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Pulmonary Surfactant Protein A Enhances Endolysosomal Trafficking in Alveolar Macrophages through Regulation of Rab7

Vicky Sender,* Christina Moulakakis,* and Cordula Stamme*†

Surfactant protein A (SP-A), the most abundant pulmonary soluble collectin, modulates innate and adaptive immunity of the lung, partially via its direct effects on alveolar macrophages (AM), the most predominant intra-alveolar cells under physiological conditions. Enhanced phagocytosis and endocytosis are key functional consequences of AM/SP-A interaction, suggesting a SP-A–mediated modulation of small Rab (Ras related in brain) GTPases that are pivotal membrane organizers in both processes. In this article, we show that SP-A specifically and transiently enhances the protein expression of endogenous Rab7 and Rab7b, but not Rab5 and Rab11, in primary AM from rats and mice. SP-A–enhanced Rab7 and Rab7b are functionally active as determined by increased interaction of Rab7 with its downstream effector RILP and enhanced maturation of cathepsin-D, a function of Rab7b. In AM and RAW264.7 macrophages, the SP-A–enhanced lysosomal delivery of GFP-E. coli is abolished by the inhibition of Rab7 and Rab7 small interfering RNA transfection, respectively. The constitutive expression of Rab7 in AM from SP-A+/+ mice is significantly reduced compared with SP-A−/− mice and is restored by SP-A. Rab7 blocking peptides antagonize SP-A–rescued lysosomal delivery of GFP-E. coli in AM from SP-A+/− mice. Activation of Rab7, but not Rab7b, by SP-A depends on the PI3K/Akt/protein kinase C (PKC) signal transduction pathway in AM and RAW264.7 macrophages. SP-A induces a Rab7/PKCζ interaction in these cells, and the disruption of PKCζ by small interfering RNA knockdown abolishes the effect of SP-A on Rab7. The data demonstrate a novel role for SP-A in modulating endolysosomal trafficking via Rab7 in primary AM and define biochemical pathways involved.

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Abbreviations used in this article: AM, alveolar macrophage; CTSD, cathepsin-D; GDI, guanine nucleotide dissociation inhibitor; LAMP-1, lysosome-associated membrane protein-1; PKCζ, protein kinase Cζ; PKCζ-PS, protein kinase Cζ pseudosubstrate; Rab, Ras related in brain; RILP, Rab7 interacting lysosomal protein; siRNA, small interfering RNA; SP-A, surfactant protein A; SP-D, surfactant protein D; WCL, whole-cell lysate; wt, wild-type.

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monocyte-derived macrophages (31). Furthermore, both SP-A and SP-D significantly increase the number of *Legionella pneumophila* colocalized with lysosome-associated membrane protein-1 (LAMP-1) in THP-1 cells (32). However, the option that SP-A influences membrane trafficking by directly modulating Rab GTPases has not been investigated so far.

In this study, we show that SP-A specifically and transiently enhances the endogenous protein levels of functionally active Rab7 and Rab7b, but not Rab5 and Rab11, in primary AM. Upregulation of Rab7, but not Rab7b, by SP-A involves the PI3K/Akt/protein kinase Cζ (PKCζ) pathway, thereby enhancing the lysosomal delivery of GFP-*Escherichia coli* in these cells.

### Materials and Methods

#### Animals and reagents

Primary cells were obtained from pathogen-free male Sprague Dawley rats, from C57BL/6J wild-type (wt) control mice (Charles River Laboratories), or from SP-A−/− mice as described previously (33). Animal care and experiments were conducted according to the Schleswig-Holstein Ministry of Environment, Nature, and Forestation. All mice used were between 6 and 12 wk of age, and were maintained at the Research Center Borstel animal facility under pathogen-free conditions.

The smooth LPS from *Salmonella* friedenau strain H909 was extracted by the phenol/water method, purified, lyophilized, and transformed into the triethylamine salt form (34). RPMI 1640 medium, PBS, and Dulbecco’s PBS were from PAA Laboratories GmbH. The PKCζ- pseudosubstrate (PKCζ-FS) was from BioSource (Invitrogen). The bicinchoninic acid reagent was from Interchim. Rabbit anti-Rab7, anti-Rab5, anti-Rab11, anti--phospho-p38 MAPK (Thr180/Tyr182), and anti-PKCζ Ab were obtained from Cell Signaling Technology. The isozyme-selective Akt inhibitor VIII, Rab7 blocking peptides, mouse anti-Rab7b, rabbit anti-Rab7, interferon gamma Abs at a 1:5000 dilution using Odyssey (Li-Cor). Band intensity was normalized to β-actin, or anti–phospho-p38 MAPK (both rabbit polyclonal, 1:1000), anti-Rab7 (mouse monoclonal, 1:1000), anti-RILP (rabbit polyclonal, 1:200, or goat polyclonal, 1:200), anti-PKCζ (rabbit polyclonal, 1:500, or mouse monoclonal, 1:200), anti-CTSD, mouse anti–β-actin, mouse anti–γ-tubulin, and HRP-conjugated goat anti-rabbit, donkey anti-goat, and donkey anti-mouse IgG were from Santa Cruz Biotechnology. IRDye700-conjugated secondary anti-rabbit Ab and IRDye800-conjugated secondary anti-mouse Ab were from BIO- TRENDE GmbH. Dynabeads were from Invitrogen. FITC-Ab labeling kit, amine-reactive Alexa Fluor 555 carboxylic acid, succinimidyl ester, DAPI, LysoTracker Red, Alexa Fluor 488 goat anti-IgM and goat anti-rabbit IgG, and Alexa Fluor 633 goat anti-mouse IgG and donkey anti-goat IgG were from Molecular Probes (Invitrogen). The Nucleofector Transfection Kit V for RAW264.7 cells was obtained from Lonza. Wortmannin and SB203580 were from Sigma-Aldrich. Clq was from Advanced Research Technologies. All other reagents (except as noted) were obtained from Carl Roth GmbH & Co. KG.

#### SP-A purification

Human SP-A was purified from bronchoalveolar lavage of patients with alveolar proteinosis, as described in detail (35). In brief, the lavage fluid was treated with butanol to extract SP-A, and the resulting pellet was sequentially solubilized in octylglucoside and 5 mM Tris-buffered water (pH 7.4). To reduce endotoxin contamination, SP-A was treated with polymyxin B agarose beads. SP-A preparations were tested for the presence of bacterial endotoxin using a Limulus amebocyte lysate assay (Haemochrom Diagnostica). All SP-A preparations used contained <0.2 pg endotoxin/μg SP-A. Collagenase-treated SP-A and deglycosylated SP-A were prepared as described previously (10).

#### Protein labeling

Purified SP-A was labeled with amine-reactive Alexa Fluor 555 carboxylic acid, succinimidyl ester according to the manufacturers’ instructions. SP-A was incubated with the respective conjugate in 100 mM sodium bicarbonate buffer (pH 9.0) for 1 h at room temperature. The reaction was stopped by adding 300 mM hydroxylamine hydrochloride (pH 8.5) and dialyzed overnight in a QuixSep Micro Dialyzer against PBS to remove unbound conjugate. Labeled proteins were analyzed by SDS-PAGE with subsequent Western blotting and Coomassie staining, and functionality of labeled SP-A was compared with that of unabeled protein as shown previously (36). Rab7 Ab was labeled with a FITC-Ab labeling kit according to the manufacturers’ instructions.
Tracker Red (100 nM) was added to GFP-

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processing was carried out with Adobe Photoshop version 10.0. Colocalization was quantified using Leica TCS NT software, and image

lysosomes. Cells were washed three times with cold PBS and were then

Western blot analysis. SP-A in a dose-dependent manner increased

Rab7 and Rab7b expression in AM (Fig. 1

SP-A enhances Rab7/RILP interaction

Both SP-A and LPS induced a strong colocalization of Rab7 with the late endosome/lysosome marker LysoTracker compared with

proteolytic processing. It is then slowly converted into a 44-kDa

to modulate Rab7b expression in human monocytes, mouse peritoneal macrophages, and RAW264.7 cells (29, 39), was used as a positive control (Fig. 1D–F). The effect of SP-A and LPS on

Rab7 (Fig. 1D) and Rab7b (Fig. 1E) expression in AM was stable for up to 10 h and declined to baseline after 18–24 h. LPS, but not SP-A, transiently increased Rab5 expression after 2–4 h with an initial peak at 2 h (256 ± 74%; Fig. 1F). Neither SP-A nor LPS treatment of AM affected Rab11 protein expression after 4, 6, 8, and 10 h (data not shown).

The Western data were confirmed by confocal microscopy. A555-labeled SP-A enhanced but did not colocalize with Rab7 after 30, 60, 90, and 120 min in primary AM (Fig. 2Ad, f, h, j). Subsequent coimmunoprecipitation experiments confirmed the lack of direct SP-A/Rab7 interaction (data not shown). Treatment of AM with LPS also enhanced Rab7 staining (Fig. 2An), but an additive effect of SP-A plus LPS on Rab7 was not observed (Fig. 2Ai). A555-labeled SP-A also enhanced but did not colocalize with Rab7b in AM (Fig. 2Bd, f, h, j). Subsequent coimmunoprecipitation experiments confirmed the lack of direct SP-A/Rab7b interaction (data not shown). When cells had been pretreated with SP-A before LPS, the enhancement of Rab7b failed to show an additive effect (Fig. 2Bl).

To determine whether the SP-A–enhanced Rab7 and Rab7b protein availability was preceded by an increase in their mRNA, quantitative RT-PCR was performed on mRNA from AM treated with SP-A or LPS for up to 24 h. However, within that time frame, compared with controls, neither SP-A nor LPS affected Rab7 or Rab7b mRNA expression (data not shown).

SP-A enhances Rab7/RILP interaction

TheaspaticpeptidaseCTSD is synthesized as prerecathespin-D precursor, which is converted into procalcitespin-D (52 kDa) and then transported toward lysosomes, where it undergoes further proteolytic processing. It is then slowly converted into a 44-kDa form and finally into the mature 32-kDa form (43). CTSD maturation is a function of Rab7b (28) regulating efficient delivery of CTSD and other lysosomal enzymes along the endocytic route. To assess whether SP-A–enhanced Rab7b is functionally active, CTSD maturation in primary AM was investigated. Because of the duration of the posttranslational modification, the maturation of CTSD was analyzed over a period of 2–6 h. AM were left untreated or treated with different concentrations of SP-A, and the

STADependent CTSD maturation

TheaspaticpeptidaseCTSD is synthesized as prerecathespin-D precursor, which is converted into procalcitespin-D (52 kDa) and then transported toward lysosomes, where it undergoes further proteolytic processing. It is then slowly converted into a 44-kDa form and finally into the mature 32-kDa form (43). CTSD maturation is a function of Rab7b (28) regulating efficient delivery of CTSD and other lysosomal enzymes along the endocytic route. To assess whether SP-A–enhanced Rab7b is functionally active, CTSD maturation in primary AM was investigated. Because of the duration of the posttranslational modification, the maturation of CTSD was analyzed over a period of 2–6 h. AM were left untreated or treated with different concentrations of SP-A, and the maturation of CTSD was determined with an anti-CTSD Ab that detects the three forms of the protease. Total CTSD expression in AM was significantly increased (p < 0.01) by 40 μg/ml SP-A

min, washed three times with PBS, followed by permeabilization with 0.25% Triton X-100 for 6 min. In experiments using LysoTracker (100 nM), cells were fixed in 2% paraformaldehyde. Subsequently, cells were blocked with 10% BSA/PBS for 30 min and then incubated with anti-Rab7, anti-Rab7, anti-RILP, anti-PKCIζ, Abs, or control IgG at a 1:50 dilution. Alexa Fluor 488 goat anti-mouse IgG and goat anti-rabbit IgG, and Alexa Fluor 633 goat anti-mouse IgG and donkey anti-goat IgG served as secondary Abs at a 1:500 dilution. Nuclei were counterstained with DAPI. Samples were analyzed using a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). Images were acquired with the Leica TCS NT software and assembled using Adobe Photoshop 10.0 (Adobe Systems).

E. coli uptake assay

GFP-E. coli (strain MJ109) were cultured at 37°C in lysogeny broth medium containing ampicillin (100 μg/ml). Rat AM, RAW264.7 cells (1 × 107 cells/well), or AM from SP-A−/− and C57BL/6j mice (1 × 107 cells/well) were treated or not treated with SP-A (40 μg/ml) or Rab7 blocking peptides (20 μg/ml) for indicated times. Subsequently, treated and untreated cells were infected with GFP-E. coli at a ratio of 1:10 (AME. coli ratio) for 15 min at 37°C. Cells were washed twice to remove unbound bacteria and chased for another 30 min at 37°C. During the chase, LysoTracker Red (100 nM) was added to GFP-E. coli–infected AM to label the lysosomes. Cells were washed three times with cold PBS and were then fixed with 2% paraformaldehyde. Subsequently, confocal microscopy was carried out. Confocal images used for quantification were scanned at the same pinhole, offset gain, and amplifier values below pixel saturation. Colocalization was quantified using Leica TCS NT software, and image processing was carried out with Adobe Photoshop version 10.0.

Statistical analysis

Data were statistically analyzed as indicated in the figure legends using GraphPad Prism (version 5.0; GraphPad). Values were considered significant when p < 0.05. Data are presented as mean ± SEM.

Results

SP-A enhances Rab7 and Rab7b protein in primary AM

To determine the role of SP-A on the protein expression of distinct Rab GTPases, primary AM were treated with different concentrations of SP-A (30 min), and the cellular contents of Rab5, Rab7, Rab7b, and Rab11 in treated and untreated AM were determined by Western blot analysis. SP-A in a dose-dependent manner increased Rab7 and Rab7b expression in AM (Fig. 1A). In the presence of 40 μg/ml SP-A, the expression of Rab7 was 178 ± 14% (p < 0.01) and Rab7b 182 ± 17% (p < 0.01) over control (no SP-A) (Fig. 1A). Western kinetics revealed that a 30-min incubation of AM with SP-A (40 μg/ml) resulted in a 154 ± 19% (p < 0.01) and 143 ± 15% (p < 0.01) enhancement of Rab7 and Rab7b levels, respectively (Fig. 1B). In contrast, the expression of Rab5 and Rab11 was not affected by treatment of the cells with SP-A, indicating a specific effect of SP-A on Rab7 and Rab7b (Fig. 1A, 1B). Similar results were obtained for Rab7 expression in RAW264.7 cells (data not shown). The expression level of Rab7b was too low to be detected in RAW264.7 cells with the available Abs. In contrast with SP-A, the homologous complement protein Clq did not affect Rab7 and Rab7b protein levels (Fig. 1C, top panel). SP-A augmented Rab7 expression in a manner that was attenuated by collagenase (p < 0.05), but not glycosidase F treatment, implicating the collagen-like but not the N-linked sugar in that function (Fig. 1C, middle panel and quantification graph).

Subcellular fractionation was performed to investigate the effect of enzyme-treated SP-A on both Rab7 protein expression in cytosolic fractions and NF-κB activity in nuclear extracts (data not shown).

For subsequent long-term experiments, isolated LPS, previously shown to modulate Rab7b expression in human monocytes, mouse peritoneal macrophages, and RAW264.7 cells (29, 39), was used as a positive control (Fig. 1D–F). The effect of SP-A and LPS on
The expression of mature CTSD was significantly enhanced by SP-A in a dose-dependent manner \( (p < 0.05; \text{Fig. 4B}) \). Western kinetics revealed that SP-A significantly increased total CTSD in a time-dependent manner after 4 and 6 h of incubation \( (p < 0.05; \text{Fig. 4E}) \) and significantly increased mature CTSD compared with control \( (p < 0.01; \text{Fig. 4F}) \). In contrast, C1q did not affect the expression of CTSD \( (\text{Fig. 4E}) \). The combined data suggest that SP-A–enhanced Rab7b is functionally active.
**FIGURE 2.** SP-A enhances Rab7 and Rab7b protein expression. A and B, Primary rat AM adhered to chamber slides at 1 \times 10^5 cells/well and were left untreated (a, b) or treated with 20 μg/ml Alexa Fluor 555-labeled SP-A (red; c–j), 100 ng/ml LPS (m, n), or both (k, l) for the times indicated. Slides were blocked and incubated with Abs against Rab7 (green; A) and Rab7b (green; B) that were detected by Alexa Fluor 488-conjugated secondary Abs. Cells were visualized by confocal microscopy. Top panels, Differential interference contrast (DIC) images. Bottom panels, Overlay of single stainings. Images are representative of three independent experiments with similar results. Scale bars, 10 μm.

**SP-A–mediated enhancement of Rab7 is wortmannin sensitive**

To determine signaling pathways involved in SP-A–enhanced Rab7 and Rab7b protein expression, we pretreated primary AM with pharmacological inhibitors of various kinases. The physical and functional link between Rab GTPases and PI3K strongly suggest that these proteins reciprocally regulate each other (44–46). Both SP-A and LPS can activate PI3K in human monocyte-derived macrophages (47) and AM (48). Primary AM were pretreated or not pretreated with wortmannin and subsequently stimulated with SP-A, LPS, or both. Subcellular fractionation was performed to observe potential shuttling of Rab7 between the cytosol and the nucleus (data not shown) in the absence of PI3K. Pretreatment of AM with wortmannin did not significantly alter the constitutive expression of Rab7 and Rab7b (Fig. 5A, 5B) but abrogated both SP-A– and LPS-enhanced Rab7 expression, indicating a role of PI3K signaling in the enhancement of Rab7 protein by both stimuli (Fig. 5A). In contrast with LPS, SP-A–enhanced Rab7b expression was wortmannin insensitive (Fig. 5B).

**SP-A induces a wortmannin-sensitive Akt1 phosphorylation at Ser473**

The activation of the PI3K signaling pathway was assessed by detecting the phosphorylation of Akt1, a known downstream target of PI3K, at serine 473. In primary AM, SP-A induced a significant and very early Akt1 Ser473 phosphorylation, reaching a transient maximum after 3–10 min and the baseline after 60 min (Fig. 5C). LPS that has been described to induce phosphorylation of Akt in primary rat AM increased in a time-dependent manner was significant after 30 min and further increased after 60 min (Fig. 5D), suggesting that the kinetics of Akt1Ser473 phosphorylation in primary rat AM increased in a time-dependent manner was significant after 30 min and further increased after 60 min (Fig. 5D), suggesting that the kinetics of Akt1Ser473 phosphorylation in response to LPS versus SP-A in AM are temporally distinct. Both LPS- and SP-A–induced Akt1Ser473 phosphorylation was completely abolished in the presence of wortmannin (Fig. 5E), indicating that Akt1Ser473 phosphorylation by both stimuli depends on PI3K.

**Inhibition of Akt1 and p38 MAPK abolishes SP-A–enhanced Rab7 stability**

To determine the role of Akt1Ser473 phosphorylation in Rab7 expression, we pretreated AM with the Akt inhibitor 1/2 VIII before stimulation with SP-A, LPS, or both, and Western analysis for Rab7 was performed. Pretreatment of the cells with the Akt inhibitor did not affect basal Rab7 levels, but abrogated SP-A– and LPS-enhanced Rab7 availability (Fig. 5F). The combined data show that the PI3K/Akt pathway is involved in LPS- and SP-A–mediated Rab7 enhancement. Similar results were obtained using RAW264.7 cells (data not shown).

Because upregulation of Rab7 activity has been shown to involve the activation of p38 MAPK (50, 51), AM were pretreated with SB203580, a selective inhibitor of p38 activity, before the addition of SP-A, LPS, or both. Pretreatment of AM with SB203580 significantly reduced both SP-A– and LPS-enhanced Rab7 expression, whereas the basal level of Rab7 was not affected (Fig. 5G). A possible cross talk between the p38 MAPK and the PI3K/Akt pathway was investigated next. We found that Akt1Ser473 phosphorized was suppressed through inhibition of p38 MAPK (Fig. 5H), and phosphorylation of p38 MAPK was reduced through inhibition of PI3K (Fig. 5I), suggesting cross talk between the p38 and the PI3K/Akt signal transduction pathway.

**SP-A induces PKCζ/Rab7 interaction in primary AM and RAW264.7 cells**

Both LPS (52) and SP-A (38, 53) have been shown to induce the activation of atypical PKCζ that needs phosphoinositides generated by PI3K for its catalytic function (54). Evidence is increasing that activation of PKCζ by PI3K is required for many cellular responses in insulin signaling and protein synthesis (55). Relevant to this study, recent data have provided evidence that PKCζ regulates vesicle trafficking by modulating cytoskeletal organization via Rho and Rac GTPases (56, 57), and binds to and activates Rab5 (58).

SP-A, significantly and in a concentration-dependent manner, increased PKCζ/Rab7 immunoprecipitation under constitutive conditions in AM (Fig. 6A) and RAW264.7 cells (Fig. 6B). The reciprocal immunoprecipitation experiments were also performed, yielding analogous results (data not shown). In AM, PKCζ/Rab7 binding was significant after 30–90 min of SP-A treatment and then declined (Fig. 6C). Pharmacological inhibition of PKCζ using an isoform-specific, cell-permeable, inhibitory myristoylated peptide derived from the pseudosubstrate motif of atypical PKCs (PKCζ-PS) significantly inhibited SP-A–induced Rab7 protein expression, whereas basal Rab7 expression was not affected (Fig.
FIGURE 3. SP-A enhanced Rab7 is functionally active. A, Primary rat AM adhered to chamber slides at 1 × 10⁵ cells/well and were left untreated (a, b) or treated with 40 μg/ml SP-A (30 min) (c, d) or 100 ng/ml LPS (60 min) (e, f). In addition, cells were incubated with LysoTracker Red (1 μg/ml, 30 min, 37°C; LysRed; red). Rab7 was detected with an FITC-labeled Rab7 Ab (green), and nuclei were counterstained with DAPI (blue). Cells were visualized by confocal microscopy. Top panels, Differential interference contrast (DIC) images. Bottom panels, Overlay of single stainings. Images are representative of three independent experiments with similar results. Scale bars, 10 μm.

B, Primary rat AM were left untreated or treated with 40 μg/ml SP-A (30–120 min) or 20 μg/ml C1q (60 min). Equal amounts of WCLs were subjected to SDS-PAGE and immunoblotted for RILP and γ-tubulin. Representative RILP and γ-tubulin Western blots are shown. Data of four independent experiments were normalized to γ-tubulin and statistically analyzed by one-way ANOVA with Dunnett’s posttest (mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001 (versus control).

C and D, Primary rat AM were left untreated or treated (C) with different concentrations of SP-A (0–60 μg/ml) for 60 min or (D) with SP-A (40 μg/ml) for the times indicated. Rab7 was immunoprecipitated from WCLs.
For this experiment, cytosolic and nuclear (not shown here) fractions were prepared to observe an eventual cytosol-nuclear shuttling of Rab7 in the absence of PKC\(\text{z}\). These data were confirmed by PKC\(\text{z}\) siRNA transfection of RAW264.7 cells. Knockdown of PKC\(\text{z}\) in RAW264.7 cells reduced PKC\(\text{z}\) protein expression by about 55% and significantly inhibited Rab7 expression in the presence of SP-A (\(p<0.001\); Fig. 6E). Confocal microscopy confirmed an SP-A–induced colocalization of Rab7 with PKC\(\text{z}\) after 60 and 90 min in AM (Fig. 6F) and a reduction of both SP-A–enhanced Rab7 expression and Rab7/PKC\(\text{z}\) colocalization in PKC\(\text{z}\) siRNA-transfected RAW264.7 cells (Fig. 6G). The data suggest that SP-A–enhanced Rab7 stabilization occurs via PKC\(\text{z}\). In contrast, SP-A did not affect PKC\(\text{z}\) coimmunoprecipitation with Rab7b in RAW264.7 cells or primary AM within the same time frame (data not shown).

SP-A–enhanced lysosomal delivery of GFP-\textit{E. coli} in primary AM and RAW264.7 cells depends on Rab7 expression

LysoTracker selectively labels late endosomes and lysosomes (59), and colocalizes with LAMP-1 (60). The maturation of phagosomes containing GFP-\textit{E. coli} was monitored by their ability to colocalize with LysoTracker-positive compartments over time. To investigate the effect of SP-A on the trafficking of GFP-\textit{E. coli} in primary AM, we pretreated the cells with SP-A for 30 and 60 min, and subsequently infected them with GFP-\textit{E. coli} at a ratio of 1:10 (AM/\textit{E. coli} ratio). The results obtained from confocal analyses (Fig. 7Ac, f, i) and their quantification (Fig. 7B) showed that the colocalization of GFP-\textit{E. coli} with lysosomes was significantly increased from 5.7 ± 1.5% (control) to 18.5 ± 2.6% (\(p<0.01\)) and 14 ± 1.3% (\(p<0.05\)) in the presence of SP-A for 30 and 60 min, respectively. To assess the role of Rab7 in SP-A’s effect, we treated and binding of RILP to Rab7 was detected by immunoblotting with the corresponding Ab. Anti-rabbit IgG was used as a control. Representative Rab7 and RILP Western blots are shown. Data of four independent experiments were normalized to Rab7 and statistically analyzed by one-way ANOVA with Dunnett’s posttest. *\(p<0.05\); **\(p<0.01\) (versus control).
FIGURE 5. Kinases involved in SP-A–induced Rab7 and Rab7b protein expression. A and B, Primary rat AM were left untreated or treated with 100 nM wortmannin (30 min), followed by the treatment with 40 µg/ml SP-A (30 min), 100 ng/ml LPS (1 h), or both. A, Cytosolic fractions and B WCLs were subjected to SDS-PAGE and immunoblotted for (A) Rab7, (B) Rab7b, and β-actin. Data of five to seven independent experiments were normalized to β-actin. C and D, SP-A and LPS induce the phosphorylation of Akt1 at Ser473. Primary rat AM were left untreated or treated with (C) SP-A (40 µg/ml) or (D) LPS (100 ng/ml) for the times indicated. Equal amounts of WCLs were immunoblotted for phospho-Akt1 (Ser473), Akt1, and β-actin. Data of six independent experiments were normalized to β-actin. E, SP-A–induced phosphorylation of Akt1 at Ser473 is wortmannin sensitive. Primary rat AM were left untreated or treated with 100 nM wortmannin (30 min) and then exposed to 40 µg/ml SP-A (30 min), 100 ng/ml LPS (1 h), or both. Equal amounts of WCLs immunoblotted for phospho-Akt1 (Ser473), Akt1, and β-actin. Data of four independent experiments were normalized to β-actin. F and G, SP-A–induced expression of Rab7 involves Akt1 and p38. Primary rat AM were left untreated or treated with 40 µM Akt1/2-inhibitor VIII (Akt-i; 30 min) or 15 µM SB203580 (p38 inhibitor; 30 min), followed by the treatment with 40 µg/ml SP-A (30 min), 100 ng/ml LPS (1 h), or both. Equal amounts of WCLs were immunoblotted for Rab7 and β-actin. Data of four independent experiments were normalized to β-actin. H, Primary rat AM were left untreated or treated with 100 nM wortmannin (30 min) and then exposed to 40 µg/ml SP-A (30 min), 100 ng/ml LPS (1 h), or both. Equal amounts of WCLs were immunoblotted for phospho-p38 (Thr180/Tyr182).
AM with cell-permeable Rab7 blocking peptides before SP-A. Pretreatment of AM with cell-permeable Rab7 blocking peptides (15 min, 25 μg/ml) abolished the SP-A–enhanced colocalization of GFP-E. coli with Lysotracker-positive compartments at 30 (p < 0.01) and 60 min (p < 0.05; Fig. 7A, 7Aa, 7B). To further clarify the role of Rab7 in SP-A–accelerated lysosomal delivery of E. coli, we inhibited the expression of Rab7 in RAW264.7 cells by RNAi. Transient transfection of the cells with specific siRNA for Rab7 reduced the expression of Rab7 by about 70% (p < 0.001) of control (mock-transfected; Fig. 7C). Rab7 siRNA transfection abrogated SP-A–enhanced colocalization of GFP-E. coli with Lysotracker-labeled membranes after 30 (4.4 ± 0.6%; ***p < 0.01) and 60 min (3.2 ± 0.9%; ***p < 0.01; Fig. 7D, 7E). The combined data indicate that SP-A–accelerated lysosomal delivery of GFP-E. coli in primary AM and RAW264.7 cells depends on Rab7.

Rab7 blocking peptides antagonize SP-A–rescued lysosomal delivery of GFP-E. coli in primary AM from SP-A+/− mice

Mice that lack SP-A are highly susceptible to intratracheally administered pathogens (1), displaying a severe pulmonary inflammation including a significantly reduced phagocytic capacity of AM and a diminished pathogen clearance (61, 62). The administration of exogenous SP-A can restore the phenotype (63). Relevant to this study, the pulmonary clearance of E. coli K12 from SP-A−/− mice is significantly reduced compared with SP-A+/+ mice (64). In this study, we found that the constitutive Rab7 protein expression in primary AM from SP-A−/− mice was significantly lower than in AM from strain-matched wt mice (***p < 0.001; Fig. 8A). The addition of exogenous SP-A significantly enhanced Rab7 expression in AM from wt-mice (***p < 0.001) and rescued Rab7 expression in AM from SP-A−/− mice (**p < 0.01; Fig. 8A). Subcellular fractionation was performed to investigate the effect of SP-A deficiency on both Rab7 protein expression in cytosolic fractions and NF-κB activity in nuclear extracts (data not shown).

The reduced lysosomal delivery of GFP-E. coli in AM from SP-A+/− mice compared with wt controls was restored by the addition of SP-A (Fig. 8Bf, 8B, 8Cf, 8Ci). Pretreatment of the cells with Rab7 blocking peptides abrogated SP-A–enhanced colocalization of GFP-E. coli with Lysotracker-labeled compartments, indicating that Rab7 is critically involved in SP-A–enhanced lysosomal delivery of GFP-E. coli in primary AM from SP-A+/− mice (Fig. 8B, 8C).

Discussion

The human pulmonary soluble C-type lectin SP-A is a pattern recognition receptor of lung host defense with important functions in lung immune homeostasis in vivo (1, 2). Resident AM have a central role in innate immune pulmonary host defense through their ability to internalize and eliminate pathogens through distinct mechanisms including receptor-mediated endocytosis and both receptor-mediated and non-specific phagocytosis (65). Both enhanced phagocytosis of pathogens and endocytosis of endogenous surfactant lipids and proteins are key functional consequences of direct AM/SP-A interaction. In phagocytic, endocytic, and exocytic trafficking, Rab GTPases play a pivotal role by mediating vesicle formation, maturation, and transport in eukaryotic cells (16, 17). In this study, it was hypothesized that SP-A directly modulates Rab-regulated membrane trafficking in AM.

The data demonstrate a role of SP-A in modulating intracellular trafficking in AM via regulation of Rab7 and delineates biochemical pathways involved. SP-A transiently enhanced the endogenous protein, but not mRNA expression, of functionally active Rab7 and Rab7b in primary AM. The effect of SP-A was specific because neither Rab5 nor Rab11 protein levels were affected within a time frame of 24 h. Collagenase treatment of SP-A, which keeps the neck and carbohydrate recognition domain intact, abolished SP-A–mediated Rab7 increase implicating the collagen domain in that effect. In line with this observation, SP-A–augmented complement receptor-3–mediated phagocytosis has been demonstrated to be reduced by collagenase treatment of SP-A (10).

Rab5 associates with phagosomes after they seal and is thought to be essential for subsequent acquisition of Rab7, which, in turn, is necessary for phagolysosome fusion (66). The finding that SP-A, in contrast with LPS, did not affect Rab5 was surprising because membrane trafficking from the early endosome to the late endosome is determined by a sequential conversion of Rab5 into Rab7 (22). Radiometric imaging studies in CFP-Rab5 and YFP-Rab5a cotransfected RAW264.7 cells revealed a slowed Rab5 accumulation and increased Rab7 levels as early as 10 min after induction of phagocytosis (67). When we investigated this very early time frame (1–10 min) in AM, SP-A induced an apparent but not significant increase in Rab5 protein stability (data not shown). The lack of comparable Rab5 and Rab11 modulations over the time under investigation suggests that the SP-A–mediated increase in Rab7 and Rab7b does not result from an overall prolongation of Rab protein half-life. The increased levels of Rab7 and the time-matched transient PKC/Thr7 interaction observed may physically stabilize the protein and protect it from or reduce lysosomal or proteasomal degradation. Little is known about what targets Rab proteins for degradation. The short-lived GTPase Rhob is targeted to a lysosomal pathway, though this is highly specific and not considered for other endosomal GTPases, including Rab5, Rab7, Rab9, and Rab11 (68). Rab protein stabilization involves membrane association that requires a C-terminal prenylation (69), activation achieved by GDP/GTP exchange, and effector interaction (70). Thus, Rab protein modification plays a central role in regulating the function.

Recent studies have shown that various cytokines and hormones can modify Rab5 and Rab7 availability in murine and human cells (51, 71–73). The enhanced expression of Rab7 in J774E cells by muramyl-dipeptide has been shown to promote the transport of live Salmonella to lysosomes, preceding efficient killing (74). Constitutively, it has been shown that Rab5 and Rab7 expression are regulated by the isoprenoid pathway (75). However, the mechanisms of Rab gene induction, protein expression, and degradation are only beginning to be understood.

The SP-A–enhanced association of Rab7 with late endosomes/lysosomes suggests that Rab7 is active, as the GTP-bound form of Rab7 is preferentially membrane associated. In addition, SP-A transiently increased the expression of the Rab7 effector RILP, a Rab7/RILP commonprecipitation, and a Rab7/RILP colocalization in AM. The combined data demonstrate that SP-A enhances a functionally active Rab7, because RILP is thought to interact exclusively with the GTP-bound form of Rab7 (40, 76).

Data of six independent experiments were normalized to β-actin. Representative Western blots are shown. Data were statistically analyzed by one-way ANOVA with Bonferroni’s posttest (mean ± SEM). *p < 0.05; ***p < 0.01; ****p < 0.001 (versus controls); **p < 0.01; ***p < 0.001 (versus SP-A); 4p < 0.05; 8p < 0.01; ***p < 0.001 (versus LPS); p < 0.01; 1p < 0.01; 1p < 0.01 (versus SP-A + LPS).
FIGURE 6. SP-A induces binding of Rab7 to PKCζ in a dose- and time-dependent manner. Primary rat AM (A) or RAW264.7 cells (B) were left untreated or treated with different concentrations of SP-A for 60 min, or with 40 μg/ml SP-A for the times indicated (C). Atypical PKCζ was immunoprecipitated from WCLs, and binding of Rab7 to PKCζ was detected by immunoblotting with the corresponding Ab. Representative Rab7 and PKCζ Western blots are shown. Data of four to eight independent experiments were normalized to total PKCζ and statistically analyzed by one-way ANOVA with Dunnett’s posttest (mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001 (versus control).

D, Primary rat AM were left untreated or treated with 10 μM PKCζ-PS (60 min) and then exposed to 40 μg/ml SP-A (30 min). Equal amounts of cytosolic fractions were subjected to SDS-PAGE and immunoblotted for Rab7 and β-actin. Data of five independent experiments were normalized to β-actin and statistically analyzed by one-way ANOVA with Bonferroni’s posttest (mean ± SEM). *p < 0.05 (versus control); **p < 0.01 (versus SP-A).

E, RAW264.7 cells were mock-transfected or transfected with PKCζ siRNA (120 pmol/2 × 10⁶ cells) as described in Materials and Methods. Left panel, Western blot analysis of WCLs from mock-transfected and PKCζ-siRNA-transfected cells were immunoblotted for PKCζ and β-actin. Mock-transfected cells were set as 100%. Right panel, Seventy-two hours posttransfection,
Rab7b shares 50% identity and 65% similarity with Rab7; therefore, it is not an isoform of Rab7. Rab7b is expressed in monocytes, mouse peritoneal macrophages, dendritic cells, HeLa cells, RAW264.7 cells, and other nonhemopoietic cell lines (28, 29, 39). Rab7b has been described to act as a negative regulator of TLR signaling in RAW264.7 cells and murine peritoneal macrophages by promoting lysosomal degradation of TLRs (29, 30). Most recent studies on Rab7b intracellular localization and function, however, have shown that Rab7b is not involved in transport along the endocytic route, but controls cycling between endosomes and the Golgi (28). In this study, we confirmed the presence of endogenous Rab7b protein in primary AM, providing further evidence for the concept that Rab7b is ubiquitously expressed. The acte protease CTSD is synthesized as a polypeptide that is proteolytically processed into the mature protease during its transport toward lysosomes. The maturation of CTSD, a function of Rab7b, was enhanced by SP-A, demonstrating that Rab7b is functionally active. As a consequence, although not determined in this study, an enhanced proteolytic activity in the lysosome may directly contribute to SP-A–enhanced pathogen clearance.

In this study, the activation of Rab7, but not Rab7b, by SP-A involved the PI3K/Akt/PKCζ pathway. SP-A induced a transient and wortmannin-sensitive Akt1Ser473 phosphorylation in primary AM within the first minutes. This is in contrast with a previous study (77) demonstrating that SP-A does not induce an increase in Akt1Ser473 phosphorylation but inhibits LPS-induced Akt1Ser473 phosphorylation in monocyte-derived macrophages. The contrasting results could be caused by the difference in Ab specificity, the cell type under investigation (i.e., 5–d-old human MDMs versus freshly isolated rat AM), the incubation conditions (i.e., adherent versus in solution), and the time points investigated. We found that, unlike SP-A, LPS-induced Akt1Ser473 phosphorylation followed a different kinetic pattern by increasing in a time-dependent manner within 60 min. The time-delayed activation of Akt1 points to distinct activation mechanisms of this kinase by SP-A versus LPS, and consequently to distinct cellular responses. Both LPS- and SP-A–induced Akt1Ser473 phosphorylation was completely abolished in the presence of wortmannin, indicating that Akt1Ser473 phosphorylation by both stimuli requires PI3K activity. The pharmacological inhibition of Akt abolished both SP-A– and LPS-enhanced Rab7 expression, further pointing at the role of the PI3K/Akt signaling pathway in Rab7 stabilization.

Several in vitro and in vivo studies have demonstrated that phosphatidylinositol-3,4,5-trisphosphate directly activates the atypical PKCs (ξ, λs/λα) in a PI3K-dependent manner (78–80). It was shown that SP-A induces a rapid activation of PKCζ in primary rat AM (38) and a rapid membrane localization of PKCζ in human myometrial cells (53). A role in regulating vesicle trafficking has been implicated for the atypical PKCs by modulating cytoskeleton reorganization via Rho and Rac small GTPases (56, 57) or by reorganization via Rho and Rac small GTPases (56, 57) or by

The authors also found that p38 regulates the formation of a complex between GDI and Rab7 (50). In this study, the pharmacological inhibition of p38abrogated Akt1Ser473 phosphorylation, and p38-Thr180/Tyr182 phosphorylation was reduced through inhibition of PI3K, suggesting cross talk between the p38 and the PI3K/Akt signal transduction pathway in AM, as previously described to occur in other cell types (83, 84).

In this study, we found that in primary AM and RAW264.7 cells, SP-A–enhanced lysosomal delivery of GFP-E. coli was abolished by the pharmacological inhibition of Rab7 and Rab7 siRNA transfection, respectively. These data support a previously hypothesized mechanism for the collectin-promoted lysosomal fusion with Legionella-containing phagosomes resulting in growth suppression of the pathogen (32). In that study, SP-A and SP-D significantly increased the number of Legionella pneumophila colocalized with LAMP-1 in THP-1 cells (32). Another study showed that coating of Mycobacterium tuberculosis with SP-D enhances phagosome-lysosome fusion in human monocyte-derived macrophages (31).

To define the relevance of Rab7 for the phenotype of intratracheally infected SP-A–/– mice, which is, reduced phagocytosis and intracellular macrophage lysis, respectively, we performed a coimmunoprecipitation of PKCζ and Rab7 in a dose- and time-dependent manner in RAW264.7 cells. Pharmacological inhibition and transient silencing of PKCζ in RAW264.7 cells abolished the effects of SP-A on Rab7 stabilization. In line with this finding, it has been shown previously that PKCζ inhibition/knockout had a significant negative effect on SP-A–mediated signaling events (38), pointing to an essential role for PKCζ in SP-A–specific modulation of AM function. In contrast, SP-A–enhanced Rab7b stabilization was wortmannin insensitive, and coimmunoprecipitation experiments failed to show an SP-A–enhanced interaction of Rab7b with PKCζ, suggesting a signaling pathway distinct from PI3K/PKCζ for SP-A–enhanced Rab7b availability.

In this study, pretreatment of AM with SB203580 abrogated both SP-A– and LPS-enhanced Rab7 levels, suggesting that both SP-A– and LPS-induced increase in Rab7 involves the activation of p38 MAPK. TLR4 ligation has been shown previously to activate p38 and to modulate phagosome maturation toward lysosomes (82). Furthermore, the pharmacological inhibition of p38 and transfection with p38 siRNA reduces the LPS-induced phagocytosis of GFP-E. coli by RAW264.7 cells (49). Interestingly, IL-12 induces Rab7 expression in J774E cells through the activation of p38 MAPK (51). Under constitutive conditions, p38 can modulate the rate of endocytic traffic by regulating the activity of GDI on Rab proteins (20, 50), thereby affecting endocytosis in vivo (50). The authors also found that p38 regulates the formation of a complex between GDI and Rab7 (50).

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FIGURE 7. SP-A–enhanced lysosomal delivery of GFP-E. coli involves Rab7. A, Primary rat AM adhered to chamber slides at $1 \times 10^5$ cells/well and were left untreated (a–c), treated with 40 $\mu$g/ml SP-A for 30 (d–f) or 60 min (g–i), or treated with 20 $\mu$g/ml Rab7 blocking peptide (15 min) before the exposure of 40 $\mu$g/ml SP-A (30 or 60 min; j–o). Cells were further infected with GFP-E. coli (green) for 15 min, then washed and incubated for another 30 min at 37°C. Lysosomes were labeled with LysoTracker Red (1 $\mu$g/ml, 30 min, 37°C; LysRed; red). Cells were visualized by confocal microscopy. Left panels, LysoTracker staining. Middle panels, GFP-E. coli. Right panels, Overlay of single stainings. Arrows show the area of colocalization. Images shown are representative of three independent experiments with similar results. Scale bars, 10 $\mu$m.

B, Quantification of GFP-E. coli colocalization with LysRed. Values are expressed in percentages ±SEM of three independent experiments in which 50–100 cells were analyzed for each condition. Quantification of colocalization was determined by Leica TCS NT software. Data were statistically analyzed by one-way ANOVA with Bonferroni’s posttest. *p < 0.05; **p < 0.01 (versus control); ##p < 0.01 (versus 30’ SP-A); $p < 0.05$ (versus 60’ SP-A).

C, RAW264.7 cells were mock-transfected or transfected with either scrambled (scr) siRNA or Rab7 siRNA (120 pmol/10^6 cells) as described in Materials and Methods. Forty-eight hours posttransfection, WCLs were prepared and analyzed for the efficiency of silencing by Western blot using Rab7 and β-actin Abs. Mock-transfected cells were set as 100%. Data of four independent experiments are expressed as percentage of Rab7 protein expression normalized to β-actin and were statistically analyzed by one-way ANOVA.
tracellular killing of pathogens (61, 62, 64), the authors determined Rab7 levels in AM from SP-A \textsuperscript{2}/\textsuperscript{2} mice ex vivo and performed functional macrophage assays. In this study, the basal Rab7 protein expression in primary AM from SP-A \textsuperscript{2}/\textsuperscript{2} mice was significantly lower than in AM from strain-matched wt mice. The addition of exogenous SP-A significantly enhanced Rab7 expression in AM from wt mice and rescued Rab7 expression in AM from SP-A–deficient mice. Functionally, the reduced lysosomal delivery of GFP-E. coli in AM from SP-A–deficient mice was rescued by the addition of SP-A, and Rab7 blocking peptides antagonized this SP-A–rescued lysosomal delivery of GFP-E. coli. The combined data demonstrate a specific regulation of Rab activities in the alveolar compartment that consists of surfactant lining fluid and resident AM. Phagosome maturation in AM is of central importance to both the constitutive homeostasis of the innate pulmonary immune system and the response to pathogens. Divergent microbial pathogens directly target with Dunnett’s posttest. ***p < 0.001 (versus Rab7 expression in mock-transfected control cells). D, Rab7 silenced and control cells (scr siRNA) adhered to chamber slides at 1 \times 10^5 cells/well and were left untreated (a–c, j–l) or treated with 40 \mu g/ml SP-A (30 min) or 60 min (d–i). Cells were further infected with GFP-E. coli (green) for 15 min, washed, and incubated for another 30 min at 37°C. Lysosomes were labeled with LysoTracker Red (LysRed; 1 \mu g/ml, 30 min, 37°C). Cells were visualized by confocal microscopy. Left panels, LysoTracker staining. Middle panels, GFP-E. coli. Right panels, Overlay of single stainings. Arrows show the area of colocalization. Images are representative of three independent experiments with similar results. Scale bars, 10 \mu m.

FIGURE 8. SP-A–enhanced lysosomal delivery of GFP-E. coli in AM from SP-A–/– mice involves Rab7. A, Primary AM from C57BL/6J and SP-A–/– mice were left untreated or treated with 40 \mu g/ml SP-A (60 min). Cytosolic fractions were subjected to SDS-PAGE and immunoblotted for Rab7 and \beta-actin. Data of five independent experiments were normalized to \beta-actin and statistically analyzed by unpaired Student t test. **p < 0.05; ***p < 0.001 (SP-A–treated versus untreated cells). \textsuperscript{a}\textsuperscript{a}p < 0.001 (C57BL/6J versus SP-A–/– mice). Primary AM from (B) C57BL/6J and (C) SP-A–/– mice adhered to chamber slides at 1 \times 10^4 cells/well and were left untreated (a–c), treated with 40 \mu g/ml SP-A for 30 (d–f) or 60 min (g–i), or treated with 20 \mu g Rab7 blocking peptide (15 min) before the exposure to 40 \mu g/ml SP-A (30 min; j–l). Cells were further infected with GFP-E. coli (green) for 15 min, washed, and incubated for another 30 min at 37°C. Lysosomes were labeled with LysoTracker Red (LysRed; 1 \mu g/ml, 30 min, 37°C). Cells were visualized by confocal microscopy. Left panels, LysoTracker staining. Middle panels, GFP-E. coli. Right panels, Overlay of single stainings. Arrows show the area of colocalization. Images are representative of three independent experiments with similar results. Scale bars, 10 \mu m.
Rab7-dependent fusion reactions to block degradation of the pathogen-containing phagosome in the lysosome (85). However, the regulation of Rab-mediated membrane trafficking in primary immune cells is only beginning to be understood. So far, there are no upstream regulators, such as Rab escort proteins, Rab geranylgeranyl transferases, GDI, or guanine-nucleotide exchange factors identified specifically for Rab7 (86). Most recently, a Rab7-selective, GTase-activating protein has been identified (87).

Taken together, this study provides insight into a novel mechanism by which SP-A modulates endolysosomal trafficking in AM. SP-A transiently regulates the protein expression of the small GTPase Rab7 and its effector RILP, and their interaction in a PI3K/Akt/PKCζ-dependent manner, thereby enhancing the lysosomal delivery of bacteria in primary AM. Understanding the specific pulmonary C-type lectin-mediated activation mechanisms of small Rab GTPases in AM will help to develop strategies that selectively target the upmodulation or downmodulation of endocytic transport.

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Disclosures

The authors have no financial conflicts of interest.

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