

# Pulmonary Surfactant Protein A Augments the Phagocytosis of *Streptococcus pneumoniae* by Alveolar Macrophages through a Casein Kinase 2-dependent Increase of Cell Surface Localization of Scavenger Receptor A\*

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Pulmonary surfactant proteins A (SP-A) and D (SP-D), members of the collectin family, play important roles in the innate immune system of the lung. Here, we show that SP-A but not SP-D augmented phagocytosis of *Streptococcus pneumoniae* by alveolar macrophages, independent of its binding to the bacteria. Analysis of the SP-A/SP-D chimeras, in which progressively longer carboxyl-terminal regions of SP-A were replaced with the corresponding SP-D regions, has revealed that the SP-D region Gly<sup>346</sup>–Phe<sup>355</sup> can be substituted for the SP-A region Leu<sup>219</sup>–Phe<sup>228</sup> without altering the SP-A activity of enhancing the phagocytosis and that the SP-A region Cys<sup>204</sup>–Cys<sup>218</sup> is required for the SP-A-mediated phagocytosis. Acetylated low density lipoprotein significantly reduced the SP-A-stimulated uptake of the bacteria. SP-A failed to enhance the phagocytosis of *S. pneumoniae* by alveolar macrophages derived from scavenger receptor A (SR-A)-deficient mice, demonstrating that SP-A augments SR-A-mediated phagocytosis. Preincubation of macrophages with SP-A at 37 °C but not at 4 °C stimulated the phagocytosis. The SP-A-mediated enhanced phagocytosis was not inhibited by the presence of cycloheximide. SP-A increased cell surface localization of SR-A that was inhibitable by apigenin, a casein kinase 2 (CK2) inhibitor. SP-A-treated macrophages exhibited significantly greater binding of acetylated low density lipoprotein than nontreated cells. The SP-A-stimulated phagocytosis was also abolished by apigenin. In addition, SP-A stimulated CK2 activity. These results demonstrate that SP-A enhances the phagocytosis of *S. pneumoniae* by alveolar macrophages through a CK2-dependent increase of cell surface SR-A localization. This study reveals a novel mechanism of bacterial clearance by alveolar macrophages.

Pulmonary surfactant is a mixture of lipids and proteins that function to keep alveoli from collapsing during expiration (1). Surfactant protein A (SP-A)<sup>1</sup> is a major constituent of the surfactant (2). SP-A belongs to the collectin subgroup of the C-type lectin superfamily (3) along with surfactant protein D (SP-D) and mannose-binding lectin. SP-A and SP-D are believed to play important roles in the innate immune system of the lung. SP-A-deficient mice exhibit reduced bacterial clearance and elevated pulmonary inflammation in response to microbial challenge (4–6). Recent studies from this and other laboratories have revealed that SP-A and SP-D modulate lung inflammation by interacting with cell surface receptors on macrophages including CD14, Toll-like receptor 2, signal-inhibitory regulatory protein  $\alpha$ , and calreticulin/CD91 (7–10). SP-A binds to and enhances the phagocytosis of bacteria including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* by immune cells (11–14). However, the relationship between the binding to the bacteria and the phagocytic effect and the mechanism of the SP-A-mediated uptake of the bacteria are not completely understood. Macrophage scavenger receptor A (SR-A) interacts with a number of ligands including the modified lipoproteins, lipopolysaccharides, lipoteichoic acid, and Gram-negative and -positive bacteria (15, 16) and functions as a pattern recognition receptor as a mannose receptor (17). These receptors are responsible for the phagocytosis of various bacteria and are essential components in the innate immune system. The purpose of this study was to investigate the mechanism of the SP-A-mediated phagocytosis of *S. pneumoniae* by alveolar macrophages. In this report, we show that SP-A augments the SR-A-mediated phagocytosis of *S. pneumoniae* by alveolar macrophages, independent of its binding to the bacteria, and that SP-A increases cell surface localization of SR-A in a CK2-dependent manner. This study reveals a novel mechanism of bacterial clearance by alveolar macrophages.

## EXPERIMENTAL PROCEDURES

**Reagents**—Fluorescein isothiocyanate (FITC) and Alexa 594-donkey anti-goat IgG were obtained from Molecular Probes. Mannan, C1q,

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<sup>1</sup> The abbreviations used are: SP-A, surfactant protein A; SP-D, surfactant protein D; SR-A, scavenger receptor A; CK2, casein kinase 2; LDL, low density lipoprotein; AcLDL, acetylated LDL; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

cycloheximide, apigenin, PD98059, bisindolylmaleimide, and  $\beta$ -casein were purchased from Sigma. H89, acetylated low density lipoprotein (AcLDL) and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  were obtained from Seikagaku Corp., Biomedical Technologies Inc., and Amersham Biosciences, respectively. Goat anti-scavenger receptor A (anti-SR-A) antibody and rabbit anti-casein kinase 2 (anti-CK2)- $\alpha$  chain antiserum were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Surfactant Proteins and Recombinant Proteins**—SP-A and SP-D were purified from rat bronchoalveolar lavage fluids as previously described (18). Recombinant wild type SP-A, mutant SP-A<sup>R197A,K201A,K203A</sup>, SP-D, and SP-A/SP-D chimeras were expressed in a baculovirus-insect cell expression system and were purified as previously described (19–21).

**Binding of SP-A to *S. pneumoniae***—*S. pneumoniae* was heat-killed (95 °C for 10 min) and used for the experiments. The binding study was performed using *S. pneumoniae* coated onto microtiter wells. *S. pneumoniae* ( $10^6$  colony-forming units) in 40  $\mu\text{l}$  of PBS was added onto the wells, and the wells were dried under vacuum, and then the bacteria was fixed in 100  $\mu\text{l}$ /well of 0.25% (v/v) glutaraldehyde in PBS for 10 min at room temperature followed by incubation with 0.1 M glycine in PBS for 30 min. Nonspecific binding was blocked by incubating the wells with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM  $\text{CaCl}_2$ , and 0.1% (w/v) BSA (buffer A). The wells were then incubated with the indicated concentrations of SP-A or the recombinant proteins in the buffer A for 1 h at 37 °C. Next, horseradish peroxidase-labeled anti-SP-A antibody was added and incubated after the wells had been washed with PBS containing 3% (w/v) skim milk and 0.1% (v/v) Triton X-100. The amount of the proteins binding to *S. pneumoniae* was determined by measuring the absorbance at 492 nm using *o*-phenylenediamine as a substrate for the peroxidase reaction. In some experiments, 5 mM EDTA was used instead of  $\text{CaCl}_2$ .  $^{125}\text{I}$ -SP-A labeled by Bolton-Hunter reagent (22) was also used for the binding study to *S. pneumoniae* coated onto microtiter wells, and the amount of  $^{125}\text{I}$ -SP-A binding to the bacteria was determined by measuring the radioactivities using a  $\gamma$ -counter.

**Phagocytosis Assay of *S. pneumoniae* by Macrophages**—FITC labeling of heat-treated *S. pneumoniae* was performed by a method based on that described by Tino and Wright (14). A 1-ml suspension of *S. pneumoniae* in 0.1 M sodium carbonate (pH 9.0) was mixed with 1  $\mu\text{l}$  of FITC (Molecular Probes; 10 mg/ml in  $\text{Me}_2\text{SO}$ ) and stirred at room temperature for 1 h in the dark. FITC-labeled bacteria was washed four times with PBS by centrifugation at 5000 rpm for 5 min. The final pellet of FITC-bacteria obtained was suspended in PBS and stored in 100- $\mu\text{l}$  aliquots at  $-80^\circ\text{C}$ .

The phagocytosis assay was carried out using macrophages in suspension by a modification of the method described by Geertsma *et al.* (11). SR-A<sup>+/+</sup> knockout mice (SR-A<sup>-/-</sup>) are described in detail elsewhere (23). Briefly, alveolar macrophages were first isolated from Sprague-Dawley rats or C57BL/6 mice by washing the lung with 0.9% NaCl containing 1 mM EDTA and centrifuging the lavage fluids at 1,000 rpm for 10 min at 4 °C. Alveolar macrophages ( $2 \times 10^5$  in 100  $\mu\text{l}$  of Hanks' balanced salt solution) were mixed with FITC-labeled *S. pneumoniae* ( $2 \times 10^6$  colony-forming units in 100  $\mu\text{l}$  of Hanks' balanced salt solution) and incubated for 30 min at 37 °C in the dark in the absence or the presence of the indicated concentrations of SP-A. The assay was stopped by the addition of ice-cold PBS to the macrophage-bacteria suspension. The bacteria that had not been associated with the cells were separated from cell-associated bacteria by centrifugation of the suspension at 2,500 rpm for 10 min at 4 °C, and the cell pellet was washed three times with ice-cold PBS. The cells were next suspended with 5  $\mu\text{l}$  of 50  $\mu\text{g}/\text{ml}$  ethidium bromide in PBS and pipetted to a microscopic slide glass. The number of macrophages with or without intracellular (green fluorescent) bacteria were counted for at least 100 macrophages in duplicate samples using a fluorescence microscope at  $\times 400$  magnification. The results were expressed as the percentage of macrophages that contained intracellular bacteria in total macrophages counted.

Mouse-derived macrophages were used only for the phagocytosis experiments with SR-A-deficient cells. Other experiments were performed using rat macrophages.

**Casein Kinase 2 Assay**—Alveolar macrophages isolated from rats were first incubated with or without 40  $\mu\text{M}$  apigenin at 37 °C for 1 h, SP-A (20  $\mu\text{g}/\text{ml}$ ) was then added, and the cells were further incubated at 37 °C for 30 min. The cells were washed with PBS and processed for the CK2 assay.

The CK2 assay was performed by the method described by Mead *et al.* (24). Briefly, alveolar macrophages ( $1 \times 10^6$ ) were suspended in 500  $\mu\text{l}$  of phosphatase-free cell extraction buffer consisting of 10 mM Tris buffer (pH 7.05), 50 mM NaCl, 50 mM NaF, 1% (v/v) Triton X-100, 30 mM

sodium pyrophosphate, 5  $\mu\text{M}$   $\text{ZnCl}_2$ , 100  $\mu\text{M}$  sodium orthovanadate, 1 mM dithiothreitol, 2.8  $\mu\text{g}/\text{ml}$  aprotinin, 2.5  $\mu\text{g}/\text{ml}$  leupeptin, 0.5 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride. The cell suspension was vortexed for 45 s at 4 °C and centrifuged at 11,000 rpm for 10 min. The supernatant was collected as the whole cell extract and stored at  $-80^\circ\text{C}$ .

The whole cell extracts were first precleared by the addition of protein A-agarose beads (20  $\mu\text{l}$ ) and the incubation at 4 °C for 1 h, followed by centrifugation at 14,000 rpm for 5 min to remove the beads. The precleared supernatant was mixed with anti-CK2- $\alpha$  chain antiserum (2  $\mu\text{g}/\text{ml}$ ) and incubated overnight at 4 °C. The CK2-antibody immune complex was isolated by the addition of protein A-agarose beads (20  $\mu\text{l}$ ) with gentle shaking for 2 h at 4 °C. The beads binding to the immune complex were collected by centrifugation at 14,000 rpm for 5 min at 4 °C and washed once with the phosphatase-free cell extraction buffer. The pellet obtained was resuspended in 25  $\mu\text{l}$  of kinase buffer consisting of 100 mM Tris buffer (pH 8.0), 100 mM sodium orthovanadate, 100 mM NaCl, 20 mM  $\text{MgCl}_2$ , 50 mM KCl, 5  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , and 5 mg/ml  $\beta$ -casein. The mixture was then incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 10  $\mu\text{l}$  of reducing solubilizing buffer (50 mM Tris buffer (pH 6.8) containing 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol). Samples were subjected to SDS-PAGE after boiling for 10 min. After electrophoresis, the gel was fixed for 20 min in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid and dried after washing once with distilled water. The phosphorylated  $\beta$ -casein was finally visualized by autoradiography.

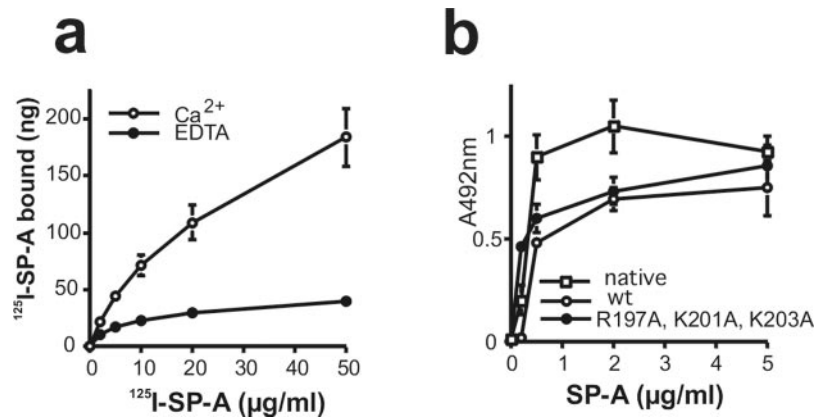
**Immunoblot Analysis**—Immunoblotting analysis was performed to examine whether the whole cell extracts under different conditions contained equal amounts of CK2. The proteins were precipitated from 10  $\mu\text{g}$  of the whole cell extracts using 4 volumes of ice-cold acetone. The precipitated proteins were subjected to SDS-PAGE, and the proteins on the gel were transferred onto polyvinylidene difluoride membrane. Nonspecific binding to the polyvinylidene difluoride membrane was blocked by the incubation with TBS (20 mM Tris buffer (pH 7.4) and 150 mM NaCl) containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20. The membrane was then incubated with anti-CK2 antiserum, followed by incubation with horseradish peroxidase-labeled goat anti-rabbit antibody. After washing the membrane with TBS containing 0.05% (v/v) Tween 20, the protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences).

**Confocal Microscope**—Alveolar macrophages ( $5 \times 10^5$ ) were suspended in Hanks' balanced salt solution and incubated with or without protein kinase inhibitors at 37 °C for 1 h and further incubated at 37 °C for 30 min on polylysine-coated coverslips in the absence or the presence of 20  $\mu\text{g}/\text{ml}$  SP-A. The cells on coverslips were washed with PBS and fixed in 4% (w/v) paraformaldehyde in PBS or in methanol at  $-20^\circ\text{C}$  for 10 min. The cells were washed with PBS containing 2% (w/v) BSA (BSA/PBS) and incubated with BSA/PBS for 30 min and then incubated with anti-SR-A antibody (2  $\mu\text{g}/\text{ml}$ ) in BSA/PBS at room temperature for 60 min. The cells were then washed three times with BSA/PBS and incubated with Alexa 594-anti-goat IgG (1:500) in BSA/PBS at room temperature for 45 min in the dark. The cells were finally washed three times with BSA/PBS and with PBS, sealed in the presence of Vectashield Antifade (Vector Laboratories), and examined using a laser microscope (LSM510; Carl Zeiss, Tokyo, Japan) with a  $\times 63$  (methanol-fixed samples) or a  $\times 20$  (for paraformaldehyde-fixed samples) oil planapochromatic lens (numerical aperture 1.4). Digital images were acquired and processed using Adobe Photoshop, version 5.0 (Mountain View, CA) and CorelDRAW software (Corel Corp.). In some experiments, alveolar macrophages were first incubated with 20  $\mu\text{g}/\text{ml}$  SP-A at 4 °C or at 37 °C for 30 min and washed with ice-cold PBS by centrifugation at 3,000 rpm for 10 min at 4 °C. The cells were then suspended with Hanks' balanced salt solution and incubated onto lysine-coated coverslips.

**Binding of  $^{125}\text{I}$ -Acetylated LDL to Alveolar Macrophages**—AcLDLs were labeled with  $^{125}\text{I}$  using Bolton-Hunter reagent (Amersham Biosciences) by the method described by Bolton and Hunter (22). The methods used in this study were adapted from those for binding of LDL to its receptor (25). Rat alveolar macrophages were cultured at a density of  $5 \times 10^5$ /well in a 24-well plate in RPMI 1640 containing 10% (v/v) fetal calf serum for 2 h. The cells were then incubated with or without SP-A (20  $\mu\text{g}/\text{ml}$ ) at 37 °C for 1 h and washed three times with 500  $\mu\text{l}$  of RPMI 1640 containing 1 mg/ml BSA. The 24-well plate was then put on ice, and the cells were incubated with the indicated concentrations of  $^{125}\text{I}$ -AcLDL in 500  $\mu\text{l}$ /well of ice-cold RPMI 1640 containing 10 mM Hepes (pH 7.4) and 10% (v/v) fetal calf serum at 4 °C for 4 h. After a 4-h incubation, the medium containing  $^{125}\text{I}$ -AcLDL was aspirated, and the cells were washed rapidly with 500  $\mu\text{l}$  of ice-cold buffer B (50 mM Tris



**FIG. 1. Binding of SP-A to *S. pneumoniae*.** Shown is binding of  $^{125}\text{I}$ -SP-A (a) or recombinant SP-As (b) to *S. pneumoniae*. *S. pneumoniae* coated onto the microtiter wells was incubated with the indicated concentrations of SP-As in the presence of  $\text{Ca}^{2+}$  (a and b) or EDTA (a) at  $37^\circ\text{C}$  for 1 h. The SP-As binding to the bacteria were determined by using a  $\gamma$ -counter (a) or by anti-SP-A antibody (b). The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples.



buffer (pH 7.4), 0.15 M NaCl, and 2 mg/ml BSA). The cell monolayer was then incubated on ice for 10 min with 500  $\mu\text{l}$  of the ice-cold buffer B. The medium was removed, and these washing steps were repeated. Next, the cell monolayer was rapidly washed with 500  $\mu\text{l}$  of ice-cold buffer containing 50 mM Tris (pH 7.4) and 0.15 M NaCl. The cells were removed from the wells by dissolution in 500  $\mu\text{l}$  of NaOH, and the amount of  $^{125}\text{I}$ -AcLDL bound to the cells was determined with a  $\gamma$ -counter.

**Flow Cytometric Analysis**—Alveolar macrophages and peritoneal macrophages isolated from rats ( $1 \times 10^6$  cells) were incubated with or without 20  $\mu\text{g/ml}$  SP-A at  $37^\circ\text{C}$  for 30 min. The cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 10 min. After fixation, the macrophages were collected by centrifugation at  $150 \times g$  for 10 min and washed once with PBS. The fixed macrophages were incubated with anti-SR-A antibody (Santa Cruz Biotechnology) or control goat IgG in PBS containing 5 mg/ml BSA and 10 mM sodium azide, followed by the incubation with FITC-conjugated anti-goat IgG. After another washing, the labeled macrophages were analyzed by using FACSCalibur and CellQuest software (BD Biosciences). In some experiments, rat alveolar macrophages were treated with 0.1% saponin at  $4^\circ\text{C}$  for 30 min after paraformaldehyde fixation, and total SR-A expression was analyzed as described above.

## RESULTS

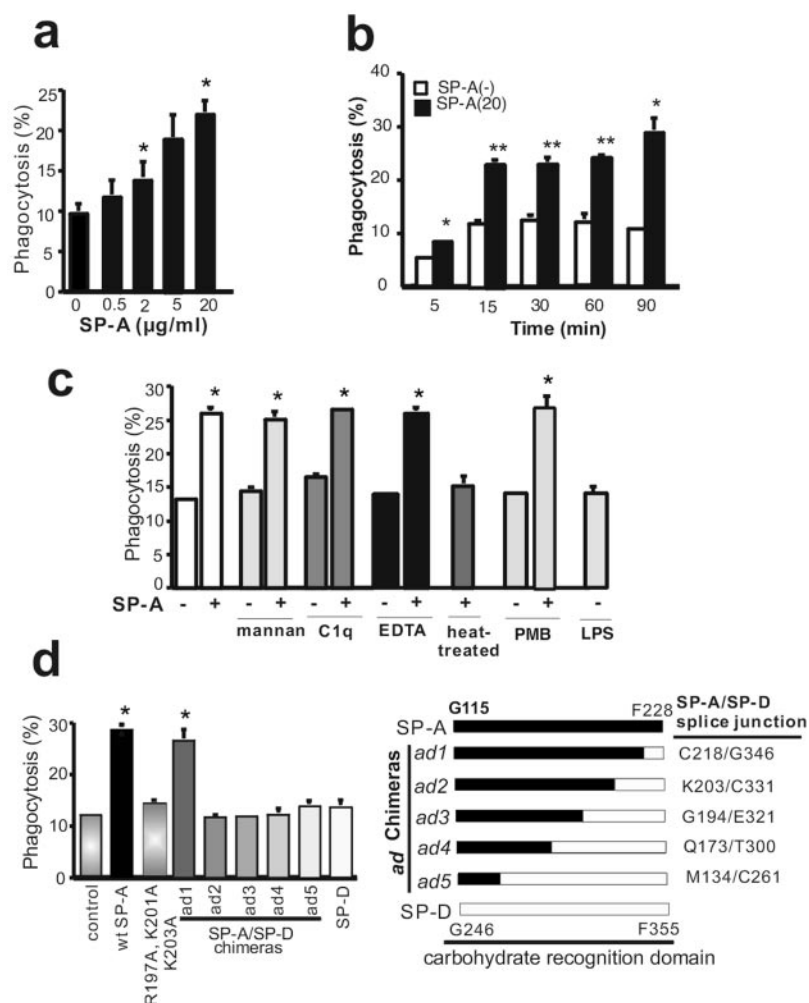
**Binding of SP-A to *S. pneumoniae***—We first examined whether SP-A bound to *S. pneumoniae* using  $^{125}\text{I}$ -labeled SP-A.  $^{125}\text{I}$ -SP-A exhibited a concentration-dependent binding to *S. pneumoniae* (Fig. 1a). Inclusion of 5 mM EDTA in the binding buffer inhibited the binding of SP-A to the bacteria, indicating that the binding of SP-A to *S. pneumoniae* is  $\text{Ca}^{2+}$ -dependent. Recombinant wild type SP-A as well as native SP-A showed a concentration-dependent and saturable binding to *S. pneumoniae* (Fig. 1b). The mutant SP-A<sup>R197A,K201A,K203A</sup> exhibited a binding comparable with that of wild type SP-A.

**SP-A Enhances the Phagocytosis of *S. pneumoniae* by Alveolar Macrophages**—The effect of SP-A on the phagocytosis of *S. pneumoniae* by alveolar macrophages was studied. The presence of SP-A significantly increased the phagocytosis of the bacteria in a manner dependent upon SP-A concentrations (Fig. 2a). SP-A augmented the phagocytosis in a time-dependent fashion (Fig. 2b). Only 5-min incubation of macrophages with SP-A significantly increased the uptake of the bacteria. At the end of the phagocytosis assay, the viabilities of alveolar macrophages were determined. No differences in the viabilities were found in the absence or the presence of SP-A ( $90.3 \pm 1.2$  versus  $89.8 \pm 0.4\%$  (means  $\pm$  S.E.),  $n = 3$ ). This result rules out a possibility that the stimulation of the phagocytosis by SP-A is due to the effect of SP-A on the macrophage viability. Excess mannan, C1q, or EDTA failed to alter the SP-A-stimulated phagocytosis of *S. pneumoniae* (Fig. 2c). This suggests that mannose receptor or C1q receptor is not involved in the SP-A-mediated enhanced phagocytosis of *S. pneumoniae*. The results also indicate that the augmentation of the phagocytosis by SP-A is independent of the binding of SP-A to *S. pneumoniae*,

since the addition of EDTA that inhibited SP-A binding to *S. pneumoniae* showed no effect on the uptake enhanced by SP-A. In addition, SP-A retained the activity of enhancing the phagocytosis in the presence of polymyxin B. Neither the heat-treated SP-A nor lipopolysaccharide stimulated the phagocytosis (Fig. 2c). These results rule out the possibility that the enhanced phagocytosis is due to the endotoxin contamination in the SP-A preparation. We also examined whether another collectin SP-D, a structural homologue to SP-A, enhanced the uptake of *S. pneumoniae* by alveolar macrophages. Unlike SP-A, SP-D failed to augment the phagocytosis of the bacteria (Fig. 2d). Thus, the effect of SP-A/SP-D chimeras, in which progressively longer carboxyl-terminal regions of SP-A were replaced with the corresponding SP-D regions, was examined to identify the SP-A region required for augmentation of the phagocytosis (Fig. 2d). Only the chimera ad1, containing the extreme carboxyl terminus of SP-D, and not the chimeras ad2–ad5 exhibited the enhanced phagocytosis, indicating the importance of the carbohydrate recognition domain in the SP-A-stimulated phagocytosis of *S. pneumoniae*. The data clearly show that the SP-D region Gly<sup>346</sup>–Phe<sup>355</sup> can be substituted for the SP-A region Leu<sup>219</sup>–Phe<sup>228</sup> without altering the SP-A activity of enhancing the phagocytosis. The results also suggest that the SP-A region Cys<sup>204</sup>–Cys<sup>218</sup> is required for the SP-A-mediated phagocytosis, since the chimera ad2 failed to augment the uptake of the bacteria. In addition, SP-A<sup>R197A,K201A,K203A</sup> did not enhance the phagocytosis of *S. pneumoniae* (Fig. 2d). Because this mutant avidly bound to *S. pneumoniae* at a level comparable with that of wild type SP-A (Fig. 1a), taken together with the results obtained from the experiments with EDTA, these results demonstrate that SP-A augments the phagocytosis of *S. pneumoniae* by alveolar macrophages, independent of its binding to the bacteria.

**SP-A Augments Scavenger Receptor A-mediated Phagocytosis of *S. pneumoniae***—When the phagocytosis assay was performed in the presence of 0–100  $\mu\text{g/ml}$  AcLDL, the increasing concentrations of AcLDL significantly reduced the uptake of *S. pneumoniae* by alveolar macrophages (Fig. 3a). SP-A exhibited almost no stimulatory effect on the phagocytosis in the presence of 20 and 100  $\mu\text{g/ml}$  AcLDL, suggesting that SP-A enhances SR-A-mediated phagocytosis. Consistent with these results, SP-A failed to stimulate the phagocytosis of *S. pneumoniae* by alveolar macrophages derived from SR-A<sup>−/−</sup> mice (Fig. 3b). These results demonstrate that SP-A stimulates SR-A-mediated phagocytosis of *S. pneumoniae*.

When FITC-labeled *S. pneumoniae* was incubated with rat alveolar macrophages at  $4^\circ\text{C}$  in the absence or the presence of fucoidan and mannan, fucoidan but not mannan significantly decreased the binding of the bacteria to macrophages (Fig. 3c). Since fucoidan is a ligand for SR-A (16), these results indicate that *S. pneumoniae* binds to SR-A. The effects of SP-A on the



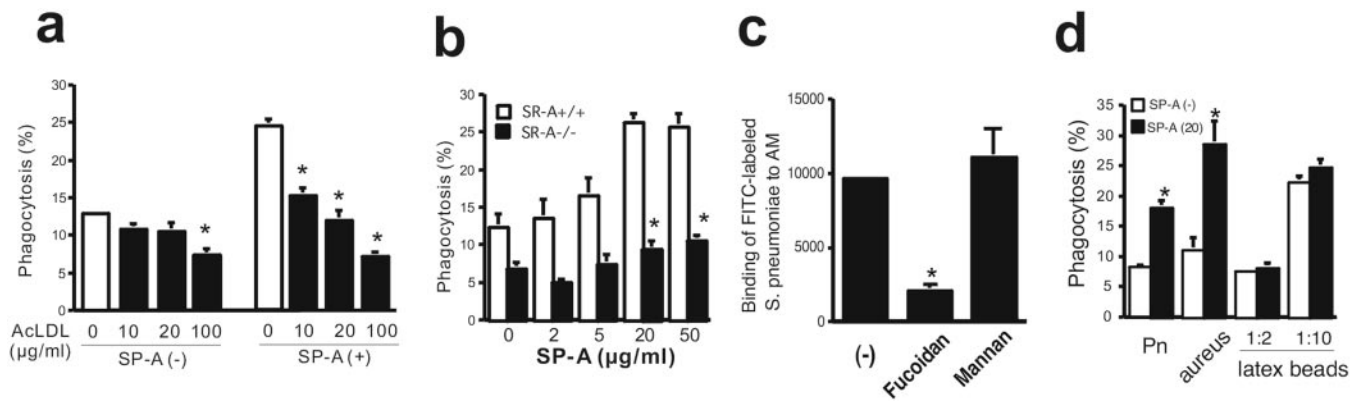
**FIG. 2. SP-A augments the phagocytosis of *S. pneumoniae* by rat alveolar macrophages.** *a* and *b*, rat alveolar macrophages ( $2 \times 10^5$ ) were incubated with FITC-labeled *S. pneumoniae* at 37 °C for 30 min (*a*) or for various periods (*b*) in the presence of the indicated concentrations of SP-A. After washing the cells to remove unbound bacteria, the cells were suspended with ethidium bromide solution, and the numbers of macrophages with or without intracellular bacteria were counted. The results are expressed as the percentage of macrophages containing intracellular bacteria in total macrophages counted (Phagocytosis (%)), as described under "Experimental Procedures." The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , when compared with the phagocytosis without SP-A. *c*, the suspension of rat alveolar macrophages and FITC-labeled *S. pneumoniae* was incubated with or without mannan (4 mg/ml), C1q (50 µg/ml), 5 mM EDTA or polymyxin B (PMB; 25 µg/ml) at 37 °C for 30 min in the absence or the presence of 20 µg/ml SP-A or heat-treated SP-A or 100 ng/ml lipopolysaccharide (LPS). The numbers of macrophages with or without intracellular bacteria were counted, and the results are expressed as percentage of phagocytosis, as described under "Experimental Procedures." The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$ , when compared with the phagocytosis without SP-A. *d*, alveolar macrophages were incubated with or without (control) 20 µg/ml wild type SP-A, the mutant SP-A<sup>R197A,K201A,K203A</sup>, SP-A/SP-D chimeras, or SP-D in the presence of FITC-labeled *S. pneumoniae* at 37 °C for 30 min. The percentage of phagocytosis was determined as described above. The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$ , when compared with control phagocytosis. The right panel shows schematic representation of the carbohydrate recognition domains of SP-A/SP-D chimeras.

phagocytosis of FITC-labeled *Staphylococcus aureus*, an SR-A ligand (15), and of FITC-labeled latex beads were also examined. SP-A significantly increased the uptake of *S. aureus* as well as *S. pneumoniae* by rat alveolar macrophages (Fig. 3d). However, SP-A did not enhance the phagocytosis of latex beads. Taken together, these results are consistent with the conclusion that SP-A augments SR-A-mediated phagocytosis. It is unlikely that SP-A stimulates the general phagocytosis phenomenon.

**Requirement of SP-A-Macrophage Interaction**—Since the SP-A-mediated phagocytosis of *S. pneumoniae* is independent of its bacterial binding, alveolar macrophages were first preincubated with SP-A at 37 °C for various lengths of time, the medium containing SP-A was removed, and the cells were washed. The cells were then incubated with FITC-labeled *S. pneumoniae*. The alveolar macrophages preincubated with SP-A still exhibited the enhanced phagocytosis even after the cells had been washed (Fig. 4a). The longer the preincubation

with SP-A, the greater the uptake of *S. pneumoniae*. However, preincubation at 4 °C failed to stimulate the uptake in the presence of SP-A (Fig. 4b). The basal uptake of the bacteria by peritoneal macrophages in the absence of SP-A is significantly greater than that by alveolar macrophages (Fig. 4c). Peritoneal macrophages did not exhibit stimulated phagocytosis in response to SP-A. The effect of cycloheximide on the phagocytosis was also examined. SP-A still enhanced the uptake of the bacteria by alveolar macrophages in the presence of 10 µg/ml cycloheximide (Fig. 4d), suggesting that new protein synthesis is not involved in the SP-A effect.

**SP-A Increases Cell Surface Localization of Scavenger Receptor A on Alveolar Macrophages**—The effect of SP-A on cell surface expression of SR-A was next examined. Alveolar macrophages were preincubated with SP-A, and the cells were processed for immunochemistry with anti-SR-A antibody after fixation in paraformaldehyde. Incubation of SP-A with the



**FIG. 3. SP-A augments scavenger receptor A-mediated phagocytosis of *S. pneumoniae*.** *a*, rat alveolar macrophages and FITC-labeled *S. pneumoniae* were incubated with or without 20  $\mu$ g/ml SP-A in the absence or the presence of the indicated concentrations of AcLDL at 37  $^{\circ}$ C for 30 min. The numbers of macrophages with or without intracellular bacteria were counted, and the results are expressed as percentage of phagocytosis, as described under "Experimental Procedures." The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$ , when compared with the phagocytosis without AcLDL. *b*, alveolar macrophages isolated from C57BL/6 mice (SR-A<sup>+/+</sup>) and scavenger receptor A-deficient mice (SR-A<sup>-/-</sup>) were incubated with FITC-labeled *S. pneumoniae* in the presence of the indicated concentrations of SP-A. The percentage of phagocytosis was determined as described above. The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$ , when compared with the phagocytosis by SR-A<sup>+/+</sup>. *c*, binding of *S. pneumoniae* to rat alveolar macrophages. Rat alveolar macrophages ( $1 \times 10^5$ ) were seeded onto 96-well microtiter plate and cultured at 37  $^{\circ}$ C for 2 h. The macrophages were then preincubated at 4  $^{\circ}$ C for 2 h with or without 500  $\mu$ g/ml fucoidan or 4 mg/ml mannan and further incubated at 4  $^{\circ}$ C for 4 h with FITC-labeled *S. pneumoniae* ( $2 \times 10^6$  colony-forming units). After washing the cells with ice-cold PBS, fluorescence intensity was measured by a fluorescence plate reader (Synergy HT; BIO-TEK). The data shown are the means  $\pm$  S.E. from three separate experiments. \*,  $p < 0.001$ , when compared with the control binding (-). *d*, effect of SP-A on the phagocytosis of *S. aureus* and latex beads. Rat alveolar macrophages ( $2 \times 10^5$ ) and FITC-labeled *S. pneumoniae*, FITC-labeled *S. aureus* ( $2 \times 10^6$  colony-forming units), or FITC-labeled latex beads ( $4 \times 10^5$ , 1:2;  $2 \times 10^6$ , 1:10) (Fluoresbrite Carboxylate Microsphere, 1  $\mu$ m; Polysciences Inc.) were incubated with or without 20  $\mu$ g/ml SP-A at 37  $^{\circ}$ C for 30 min. The numbers of macrophages with or without intracellular bacteria or beads were counted, and the results are expressed as percentage of phagocytosis, as described under "Experimental Procedures." The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$ , when compared with the phagocytosis in the absence of SP-A.

macrophages clearly increased the expression of SR-A (Fig. 5a). Observation of the immunostained cells that had been fixed in methanol revealed that SP-A induced translocation of SR-A from cytoplasmic vesicles to plasma membrane. Consistent with these results, the binding of acetylated LDL, an SR-A ligand, to alveolar macrophages that had been preincubated with SP-A was significantly increased (Fig. 5b). These data support the concept that SP-A increases cell surface localization of SR-A.

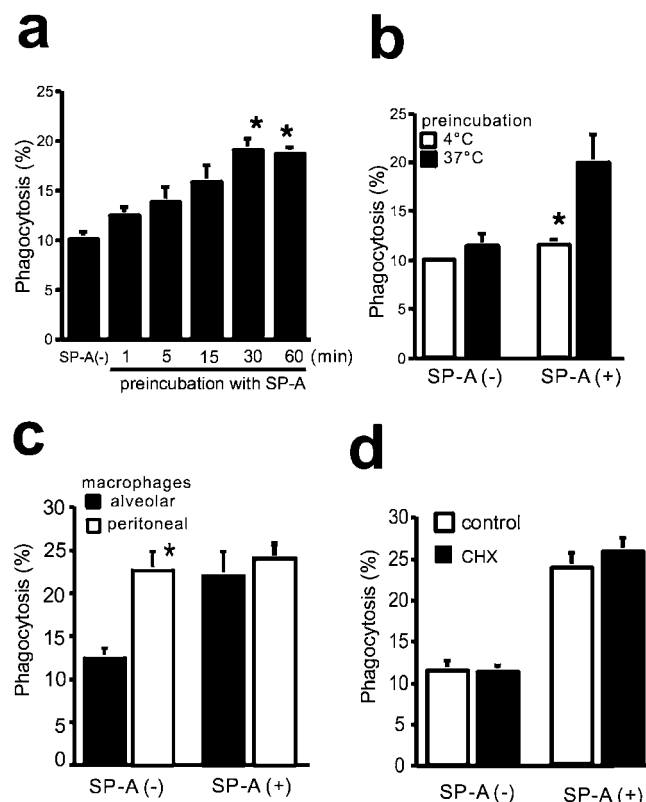
SP-A still increased cell surface localization of SR-A even in the presence of cycloheximide (Fig. 5c). In addition, preincubation of the cells with SP-A at 4  $^{\circ}$ C failed to enhance the immunostaining of SR-A (Fig. 5c). These results correlate well with those obtained from the phagocytosis assay (Fig. 4, *b* and *d*) and suggest that SP-A stimulates the phagocytosis and the cell surface SR-A localization by the mechanism that is temperature-dependent but is not involved in the new protein synthesis.

We further assessed the cell surface expression of SR-A on alveolar and peritoneal macrophages by flow cytometry. SR-A was constitutively expressed on cell surfaces of alveolar macrophages (Fig. 6a, gray line). After exposure of 20  $\mu$ g/ml SP-A for 30 min, cell surface expression of SR-A on alveolar macrophages was enhanced (Fig. 6a, solid black line). When the mean fluorescence intensity was calculated as a ratio to control, SP-A significantly increased the mean fluorescence intensity ratio (Fig. 6a, inset). These results support the conclusion obtained from confocal microscopy that SP-A increases cell surface SR-A localization on alveolar macrophages. However, the flow cytometric analysis of peritoneal macrophages revealed that SP-A failed to stimulate cell surface expression of SR-A (Fig. 6b). In addition, the mean fluorescence intensity ratio of peritoneal macrophages without SP-A treatment was higher than that of untreated alveolar macrophages (Fig. 6, *a* and *b*, insets), indicating the cell surface expression of SR-A on untreated peritoneal macrophages is stronger than that on untreated alveolar macrophages. These results are consistent

with those obtained from the phagocytosis assay by peritoneal macrophages (Fig. 4c), showing that the percentage of phagocytosis without SP-A by peritoneal macrophages is greater than that by alveolar macrophages and that SP-A did not stimulate the phagocytosis by peritoneal macrophages. In order to examine total SR-A expression in alveolar macrophages, the cells were treated with saponin after paraformaldehyde fixation, and the flow cytometric analysis was performed (Fig. 6c). The fluorescence intensity in the saponin-treated cells was clearly increased when compared with that in the saponin-untreated cells, and total SR-A expression was not altered in the cells with or without SP-A exposure. In addition, immunoblotting analysis of the whole cell lysate of macrophages with SR-A antibody showed that total content of SR-A protein in the cells with SP-A exposure was not different from that in the cells without SP-A exposure (Fig. 6d). These results indicate that SP-A did not affect total SR-A expression in macrophages. Taken together, these results support the conclusion that SP-A increases cell surface expression of SR-A but not total SR-A expression in alveolar macrophages and are consistent with the idea that new protein synthesis is not involved (see Figs. 4d and 5c).

The effects of protein kinase inhibitors were next examined to determine whether protein phosphorylation is involved in the stimulatory activity of SP-A on the phagocytosis and the cell surface localization of SR-A. The increased SR-A cell surface localization by SP-A was not blocked in the presence of PD98059, bisindolylmaleimide, and H89 (Fig. 7a), which inhibit mitogen-activated protein kinase kinase, protein kinase C, and protein kinase A, respectively. In contrast, apigenin, a selective CK2 inhibitor, abolished the SP-A-mediated enhancement of cell surface SR-A localization. We attempted to quantify fluorescence intensity in alveolar macrophages after subtracting background intensity from the intensity of the cells in each image. No difference in fluorescence intensity was observed in the absence or the presence of SP-A when apigenin was coin-

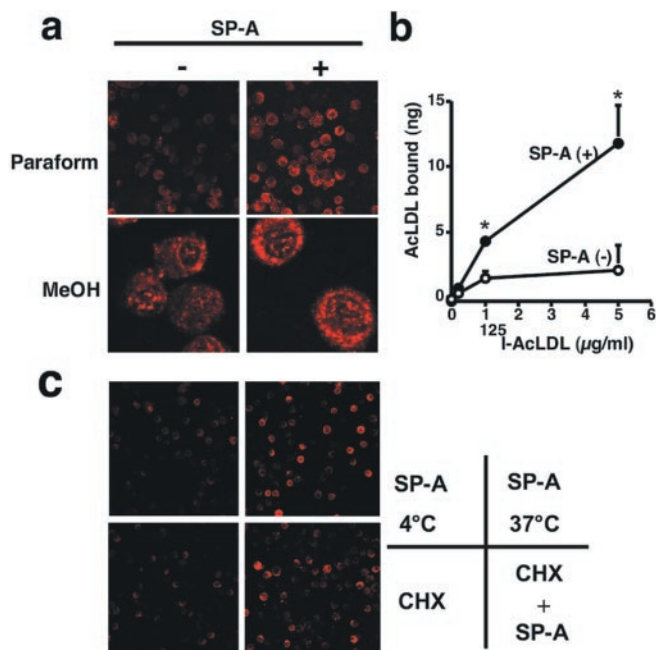




**FIG. 4. Effects of preincubation with SP-A, temperature, cell type, and cycloheximide on SP-A-stimulated phagocytosis.** *a*, rat alveolar macrophages were first preincubated with 20  $\mu$ g/ml SP-A for the indicated length of time, the medium containing SP-A was removed, and the cells were washed. The cells were then incubated with FITC-labeled *S. pneumoniae* at 37 °C for 30 min. The numbers of macrophages with or without intracellular bacteria were counted, and the results are expressed as percentage of phagocytosis, as described under "Experimental Procedures." The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$  when compared with the phagocytosis without SP-A preincubation (-). *b*, preincubation of rat alveolar macrophages with 20  $\mu$ g/ml SP-A was performed at 4 °C or 37 °C for 30 min. The cells were then washed and further incubated at 37 °C with FITC-labeled *S. pneumoniae*, and percentage of phagocytosis was determined as described above. The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.05$ , when compared with the phagocytosis after 37 °C preincubation with SP-A. *c*, alveolar macrophages or peritoneal macrophages ( $2 \times 10^5$ ) isolated from rats were incubated with or without 20  $\mu$ g/ml SP-A in the presence of FITC-labeled *S. pneumoniae*, and percentage of phagocytosis was determined as described above. The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples. \*,  $p < 0.02$ , when compared with the phagocytosis without SP-A by alveolar macrophages. *d*, cycloheximide (10  $\mu$ g/ml) was first incubated with rat alveolar macrophages at 37 °C for 1 h, FITC-labeled *S. pneumoniae* was added, and the suspension of macrophages and bacteria was further incubated in the presence or the absence of 20  $\mu$ g/ml SP-A at 37 °C for 30 min. The percentage of phagocytosis was determined as described above. The data shown are the means  $\pm$  S.E. from three separate experiments with duplicate samples.

cubated with the cells (Fig. 7*b*). However, SP-A significantly increased the fluorescence intensity in the control and in the experiments with PD98059, bisindolylmaleimide, and H89.

Likewise, apigenin also inhibited the SP-A-stimulated phagocytosis in a concentration-dependent fashion (Fig. 7*c*). SP-A still enhanced the phagocytosis in the presence of PD98059 and H89, although bisindolylmaleimide somewhat augmented the basal level of phagocytosis. Taken together, these results indicate that protein phosphorylation by CK2 is involved in the increased cell surface localization of SR-A and in the enhanced phagocytosis of *S. pneumoniae* that have been caused by SP-A. In addition, the results also suggest the idea that SP-A stimulates CK2 activity.



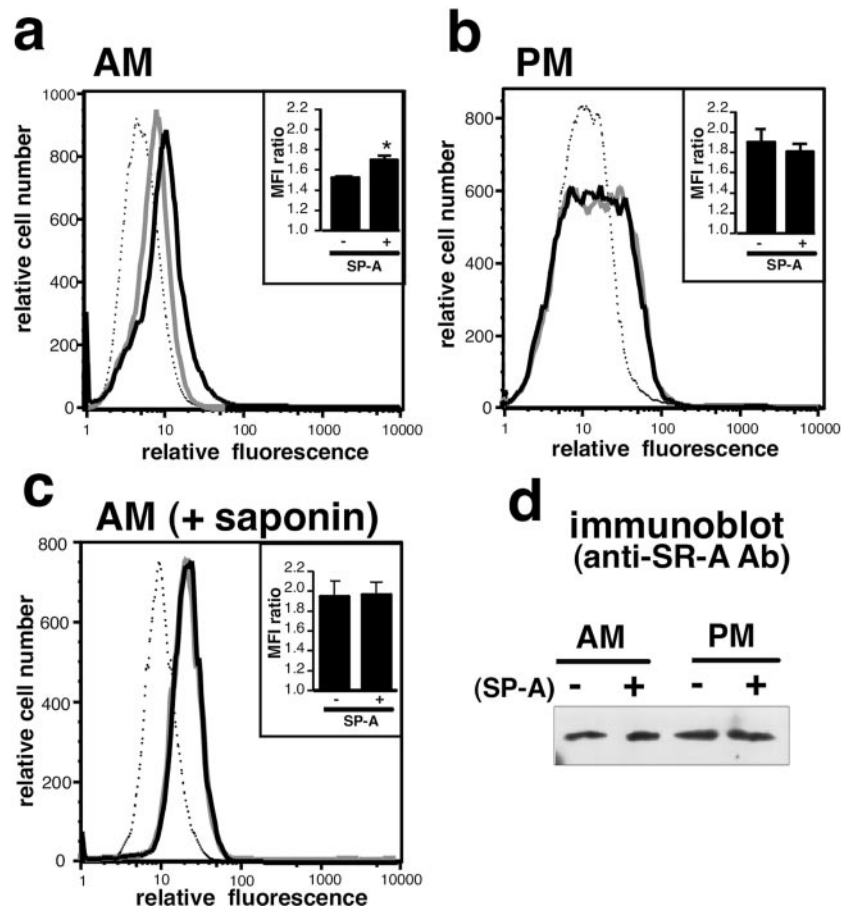
**FIG. 5. SP-A increases localization of scavenger receptor A on alveolar macrophage cell surface.** *a*, rat alveolar macrophages were preincubated with or without 20  $\mu$ g/ml SP-A at 37 °C for 30 min on polylysine-coated coverslips, and the cells were then washed with PBS and fixed in 4% (w/v) paraformaldehyde (Paraform) or methanol (MeOH). SR-A was detected by anti-SR-A antibody and analyzed using a confocal microscope. *b*, binding of acetylated LDL to rat alveolar macrophages. The indicated concentrations of  $^{125}$ I-AcLDL were incubated at 4 °C for 4 h with rat alveolar macrophages ( $5 \times 10^5$ ) that had been preincubated with or without 20  $\mu$ g/ml SP-A at 37 °C for 1 h and had been washed with the ice-cold buffer. The amounts of  $^{125}$ I-AcLDL binding to the cells were finally determined using a  $\gamma$ -counter. The data shown are the means  $\pm$  S.E. of three separate experiments. \*,  $p < 0.01$ , when compared with the AcLDL binding to the cells preincubated without SP-A. *c*, cell surface localization of SR-A on macrophages. In the upper panels, rat alveolar macrophages were preincubated with 20  $\mu$ g/ml SP-A at 4 or 37 °C for 30 min and were further incubated on polylysine-coated coverslips at 37 °C for 30 min after washing the cells with PBS. In the lower panels, the cells were first preincubated with 10  $\mu$ g/ml cycloheximide (CHX) at 37 °C for 1 h and then further incubated with or without 20  $\mu$ g/ml SP-A. The cells were fixed in 4% (w/v) paraformaldehyde and immunostained with anti-SR-A antibody and were analyzed using a confocal microscope.

**SP-A Stimulates Casein Kinase 2 Activity**—Thus, we examined whether SP-A stimulated CK2 activity. Immunoblotting analysis revealed that the cell extracts of alveolar macrophages under different conditions contained almost equal levels of CK2 protein (Fig. 8). Next, *in vitro* casein kinase activity was examined by using immunoprecipitated CK2 and  $\beta$ -casein.  $\beta$ -Casein was phosphorylated only when macrophages were preincubated with SP-A. These results clearly demonstrate that SP-A stimulates CK2 activity. Incubation of macrophages with apigenin completely abolished the CK activity in the presence of SP-A. These results support the idea that the protein phosphorylation caused by SP-A-stimulated CK2 increases cell surface SR-A localization and *S. pneumoniae* phagocytosis.

#### DISCUSSION

The present study shows that SP-A augments the scavenger receptor A (SR-A)-mediated phagocytosis of *S. pneumoniae* by alveolar macrophages, independent of its binding to the bacteria, and that SP-A increases cell surface localization of SR-A in a manner dependent upon the CK2 activity. This study reveals a novel mechanism of bacterial clearance by alveolar macrophages.

A previous study (11) has shown that C1q inhibits the binding of SP-A to monocytes and the SP-A-enhanced uptake of

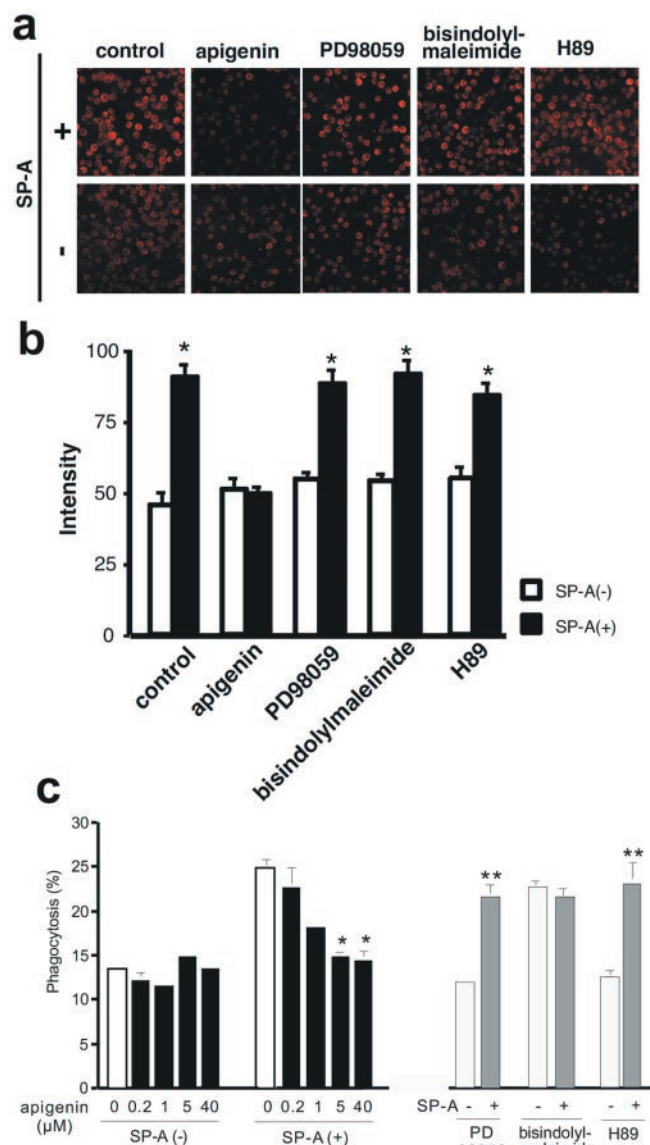


**FIG. 6. SP-A increases cell surface expression of SR-A on alveolar macrophages but not on peritoneal macrophages.** *a* and *b*, rat alveolar macrophages ( $1 \times 10^6$ ) (*a*, AM) and peritoneal macrophages (*b*, PM) were incubated with or without 20  $\mu\text{g/ml}$  SP-A at 37 °C for 30 min. After washing, the cells were fixed with 4% paraformaldehyde and were incubated with anti-SR-A antibody, followed by incubation with FITC-conjugated anti-goat IgG. The stained cells were analyzed by flow cytometry. The histograms shown are representatives from three experiments. The solid black line shows cytometric analysis of the SP-A-treated cells, and the gray line shows the untreated cells. The dotted line shows the control without the first antibody. The insets indicate the mean fluorescence intensity ratio to the control. The data shown are the mean  $\pm$  S.E. from three separate experiments. \*,  $p < 0.05$ , when compared with SP-A (-). *c*, total SR-A expression in rat alveolar macrophages (AM (+ saponin)). Alveolar macrophages were incubated with or without 20  $\mu\text{g/ml}$  SP-A at 37 °C for 30 min. The cells were treated with 0.1% saponin after fixation with 4% paraformaldehyde. Total SR-A expression in alveolar macrophages was then analyzed as described above. *d*, immunoblot analysis of macrophages with anti-SR-A antibody. Alveolar macrophages (AM) ( $2 \times 10^6$ ) and peritoneal macrophages (PM) from rats were incubated at 37 °C for 30 min with (+) or without (-) 20  $\mu\text{g/ml}$  SP-A. The macrophages were washed, and the whole cell extracts were obtained by using the lysis buffer containing 1% (v/v) Triton X-100, as described for the CK2 assay. 5  $\mu\text{g/lane}$  of the cell extract was analyzed with anti-SR-A antibody and horseradish peroxidase-conjugated anti-goat IgG, as described under "Experimental Procedures."

*S. aureus* by monocytes, indicating that the binding of SP-A to C1q receptor mediates phagocytosis of the bacteria by monocytes. However, another (14) and the present (see Fig. 2c) studies have revealed that C1q failed to attenuate the SP-A-stimulated phagocytosis by alveolar macrophages. This may imply that the stimulatory effect of SP-A on phagocytosis was cell type-specific.

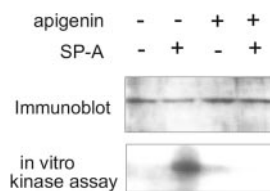
Although SP-A does not stimulate the uptake of *S. pneumoniae* by peritoneal macrophages, the level of phagocytosis without SP-A by peritoneal macrophages is significantly greater than that by alveolar macrophages (see Fig. 4c). This corresponds to the results obtained from flow cytometric analysis of cell surface SR-A expression (see Fig. 6, *a* and *b*, insets). Fluorescence intensity obtained for peritoneal macrophages without SP-A treatment is stronger than that for alveolar macrophages. In addition, the phagocytosis without SP-A by peritoneal macrophages is at the level almost equivalent to that with SP-A by alveolar macrophages (see Fig. 4c). Thus, it is possible to assume that the nonstimulatory effect of SP-A on the phagocytosis by peritoneal macrophages is due to the saturated cell surface expression of SR-A on peritoneal macrophages.

EDTA significantly inhibits the binding of SP-A to *S. pneumoniae* but does not completely block its binding (see Fig. 1a). Thus, there is a possibility that the failure of inhibiting the SP-A-stimulated phagocytosis by EDTA (see Fig. 2c) is due to the weak binding of SP-A to the bacteria in the presence of EDTA still affecting the phagocytosis. However, the preincubation experiments (see Fig. 4, *a* and *b*) clearly demonstrate that the stimulatory effect of SP-A on the phagocytosis is independent of its binding to the bacteria. In addition, the mutant SP-A<sup>R197A,K201A,K203A</sup>, which exhibits comparable binding to the bacteria, fails to stimulate the phagocytosis by alveolar macrophages (see Figs. 1b and 2d). These results also support the conclusion that the phagocytosis and the bacterial binding are independent processes. Thus, it is likely that SP-A does not serve as an opsonin. In contrast, SP-A has been shown to bind *S. aureus* and induce C1q receptor-mediated phagocytosis by monocytes (11) and to bind to the capsular polysaccharides of *K. pneumoniae* and to a putative SP-A receptor on macrophages (13), suggesting an opsonic activity of SP-A. SP-A also enhances the phagocytosis of sheep erythrocytes opsonized with IgG or IgM and complement by monocytes and monocyte-derived macrophages (26), indicating that SP-A augments FcR-



**FIG. 7. Apigenin inhibits the SP-A-mediated increase of cell surface SR-A localization and the SP-A-mediated augmentation of *S. pneumoniae* phagocytosis.** *a*, rat alveolar macrophages were preincubated at 37 °C for 1 h in the absence (control) or the presence of apigenin (40 μM), PD98059 (50 μM), bisindolylmaleimide (1 μM), or H89 (20 μM) and were further incubated with or without 20 μg/ml SP-A at 37 °C for 30 min on polylysine-coated coverslips. The cells were then washed with PBS, fixed in 4% (w/v) paraformaldehyde, and immunostained with anti-SR-A antibody and were analyzed using a confocal microscope. *b*, fluorescence intensity in confocal microscopic images. Fluorescence intensity of alveolar macrophages was quantified in confocal microscopic images after subtracting background intensity from the intensity of the cells in each image. Intensity of more than 50 macrophages was determined. The data shown are the means ± S.E. of counted macrophages. \*,  $p < 0.01$ , when compared with the experiments in the absence of SP-A. *c*, rat alveolar macrophages were preincubated at 37 °C for 1 h with apigenin (0–40 μM), PD98059 (50 μM), bisindolylmaleimide (1 μM), or H89 (20 μM) and were further incubated with FITC-labeled *S. pneumoniae* in the absence or the presence of 20 μg/ml SP-A at 37 °C for 30 min. The numbers of macrophages with or without intracellular bacteria were counted, and the results are expressed as percentage of phagocytosis, as described under "Experimental Procedures." The data shown are the means ± S.E. from three separate experiments with duplicate samples. \*,  $p < 0.01$ , when compared with the phagocytosis without apigenin in the presence of SP-A. \*\*,  $p < 0.01$ , when compared with the phagocytosis without SP-A.

and CR1-mediated phagocytosis. SP-A may increase the phagocytosis by two mechanisms, one of which is by serving as an opsonin and the other by activating macrophages.



**FIG. 8. SP-A stimulates casein kinase 2 activity.** Rat alveolar macrophages were first incubated with or without 40 μM apigenin at 37 °C for 1 h and were further incubated at 37 °C for 30 min in the absence or the presence of 20 μg/ml SP-A. After washing the cells, the whole cell lysate was isolated, and 10 μg of the cell lysate was analyzed by immunoblotting with anti-CK2-α chain antiserum (Immunoblot). CK2 was immunoprecipitated from the whole cell lysates (100 μg) and subjected to an *in vitro* kinase assay by using immunoprecipitated CK2, [ $\gamma$ - $^{32}$ P]ATP, and  $\beta$ -casein as a substrate (*in vitro* kinase assay).

SP-D, a structural homologue to SP-A, does not stimulate the phagocytosis of *S. pneumoniae* by alveolar macrophages (see Fig. 2d). Only one SP-A/SP-D chimera, ad1, in which the SP-A region Leu<sup>219</sup>-Phe<sup>228</sup> is replaced with the corresponding SP-D region Gly<sup>346</sup>-Phe<sup>355</sup>, possesses the stimulatory activity on the phagocytosis, indicating that the extreme carboxyl-terminal SP-D region can be replaced with that of the SP-A region without altering the SP-A function on the phagocytosis. The results also suggest that the SP-A region Cys<sup>204</sup>-Cys<sup>218</sup> is required for the SP-A-mediated phagocytosis, since the chimera ad2, in which the SP-D region Cys<sup>331</sup>-Phe<sup>355</sup> is substituted for the corresponding SP-A region Cys<sup>204</sup>-Phe<sup>228</sup>, fails to augment the uptake of *S. pneumoniae* by alveolar macrophages. The results demonstrate the importance of the carbohydrate recognition domain of SP-A in the augmentation of the phagocytosis. A previous study (19) from this laboratory has shown that the chimera ad1 fails to enhance the uptake of liposome containing dipalmitoylphosphatidylcholine by alveolar type II cells. Thus, taken together with this study, structural requirements for interaction of SP-A with alveolar macrophages are different from those with alveolar type II cells. Since the stimulatory effect of SP-A on *S. pneumoniae* phagocytosis occurs through SP-A-cell interaction and is specific for alveolar macrophages, it is presumed that the SP-A carbohydrate recognition domain recognizes the putative SP-A receptor on alveolar macrophages.

One recent study (7) has shown that the collagenous tails of SP-A and the SP-A mutant lacking functional head groups enhance inflammatory cytokine production by binding to calreticulin/CD91, suggesting a proinflammatory function of SP-A. They propose that the binding of SP-A to a foreign organism and cell debris via the globular heads of the protein and the presentation of the collagenous tails to calreticulin/CD91 stimulate phagocytosis and proinflammatory responses. The present study also supports the concept that SP-A exhibits proinflammatory functions. However, the mutant SP-A<sup>R197A,K201A,K203A</sup> with intact collagenous tails, in which the amino acid residues in the head group are mutated, fails to stimulate the bacterial phagocytosis by alveolar macrophages (see Fig. 2d). In addition, the SP-A/SP-D chimera ad2 containing the SP-A tails, in which the SP-D region Cys<sup>331</sup>-Phe<sup>355</sup> is substituted for the corresponding SP-A region Cys<sup>204</sup>-Phe<sup>228</sup>, also fails to augment the uptake of *S. pneumoniae* (see Fig. 2d). These results indicate the importance of the SP-A head groups in the SP-A-stimulated phagocytosis of *S. pneumoniae*. Thus, it is unlikely that the presentation of the SP-A tails to calreticulin/CD91, as reported by Gardai *et al.* (7), causes the increased phagocytosis of *S. pneumoniae* by alveolar macrophages. These studies support the idea that SP-A exhibits proinflammatory functions by different mechanisms.

Macrophage SR-A (16, 27) recognizes a number of ligands including chemically modified lipoprotein such as AcLDL and



oxidized LDL. A study using SR-A-deficient mice (23) has revealed that SR-A contributes to the generation of atherosclerotic lesions *in vivo* and also that it is an element of the innate immune system. SR-A recognizes a variety of polyanions and bacterial cell wall components (15, 28, 29). In addition, a study with macrophages derived from SR-A<sup>-/-</sup> mice has revealed that SR-A mediates opsonin-independent phagocytosis of Gram-positive bacteria (30), indicating that SR-A serves as a phagocytic receptor. The present results (see Fig. 3) clearly demonstrate that SP-A increases the SR-A-mediated phagocytosis of *S. pneumoniae*. We then pursued the mechanism of SP-A-stimulated phagocytosis. Immunocytochemical observation of SP-A-treated macrophages by anti-SR-A antibody revealed increased cell surface localization of SR-A, and this was confirmed by the binding study at 4 °C with an SR-A ligand, AcLDL (see Fig. 5, *a* and *b*). The stimulatory effect of SP-A on the uptake of *S. pneumoniae* was observed after only a 5-min incubation of the cells with SP-A (see Fig. 2*b*). In addition, preincubation at 4 °C does not confer the SP-A-stimulated phagocytosis on alveolar macrophages, and the presence of cycloheximide failed to inhibit the SP-A-mediated enhanced phagocytosis (see Fig. 4, *b* and *d*). The results are consistent with those showing the cell surface localization of SR-A (see Fig. 5*c*). These data rule out a possibility that the stimulatory effect of SP-A on the phagocytosis is a consequence of new protein synthesis, leading us to examine the effect of protein kinase inhibitors on phagocytosis and SR-A localization. Apigenin inhibits both the increase of cell surface SR-A localization and the stimulated phagocytosis by SP-A (see Fig. 7), indicating the involvement of CK2 in the increased cell surface localization of SR-A. Bisindolylmaleimide somewhat increased the basal uptake of *S. pneumoniae*. This does not appear to have been due to the enhanced SR-A expression, since the cell surface SR-A immunostaining was not increased in the absence of SP-A when this inhibitor was included.

Preincubation of SP-A with alveolar macrophages at 37 °C increases the cell surface SR-A localization and enhances the phagocytosis (see Figs. 4, *a* and *b*, and 5), indicating that the direct interaction of SP-A with the cells is required. However, 4 °C preincubation of SP-A with the cells neither induces the recruitment of SR-A to the cell surface nor stimulates the uptake of the bacteria. It is unlikely that 4 °C preincubation affects the general mechanism of phagocytosis, because preincubation was performed at 4 °C in these experiments but the phagocytosis assay was carried out at 37 °C after washing out SP-A. These results support the idea that 37 °C preincubation of SP-A induces the SR-A migration to the plasma membrane. It is possible to assume that 4 °C preincubation with the cells does not cause phosphorylation of the protein by CK2, which could be involved in the SR-A recruitment, since the phosphorylation reaction by protein kinase does occur at 37 °C but not at 4 °C. In addition, the binding kinetics at 4 °C could be different from that at 37 °C. The weak binding of SP-A to the cells at 4 °C, compared with that at 37 °C, may cause weaker signals, resulting in the failure of increasing cell surface SR-A expression and of stimulating the phagocytosis.

Newly synthesized SR-A subunits are inserted into the membrane of the rough ER, and a precursor form of the receptor is generated in the lumen of the ER to undergo a series of post-translational modifications (28). The precursor trimeric forms are then transported to and through the Golgi apparatus and *trans*-Golgi network en route to the plasma membrane. However, the precise mechanism of intracellular transport of SR-A to the plasma membrane remains unknown. In bovine alveolar macrophages, SR-A are localized on the cell surface, vesicles, and endosomes (31). Since Chinese hamster ovary cells trans-

fectured with SR-A cDNA exhibit the localization of SR-A protein in the ER, nuclear envelope, and Golgi apparatus but not on the cell surface, there may be a macrophage-specific transport system of SR-A to the plasma membrane. In this study, we showed that SP-A stimulates CK2 activity, which induces protein phosphorylation that presumably mediates increased cell surface localization of SR-A, resulting in the enhanced phagocytosis of *S. pneumoniae*. Protein phosphorylation by CK2 may be involved in this transport system of SR-A in macrophages. Although the target protein of CK2 that regulates the SR-A localization is unknown, it is possible to conclude that SP-A stimulates the CK2 activity through a putative SP-A receptor on alveolar macrophages and that CK2-dependent protein phosphorylation up-regulates the cell surface localization of SR-A, resulting in the enhanced phagocytosis of the bacteria.

This study reveals that the direct interaction of SP-A with alveolar macrophages causes the increased cell surface expression of SR-A and the enhanced phagocytosis of *S. pneumoniae*. However, an SP-A receptor responsible for these events has not been yet identified. It is unlikely that SP-A binds to SR-A, because Chronos *et al.* (32) have reported that fucoidan and polyinosinic acid, SR-A ligands, do not compete with SP-A for the binding to rat bone marrow-derived macrophages. They have purified a 210-kDa protein as a putative receptor for SP-A from macrophage-like cell line U937. Although antibody against this protein inhibits the SP-A binding to both alveolar type II cells and macrophages and also blocks the SP-A-mediated inhibition of surfactant lipid secretion from type II cells, it is unclear whether this protein is involved in the SP-A-mediated SR-A recycling and the phagocytosis of *S. pneumoniae*.

Phosphorylation and/or dephosphorylation have been reported to be involved in the recycling of several receptors. The recycling of the T cell receptor back to the membrane is dependent on dephosphorylation of the receptor subunit that is mediated by a serine/threonine phosphatase (33). Vasopressin V2 receptor recycling has been shown to be regulated by dephosphorylation of a specific C-terminal domain (34). Fong and Le (35) have reported that prevention of phosphorylation at Ser<sup>49</sup> in mouse SR-A reduces acetylated LDL internalization rate. In addition, the human SR-A mutant, in which Ser<sup>49</sup> is replaced with Asp and which mimics phosphorylation at Ser<sup>49</sup>, reduces cell surface expression of SR-A (36). These studies suggest that phosphorylation at Ser<sup>49</sup> in SR-A rather decreases cell surface SR-A localization. Thus, it is unlikely that the target protein of CK2 is SR-A. The protein kinase that phosphorylates SR-A has not been yet identified. Several complicated steps may constitute the mechanism of SR-A recycling. The phosphorylation of the target protein by CK2 is likely to be an earlier event for the SR-A recycling. One study (37) has revealed the interaction of CD163 with the regulatory subunit of CK2. Although CD163 belongs to the family of the scavenger receptor cysteine-rich proteins characterized by a cysteine-rich domain (38), evidence of the direct interaction of SR-A with CK2 has not been demonstrated. The identification of the substrate for CK2 that may be related to the SR-A recycling could be an important step to elucidate the mechanism of the SP-A-mediated cell surface expression of SR-A in alveolar macrophages.

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**Pulmonary Surfactant Protein A Augments the Phagocytosis of *Streptococcus pneumoniae* by Alveolar Macrophages through a Casein Kinase 2-dependent Increase of Cell Surface Localization of Scavenger Receptor A**

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