performed. We made GST fusion proteins including the amino acids 25–262 of CPj0783 and purified these recombinant proteins from *Escherichia coli*. When the protein from HEp-2 cells expressing HIP14-GFP was mixed with GST-CPj0783, HIP14 was pulled down by CPj0783 and was specifically detected on the immunoblots (Fig. 5C). These results demonstrated that CPj0783 specifically interacted with HIP14 in vitro.

## Discussion

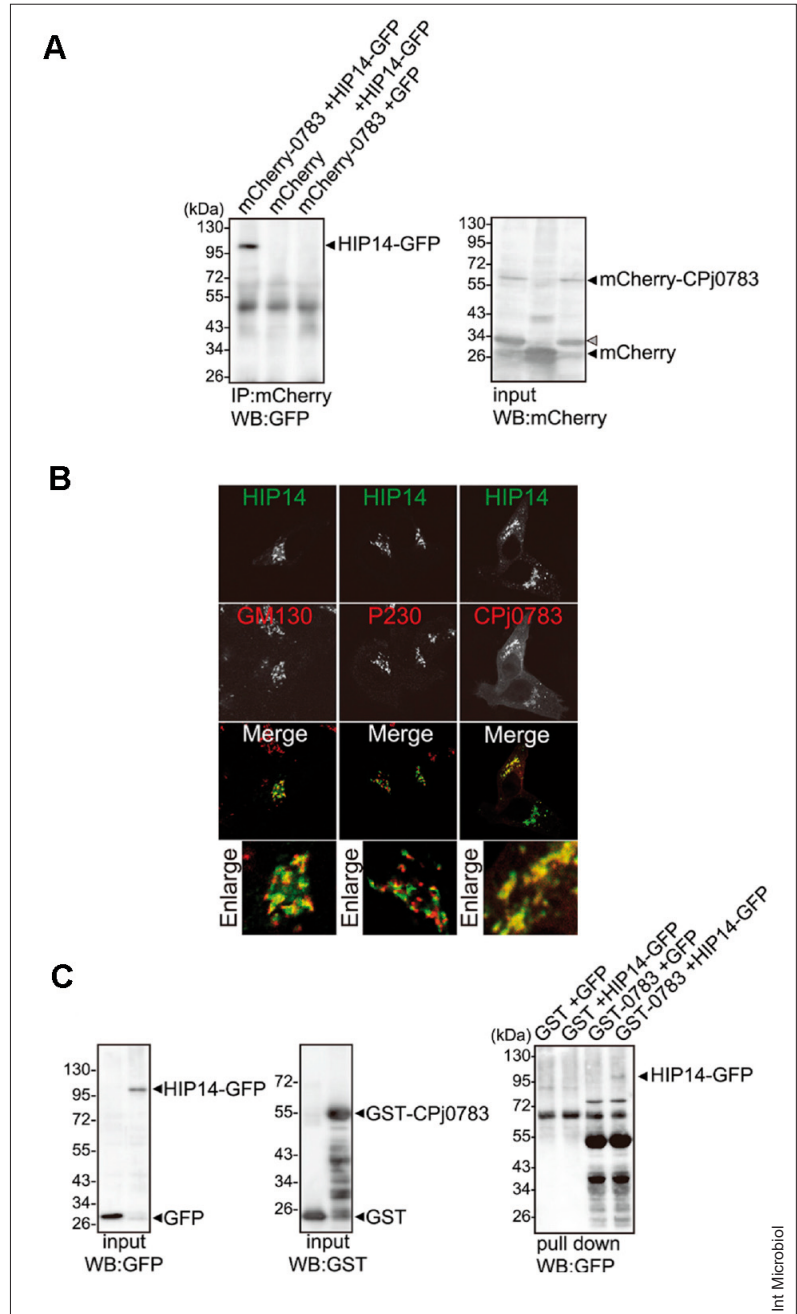
*Chlamydia pneumoniae* is an obligate intracellular pathogen. It is necessary for *C. pneumoniae* to escape the host immune response. Upon infection, the nascent inclusion membrane surrounding the infectious EB is plasma membrane-derived, but within a few hours, chlamydial translocated proteins modify the inclusion membrane. These modifications result in inclusion trafficking to the microtubule organizing center and



**Fig. 4.** Identification of CPj0783-binding proteins by a yeast two-hybrid screen. **(A)** The predicted structures of CPj0783 and HIP14. The schematic presentation of HIP14 shows its five ankyrin repeats, six transmembrane regions and one DHC palmitoyl acyl transferase domain. Amino acids 25–262 of CPj0783 were cloned into the vector containing the GAL4 DNA-binding domain (bait vector). As prey, Mate & Plate Library-HeLa S3 (normalized) cDNA library was used. After mating, yeast were grown on both SD/-Trp/-Leu and SD/-Trp/-Leu/-His/-Ade + X-gal selection medium and incubated at 30°C for 48 h. The vector served as a negative control, and p53 and T7 served as positive controls, as suggested in the instruction manual. **(B)** To confirm the yeast two-hybrid screening results, the cDNAs in the baits and preys used in the screening were exchanged. Amino acids 8–182 of HIP14 were cloned into the bait vector and amino acids 25–262 of CPj0783 were cloned into the prey vector. The expression of recombinant HIP14 (amino acids 8–182) in the bait vector and CPj0783 (amino acids 25–262) in the prey vector was confirmed by immunoblotting. **(C)** The interaction between CPj0783 and the total cytoplasmic region of HIP14 was examined. Amino acids 1–303 of HIP14 were cloned into a prey vector. Expression of recombinant HIP14 (amino acids 1–303) in the prey vector and CPj0783 (amino acids 25–262) in the bait vector were confirmed by immunoblotting.

the separation of the inclusion from the classical endosomal/lysosomal pathway [10]. However, the detailed mechanism of how *Chlamydia* could disturb vesicle trafficking in host cells remained unclear. We used the properties of a hybrid protein resulting from a fusion (CPY-Inv) between carboxypeptidase Y (CPY) and invertase (Inv), both of which are originally encoded by the yeast genome in nature. The CPY gene encodes a vacuolar hydrolase that undergoes post-translational modifications in the Golgi apparatus, followed by trafficking to the late endosome and sorting to the vacuole, which functions as the lysosome in yeast cells. The CPY-Inv fusion protein is normally translocated from the endoplasmic reticulum to the Golgi apparatus and subsequently to the vacuole via the late endosome. The fusion protein is strictly sequestered in the vacuole and cannot reach the yeast cell surface at all. Thus, the cell cannot hydrolyze exogenously

provided sucrose in this screening system. Once the normal trafficking becomes perturbed, cargo vesicles are blocked from getting to the vacuole and then mis-sorting of the fusion protein is induced. As a consequence, spillover of the fusion proteins is evoked, and they are translocated into vesicles destined for the yeast cell surface. When abnormal secretion of the fusion protein occurs in yeast cells, it allows the cell to hydrolyze exogenous sucrose [17,20]. In this study, we found ten *C. pneumoniae* candidate molecules that caused the mis-sorting of the CPY-Inv fusion protein in yeast cells. One of these candidates, CPj0186, showed relatively strong colorimetric changes in the assay system for the mis-sorting phenotype. It had been previously reported that CPj0186 is inclusion membrane protein A (IncA) and has similarity to CT119 in the *C. trachomatis* genome [2]. The IncA protein of *C. trachomatis* has been well studied, and it has been reported



**Fig. 5.** CPj0783 interaction with HIP14 in vivo and in vitro. **(A)** mCherry-CPj0783 and HIP14-GFP were expressed in HEp-2 cells. The proteins were extracted in TNE buffer. The samples were incubated with a protein G-Sepharose slurry to preclear the sample of nonspecific binding to proteins. Co-immunoprecipitation studies were carried out with these samples. The precipitates were analyzed by immunoblotting with anti-GFP pAb. Similar results were obtained from at least three independent experiments. **(B)** mCherry-CPj0783 and HIP14-GFP were transfected in HEp-2 cells and incubated at 37°C for 48 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 in PBS. Cells were incubated with anti-P230 or GM130 mAb 594 was incubated at room temperature for 60 min. A secondary antibody coupled to Alexa 594 was incubated on cells at room temperature for 60 min. Images were obtained using a Zeiss LSM 700 confocal laser-scanning microscope system. **(C)** GST pull-down assay. GST-CPj0783 was expressed in *E. coli* JM109. Purified GST-CPj0783 was mixed with the crude extract obtained from HEp-2 cells expressing HIP14-GFP and precipitated with glutathione-Sepharose 4B beads. Eluates from the beads were subjected to SDS-PAGE and analyzed by immunoblotting with anti-GFP pAb. Similar results were obtained from at least three independent researchers.

that IncA interacts with eukaryotic proteins called SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) that catalyze membrane fusion during intracellular vesicular transport [14]. We have previously reported that CPj0572 is recognized by three types of immunoglobulins, IgA, IgM and IgG, derived from hosts who suffer from primary acute *C. pneumoniae* infection [21]. In the present study, CPj0572 showed relatively strong colorimetric changes in this screening system and could be one of the candidates.

It has been reported that CPj0572 is an ortholog molecule of *C. trachomatis* translocated actin recruiting phosphoprotein (Tarp), containing the alpha helix structure that is associated with host actin, and could actually induce actin nucleation [3,8]. Therefore we could accurately detect CPj0186/IncA and CPj0572/Tarp as molecules that caused the CPY mis-sorting in this screening system, and this indicated that the system itself was functioning extremely well to identify the molecules altering vesicle trafficking pathways. Another

candidate molecule, CPj1027, which showed a strong colorimetric change in this assay system, has been reported to be a newly identified inclusion membrane protein unique to *C. pneumoniae* [5]. The CPj1027 protein was detected as early as 12 h after *C. pneumoniae* infection and remained in the inclusion membrane throughout the infection cycle. The function of CPj1027 has not yet been clarified. CPj1027 might interact with some proteins that are related to vesicle trafficking. It is thus necessary to investigate the function of this molecule in future studies.

CPj0783 showed a moderate colorimetric change for the mis-sorting phenotype. The *C. trachomatis* ortholog of CPj0783 is CT598; the functions of CPj0783 and CT598 are completely unknown. The amino acid sequence similarity between CPj0783 and CT598 is merely 35%, and both molecules might have the similar function. CT598 is a hypothetical protein and its expression in *C. trachomatis* has not been proved yet. Analysis of CT598 would be necessary and we plan to investigate this issue. We have previously reported that 58 clones expressing *C. pneumoniae* ORFs are antigens using serum samples obtained from *C. pneumoniae*-infected patients as the primary antibodies [21]. Thus, we examined the immunoreactivities of CPj0783 and revealed that *C. pneumoniae* definitely produced CPj0783 during infection; this molecule was recognized by three types of immunoglobulins, IgA, IgM and IgG, derived from hosts who suffered from primary acute *C. pneumoniae* infection. These results suggest that CPj0783 could be secreted out of the infected cells or released from the extruded inclusion and/or lysis of the host cell, and then the host immune system could recognize CPj0783 and produce specific antibodies. Based on these data, we chose CPj0783 and investigated it in detail.

It is thought that *C. pneumoniae* exchanges its morphology from RB to EB at approximately 48 h post-infection. The MOMP expression level increased depending on the number of *C. pneumoniae* cells without being affected by the *C. pneumoniae* cell cycle. The protein expression of CPj0783 was detected during the period of 24 h to 66 h post-infection and its peak expression occurred in 48 h post-infection. Then it rapidly decreased. Immunostaining of *C. pneumoniae*-infected cells showed the presence of CPj0783 on chlamydial inclusions. These data suggest that CPj0783 might be generated in RBs, but not EBs, and would be necessary for growth in the inclusion body.

To investigate the function of CPj0783, we performed yeast two-hybrid screening and identified the CPj0783-interacting protein. Using amino acids 25–262 of CPj0783 as bait, we found positive clones containing amino acids 8–182

of HIP14. To confirm the interaction between CPj0783 and HIP14 in yeast cells, we exchanged the DNAs in the bait and prey used in the initial screening and found a positive interaction between the two. In addition, we used the total HIP14 cytoplasmic region (amino acids 1–303) as prey and still obtained a positive interaction with CPj0783 in yeast cells. By using an in vivo co-immunoprecipitation assay, we investigated the interaction between CPj0783 and HIP14. It was reported that HIP14 localized to the Golgi apparatus and cytoplasmic vesicles [6,7]. We made four types of HIP14 constructs; N-terminal GFP- or mCherry-tagged HIP14 and C-terminal GFP- or mCherry-tagged HIP14. We found that all four types of HIP14 recombinants localized to the Golgi apparatus without any influence from the tagging molecules (data not shown). Then, subcellular localizations of CPj0783 and HIP14 were examined by immunostaining, both of which were confirmed to be present in the Golgi apparatus. It was also demonstrated that HIP14 could be co-immunoprecipitated with CPj0783 in vivo (Fig. 5A) and the interaction between CPj0783 and HIP14 was also found by an in vitro GST-pull down assay (Fig. 5C). These results demonstrably indicate that CPj0783 interacted with HIP14. Recently, it has been reported that the palmitoylation and distribution of HTT are regulated by the palmitoyl transferase of HIP14 [1,12,18]. There are multiple key functional domains identified: the ankyrin repeat domain in its N-terminal half (amino acids 1–303) and the DHHC-palmitoyl acyl transferase domain and six transmembrane regions in its C-terminal half (amino acids 304–632) [15]. According to the results obtained from the yeast two-hybrid screening and the in vivo and in vitro binding assays, CPj0783 can interact with a part of the HIP14 ankyrin repeat.

In this study, we focused on one of the *C. pneumoniae* proteins, CPj0783, which caused an intracellular trafficking abnormality in yeast cells. CPj0783 was detected by immunoglobulins derived from *C. pneumoniae*-infected patients and was certainly recognized by human immune cells. After the infection, CPj0783 might be generated in RBs and then transported in host cells to the Golgi apparatus where CPj0783 interacted with HIP14. The immunostaining assay could not reveal the existence of CPj0783 in the Golgi apparatus in *C. pneumoniae*-infected cells but did find CPj0783 on the inclusion bodies. As shown in the case of CPj0186/IncA, the molecule on the inclusion could interact with SNAREs localized to various intracellular organelles and membranes in human cells, and it caused the CPY mis-sorting in yeast cells [14]. In the case of *C. trachomatis* infection, the bacteria hijack the conserved oligomeric Golgi (COG)



complex, which is orchestrating the vesicular trafficking in the Golgi apparatus. Interaction of COG complex with chlamydial inclusion has been observed with *C. trachomatis* and this interaction was maintained throughout the entire development cycle [13]. It has not been yet clarified what molecules on the inclusion could recruit COG complex. After recruitment of COG complex to the inclusion, CPj0783, expressed on the inclusion, could contact with HIP14. In the *C. pneumoniae*-infected patients, CPj0783 might be translocated from the inclusion into host cells and subsequently induce the immunological response in human body [21]. It can be speculated that all molecules of CPj0783 expressed in RB do not translocate into host cells and that the translocated CPj0783 cannot be detected by immunostaining. Future work will be needed to further define the subcellular localization by more sensitive means of detection. Further studies on the function of CPj0783 in the *C. pneumoniae* infected cells are warranted, especially with regards to its function in the vesicle sorting machinery, which includes HIP14.

**Acknowledgements.** We thank Yumiko Noguchi for her technical assistance. This work was supported by JSPS KAKENHI Grant Number 26860223 and Research Project Grants from Kawasaki Medical School.

**Competing interests.** None declared.

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