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Sorting nexin 8 regulates endosome-to-Golgi transport

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ABSTRACT

Sorting nexin 8 (SNX8) belongs to the sorting nexin protein family, whose members are involved in endocytosis and endosomal sorting and signaling. The function of SNX8 has so far been unknown. Here, we have investigated the role of SNX8 in intracellular transport of the bacterial toxin Shiga toxin (Stx) and the plant toxin ricin. After being endocytosed, these toxins are transported retrogradely from endosomes, via the Golgi apparatus and the endoplasmic reticulum (ER), into the cytosol, where they exert their toxic effect. Interestingly, our experiments show that SNX8 regulates the transport of Stx and ricin differently; siRNA-mediated knockdown of SNX8 significantly increased the Stx transport to the *trans*-Golgi network (TGN), whereas ricin transport was slightly inhibited. We also found that SNX8 colocalizes with early endosome antigen 1 (EEA1) and with retromer components, suggesting an endosomal localization of SNX8 and supporting our finding that SNX8 is involved in endosomal sorting.

Introduction

Several protein toxins exploit the cellular machinery for internalization and transport to their target destination, and these toxins can therefore be used as tools to study intracellular transport. The bacterial Stx and the plant toxin ricin both consist of a catalytic A-subunit and a B-moiety responsible for binding to the cell surface (reviewed in [1]). The pentameric B-moiety of Stx binds to the glycosphingolipid globotriaosyl ceramide (Gb3), whereas the ricin B-subunit binds to all glycolipids and glycoproteins with terminal galactose residues. After internalization by various endocytic mechanisms [2,3], the toxins are transported to early endosomes. Here, they can either be recycled back to the plasma membrane, sent to lysosomes for degradation, or sorted into the retrograde pathway. In the retrograde pathway, the toxins are sent to the TGN, transported through the Golgi apparatus and into the ER. In the ER, the A- and B-moieties are separated and the A-subunit is translocated into the cytoplasm. Here, the A-subunit exerts its toxic effect by inactivating the 60S ribosomal subunit, and thereby inhibiting protein synthesis. Even though both Stx and ricin follow the retrograde pathway, they require different proteins for sorting [4–8].

Endosomes function as cellular sorting stations where proteins are being distributed into transport vesicles and sent to their correct destinations. This sorting is regulated by a multitude of proteins, amongst them sorting nexins (SNXs). The SNXs belong to a protein family characterized by a distinct phosphoinositide (PI)-binding domain, the SNX-PX domain [9], which preferentially targets these proteins to phosphatidylinositol-3-phosphate (PI(3)P) on endosomes [10]. Some of the SNXs also contain a BAR (Bin/amphiphysin/Rvs) domain, which can sense, stabilize and induce membrane curvature [11,12]. Several of the SNXs have been found to be regulators of membrane trafficking (for a recent review of SNXs, see [13]). SNX1 and SNX2 are members of a membrane coat complex called the retromer complex, which is involved in organizing endosome-to-Golgi retrieval of various cargos, such as the cation-independent mannose-6-phosphate receptor (CI-M6PR) [14,15]. SNX1 and SNX2 contribute to the targeting of the retromer to highly tubulated endosomal membranes, whereas the vacuolar protein sorting (VPS) proteins VPS26, VPS29, and VPS35 form the cargo-recognition subunit. Recently, it has been shown that also SNX5 and SNX6 participate in the membrane-bound retromer subcomplex in specific combinations with SNX1 and SNX2 [16,17]. The SNXs are also involved in other transport pathways. For instance, SNX3 is involved in recycling of the reductive iron transporter Fet3p-Ftr1p in iron-starved yeast cells [18] and in the formation of multivesicular bodies in mammalian cells [19], whereas SNX9 has a role in several endocytic processes (reviewed in [20]). SNX4 was recently found to regulate endosome-to-Golgi transport of ricin [21], and it has also been shown to play a role in recycling of the transferrin receptor [22]. SNX8 is another SNX related to SNX1 [9]. So far, the function of SNX8 has been unknown, but its yeast homolog, Mvp1p [9], is required for transporting carboxypeptidase Y (CPY) to the vacuole and

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interacts with the dynamin-like protein Vps1p [23]. As SNX8 and SNX1 have similar domain structure, it was conceivable that also SNX8 contributes in endosomal sorting. In agreement with this, we found that SNX8 localizes to early endosomes and that it partially colocalizes with retromer components.

In this study we have investigated the role of SNX8 in the retrograde transport of Stx and ricin. Interestingly, we found that knockdown of SNX8 had opposite effects on Golgi transport of the toxins. We show that the transport of Stx from endosomes to the TGN is increased in the absence of SNX8, whereas ricin transport is slightly reduced. These findings illustrate that parallel pathways to the Golgi can be under differential regulation by one and the same member of the SNX family.

Materials and methods

Reagents and antibodies. $Na_2{}^{35}SO_4$ was from Hartman Analytics and $Na^{125}I$ from Perkin Elmer. The plasmid expressing ShigaB-sulf2 was a kind gift from Dr. B. Goud (Institut Curie, Paris, France). ShigaB-sulf2 was prepared as earlier described [24]. Shiga holotoxin was provided by Dr. J.L. Koslov (Academy of Sciences of Russia, Moscow, Russia) and Dr. J.E. Brown (USAMRIID, Fort Detrick, MD, USA). A modified ricin A-chain containing a tyrosine sulfation site was produced, purified and reconstituted with ricin B-chain to form ricin-sulf1 as previously described [25]. Ricin and wortmannin were from Sigma–Aldrich. The following antibodies were used: Mouse anti-Stx 3C10 and 13C4 (Toxin Technology), rabbit anti-ricin (Sigma–Aldrich), mouse anti-SNX1 and mouse anti-SNX2 (BD Biosciences), rabbit anti-VPS26 and goat anti-VPS35 (Abcam), rabbit anti-EEA1 (Cell Signaling Technology), rabbit anti-giantin (Berkeley Antibody Company), and Cy2-, Cy3-, and Cy5-conjugated secondary antibodies from Jackson Immunoresearch. Other chemicals used were from Sigma–Aldrich unless otherwise stated.

Construction of GFP-SNX8 fusion gene. SNX8 was amplified from wild-type cDNA using 5′-GGAACTCGAGCTATGACTGGCCGCGCGATG-3′ forward primer and 5′-GGAAGGTACCCTAGTGAGGACACAGGCCGTCC-3′ reverse primer from Eurofins MWG Operon, which added XhoI and KpnI restriction sites (underlined), respectively, to the ends of the amplicon. Using the corresponding restriction enzymes, the amplified product was cloned into pEGFP-C1 vector (Clontech), resulting in a recombinant GFP-SNX8 gene. The construct was sequenced for verification.

Cell lines and transfection. HeLa cells were grown at 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 10% v/v fetal calf serum (FCS; PAA Laboratories) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen). Cells were seeded 24 h prior to transfection. The cells were transiently transfected for 4–12 h with the appropriate plasmid DNA using Fugene 6 (Roche Diagnostics) according to the manufacturer's instructions. siRNA oligos were transfected into cells using DharmaFECT 1 transfection reagent (Dharmacon) following the manufacturer's protocol. Cells were transfected with 25 nM siRNA for 4 h and then grown in complete medium containing serum and antibiotics for 3 days. The following siRNA sequences were purchased from Dharmacon: SNX8-1: GCUCGGAUGUGCAGAACAA and SNX8-2: GUAAGCAGGAAGAGAACGA.

Real-time RT-PCR. The knockdown efficiency of the siRNA oligos were determined by real-time RT-PCR. Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen) and the automated sample preparation system QiaCube (Qiagen). cDNA synthesis (from 1 μg RNA) was performed using iScript™ cDNA Synthesis Kit (Bio-Rad), and was followed by real-time PCR using LightCycler 480 SYBR green 1 master mix (Roche) with TBP (TATA box binding protein) as internal control. The QuantiTect Primer Assay for SNX8 was from Qiagen, whereas the TBP primer pair (5′-GCCCGAAACGCCGAATAT-3′ and 5′-CGTGGCTCTCTTATCCTCATGA-3′) was from MWG Biotech. The real-time PCR analysis was run on a LightCycler 480 Real-Time PCR System using the following program: Preincubation 5 min 95 °C, amplification 45 cycles (10 s denaturation 95 °C, 20 s annealing 60 °C and 10 s extension 72 °C), followed by melt-curve analysis. Quantification was done with LightCycler 480 software.

Sulfation of ShigaB-sulf2 and ricin-sulf1. The cells were washed twice with sulfate-free medium and subsequently incubated with 0.2 mCi/ml $Na_2{}^{35}SO_4$ for 3 h at 37 °C in the same medium. ShigaB-sulf2 (StxB-sulf2) or ricin-sulf1 was added and the incubation continued for 1 and 2 h, respectively. The cells treated with StxB-sulf2 were then washed in ice-cold PBS and lysed in lysisbuffer (0.1 M NaCl, 10 mM Na_2HPO_4 (pH 7.4), 1 mM EDTA, 1% Triton X-100, supplemented with a mixture of complete protease inhibitors (Roche Diagnostics) and 60 mM *n*-octyl-β-pyranoside). Ricin-sulf1 treated cells were washed twice in 0.1 M lactose in Hepes-buffered medium to remove cell surface-bound ricin-sulf1 before being washed in ice-cold PBS and lysed. StxB-sulf2 or ricin-sulf1 were then immunoprecipitated from cleared lysates overnight at 4 °C using Protein A Sepharose beads (GE Healthcare) with the appropriate antibody adsorbed. The immunoprecipitate was washed twice in 0.35% Triton X-100 in PBS, resuspended in sample buffer and boiled. The immunoprecipitate was then separated by SDS–PAGE, transferred to a PVDF membrane and investigated by autoradiography. Band intensities were quantified using the Quantity One® 1-D Analysis Software (Bio-Rad Laboratories Inc.). The total amount of sulfated proteins was determined by TCA precipitation of the remaining lysates.

Endocytosis of Stx and ricin. The endocytosis of Stx was quantified as previously described [7]. The amount of cell-associated ricin was measured by γ-counting after incubating with ^{125}I-labeled ricin for 20 min at 37 °C. The amount of internalized ricin was determined as the fraction of ^{125}I-ricin remaining after incubating with 0.1 M lactose in Hepes-buffered medium for 5 min and washing twice in the same solution.

Immunofluorescence confocal microscopy. Cells were washed with Hepes-buffered medium before incubation with or without 500 ng/ml StxB-sulf2 for the times indicated in the figures. All samples were fixed in 10% formalin (Sigma–Aldrich) and permeabilized with 0.1% Triton X-100, before blocking in 5% FCS. The samples were incubated with primary antibody in 5% FCS for 1 h at room temperature or overnight at 4 °C, followed by 30 min incubation with fluorophore-conjugated secondary antibodies. The samples were mounted in Mowiol (Calbiochem) and investigated using a Zeiss LSM 510 Meta laser scanning confocal microscope. Images were prepared using the LSM 510 Image Examiner (Carl Zeiss) and ImageJ (NIH) software. LSM 510 Image Examiner was also used to quantify the signal intensity in the colocalization analysis.

Statistics. All experiments were performed with duplicates. The experimental results are presented as mean values + SEM of n independent experiments, where n is indicated in each figure legend. The paired Student's t test was used to determine the difference between means of two groups and the minimum level of significance was set at $p \leqslant 0.05$.

Results

Knockdown of SNX8 has opposite effects on endosome-to-Golgi transport of Stx and ricin

It has previously been shown that the retromer components SNX1 [26,27], SNX2 [26], and VPS26 [28] are required for efficient retrograde transport of Stx to the Golgi apparatus. As the domain structure of SNX8 is similar to SNX1, we wanted to study the role of SNX8 in the intracellular transport of Stx and ricin. We started

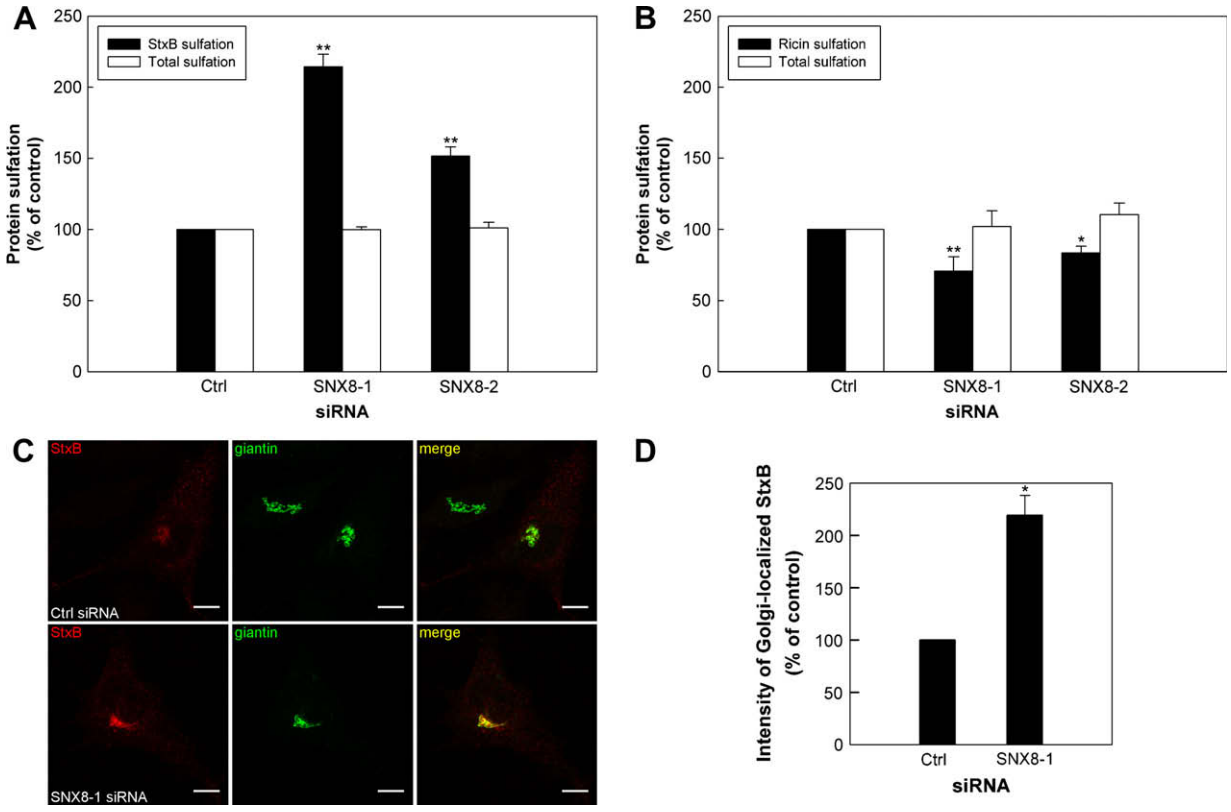

Fig. 1. SNX8 depletion alters the transport of Stx and ricin to the TGN. HeLa cells were transfected with 25 nM of the indicated siRNA oligos 3 days prior to the experiment. (A + B) The cells were incubated with StxB-sulf2 (A) or ricin-sulf1 (B) in sulfate-free medium supplemented with 0.2 mCi $Na_2{}^{35}SO_4$ at 37 °C for 1 and 2 h, respectively. The cells were then lysed and the toxins were immunoprecipitated. After SDS–PAGE the amount of sulfated toxin was determined by autoradiography. The total protein sulfation was determined by TCA precipitation, followed by β-counting. The toxin sulfation and the total protein sulfation are expressed relative to the control and plotted as mean values + SEM, with $n \geqslant 3$. (C) Stx transport to the Golgi was investigated by incubating cells with StxB for 15 min and staining with Stx and giantin antibodies. Representative images are presented. Scale bars: 10 μm. (D) The intensity of Stx colocalizing with giantin (as illustrated in C) was quantified and is shown as mean values + SEM relative to control. $n = 3$ with at least six cells analyzed for each condition. $^{*}p \leqslant 0.05$, paired Student's t test compared to control. $^{**}p \leqslant 0.01$, paired Student's t test compared to control.

by analyzing the effect of siRNA-mediated knockdown of SNX8 on endosome-to-TGN transport of Stx. Sulfotransferases localized in the TGN add sulfate to proteins containing specific sulfation sites. By measuring the amount of radioactive sulfate incorporated in a modified StxB molecule containing two sulfation sites, StxB-sulf2, we were able to determine the amount of toxin transported to the TGN. After knockdown of SNX8, the Stx transport after 1 h of incubation was increased to 214 ± 23% and 152 ± 7% of control in cells treated with siRNA SNX8-1 and SNX8-2, respectively (Fig. 1A, black bars). To check whether SNX8 influences retrograde

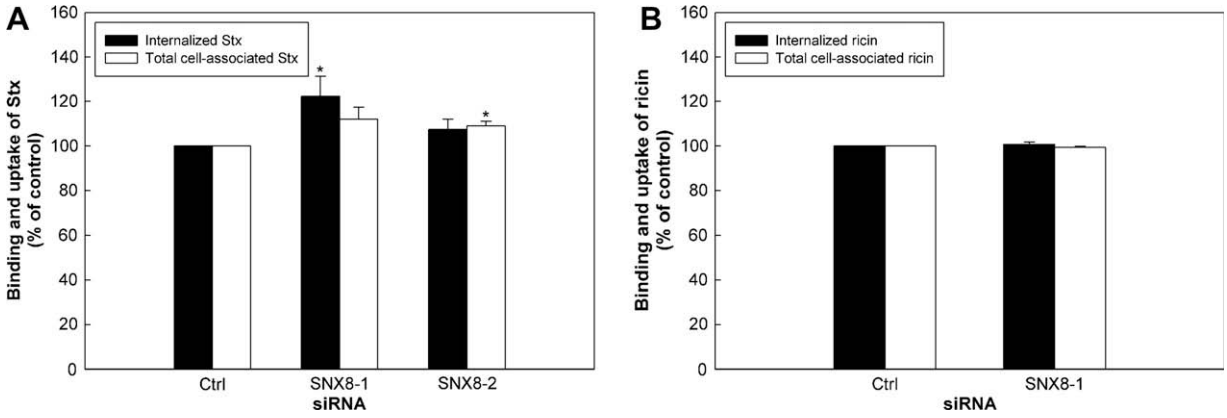

Fig. 2. Knockdown of SNX8 does not affect endocytosis of Stx and ricin. HeLa cells were transfected 3 days prior to the experiment with 25 nM of the indicated siRNA oligos. (A) Cells were incubated with biotinylated Stx for 20 min at 37 °C and subsequently treated with or without MESNa to determine the amount of internalized and total cell-associated toxin, respectively. The cells were then lysed and immunoprecipitated by streptavidin-coated beads in the presence of a Ru(II)-tag labeled Stx antibody. The amount of internalized and total cell-associated Stx was measured as the electrochemiluminescence produced by the Ru(II)-tag, and the mean values + SEM are plotted as the percentage of control, with $n \geqslant 4$. $^{*}p \leqslant 0.05$, paired samples Student's t test compared to control. (B) Cells were incubated with ^{125}I-ricin for 20 min at 37 °C. Half of the cells were then incubated with 0.1 M lactose to remove membrane-bound ricin. After TCA precipitation, the amount of internalized and total cell-associated ricin was determined by γ-counting. The mean values (from 2 independent experiments, each with duplicates) + deviation from the mean, expressed relative to the control, are shown.

transport to the Golgi in general, or if it specifically regulates the pathway taken by Stx, we also investigated the effect of SNX8 knockdown on ricin transport. In contrast to Stx, ricin sulfation was reduced by 29 ± 4% and 16 ± 3% using siRNA SNX8-1 and SNX8-2, respectively (Fig. 1B, black bars). The total protein sulfation was measured by TCA precipitation and was not affected or only slightly affected (⩽10%) in both experiments (Fig. 1A, B, white bars). The knockdown of SNX8 was determined by real-time RT-PCR and was at least 80% at the mRNA level (not shown). The Stx transport to the TGN was also studied by immunofluorescence microscopy. After 15 min of incubation with StxB-sulf2, the intensity of the toxin colocalizing with the Golgi marker giantin was higher in SNX8-depleted cells than in control cells (Fig. 1C, D). This finding supports the sulfation data, showing an increased Stx transport to the TGN in SNX8 knockdown cells.

SNX8 depletion does not affect the endocytosis of Stx and ricin

To investigate if the increased Stx sulfation and the reduced ricin sulfation are caused by an altered uptake of toxins, we measured the internalization of Stx and ricin. HeLa cells were transfected with SNX8 siRNA 3 days before the experiment and biotin-labeled Stx or ^{125}I-labeled ricin were added to the cells. The amounts of total cell-associated toxin and internalized toxin were measured after 20 min of incubation, and the results are shown in Fig. 2. Although there is a very small, but statistically sig-

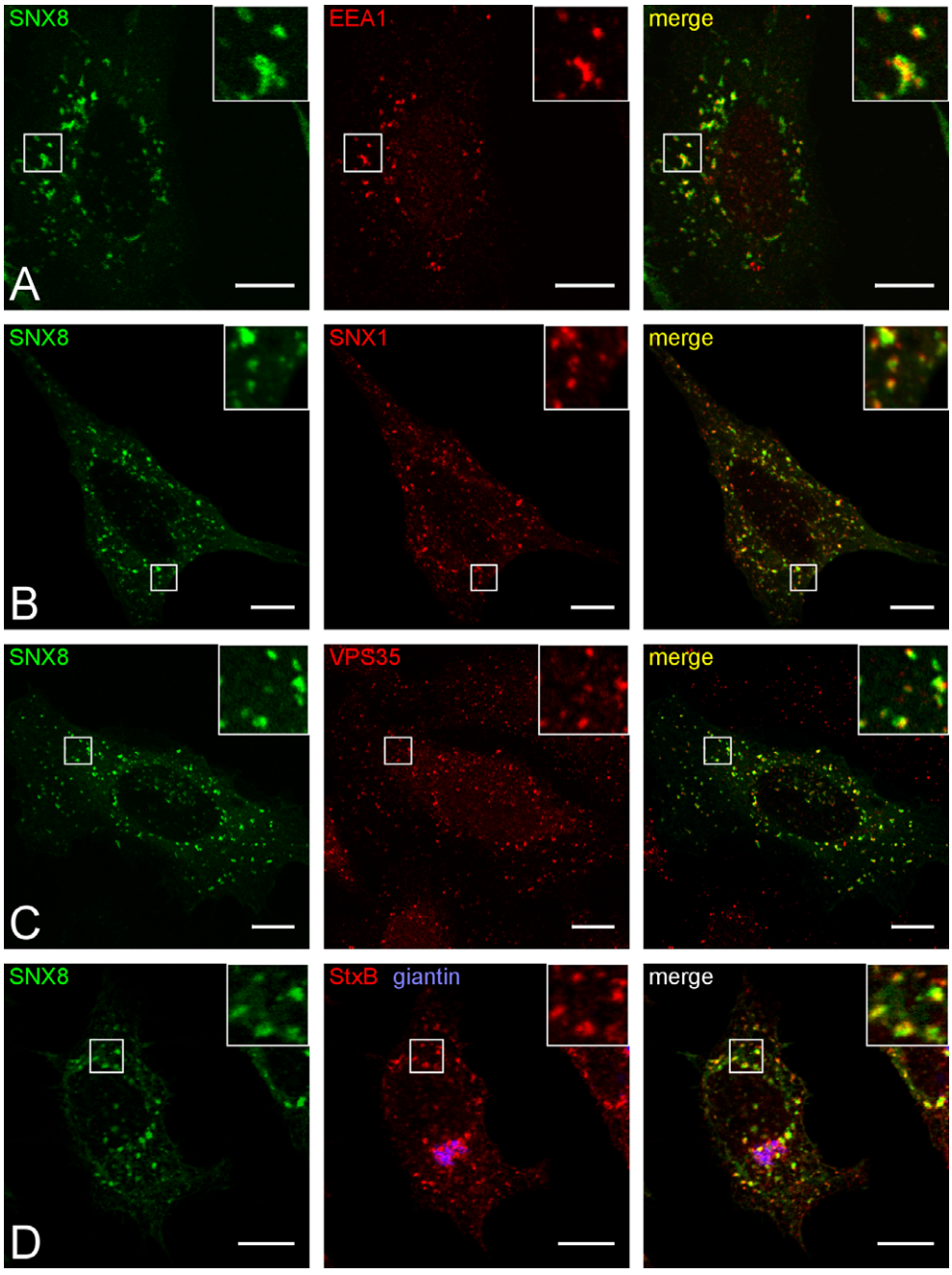

Fig. 3. SNX8 is localized to early endosomes. HeLa cells expressing GFP-SNX8 were stained with various antibodies and investigated by confocal microscopy. There was a partial colocalization of GFP-SNX8 with both the early endosome marker EEA1 (A) and the retromer components SNX1 (B) and VPS35 (C). Cells expressing GFP-SNX8 were incubated with StxB-sulf2 for 15 min and stained with Stx and giantin antibodies (D). A large fraction of the toxin reached the giantin-positive structures (shown in blue), while vesicular StxB-sulf2 partly colocalized with SNX8. Scale bars: 10 μm.

nificant increase in Stx internalization after SNX8 knockdown with one of the siRNA oligos, this increase is far too small to account for the increased Stx sulfation. We did not observe any differences in binding or uptake of ricin. These findings support the hypothesis that SNX8 act on the endosome-to-Golgi transport step.

SNX8 colocalizes with StxB in early endosomes

SNXs contain a PX domain, which targets the proteins to PI-enriched membrane domains. In general, PX domains preferentially interacts with PI(3)P, although in some cases the affinity towards other PIs is higher [29]. To determine the subcellular localization of SNX8, we investigated cells expressing a GFP-tagged SNX8 protein by confocal microscopy. The GFP-SNX8 protein was mainly visible as punctate, vesicular structures, but in cells expressing high levels of the construct there was a change in the morphology of these structures into enlarged vesicles or compartments. Similar observations have been made after overexpression of other sorting nexins [30,31]. After treatment with the PI3K inhibitor wortmannin, the punctate staining was lost and SNX8-GFP was evenly distributed throughout the cytoplasm (not shown). By costaining with EEA1 it was revealed that SNX8 is targeted to EEA1-positive endosomal structures (Fig. 3A). Immunofluorescence experiments also showed that there is colocalization between SNX8 and the retromer components SNX1 (Fig. 3B), VPS35 (Fig. 3C), SNX2 (not shown), and VPS26 (not shown), which are all involved in sorting Stx from early endosomes to the TGN. To see if Stx is targeted to SNX8-positive structures, cells expressing SNX8-GFP were incubated with StxB-sulf2 for 15 min and stained with anti-Stx antibody. This revealed that SNX8 colocalizes with StxB-sulf2 in vesicular structures, and that the accumulation of StxB-sulf2 in perinuclear structures is not perturbed by the expression of GFP-SNX8 (Fig. 3D). Thus, SNX8 seems to be localized to the sorting endosomes from where Stx is targeted to the retrograde pathway by the retromer.

Discussion

The main finding in the present article is that SNX8 plays a role in the sorting of endosomal content. Surprisingly, knockdown of SNX8 results in an increase in Stx transport to the Golgi whereas there is a reduction in ricin transport. In contrast to SNX1 and SNX2 which promote Golgi transport of Stx, SNX8 therefore seems to work as a brake and downregulates this pathway. Perhaps cellular signaling can regulate Golgi transport through SNX8.

Our localization studies show that SNX8-GFP partially colocalizes with the retromer components SNX1, SNX2, VPS26, and VPS35 and with EEA1, which indicates an endosomal localization of SNX8. SNX8 contains both the SNX-PX and the BAR domain, which is known to target proteins to highly tubular membranes containing PI(3)P. The binding of SNX8 to PI(3)P is further supported by the loss of punctate staining upon PI(3)P depletion using the PI3K inhibitor wortmannin. Additionally, it has been found in a protein-fragment complementation assay that Mvp1p, the yeast homolog of SNX8, interacts with the yeast retromer components Vps17p and Vps35p [32].

The observation that SNX8 colocalizes with components of the retromer complex is interesting, as the retromer complex is required for efficient retrograde transport of Stx. It has been shown that knockdown of the retromer components inhibits endosome-to-TGN transport of Stx [26–28], whereas ricin transport is not affected by SNX1 knockdown in HEK293 cells [21]. In contrast, we found that SNX8 knockdown significantly increased the Stx transport to the TGN, but only slightly inhibited ricin transport. The increase in retrograde transport of Stx to the Golgi is not caused by an altered endocytic uptake, as there is only a slight increase in Stx endocytosis after SNX8 depletion. Moreover, as we have shown that SNX8 is localized mainly to the endosomes, it is more likely that it functions at the endosomal level. As discussed, SNX8 might function as an adaptor protein that negatively regulates transport mediated by the retromer, retaining Stx in the endosomes. The yeast homolog of SNX8, Mvp1p, is mediating transport to the vacuole, the yeast equivalent of lysosomes, and it would therefore not be surprising if SNX8 acts also in the degradative pathway. Recently, SNX8 was found to be up-regulated in HEp-2 cells after activating the pregnane X receptor, which regulates pathways involved in metabolism of toxic xenobiotics and endogenous metabolites [33]. Furthermore, it has been discovered that SNX8 is an activator of the sterol regulatory element binding protein (SREBP) [34]. Apparently, a given SNX protein can have more than one function, and the results shown here indicate that SNX8 regulates Golgi transport.

Acknowledgments

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