A Peptide Derived from Phosphoinositide 3-kinase Inhibits Endocytosis and Influenza Virus Infection

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ABSTRACT. Endocytosis mediates the internalization and ingestion of a variety of endogenous or exogenous substances, including virus particles, under the control of intracellular signaling pathways. We have previously reported that the complex formed between the small GTPase Ras and phosphoinositide 3-kinase (PI3K) translocates from the plasma membrane to endosomes, signaling from which thereby regulates clathrin-independent endocytosis, endosome maturation, influenza virus internalization, and infection. However, the molecular mechanism by which the Ras-PI3K complex is recruited to endosomes remains unclear. Here, we have identified the amino acid sequence responsible for endosomal localization of the Ras-PI3K complex. PI3K lacking this sequence failed to translocate to endosomes, and expression of the peptide comprising this PI3K-derived sequence inhibited clathrin-independent endocytosis, influenza virus internalization, and infection. Moreover, treatment of cells with this peptide in an arginine-rich, cell-penetrating form successfully suppressed influenza virus infection in vitro and ex vivo, making this peptide a potential therapeutic agent against influenza virus infection.

Key words: signal transduction, endocytosis, endosome, imaging, influenza virus

Introduction

Endocytosis is a cellular mechanism by which a variety of substances are engulfed and transported from outside a cell to the inside. Several taxonomies are available to characterize endocytosis. For instance, endocytosis can be roughly divided into pinocytosis (cell drinking) and phagocytosis (cell eating) based on the type of substance incorporated and their mechanisms. Endocytosis pathways can also be subdivided into four categories, including receptor-mediated (or clathrin-mediated), caveolae-dependent, clathrin-independent (including macropinocytosis and micropinocytosis), and phagocytosis, based on the regulatory mechanism utilized, such as coated pits lined with proteins (Godlee and Kaksonen, 2013; Maldonado-Báez et al., 2013; McMahon and Boucrot, 2011; Merrifield and Kaksonen, 2014). Endocytosis is a mandatory process in which cells uptake important chemical substances, as large polar molecules (such as proteins) cannot penetrate the aliphatic plasma membrane in a passive manner. Low-density lipoproteins, transferrin, growth factors, antibodies, and many other ligands can be internalized into cells by binding their specific receptors and then undergoing the subsequent coated pit formation and clathrin-mediated endocytosis (Marsh and McMahon, 1999).

Pathogens are also internalized into cells via endocytosis. In antigen-presenting cells, bacteria or other pathogenic microorganisms are internalized via phagocytosis, digested, and degraded in lysosomes, the products of which are then...
presented at the cell surface for recognition by helper T cells. *Shigella*, *Salmonella enterica*, and *Mycobacterium tuberculosis* enter cells via endocytosis to reach a milieu in which they can proliferate. In addition to bacteria, both non-enveloped viruses (e.g., *Adenoviridae*) and enveloped viruses (e.g., influenza viruses) are internalized into cells via endocytosis, after which they release their genomes into the cytoplasm for replication.

In addition to extracellular and exogenous substances, membrane proteins are also internalized into cells via endocytosis, which eventually contributes to the regulation of signal transduction pathways. For instance, endocytosis promotes the translocation of membrane receptors from the plasma membrane to endosomes, resulting in a decreased probability of receptors associating with ligands by sequestering receptors from the cell surface. This thereby results in desensitization to the ligands and signaling downregulation. Moreover, endocytosis regulates the fate of signaling molecules via endosomal sorting (i.e., recycling versus degradation). Endosomes are also attracting attention not only as organelles that carry many substances but also as platforms that positively emit signals themselves. For example, nerve growth factor receptors are known to activate different downstream factors depending on their localization; activation at the cell membrane and in endosomes is responsible for cell proliferation and differentiation, respectively (Grimes et al., 1996; Murphy et al., 2009). On the other hand, endocytosis machinery is regulated by a variety of signaling pathways, including phosphoinositide 3-kinase (PI3K) signaling.

The lipid kinase PI3K phosphorylates phosphoinositides at the 3’ position of the inositol ring (Whitman et al., 1988) and plays roles in a variety of cellular functions, including cell growth, proliferation, differentiation, motility, survival, and intracellular membrane trafficking, through the recruitment of pleckstrin homology (PH) domain-containing proteins (Vanhaesebroeck et al., 2012). Activation of PI3K is promoted by at least four independent pathways. First, the p110β catalytic subunit of PI3K can be activated by G protein-coupled receptors (Katso et al., 2001). The other three pathways are initiated by the activation of receptor tyrosine kinases (RTKs) via ligand binding. The Src homology 2 (SH2) domain in the regulatory subunit p85 of PI3K is directly recruited to phosphorylated tyrosines (YXXM) within RTKs (Domechek et al., 1992), triggering the catalytic activation of PI3K. p85 can also be recruited to another phosphorylated tyrosine-containing motif, YXN, via adaptor proteins, including growth factor receptor-bound protein 2 (Grb2) and Grb2-associated binder 1 (GAB1) (Pawson, 2004). Finally, the fourth way to activate PI3K is via Ras. Recruitment of Grb2, some fraction of which constitutively binds the Ras activator son of sevenless (Sos), results in activation of Ras, which activates p110 independently of p85 by the binding of Ras to the Ras-binding domain (RBD) of p110 (Ong et al., 2001).

We have previously reported that the Ras-PI3K complex is translocated to endosomes, and calcium-dependent activation of Ras-PI3K signaling are required for the regulation of clathrin-independent endocytosis, endosomal maturation, influenza virus internalization, and infection (Fujikura et al., 2018, 2011, 2013; Tsutsumi et al., 2009). However, the molecular mechanism by which the Ras-PI3K complex is recruited to the endosome remains unclear. Here, we have identified the amino acid sequence that is crucial for endosomal localization of the Ras-PI3K complex, and we named this sequence RAPEL (Ras-PI3K endosomal localization). The forced expression of this peptide inhibited endocytosis, influenza virus internalization, and infection without affecting canonical Ras or PI3K-related signaling. Given that its introduction in peptide form was also effective in inhibiting influenza virus infection, we offer a novel peptide-based therapy targeting influenza virus infection and other endocytosis-related diseases.

### Materials and Methods

#### Sequence analysis

The amino acid sequences of PI3K p110α (NP_006209.2), PIK3 p110β (NP_006210.1), p110γ (NP_001269356.1), p110δ (NP_004752.1), c-Raf (NP_002871.1), B-Raf (NP_004324.2), A-Raf (NP_001645.1), RafGDS (NP_006257.1), Rgl1 (NP_055964.3), Rgl2 (NP_004752.1), Rgl3 (NP_001155088.2), and Rif (AAC52724.1) were obtained from the NCBI Protein Database. From these sequences, the RBDs were identified with the use of the CD Search tool of the NCBI Conserved Domain Database. Multiple sequence alignment was performed with the use of ClustalW.

#### Reagents and antibodies

Recombinant human EGF was purchased from PeproTech (Rocky Hill, NJ, USA). Hoechst 33342, Alexa Fluor 546-labeled dextran (10 kDa), rhodamine B-labeled dextran (70 kDa), Alexa Fluor 546-labeled transferrin, and Alexa Fluor 594- and Alexa Fluor 647-conjugated antibodies against mouse, rabbit, and rat immunoglobulin G were obtained from Thermo Fisher Scientific (Carlsbad, CA, USA). Vps34-IN1 was purchased from Funakoshi (Tokyo, Japan). Horseradish peroxidase-conjugated sheep antibodies against mouse, donkey, and rabbit immunoglobulin G were obtained from Thermo Fisher Scientific (Carlsbad, CA, USA). Vps34-IN1 was purchased from Funakoshi (Tokyo, Japan). Horseradish peroxidase-conjugated sheep antibodies against mouse, donkey, and rabbit immunoglobulin G were obtained from GE Healthcare (Little Chalfont, UK), and horseradish peroxidase-conjugated donkey antibodies against goat immunoglobulin G were purchased from Promega (Madison, WI, USA). Antibodies against EEA-1 (610456) were obtained from BD Biosciences (San Jose, CA, USA). Antibodies against GST (5A7) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Antibodies against ERK (9102S), phosphor-ERK (9101S), Akt (9272S), phosphor-Akt (9271S), and Rab7 (9367S) were purchased from Cell Signaling Technology (Danvers, MA).
Antibodies against GFP (598), HA (3F10), NP (ab20343), β-actin (sc-7778), and Ras (Ab-3) were obtained from Medical & Biological Laboratories (MBL; Nagoya, Japan), Roche (Indianapolis, IN, USA), Abcam (Cambridge, UK), Santa Cruz Biotechnology (Santa Cruz, CA), and Oncogene (Cambridge, MA, USA), respectively.

### Plasmids

pCAGGS-VN-H-Ras WT and G12V, pCXN2-Flag-p110γ RBD-VC, pCXN2-Flag-H-Ras WT, pCAGGS-EGFP-H-Ras G12V, pCAGGS-Venus-H-Ras G12V, pCXN2-3×HA-p110γ, pCAGGS-ECP-Rab5, and pRaichu-Ras 146X were described previously (Fujioka et al., 2011; Mochizuki et al., 2001; Ohba et al., 2003; Tsutsumi et al., 2009). cMV-TagRFP-EFA-1 was purchased from Addgene (Cambridge, MA, USA). cDNA for p110γ RBD ΔRAPEL was generated by PCR with the following primers: ΔRAPEL_F (5'-CCCTCGAGACGACATTAAGGTCTCACC-3') and ΔRAPEL_R (5'-GGGCGGCCGCCGGTGCAGGTACTCC-3') and RBDR (5'-GGCCGGCCGCCGCTTGGAGGCGTGTCCAGT-3').

These sequences were then subcloned into the Xhol/Not I sites of pCXN2-Flag-p110γ RBD-VC to obtain pCXN2-Flag-p110γ RBD ΔRAPEL-VC. The coding sequences of wild-type p110γ RBD and p110γ RBD ΔRAPEL were also subcloned into the XhoI/Not I sites of the pCAGGS-ECFP, pCAGGS-mRFP, or pCAGGS-GST vectors to obtain pCAGGS-ECFP-p110γ RBD, pCAGGS-mRFP-p110γ RBD, or pCAGGS-GST-p110γ RBD ΔRAPEL, pCAGGS-mRFP-p110γ RBD ΔRAPEL, or pCAGGS-GST-p110γ RBD ΔRAPEL, respectively.

The coding sequences of RAPEL and its derivatives (CT9, CT20, NT8, and M11) were amplified by PCR with the following primers and pBlueScript II SK(+)-p110γ (Fujioka et al., 2009). pCMV-TagRFP-EEA-1 was purchased from Addgene (Cambridge, MA, USA). cDNA for p110γ RBD ΔRAPEL was generated by PCR with the primers ΔRAPEL_F and PI3K_R (5'-CCGGGCCCTCAGCTAGTTAGCGGCCGCCGGCTGAATGTTCCAGTCC-3') and RBDR (5'-GGCCGGCCGCCGGTGCAGGTACTCC-3') and RBDR (5'-GGCCGGCCGCCGGTGCAGGTACTCC-3').

These sequences were then subcloned into the Xhol/Not I sites of pCXN2-Flag-p110γ RBD-VC to obtain pCXN2-Flag-p110γ RBD ΔRAPEL-VC. The coding sequences of wild-type p110γ RBD and p110γ RBD ΔRAPEL were also subcloned into the XhoI/Not I sites of the pCAGGS-ECFP, pCAGGS-mRFP, or pCAGGS-GST vectors to obtain pCAGGS-ECFP-p110γ RBD, pCAGGS-mRFP-p110γ RBD, or pCAGGS-GST-p110γ RBD ΔRAPEL, pCAGGS-mRFP-p110γ RBD ΔRAPEL, or pCAGGS-GST-p110γ RBD ΔRAPEL, respectively.

### Cell culture

HEK293T (CRL-11268), MDCK (CCL-34), BEAS-2B (CRL-9609), and A549 (CCL-185) cells were obtained from American Type Culture Collection (Manassas, VA, USA), and Cos-1 cells (JCRB9082) were from purchased from the Japan Collection of Research Bioresources Cell Bank of the National Institutes of Biomedical Innovation, Health and Nutrition (Ibaraki, Osaka, Japan). These cells were maintained under a 5% CO₂ humidified atmosphere at 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific). Expression vectors were introduced into cells by transfection with the use of Polyethylenimine “Max” (Polysciences, Warrington, PA, USA). Mouse embryonic fibroblasts deficient in PIk3CG were prepared as described (Sasaki et al., 2000), and maintained in DMEM supplemented with 10% FBS. Expression vectors for wild-type and mutant PI3Kγ were linearized by Scal and introduced into the knockout cells using nucleofection according to the manufacturer’s recommendations (Amazx Biosystems, Cologne, Germany). Starting at 2 days, the cells were cultured in DMEM containing 0.5 mg/ml G418 sulfate (Sigma), and the resistant colonies were collectively isolated. Expression of each protein was confirmed by immunoblotting (see Fig. S5B). Stably transfected cells were maintained in DMEM supplemented with 10% FBS and 0.2 mg/ml G418. The absence of mycoplasma contamination was confirmed using a PCR Mycoplasma Test Kit (Takara, Shiga, Japan).

### Virus preparation and plaque assay

MDCK cells were infected with IAV strains A/Puerto Rico/8/34 (H1N1; PR8) or A/Aichi/2/68 (H3N2; Aichi) at a multiplicity of infection (MOI) of 0.0001 plaque-forming units (PFUs) per cell for 48 hours at 35°C. Viruses isolated by high-speed centrifugation were resuspended in phosphate-buffered saline (PBS) and stored at −80°C until use. The amount of infectious virus in the
virus solution or culture media was determined with a plaque assay (Pleschka et al., 2001). In brief, confluent MDCK cell monolayers in 12-well plates were inoculated with conditioned medium of virus-infected cells. After 1 hour of culturing, the supernatants were removed, and the cells were overlaid with minimal essential medium containing 1% Bacto-agar (Sigma-Aldrich) and trypsin (5 mg/ml, Sigma-Aldrich). Plaques were counted after incubation at 35°C under 5% CO₂ for 2 days.

**Peptides**

The peptides used in this study were chemically synthesized using (9-fluorenylmethyloxycarbonyl)-solid phase peptide synthesis. Fluorescent labeling of the peptides was conducted by treatment with 5-maleimidofluorescein isothiocyanate in dimethylformamide methanol followed by reverse phase high-pressure liquid chromatography purification (purity >90%). The fidelity of the products was ascertained by time-of-flight mass spectrometry. Peptide synthesis and purification were performed by BEX (Tokyo, Japan). For peptide delivery, 1 × 10⁶ MDCK or 293T cells were seeded in a collagen-coated, 35-mm-diameter glass-base dish (Asahi Techno Glass Co., Yoshiida, Shizuoka, Japan) and grown to 60–70% confluency. Each well was washed three times with PBS and then incubated with 100 μM peptide in serum-free DMEM for 1 hour at 37°C. Cells were then washed three times with PBS, incubated for 24 hours, and subjected to analyses.

**Wide-field fluorescence microscopy**

Cells were imaged with an IX-83 microscope (Olympus, Tokyo, Japan) equipped with a BioPoint MAC6000 filter and shutter control unit (Ludl Electronic Products, Hawthorne, NY, USA), an automated XY-stage (Chuo Precision Industrial, Tokyo, Japan), an sDISK spinning disk confocal microscope (Andor Technology, Belfast, UK), and a Rolera EM-C2 electron multiplying cooled charge-coupled device camera (QImaging, Surrey, BC, Canada). For epifluorescence microscopy, cells were illuminated with a SOLA Light Engine (Lumencor, Beaverton, OR, USA) and a Rolera EM-C2 electron multiplying cooled charge-coupled device camera (QImaging, Surrey, BC, Canada). For epifluorescence microscopy, cells were illuminated with a SOLA Light Engine (Lumencor, Beaverton, OR, USA) and a Rolera EM-C2 electron multiplying cooled charge-coupled device camera (QImaging, Surrey, BC, Canada). 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**Immunofluorescence-based influenza virus internalization and infection assay**

Immunofluorescence-based virus internalization and infection assays were performed as described previously (Fujioka et al., 2013, 2018). In brief, serum-deprived cells were incubated with IAV at an MOI of 10 PFU per cell for 1 hour (internalization assay) or at an MOI of 1 PFU per cell for 4 hours (infection assay). Cells were washed with acidic buffer, fixed with 3% paraformaldehyde for 15 min at room temperature, and then subjected to immunofluorescence with the use of an anti-NP antibody (1:1,000) and an anti-Rab7 antibody (1:500). Nuclei were visualized by staining with Hoechst 33342. Images were acquired with the FV10i confocal microscope for the internalization assay and for the infection assay in a 3D culture system or with the widefield epifluorescence microscope for the infection assay in 2D cultures.

To quantify virus internalization, confocal sections were acquired from the top to the bottom of the cells. Vesicles positive for NP or Rab7 were extracted with the use of the ‘granularity’ module of MetaMorph software, and the colocalization of NP and Rab7 was evaluated with the use of the ‘measure colocalization’ function. For analysis of virus infection, a region of each individual cell was identified by the “watershed” algorithm based on the nuclear staining and total fluorescence intensities of NP were gauged within the region.

**Immunoblotting**

Unless otherwise specified, cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM Na3VO4] for 30 min on ice. The lysates were centrifuged at 20,000 g for 10 min at 4°C, and the resulting supernatants were subjected to SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) and subjected to immunoblot analysis. Immune complexes were detected with the use of ECL Western Blotting Detection Reagent (GE Healthcare) and an LAS-1000UVmini image analyzer (Fujifilm, Tokyo, Japan).

**Pull-down assays**

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4]. After centrifugation, the resulting supernatants were incubated for 60 min at 4°C with glutathione Sepharose beads (GE Healthcare), washed three times with lysis buffer, eluted in SDS sample buffer, and subjected to immunoblot analysis.

**3D culture**

BEAS-2B cells were cultured in Matrigel (CORNING, Corning, NY, USA) in a 35-mm-glass-base dish for 5 days. After confirming monolayer formation, the cells were further cultured in the presence or absence of the synthetic peptides for 1 hour, and after washing, further incubated for 24 hours. The cells were then infected with PR8 at an MOI of 1 PFU per cell for 4 hours and subjected to an immunofluorescence-based infection assay with the use of antibodies to IAV NP. Nuclei were counterstained with Hoechst 33342. The samples were observed under the confocal microscope FV10i.

**Statistical analysis**

For data presented as box-and-whisker plots, the highest and lowest boundaries of the box represent the 25th and 75th percentiles, respectively, the whiskers above and below the box designate the 5th and 95th percentiles, respectively, and the line and dot within the box indicate the median and mean, respectively. Other data are presented as the mean±s.e.m. Such data were obtained from at least three independent experiments (unless indicated otherwise) and were compared by Student’s t-test (parametric test between two conditions), Welch’s t-test (nonparametric test between two conditions) or one-way analysis of variance (ANOVA) followed by post-hoc Tukey honestly significant difference (HSD) test (among multiple conditions). Time series data sets were compared by repeated-measures ANOVA (one condition), multivariate analysis of variance (MANOVA) (between two conditions), or MANOVA with Bonferroni correction (among multiple conditions). No statistical methods were used to predetermine sample size. Studies were performed unblinded.

**Results**

**Identification of the amino acid sequence required for endosomal localization of the Ras-PI3K complex**

We have previously reported that the Ras-PI3K complex, but not complexes between Ras and other effectors, specifically localizes to endosomes (Tsutsumi et al., 2009), which contributes to the regulation of clathrin-independent endocytosis and influenza virus infection (Fujioka et al., 2011). To understand the molecular mechanism by which the Ras-PI3K complex is translocated to endosomes, RBD amino acid sequences were compared. The amino acid sequences of three major Ras effector families, including Raf (c-Raf, B-Raf, and A-Raf), PI3Ks (PI3K p110α, p110β, p110γ, and p110δ), and RalGEFs (RalGDS, Rif, Rgl1, Rgl2, and Rgl3), were obtained from the National Center for Biotechnology Information (NCBI) Protein Database. The RBDs were identified by the NCBI Conserved Domain Database, and multiple sequence alignment was performed with the use of ClustalW (Fig. 1A and Fig. S1). A phylogenetic tree revealed that each family member forms distinct sister groups (same node), as expected. Interestingly, the PI3K group is the outgroup to Raf and RalGEF groups (Fig. S1), indicating that the RBD of PI3K is evolutionarily distinct.
Fig. 1. Deletion of RAPEL in PI3K RBD resulted in the inability of the Ras-PI3K complex to undergo endosomal localization. (A, B) Multiple alignments of amino acid sequences of the RBDs of Ras effectors (A) and of 28 N-terminal amino acids of PI3K RBDs (B). "•", "••", and "•••" indicate low homology, high homology, and identical amino acids, respectively. (C, D) Cos-1 cells were transfected with expression vectors for TagRFP-EEA-1 and VN-H-Ras together with either wild-type (WT) or RAPEL-deleted (ΔRAPEL) PI3K RBD-VC vectors. Twenty-four hours after transfection, the cells were serum-starved for 4 hours and subjected to time-lapse confocal microscopy. At time 0, the cells were exposed to 100 ng/ml EGF. Representative images at 30 min after EGF stimulation are shown (C). Bar, 10 μm. The extent of colocalization of the Ras-PI3K complexes with EEA-1 was quantified as described in the Materials and Methods and plotted over time (D). Data are presented as the mean±s.e.m. (n ≥20 from three independent experiments). *P<0.0001 as calculated by MANOVA. (E, F) Cos-1 cells were transfected with expression vectors for HA-tagged wild-type (WT) or RAPEL-deleted (ΔRAPEL) PI3K p110γ. After 24 hours, the cells were subjected to immunofluorescence with the use of anti-EEA-1 and anti-HA antibodies. Representative images are shown (E). Left panels are higher magnification images of the inset indicated in the most right entire-cell images. Bar, 10 μm. The extent of colocalization of PI3K p110γ with EEA-1 was quantified (F). Data are presented as the mean±s.e.m. (n ≥40 from three independent experiments). *P<0.0001 versus WT PI3K p110γ expressing cells as calculated by Welch's t-tests. (G, H) 293T cells were transfected with a control vector (Ctrl) or an expression vector for either WT or ΔRAPEL PI3K RBD tagged with glutathione S-transferase (GST) together with a vector for Venus-H-Ras G12V (the constitutively active form). After 24 hours, the cells were subjected to pull-down assay, followed by immunoblotting with antibodies indicated at the right. An aliquot of the cell lysate was also analyzed as a loading control. Representative immunoblots are shown (G). Band intensities were quantitated and plotted (H). Data are means±s.e.m from three independent experiments. F=0.400 between WT and ΔRAPEL as calculated by Student’s t-test.
from those of Raf and RaLGDS. When the RBD sequences of PI3K p110α, p110β, and p110γ were directly compared with those of c-Raf and RaLGDS, the PI3K RBDs were found to possess a unique N-terminal sequence of approximately 20 amino acids (Fig. 1A). Furthermore, 28-amino acid regions in the N-terminus of PI3K RBDs displayed high homology, leading us to examine whether this region plays a role in endosomal localization of the Ras-PI3K complex (Fig. 1B).

We thus prepared a PI3K p110γ RBD (simply termed PI3K RBD hereafter) that lacks these N-terminal 28 amino acids, and localization of the Ras-PI3K RBD complex was analyzed with the use of a bimolecular fluorescence complementation (BiFC) assay (Fig. 1C, D). In a quiescent state, Ras complexes with both wild-type and mutant PI3K RBD localized to the plasma membrane. Upon epidermal growth factor (EGF) stimulation, the Ras complex with wild-type RBD translocated to endosomes (Fig. 1C and Movie S1), as reported previously (Tsutsumi et al., 2009). Quantitative analysis revealed that EGF promoted colocalization of the complex with early endosomes (visualized by early endosomal antigen-1 [EEA-1]) approximately 2-fold higher (at 30 min after EGF stimulation) than that in the unstimulated state (Fig. 1D). In contrast, the Ras complex with the mutant PI3K RBD was not retained in endosomes but rather accumulated in the perinuclear region (Fig. 1C, D, and Movie S2). The EGF-dependent recruitment of full-length PI3K p110γ (PI3Kγ) to endosomes was also inhibited by deletion of this region (Fig. 1E, F). Given that the mutant RBD could bind Ras to a similar extent as wild-type RBD (Fig. 1G, H), the N-terminal 28-amino acid region of RBD might possess a unique function in regulating localization of the Ras-PI3K complex without affecting its ability to bind Ras. The peptide (derived from PI3Kγ) was therefore named the Ras-PI3K endosomal localization (RAPEL) sequence.

**Inhibition of endocytosis and influenza virus infection by RAPEL expression**

Overexpression of a protein fragment that contains a regulatory domain often exerts a negative effect on downstream signaling by sequestering upstream factors, termed a dominant-negative effect. Thus, we next investigated whether the overexpression of RAPEL affects endocytosis. To evaluate the effects of RAPEL expression on clathrin-independent endocytosis at the single-cell level, cells were transfected with an expression vector harboring CFP-tagged RAPEL and challenged with fluorescently labeled 10-kDa dextran (Fig. 2A, B) and 70-kDa dextran (Fig. S2A, B) that were reported to enter cells via different clathrin-independent pathways (Li et al., 2015). As expected, RAPEL expression significantly inhibited the uptake of both dextrans in MDCK cells (Fig. 2A, B, and Fig. S2A, B). This inhibition was also observed in A549, Cos-1, and 293T cells (Fig. 2A, B), indicating that the inhibitory effect of RAPEL on endocytosis is functional among several cell contexts. In contrast, RAPEL expression did not affect transferrin uptake (Fig. 2C, D), demonstrating that RAPEL plays a role in the regulation of clathrin-independent endocytosis, including macropinocytosis and micropinocytosis, but not in clathrin-mediated endocytosis. This notion is consistent with our previous report that Ras-PI3K signaling is preferentially involved in the regulation of clathrin-independent endocytosis (Fujioka et al., 2011).

We have previously reported that influenza A virus (IAV) enters host cells via both clathrin-mediated and clathrin-independent endocytosis (Fujioka et al., 2013), and Ras-PI3K signaling plays a role in virus internalization and infection (Fujioka et al., 2011). Therefore, we investigated whether RAPEL overexpression inhibited IAV infection. An MDCK plaque assay revealed that RAPEL overexpression suppressed the plaque-forming activity of the IAV strain A/Puerto Rico/8/34 (H1N1; PR8) in 293T cells (Fig. S2C, D). An essentially similar inhibitory effect of RAPEL on replication of another IAV strain A/Aichi/2/68 (H3N2; Aichi) was observed (Fig. S2E). Next, to further examine the effect of RAPEL expression on the infection in other cell lines, we utilized immunofluorescence-based infection assay. This assay allows for visualization of single-round replication of IAV at a single cell level (Fujioka et al., 2018), and the percentage of nucleoprotein (NP)-positive cells is well correlated with the titer of infected viruses (Fig. S2F, compare with Fig. S2C) (Fujioka et al., 2018). It was thereby revealed that IAV infection was inhibited by RAPEL expression in A549, Cos-1, and 293T cells, in addition to MDCK cells (Fig. 2E, F). Given that recruitment of the Ras-PI3K complex to endosomes plays a role in IAV internalization (Fujioka et al., 2011), RAPEL is thought to hamper influenza virus infection as an inhibitor of the virus internalization step. Indeed, RAPEL overexpression also inhibited virus internalization (Fig. S2G). Moreover, endosomal localization of the Ras complex with wild-type PI3K RBD was inhibited in RAPEL-expressing cells, as visualized by BiFC (Fig. 2G, H). Notably, RAPEL expression did not affect EGF-induced Ras activation (Fig. S3A), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) phosphorylation, or Akt phosphorylation (Fig. S3B–D). Therefore, RAPEL might specifically impede Ras-PI3K localization and inhibit endocytosis without affecting Ras or its downstream signaling emitted at the plasma membrane.

**Determination of the minimal essential and critical residues in RAPEL**

We next aimed to minimize the peptide length of RAPEL aiming at its facile synthesis and thus prepared a variety of RAPEL truncation mutants (Fig. 3A). Expression of peptides with an 8-amino acid deletion at the N-terminus (CT20)
Fig. 2. RAPEL expression inhibited endocytosis and IAV infection. (A–D) MDCK, A549, Cos-1, and 293T cells were transfected with expression vectors for CFP (Control) or CFP-RAPEL. Twenty-four hours after transfection, the cells were incubated with either Alexa Fluor 546-labeled dextran (10 kDa) for 10 min (A, B) or transferrin for 30 min (C, D) at 37°C and then washed with acidic buffer and PBS. Representative images of MDCK cells are shown (A, C). Bar, 10 μm. Total fluorescence intensity within cells was quantitated and plotted (B, D). Data are presented as the mean±s.e.m. (n≥30 from three independent experiments). *P<0.0001 versus control as calculated by Student’s t-tests. (E, F) MDCK, A549, Cos-1, and 293T cells were transfected with expression vectors for CFP or CFP-RAPEL. Twenty-four hours after transfection, the cells were infected with PR8 at an MOI of 1 PFU per cell for 4 hours and then subjected to an immunofluorescence-based virus infection assay. Representative images of MDCK cells are shown (E). Bar, 10 μm. Total fluorescence intensities within cells were quantitated for at least 55 cells in each of three independent experiments and plotted in box-and-whisker plots (F). *P<0.0001 versus control as calculated by Student’s t-tests. (G, H) Cos-1 cells were transfected with expression vectors for CFP or CFP-RAPEL together with those for TagRFP-EEA-1, VN-H-Ras, and wild-type PI3K RBD-VC. Twenty-four hours after transfection, the cells were serum-starved for 4 hours and subjected to time-lapse confocal microscopy. At time 0, the cells were exposed to 100 ng/ml EGF. Representative images at 30 min after EGF stimulation are shown (G). Bar, 10 μm. The extents of colocalization of the Ras-PI3K complexes with EEA-1 were quantified as described in the Materials and Methods and plotted over time (H). Data are presented as the mean±s.e.m. (n≥15 from three independent experiments). P<0.0004 as calculated by MANOVA.
inhibited virus infection to a similar extent as full-length RAPEL (Fig. 3B, C), whereas expression of 8 N-terminal amino acids and 9 C-terminal amino acids (NT8 and CT9) failed to do so, indicating that the middle 11 amino acids (M11) are required for RAPEL function. In fact, M11 expression inhibited virus infection to an extent comparable to that of full-length RAPEL (Fig. 3B, C).

We further aimed to identify amino acids that are critical for the inhibitory effect of RAPEL on endocytosis and virus infection. The middle region of RAPEL (M11) consists of 7 nonpolar amino acids, two basic amino acids (lysine), and two polar amino acids (asparagine) (Fig. 4A). In addition, one of the two lysine residues is conserved among p110α, p110β, and p110γ (see Fig. 1B). Therefore, we decided to substitute this lysine with the acidic amino acid glutamate (Fig. 4A). All mutant RAPEL peptides (M11-EK, M11-KE, and M11-EE) in which either or both lysine residues were substituted by glutamate partially lost the ability to inhibit influenza virus infection (Fig. 4B and Fig. S4A). These RAPEL M11 mutants also failed to inhibit clathrin-independent endocytosis (Fig. 4C and Fig. S4B) and endosomal localization of the Ras-PI3K complex (Fig. 4D and Fig. S4C). These results indicated that lysine residues within RAPEL are critical for endosomal localization of the Ras-PI3K complex and the subsequent regulation of clathrin-independent endocytosis. Therefore, substituting the lysine residues with glutamate resulted in a decreased ability to inhibit endocytosis and influenza virus infection.

To further examine whether RAPEL sequence and the lysine residues contained therein are required for IAV internalization, we performed rescue experiments, in which wild-type PI3K or its mutants were introduced into embryonic fibroblasts from mice deficient in PIK3CG, the gene encoding the catalytic subunit of PI3K γ (p110γ) (Fujioka et al., 2011; Sasaki et al., 2000). Constructed PI3K mutants include PI3K in which either or both lysine residues were replaced with asparagine (PI3K-EK, PI3K-KE, and PI3K-EE), in addition to PI3K lacking RAPEL sequence (PI3K-ΔRAPEL). Whereas wild-type PI3K restored IAV internalization in PI3K-knockout mouse embryonic fibroblasts, which is consistent with our previous report (Fujioka et al., 2011), the mutants failed to do so (Fig. S5). These
results indicated that RAPEL sequence, particularly the lysine residues in RAPEL, is involved in the regulation of IAV infection at the entry step.

**RAPEL as a peptide therapeutic**

We next examined whether RAPEL can be applied as a peptide therapeutic using cell-penetrating peptides (CPPs). The synthetic peptides consisted of 11 arginine residues (R$_{11}$) presented as CPP, RAPEL-M11 (either wild type or EE), and an extra lysine residue for the covalent conjugation of a fluorescent dye (fluorescein-5-isothiocyanate, FITC) from the N-terminus (Fig. 5A). Treatment of cells with these peptides resulted in cytoplasmic delivery and accumulation to the perinuclear region to some extent, as visualized by fluorescence microscopy (Fig. 5B, D, and Fig. S6A). When MDCK cells were treated with R$_{11}$-RAPEL-M11, IAV infection was modestly inhibited, as detected by NP staining (Fig. 5B, C). Similarly, R$_{11}$-RAPEL-M11 also inhibited IAV infection in 293T cells significantly (Fig. 5D, E). In contrast, R$_{11}$-RAPEL-M11-EE did not significantly impact IAV infection in MDCK cells (Fig. 5B, C). This EE mutant inhibited the infection to some extent in 293T cells; however, this inhibitory effect was significantly less effective than that of wild-type R$_{11}$-RAPEL-M11 (Fig. 5D, E). Essentially similar results were obtained when we evaluated the effect of RAPEL peptides on the titer of infectious virus particles by plaque assay (Fig. S6B and C).

We further investigated the effect of RAPEL peptide on IAV infection in an ex vivo model with the use of the human respiratory epithelial cell line BEAS-2B. When cultured in Matrigel, these cells form a monolayer that mimics bronchial epithelium (Fujioka et al., 2018; Shen et al., 2018).

Fig. 4. Lysine residues in RAPEL were crucial for its function. (A) Amino acid sequences of RAPEL-M11 and RAPEL-M11 mutants in which one or two lysine residues were replaced with glutamate. (B–D) Cos-1 cells were transfected with expression vectors for CFP (Ctrl), CFP-RAPEL-M11, or CFP-RAPEL-M11 mutant as indicated. In (D), the cells were further transfected with expression vectors for VN-H-Ras and wild-type PI3K RBD-VC. After 24 hours, the cells were exposed to PR8 at an MOI of 1 PFU per cell for 4 hours (B), to Alexa Fluor 546-conjugated dextran for 10 min (C), or to 100 ng/ml EGF for 50 min (D). The cells were then subjected to infection (B), endocytosis (C), and BiFC (D) assays. The fluorescence intensities of NP (B) and dextran (C) were quantitated and plotted. *$P<0.002$ and **$P<0.0001$ as calculated by one-way ANOVA with post-hoc Tukey HSD test. In (D), intracellular granules formed by the BiFC signal with EEA-1 were quantified as described in the Materials and Methods and plotted over time. Data are presented as the mean±s.e.m. ($n\geq10$ from three independent experiments). $P<0.0001$ between control and M11; $P=0.842$ between control and EE; $P=0.0059$ between control and KE; $P=0.0455$ between control and EE; $P=0.0006$ between M11 and KE, as calculated by MANOVA and Bonferroni correction. See Fig. S4 for imaging data used to obtain the quantitative data shown here.
Fig. 5. Introduction of RAPEL as a synthetic peptide inhibited IAV infection. (A) Amino acid sequences of the synthetic peptides R11-RAPEL-M11 and R11-RAPEL-M11-EE. FITC, fluorescein isothiocyanate. (B–E) MDCK (B, C) and 293T (D, E) cells were grown to 80% confluency, treated with 100 μM R11-RAPEL-M11 or R11-RAPEL-M11-EE or left untreated for 1 hour, and after washing, further incubated for 24 hours. The cells were then infected with PR8 at an MOI of 1 PFU per cell for 4 hours and subjected to an immunofluorescence-based infection assay. Representative images are shown (B, D). Bar, 10 μm. The fluorescence intensities of NP were quantitated for at least 180 cells for MDCK and 1,400 cells for 293T in each of three independent experiments and plotted in box-and-whisker plots (C, E). *P<0.0001 as calculated by one-way ANOVA with post-hoc Tukey HSD test.
2015). Such epithelial monolayers were pretreated with RAPEL peptides and exposed to PR8. Whereas treatment with R₁₁-RAPEL-M11 inhibited IAV infection, that with R₁₁-RAPEL-M11-EE had no significant inhibitory effect on the infection (Fig. S7). Together, these results demonstrate the significance of RAPEL-M11 with CPPs for peptide therapy applications against IAV.

**Participation of PI3P in the regulation of endosomal localization of the Ras-PI3K complex**

Finally, we endeavored to explore the molecular mechanism by which the Ras-PI3K complex is translocated to the endosome via RAPEL sequence. To do this, the effect of inhibitor of the class III PI3K Vps34 (Vps34-IN1) on Ras-PI3K translocation. Vps34 is an enzyme that produces phosphatidylinositol 3-phosphate (PI3P, the most abundant phosphoinositide on the cytoplasmic leaflets of the early endosomal membrane) (Lemmon, 2008). The treatment with Vps34-IN1 partially, but significantly reduced the amount of the Ras-PI3K complex in the endosome, as visualized by BiFC (Fig. 6). Under this condition, the fluorescence signal in endosomes of RFP-tagged EEA-1, which is recruited to the endosomes through interaction with PI3P (Law et al., 2017), was dramatically dispersed by the treatment. In contrast, CFP-tagged Rab5, which is deactivated and dispersed by PI3P production, accumulated in the endosome, confirming PI3P depletion on the endosomal membrane. Taken together, PI3P might be involved, albeit in part, in RAPEL-dependent localization of the Ras-PI3K complex in the endosome.

**Discussion**

In this study, we identified RAPEL as the region of PI3K that is important for endosomal localization of the Ras-PI3K complex. Deletion of this region resulted in an inability of the complex to be retained in the endosome and was instead fast trafficked to the perinuclear region (recycling endosomes). Overexpression of RAPEL inhibited clathrin-independent endocytosis as well as IAV internalization and infection. In total, 11 amino acids were deemed essential for primary RAPEL function, among which the lysine residues appeared to play a role in the dominant-negative effect of RAPEL. IAV infection was inhibited by not only RAPEL expression as a genetically encoded peptide but also by its application in peptide form, demonstrating the significance of RAPEL as a therapeutic agent.

Endocytosis, a fundamental cellular mechanism, is involved in a wide range of pathophysiological phenomena, including substance uptake, receptor downregulation, epithelial polarity, barrier function maintenance, microbial infection, and apoptotic cell phagocytosis (Miller and Krijnse-Locker, 2008). Lipid content, particularly that of phosphatidylinositol, at endosomal membranes is specific for the compartment. The relative abundances of phosphatidylinositol are altered during endosomal maturation, which thereby recruits different proteins to different endosomal components. The most abundant phosphatidylinositol in endosomes is PI3P, which promotes the endosomal localization of EEA-1, a marker for early endosomes. This phosphoinositide also participated in the recruitment of Ras-PI3K complex (Fig. 6); however, it remains controversial whether the enzymatic activity of type I PI3K is indeed required for this phenomenon. The requirement for type I PI3K enzymatic activity in endosomes is supported by reports that switching from phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃) is crucial for the maturation of macropinocytosis and that the subsequent loss of PIP₃ promotes the acquisition of PI3P (Porat-Shliom et al., 2008; Zoncu et al., 2009). On the other hand, the possibility of PI3K functioning as something other than a lipid kinase, such as a scaffold protein, may also play a role therein.
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Future work will be needed to clarify this possibility. Although we demonstrated the requirement for RAPEL in recruiting (retaining) the Ras-PI3K complex to the endosome, the molecular mechanism by which this complex is recruited via RAPEL remains unknown. A possible mechanism is that molecule(s) that binds to RAPEL in the endosome tethers the Ras-PI3K complex to the endosome (Fig. S8). Overexpression of RAPEL might sequester this molecule, thereby inhibiting endocytosis and IAV infection. Such a molecule is not necessarily localized to endosomes in quiescent conditions but is rather transiently translocated in a manner dependent on stimulation. Screening the molecules that bind to RAPEL in the endosomes, would be a useful approach for answering these questions in the future.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

Domech, S.M., Auger, K.R., Chatterjee, S., Burke, T.R., and Shoelson, S.E. 1992. Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphonopeptide. Biochemistry, 31: 9865–9870.

Fujiooka, Y., Tsuda, M., Hattori, T., Sasaki, J., Sasaki, T., Miyazaki, T., and Ohba, Y. 2011. The Ras-PI3K signaling pathway is involved in clathrin-independent endocytosis and the internalization of influenza viruses. PLoS ONE, 6: e16324.

Fujiooka, Y., Tsuda, M., Nanbo, A., Hattori, T., Sasaki, J., Sasaki, T., Miyazaki, T., and Ohba, Y. 2013. A Ca2+-dependent signalling circuit regulates influenza A virus internalization and infection. Nat. Commun., 4: 2763.

Fujiooka, Y., Nishide, S., Ose, T., Suzuki, T., Kato, I., Fukuura, H., Fujiooka, M., Horiuchi, K., Satoh, A.O., Nepal, P., et al. 2018. A Stably-expressed Voltage-Dependent Ca2+ Channel Binds Hemagglutinin and Mediates Influenza A Virus Entry into Mammalian Cells. Cell Host Microbe, 23: 809–818.

Godlee, C. and Kaksonen, M. 2013. Review series: From uncertain beginnings: initiation mechanisms of clathrin-mediated endocytosis. J. Cell Biol., 203: 717–725.

Grimes, M.L., Zhou, J., Beattie, E.C., Yuen, E.C., Hall, D.E., Valletta, J.S., Topp, K.S., LaVail, J.H., Bunnett, N.W., and Mobley, W.C. 1996. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. J. Neurosci., 16: 7950–7964.

Kameyama, S., Horie, M., Kikuchi, T., Omura, T., Tadokoro, A., Takeuchi, T., Nakase, I., Sugiyama, Y., and Futaki, S. 2007. Acid wash in determining cellular uptake of Fab/cell-permeating peptide conjugates. Biopolymers, 88: 98–107.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., and Waterfield, M.D. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu. Rev. Cell Dev. Biol., 17: 615–675.

Law, F., Seo, J.H., Wang, Z., DeLeon, J.L., Bolis, Y., Brown, A., Zong, W.-X., Du, G., and Rocheleau, C.E. 2017. The VPS34 PI3K negatively regulates RAB5 during endosome maturation. J. Cell Sci., 130: 2007–2017.

Lemmon, M.A. 2008. Membrane recognition by phospholipid-binding domains. Nat. Rev. Mol. Cell Biol., 9: 99–111.

Li, L., Wan, T., Wan, M., Liu, B., Cheng, R., and Zhang, R. 2015. The effect of the size of fluorescent dextran on its endocytic pathway. Cell Biol. Int., 39: 531–539.

Maldonado-Baez, L., Williamson, C., and Donaldson, J.G. 2013. Clathrin-independent endocytosis: a cargo-centric view. Exp. Cell Res., 319: 2759–2769.

Marsh, M. and McMahon, H.T. 1999. The structural era of endocytosis. Science, 285: 215–220.

McMahon, H.T. and Boucrot, E. 2011. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol., 12: 517–533.

Merrifield, C.J. and Kaksonen, M. 2014. Endocytic accessory factors and regulation of clathrin-mediated endocytosis. Cold Spring Harb. Perspect. Biol., 6: a016733–a016733.

Miller, S. and Krijnse-Locker, J. 2008. Modification of intracellular membrane structures for virus replication. Nat. Rev Microbiol., 6: 363–374.

Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. 2001. Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. Nature, 411: 1065–1068.

Murphy, J.E., Padilla, B.E., Hasdemir, B., Cottrell, G.S., and Bunnett, N.W. 2009. Endosomes: a legitimate platform for the signaling train. Proc. Natl. Acad. Sci. USA, 106: 17615–17622.

Ohba, Y., Kurokawa, K., and Matsuda, M. 2003. Mechanism of the spatio-temporal regulation of Ras and Rap1. EMBO J., 22: 859–869.

Ong, S.H., Hadari, Y.R.,goto, N., Guy, G.R., Schlessinger, J., and Lax, I. 2001. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc. Natl. Acad. Sci. USA, 98: 6074–6079.

Pawson, T. 2004. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell, 116: 191–203.

Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rupp, U., and Ludwig, S. 2001. Influenza virus propagation is impared by inhibition of the Raf/MEK/ERK signalling cascade. Nat. Cell Biol., 3: 301–305.

Porat-Shliom, N., Kloo, Y., and Donaldson, J. 2008. A Unique Platform for H-Ras Signaling Involving Clathrin-independent Endocytosis. Mol. Biol. Cell, 19: 765–775.

Sasaki, T., Irie-Sasaki, J., Jones, R., Oliveira-dos-Santos, A., Stanford, W., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., and Kozieradzki, I., et al. 2000. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science, 287: 1040–1046.

Shen, Y., Hou, Y., Yao, S., Huang, P., and Yobas, L. 2015. In vitro epithelial organoid generation induced by substrate nanotopography. Sci. Rep., 5: 9293.

Tatsunisi, K., Fujioka, Y., Tsuda, M., Kawaguchi, H., and Ohba, Y. 2009. Visualization of Ras-PI3K interaction in the endosome using BiFC. Cell. Signal., 21: 1672–1679.
Vanhaesebroeck, B., Stephens, L., and Hawkins, P. 2012. PI3K signalling: the path to discovery and understanding. Nat. Rev. Mol. Cell Biol., 13: 195–203.

Whitman, M., Downes, C.P., Keeler, M., Keller, T., and Cantley, L. 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature, 332: 644–646.

Zoncu, R., Perera, R., Balkin, D., Pirruccello, M., Toomre, D., and De Camilli, P. 2009. A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell, 136: 1110–1121.

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