Structural mechanism for regulation of Rab7 by site-specific monoubiquitination

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1. Introduction

Ubiquitination is an extensively studied posttranslational modification regulating various cellular processes, including immune response, protein degradation, intracellular signaling, and membrane trafficking [1–4]. The C-terminal glycine residue of ubiquitin is conjugated to primary amines of target proteins via isopeptide bond formation by the cascade action of three types of enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) [5,6]. Through this enzymatic machinery, ubiquitin is attached to the substrate in the form of monomer (mono- or multi-ubiquitination) or polymer (polyubiquitination) [7,8].

Rab GTPases are important regulators of intracellular membrane trafficking pathway and constitute the largest family of small GTPases [9]. Switching between the active and inactive states of Rab GTPases is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [10,11]. Rab5 is the core protein that is recruited to form early endosomes. GDP-Rab5 is activated by Rabex-5 and GTP-Rab5 is recruited to early endosome, exerting its effects. GAP hydrolyzed GTP to GDP on Rab5 for new cycle [12]. Conversion of Rab5A to Rab7 leads to the maturation of early to late endosomes [13–15]. Rab7 is another Rab GTPases that regulate intracellular membrane trafficking. Rab7 regulates the maturation of early endosome to late endosomes which turn into lysosomes or multi-vesicular bodies (MVB) destined for degradation of cargo proteins. For example, Rab7 is involved in regulating endo-lysosomal trafficking of the membrane-bound receptors such as epidermal growth factor (EGF)-EGF receptor (EGFR) complex [16,17]. Rab7 on endosomal membrane recruits
various effector protein to promote ongoing trafficking process. These effector proteins include Rab7-interacting protein (RILP) which is used for anterograde vesicle transport [18]. Rab7 is also known as regulating retrograde transport from late endosome to trans-Golgi network (TGN) and plasma membrane [19]. Interestingly, Rab7 is involved in viral egression. Beta-coronaviruses reportedly utilize lysosomal pathway for their egression. During the lysosomal egression pathway, ORF3a colocalizes with Rab7 and a competitive inhibitor for Rab7 ameliorates the viral egression [20].

Regulation of activities of Rab GTPases by ubiquitination is now well established. For instance, ubiquitination can activate Rab11a. In lieu of regulation of Rab GTPases by ubiquitination, we previously reported that site-specific monoubiquitination of Rab5A (hereafter referred to as “Rab5”) acts as an inhibitor signal to its activities [21]. This study raises the question whether the regulatory mechanism by ubiquitination of Rab5 is conserved in other GTPases. Rab7A (hereafter referred to as “Rab7”) has been reported to be regulated by site-specific ubiquitination [22,23]. Ubiquitination on K191 can inhibit the recycling from late endosomes to TGN while ubiquitination on K38 can increase affinity for an effector RILP [22,23]. However, no extensive survey of site-specific ubiquitination on Rab7 nor structural basis for ubiquitination-regulated activity of Rab7 has been elucidated. Here we comprehensively survey ubiquitination sites of Rab7A to uncover structural basis for regulation of Rab7 by site-specific ubiquitination. Structural ensemble analysis derived from small-angle X-ray scattering and molecular dynamics simulation, complemented by biochemical and cellular assays demonstrated that site-specific monoubiquitination interferes with the recruitment of Rab7 to endosomal membrane, resulting the deficiency on endosomal trafficking shown as immunofluorescence studies and EGFR degradation.

2. Materials and methods

2.1. Plasmids and cell culture

The Rab7 gene was chemically synthesized (Bioneer, Korea), and cloned into parallel-His, a bacterial expression vector, and pcDNA-FLAG, a mammalian expression vector. The ORF3a-EGFP was purchased from Addgene (RRID: Addgene_165121). Human HEK293FT and HeLa cells were purchased from American Type Culture Collection (RRID: CVCL_0063 and CVCL_0030, respectively). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (heat-inactivated), streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37 °C in 5% CO₂ atmosphere. Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) for HeLa cells or PEI 25000 (Polysciences, 23966) for HEK293FT cells. The ratio of a transfection reagent to DNA was 1:2 or 1:3 for Lipofectamine-mediated transfection and 1:4 for PEI-mediated transfection, respectively. Lipofectamine-mediated transfection was performed following the manufacturer’s protocol. For PEI-mediated transfection, PEI was added to serum-free DMEM with plasmids. Solutions were vortexed thoroughly, incubated at room temperature for 20 min, and subsequently added to the plate with cells. No mycoplasma contamination was detected by mycoplasma detection kit (Lonza, LT07-318).

2.2. Protein expression and purification

Hexahistidine-tagged Rab7 (wild type and K to R mutants) were expressed in E. coli BL21 (DE3) cells. Cells were inoculated and grown to an OD₆₀₀ of 0.6–0.8 at 37 °C. To induce protein expression, 0.5 mM isopropyl β-D-thiogalactoside (IPTG) was added to the cultures, and they were further incubated at 20 °C overnight. Induced cells were harvested by centrifugation at 4000 rpm. Cells were resuspended in buffer A (50 mM Tris-HCl pH 7.5 and 150 mM NaCl). Resuspended cells were lysed by sonication and centrifuged at 13,000 rpm. The supernatant was applied to Ni-NTA-agarose resin (Qiagen) for His-tagged proteins. Non-specific proteins were washed with buffer B (50 mM Tris-HCl pH 7.5, 500 M NaCl, and 20 mM imidazole) for His-tagged proteins. To elute target proteins, buffer C (50 mM Tris-HCl pH 7.5, 300 mM imidazole, and 500 mM NaCl) was used for His-tagged proteins. The His-tag were cleaved from the fusion proteins using GFP-tagged tobacco etch virus protease in cleavage buffer (25 mM Tris-HCl pH 7.5, 75 mM NaCl, and 0.5 mM EDTA) [24]. Cleaved samples were applied to Ni-NTA-agarose to remove the released His-tags. For Ub G76C, cells were inoculated and grown to an OD₆₀₀ of 0.8–1.0 at 37 °C and further incubated at 37 °C for 4 h. Harvested cells were resuspended in buffer A and lysed by sonication and centrifuged at 2000g for 30 min. Supernatants were filtered with 0.4 μm syringe filter and were added 35% perchloric acid until pH is 4–4.5. After supernatants turned to milky solutions, milky solutions were subjected to centrifuge 20,000g for 40 min. Supernatants were dialyzed in 0.5 M ammonium acetate (pH 4.5) for overnight. Proteins were further purified by size exclusion chromatography on a Superdex 75 or 200 prep grade 16/60 column (GE HealthCare). Fractions were analyzed by SDS-PAGE, and samples were pooled and concentrated by centrifugal concentrators (Amicon Ultra 3, 10, or 30 kDa, Millipore). Final concentration was determined by either absorbance at 280 nm or Bradford assay. For Ub G76C and His-Rab7 K-to-C mutants, 2 mM TCEP was added during the purification procedure.

2.3. Ubiquitination assay

Ubiquitination assay was performed based on previously described methods [25,26]. Briefly, HEK293FT and HeLa cells were harvested in cold PBS at 24 h after transfection with indicated plasmids, and then lysed with lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl pH 8.0, 2 mM sodium ortho-vanadate, 50 mM sodium fluoride, and a protease inhibitor cocktail from Roche). The lysates were boiled for 10 min at 95 °C and then sonicated. After sonication, sample were diluted with dilution buffer (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA, and 1% Triton X-100) and centrifuged at 16,200g for 10 min. After pre-clearing using normal mouse IgG and protein G-beads, the supernatants were incubated with an anti-FLAG antibody for 12 h at 4 °C and precipitated with protein G for 1 h at 4 °C. The beads were washed with wash buffer (10 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, and 1% NP-40) three times, and then boiled with SDS loading buffer.

2.4. Subcellular fractionation assay

Subcellular fractionation assay was carried out according a published method [27]. Briefly, 3 μg of pcDNA-Flag-Rab7 and 5 μg of pC33-HA-Ub were transfected to HEK293FT cells. The cells were harvested and washed with ice-cold PBS. 400 μl of buffer A (150 mM NaCl, 50 mM HEPES pH 7.4, 1 M hexylene glycol and 100 μg/ml digitonin) supplemented with 1× protease inhibitor cocktail (P8340, Sigma) was added to the cell. The buffer-resuspended cells were incubated on an end-over-end rotator at 4 °C for 15 min, and then centrifuged at 2000g for 10 min. Collected was the supernatant 1 which contained cytosolic proteins. Subsequently, 400 μl of buffer B (150 mM NaCl, 50 mM HEPES pH 7.4, 1% (v/v) Nonidet-P40, and 1 M hexylene glycol) supplemented with 1× protease inhibitor cocktail (P8430, Sigma) was added to the pellet. The pellet was dismantled on a vortex mixer. After 30 min incubation on ice, the buffer-resuspended pellet was centrifuged at 7000g for 10 min. Collected was the supernatant 2 which contained membrane proteins. Both cytosolic and membrane fractions were further immunoprecipitated and analyzed by immunoblotting.

2.5. Chemical ubiquitination of Rab7

Chemical synthesis of a monoubiquitinated Rab7 (mUbRab7) was carried out according a published method [21,28–30]. Production of mUbRab7 was confirmed using SDS-PAGE under non-reducing conditions.
2.6. Small angle X-ray scattering (SAXS) data collection and processing

SAXS data from proteins in solution were collected at 4C beamline of Pohang Accelerator Laboratory (PAL) in Korea with the sample-detector distance set at 3 m and 1 m [31]. Protein concentrations were determined by measuring absorbance at 280 nm. Each sample was measured ten times and radiation damage was checked immediately. Concentration dependence of the sample was confirmed using serially diluted samples. Data were normalized against concentration and processed using an in-house program and PRIMUS. (0) values from Guinier plot were calculated using AUTORG. Protein flexibility was assessed by Kratky analysis. Molecular ensemble models were generated by EOM. EOM analysis was conducted according a published method [21,32]. In brief, we generated the sequences and atomic coordinates of the mUbRab7s in the following steps. We firstly used sequence started with the ubiquitin sequence followed by the partial Rab7 sequence for the sample. Data were normalized against concentration and processed in the same way with the preparation of the input sequence. Structure models from PDB were used for domain 1 and domain 2 (PDB: 1VGB, 1UBQ). The model for rat Rab7 (PDB: 1VG8) was employed because this model contained residues up to residue 190 while all the models for human Rab7 (PDB: 1TP1, 1YHN, 3LAW, 6IYB and 6WCW) covered at most to residue number 182, far from the major monoubiquitination sites unequivocally identified in this study (residue numbers 191 and 194).

2.7. Molecular dynamics simulation

2.7.1. System preparation

All systems were built using MODELLER 10.0 [33]. Amino acid sequences of Rab7 and ubiquitin were obtained from UniProt database [34] (entry numbers: P51149 and POCG48, respectively). The previously published Rab7 structures (PDB: 1VGB and 1VGA) and ubiquitin structures from the EOM models (Section 2.6) were used as template for model building for molecular dynamics (MD) simulation. Alignment was performed by python codes with Pymol library. During the full sequence homologous modeling, the specific lysine residues (K38, K175, K191 and K194) of Rab7 and the C-terminal glycine residue of ubiquitin were mutated to cysteine, yielding ‘K-to-C’ Rab7 mutants and G76C ubiquitin mutant, respectively. The isopeptide bond in a site-specifically ubiquitinated Rab7 was substituted by a disulfide bond between a Rab7 K-to-C and ubiquitin G76C mutants. The cysteine residues of a Rab7 K-to-C and ubiquitin G76C were applied to the disulfide bond and its loop refinement was performed with MODELLER. For each step, the quality of the models was estimated with the discrete optimized protein energy (DOPE) score [35] and the model with the lowest DOPE score was chosen. Membrane buildings and protein geranyl-geranylation (GG) on C205 and C207 of the modelled Rab7 were performed using CHARMM-GUI [36]. The compositions of membrane were emulated as described in a reported study [37]. For insertion of the geranylgeranylated moiety to Rab7, 2 palmitoyloleoylphosphatidylcholine (POPC) molecules were eliminated in the protein side of the model membrane. Salt concentrations of all systems were set at 150 mM.

2.7.2. Minimization, equilibration and production

All simulations were performed in AMBER20 MD simulation package [38], CHARMM36m force field [39] was used. All initial inputs of the systems were generated by CHARMM-GUI input generator [40]. Hydrogen atoms were constrained by SHAKE algorithm [41,42]. All systems were solvated by TIP3P water model [43] with periodic boundary condition (PBC). Those systems including 38,930 water molecules were neutralized. Each system was minimized with 2500 steepest descent and maximum 2500 conjugate gradient minimization steps. Sequentially, each system was heated to 310 K, equilibrated for 10 ns and produced for 90 ns. Temperature was regulated by Langevin thermostat with 1.0 ps collision frequency. Nonbonded long-range interaction was regulated by particle-mesh Ewald (PME) method [44] with 12 Å cutoff distance. To use ionic strength effects, salt concentration was set at 150 mM based on Debye-Hückel screening [45].

2.7.3. MD trajectories analysis.

All trajectories were processed and analyzed using CPPTRAJ [46] provided by AMBER20 package [38]. The trajectories were aligned to the first frame for catalytic ligand-binding domain of Rab7 (G domain, residues 1–174). Root mean square deviation (RMSD) was calculated using the backbone atoms of the Rab7 G domain without hydrogen atoms. Each protein distance was calculated as the distance between each center of mass of the Rab7 G domain and ubiquitin without proton and the membrane plane which comprises the phosphorus atoms of protein-side membrane layer. The insertion depth of GG anchors, geranyl-geranylated cysteines (C205 and C207 of Rab7) was calculated as the distance between the center of mass of the GG anchors and the membrane plane. All visualizations of the MD trajectory analysis were performed by NumPy [47] and Matplotlib [48] modules in Python 3.6.

2.8. Immunofluorescence

Transfected HeLa cells grown on coverslips were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature and the rinsed in PBS, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 2% BSA in PBS for 30 min at room temperature. Cells were immune-stained with primary antibodies in PBS with 2% BSA for 2 h at room temperature, then rinsed with PBS and incubated for 30 min with fluorescence-tagged secondary antibodies in PBS with 2% BSA. Images were obtained using a confocal microscope (Zeiss, LSM 700). Anti-Flag (Sigma, F1804), anti-HA (Roche, 1186743001), anti-GFP (Invitrogen, A11122), and anti-LAMP-2 (Abcam, ab13524) were used for primary antibody. We used 1/400 diluted anti-Flag, 1/200 diluted anti-HA, 1/200 diluted anti-GFP, 1/100 diluted anti-Lamp2, and 1/1000 diluted secondary antibodies.

2.9. EGF-induced EGFR degradation assay

FLAG-Rab7 WT, K38R, K157R, and HA-ubiquitin were transfected in HeLa cells. 1 μg of pcDNA-Flag-Rab7 and 1 μg of pCS3-HA-Ub were transfected. After the 24 h of transfection, EGF (20 ng/ml) was treated on the transfected cells. Cells were harvested at indicated time points after transfection. The harvested cells were lysed with RIPA II cell lysis buffer (GeneDepot, R4200-010) supplemented with a protease inhibitor cocktail (GeneDepot, P3100-001) and a phosphatase inhibitor cocktail (GeneDepot, P3200-010), and subjected to immunoblotting with an anti-EGFR antibody (Abcam, ab58284), 1/1000 diluted anti-EGFR antibody was used as primary antibody and 1/5000 mouse anti-rabbit IgG-HRP antibody (Santa Cruz, sc-2357) as secondary antibody. Primary antibody was incubated for overnight in 4 °C and secondary antibody for 2 h at room temperature.

2.10. Immunoprecipitation

HEK293FT cells were harvested in cold PBS at 24 h after transfection with indicated plasmids, and then lysed with lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, and a protease inhibitor cocktail from Roche). The lysates were centrifuged at 16,200g for 10 min and the supernatants were precleared with normal mouse IgG and protein G beads. After pre-clearing, the supernatants were incubated with an anti-flag M2 affinity gel (Millpore, A2220) for 16 h at 4 °C. The beads were washed with wash buffer (10 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM EDTA, and 1% Triton X-100) three times, and then boiled with SDS loading buffer.
3. Results

3.1. Rab7 is predominantly monoubiquitinated in the membrane fraction of cultured cells

In a previous study, we demonstrated that the site-specific ubiquitination of Rab5 plays a negative role in early endosome formation [21]. Although ubiquitination of Rab7 was noted in cultured, no systematic survey of Rab7 ubiquitination is available. To explore systematically how Rab7, acting as a downstream molecule of Rab5 along the endocytic pathway, undergoes ubiquitination and analyzed by western blotting. (c, d) Immunofluorescence assay of Rab7 WT. FLAG-Rab7 WT was co-transfected with or without HA-ubiquitin into HeLa cells. FLAG was stained with Alexa-fluor-488 (green). HA and TMEM192 were stained with rhodamine (red). DNA in the nucleus was stained with DAPI (blue).

Fig. 1. Rab7 is predominantly monoubiquitinated in cells. (a) HEK293FT and HeLa cells were transfected with FLAG-Rab7 in combination with either HA-ubiquitin or empty vector, followed by immunoprecipitation (IP) with the FLAG antibody (α-FLAG). (b) Flag-Rab7 WT, HA-empty vector and HA-ubiquitin was transfected as indicated combination. Cytosol and membrane fractions were subjected to immunoprecipitation and analyzed by western blotting. (c, d) Immunofluorescence assay of Rab7 WT. FLAG-Rab7 WT was co-transfected with or without HA-ubiquitin into HeLa cells. FLAG was stained with Alexa-fluor-488 (green). HA and TMEM192 were stained with rhodamine (red). DNA in the nucleus was stained with DAPI (blue).

endosomal puncta. Using immunofluorescence assay, we investigated whether ubiquitination of Rab7 also affects Rab7-positive intracellular structure formation. First, we ensured that overexpression of Rab7 in HeLa cells did not cause any unusual artifact upon imaging (Supplementary Fig. S1). Rab7 WT overexpression featured round-shaped intracellular structures that were reduced when HA-ubiquitin was co-transfected (Fig. 1c). To corroborate our findings, we investigated co-localization of Rab7 with transmembrane protein 192 (TMEM-192), an abundant membrane glycoprotein, which is ubiquitously expressed in late endosome and lysosomes [49]. Investigation of the cellular distribution of Rab7 and TMEM-192 upon transfection of HA-ubiquitin

Fig. 2. Identification of monoubiquitination sites on Rab7. (a) Ubiquitination assay of selected single K-to-R mutants (K21R and K126R) and multiple K-to-R mutants (6KR and 15KR) of Rab7. The 6KR mutant (K32R/K38R/K48R/K137R/K175R/K191R) contains mutations suggested to be ubiquitinated by proteomics studies. The 15KR mutant has 15 out of 17 lysine residues on Rab7 mutated to arginine residues: K5R/K6R/K10R/K31R/K32R/K38R/K48R/K97R/K137R/K146R/K157R/K175R/K191R/K194R/K199R. (b) Ubiquitination assay with single R to K mutants in the background of 15KR. 15KR mutant was used as a negative control. (c) Quantitation of the monoubiquitinated Rab7: total Rab7 ratio, represented by a bar graph. Data are presented as mean ± standard deviation from three replicates (*p < 0.1, **p < 0.01, ***p < 0.001, by one-way ANOVA). (d) Flag-Rab7 WT, K-to-R mutants, HA-empty vector and HA-ubiquitin was transfected as indicated combination. Cytosol and membrane fractions were subjected to immunoprecipitation and analyzed by western blotting. (e) Quantitation of the monoubiquitinated Rab7 in membrane fractions represented by a bar graph. Data are presented as mean ± standard deviation from three replicates (**p < 0.01, by one-way ANOVA). (f) Quantitation of the Rab7 in the membrane represented by a bar graph. Data are presented as mean ± standard deviation from three replicates (**p < 0.01, by one-way ANOVA).
showed that the cellular distributions of TMEM-192 are similar with those of Rab7 (Fig. 1d), suggesting that the cellular structures observed by Rab7 WT would be late endosomes or lysosomes.

3.2. Comprehensive survey of monoubiquitination sites on Rab7 reveals K191 and K194 as the major ones

Rab7 functions in the downstream of Rab5 in the maturation of endosomes and both Rab5A and Rab7 belong to Rab small GTPase family [17]. These commonalities between Rab7 and Rab5 allowed us to hypothesize that similarity in ubiquitination site between Rab5A and Rab7 might exist. Pairwise sequence alignment of Rab7 with Rab5A revealed that only two lysine residues (K157 and K175 in Rab7) are conserved (Supplementary Fig. S2a). Among the three ubiquitination sites on Rab5A discovered in the previous study [21], K116 and K140 of Rab5A correspond to D104 and D132 in Rab7, respectively. Only K157 is conserved in Rab7 (corresponding to K165 of Rab5A). K175 of Rab7 corresponds to K183 in Rab5A, a polyubiquitination site identified with 11KR-single K mutant of Rab5A. Some non-conserved ubiquitination sites on Rab7 are negatively charged residues: K38/Rab7 to E50/Rab5A and K116/Rab5A to D104/Rab7. These data implie that the ubiquitination sites are likely to be specific to each Rab GTPase despite Rab GTPases being monoubiquitinated. To comprehensively identify monoubiquitination sites on Rab7, we mutated each of 17 lysine residues to arginine. Ubiquitination assay using single K-to-R mutants of Rab7 showed no substantial decrease in band intensities among the single K-to-R mutants (Supplementary Fig. S2b, c). Taken together, these results suggested that Rab7 has multiple ubiquitination sites.

Since we could not unequivocally find potent residues of monoubiquitination with K-to-R mutants, we prepared a Rab7 15KR mutant in which 15 out of 17 lysine residues was mutated to arginine, leaving K21 and K126 intact. The lysine residues K21 and K126, corresponding to K33 and K134 of Rab5A, are crucial for contact with the guanine nucleotides. Proteomic studies, as compiled in mUbiSiDa [50], revealed that at least six ubiquitination sites (K32, K38, K48, K137, K175, and K191) out of the total 17 lysine residues on Rab7. To test potential effect of these sites, we also prepared a 6KR mutant (K32R/K38R/K48R/K137R/K175R/K191R). We performed ubiquitination assay using the 6KR and 15KR mutants to check whether monoubiquitination of the Rab7 mutants was impaired. Monoubiquitinated Rab7 band completely disappeared in the case of the 15KR mutant, but K21R, K126R and the 6KR mutants showed weakly monoubiquitinated Rab7 bands (Fig. 2a). Given the clear disappearance of the monoubiquitinated band by the 15 KR mutant, we mutated each arginine of the 15KR mutant back to lysine, single R-to-K mutants, to identify which lysine residue(s) was major monoubiquitination site(s). When Rab7 monoubiquitination level was normalized by the expression level of Rab7, R191K and R194K showed marked increase in the level of monoubiquitinated Rab7. When comparing with Rab7 15KR, the monoubiquitinated Rab7 levels of R175K, R191K and R194K were statistically significant (Fig. 2b, c). These results implicate that the other mutants are unlikely to be major ubiquitination sites. By contrast, three mutants (R175K, R191K and R194K) showed restoration of the monoubiquitination level to that of 15KR, suggesting that these sites are candidates for major monoubiquitination sites. Among these three R-to-K mutants, the R191K mutant showed highest degree of restoration in monoubiquitination, followed by the R194K and R175K mutants (Fig. 2b, c). Next, we conducted fractionation assay to investigate whether monoubiquitination of Rab7 occurs in a localization-dependent manner. To check the relationship between enzymatic activity of Rab7 and monoubiquitination in the membrane, we transfected cells with a FLAG-Rab7 (WT), a dominant-active mutant (Q67L), or a dominant-negative mutant (T22N) and HA-ubiquitin [51]. β-Actin and E-cadherin were used as a marker for the cytosol and the membrane fraction, respectively [52]. mUbRab7ΔQ67L was recruited to the membrane fraction than mUbRab7ΔT22N (Supplementary Fig. S3). This observation implicates that functionally active form of Rab7 is likely to be monoubiquitinated in the membrane fraction when overexpressed in cells. We employed K191R, K194R, and 2KR mutants for fractionation assay because it was suggested that K191 and K194 are the target sites for deubiquitination in late endosomes by USP32 [22]. The 2KR double mutant of Rab7 showed a mono- ubiquitinated Rab7 band with reduced intensity in the membrane fraction in comparison to WT while either K191R or K194R single mutant did not (Fig. 2d, e). We also checked whether monoubiquitination on K38 and K175 affect the localization of Rab7 to the membrane using the fractionation assay. Rab7 K38R and K175R mutant showed no significant change in intensities of mUbRab7 and Rab7 bands in the membrane fractions (Supplementary Fig. S4). These results suggest that K191 and K194 are the major monoubiquitinated residues of membrane-localized Rab7. Since we observed the reduced level of the monoubiquitinated 2KR mutant, we investigated the changes in the overall expression level of the 2KR mutant in the membrane fraction. The total expression level of the 2KR mutant in the membrane fraction was increased in comparison to those of WT, K191R and K194R, implicating that mono- ubiquitination on K191 and K194 interrupt the membrane localization of Rab7 (Fig. 2d, f).

In all the ubiquitination assays, we noticed the polyubiquitination of Rab7 to some extent. In case of some Rab7 mutants, polyubiquitination of Rab7 markedly increased. To further investigate the bands apparently corresponding to polyubiquitinated Rab7, we repeated the ubiquitination assay with HA-Ub_7KR in which all lysine residues on ubiquitin were mutated to arginine, thereby incapable of generating polyubiquitin chains. We noticed that there was still some polyubiquitinated Rab7 remaining (Supplementary Fig. S5a). In fractionation assay with HA-Ub_7KR, we detected high molecular mass bands upon longer exposure (Supplementary Fig. S5b), implicating that HA-Ub_7KR was likely to be conjugated to endogenous polyubiquitin chains. Alternatively, a cell lysis condition in the fractionation assay is different from that in a typical ubiquitination assay, leaving non-covalent bonds intact.

3.3. Solution structural models of mUbRab7s derived by small angle X-ray scattering

To investigate how site-specific monoubiquitination of Rab7 affects its function at molecular level, we derived solution structural models by small-angle X-ray scattering (SAXS) for site-specifically mono- ubiquitinated Rab7 on K38, K175, K191, and K194 (hereafter called as mUbRab7K38, mUbRab7K175, mUbRab7K191 and mUbRab7K194) (Supplementary Table 1). R38K mutant was one of the R-to-K mutants which did not restore the monoubiquitination level significantly while K175, K191 and K194 exhibited statistically significant increases in the monoubiquitination level (Fig. 2b, c). Notably, K38, a non-conserved ubiquitination site, is structurally important in releasing GDP. The GDP release mechanism of Ypt7, yeast orthologue of Rab7, involving K38 was elucidated from analysis of Ypt7:Mon1-Ccz1 complex structure [53]. Site-specific mUbRab7s were prepared by formation of a disulfide bond between cysteine of lysine-to-cysteine mutants Rab7 and G76C of ubiquitin in place of the isopeptide bond by iterative addition of ubiquitin [50]. To prevent the formation of a non-specific disulfide bond, we mutated naturally occurring cysteine residues in Rab7 to either serine (C143S) or alanine (C83A and C84A) and deleted C-terminal residues (C205, S206, and C207) involved in membrane association. Kratky plots from SAXS measurements of all four mUbRab7s revealed that mUbRab7s were flexible in solution (Supplementary Fig. S6). To define conformational flexibility of mUbRab7s at molecular level, we opted to employ ensemble-optimized method (EOM) using models derived from the SAXS data [32]. EOM is designed for defining a multi-domain protein with flexible linkers [32]. Input files for EOM are single linear protein sequence, atomic coordinates for each domain, and SAXS data [32]. We prepared the sequences and atomic coordinates of mUbRab7s as described in the Materials and methods. Firstly, a pool of 10,000 or 50,000 models are generated based on the sequence and
structural information from a SAXS curve by RANCH (embedded in EOM, ATSAS package). Secondly, a genetic algorithm for the selection of models was performed by GAJOE with 100 iterations (embedded in EOM, ATSAS package). Finally, the best ensemble with the lowest $\chi^2$ was selected.

Ensemble models of mUbRab7$_{K191}$ and mUbRab7$_{K194}$ revealed that they have more flexibility than mUbRab7$_{K38}$ and mUbRab7$_{K175}$. mUbRab7$_{K38}$ exhibited single population with radius-of-gyration ($R_g$) of 24 Å and maximum distance ($D_{max}$) of 79 Å (Fig. 3a, e). mUbRab7$_{K175}$ also showed almost single population, with two populations being close to each other (Fig. 3b, f). By contrast, mUbRab7$_{K191}$ has two distinct populations of conformational ensemble, with $R_g$ of 24 and 32 Å and $D_{max}$ of 76 and 102 Å, respectively (Fig. 3c, h). Two conformations were derived for mUbRab7$_{K191}$ and three conformations were derived for mUbRab7$_{K194}$, but these conformations were converged to specific orientations of the ubiquitin moiety (Fig. 3i, j), consistent with largely single populations in analyses of $R_g$ and $D_{max}$. However, mUbRab7$_{K191}$ and mUbRab7$_{K194}$ revealed distinct orientations of the ubiquitin moiety, indicating that the ubiquitin moieties on K191 and K194 are more flexible than those on K38 and K175 (Fig. 3k, l). K191 and K194 are located in the very flexible loop of C-terminus Rab7 which is geranyl-geranylated and anchored in the endosomal membrane. Considering juxtaposition of the C-terminal loop to the endosomal membrane, structural analyses of the EOM models of mUbRab7$_{K191}$ and mUbRab7$_{K194}$ corroborate that ubiquitination on K191 and K194 would interfere with the localization of Rab7 on the endosomal membrane.

Consistent with the aforementioned structural insights, the 2KR (K191R/K194R) mutant showed an increased level of Rab7 in the membrane (Fig. 2d, f).

3.4. Molecular dynamic simulation studies of site-specific mUbRab7 models

To study the interaction between Rab7 and the endosomal membrane, we performed MD simulation similarly as in a previous MD study [37]. We built total ten ensemble models selected from the SAXS EOM models of four monoubiquitinated Rab7 (mUbRab7$_{K38}$, mUbRab7$_{K175}$, mUbRab7$_{K191}$, and mUbRab7$_{K194}$) accounting for ten conformations with the highest EOM fractions, implemented in a model endosomal membrane whose composition was taken from the previous MD study [37]. Subsequently we performed 90 ns conventional molecular dynamics simulation at 310 K for each system after the 10 ns equilibration, and total simulation time was one microsecond. The root mean standard deviation values (RMSDs) of the backbone of Rab7 were saturated in MD simulations after 30 ns, suggesting the system was equilibrated (Fig. 4a). We selected last 60 ns data after 30 ns for the further calculation. To investigate the interaction between Rab7 and the endosomal membrane, the distances of Rab7 G domain (residues 1–174) and ubiquitin from the membrane plane were calculated (Fig. 4b). The distribution of Rab7-membrane distances weighted with the fractions of EOM models showed that the Rab7 moieties of mUbRab7$_{K38}$ and mUbRab7$_{K175}$ were close to the membrane and that those of mUbRab7$_{K191}$ and mUbRab7$_{K194}$ were far from the membrane (Fig. 4b). These results implicate that the ubiquitin moieties of some mUbRab7$_{K191}$ and
and ubiquitin were co-transfected compared to when only Rab7 WT was transfected (Fig. 5b). By contrast, transfection of the Rab7 mutants with ubiquitin led to the increase in the proportion of the enlarged endosomes compared to transfection of the Rab7 WT with ubiquitin (Fig. 5b). The proportion of the enlarged puncta by Rab7 K191R mutant increased by ~4-fold in comparison to the numbers by WT and K194R and K191R/ K194R mutants (Fig. 5b). Since enlarged endosomes are observed when Rab7 is hyperactivated [54], the K191R mutant may represent a hyperactive state of Rab7. Since Rab7 in the membrane is dominantly monoubiquitinated supported by results from the fractionation assay and MD simulation, monoubiquitination on K191 and K194 may differentially contribute to the hyperactive state of Rab7. These results demonstrate that ubiquitination of Rab7 can affect the Rab7 activity on the endosomal membrane.

Rab7 regulates endocytosis of membrane proteins including EGFR [16]. Having established that ubiquitination on K191 of Rab7 exhibited profound effects in the morphology of late endosomes, we investigated whether site-specific ubiquitination of Rab7 has effects on the trafficking of EGFR. In HeLa cells, EGFR degraded 90 min after EGF treatment. While overexpression of ubiquitin prevented EGF degradation, over-expression of Rab7 alone promoted EGF degradation in HeLa cells. By contrast, overexpression of Rab7 with ubiquitin attenuated EGF degradation (Fig. 5c, d), suggesting that ubiquitination of Rab7 down-regulates the Rab7-mediated EGF endocytic pathway. We also observed EGFR degradation when Rab7 K191R, K194R and K191R/ K194R mutants were transfected with ubiquitin (Fig. 5c, e). Collectively, these results suggested that K191 and K194 of Rab7 are a major monoubiquitination sites, conferring negative impacts on not only late endosomal formation but also endocytosis of membrane proteins such as EGFR.

3.6. Monoubiquitinated Rab7 can prevent the egression of SARS-CoV-2

It has been recently reported that betacoronavirus to which severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) responsible for the current global coronavirus disease 2019 (COVID-2019) pandemic employs lysosomal trafficking pathway for its egression instead of biosynthetic secretory pathway [20]. Interestingly, the egression of mouse hepatitis virus, a β-coronavirus, is inhibited by a Rab7 competitive inhibitor, implicating that SARS-CoV-2 may also utilize lysosomal trafficking pathway for its egression in a Rab7-dependent manner. Open reading frame 3a (ORF3a) from SARS-CoV-2 is a viral calcium-permeable, nonselective cation channel [55]. SARS-CoV-2 ORF3a is localized with Rab7 in late endosomes and lysosomes and inhibits fusion of autophagosomes with lysosomes [56,57]. We postulated that mono-ubiquitinated Rab7 can interfere with SARS-CoV-2 egression. To test our hypothesis, we investigated the colocalization of Rab7 with ORF3a with or without ubiquitin. Firstly, to confirm the previous results, we examine the colocalization of Rab7 and ORF3a with lysosome-associated membrane protein 2 (Lamp-2). Lamp-2, one of abundant membrane glycoproteins, is ubiquitously expressed in late endosome and lysosomes [58]. Rab7 is colocalized with Lamp-2 and ORF3a is also colocalized with Lamp-2 (Fig. 6a). To examine whether Rab7 ubiquitination altered the colocalization of Rab7 and ORF3a, we transfected Flag-Rab7 and GFP-ORF3a together with HA-Ub. Without HA-Ub, Rab7 wild-type and ORF3a were colocalized. However, when HA-Ub was cotransfected, colocalization of Rab7 wild-type and ORF3a was decreased. Such decreased level of colocalization was restored when HA-Ub was cotransfected with Rab7 2KR mutant (Fig. 6b, Supplementary Fig. S7). These results suggest that site-specific ubiquitination of Rab7 can down-regulate the function of ORF3a, thereby interfering with the egression of SARS-CoV-2.

ORF3a is reported to interact weakly with endogenous Rab7 [56]. Our structural model derived from SAXS analysis and MD simulation (Figs. 3, 4) suggested that ubiquitination on K191 and K194 would propel Rab7 from the endosomal membrane. To corroborate the effect of
Fig. 5. Site-specific ubiquitination of Rab7 affects intracellular structure formation and EGFR endocytosis. (a) Immunofluorescence assay of Rab7 WT, R191K, and R194K mutants. FLAG-Rab7 WT and mutants were co-transfected with or without HA-ubiquitin into HeLa cells. FLAG and HA were stained with Alexa-fluor-488 (green) and Rhodamine (red), respectively. Nuclei were stained with DAPI (blue). (b) Quantification of immunofluorescence results. The size of the Rab7-positive endosomes was measured from cells transfected with ubiquitin and the distribution of endosome size is shown with the bar graph. (c) EGF-induced EGFR degradation assay was conducted in HeLa cell. Cells were harvested at indicated time after treatment of EGF (20 ng/ml) and analyzed by immunoblotting. (d, e) The normalized relative amounts of EGFR in the immunoblots are quantitatively represented in graph. Data are presented as mean ± standard deviation from three replicates (*p < 0.1, **p < 0.01, ***p < 0.001, by one-way ANOVA).
4. Discussion

Regulation of Rab GTPases by ubiquitination is an emerging theme. Having established the role of ubiquitination on the function of Rab5, we expanded our investigation to Rab7 which acts in the downstream of Rab5 in the endocytic pathway. We observed disappearance of vesicle-like structures that was shown in transfection of Rab7 WT when these residues were co-transfected with ubiquitin. We also discovered that Rab7 can be monoubiquitinated and K175, K191, and K194 among 17 lysine residues of Rab7 are major monoubiquitination sites. The majority of the monoubiquitinated Rab7 WT was localized in the membrane fractions (Fig. 1b). It is most likely that vesicle-like structures observed when Rab7 WT was overexpressed were either late endosomes or lysosomes evidenced by co-localization with TME-M192 (Fig. 1c).

We provide a structural mechanism for the functional consequences of ubiquitination on K191 and K194 of Rab7. A recent study reported that Rab7 2KR (K191R/K194R) preferentially interacts with Rab-interacting lysosomal protein (RILP) than WT, and that deubiquitination of Rab7 on K191 promotes release of Rab7 from the membrane and enables new functional cycles of Rab7 [22]. The structural models of mUbRab7K191 and mUbRab7K194 derived from SAXS analysis showed conformational flexibility and diversity of the ubiquitin moiety (Fig. 3). MD simulation revealed that proximity of both K191 and K194 apparently caused Rab7 to dissociate from the endosomal membrane upon ubiquitination (Fig. 4). We conclude that the conformational flexibility and membrane proximity of the ubiquitin moiety on K191 and K194 intervened the membrane recruitment of Rab7. Consistent with our structural mechanism on the roles of the ubiquitination on K191 and K194 in the effector interaction, the portion of the monoubiquitinated Rab7 was the least and that of the membrane-localized Rab7 the most for the 2KR mutant (Fig. 2d, e, f). Accelerated EGFR degradation by K191R and K194R can be explained by the increased chances of either mutant to stay recruited to the endosomal membrane (Fig. 5c, d). However, our structural models for mUbRab7K191 and mUbRab7K194 reveal some limits. In the immunofluorescence assay, the K191R mutant featured enlarged endosomes while the 2KR mutant did not (Fig. 5a, b). The 2KR mutant might show other phenotypes such as tubule in different cell lines as shown in USP32-knock-down cells [22]. It is also plausible that ubiquitination on K191 might have another regulatory role not explored yet. It should be noted that all the experiments assessing the functional consequences of monoubiquitination on Rab7 were performed in cell overexpressing Rab7 and ubiquitin (Fig. 5). In case of endogenous Rab7, it is composed of active Rab7 predominantly in the membrane and inactive Rab7 predominantly in the cytosol. Interestingly, Rab7 K-to-R mutants (K191R, K194R, and K191R/K194R), deficient of monoubiquitination on specific residue(s), were apparently more active in the cells overexpressing the corresponding Rab7 mutant with ubiquitin. We speculated that overexpressed active Rab7 can form endosome more effectively and accelerates EGFR degradation (Fig. 5). Nevertheless, endogenous Rab7 may have affected the endosome formation or EGFR degradation. Experiments in cells overexpressing Rab7 mutants and ubiquitin with endogenous Rab7 being knocked down would reveal the effects of monoubiquitination on Rab7 more accurately.

The SAXS-derived structural model for mUbRab7K38 gives us hints on the role of ubiquitination on K38. A previous study reported that ubiquitination on K38 of Rab7 increased its effector binding and membrane association [23]. According to the complex structure of Rab7 with RILP (PDB: 1YHN) [59], K38 of Rab7 is located on switch 1 region which interacts with RILP (Supplementary Fig. S8a). K38 of Rab7 was known to be the essential residue in interaction of RILP and Rab7 [59]. We aligned the complex structure with our mUbRab7K38 models derived from EOM (Supplementary Fig. S8b). No steric clash was observed between mUbRab7K38 models and RILP in complex with Rab7, corroborating that monoubiquitination on K38 of Rab7 does not interfere with its binding to RILP. However, no obvious contact between the ubiquitin moiety of
**Fig. 7.** A speculative model for the regulation of Rab7 function by site-specific ubiquitination. (a) Normal recruitment of Rab7 to the endosomal membrane. Rab7 is geranyl-geranylated on the C-terminal cysteines and recruited on the endosomal membrane. RILP, an effector protein, is recruited to Rab7. (b) Rab7 is monoubiquitinated on 4 lysine residues (K38, K175, K191, and K194). When Rab7 is monoubiquitinated on K191 or K194, Rab7 recruitment to the endosomal membrane decreases. This leads to downregulating intracellular trafficking of a cargo protein such as EGFR and egression of beta-coronavirus including SARS-CoV-2.

mUbRa7K38 models and RILP. Further high-resolution structural data would be required to fully explain the enhancement of affinity toward RILP upon ubiquitination on K38 of Rab7.

Our study shed lights on the roles of site-specific ubiquitination in viral egression. Involvement of ubiquitin ligases such as HECT-type ligases has been shown to promote viral assembly and budding [60,61]. In our study, we observed that site-specific ubiquitination of Rab7 is likely to deteriorate viral egress from colocalization and immunoprecipitation between Rab7 and ORF3a from SARS-CoV-2 (Fig. 6). The colocalization of Rab7 WT and ORF3a was reduced when ubiquitin is co-transfected. However, Rab7 2KR which could not be ubiquitinated on K191 and K194 exhibited increased colocalization with ORF3a in HEK293FT cell lines (Fig. 6b). Immunoprecipitation results also showed decreased interaction of Rab7 and ORF3a with transfection of ubiquitin (Fig. 6c, d). Since the colocalization of Rab7 with ORF3a is a hallmark of the egression of beta-coronavirus [56,57], our data strongly suggests that site-specific ubiquitination of Rab7 can deteriorate the viral egression. Immunoprecipitation results using Rab7 2KR (K191R/K194R) implicate that ubiquitination on these two lysine residues is likely to be determinants for retardation of viral egression. SAXS-based structural analysis and molecular dynamics simulation suggested that monoubiquitination of Rab7 on K191 and K194 disturbed the membrane recruitment of Rab7, resulting in the inhibition of fusion of late endosomes with lysosomes (Figs. 3, 6). Our data demonstrates that site-specific ubiquitination of Rab7 can interfere with viral egress by means of exclusion of the ubiquitinated protein from membrane recruitment, potentially providing another point of intervention in COVID-19. A recent multi-omics study revealed that the phosphorylation level of Rab7 is increased upon infection by SARS-CoV-2 [62]. Taken together, posttranslational modifications of Rab7 is a key regulator for the egression of SARS-CoV-2.

Based on the results presented in this study, we propose a plausible model on how site-specific monoubiquitination regulates the function of Rab7 (Fig. 7). In the normal pathway, Rab7 is geranyl-geranylated on its C-terminus and recruited to the endosomal membrane. Effector proteins such as RILP are recruited to Rab7 on endosome and matured late endosome fused with lysosome (Fig. 7a). However, site-specific mono- ubiquitination of Rab7 alters its activity. Monoubiquitination on K191 and K194 of Rab7 inhibits the endosomal recruitment of Rab7, preventing RILP from binding to Rab7 on the endosome (Fig 7b). Disruption of membrane recruitment of site-specifically monoubiquitinated Rab7 can downregulate the lysosomal degradation and the viral egression (Fig. 7b).

**CRediT authorship contribution statement**

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.11.074.

**References**


