Human SCAMP5, a Novel Secretory Carrier Membrane Protein, Facilitates Calcium-Triggered Cytokine Secretion by Interaction with SNARE Machinery[1]

Chaofeng Han,[2]* Taoyong Chen,[2†] Mingjin Yang,[†] Nan Li,[†] Haibo Liu,[†] and Xuetao Cao[3]*[†]

Cytokines produced by immune cells play pivotal roles in the regulation of both innate and adaptive immunity. However, the mechanisms controlling secretion of cytokines have not been fully elucidated. Secretory carrier membrane proteins (SCAMPs) are widely distributed integral membrane molecules implicated in regulating vesicular transport. In this study, we report the functional characterization of human SCAMP5 (hSCAMP5), a novel SCAMP protein that is widely expressed by a variety of neuronal and nonneuronal tissues and cells. By measuring the cytokine secretion (RANTES/CCL5 and IL-1β) as an exocytotic model, we show that hSCAMP5 can promote the calcium-regulated signal peptide-containing cytokine (CCL5 but not IL-1β) secretion in human epithelial cancer cells, human monocytes, and mouse macrophages. By using subcellular fractionation, immunofluorescence confocal microscopy, and membrane vesicle immunoisolation methods, we find that hSCAMP5 is mainly localized in the Golgi-associated compartments, and the calcium ionophore ionomycin can trigger a rapid translocation of hSCAMP5 from Golgi apparatus to plasma membrane along the classical exocytosis pathway. During the translocation of hSCAMP5 from Golgi apparatus to plasma membrane, hSCAMP5 can codistribute and complex with local soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) molecules. We further demonstrate that hSCAMP5 can directly interact with the calcium sensor synaptotagmins via the cytosolic C-terminal tail of hSCAMP5, thus providing a potential molecular mechanism linking SCAMPs with the SNARE molecules. Our findings suggest that hSCAMP5, in cooperation with the SNARE machinery, is involved in calcium-regulated exocytosis of signal peptide-containing cytokines. ***The Journal of Immunology,*** **2009, 182: 2986–2996.**

From yeast to mammals, all types of cells secrete proteins along the secretory pathway that consists of various membrane organelles or vesicles via constitutive or regulated mechanisms (1–4). Genetic and in vitro experiments have revealed the molecular mechanisms of exocytosis in many well-characterized cell types including neurons, neuroendocrine, endocrine, exocrine, and hematopoietic cells (1–4). Soluble *N*-ethylmaleimide sensitive factor (NSF)[4] attachment protein receptors (SNAREs) have been suggested to play a fundamental and core role in membrane trafficking (5–7). According to the SNARE hypothesis, vesicles dock to a target membrane through the interaction of complementary sets of vesicular (v-SNARE) and target (t-SNARE) membrane proteins (8–10). In synaptic vesicle exocytosis, the vesicular protein synaptobrevin (also called vesicle-associated membrane proteins (VAMPs)) is the v-SNARE, and the plasma membrane-associated proteins SNAP-25 (synaptosomal-associated protein of 25 kDa) and syntaxin 1 function as t-SNAREs (11). Formation of the SNARE complex (or core complex) in two adjacent membranes brings the membranes into close opposition, and is followed by recruitment of the cytosolic proteins NSF and α-, β-, and γ-SNAP (soluble NSF attachment protein), which are required for dissociation of the core complex and for membrane fusion (12, 13). The SNAREs, particularly VAMPs and syntaxins, are specifically localized throughout the mammalian vesicular compartments, including endoplasmic reticulum (ER), *trans*-Golgi network (TGN), early endosomes (EE), secretory vesicles, and the plasma membrane (PM) (1–4). Up to now, at least 18 syntaxins and 8 VAMPs have been characterized, which are localized to various subcellular compartments and mediate the docking and fusion of vesicles therein (2–4). Other families of proteins, such as Rab small GTPases and secretory carrier membrane proteins (SCAMPs), have also been implicated in the regulation of membrane trafficking in eukaryotic cells (1–4).

SCAMPs are a family of conserved membrane proteins that were initially discovered as components of regulated secretory carriers in exocrine, neural, and endocrine cells (14–17). SCAMPs are widely distributed as components of post-Golgi membranes, synaptic vesicles, secretion granules, and transporter vesicles (14–17). SCAMPs (SCAMPs 1–5) from diverse species have been characterized (18–20). SCAMPs 1–3, which contain cytoplasmic N-terminal domain with multiple NPF (asparagine-proline-phenylalanine) repeats, are implicated in endocytosis and signaling,

*Institute of Immunology, Tsinghua University School of Medicine, Beijing, People's Republic of China; and †Institute of Immunology and National Key Laboratory of Medical Immunology, Second Military Medical University, Shanghai, People's Republic of China

Received for publication June 19, 2008. Accepted for publication December 24, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Foundation for the Author of Excellent Doctoral Dissertation of China (200775), National Natural Science Foundation of China (30572122, 30771118, 30721091), National Key Basic Research Program of China (2007CB512403), and National Specific Program of New Drug Development and Shanghai Committee of Science and Technology (07QA14067).

[2] These authors contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Xuetao Cao, Institute of Immunology, Tsinghua University School of Medicine, Beijing, People's Republic of China. E-mail address: caoxt@public3.sta.net.cn

[4] Abbreviations used in this paper: NSF, *N*-ethylmaleimide sensitive factor; SNARE, soluble NSF attachment protein receptor; v-SNARE, vesicular SNARE; t-SNARE, target SNARE; VAMP, vesicle-associated membrane protein; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; EE, early endosome; PM, plasma membrane; SCAMP, secretory carrier membrane protein; EGF, epidermal growth factor; TMR, transmembrane region; hSCAMP5, human SCAMP5; BMSC, bone marrow stromal cell; siRNA, small interfering RNA; HDM, high density microsome; LDM, low density microsome; GDV, Golgi-derived vesicles; NPF, asparagine-proline-phenylalanine; SNAP-23, synaptosomal-associated protein of 23 kDa.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802002

evidenced by their phosphorylations on tyrosine residues after epidermal growth factor (EGF) stimulation and the interaction of SCAMP1 and SCAMP3 with EGF receptor (21, 22). Moreover, SCAMPs 1–3 are also involved in the regulation of exocytosis (23–28). SCAMPs 1–3 are colocalized with SNARE machinery in secretory vesicles and have been detected in the glucose transporter 4-containing vesicles (23–26). The topology structure of SCAMP1 has been analyzed in detail, which shows that the N- and C-terminal segments as well as loops between the transmembrane regions (TMR) that face the cytosol may be functionally involved in regulation by agonists (27–30). Two functional segments of SCAMPs have been identified (27, 28, 30, 31). The NPF repeats contained in SCAMPs 1–3 can interact with intersectin-1 and γ-synergin (31), and a synthetic 11-residue-peptide (E peptide) corresponding to the region between TMR 2 and 3 of SCAMP2 can inhibit the fusion of secretory vesicles with plasma membrane in mast cells and PC12 cells (27, 28, 30). Recently, mouse (mSCAMP5 or mSC5) and rat SCAMP5 have been cloned and reported (18). It is shown that SCAMP5 is expressed in synaptic vesicles of neuroendocrine tissues, such as brain and neurohypophysis, and is codistributed with synaptotagmin I, SCAMPs 1–3, Rab3 and synaptophysin in synaptic vesicles, suggesting a role of SCAMP5 in exocytosis of neuronal cells (18). However, up to now there is no report about human SCAMP5, and whether SCAMP5 is involved in exocytosis or in endocytosis lacks direct evidence.

Cytokines play important roles in the innate and adaptive immunity. Secretion of the signal peptide-containing cytokines (such as RANTES/CCL5 and IL-6) is via a classical pathway, that is, from ER to Golgi apparatus and then to plasma membrane (1–4), while secretion of cytokines without signal peptide (such as IL-β and FGF) is dependent on a nonclassical pathway that remains elusive (32–34). Recently, the involvement of SNARE molecules in cytokine production of immune cells has been the focus of investigations (33–35). Newly synthesized TNF-α precursors accumulate in the Golgi complex of macrophages where it is colocalized with SNARE molecules syntaxin 6 and Vit1b (36). Upon LPS or IFN-γ stimuli, TNF-α is translocated to plasma membrane where it colocalizes with syntaxin 2 and syntaxin 4 (36, 37). In HUVEC cells derived from human umbilical cords, chemokines are codistributed with Rab4, Rab32, and VAMP3 (38). In $CD4^+$ Th cells, various cytokines and chemokines are codistributed with GM130, Rab3D, Rab19, Rab37, and the SNARE proteins syntaxin 6 and Vit1b upon the synaptic interaction between Th and B cells (39). These results have suggested that key SNAREs at the levels of the TGN and the secretory vesicles, together with the cell surface SNARE complexes, are rate-limiting for cytokine secretion. Macrophages are competent in secretion of various cytokines that are implicated in the regulation of both innate and adaptive immunity. Although many SNARE-associated molecules have been reported to regulate the cytokine secretion of macrophages (36, 37), the associated definite mechanisms and the roles of SCAMPs in controlling macrophage cytokine secretion need to be fully elucidated.

We have cloned a novel human SCAMP5 (hSCAMP5) molecule, designated on the basis of high homology with murine and rat SCAMP5 (18), from a bone marrow stromal cell (BMSC)-derived cDNA library (40). We find that hSCAMP5 is widely distributed in neuronal and nonneuronal tissues and cells. We show that hSCAMP5 can promote the exocytosis of signal-peptide containing cytokines (e.g., CCL5, TNF-α) but not the cytokines without signal peptide (e.g., IL-1β) upon ionomycin treatments. We also provide evidence that hSCAMP5 can colocalize with and interact with different SNARE pairs and synaptotagmins during the translocation of hSCAMP5 from Golgi-associated compartments to plasma membrane. Our data suggest that hSCAMP5 is involved in the regulation of cytokine secretion along the classical exocytosis pathway by interaction with SNARE molecules.

Materials and Methods

Cells, Abs, and reagents

All the cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured under standard conditions as described (40–42). The Abs against calnexin (Ab31290, mouse), EEA1 (Ab15846, mouse), Hexa-His (Ab18184, mouse), sodium potassium ATPase (Na^+/K^+ ATPase, Ab7671, mouse), Rab4 (Ab13252, rabbit), Rab5 (Ab18211, rabbit), syntaxin 4 (Ab18010, mouse), VAMP2 (Ab18014, rabbit), and VAMP4 (Ab3348, rabbit), and the pre-adsorbed HRP-linked secondary Abs were from Abcam. The Abs against synaptosomal-associated protein of 23 kDa (SNAP-23) (sc-22643, goat), synaptotagmin I (sc-7753, goat), synaptotagmin II (sc-12465, goat), syntaxin 8 (sc-101304, mouse), TGN38 (sc-27680, goat), and VAMP3 (sc-18208, goat) were obtained from Santa Cruz Biotechnology. The Ab against syntaxin 6 (no. 2417, rabbit) was from Cell Signaling Technology. The Abs against Flag (F1804, mouse) and β-actin (A3853, mouse), the anti-Flag agarose, ionomycin, and LPS (*Escherichia coli* 0111:B4) were from Sigma-Aldrich. Platelet-activating factor, poly (I:C) and BAPTA were from Calbiochem. The Ab against SCAMP5 (S0442-05, rabbit) was obtained from U.S. Biological. Fluorescent dyes for Golgi apparatus (BODIPY-FBA, 558/568) and lysosomes (Lyso Tracker Red DND-99, 577/590), and pre-adsorbed Oregon Green 488- and Alexa Fluor 555-conjugated secondary Abs, were from Molecular Probes. CpG ODN was synthesized by us as described (42).

Isolation and sequence analysis of full-length hSCAMP5

The full-length cDNA of hSCAMP5 was directly isolated from cDNA library of human BMSC prepared by us as described previously (40). Bioinformatic analysis was performed with the BLAST program provided by NCBI or ClustalW program provided by EBI. The full-length sequence of hSCAMP5 is available at GenBank (No. AF495715).

Recombinant expression of GST fusion proteins of hSCAMP5

The cDNA encoding the full-length, the C-terminal 64 residues (hSCAMP5C64), and the deletion of C-terminal 64 residues (hSCAMP5ΔC64) of hSCAMP5 was cloned into pGEX-2T expression vector (Pharmacia Biotech). GST fusion proteins expressed in *E. coli* strain BL21 was purified as described previously (41).

Expression pattern of hSCAMP5

RT-PCR, Northern blot, and quantitative RT-PCR assays were performed as described previously (41, 42). Primers for RT-PCR assays of hSCAMP5 were 5′-GCAGAGAAAGTGAACAACTTCC-3′ (sense) and 5′-GGT TCACATCTCATTGGAGTAC-3′ (antisense). Primers 5′-TGGACATC CGCAAAGACCTGTAC-3′ (sense) and 5′-TCAGGAGGAGCAATG ATCTTGA-3′ (antisense) were used for human β-actin amplification. Northern blot filters containing multiple human poly(A)$^+$ RNA (2 μg/lane) from various tissues were purchased from Clontech Laboratories. For immunohistochemistry assay of hSCAMP5 expression in normal human thyroid and adrenal gland, microarrays containing indicated human tissues (1.5 mm in diameter) were obtained from Cybrdi. hSCAMP5 Ab was used at dilutions of 1/100 to 1/200. The EnVision$^+$ detection system (Dako-Cytomation) was used per the manufacturer's instructions. The other primer sequences used in quantitative RT-PCR assays were available upon request.

RNA interference

For transient transfection, 21-nucleotide small interfering RNA duplexes (siRNA) were synthesized for hSCAMP5. The sequences used were: 5′- GCGCCUCUACUACCUCUGGUU-3′ (for both hSCAMP5 and mSCAMP5, hSC5 siRNA1), 5′-AUUCAUCCCGCUGAAGC CAUU-3′ (for hSCAMP5, hSC5 siRNA2), 5′-GCGCGUCUAC UACGUCUCGUU-3′ (scrambled control for hSC5 siRNA1, Ctrl siRNA1), and 5′-AUACAUCGCGCUGAAGGCAUU-3′ (scrambled control for hSC5 siRNA2, Ctrl siRNA2). siRNA duplexes were transfected into MCF-7 cells and RAW264.7 cells using INTERFERin reagent (Polyplus-transfection Company) according to the standard protocol and as described (42).

Western blot

Western blot was performed as described by us previously (43). Bands were revealed using Supersignal West Femto Maximum Sensitivity substrate (Pierce), following the manufacturer's instructions.

Mammalian expression vector construction, transfection and stable selection

For construction of hSCAMP5 expression vectors with Flag Tag, pcDNA3.1-Flag (Invitrogen) were used. For the construction of human RANTES/CCL5 (GenBank No. NM_002985) or IL-1β (GenBank No. NM_000576) expression vector with hexa-His tag, the pcDNA6.2-DEST vector (Invitrogen) was used. cDNA fragments were amplified via PCR using human dendritic cell cDNA or hSCAMP5 plasmid as templates and in-frame cloned into indicated expression vectors under the promoter of CMV. All the expression vectors used in this study were confirmed by sequencing and then prepared using Endofree Plasmid Maxi kit (Qiagen) according to the manufacturer's instructions. For the transfection of expression vectors in MCF-7 and HeLa cells, the jetPEI reagent (Polyplus-transfection Company) was used; for the transfection of RAW264.7 cells, the jetPEI-Macrophage reagent (Polyplus-transfection Company) was used and for the transfection of THP-1 monocytes, the Nucleofector and the related reagents (Amaxa) were used. For the establishment of stable expression cell lines, the transiently transfected cells were selected under 400–600 μg/ml neomycin (for pcDNA3.1-Flag) and/or blasticidin (for pcDNA6.2-DEST) for 2 wk as described previously (42).

Immunofluorescence confocal microscopy

Cells growing on glass coverslips placed in 6-well plates (Falcon, BD Biosciences) were incubated with 0.1 μM BODIPY 558/568 BFA derivatives or LysoTracker Red DND-99 (577/590) for 15 min at room temperature in the dark. Samples were washed briefly in PBS and fixed in 4% paraformaldehyde before Ab staining as described (41). For colocalization assays, slides that have been fixed in 4% paraformaldehyde were sequentially incubated with primary Abs as indicated and Oregon Green 488-conjugated or Alexa Fluo 555-conjegated secondary Ab in PBS containing 1% saponin, 0.5% BSA, and 10% FCS as described previously (42). Slides were finally examined under a Zeiss LSM 510 confocal microscope (Carl Zeiss). Images were acquired under a ×40/0.75 NA oil objective and processed using Zeiss LSM Image Browser Version 3.1.0.99 (Carl Zeiss).

Membrane protein preparation and subcellular fractionation

For the subcellular localization assays of the hSCAMP5, we used an established differential centrifugation as described (44). This procedure generates four membrane-enriched fractions as follows: mitochondria/nuclei, PM, and high and low density microsomes (HDM and LDM, respectively). The HDM is enriched in ER markers and fluid phase markers; the LDM contains Golgi markers, recycling endosomes, and the majority of the intracellular small vesicles.

To determine the membrane fractions in which hSCAMP5 was localized, an established sucrose equilibrium density centrifugation procedure was used (45). The postnuclear supernatants were fractionated on sucrose equilibrium density as described (45). Twelve 1-ml fractions were collected from the top of the gradient using a fractionator, and 30 μl of each fraction were analyzed by Western blotting.

Cytokine secretion assay

HeLa, MCF-7, THP-1, or RAW264.7 cells were cultured in 24-well plates and treated as indicated. Then cytokines contained in the medium were determined by ELISA as instructed by the manufacturer (R&D System). To determine the roles of hSCAMP5 in cytokine secretion, cells overexpressing hSCAMP5 and cells silenced with siRNAs were serum starved for 24 h, cultured in nominal calcium-free HEPES-buffered OPTI-MEM-reduced serum medium for 6 h (Invitrogen), and then treated with ionomycin (10 μM), LPS (100 ng/ml), CpG ODN (2 μM), poly (I:C) (p(I:C), 10 μg/ml), ATP (2 mM), or PAF (100 nM) as indicated.

Immunoisolation of membrane vesicles and immunoprecipitation

To identify the colocalization of hSCAMP5 with multiple subcellular compartments markers, the indicated membrane fractions were collected, resuspended in homogenization buffer (45), and preadsorbed with protein A agarose (Sigma-Aldrich) for 4 h at 4°C. Then the membrane fractions supplemented with 0.2% BSA were incubated with Flag Ab-conjugated agarose at 4°C with gentle agitation for 1 h. The immunoisolated membrane/vesicles were precipitated by centrifugation and washed four times in homogenization buffer.

For immunoprecipitation assays, membrane fractions were solubilized in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1 mM PMSF at a protein concentration of 0.5 mg/ml for 1 h at 4°C. The supernatants were collected by centrifugation at 200,000 × *g* for 1 h and subjected to immunoprecipitation in the presence of Flag Ab-conjugated agarose or IgG plus protein A/G agarose (Pierce).

Finally, the immunoisolated membranes/vesicles and the immunoprecipitated immune complexes were solubilized in 2 × SDS sample buffer, separated on SDS-PAGE gel, and examined by Western blotting.

Statistical analysis

All the experiments were repeated at least three times. Results are given as mean ± SE or mean ± SD. Comparisons between two groups were done using Student's *t* test analysis. Multiple comparisons were done with a one-way ANOVA followed by Fisher's least significant difference analysis. Statistical significance was determined as $p < 0.05$.

Results

Identification and bioinformatic analysis of hSCAMP5

The full-length cDNA of hSCAMP5 was composed of 2882 bp and contained a complete ORF of 708 bp (140–847 bp), potentially encoding a 235-residue protein with a calculated molecular mass of 26 kDa. hSCAMP5 protein was highly homologous to mouse SCAMP5 and other human SCAMPs (Supplemental Fig. S1).[5] By comparison with human genome sequence, we found that the ORF region of hSCAMP5 was overlapped with the Unigene cluster Hs.7934, which was mapped to 15q23. By comparison with the topology structure of SCAMP1 (29), we predicted that the structure of hSCAMP5 consisted of a short N-terminal cytoplasmic tail, four TMRs, and a C-terminal tail (172–235 aa) (Supplemental Fig. S1). Therefore, hSCAMP5 is a novel human SCAMP molecule.

hSCAMP5 is distributed in neuronal and nonneuronal tissues and cells

To investigate the expression pattern of hSCAMP5 mRNA, we performed Northern blot and RT-PCR assays. The Northern blot assays showed that hSCAMP5 mRNA was expressed as a single transcript ~3.4 kb in brain, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, bone marrow, and the different parts of human brain (Fig. 1*A*). The RT-PCR assays showed that hSCAMP5 mRNA could be detected in BMSC and several hematological leukemia cell lines (Fig. 1*B*). But in most of the solid tumor cells, expression of hSCAMP5 mRNA was mainly detected in adenocarcinoma and epithelial cancer cell lines, including MCF-7, PC-3, LoVo, U251, CaoV-3, SW13, and SW579 (Fig. 1*C*).

To verify the expression pattern of hSCAMP5 at protein level, we examined the expression of hSCAMP5 by using Western blot and immunohistochemistry assays. We found that hSCAMP5 protein could be detected in human BMSC, breast cancer cells (MCF-7), adrenal gland small cell carcinoma cells (SW13), and Burkitt's lymphoma (Daudi), but not in parental HeLa cells and THP-1 cells (Fig. 1*D*). In the human thyroid tissues, we found that hSCAMP5 was mainly expressed by the follicular epithelial cells (*upper panel*, Supplemental Fig. S2). In the adrenal gland tissues, we specifically detected hSCAMP5 in the zona fasciculate of the cortex region (*lower panel*, Supplemental Fig. S2). These data suggest that hSCAMP5 is not only expressed by neuronal tissues but also expressed by other nonneuronal tissues and cells.

hSCAMP5 is an integral membrane protein mainly localized to Golgi-associated compartments

To examine the subcellular distribution of hSCAMP5 within MCF-7 and SW13 epithelial cells, four different fractions were

[5] The online version of this article contains supplementary material.

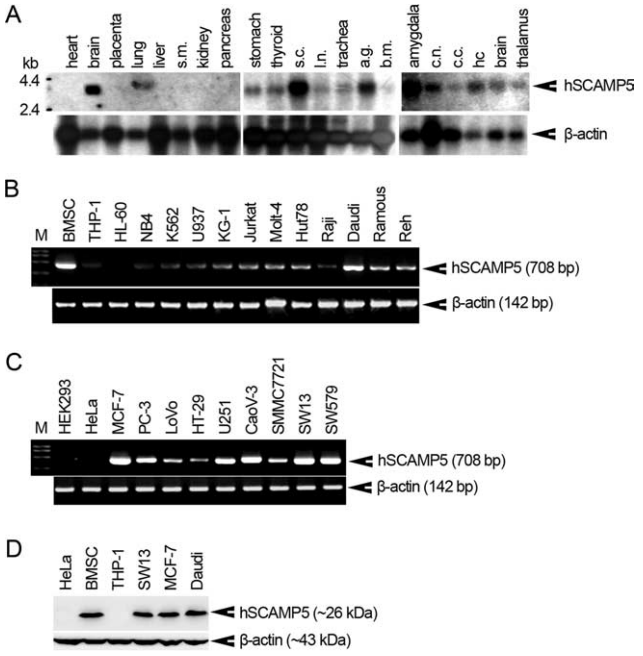

FIGURE 1. Expression pattern of hSCAMP5. *A*, Northern blot analysis for tissue distribution of hSCAMP5 mRNA. Northern blots were analyzed with a probe corresponding to encoding region of hSCAMP5. β-actin was probed as control. s.m., skeletal muscle; s.c., spinal cord; l.n., lymph node; a.g., adrenal gland; b.m., bone marrow; c.n., caudate nucleus; c.c., corpus callosum; hc, hippocampus. *B* and *C*, RT-PCR assays of hSCAMP5 mRNA expression in human hematopoietic cell lines (*B*) and solid tumor cell lines (*C*). M, marker; BMSC, bone marrow-derived stromal cells. *D*, Western blot assays of hSCAMP5 expression in cell lines. β-actin was examined as loading control.

analyzed for the expression of hSCAMP5 by Western blot. To ensure the effectiveness of the isolation procedure (44), markers for Golgi (TGN38), ER (Calnexin), and PM (Na^+/K^+ ATPase) were also examined. We found that hSCAMP5 was mainly distributed in the LDM fractions, and minimally detected in the PM fractions, but not in the HDM fractions enriched of ER and fluid phase markers and the mitochondria/nuclei fractions (Supplemental Fig. S3*A*). In SW13 and MCF-7 cell lines, we examined the subcellular distribution of hSCAMP5 by immunofluorescence confocal microscopy. We found that hSCAMP5 was colocalized with Golgi marker (BODIPY-BFA), but not with the ER marker (calnexin) and lysosome marker (Lyso Tracker) (Supplemental Fig. S3*B*). Therefore, endogenous hSCAMP5 may be mainly localized in the Golgi-associated compartments.

To investigate the roles of hSCAMP5 in the regulation of exocytotic pathways, we first used hSCAMP5-negative HeLa epithelial cells as a cell model because HeLa cells have been widely used in the membrane transport studies and are equipped with diverse definite SNARE molecules (1–4). We stably overexpressed hSCAMP5-Flag in HeLa cells and examined its localization by differential centrifugation. We found that hSCAMP5-Flag was mainly localized in the LDM fraction (Supplemental Fig. S4*A*), which was consistent with the distribution of hSCAMP5 in SW13 and MCF-7 cells (Supplemental Fig. S3*A*). As predicted by the protein sequence, hSCAMP5 contained four TMRs and was a membrane-bound molecule (Supplemental Fig. S1). To test this possibility, we isolated the total membranes from HeLa cells stably overexpressing hSCAMP5-Flag and treated the membranes with indicated reagents. We found that these treatments could not extract hSCAMP5 from membrane fractions (Supplemental Fig. S4*B*), suggesting that hSCAMP5 was indeed an integral membrane protein.

To further confirm the hSCAMP5 localization, we performed sucrose equilibrium density centrifugation in hSCAMP5-Flag-stably overexpressed HeLa cells. We found that the PM markers (Na^+/K^+ ATPase, SNAP-23, and syntaxin 4) were mainly distributed in fractions 1 and 2; the ER marker calnexin was mainly distributed in fractions 11 and 12; and the markers for Golgi and Golgi-derived vesicles (GDV) (TGN38, Mannosidase II, VAMP4, syntaxin 6, syntaxin 8, LAMP-1, and EEA-1, etc.) were mainly distributed in fractions 5–7 (Supplemental Fig. S4*C*), suggesting that the isolation procedure was efficient in separation of the indicated fractions. For hSCAMP5, we found that the peak was present in fraction 6, suggesting that hSCAMP5-Flag was mainly distributed in Golgi-associated compartments.

hSCAMP5 promotes calcium-triggered exocytosis of signal peptide-containing cytokines in epithelial cancer cells

According to the protein sequence, soluble cytokines can be classified into signal peptide-containing cytokines (e.g., RANTES/CCL5) and cytokines without signal peptide (e.g., IL-1β). To investigate the possible roles of hSCAMP5 in exocytosis, we used the cytokine secretion model to examine the involvement of hSCAMP5 in exocytosis and the associated exocytotic machinery.

In hSCAMP5-negative HeLa cells, we cotransfected the hSCAMP5-Flag plasmid (neomycin-resistant) with CCL5-His or IL-1β-His plasmid (blasticidin-resistant) and selected the transfected cells with both neomycin and blasticidin for 2 wk. The established cell lines were examined by both Western blot (for hSCAMP5-Flag expression, Supplemental Fig. S5*A*) and by ELISA (for cytokine contained in lysates, Supplemental Fig. S5*B* and S5*C*). The stable expression of hSCAMP5-Flag in combination with or without CCL5-His or IL-1β-His could lead to significant increase in the production of CCL5 but not IL-1β (Supplemental Fig. S5*D* and S5*E*) under normal culture conditions, suggesting that hSCAMP5 may be involved in the constitutive release of cytokines. We next cultured serum-starved HeLa cells in nominal calcium-free medium for 6 h and stimulated cells with ionomycin to initiate the calcium-triggered exocytotic machinery. We found that ionomycin could promote a rapid release of CCL5 and IL-1β from HeLa cells (Supplemental Fig. S5*F* and S5*G*). More importantly, we found that HeLa cells overexpressing hSCAMP5-Flag showed a more rapid and robust release of CCL5 than mock cells (Supplemental Fig. S5*F*), but hSCAMP5-Flag overexpression exerted no significant effects on the calcium-regulated release of IL-1β (Supplemental Fig. S5*G*) upon ionomycin treatments. Therefore, hSCAMP5 may be involved in the exocytotic regulation of signal peptide-containing cytokines.

To confirm the differential roles of hSCAMP5 in exocytosis of signal peptide-containing cytokines and cytokines without signal peptide, we stably overexpressed CCL5-His or IL-1β-His in MCF-7 epithelial cells and then silenced hSCAMP5 expression with hSCAMP5 siRNAs. The efficiency of silencing was determined by quantitative RT-PCR, Western blot, and confocal microscopy assays, which showed that the hSC5 siRNA1 could efficiently decrease the expression of hSCAMP5 (Fig. 2, *A–C*). In the hSCAMP5-silenced MCF-7 cells, ionomycin-triggered release of CCL5 was significantly impaired as compared with mock cells (Fig. 2*D* and Supplemental Fig. S6*A*). However, the release of IL-1β was not significantly affected by hSCAMP5 silencing (Fig. 2*E* and Supplemental Fig. S6*B*).

To further verify the roles of SCAMP5 in promoting CCL5 release in response to calcium signal, we examined the CCL5 release in response to extracellular ATP (2 mM) and PAF (100 nM),

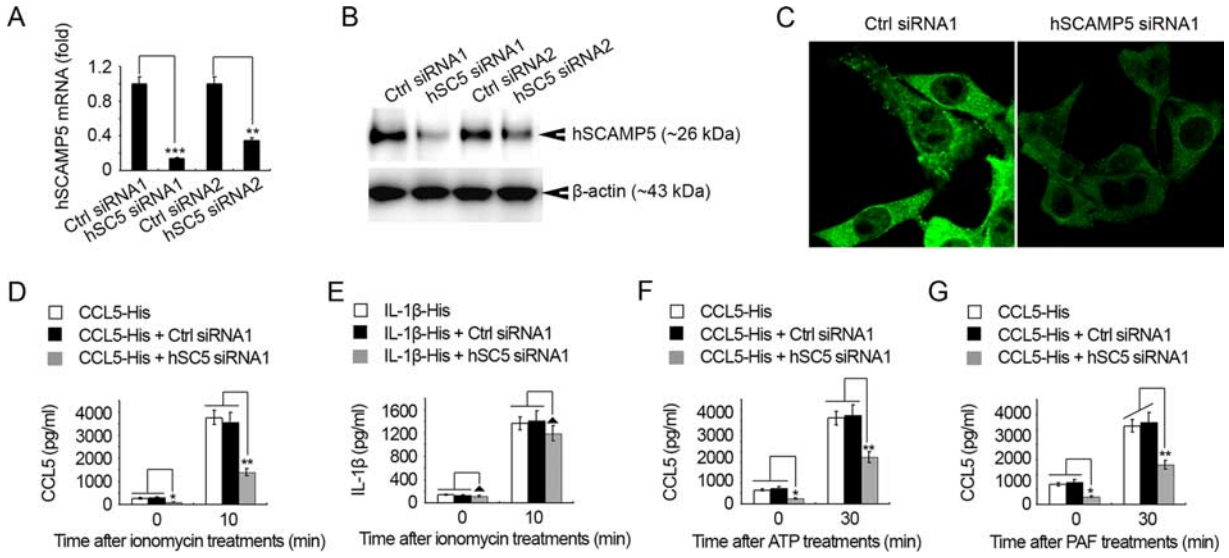

FIGURE 2. hSCAMP5 silence inhibits calcium-triggered exocytosis of CCL5 in epithelial cancer cells. *A–C*, Efficiency of hSCAMP5 siRNAs in MCF-7 cells. MCF-7 cells were transiently transfected with indicated siRNAs. Forty-eight hours later, the expression of hSCAMP5 was examined by quantitative RT-PCR (*A*), Western blot (*B*), and immunofluorescence confocal microscopy (*C*). For quantitative RT-PCR, results were presented as mean ± SE of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$. *D–G*, ELISA of CCL5 and IL-1β release in hSCAMP5-silenced MCF-7 cells (5×10^5 cells/ml). MCF-7 cells stably transfected with CCL5-His vector (CCL5-His) or IL-1β-His vector (IL-1β-His), and CCL5-His-transfected MCF-7 cells transiently transfected with Ctrl siRNA1 (CCL5-His plus Ctrl siRNA1) or hSC5 siRNA1 (CCL5-His plus hSC5 siRNA1) for 48 h, were serum starved for 24 h, cultured in OPTI-MEM for 6 h, and treated with 10 μM ionomycin (*D* and *E*), 2 mM ATP (*F*), or 100 nM PAF (*G*) as indicated. The culture supernatants were collected and measured for the CCL5 and IL-1β production via ELISA. Results were presented as mean ± SD of triplicate samples. *, $p < 0.05$; **, $p < 0.01$.

reagents that trigger sustained and transient Ca^{2+} mobilization, respectively, in hSCAMP5-silenced MCF-7 cells overexpressing CCL5-His. We found that the release of CCL5 was significantly inhibited by SCAMP5 silence (Fig. 2, *F* and *G*).

Given that the effects of ionomycin, ATP, and PAF on cytokine release were all inhibited by calcium chelator BAPTA pretreatments (data not shown), it may be concluded that hSCAMP5 was involved in the regulation of calcium-triggered release of signal peptide-containing cytokines.

hSCAMP5 promotes calcium-triggered exocytosis of signal peptide-containing cytokines in monocytes and macrophages

The above results (Supplemental Fig. S5 and Fig. 2) indicated that hSCAMP5 was involved in the regulation of cytokine secretion of epithelial cancer cells. Cancer cells can produce chemokines and cytokines, contributing to tumor immune escape. However, epithelial cancer cells are not well-defined model for cytokine secretion, and the molecular machinery controlling cytokine secretion of epithelial cancer cells was poorly understood. We, therefore, investigated the roles of SCAMP5 in cytokine secretion of human monocytes (THP-1 cells) and mouse macrophages (RAW264.7 cells).

Previous studies have suggested that monocytes and macrophages are rather competent in producing various inflammatory cytokines upon TLR agonists treatments. We went first to examine the expression of exocytosis-associated molecules in THP-1 cells and RAW264.7 cells before and after TLR agonists treatments by quantitative RT-PCR assays. We found that SCAMP5, together with SNAP-23, syntaxin 4, syntaxin 6, VAMP3, VAMP4, synaptotagmin I, and synaptotagmin II, was rapidly up-regulated by various TLR agonists (Fig. 3, *A* and *B* and Supplemental Fig. S7),

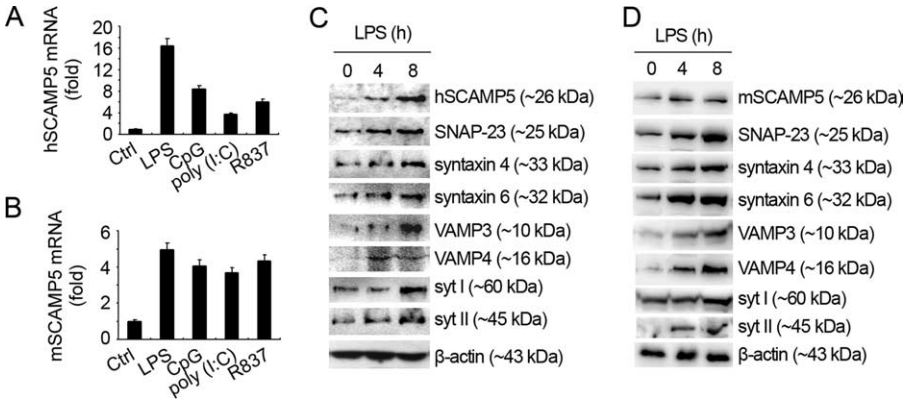

FIGURE 3. SCAMP5 and SNARE molecules are regulated by TLR agonists in THP-1 monocytes and RAW264.7 macrophages. *A* and *B*, Quantitative RT-PCR assays of SCAMP5 mRNA expression. THP-1 cells (*A*) and RAW264.7 cells (*B*) were treated with 100 ng/ml LPS (TLR4), 2 μM CpG ODN (TLR9), 10 μg/ml poly (I:C) (TLR3), or 20 μg/ml R837 (TLR7) for 2 h, then the expression of SCAMP5 was determined by quantitative RT-PCR. Results were presented as mean ± SE of three independent experiments. *C* and *D*, Western blot assays of SCAMP5 and SNARE expression. THP-1 cells (*C*) and RAW264.7 cells (*D*) were treated with 100 ng/ml LPS (TLR4) for 4 or 8 h, then the expression of indicated molecules in lysates was determined by Western blot.

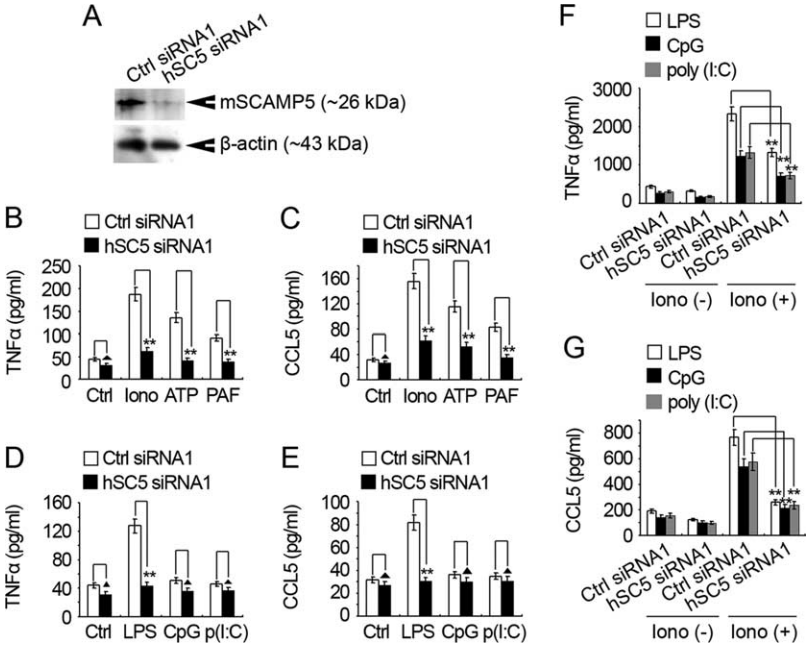

FIGURE 4. SCAMP5 silence inhibits calcium-regulated exocytosis of CCL5 in RAW264.7 macrophages. *A*, Efficiency of SCAMP5 silence in RAW264.7 cells. RAW264.7 cells were transiently transfected with hSC5 siRNA1 or Ctrl siRNA1, and the expression of mSCAMP5 was examined by Western blot. *B–E*, Cytokine exocytosis in SCAMP5-silenced RAW264.7 cells (2×10^5 cells/ml). Ctrl siRNA1- or hSC5 siRNA1-transfected RAW264.7 cells were starved for 24 h, cultured in OPTI-MEM for 6 h and then treated with calcium agonists (*B* and *C*) or TLR agonists (*D* and *E*). The agonists used were 10 μM ionomycin (Iono, 10 min), 2 mM ATP (30 min), 100 nM PAF (30 min), 100 ng/ml LPS (30 min), 2 μM CpG ODN (30 min), and 10 μg/ml poly (I:C) (p(I:C), 30 min). Then, indicated cytokines in the supernatants were measured by ELISA. The results were presented as mean ± SD of triplicate samples. ▲, $p > 0.05$; **, $p < 0.01$. *F* and *G*, Cytokine exocytosis in TLR ligands-stimulated SCAMP5-silenced RAW264.7 cells. The indicated RAW264.7 cells were treated with TLR ligands for 4 h in OPTI-MEM medium, and then stimulated with 10 μM ionomycin (Iono, 10 min). Then, TNF-α (*F*) and CCL5 (*G*) in the supernatants were measured by ELISA. The results were presented as mean ± SD of triplicate samples. **, $p < 0.01$.

suggesting that these SNARE molecules may be involved in the regulation of inflammatory cytokine production induced by TLR agonists. To verify the TLR-induced expression of SNARE molecules in RAW264.7 and THP-1 cells, we performed Western blot assays of these molecules after TLR4 agonist LPS treatments. We found that TLR4 signaling activation could up-regulate SCAMP5 and these SNARE molecules (Fig. 3, *C* and *D*). These results together indicate that SCAMP5 may be involved in the regulation of cytokine secretion of monocytes and macrophages in response to TLR agonists.

To investigate the roles of SCAMP5 in the regulation of cytokine secretion, we stably overexpressed hSCAMP5-Flag in THP-1

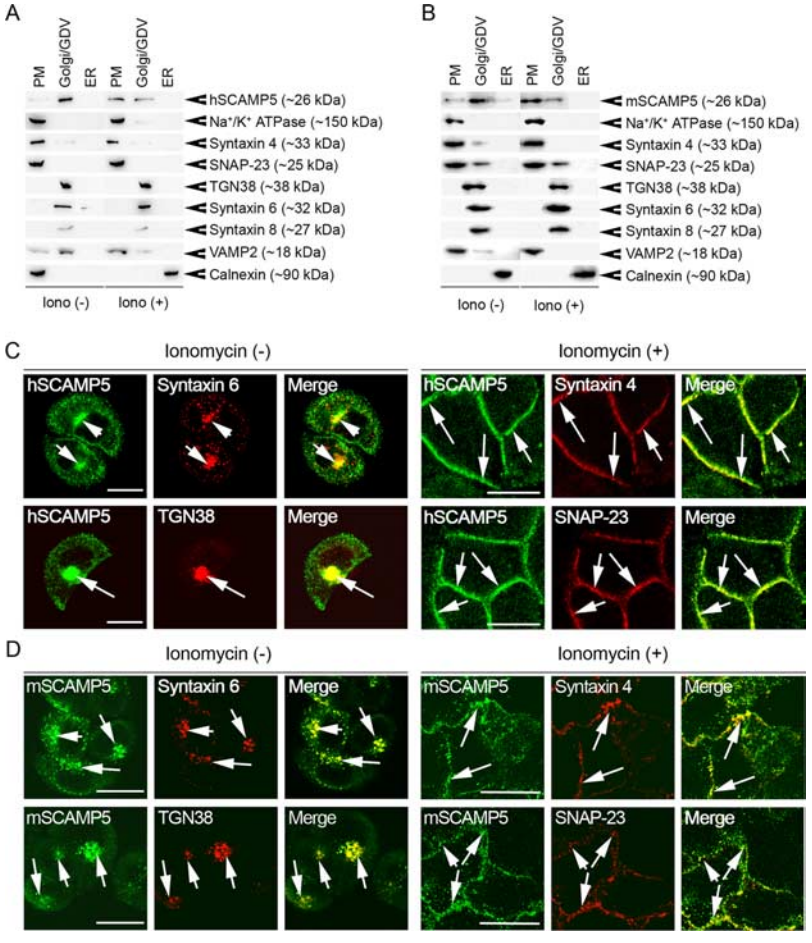

FIGURE 5. SCAMP5 translocates from Golgi-associated compartments to plasma membrane after ionomycin treatments. *A* and *B*, Distribution of SCAMP5 in MCF-7 and RAW264.7 cells before and after ionomycin treatments. Parental MCF-7 cells (*A*) or wild-type RAW264.7 cells (*B*) were treated with (Iono (+)) or without (Iono (−)) 10 μM ionomycin for 10 min. Then, cells were subjected to sucrose equilibrium density centrifugation. Twelve 1-ml fractions were obtained. Then, equal volume of fractions corresponding to PM (combination of Fractions 1–2), Golgi/GDV (combination of Fractions 5–7), and ER (combination of Fractions 11–12) were examined by Western blot for indicated SNARE molecules and fraction markers. GDV, Golgi-derived vesicles. *C* and *D*, hSCAMP5 translocates to plasma membrane in MCF-7 cells after ionomycin treatments. Parental MCF-7 cells (*C*) and wild type RAW264.7 cells (*D*) were treated with 10 μM ionomycin for 10 min. Then, cells were sequentially stained with SCAMP5 Ab, Abs for indicated markers, and Oregon Green 488-conjugated (green) and Alexa Fluor 555-conjugated (red) secondary Abs as indicated. The bright yellow region indicated for the colocalization sites (indicated by white arrows). Bar, 100 μm.

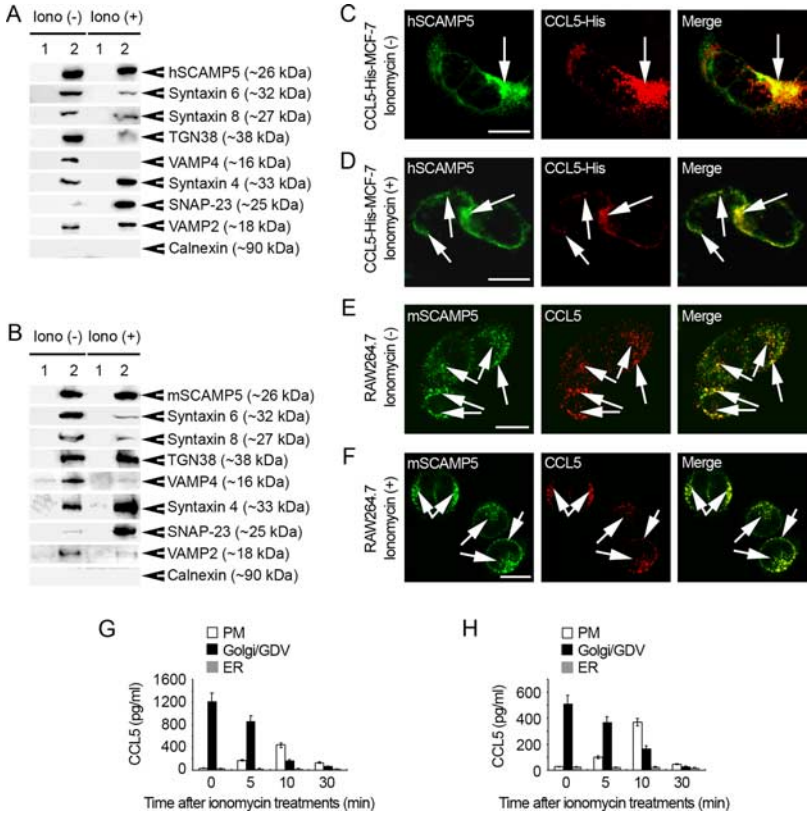

FIGURE 6. SCAMP5 codistributes with SNARE pairs and CCL5 during the translocation along the exocytotic pathway. *A* and *B*, SNARE molecules in SCAMP5-positive vesicles. CCL5-His stably transfected MCF-7 cells (CCL5-His-MCF-7, *A*) and wild-type RAW264.7 cells (*B*) were treated with (Iono (+)) or without (Iono (−)) 10 μM ionomycin for 10 min. Then, cells were isolated for total membrane vesicles. SCAMP5-positive vesicles were isolated by immunoisolation methods using isotype control IgG (*Lane 1*) or anti-SCAMP5 Ab (*Lane 2*) plus protein A/G beads. The components in the immunoisolated vesicles were examined by Western blot. *C–F*, Codistribution of SCAMP5 with CCL5 during the ionomycin-induced translocation process. CCL5-His-MCF-7 cells (*C* and *D*) and wild-type RAW264.7 cells (*E* and *F*) were treated with 10 μM ionomycin for 10 min. Then, endogenous SCAMP5 (green) and CCL5 (red) were double stained as indicated (anti-His Ab for CCL5-His-MCF-7 cells and anti-CCL5 Ab for RAW264.7 cells). The bright yellow region indicated for the colocalization sites (white arrows). Bar = 100 μm. *G* and *H*, ELISA of CCL5 in different fractions before and after ionomycin treatments. CCL5-His-MCF-7 cells (*G*) and wild-type RAW264.7 cells (*H*) were treated with 10 μM ionomycin for as indicated. Then, cells were subjected to sucrose equilibrium density centrifugation. Twelve 1-ml fractions were obtained. Then, equal volume of fractions corresponding to PM (combination of Fractions 1–2), Golgi/GDV (combination of Fractions 5–7), and ER (combination of Fractions 11–12) were examined by ELISA. Results were presented as mean ± SD of triplicate samples.

cells, and examined the cytokine secretion after various treatments (Supplemental Fig. S8*A*). We found that hSCAMP5 overexpression could promote the secretion of TNF-α and CCL5, but not IL-1β, upon ionomycin, ATP and PAF treatments (Supplemental Fig. S8*B*–S8*D*). To further verify the roles of SCAMP5 in cytokine release under physiological stimulus, we treated THP-1 cells with LPS, CpG ODN, and poly (I:C). We found that hSCAMP5 could increase the release of TNF-α and CCL5 by LPS, but not CpG ODN and poly (I:C) (Supplemental Fig. S8*E*). Interestingly, we found that the calcium chelator BAPTA could block the effects of SCAMP5 on promotion of cytokine release induced by LPS (data not shown), indicating that the effects of SCAMP5 on cytokine release were dependent on the calcium-regulated signals.

In RAW264.7 macrophages, we silenced the expression of SCAMP5 by transient transfection of SCAMP5 siRNAs (Fig. 4*A*). Upon ionomycin, ATP, and PAF treatments, the rapid release of TNF-α and CCL5 was significantly inhibited in SCAMP5-silenced cells (Fig. 4, *B* and *C* and Supplemental Fig. S9*A*). When the cells were treated with LPS, CpG ODN, and poly (I:C), we found that the release of TNF-α and CCL5 was inhibited in SCAMP5-silenced cells in response to LPS (Fig. 4, *D* and *E* and Supplemental Fig. S9*B*). As further evidence, we primed RAW264.7 cells with 100 ng/ml LPS, 2 μM CpG ODN, or 10 μg/ml poly (I:C) for 4 h to up-regulate the cytokines, and then treated the cells with ionomycin, ATP, and PAF, respectively. We found that the calcium-triggered release of TNF-α and CCL5 was also inhibited in SCAMP5-silenced RAW264.7 cells (Fig. 4, *F* and *G* and Supplemental Fig. S9*C*).

Ionomycin promotes the translocation of SCAMP5 from Golgi-associated compartments to plasma membrane in both epithelial cancer cells and in macrophages

The above results (Supplemental Fig. S8 and Fig. 4) suggest that SCAMP5 is involved in the regulation of cytokine exocytosis. However, the molecular mechanisms responsible for the roles of SCAMP5 in promotion of signal peptide-containing cytokines remain elusive. To resolve this problem, we observed the subcellular distribution of SCAMP5 in MCF-7 cells and RAW264.7 cells during ionomycin treatments by sucrose equilibrium density centrifugation. Before ionomycin treatments, endogenous SCAMP5 was codistributed with Golgi-associated organelles/vesicles (combination of Fractions 5–7, Golgi/GDV) in both MCF-7 cells and RAW264.7 cells (Fig. 5, *A* and *B*). However, after ionomycin treatments, we found that hSCAMP5 is concentrated on plasma membrane (combination of Fractions 1–2, PM) and codistributed with SNAP-23 and syntaxin 4 (Fig. 5, *A* and *B*). By immunofluorescent confocal microscope, we found that endogenous SCAMP5 was

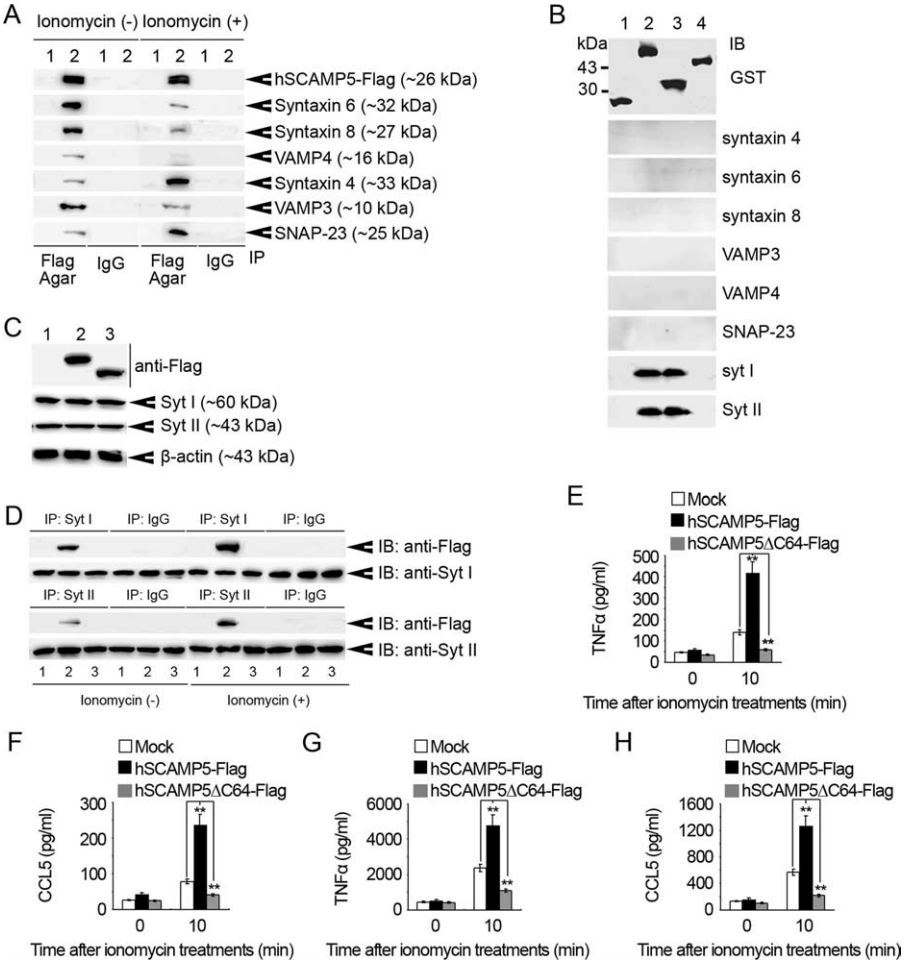

FIGURE 7. hSCAMP5 complexes with SNARE molecules and directly interacts with synaptotagmins. *A*, hSCAMP5 complexes with SNARE molecules. MCF-7 cells stably transfected with mock (*Lane 1*) or hSCAMP5-Flag (*Lane 2*) vectors were treated with (Iono (+)) or without (Iono (−)) 10 μM ionomycin for 10 min. Then, lysates were immunoprecipitated with anti-Flag agarose (Flag Agar) or IgG plus protein A/G agarose (IgG). Then, indicated components were examined by Western blot assays. *B*, GST pull-down assays. One hundred nanograms of GST fusion proteins were incubated with 100 μg lysates derived from LPS-treated RAW264.7 cells (100 ng/ml for 4h) for 1 h, and then pulled down with Sepharose 4B beads. Then, the components interacting with the GST fusion proteins were examined by Western blotting (IB) with indicated Abs. *Lane 1*, GST; *Lane 2*, GST-hSCAMP5; *Lane 3*, GST-hSCAMP5C64; *Lane 4*, GST-hSCAMP5ΔC64. *C* and *D*, hSCAMP5 complexes with synaptotagmin I (Syt I) and II (Syt II). RAW264.7 cells stably transfected with mock (*Lane 1*), hSCAMP5-Flag (*Lane 2*), or hSCAMP5ΔC64-Flag (*Lane 3*) vectors were prepared for whole cell lysates. Then the expression of indicated molecules were examined by Western blot (*C*). Otherwise, the cells were treated with 10 μM ionomycin for 10 min. Then, lysates were immunoprecipitated (IP) with anti-synaptotagmin I or anti-synaptotagmin II (Syt II) Abs plus protein A/G beads (*D*). IgG was used as isotype controls. The indicated components in the immune complexes were examined by Western blot assays (IB). *E–H*, hSCAMP5ΔC64-Flag overexpression inhibits ionomycin-triggered cytokine release. RAW264.7 cells (2×10^5 cells/ml) stably transfected with indicated vectors were starved for 24 h, cultured in OPTI-MEM for 6 h and then treated with 10 μM ionomycin for 10 min (*E* and *F*). Alternatively, the selected RAW264.7 cells (2×10^5 cells/ml) were treated with 100 ng/ml LPS for 4 h in OPTI-MEM medium and then with 10 μM ionomycin for 10 min (*G* and *H*). Finally, the TNF-α (*E* and *G*) and CCL5 (*F* and *H*) contained in the supernatants were examined by ELISA, and the results were presented as mean ± SD of triplicate samples. **, $p < 0.01$.

colocalized with syntaxin 6 and TGN38 before ionomycin treatments and with Syntaxin 4 and SNAP-23 after ionomycin treatments in both MCF-7 cells (Fig. 5*C*) and RAW264.7 cells (Fig. 5*D*). These data together suggest that hSCAMP5 is translocated from Golgi-associated compartments to plasma membrane during calcium-triggered exocytosis.

hSCAMP5 codistributes with SNARE pairs and CCL5 during the translocation along the exocytotic pathway

It is widely accepted that the regulation of exocytotic pathway is under the control of SNARE machinery (1–4). Therefore, we next determined the hSCAMP5-associated SNARE components by immunoisolation of hSCAMP5-containing vesicles after isolation of total membrane fractions. In MCF-7 cells, hSCAMP5 was codistributed with the v-SNARE molecules syntaxin 6 and the t-SNARE molecule VAMP4 under normal culture conditions (Fig. 6*A*). After ionomycin treatments, hSCAMP5 was codistributed with syntaxin 4, SNAP-23, and VAMP2 (Fig. 6*A*). Similar alterations in SCAMP5 distribution were observed in RAW264.7 cells (Fig. 6*B*). These data, together with the localization data (Fig. 5), indicated that SCAMP5 was codistributed with different SNARE molecules when it was translocated from Golgi-associated compartments to plasma membrane.

To investigate the relationship between hSCAMP5 translocation and CCL5 exocytosis, we examined the localization of both SCAMP5 and CCL5 before and after ionomycin treatments. In CCL5-His stably transfected MCF-7 cells, CCL-His was capable of translocating from perinuclear region to plasma membrane and then released to extracellular medium in response to ionomycin within 30 min (Supplemental Fig. S10). SCAMP5 was colocalized

with CCL5-His before ionomycin treatments, and SCAMP5 was translocated to plasma membrane together with CCL5-His after ionomycin treatments in both MCF-7 cells (Fig. 6, *C* and *D*) and RAW264.7 macrophages (Fig. 6, *E* and *F*). These data suggest that ionomycin could trigger the translocation of hSCAMP5-containing compartments together with CCL5 from Golgi-associated organelles/vesicles to plasma membrane to facilitate the final release of CCL5 into extracellular medium. As further evidence, we examined the levels of CCL5 in fractions derived from sucrose equilibrium density centrifugation, in lysates and in extracellular medium respectively before and after ionomycin treatments. We found that the distribution of CCL5 was consistent with that of SCAMP5 in MCF-7 cells and RAW264.7 cells (Fig. 6, *G* and *H* vs Fig. 5, *A* and *B*, and data not shown).

hSCAMP5 complexes with SNARE molecules by directly binding synaptotagmin via the C-terminal tail

Previous studies have suggested that SCAMPs could oligomerize between SCAMPs and interact with SNAP-23, ARF6 and phospholipase D. We found that hSCAMP5 could complex with SNARE pairs in hSCAMP5-Flag-overexpressed MCF-7 cells (Fig. 7*A*). Similarly, we found that hSCAMP5-Flag overexpressed in RAW264.7 cells could also coimmunoprecipitate with SNARE molecules (data not shown). However, it remains unknown how hSCAMP5 interacts with the observed SNARE molecules. We thus performed in vitro GST pull-down assays. We found that GST-hSCAMP5 could not bind syntaxins, VAMPs, and SNAP-23 (Fig. 7*B*), indicating that the complexes formed by hSCAMP5 and the SNARE molecules was not due to a direct interaction between hSCAMP5 and the SNARE molecules. We then examined the other molecules that have been detected in SNARE complexes (1–4). We found that GST-hSCAMP5 could bind synaptotagmin I and II derived from lysates of LPS-treated RAW264.7 cells (Fig. 7*B*), indicating that hSCAMP5 complexed with SNARE molecules via direct interaction with synaptotagmins that can interact with SNAP-23, syntaxins, and VAMPs (1–4).

Two functional regions, the N-terminal NPF repeats and the cytosolic E peptide, have been identified in SCAMPs. Structurally, hSCAMP5 contains no NPF repeats, but contains the E peptide and the C-terminal cytosolic region (Supplemental Fig. S1). In the presence of E peptide (CWFRPIYKAFK), the association of hSCAMP5 with synaptotagmin I/II still existed (data not shown), indicating that the interaction between hSCAMP5 and synaptotagmins was not due to the E peptide region. Therefore, we examined whether hSCAMP5 interacts with synaptotagmins via the C-terminal cytosolic tail because only the cytosolic regions of SCAMPs were exposed to regulatory factors. We purified the GST-hSCAMP5C64 (containing 172–235 aa of hSCAMP5) and GST-hSCAMP5ΔC64 (deletion of the 172–235 aa of hSCAMP5). We found that GST-hSCAMP5C64, but not the GST-hSCAMP5ΔC64, could bind synaptotagmin I and II (Fig. 7*B*). As further evidence, we immunoprecipitated endogenous synaptotagmin I and II in RAW264.7 cells stably transfected with hSCAMP5-Flag and hSCAMP5ΔC64-Flag (Fig. 7*C*), and examined the SCAMP5-synaptotagmin association in ionomycin-treated cells. We found that synaptotagmin I and II could coimmunoprecipitate with hSCAMP5-Flag, but not hSCAMP5ΔC64-Flag (Fig. 7*D*). These data suggest that hSCAMP5 complexes with SNARE pairs through the C-terminal cytosolic tail of hSCAMP5.

To further elucidate the roles of hSCAMP5-synaptotagmin interaction in cytokine secretion, we stably overexpressed hSCAMP5ΔC64 in RAW264.7 cells (Fig. 7*C*) and performed the cytokine exocytosis assays. We found that the mutant hSCAMP5ΔC64 inhibited the ionomycin-induced TNF-α and CCL5 secretion (Fig. 7, *E–H*) in macrophages, which indicated that hSCAMP5 may facilitate cytokine secretion by interaction with synaptotagmins. In RAW264.7 cells, SCAMP1, 2, 3, but not 4, were also highly expressed (data not shown). These data may also indicate that SCAMP 1–3 cannot compensate for the deficiency in SCAMP5 functions during calcium-triggered cytokine secretion.

Discussion

To date, five distinct SCAMPs have been reported in mammalian cells with SCAMPs 1–4 being ubiquitously expressed and mouse/rat SCAMP5 being neuron-preferred (14–31, 46–48). hSCAMP5 is abundantly detected not only in neural tissues including different parts of brain and spinal cord, but also in endocrine tissues including adrenal gland and thyroid, in exocrine tissues including stomach and trachea, and in hematopoietic tissues including bone marrow and lymph nodes. Though the nonneuronal tissues as trachea, thyroid and adrenal gland contain neuronal tissues, the detection of hSCAMP5 in the cortex region of adrenal gland and the follicular epithelial cells of thyroid indicates that hSCAMP5 may be expressed in both neuronal and nonneuronal tissues and cells. Therefore, hSCAMP5 may play a ubiquitous role in regulating membrane transport.

SCAMPs have been characterized as a family of integral membrane proteins with four transmembrane spans that are associated with regulated secretory carriers and with Golgi-derived and endosomal membranes (14–19, 22–30, 46–48). Functional studies have detected a region between TMR 2/3 (E peptide), which regulates membrane transport during exocytosis and endocytosis by interacting with the small GTPase ARF6, phospholipase D1, (Na^+, K^+)/H^+ exchanger NHE7 and the serotonin transporter (27, 28, 30, 46, 48). The potential interaction of SCAMP1 with intersectin-1 and γ-synergin via the NPF repeats, and the phosphorylation of SCAMP1 and SCAMP3 by EGF receptor in the NPF repeats together suggest that NPF-containing SCAMPs may be involved in endocytosis (21, 31). Recently, it has been shown that the SCAMP4 without NPF repeats is detected in recycling endosomes (47), indicating that hSCAMP5 may similarly be involved in intracellular membrane transport, which is supported by our findings that a portion of hSCAMP5 can be codistributed with EEA1, Rab4, and Rab11 (data not shown), two markers of the recycling endosomes.

hSCAMP5 contains the conventional E region but not the NPF repeats of SCAMPs 1–3, suggesting that hSCAMP5 may be involved in exocytosis. However, whether SCAMP5 is involved in endocytosis need further investigations. Previous studies suggest that the E-peptide of SCAMP2 plays crucial roles in the regulation of exocytosis (27, 28), which is later explained by the findings that the E-peptide can bind ARF6 and phospholipase D (30), suggesting that the E-peptide region is subjected to the regulation of phosphatidic acid and inositol phospholipids. In our study, we show that hSCAMP5 can promote the ionomycin-triggered cytokines release in epithelial cells and macrophages. As we know, ionomycin is a kind of calcium ionophore that can elevate intracellular calcium level, in turn, leading to ubiquitous membrane targeting, docking, and fusion processes (1–4). The involvement of hSCAMP5 in calcium-triggered exocytosis suggests that hSCAMP5 may somehow sense the calcium elevation. Our data have shown that hSCAMP5 can bind synaptotagmins via the C-terminal tail, and hSCAMP5 without the C-terminal tail fail to translocate to plasma membrane upon ionomycin treatments (data not shown). Synaptotagmin is a Ca^{2+}-binding membrane protein of vesicles that is required for fast Ca^{2+}-activated exocytosis and

serves as a calcium sensor during exocytosis (1–4). The SCAMP5-synaptotagmin interaction may provide a mechanism for the involvement of hSCAMP5 in calcium-triggered exocytosis. Therefore, taken together, the previous report on mouse/rat SCAMP5 (18), our studies have provided functional evidence for a role of hSCAMP5 in exocytosis.

In our study, we find that hSCAMP5 can promote the release of CCL5 but not IL-1β upon the calcium triggering, suggesting that hSCAMP5 may be localized along the classical secretory routes (1–4). Our subcellular fractionation assays and the immunofluorescent assays together show that hSCAMP5 is mainly distributed in Golgi, *trans*-Golgi network, and Golgi-derived vesicles, and is colocalized with CCL5, indicating a role of hSCAMP5 in targeting vesicles containing signal peptide-containing cytokines. The findings that hSCAMP5 can rapidly translocate to plasma membrane together with CCL5 upon ionomycin stimuli confirm that hSCAMP5 is translocated along the classical secretory routes. Taking together, the previous reports (1–4, 14–31, 46–48), we suggest in this study that hSCAMP5 is involved in the regulated cytokine exocytosis. Interestingly, we find that SCAMP5 can facilitate the release of cytokines in response to ionomycin, ATP, PAF, and TLR agonists LPS (but not CpG ODN and poly (I:C), which is possibly due to the differential effects of these reagents on calcium mobilization (1–4, 49–51), and thus confirms that the roles of SCAMP5 in cytokine release are regulated by calcium signals. The expression of SCAMP5 in various immune cells and the promotion of cytokine release of macrophages and monocytes by SCAMP5 in response to TLR agonists, together suggest a physiological involvement of SCAMP5 in regulation of immune response, which need to be further verified by transgenic and knockout experiments.

SNARE proteins play crucial and universal roles in membrane vesicle docking and fusion (1–4). Previous studies have found that cytokines synthesized by various immune cells are always colocalized with Rab proteins and SNARE molecules, and are translocated to plasma membrane upon extracellular stimuli (36–39). Our studies show that hSCAMP5 is codistributed with Rab3, Rab4, Rab11, syntaxin 6, syntaxin 8, and VAMP4 before ionomycin treatments and with Rab3, syntaxin 4, VAMP2, and SNAP-23 after ionomycin treatments, convincingly demonstrating an involvement of hSCAMP5 in the cytokine exocytosis. More importantly, we find that hSCAMP5 can complex with syntaxin 6-VAMP4 SNARE pairs before calcium triggering and with SNAP-23-syntaxin 4-VAMP2 SNARE pairs after ionomycin treatments, which indicates that the hSCAMP5-associated exocytotic machinery for cytokine release is tightly regulated by SNARE proteins for the specific targeting, docking and fusion processes during the transport of cytokine-containing membrane structures. Upon calcium elevation, the integration of complxes formed by synaptotagmins, syntaxins, VAMPs, SNAP-23/SNAP-25, and SNAPs were rapidly and accurately regulated (1–4). The codistribution and coimmunoprecipitation of hSCAMP5 with SNARE molecules strongly suggest that hSCAMP5 may work together with the SNARE machinery to facilitate classical cytokine exocytosis.

However, the model in our study has greatly simplified the exocytotic machinery of cytokines. One of the questions remain unsolved in our study is about the intermediate intracellular structures between the Golgi and the plasma membrane because little is known about the cytokine-containing structures in these cells. In neutrophils, eosinophils, mast cells, and pancreatic β cells and so forth, various secretory structures have been identified, e.g., large dense core granules, synaptic-like vesicles, azurophil granules, specific secretory granules, secretory vesicles, secretory lysosomes, and multivesicular bodies etc (4). In eosinophils, CCL5 is detected in the eosinophil granule crystalline core and is codistributed with the late endosomal and lysosomal marker CD63 (52). In addition to the SNARE proteins and specific organellar markers that codistributed with hSCAMP5, components in early endosomes (EEA-1), late endosomes and lysosomes (LAMP-1), recycling endosomes (Rab4 and Rab11), and secretory vesicles (Rab3) can also be detected in hSCAMP5-containing membrane vesicles. Therefore, we suggest in this study that hSCAMP5 may be involved in multiple intracellular secretory machineries that facilitate the release of cytokines. Considering that SCAMP5 is partially codistributed with endosome markers (e.g., EEA1, Rab4, Rab5, etc.) and can facilitate the release of cytokines within 10–30 min after calcium agonists treatments, it may be inferred that part of SCAMP5 may be localized to endosomes (e.g., recycling endosome). The recycling endosome is identified as an intermediate compartment for the post-Golgi trafficking and exocytosis process (1–4). Recently, it has been reported that recycling endosomes may be involved in the exocytosis of endocytosed receptors and some but not all cytokines (1–4, 36, 37, 53–55). Further in vitro reconstitution experiments using recycling endosomes may help to finally elucidate the contribution of SCAMP5 to membrane transport to/from recycling endosomes.

In summary, we have identified a human homologue of mouse SCAMP5, hSCAMP5, which is involved in calcium-regulated exocytosis of signal peptide-containing cytokines by localizing and transporting along the classical exocytotic pathway and by interacting with SNARE components. Our results provide direct evidence for the involvement of hSCAMP5 in exocytosis and suggest a mechanism for SCAMPs interaction with SNARE proteins. Therefore, our study will be of value in understanding the mechanisms mediating SCAMPs involvements in the regulation of putative vesicle docking and fusion processes.

Acknowledgments

We thank Dr. Qiangsu Guo, Mei Jin, and Yan Li for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Lippincott-Schwartz, J., T. H. Roberts, and K. Hirschberg. 2000. Secretory protein trafficking and organelle dynamics in living cells. *Annu. Rev. Cell Dev. Biol.* 16: 557–589.
2. Jahn, R., and T. C. Sudhof. 1999. Membrane fusion and exocytosis. *Annu. Rev. Biochem.* 68: 863–911.
3. Mellman, I., and G. Warren. 2000. The road taken: past and future foundations of membrane traffic. *Cell* 100: 99–112.
4. Rettig, J., and E. Neher. 2002. Emerging roles of presynaptic proteins in Ca^{2+}-triggered exocytosis. *Science* 298: 781–785.
5. Bennett, M. K., and R. H. Scheller. 1993. The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. USA* 90: 2559–2563.
6. Weber, T., B. V. Zemelman, J. A. McNew, B. Westermann, M. Gmachl, F. Parlati, T. H. Sollner, and J. E. Rothman. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell* 92: 759–772.
7. McNew, J. A., F. Parlati, R. Fukuda, R. J. Johnston, K. Paz, F. Paumet, T. H. Sollner, and J. E. Rothman. 2000. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* 407: 153–159.
8. Sollner, T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J. E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362: 318–324.
9. Bennett, M. K., J. E. Garcia-Arraras, L. A. Elferink, K. Peterson, A. M. Fleming, C. D. Hazuka, and R. H. Scheller. 1993. The syntaxin family of vesicular transport receptors. *Cell* 74: 863–873.
10. Fukuda, R., J. A. McNew, T. Weber, F. Parlati, T. Engel, W. Nickel, J. E. Rothman, and T. H. Sollner. 2000. Functional architecture of an intracellular membrane t-SNARE. *Nature* 407: 198–202.
11. Sollner, T., M. K. Bennett, S. W. Whiteheart, R. H. Scheller, and J. E. Rothman. 1993. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75: 409–418.

12. Weidman, P. J., P. Melancon, M. R. Block, and J. E. Rothman. 1989. Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J. Cell Biol.* 108: 1589–1596.
13. Clary, D. O., I. C. Griff, and J. E. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* 61: 709–721.
14. Brand, S. H., S. M. Laurie, M. B. Mixon, and J. D. Castle. 1991. Secretory carrier membrane proteins 31–35 define a common protein composition among secretory carrier membranes. *J. Biol. Chem.* 266: 18949–18957.
15. Brand, S. H., and J. D. Castle. 1993. SCAMP 37, a new marker within the general cell surface recycling system. *EMBO J.* 12: 3753–3761.
16. Wu, T. T., and J. D. Castle. 1997. Evidence for colocalization and interaction between 37 and 39 kDa isoforms of secretory carrier membrane proteins (SCAMPs). *J. Cell Sci.* 110: 1533–1541.
17. Singleton, D. R., T. T. Wu, and J. D. Castle. 1997. Three mammalian SCAMPs (secretory carrier membrane proteins) are highly related products of distinct genes having similar subcellular distributions. *J. Cell. Sci.* 110: 2099–2107.
18. Fernandez-Chacon, R., and T. C. Sudhof. 2000. Novel SCAMPs lacking NPF repeats: ubiquitous and synaptic vesicle-specific forms implicate SCAMPs in multiple membrane-trafficking functions. *J. Neurosci.* 20: 7941–7950.
19. Wen, G., T. Leeb, D. Hui, B. G. Baumgartner, A. Robic, H. Hameister, and B. Brenig. 1998. Structural and functional analysis of the porcine secretory carrier membrane protein 1 gene (SCAMP1). *Mamm. Genome* 9: 536–539.
20. Lam, S. K., C. L. Siu, S. Hillmer, S. Jang, G. An, D. G. Robinson, and L. Jiang. 2007. Rice SCAMP1 defines clathrin-coated, trans-golgi-located tubular-vesicular structures as an early endosome in tobacco BY-2 cells. *Plant Cell* 19: 296–319.
21. Wu, T. T., and J. D. Castle. 1998. Tyrosine phosphorylation of selected secretory carrier membrane proteins: SCAMP1 and SCAMP3, and association with the EGF receptor. *Mol. Biol. Cell* 9: 1661–1674.
22. Fernandez-Chacon, R., G. Alvarez de Toledo, R. E. Hammer, and T. C. Sudhof. 1999. Analysis of SCAMP1 function in secretory vesicle exocytosis by means of gene targeting in mice. *J. Biol. Chem.* 274: 32551–32554.
23. Laurie, S. M., C. C. Cain, G. E. Lienhard, and J. D. Castle. 1993. The glucose transporter GluT4 and secretory carrier membrane proteins (SCAMPs) colocalize in rat adipocytes and partially segregate during insulin stimulation. *J. Biol. Chem.* 268: 19110–19117.
24. Brumell, J. H., A. Volchuk, H. Sengelov, N. Borregaard, A. M. Cieutat, D. F. Bainton, S. Grinstein, and A. Klip. 1995. Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. *J. Immunol.* 155: 5750–5759.
25. Fischer, Y., J. Thomas, L. Sevilla, P. Munoz, C. Becker, G. Holman, I. J. Kozka, M. Palacin, X. Testar, H. Kammermeier, and A. Zorzano. 1997. Insulin-induced recruitment of glucose transporter 4 (GLUT4) and GLUT1 in isolated rat cardiac myocytes: evidence of the existence of different intracellular GLUT4 vesicle populations. *J. Biol. Chem.* 272: 7085–7092.
26. Sevilla, L., E. Tomas, P. Munoz, A. Guma, Y. Fischer, J. Thomas, B. Ruiz-Montasell, X. Testar, M. Palacin, J. Blasi, and A. Zorzano. 1997. Characterization of two distinct intracellular GLUT4 membrane populations in muscle fiber: differential protein composition and sensitivity to insulin. *Endocrinology* 138: 3006–3015.
27. Liu, L., Z. Guo, Q. Tieu, A. Castle, and D. Castle. 2002. Role of secretory carrier membrane protein SCAMP2 in granule exocytosis. *Mol. Biol. Cell* 13: 4266–4278.
28. Guo, Z., L. Liu, D. Cafiso, and D. Castle. 2002. Perturbation of a very late step of regulated exocytosis by a secretory carrier membrane protein (SCAMP2)-derived peptide. *J. Biol. Chem.* 277: 35357–35363.
29. Hubbard, C., D. Singleton, M. Rauch, S. Jayasinghe, D. Cafiso, and D. Castle. 2000. The secretory carrier membrane protein family: structure and membrane topology. *Mol. Biol. Cell* 11: 2933–2947.
30. Liu, L., H. Liao, A. Castle, J. Zhang, J. Casanova, G. Szabo, and D. Castle. 2005. SCAMP2 interacts with Arf6 and phospholipase D1 and links their function to exocytotic fusion pore formation in PC12 cells. *Mol. Biol. Cell* 16: 4463–4472.
31. Fernandez-Chacon, R., M. Achiriloaie, R. Janz, J. P. Albanesi, and T. C. Sudhof. 2000. SCAMP1 function in endocytosis. *J. Biol. Chem.* 275: 12752–12756.
32. Cleves, A. E. 1997. Protein transports: the nonclassical ins and outs. *Curr. Biol.* 7: R318–R320.
33. Stephens, D. J., and R. Pepperkok. 2001. Illuminating the secretory pathway: when do we need vesicles? *J. Cell Sci.* 114: 1053–1059.
34. Moqbel, R., and J. J. Coughlin. 2006. Differential secretion of cytokines. *Sci. STKE* 2006: pe26.
35. Stow, J. L., A. P. Manderson, and R. Z. Murray. 2006. SNAREing immunity: the role of SNAREs in the immune system. *Nat. Rev. Immunol.* 6: 919–929.
36. Murray, R. Z., F. G. Wylie, T. Khromykh, D. A. Hume, and J. L. Stow. 2005. Syntaxin 6 and Vti1b form a novel SNARE complex, which is up-regulated in activated macrophages to facilitate exocytosis of tumor necrosis factor-α. *J. Biol. Chem.* 280: 10478–10483.
37. Kay, J. G., R. Z. Murray, J. K. Pagan, and J. L. Stow. 2006. Cytokine secretion via cholesterol-rich lipid raft-associated SNAREs at the phagocytic cup. *J. Biol. Chem.* 281: 11949–11954.
38. Oynebraten, I., N. Barois, K. Hagelsteen, F. E. Johansen, O. Bakke, and G. Haraldsen. 2005. Characterization of a novel chemokine-containing storage granule in endothelial cells: evidence for preferential exocytosis mediated by protein kinase A and diacylglycerol. *J. Immunol.* 175: 5358–5369.
39. Huse, M., B. F. Lillemeier, M. S. Kuhns, D. S. Chen, and M. M. Davis. 2006. T cells use two directionally distinct pathways for cytokine secretion. *Nat. Immunol.* 7: 247–255.
40. Sun, H., N. Li, X. Wang, S. Liu, T. Chen, L. Zhang, T. Wan, and X. Cao. 2003. Molecular cloning and characterization of a novel cystatin-like molecule. CLM, from human bone marrow stromal cells. *Biochem. Biophys. Res. Commun.* 301: 176–182.
41. Yang, M., T. Chen, C. Han, N. Li, T. Wan, and X. Cao. 2004. Rab7b, a novel lysosome-associated small GTPase, is involved in monocytic differentiation of human acute promyelocytic leukemia cells. *Biochem. Biophys. Res. Commun.* 318: 792–799.
42. Wang, Y., T. Chen, C. Han, D. He, H. Liu, H. An, Z. Cai, and X. Cao. 2007. Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4. *Blood* 110: 962–971.
43. Chen, T., J. Guo, M. Yang, C. Han, M. Zhang, W. Chen, Q. Liu, J. Wang, and X. Cao. 2004. Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. *Blood* 103: 413–421.
44. Simpson, I. A., D. R. Yver, P. J. Hissin, L. J. Wardzala, E. Karnieli, L. B. Salans, and S. W. Cushman. 1983. Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochim. Biophys. Acta* 763: 393–407.
45. Greenfield, J. P., J. Tsai, G. K. Gouras, B. Hai, G. Thinakaran, F. Checler, S. S. Sisodia, P. Greengard, and H. Xu. 1999. Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer β-amyloid peptides. *Proc. Natl. Acad. Sci. USA* 96: 742–747.
46. Lin, P. J., W. P. Williams, Y. Luu, R. S. Molday, J. Orlowski, and M. Numata. 2005. Secretory carrier membrane proteins interact and regulate trafficking of the organellar $(Na^+,K^+)/H^+$ exchanger NHE7. *J. Cell Sci.* 118: 1885–1897.
47. Castle, A., and D. Castle. 2005. Ubiquitously expressed secretory carrier membrane proteins (SCAMPs) 1–4 mark different pathways and exhibit limited constitutive trafficking to and from the cell surface. *J. Cell Sci.* 118: 3769–3780.
48. Muller, H. K., O. Wiborg, and J. Haase. 2006. Subcellular redistribution of the serotonin transporter by secretory carrier membrane protein 2. *J. Biol. Chem.* 281: 28901–28909.
49. Supajatura, V., H. Ushio, A. Nakao, S. Akira, K. Okumura, C. Ra, and H. Ogawa. 2002. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.* 109: 1351–1359.
50. Varadaradjalou, S., F. Féger, N. Thieblemont, N. B. Hamouda, J. M. Pleau, M. Dy, and M. Arock. 2003. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *Eur. J. Immunol.* 33: 899–906.
51. Buczynski, M. W., D. L. Stephens, R. C. Bowers-Gentry, A. Grkovich, R. A. Deems, and E. A. Dennis. 2007. TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *J. Biol. Chem.* 282: 22834–22847.
52. Mahmudi-Azer, S., G. P. Downey, and R. Moqbel. 2002. Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. *Blood* 99: 4039–4047.
53. Murray, R. Z., J. G. Kay, D. G. Sangermani, and J. L. Stow. 2005. A role for the phagosome in cytokine secretion. *Science* 310: 1492–1495.
54. Ha, S. D., A. Martins, K. Khazaie, J. Han, B. M. Chan, and S. O. Kim. 2008. Cathepsin B is involved in the trafficking of TNF-α-containing vesicles to the plasma membrane in macrophages. *J. Immunol.* 181: 690–697.
55. Manderson, A. P., J. G. Kay, L. A. Hammond, D. L. Brown, and J. L. Stow. 2007. Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFα. *J. Cell Biol.* 178: 57–69.