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Autophagy regulation revealed by SapM-induced block of autophagosome-lysosome fusion via binding RAB7

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ABSTRACT

The mechanism underlying autophagy alteration by mycobacterium tuberculosis remains unclear. Our previous study shows LpqH, a lipoprotein of mycobacterium tuberculosis, can cause autophagosomes accumulation in murine macrophages. It is well known that SapM, another virulence factor, plays an important role in blocking phagosome-endosome fusion. However, the mechanism that SapM interferes with autophagy remains poorly defined. In this study, we report that SapM suppresses the autophagy flux by blocking autophagosome fusion with lysosome. Exposure to SapM results in accumulations of autophagosomes and decreased co-localization of autophagosome with lysosome. Molecularly, Rab7, a small GTPase, is blocked by SapM through its CT domain and is prevented from involvement of autophagosome-lysosome fusion. In conclusion, our study reveals that SapM takes Rab7 as a previously unknown target to govern a distinct molecular mechanism underlying autophagosome-lysosome fusion, which may bring light to a new thought about developing potential drugs or vaccines against tuberculosis.

1. Introductions

Autophagy is a conserved mechanism that eukaryotic cells possess to dispose of intracellular organelles, protein aggregates and microbes that are hard to be degraded only by the proteasome [1]. This process is mainly divided into two stages [1,2]: the early stage in which the major event is the biogenesis of autophagosome that has been well studied for identifying regulatory molecules and signal pathways, and the late stage in which the autophagosome needs to fuse with lysosome for degradation of the enclosed materials. It is clear that the fusion between autophagosome and lysosome takes a key role in the elimination of intracellular parasites, because some microbes can persist within immature autophagosome due to the compromised fusion [2,3]. At the last few years, RAB7 has been identified as one key element to be involved in the fusion process [4–7], but its role in autophagy mediated by microbes remains to be identified.

Mtb is a facultative intracellular microbe that usually survivals in host macrophages. Whereas this pathogen appears to be deleted more easily in the context that autophagy is activated by some external stimulation, such as nutrient starvation, rapamycin or cytokines [8–10]. An important issue is how Mtb regulates autophagy by its self. An interesting finding has shown that both live BCG (Bacillus Calmette–Guerin) and its culture filtrate proteins (CFP) can inhibit lysosome fusion with other vesicles by inactivating RAB7 which prevents the recruitment of RAB7-interacting lysosomal protein (RILP) [11]. Recently, we report LpqH that is a lipoprotein secreted by Mtb has been characterized by inducing the accumulation of autophagosomes [12], and being recognized by receptors on the surface of host cells for providing signals to positively regulate autophagy [13,14]. However, there is a lot to be discovered about the negative regulation of autophagy in which Mtb might inhibit or compromise normal function of autophagy.

SapM is an acid phosphatase secreted by Mtb or BCG and has been characterized as a virulence factor to be employed to block phagosome-endosome fusion [15], which leads to the hard-to-

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eradication of this intracellular microbe [16]. In the last few years, a few experiments *in vivo* indicated that SapM also might be involved in the negative regulation of autophagy [8,10], however, the molecular mechanism is still little known. In this study, we present evidences that SapM negatively regulates autophagy through blocking the fusion between the autophagosome and lysosome, and this block is dependent on the interaction of SapM with RAB7 through its CT domain.

2. Materials and methods

2.1. Cell culture, antibodies, and plasmids

Raw 264.7 cells and stable GFP-LC3-Raw264.7 cells [] were cultured in DMEM supplemented with 10% FBS (GIBCO) or in earle's balanced salts solution for starvation. Antibodies used in this study include anti-LC3 (PTG), anti-HA (SIGMA), anti-flag (SIGMA), anti-Rab7 (SIGMA), anti-Beclin 1 (PTG), anti-β actin (PTG), anti-P62 (PTG). Genetic constructed plasmids includes: pCMV-flag-SapM, pEGFP-SapM, mCherry-SapM and pRFP-GFP-LC3. Based on the vector expressing SapM-flag, a series of mutants were constructed via recombinant PCR including pCMV-flag-SapM$^{\triangle arcA}$, -SapM$^{\triangle FReD}$ and -SapM$^{\triangle CT}$. Cellular transient transfection was performed with the nucleoporation protocol (Amaxa). In brief, 10 μg plasmid DNA was mixed with 0.1 ml of cell suspension, and transferred to an electroporation cuvette to nucleofecte. After electroporation, cells were transferred to complete medium for recovery. Targeting murine Rab7, BECN1, ATG5 and non-sensitive siRNAs (siNS) were synthesized from INVITROGEN.

2.2. LC3 puncta assay

GFP-LC3-Raw264.7 cells were washed in PBS and treated with complete media, EBSS, 50 nM wortmannin, or 20 μM chloroquine for 1 h unless otherwise indicated. At least 100 cells in each experiment were imaged by Olympus IX73 inverted fluorescent microscope, in which GFP-LC3 puncta (autophagosome) up to 0.5 μm in diameter were counted. Raw cells expressing RFP-GFP-LC3 and Flag-SapM were incubated in either normal medium or EBSS for 1 h, and were imaged by confocal microscopy using the Zeiss 510 Laser Scanning microscope. During which, RFP-GFP-LC3 puncta were counted with several colors: green or yellow dots (autophagosomes) or red dots (autolysosomes), because green fluorescence usually is quenched in lysosome due to very acid condition [17,25]. In all experiments, a minimum of 100 cells per sample were counted, and triplicate samples were counted per experimental condition.

2.3. Western blot

Cells were washed in PBS and lysed in ice cold lysis buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 2 mM EDTA, 2% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Roche)) for 10 min (about 10^6 cells/200 μl). Whole cells and cell debris were pelleted by low speed centrifugation (400 g, 3 min) and cleared supernatants were transferred to a new tube. Protein concentration was determined by BCA protein assay (Pierce). Samples were boiled for 5 min in the presence of 4x SDS-PAGE-loading buffer (250 mM Tris–HCl pH 6.8, 40% glycerol, 8% SDS, 0.57 Mβ-mercaptoethanol, 0.12% bromophenol blue). Equal amounts of protein were loaded on 12%SDS-PAGE gels and transferred onto a PVDF membrane. The membrane was blocked overnight at 4 °C in 5% milk in PBS/Tween 20 (0.1%) and probed with primary antibodies for 1 h at room temperature. After washing with PBS/Tween, the blot was probed with appropriate HRP-conjugated secondary antibody for 1 h at room temperature and stained with SuperSignal West Dura chemiluminescent substrate (Pierce). Actin was used as a loading control.

2.4. LC3 turnover assay

If cells are treated with chloroquine (Sigma), an agent impairing lysosomal acidification to inhibit autophagosome-lysosome fusion, the degradation of LC3-II is blocked, resulting in the accumulation of LC3-II [4]. In the turnover assay of autophagosomes, the ratio of LC3-II was calculated between chloroquine-treated and −untreated cells.

2.5. Immunofluorescence assay

After transfection of mCherry-SapM for 35 h plus starvation for 1 h, Raw264.7 cells were fixed with 2% paraformaldehyde in PBS, permeabilized in 0.5% Triton X-100 in PBS, blocked with 1% BSA in PBS, stained with anti-Rab7 (1:50 dilution) and labeled with FITC-conjugated goat anti-rabbit antibodies (1:100 dilution). All steps were carried out at room temperature. The cells were imaged by confocal microscopy using the Zeiss 510 Laser Scanning microscope and analyzed with LSM Image Browser program (Zeiss).

2.6. Immunoprecipitations

Cells transfected with vectors expressing SapM, SapM$^{\triangle ARCA}$, SapM$^{\triangle FRED}$ and SapM$^{\triangle CT}$ were lysed with lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1% NP40 and 1% Triton X-100 and protease inhibitor cocktail) for 1hr, followed by centrifugation at 15,000*g* for 10 min at 4 °C. After pre-cleared using Protein-G for 1 h, supernatants were immunoprecipitated overnight at 4 °C with the antibody of anti-flag, anti-RAB7, or IgG to form the immunocomplexes which were captured with Protein G plus-sepharose beads (GE Healthcare, 17-0618-02), washed 4 times and subjected to western-blotting analyses with anti-Flag or anti-RAB7.

2.7. Statistical analysis

All graphs were produced using GraphPad Prism® software and error bars represent the standard deviation. Data were expressed as Mean ± SEM. Statistics was calculated using either unpaired student *t*-test or one-way Anova followed by a Bonferroni multiple comparison *post-hoc*-test. The significant difference was considered if $p < 0.05$.

3. Results

3.1. SapM induces accumulation of autophagosomes in phosphatidylinositol 3-kinase activity-dependent manner

Autophagy is operational in many immune cells, especially in macrophage as one host defense mechanism. However, intracellular pathogens are able to produce some special weapons, such as many virulence factors, to antagonize autophagical functions for their own replication or survival [2]. To test whether SapM could affect autophagy, we treated Raw264.7 cells (mouse macrophages) with SapM, wortmanin or starvation, and analyzed autophagosomes formation and LC3II levels (Fig. 1). In Raw264.7 cells expressing GFP-LC3, both starvation and SapM resulted in an increased numbers of GFP-LC3 puncta (Fig. 1A) and LC3II(Fig. 1B), suggesting accumulation of autophagosomes.

To test whether SapM induced autophagosome accumulation and increased LC3II require the autophagical upstream, we analyzed the effect of wortmanin, an agent that inhibits

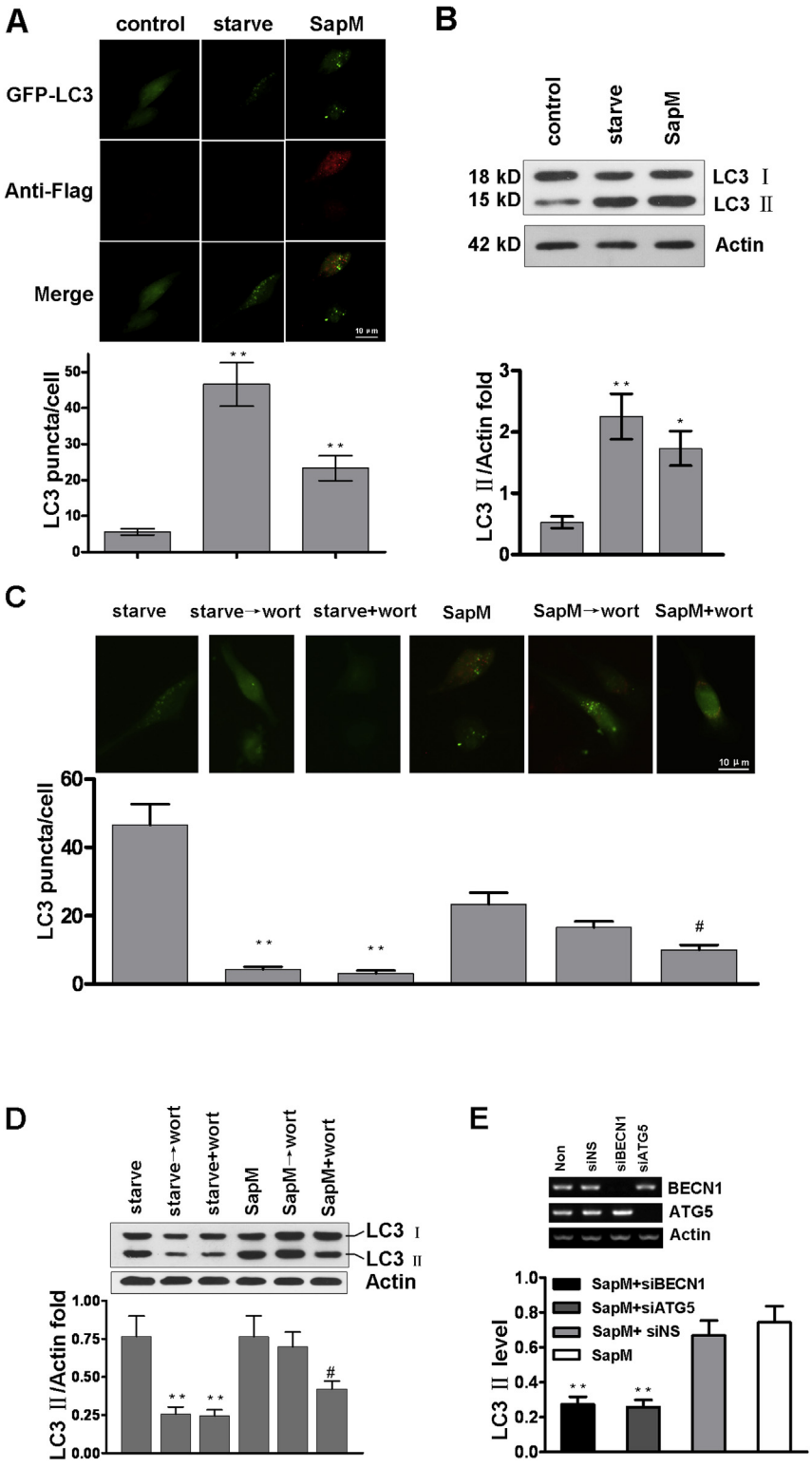

Fig. 1. SapM induces autophagosome accumulation. (A) Raw264.7 cells expressing GFP-LC3 were treated with EBSS for 1 h (starve), transfection of pCMV-flag-SapM (SapM) or pCMV vector (control) for 36 h, then labled with anti-flag. Autophagosome puncta with diameter above 0.5 μm were counted. Representative fluorescence images were presented in upper panel. And quantitative analysis was shown in lower panel (**$p < 0.01$ *vs* control). (B) Raw264.7 cells were treated as in panel (A), LC3II levels were detected using western blot and quantitatively analyzed (*$p < 0.05$, **$p < 0.01$ *vs* control). (C) GFP-LC3-expressing Raw264.7 cells were treated with EBSS for 1 h (starve), together (starve + wort) or followed by 50 nM wortmannin for 1 h (starve → wort), or pCMV-SapM transduction for 36 h (SapM), together (SapM + wort) or followed by 50 nM wortmannin for 1 h (SapM → wort). Then cells were labled with anti-flag and imaged for counting LC3 puncta number (**$p < 0.01$ *vs* starve, # $p < 0.05$ *vs* SapM). (D) Raw264.7 cells were treated as in panel (C), and LC3II level was quantified (**$p < 0.01$ *vs* starve, # $p < 0.05$ *vs* SapM). (E) Raw264.7 cells transduced with non-sensitive siRNA (siNS) or siRNA targeting BECN1 (siBECN1) or ATG5 (siATG5) were transfected with pCMV-SapM for 36 h. The interfering efficiency was analyzed by RT-PCR as shown in upper panel. LC3II level was detected by western blotting and quantified as seeing in bottom panel (**$p < 0.01$ *vs* SapM). Data shown represent the means ± SD from three independent experiments.

phosphatidylinositol 3-kinase (PI3K) activity to block the autophagosome formation at autophagical upstream. In the presence of wortmanin, either GFP-LC3 puncta or LC3II was drastically reduced in the cells treated by starvation (Fig. 1C), conforming that starvation induced autophagy is dependent on PI3K activity. This is different from SapM, because later addition of wortmanin causes no further reduction in GFP-LC3 puncta or LC3 lipitation, but simultaneous addition of wortmanin results significant reduce of them (Fig. 1C). This difference in autophagy caused by SapM indicated that it does not activate autophagy, but blocks autophagy flux in PI3K-dependent manner. To further clarify targeting autophagy of SapM, we knocked down BECN1 and ATG5 with siRNA, and found that SapM-induced LC3II reduction is dependent on autophagy (Fig. 1E).

3.2. SapM blocks the autophagy flux by inhibit autophagosome-lysosome fusion

The next logical step is to determine whether SapM suppress autophagy flux. Additional treatment was performed with

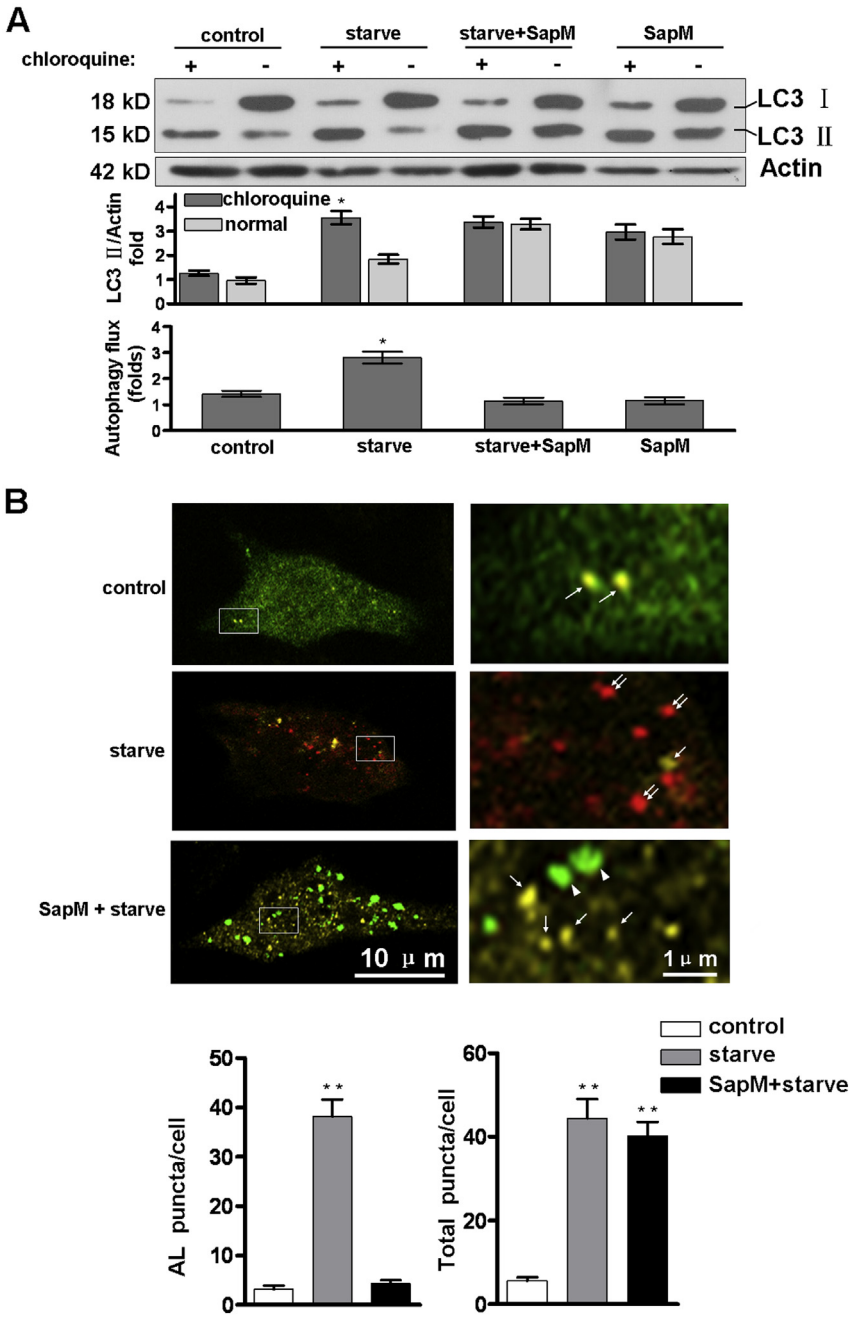

Fig. 2. SapM inhibits autophagy flux through blocking autophagosome-lysosome fusion. (A) LC3 turnover assay. Raw264.7 cells were treated with EBSS, SapM or both as described in Fig. 1B, with or without addition of 20 μM chloroquine for the final hour. The LC3IIwas detected using western blotting (upper) and the difference between chloroquine-treated and -untreated cells was statistically analyzed (middle, $^{*}p < 0.05$ *vs* normal) and the ratio of LC3II representing autophagy flux is calculated (bottom, $^{*}p < 0.05$ *vs* control). (B) SapM blocks autophagosome-lysosome fusion. RFP-GFP-LC3 expressing Raw264.7 cells were treated with EBSS, SapM or both as described in (A). Three types of puncta that differ in color were imaged: green or yellow puncta represented autophagosomes and red ones represented autolysosomes (AL). These puncta were counted and statistically analyzed in at least 100 cells ($^{**}p < 0.01$ *vs* control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

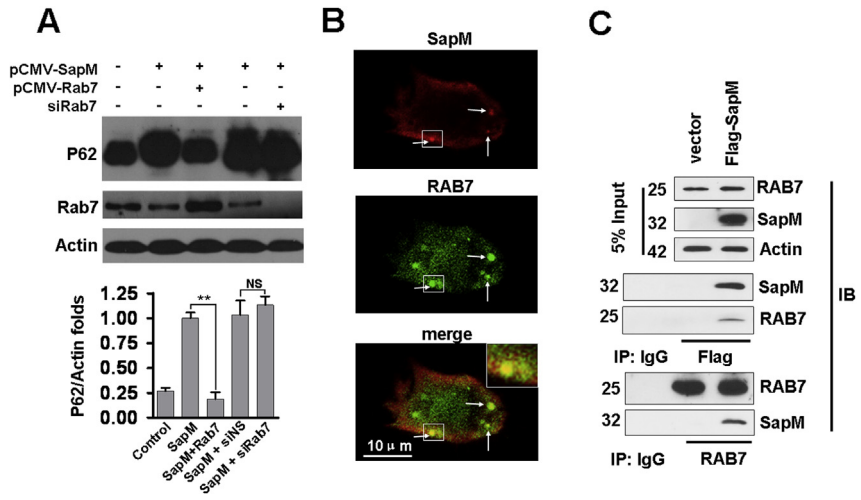

Fig. 3. Rab7 is required for SapM-hindered P62 degradation and interacts with SapM. (A) Raw264.7 cells were transduced with pCMV-SapM, -Rab7, siRab7 or vectors. Rab7 and P62 were detected using western blot, and P62 was statistically analyzed (**$p < 0.01$, NS: no significance). (B) Co-localization of SapM with RAB7. Raw264.7 cells were transfected with mCherry-SapM (red) for 35 h. After starvation for 1 h, these cells were fixed and immuno-labeled with the anti-Rab7 antibodies, followed by an addition of FITC-conjugated goat anti-rabbit IgG (green), and imaged using confocal microscope. The representative fluorescence images were presented, in which the co-localization of SapM and RAB7 was marked with white arrows, and magnified in a white frame for more precise observation. (C) Co-immunoprecipitation of SapM with endogenous RAB7. Raw264.7 cells were transfected with only pCMV-flag-SapM for 35 h. After starvation for 1 h, these cells were lysed for co-immunoprecipitation assay. Both endogenous RAB7 fished by anti-flag antibodies and exogenous SapM fished by anti-RAB7 were detected using western blotting. Specific protein levels in cell lysates (input) and immunoprecipitation with IgG controls were shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chloroquine which can impair autophagosome-lysosome fusion to block LC3II degradation [17]. Chloroquine caused further elevation of LC3II in starvation-treated cells, confirming that starvation induces a complete autophagy flux. Contrarily, no more increase in LC3IIwas found in SapM-treated cells plus chloroquine (Fig. 2A), demonstrated that SapM suppressed autophagy flux. These evidences that SapM blocks autophagical downstream (Figs. 1 and 2A) suggest that SapM may disrupt autophagosome-lysosome fusion. To confirm this, cells were transfected with mRFP-GFP-LC3 plasmid for confocal microscopy assay. We found increased red puncta representing more autolysome formation and yellow puncta representing autophagosome in starvation treated cells (Fig. 2B), indicating that starvation induces autophagy levels in whole process. With SapM treatment, most puncta were yellow or green representing the accumulation of autophagosomes without fusion with lysosomes (Fig. 2B), demonstrated SapM blocks the autolysosomes formation. This is reinforced by the treatment of starvation plus SapM, as only yellow and green puncta were increased but not red punctae even upon starvation (Fig. 2B), indicating that SapM blocks lysosomes fusion with new formed autophagosomes induced by starvation.

3.3. *Rab7 is required for SapM-induced autophagical inhibition*

Previous studies suggest that Rab7 is responsible for coordinating autophagosome fusion with a variety of vesicles [7,18]. A recent study implicates that Rab7 is required for autophagosomes to fuse with lysosomes [6]. To clarify the role of Rab7 in SapM-induced autophagy block, we either knock down or over-express Rab7 in SapM-treated cells. As a classic marker to evaluate autophagic function on degradation, P62 levels were detected in these cells. Overexpression of Rab7 reduced P62 in SapM-treated cells with statistical significance, indicating that Rab7 attenuates SapM-induced autophagy inhibition of degradation (Fig. 3A). In addition, siRNA targeting Rab7 did not increase P62 compare to non-sensitive siRNA (siNS) in SapM-treated cells, suggesting that SapM has an overlapping effect with Rab7-downregulation on inhibiting P62-degradation (Fig. 3A). Consistent to SapM-blocked

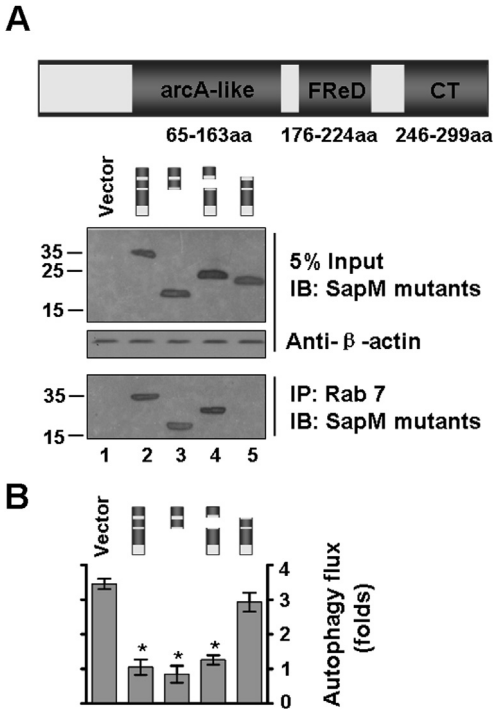

Fig. 4. SapM interacts with RAB7 via its C-terminus that is required for SapM-induced autophagy block. (A) The CT domain is required for SapM to bind RAB7. According to upper diagram, three SapM-mutants lacking ARCA-like, FRED or CT domains were constructed into pCMV-flag. Raw264.7 cells were transfected with these mutants for 35 h and followed by starvation for 1 h. Then these cells were lysed and subject to co-immunoprecipitation assay as described in Fig. 3C. Only the mutant lacking CT domain was absent from the precipitation by anti-RAB7. (B) CT domain is required for SapM to block autophagy flux. These treated cells were lysed and subject to LC3 turnover assay as shown in Fig. 2A. CT domain-defected SapM also lost the function in suppressing autophagy flux (*$p < 0.05$ *vs* vector).

autophagosome-lysosome fusion (Fig. 2B), more P62 was found in SapM-treated cells than control (Fig. 3A). Because evident change of Rab7 in quantity was not found in SapM-treated cells compared to control cells (Fig. 3A), we deducted that SapM may inhibit Rab7 ability. To confirm this, we investigated whether SapM interacts with Rab7. First, SapM co-staining with Rab7 was observed in cells that were transfected with RFP-SapM and subjected to immunocytofluorescence to probe Rab7 with FITC (Fig. 3B), suggesting SapM is able to interact with Rab7 *in vivo*. To verify this interaction, *in vitro* immunoprecipitation was performed. Rab7 in extracts from cells transfected with SapM-Flag was present in the precipitate by anti-Flag. Consistently, SapM was also precipitated by anti-Rab7 (Fig. 3C). Consequently, these results demonstrated that SapM has a directly or indirectly interaction with Rab7 which contributes to the functional suppression of autophagical degradation.

3.4. CT-domain is required for SapM-mediated interaction with Rab7 and autophagical block

To determine which domain is responsible for SapM to regulate autophagy, we constructed three SapM mutants including SapM$^{\triangle arcA}$, SapM$^{\triangle FReD}$ and SapM$^{\triangle CT}$. In immunoprecipitation experiments, only lacking CT-domain results in the failure to interact with Rab7, but arcA and FReD domain have no such influence (Fig. 4A). Furthermore, the deficiency of CT-domain also dismisses the inhibition of autophagy flux induced by SapM (Fig. 4B). Consistently, such elevated autophagy flux was not found in SapM$^{\triangle arcA}$ or SapM$^{\triangle FReD}$ (Fig. 4B). Collecting these results, CT-domain mediated interaction with Rab7 is required for SapM to reduce autophagy. Infurther refined the interaction domain between SapM and Rab7.

4. Discussion

One way by which Mtb regulates autophagy is to produce virulence factors. For example, it has been discovered that LpqH as one virulence factor can upregulate autophagy when this protein antigen extracted from bacteria lysate is sensed by Toll like receptors displayed on cell surface [13,14]. From our study, we reveal that another virulence factor, SapM, inhibited autophagy by blocking Rab7 to involve in autophagosome-lysosome fusion. We also determined that CT-domain of SapM is indispensible for the incomplete autophagy.

The reason to begin this study is we found that obvious accumulation of autophagosomes was induced by SapM in macrophages, and it's not like starvation induced complete autophagy during delayed addition of wortmannin. Following this result, we found that the increased autophagosomes results from reduction of autophagy flux, because SapM prevented the autophagosomes from incorporating lysosomes. These results are consistent with the finding that Mtb inhibits autophagosomal colocalization with lysosomes [19]. As we known, this special attributes of SapM in autophagy inhibition is quite different from that of other virulence factors produced by microbes [13,20–22], but resembles thapsigargin which is recently characterized in arresting autophagosome-lysosome fusion [6,23].

These results bring up an important question what is the target of SapM to arrest autophagy. RAB7 that has been shown to be essential for autophagosome fusion with lysosome [4,24]. Consistently, we also found that increasing Rab7 can rescue impaired autophagy by SapM. Rab7 may be targeted to block autophagosome-lysosome fusion in two ways: inhibiting its expression and blocking its function [7,23]. Contrary to reducing Rab7 expression, we found that SapM can interact with Rab7 to impede autophagosome-lysosome fusion. To the best of our knowledge, this is the first study to identify CT-domain as an indispensable element for SapM to bind Rab7 and inhibit autophagy. Our study can explain partially the enhanced immunoprotection and the attenuated virulence of *Mycobacterium* deleting SapM [8,10] and why live Mtb could impair autophagy at the step of autophagosome-lysosome fusion [19].

In summary, we reveal a new mechanism undergoing SapM-induced autophagy inhibition by which SapM targets RAB7 via its CT domain for blocking autophagosome-lysosome fusion. However, further investigations based on whole bacillus and *in vivo* model are needed to clarify the function of SapM on autophagy regulation. Our study brings new light to understanding the “cross-talking” between autophagy and invaders, and may contribute to develop new vaccines or therapy targeting SapM-blocked autophagy.

Conflict of interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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