Sec22b Is a Negative Regulator of Phagocytosis in Macrophages

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The endoplasmic reticulum (ER) is proposed to be a membrane donor for phagosome formation. In support of this, we have previously shown that the expression level of syntaxin 18, an ER-localized SNARE protein, correlates with phagocytosis activity. To obtain further insights into the involvement of the ER in phagocytosis we focused on Sec22b, another ER-localized SNARE protein that is also found on phagosomal membranes. In marked contrast to the effects of syntaxin 18, we report here that phagocytosis was nearly abolished in J774 macrophages stably expressing mVenus-tagged Sec22b, without affecting the cell surface expression of the Fc receptor or other membrane proteins related to phagocytosis. Conversely, the capacity of the parental J774 cells for phagocytosis was increased when endogenous Sec22b expression was suppressed. Domain analyses of Sec22b revealed that the R-SNARE motif, a selective domain for forming a SNARE complex with syntaxin18 and/or D12, was responsible for the inhibition of phagocytosis. These results strongly support the ER-mediated phagocytosis model and indicate that Sec22b is a negative regulator of phagocytosis in macrophages, most likely by regulating the level of free syntaxin 18 and/or D12 at the site of phagocytosis.

INTRODUCTION

Phagocytosis is an essential property of macrophages whereby large foreign particles are internalized into the cytoplasm, and it is critical for the ingestion of dead cells and invoking immune responses. This process is initiated by the engagement and clustering of specific receptors on the cell surface, leading to actin polymerization; the actin filaments then propel the extension of the phagocytic cup around the forming phagosome (Desjardins, 2003). After particle internalization, phagosomes gradually grow into phagolysosomes by fusing with different endomembrane systems, such as endosomes and lysosomes. The mature phagolysosome eventually acquires an acidic environment so that hydrolytic enzymes can degrade internalized microbes (Jutras and Desjardins, 2005; Haas, 2007).

When phagosomes take up large particles, the phagosome membranes are thought to be supplied not only from invaginated plasma membrane but also from the membranes of intracellular organelles (Desjardins, 2003), including early endosomes or recycling endosomes. Exocytosis at the site of phagosome formation in these organelles is mediated by a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein, VAMP3 (also called cellubrev-

in; Bajno *et al.*, 2000; Coppolino *et al.*, 2001). Another intracellular source of membrane is late endosomes, which are exocytosed by a VAMP7 (also called TI-VAMP)-related fusion process during Fc receptor-mediated phagocytosis (Braun *et al.*, 2004). However, in dendritic cells, VAMP7 as well as VAMP8 (also called endobrevin) are negative regulators of phagocytosis (Ho *et al.*, 2008). Also, in recent studies on the ingestion of large particles (3.0–6.0 μ m) by macrophages, the recruitment of the peripheral domains of lysosomes to nascent phagosomes and the mobilization of recycling endosomes as a source of endomembrane during phagocytosis occurred in a synaptotagmin VII-dependent (Czibener *et al.*, 2006) and a synaptotagmin V-dependent (Vinet *et al.*, 2008) manner, respectively.

In addition to organelles related to the endosome/lysosome pathway, the endoplasmic reticulum (ER) has also been implicated in phagosome formation. ER-derived membrane has been observed in the bottom of forming phagosomes, and some ER-resident proteins were detected in a proteomics analysis of isolated phagosomes (Gagnon *et al.*, 2002; Desjardins, 2003). Also, all constituents of the major histocompatibility complex (MHC) class I processing and presentation machinery, which are ER-resident proteins, have been found in phagosomes (Ackerman *et al.*, 2003; Guernonprez *et al.*, 2003; Houde *et al.*, 2003), indicating that the cross-presentation of foreign antigens could be initiated in phagosomes. Despite these findings, the existence of ER-mediated phagocytosis per se remains highly controversial (Groothuis and Neefjes, 2005; Touret *et al.*, 2005).

Fusion between the vesicle and the target membrane is mediated by SNARE proteins extended from each membrane. The SNARE complex produces a parallel four-helix bundle, of which three helices are extended from one membrane by proteins of the syntaxin and synaptosomal associated protein of 25 kDa (SNAP-25) families (Q-SNAREs) that contain a conserved glutamine residue at a central position

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Abbreviations used: ECL, enhanced chemiluminescence; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; i-SNARE, inhibitory SNARE; mVenus, monomeric Venus; SNAP-25, synaptosomal associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TMD, transmembrane domain; VAMP, vesicle-associated membrane protein.

called the “0” layer, and one helix is extended from the opposite membrane by a protein of the VAMP or synaptobrevin family (an R-SNARE) that contains a conserved arginine residue at the same position (Fasshauer *et al.*, 1998; Jahn *et al.*, 2003; Jahn and Scheller, 2006). In the ER, syntaxin 18, D12 (also called p31), and BNIP1 are Q-SNARE proteins, and Sec22b (also called ERS-24) is classified as an R-SNARE (Hatsuzawa *et al.*, 2000; Nakajima *et al.*, 2004; Jahn and Scheller, 2006; Okumura *et al.*, 2006). Recently, we have shown that syntaxin 18 is involved in membrane fusion between the ER and the plasma membrane during phagocytosis in macrophages (Hatsuzawa *et al.*, 2006). We found the ER-localized SNARE proteins syntaxin 18, D12, and Sec22b in phagosomes from the earliest stage of phagosome formation. Interestingly, Becker *et al.* (2005) observed that introduction of a dominant-negative form (cytoplasmic region) of Sec22b or anti-Sec22b antibodies into macrophages selectively inhibited phagocytosis of relatively large 3- μ m latex beads. These observations support the involvement of the ER in phagocytosis by macrophages. However, the molecular mechanism by which the ER-localized SNARE proteins regulate phagocytosis is not clear. Here, we show that Sec22b plays an inhibitory role in phagocytosis through an interaction with syntaxin 18 and/or D12 in J774 macrophages.

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies to Sec22b, syntaxin 18, and D12 were prepared as described previously (Hatsuzawa *et al.*, 2006). The polyclonal antibody to enhanced green fluorescent protein (EGFP) was raised against bacterially expressed glutathione S-transferase (GST)-tagged EGFP and purified using EGFP-coupled affinity beads. The purified antibody recognizes mVenus as well as EGFP. The monoclonal c-Myc antibody was prepared from 9E10 hybridoma cells (American Type Culture Collection, Manassas, VA). The remaining antibodies were obtained from commercial sources as follows: syntaxin 4 (Sigma-Aldrich, St. Louis, MO), CD64 (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Ambion, Austin, TX), and fluorescent-labeled secondary antibodies (Molecular Probes, Eugene, OR).

Cell Culture

J774 cells were obtained from the Riken Cell Bank (Tsukuba, Japan) and grown in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal calf serum (FCS). J774 cells stably expressing mVenus-tagged proteins were maintained in RPMI 1640 with 10% FCS containing puromycin (final concentration 2 μ M). 293T cells were cultured in DMEM-high glucose (Sigma-Aldrich) supplemented with 10% FCS. All cell lines were cultured at 37°C under 5% CO₂.

Expression Vectors and Establishment of Stable Transformsants

The expression plasmids for syntaxin 18, D12, and Sec22b were constructed by subcloning cDNA fragments by PCR into pmVenus-C1 vector as described previously (Hatsuzawa *et al.*, 2006). The J774 cell lines expressing mVenus-tagged proteins [J774/mVenus, J774/mVenus-syntaxin 18, J774/mVenus-D12, J774/mVenus-Sec22b (1-215), J774/mVenus-Sec22b Δ TMD (1-195), and J774/mVenus-R-SNARE (100-195)] were established by infection with recombinant retroviruses generated using cDNAs of mVenus-tagged proteins cloned into the pCX4puro vector as described (Akagi *et al.*, 2003; Hatsuzawa *et al.*, 2006).

Opsonized Fluorescein Isothiocyanate-conjugated Zymosan Assay

J774 cells stably expressing mVenus-tagged proteins were plated at a density of 0.75×10^6 cells per well in a 24-well dish and cultured overnight. The culture medium was exchanged with fresh medium, and 30 min later the cells were incubated for 1 h in the presence or absence of 2.25×10^7 fluorescein isothiocyanate (FITC)-conjugated zymosan A particles (Cat. no. Z2841; Molecular Probes) which had been treated with opsonizing reagent (rabbit anti-zymosan IgG, Cat. no. Z2850; Molecular Probes). The cells were then washed extensively with ice-cold PBS to remove free particles. Trypan blue solution (0.25 mg/ml in 20 mM sodium citrate, pH 4.4, containing 150 mM NaCl) was

then added to quench the fluorescence of noninternalized particles. After 3 min the solution was removed, and fluorescence was quantitatively measured in a Plate Chameleon V microplate reader (Hidex, Turku, Finland) using 485 nm for excitation and 535 nm for emission. Arbitrary fluorescence units were obtained by subtracting the fluorescence intensity observed in the absence of IgG-opsonized FITC-conjugated zymosan particles from that observed in the presence of the particles and normalized to the maximal value obtained for J774/mVenus cells within the same experiment, which was set to 100%. Alternatively, cells were incubated for 30 min on ice with IgG-opsonized FITC-conjugated zymosan particles in the presence of cytochalasin B (final concentration 10 μ M) and then washed thoroughly with ice-cold PBS to remove free particles. The fluorescence of the particles associated with the cells was then measured as described above.

Small Interfering RNA Experiment

The RNA duplex used for targeting was mouse Sec22b siRNA (5'-GC-CCGUCUCGGAGAAAUCUAG-3'; RNAi Co., Tokyo, Japan). A small interfering RNA (siRNA) duplex containing 47% GC content (Cat. no. D-001206-09-05; Dharmacon, Lafayette, CO) was used as a control. J774 cells were transfected with either control or Sec22b siRNA using Lipofectamine (Invitrogen, Tokyo, Japan). Twenty-four hours after transfection, the siRNAs were transfected into the cells again under the same conditions. Forty-eight hours after the first transfection, the cells were split into two dishes for Western blot analysis and an opsonized FITC-conjugated zymosan assay.

Immunoprecipitations

Cell lysates were prepared from either J774 cells stably expressing monomeric Venus (mVenus)-tagged proteins or 293T cells transiently cotransfected with mVenus-tagged proteins and Myc-tagged syntaxin 18 constructs as described (Hatsuzawa *et al.*, 2006). The lysates were incubated with an anti-EGFP antibody for 30 min at 4°C. protein A-Sepharose (GE Healthcare Bio-Sciences, Tokyo, Japan) was then added, and the mixture was allowed to incubate for 16 h at 4°C with gentle rotation. The beads were washed four times with extraction buffer (20 mM HEPES-KOH, pH 7.2, 100 mM KCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and a protease inhibitor cocktail; Nakarai Chemicals, Kyoto, Japan). The immune-complexes were eluted from the Sepharose beads with SDS-PAGE sample buffer. In addition, 10% of the total lysate volume was mixed with 5 $\times$ SDS-PAGE sample buffer and heated at 95°C for 5 min. After SDS-PAGE, the samples were analyzed by Western blotting using ReliaBLOT (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions. Immunoreactive proteins were visualized using ECL Western Blotting Detection Reagents (GE Healthcare Bio-Sciences).

Proteinase K Digestion

J774 cells stably expressing mVenus-Sec22b Δ TMD and 293T cells transiently transfected with an mVenus-Sec22b Δ TMD construct were homogenized by fifteen passages through a 25-gauge needle in ice-cold homogenization buffer (20 mM HEPES-KOH, pH 7.2, 0.25 M sucrose, and a protease inhibitor cocktail; Nakarai Chemicals). The homogenates were centrifuged at 2500 rpm for 10 min at 4°C in a microcentrifuge to remove nuclei and unbroken cells. An aliquot of the postnuclear supernatant was incubated with proteinase K (ProK) at a final concentration of 5, 10, 20, 25, or 50 μ g/ml for 15 min on ice. The reaction was stopped by adding phenylmethylsulfonyl fluoride (final concentration 5 mM). The samples were mixed with 5 $\times$ SDS-PAGE sample buffer and heated at 95°C for 5 min. After SDS-PAGE, the samples were analyzed by Western blotting using an anti-Sec22b antibody.

Immunofluorescence

Immunofluorescence microscopy was performed as described previously (Hatsuzawa *et al.*, 2006). Briefly, J774 cells expressing mVenus-tagged proteins were fixed with 4% paraformaldehyde for 15 min on ice. The cells were then incubated with goat polyclonal anti-CD64 diluted 1:50, followed by incubation with secondary antibody conjugated to Alexa594 (Molecular Probes) diluted 1:200. Confocal microscopy was performed on an LSM510meta laser scanning microscope using a plan-Apochromat 63 $\times$ NA 1.4 oil immersion objective (Zeiss, Oberkochen, Germany).

Statistics

Data are presented as mean $\pm$ SE for the number of experiments indicated in the figure legends. Statistical significance was determined with Student's paired *t* test (one-tailed) using GraphPad Prism (GraphPad Software, San Diego, CA). Differences between the analyzed samples were considered significant at *p* < 0.05.

RESULTS

Stable Overexpression of mVenus-Sec22b Lessens the Capability of Phagocytosis in J774 Macrophages

We have previously demonstrated that syntaxin 18 positively regulates phagocytosis (Hatsuzawa *et al.*, 2006). To

elucidate the exact mechanisms, we investigated the role of another ER-localized SNARE protein, Sec22b, which is also recruited to phagosomes (Hatsuzawa *et al.*, 2006). We first analyzed J774 macrophages stably expressing mVenus-tagged Sec22b (J774/mV-22b); mVenus is a variant of EGFP. Western blot analysis revealed that mV-22b (Figure 1A, left panel lane 3 and right panel lane 3, top band) was expressed at two- to fourfold higher levels than the endogenous Sec22b (Figure 1A, right panel, asterisk). A similar result was observed in J774 cells stably expressing mVenus-tagged syntaxin 18 (Figure 1A, middle panel). The fluorescent signal for mV-22b was predominantly detected in the ER (Figure 2B; Hatsuzawa *et al.*, 2006). We then examined the effects of overexpressing mV-22b on Fc receptor (FcR)-mediated phagocytosis by measuring the uptake of FITC-conjugated zymosan A particles, which were opsonized with IgG (see *Materials and Methods*). The analysis showed that phagocytosis was dramatically impaired in J774/mV-22b cells, with a reduction of more than 35% from the level observed in control J774/mVenus (mV) cells (Figure 1C), whereas overexpression of mVenus-tagged syntaxin 18 (mV-syx18) enhanced the efficiency of phagocytosis (Figure 1C) as we have previously reported (Hatsuzawa *et al.*, 2006). The overexpression of these mVenus-tagged proteins had no effect on the association of IgG-opsonized zymosan particles with J774 cells (Figure 1B). These effects were not limited to the receptor-mediated process and were also observed in FcR-independent phagocytosis as quantified using nonopsonized luminol beads, which give off chemiluminescence by reacting with reactive oxygen species (ROS) generated in phagosomes (Supplementary Figure S1). D12, another ER-localized SNARE protein, had the same positive effect on phagocytosis as syntaxin 18 (Supplementary Figure S1 and unpublished data).

These results suggest that the ER-localized SNARE proteins differentially regulate phagocytosis in J774 cells. However, because Sec22b has been shown to play an important role in the secretory pathway (Hay *et al.*, 1997; Paek *et al.*, 1997), it was also possible that overexpression of mV-22b alters the expression levels of some plasmalemmal proteins responsible for phagocytosis. To eliminate this possibility, we examined the expression of CD64 (Fc receptor 1a) and syntaxin 4, a plasmalemmal SNARE protein required for VAMP3-mediated delivery of TNF- α to the cell surface at the site of phagocytic cup formation in activated macrophages (Murray *et al.*, 2005). Western blot analysis of J774/mV and J774/mV-22b cells (Figure 2A) showed no difference in the expression levels of CD64 or syntaxin 4. Furthermore, immunofluorescence microscopy revealed a similar expression and localization of CD64 on the plasma membrane in J774/mV-22b cells and J774/mV cells (Figure 2B).

To investigate which stage of phagocytosis is impaired by Sec22b overexpression, we measured the kinetics of phagocytosis using IgG-opsonized FITC-zymosan particles. As shown in Figure 2C, J774/mV-22b cells showed a markedly reduced rate of particle uptake compared with J774/mV cells as early as 5 min of incubation and reached a near plateau at 30 min, indicating that the J774/mV-22b cells are impaired in membrane fusion, most likely between the ER and the plasma membrane. This data suggests that overexpression of Sec22b inhibits an early step in phagocytosis rather than phagosome maturation.

Knockdown of Sec22b Expression Augments Phagocytosis Activity in J774 Macrophages

To test whether the observed suppression of phagocytosis in J774/mV-22b cells was the result of augmented Sec22b function, we examined the effects of a siRNA against Sec22b. Transfection of the cells with Sec22b siRNA decreased Sec22b expression to ~50% of the control value (Figure 3, A and B). A slight decrease was also observed for endogenous proteins such as CD64, syntaxin 4, and syntaxin 18 (Figure 3, A and B). The opsonized FITC-zymosan assay showed a significant increase in phagocytosis activity in the Sec22b siRNA cells to ~130% of control (Figure 3D), although the siRNA treatment had no effect on the association of zymosan particles with the cells (Figure 3C). Likewise, Sec22b siRNA enhanced phagocytosis activity using the luminol bead assay (Supplementary Figure S2). These findings strongly suggest that the inhibition of phagocytosis as shown in Figures 1 and 2 is due to a dominant-active effect of the expressed mV-22b rather than a dominant-negative one, thus implying that endogenous Sec22b negatively regulates phagocytosis in J774 cells.

The R-SNARE Motif of Sec22b Is Responsible for the Inhibition of Phagocytosis

Similar to other SNARE proteins, Sec22b possesses a predicted coiled-coil region at the N-terminus of its transmembrane domain (TMD). This region, referred to as the SNARE motif (R-SNARE; Figure 4A), is thought to be important for fusion events. We therefore investigated whether expression of the cytoplasmic domain (1-195) of Sec22b (mV-22b Δ TMD) or the R-SNARE motif (100-195) of Sec22b (mV-22b-R-SNARE) N-terminally fused to mVenus had a dominant-negative effect on phagocytosis in J774 cells. To this end, we generated J774 cells stably expressing each mutant (Figure 4B). Immunofluorescence analysis showed that mV-, mV-22b Δ TMD-, and mV-22b-R-SNARE were distributed throughout the cytoplasm (Figure 4B), whereas mV-22b was largely confined to the ER. Overexpression of these proteins had no effect on the expression of endogenous plasmalemmal proteins such as CD64 (Supplementary Figure S3) and had no effect on the association of opsonized zymosan particles with the cells (Figure 4C). Unexpectedly, when the opsonized FITC-zymosan assay was performed with the mutant cells, a potent inhibition of phagocytosis was observed in the J774/mV-22b-R-SNARE cells (Figure 4D), suggesting that the inhibitory effects of mV-22b on phagocytosis are mediated through the R-SNARE motif. Interestingly, J774/mV-22b Δ TMD cells exhibited an apparent augmentation of phagocytosis activity compared with J774/mV control cells, despite the presence of the R-SNARE motif (Figure 4D). However, this result seemingly contradicts our previous data from the same J774/mV-22b Δ TMD cells using the luminol bead assay (see supplementary Figure S1C of our previous report; Hatsuzawa *et al.*, 2006). Because the luminol bead assay is an indirect method to measure the phagocytosis activity by ROS generation in phagosomes, we investigated whether ROS generation is affected in the J774/mV-22b Δ TMD cells. As shown in Supplementary Figure S4, ROS generation by phorbol 12-myristate 13-acetate (PMA) stimulation was strongly reduced in the J774/mV-22b Δ TMD cells compared with the J774/mV cells. Therefore, our previous inhibitory data observed in the J774/mV-22b Δ TMD cells was due to a defect in ROS generation ability rather than phagocytosis activity.

SNARE motifs are generally involved in interactions between a specific set of SNARE partner proteins that leads to

Figure 1. Overexpression of mVenus-Sec22b reduces phagocytosis of IgG-opsonized zymosan particles in J774 macrophages. (A) Western blot analysis of total lysates from J774 macrophages stably expressing mVenus-SNARE proteins. Western blotting was carried out using antibodies against EGFP (which recognize mVenus), syntaxin 18 (syx18), and Sec22b. Asterisks (*) denote the bands containing endogenous SNARE proteins. A 50-kDa band (#) represents a nonspecific protein recognized by anti-syntaxin 18 antibodies. (B and C) J774/mVenus (mV), J774/mVenus-syntaxin 18 (mV-syx18), and J774/mVenus-Sec22b (mV-22b) cells were incubated with IgG-opsonized FITC-zymosan particles and then measured for the efficiency of association (B) and phagocytosis (C) as described in *Materials and Methods*. Arbitrary fluorescence units were normalized to the maximal value obtained for mV cells within the same experiment, which was set to 100%. Data presented are the mean $\pm$ SE of three independent experiments. * $p < 0.001$, compared with mV cells. Student's paired *t* test, one-tailed.

Figure 2. J774/mVenus-Sec22b cells inhibit FcR-mediated phagocytosis at an early time point without altering the expression and localization of CD64 (Fc γ RIa receptor). (A) Total cell lysates from mV and mV-22b cells were analyzed by Western blotting using the indicated antibodies. (B) J774 cells expressing mV or mV-22b were fixed with paraformaldehyde and stained with an antibody against CD64 and then visualized with an anti-goat IgG antibody conjugated to Alexa594 (Invitrogen). Bar, 10 μ m. (C) J774/mVenus and J774/mVenus-Sec22b (mV-22b) cells were incubated with IgG-opsonized FITC-zymosan particles for various times, and the efficiency of phagocytosis was then measured as described for Figure 1. Data presented are the mean $\pm$ SE of three independent experiments. * $p < 0.05$ and ** $p < 0.005$, compared with mV cells. Student's paired *t* test, one-tailed.

proper membrane fusion. We therefore postulated that the observed inhibition of phagocytosis by the R-SNARE motif of Sec22b may have been caused by the sequestration of other ER-localized SNARE proteins from the phagocytosis event. To investigate this possibility, lysates from J774 cells stably expressing mVenus-Sec22b mutants were incubated with anti-EGFP antibodies, and the immunoprecipitates were subjected to SDS-PAGE followed by Western blot analysis with anti-syntaxin 18, anti-D12, anti-syntaxin 4, and anti-EGFP antibodies (Figure 5A). Endogenous syntaxin 18 and D12 were coprecipitated with all of the Sec22b mutants, although the amount was considerably lower with mV-22b Δ TMD compared with mV-Sec22b wild type or mV-22b-R-SNARE (Figure 5A). These results mirror the profile of phagocytosis activity shown in Figure 4D. The low level of interaction with syntaxin 18 and/or D12 observed in J774/mV-22b Δ TMD cells suggests that the R-SNARE motif of mV-22b Δ TMD is masked by its N-terminal, profilin-like, longin domain and takes a closed conformation, similar to that of Ykt6 (Fukasawa *et al.*, 2004; Hasegawa *et al.*, 2004) or VAMP7 (Pryor *et al.*, 2008). No detectable interaction between syntaxin 4 and mVenus-Sec22b mutants was observed (Figure 5A).

Figure 3. Knockdown of Sec22b expression enhances phagocytosis of IgG-opsonized zymosan particles in J774 cells. (A and B) J774 cells were transfected with a Sec22b siRNA or a nonspecific siRNA as a control. Total cell lysates from siRNA transfected cells of three independent experiments (exp #1, #2, and #3) were analyzed by Western blotting using the indicated antibodies. The bands were quantified by using NIH ImageJ version 1.41 (<http://rsb.info.nih.gov/ij/>), and the values for each protein were expressed as a ratio of Sec22b siRNA cells to control siRNA cells, which were then normalized to that of GAPDH, which was set to 100%. Data presented are the mean $\pm$ SE of three independent experiments. *p* values indicate statistical significance compared with GAPDH. Student's paired *t* test, one-tailed. (C and D) J774 cells transfected with control siRNA or Sec22b siRNA were incubated with IgG-opsonized FITC-zymosan particles and then measured for the efficiency of association (C) and phagocytosis (D) as described in Figure 1. Arbitrary fluorescence units from each cell line were normalized to the maximal value obtained for control siRNA cells within the same experiment, which was set to 100%. Data presented are the mean $\pm$ SE of three independent experiments. * *p* < 0.005, compared with control siRNA cells. Student's paired *t* test, one-tailed.

In contrast to our results with mVenus-tagged Sec22bΔTMD (Figure 4D), it has been previously reported that transient introduction of GST-tagged Sec22bΔTMD into J774 cells decreased the efficiency of phagocytosis (Becker *et al.*, 2005). This apparent discrepancy could be due to a difference in the affinity between the expressed Sec22bΔTMDs and syntaxin 18. To confirm this, we transiently coexpressed mVenus-Sec22b mutants with Myc-syntaxin 18 (Myc-syx18) and then incubated the lysates from these cells with anti-EGFP antibodies. Immunoprecipitates were analyzed by Western blotting with anti-Myc and anti-EGFP antibodies (Figure 5B). In contrast to the results in Figure 5A, all Sec22b mutants including mV-22bΔTMD interacted with Myc-syx18 efficiently (Figure 5B, top panel). However, because

Sec22b mutants with Myc-syntaxin 18 (Myc-syx18) and then incubated the lysates from these cells with anti-EGFP antibodies. Immunoprecipitates were analyzed by Western blotting with anti-Myc and anti-EGFP antibodies (Figure 5B). In contrast to the results in Figure 5A, all Sec22b mutants including mV-22bΔTMD interacted with Myc-syx18 efficiently (Figure 5B, top panel). However, because

Figure 4. Phagocytosis of IgG-opsonized zymosan particles in J774 cells is strongly inhibited by overexpression of mV-22b-R-SNARE but not by mV-22bΔTMD. (A) Schematic representation of the mVenus-Sec22b constructs. Sec22b is composed of three domains: the N-terminal domain, the coiled-coil domain (also called the R-SNARE motif), and a domain comprising the transmembrane domain and a short luminal domain (TMD). Sec22b lacking its TMD and the R-SNARE motif of Sec22b were tagged with mVenus as depicted. (B) J774 cells stably expressing mVenus-tagged proteins were fixed and analyzed by confocal microscopy. Bar, 10 μ m. (C and D) J774 cells stably expressing mVenus-tagged proteins were incubated with IgG-opsonized FITC-zymosan particles and the efficiency of association (C) and phagocytosis (D) measured as described in Figure 1. Data presented are the mean $\pm$ SE of three independent experiments. * *p* < 0.01 and ** *p* < 0.001, compared with mV cells. Student's paired *t* test, one-tailed.

Figure 5. Sec22b interacts with syntaxin 18 and D12 through its R-SNARE motif but stably expressed Sec22b Δ TMD does not. (A) J774 cells stably expressing mV, mV-22b, mV-22b Δ TMD, or mV-22b-R-SNARE were lysed and immunoprecipitated (IP) with anti-EGFP antibodies. The immunocomplexes were subjected to SDS-PAGE, followed by Western blot (WB) analysis using the indicated antibodies. Asterisks (*) denote the bands containing mVenus-tagged proteins. (B) 293T cells were transiently cotransfected with mV, mV-22b, or mV-22b mutants and the indicated Myc-tagged constructs. Twenty-four hours after transfection, the cells were harvested and lysed. mVenus-tagged proteins were immunoprecipitated (IP) from the cell lysates with anti-EGFP antibodies. The immunocomplexes were separated on SDS-PAGE and analyzed by Western blotting using anti-EGFP and anti-Myc antibodies. (C) Postnuclear supernatants from stably transfected J774/mV-22b Δ TMD cells and 293T cells transiently expressing mV-22b Δ TMD were subjected to proteinase K (ProK) digestion at the indicated final concentrations for 15 min on ice. The digested samples were separated by SDS-PAGE and then analyzed by Western blotting using an anti-Sec22b antibody as described in *Materials and Methods*. In J774/mV-22b Δ TMD cells, a ~30-kDa proteinase K resistant fragment was observed. (D) The bands in Panel C for the full-length mV-22b Δ TMD (circles) or the endogenous Sec22b (diamonds) from stably expressed mV-22b Δ TMD cells (open) or transiently expressed mV-22b Δ TMD cells (filled) were quantified using NIH ImageJ version 1.41 and plotted as a fraction of the total.

overexpression of full-length syntaxin 18 tends to cause aggregation of the ER membranes (Hatsuzawa *et al.*, 2000), mV-22 Δ TMD may have been nonspecifically trapped in the aggregates. To exclude this possibility, we performed the same immunoprecipitation experiment using Myc-syntaxin 18 Δ TMD (Myc-syx18 Δ TMD) whose overexpression does not cause aberrant changes in the apparent ER structure (Hatsuzawa *et al.*, 2000). We observed essentially the same coprecipitation profile as that obtained using Myc-syx18 (Figure 5B, middle panel). Therefore transiently expressed mV-22b Δ TMD, unlike mV-22b Δ TMD in the stable transformant, may form an open conformation to allow the R-SNARE motif to interact with syntaxin 18, resulting in negative regulation of phagocytosis (Becker *et al.*, 2005). To

test the possibility that there is a conformational difference between the stably expressed and the transiently expressed mV-22b Δ TMD, postnuclear supernatants from J774/mV-22b Δ TMD cells (stably expressed) and 293T/mV-22b Δ TMD cells (transiently expressed) were subjected to ProK digestion (Figure 5C). Quantification of the full-length band ("mV-22b Δ TMD") revealed that indeed transiently expressed mV-22b Δ TMD was more sensitive to ProK digestion than the stably expressed mV-22b Δ TMD (Figure 5D). Endogenous Sec22b, in both stably (J774) and transiently expressing (293T) cells, showed similar resistance to ProK digestion (Figure 5D). Of note, is a fragment of ~30-kDa, which was detected in the stably expressed J774/mV-22b Δ TMD cells (Figure 5C). These data indicate that the

stably expressed mV-22b Δ TMD in J774 cells forms a more compact conformation than the transiently expressed mV-22b Δ TMD and that presumably the former would represent a closed conformation and the latter an open one.

These data indicate that Sec22b interacts with other ER-localized SNARE proteins through its R-SNARE motif and that these interactions may reduce the fusion-competent pools of syntaxin 18 and D12, which are necessary for ER-mediated phagocytosis (Hatsuzawa *et al.*, 2006). Taken together, our results strongly suggest that Sec22b is a negative regulator of phagocytosis in macrophages.

DISCUSSION

The contribution of the ER to phagocytosis and phagosome formation has been demonstrated by proteomic analysis of phagosomes (Garin *et al.*, 2001), electron microscopy (EM; Gagnon *et al.*, 2002; Guermonprez *et al.*, 2003), the discovery of MHC class I molecules required for cross-presentation in phagosomes (Ackerman *et al.*, 2003; Guermonprez *et al.*, 2003; Houde *et al.*, 2003), and demonstration of the export of antigens out of the phagosome by ER components (Ackerman *et al.*, 2006). Furthermore, in *Dictyostelium discoideum* two major ER proteins (calnexin and calreticulin) have been shown to be involved in the outgrowth of phagocytic cups (Muller-Taubenberger *et al.*, 2001). Nonetheless, the involvement of the ER in this process is controversial. Some groups were not able to detect the involvement of the ER in phagosome formation using techniques such as morphological analyses by EM (Groothuis and Neefjes, 2005; Touret *et al.*, 2005). For example, immunogold EM using antibodies against ER marker proteins in J774 cells, RAW264.7 cells, and dendritic cells and an EM analysis of in situ glucose-6-phosphate staining in J774 cells and bone marrow-derived macrophages barely detected ER markers on phagosomes (Touret *et al.*, 2005). On the other hand, studies on the membrane fusion machinery, such as Sec22b and syntaxin 18, have provided additional evidence for the involvement of the ER in phagocytosis in macrophages (Becker *et al.*, 2005; Hatsuzawa *et al.*, 2006). However, although it is clear that syntaxin 18 is actively engaged in phagocytosis (Hatsuzawa *et al.*, 2006), the contribution of Sec22b to this process had not been well elucidated (Becker *et al.*, 2005). Here, we have demonstrated that J774 cells overexpressing Sec22b inhibited the phagocytosis of both IgG-opsonized zymosan particles (Figures 1 and 2) and nonopsonized luminol microbeads (Supplementary Figure S1). Analyses of deletion mutants using the opsonized FITC-zymosan assay (Figure 4D) and immunoprecipitation (Figure 5) showed that this inhibition is mediated by the interaction of the Sec22b R-SNARE motif with syntaxin 18 and/or D12.

Overexpression of a truncated SNARE protein lacking the TMD (Δ TMD) often inhibits specific membrane fusion. For example, overexpression of syntaxin 18 Δ TMD or D12 Δ TMD inhibited phagocytosis (Hatsuzawa *et al.*, 2006), whereas overexpression of their full-length forms enhanced the efficiency of phagocytosis (Figure 1C and Supplementary Figure S1). In this context, Becker *et al.* (2005) reported that the introduction of GST-tagged Sec22b Δ TMD and/or an anti-Sec22b Fab fragment into J774 cells inhibited phagocytosis of IgG-opsonized 3.0- μ m beads. The authors concluded that a functional Sec22b is required for membrane fusion between the ER and the plasma membrane during Fc-receptor-mediated phagocytosis (Becker *et al.*, 2005). However, this conclusion contradicts our observation that J774 cells overexpressing mVenus-tagged full-length Sec22b, unlike cells expressing syntaxin 18 or D12, were deficient in phagocytosis

Figure 6. Schematic representation of the regulation of a SNARE complex containing Sec22b with syntaxin 18 and D12 in phagocytosis. ER-mediated phagocytosis requires the participation of syntaxin 18 and D12, which exist in a free state or in an acceptor complex with Sec22b for ER-Golgi transport. We propose that phagocytosis activation signal(s) emanated from the binding of foreign particles cause a conformational change of Sec22b to a closed form locally. This form of Sec22b is no longer able to assemble the acceptor complex, resulting in release of syntaxin 18 and D12 from the complex. The liberated syntaxin 18 and D12 would work on membrane fusion between the ER and the plasma membrane at the site of phagocytosis. At present, the plasmalemmal cognate Q- and R-SNARE proteins involved in ER-mediated phagocytosis remain unknown (see Discussion).

sis (Figure 1C and Supplementary Figure S1). Furthermore, the siRNA-mediated suppression of Sec22b enhanced phagocytosis activity (Figure 3D and Supplementary Figure S2). One possible explanation for the difference in the effect on phagocytosis between our siRNA data and Becker *et al.*'s data using an anti-Sec22b Fab fragment is that the introduced Fab fragment could enhance the assembly of the SNARE complex through interrupting the entry of NSF/ α -SNAP by its binding to Sec22b, resulting in depletion of a pool of free syntaxin 18 and/or D12. If this occurs, reduction of free syntaxin 18 and/or D12 would cause phagocytosis inhibition. In contrast, knockdown of Sec22b by siRNA could increase the pool of free syntaxin 18 and/or D12, thereby enhancing phagocytosis activity. Alternatively, steric hindrance of the Fab fragment bound to Sec22b might have caused indirect effects, or it is even possible that the reductive environment of the cytoplasm may have denatured the Fab fragment and unrelated effects were exerted.

Hence, we postulate that Sec22b functions as a negative regulator of phagocytosis, a function that is consistent with the general idea of inhibitory SNAREs (i-SNAREs), which form nonfusogenic SNARE complexes that have an important role in fine tuning the specificity of fusion (Varlamov *et al.*, 2004). Considering the results of our coimmunoprecipitation experiments with syntaxin 18 or D12 and Sec22b mutants (Figure 5, A and B), we propose that Sec22b specifically sequesters these ER-localized free SNARE proteins, thereby preventing excessive membrane fusion at the site of phagocytosis (Figure 6). This model predicts that activation signal of phagocytosis triggers formation of the closed conformation of Sec22b, thereby increasing the pool of free SNARE proteins such as syntaxin 18 and/or D12, probably through stimulation of the acceptor complex disassembly (Figure 6). In this context, stably expressed mV-22b Δ TMD might represent the "closed," physiologically active form for phagocytosis. The closed conformation is thought to be

formed when the R-SNARE motif of Sec22b binds back onto its N-terminal longin domain under certain conditions without interacting with other SNARE partners (Mancias and Goldberg, 2007). Only the stably expressed mV-22b Δ TMD would be in a closed rather than open conformation (Figure 5A), whereas the transiently expressed mV-22b Δ TMD would be in an open conformation (Figure 5B). The results of ProK treatment (Figure 5, C and D) are consistent with the presence of a compact form in stably expressed mV-22bTMD. The precise reason for this is currently unclear. Although it is possible that formation of the proper "closed conformation" may be a slow process, we think it more likely that the open form is actively converted to the closed one by some unknown machinery and that the function is up-regulated in the J774 cells stably expressing mV-22b Δ TMD. This hypothesis would explain why phagocytosis occurs more efficiently in the J774/mV-22b Δ TMD cells. When phagocytosis signal is emanated, the up-regulated conversion machinery would cause an efficient disassembly of the acceptor complex, and then the released syntaxin 18 and D12 would stimulate phagocytosis. We do not exclude the possibility that there may be a route whereby mV-22b Δ TMD stimulates phagocytosis independently of syntaxin 18 or D12. It is also possible that some modification such as focal regulation of NSF/ α -SNAP (Zhao *et al.*, 2007) and/or phosphorylation of SNARE proteins might cause a structural change (Snyder *et al.*, 2006) to modify the availability of SNARE proteins.

Although our studies indicate that ER membranes play a role in phagocytosis, involvement of endocytic organelles in phagocytosis has been firmly established (Haas, 2007). VAMP3-positive recycling endosomes (Bajno *et al.*, 2000; Coppolino *et al.*, 2001) and VAMP7-positive late endosomes/lysosomes (Braun *et al.*, 2004) have been shown to contribute to the formation of phagosomes. However, it is unknown how the two membrane systems are integrated in the process of phagocytosis. To address this question, we compared the content of phagosomes isolated using latex beads. If incorporation occurs independently, then endocytic markers should be enhanced in Sec22b-R-SNARE mutant-overexpressing cells, which exerted a strong suppression of phagocytosis activity (Figure 4D). When latex bead-loaded phagosomes were isolated 30 min after the initiation of phagocytosis, we observed little difference in the phagosome content from J774/mV control cells and J774/mV-22b-R-SNARE cells (Supplementary Figure S5). ER-marker proteins (syntaxin 18, Sec22b, and calnexin), endocytic marker proteins (VAMP3 and VAMP7), and the phagosome maturation marker protein LAMP-1 were recruited to phagosomes from J774/mV-22b-R-SNARE cells at the almost same rate as that detected in the phagosomes from J774/mV cells (Supplementary Figure S5). The amount of isolated phagosomes from J774/mV-22b-R-SNARE cells actually decreased compared with that from J774/mV cells (data not shown), reflecting their phagocytosis activity. These data indicate that proper phagosomes may not be formed unless enough ER membranes are supplied, suggesting that the step requiring the ER membranes precedes the fusion step with endocytic components. Alternatively, it is still possible that Sec22b also regulates the endocytic pathway through unidentified mechanism(s) during phagocytosis. A critical issue in understanding phagocytosis will be to elucidate the precise mechanisms of how incorporation of the two membrane integration processes is organized.

SNARE complexes generally consist of a parallel helix bundle composed of four heptad repeat-containing SNARE motifs, three from Q-SNARE proteins and one from an R-SNARE

protein (Jahn and Scheller, 2006). Syntaxin 18 and D12 are Q-SNARE proteins involved in ER-mediated phagocytosis. However, Sec22b is apparently not a cognate R-SNARE protein because it inhibits syntaxin-mediated membrane fusion for phagocytosis as an i-SNARE (Varlamov *et al.*, 2004). Thus, the cognate R-SNARE protein that actively participates in this process remains unknown. A possible candidate is VAMP5, an R-SNARE protein localized to the plasma membrane (Zeng *et al.*, 1998), and/or Ykt6, an R-SNARE protein localized to both the cytosol and membrane (Figure 6). Ykt6 contains a palmitoylation site at its C-terminus and analyses using various mutants have indicated that some Ykt6 mutants are targeted to the plasma membrane (Fukasawa *et al.*, 2004; Hasegawa *et al.*, 2004). The cognate plasma membrane Q-SNARE(s) involved in this process also remains unidentified, although syntaxins 2–4 can interact with syntaxin 18 in vitro (Hatsuzawa *et al.*, 2006). Further studies are needed to elucidate the details of ER-mediated phagocytosis.

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