Live-cell imaging of exocyst links its spatiotemporal dynamics to various stages of vesicle fusion

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Tethers play ubiquitous roles in membrane trafficking and influence the specificity of vesicle attachment. Unlike soluble N-ethyl-maleimide–sensitive fusion attachment protein receptors (SNAREs), the spatiotemporal dynamics of tethers relative to vesicle fusion are poorly characterized. The most extensively studied tethering complex is the exocyst, which spatially targets vesicles to sites on the plasma membrane. By using a mammalian genetic replacement strategy, we were able to assemble fluorescently tagged Sec8 into the exocyst complex, which was shown to be functional by biochemical, trafficking, and morphological criteria. Ultrasensitive live-cell imaging revealed that Sec8-TagRFP moved to the cell cortex on vesicles, which preferentially originated from the endocytic recycling compartment. Surprisingly, Sec8 remained with vesicles until full dilation of the fusion pore, supporting potential coupling with SNARE fusion machinery. Fluorescence recovery after photobleaching analysis of Sec8 at cell protrusions revealed that a significant fraction was immobile. Additionally, Sec8 dynamically repositioned to the site of membrane expansion, suggesting that it may respond to local cues during early cell polarization.

Introduction

Tethering factors play important roles in membrane traffic. At many different trafficking steps, they provide an initial long-distance interaction between the vesicle and target membrane that can influence the fidelity of vesicle delivery, upstream of SNARE-mediated fusion (Sztul and Lupashin, 2009; Bröcker et al., 2010; Yu and Hughson, 2010). One of the most extensively studied tethering factors is the exocyst, an octomeric protein complex composed of Sec3/5/6/8/10/15/Exo70/84 proteins that targets vesicles to specific sites on the plasma membrane (PM; Heider and Munson, 2012; Liu and Guo, 2012). In budding yeast, where exocyst proteins were first identified (Novick et al., 1980), the exocyst is required for vesicle targeting to the bud tip and cleavage furrow (TerBush et al., 1996). Specifically, Sec15 is anchored to the vesicle through the Rab GTPase Sec4, whereas Sec3 and Exo70 act as “spatial landmarks” on the PM for complex assembly (Finger et al., 1998) or polarity (Heider and Munson, 2012). In mammalian cells, perturbation of the exocyst (using blocking antibodies, overexpressed mutants, or RNAi) revealed that it is essential in a myriad of cellular processes, such as cell division, secretion, migration, and ciliogenesis and formation of junctions, invadopodia, and nanotubes (Liu and Guo, 2012; Heider and Munson, 2012).

Although the exocyst has the molecular interactions (Munson and Novick, 2006) and physical size (Hsu et al., 1998) to bridge the vesicle to the PM, precisely how it is linked in space and time to vesicle fusion is not understood. This is because the exocyst (or any other tether) has not been directly observed relative to vesicle fusion. Biochemical assays provide a means to dissect vesicle tethering from fusion and have shown that two multisubunit tethers (HOPS and TRAPP) bind vesicles to their target (Cai et al., 2007; Stroupe et al., 2009), but these ensemble techniques cannot provide the temporal and spatial information to fully elucidate the tethering process.

A key open question regarding exocyst dynamics is when and where are exocyst components recruited and released. In mammalian cells it has been speculated that three subunits (Sec10/Sec15/Exo84) ride on the vesicle for complex assembly (Finger et al., 1998) or polarity (Heider and Munson, 2012). In mammalian cells, perturbation of the exocyst (using blocking antibodies, overexpressed mutants, or RNAi) revealed that it is essential in a myriad of cellular processes, such as cell division, secretion, migration, and ciliogenesis and formation of junctions, invadopodia, and nanotubes (Liu and Guo, 2012; Heider and Munson, 2012).
get close enough to the PM to fuse (Heider and Munson, 2012). Yet many multisubunit tethers, including the exocyst, interact with the SNARE machinery (Sivaram et al., 2005; Yu and Hughson, 2010; Morgera et al., 2012), suggesting that they may be linked to the fusion step. Although biochemical and genetic data have shown interactions between the exocyst and SNAREs (Wiederkehr et al., 2004; Grosshans et al., 2006), this binding could either stabilize the interaction of the tether to the acceptor compartment, promote vesicle fusion, or both (Bröcker et al., 2010).

Dynamic imaging of tethers relative to vesicle exocytosis would be a direct means to elucidate the orchestration of tethering and fusion. Unfortunately, current methods cannot probe the transitory nature of tethers. Exocyst subunits were systematically tagged with GFP in MDCK cells a decade ago, but most GFP fusions produced diffuse cytosolic labeling, incongruent with localization to vesicles (Matern et al., 2001). However, in budding yeast, imaging and photobleaching studies showed that triple GFP-tagged subunits moved on puncta into the bud tip (Boyd et al., 2004). This was not studied in relationship to vesicle tethering or fusion, which may be technically challenging because of the rapid flux and high density of vesicles in the yeast bud. Exocyst subunits have also been imaged in fission yeast (Bendezú and Martin, 2011), Arabidopsis thaliana (Pecenková et al., 2011), Dictyostelium discoideum (Essid et al., 2012), and Drosophila melanogaster (Guichard et al., 2010), but it is not clear if the probes were bona fide reporters of the exocyst complex.

To study the role of the exocyst in constitutive exocytosis, we combined a genetic replacement strategy to label the exocyst with a fluorescently tagged version of Sec8 in mammalian cells with sensitive imaging of vesicle arrival and fusion by total internal reflection fluorescence microscopy (TIRFM). We show that Sec8 arrived on recycling vesicles and surprisingly remained until full dilation of the fusion pore, indicating a potential link with the SNARE fusion machinery. In live migrating cells, Sec8 dynamically repositioned to sites where membrane outgrowth subsequently occurred, suggesting that it may act during early stages of cell polarization.

Results and discussion

Genetic replacement strategy to study exocyst dynamics in mammalian cells

It has been shown that GFP-tagged exocyst subunits mislocalize to the cytosol when expressed in MDCK cells; an exception was Exo70-GFP, yet its overexpression decreased transepithelial resistance (Matern et al., 2001). Although a trivial explanation is that the tags rendered the subunits nonfunctional, another is that most overexpressed GFP-tagged subunits were not incorporated into the complex. We favor the latter possibility. First, in yeast most GFP-tagged exocyst subunits genetically rescued ts phenotypes (Boyd et al., 2004). Additionally, single particle studies of the conserved oligomeric Golgi subcomplex, a tethering complex with subunits structurally similar to the exocyst, showed that most GFP-tagged subunits were assembled in the complex (Lees et al., 2010).

We designed experiments to test whether a fluorescently tagged exocyst subunit would become incorporated into the holocomplex if its endogenous counterpart is selectively knocked down. We observed that exogenous Sec8 tagged at the C terminus with TagRFP (rSec8-TagRFP) was partially degraded in HeLa cells at low expression and further degraded at higher expression (Fig. 1 A). In contrast, when endogenous Sec8 was simultaneously depleted (~80% knockdown [KD] efficiency; Fig. 1 A, lane 1 vs. 7) and rescued with RNAi-resistant rat Sec8-TagRFP, the tagged Sec8 became more stable, especially at low expression (0.1 µg), with levels similar (~98%) to endogenous Sec8 in control cells (Fig. 1 A, lane 8 vs. 1). Immunoprecipitated (IP) rSec8-TagRFP was able to pull down other exocyst subunits in Sec8KD cells (Fig. 1 B), supporting that it was incorporated into the functional complex.

We next performed the corresponding imaging experiments. In control cells, overexpressed rSec8-TagRFP appeared either cytosolic (Fig. 1 C, asterisk) or in large aggregates (arrows), as reported previously (Matern et al., 2001). In striking contrast, when Sec8 was knocked down, rSec8-TagRFP appeared as dim diffraction-limited puncta by TIRFM (Fig. 1 C, right). Live-cell movies (Video 1) and corresponding kymographs (Fig. 1 D) revealed that small (~250 nm) Sec8 puncta moved into the evanescent field, stayed in a fixed position (~500 nm xy displacement), and then rapidly disappeared (Fig. 1 D, arrowheads). The size and dynamics of Sec8 spots are consistent with a putative vesicle tether at the PM. Importantly, these dim, dynamic punctae were only observed when endogenous Sec8 was knocked down and only by using sensitive live TIRFM cell imaging (and not by confocal microscopy; unpublished data).

Sec8 arrives on vesicles that tether to the PM and fuse

To test if the appearance of Sec8 at the surface corresponded to vesicle tethering, “Sec8-replaced” cells were cotransfected with Vamp2-GFP (a type II membrane protein); Vamp2 was chosen because it is involved in trafficking pathways that interface with the exocyst in adipocytes (Kanzaki and Pessin, 2003). As seen in Video 2 and its maximum projection image in Fig. 2 A, many peripheral Vamp2-GFP spots colocalized with rSec8-TagRFP (arrows). The corresponding kymograph (Fig. 2 B) revealed that rSec8-TagRFP puncta appeared and disappeared concurrently with Vamp2-GFP puncta (open and closed arrowheads, respectively); the bright static Vamp2-GFP structures that were negative for Sec8 may represent endosomes or clathrin patches on the PM. The lifetime of rSec8-TagRFP spots (n = 3,000 objects) showed an interarrival interval of ~7.5 s (Fig. 2 C). Imaging of deeper TIRFM (>300-nm penetration depth) indicated that Sec8 was on vesicles, as many of the small puncta exhibited long-range motion along curvilinear paths, which is consistent with trafficking along microtubules (Fig. S1 A and Video 2). Additional analysis of rSec8-TagRFP colocalization with other vesicle markers (Fig. S1, C and D) showed a high colocalization with the recycling endosome marker Rab11 (~60%) but only ~20% colocalization with the post-Golgi markers VSVG and NPY (Fig. 2 D). Nearer to the cell surface (~150-nm penetration depth), rSec8-TagRFP appeared with the arrival of Vamp2-GFP vesicles (Fig. 2 E, open arrowhead) and disappeared when the vesicles fused (closed arrowhead). Sometimes Vamp2-GFP rapidly brightened (Fig. 2 E, asterisk;
Strikingly, the Sec8 signal did not disappear at the opening of the fusion pore, but instead persisted until the vesicle fully fused, as assayed by measuring the lateral diffusion of Vamp2 into the PM (Xu et al., 2011). This indicates that Sec8 remains at fusion sites until the cargo disperses into the PM. We next imaged the release of pHluorin-tagged transferrin receptor (TfRc-pH; Fig. 3 B, second panel). To obtain a quantitative temporal profile of Sec8 recruitment, we temporally aligned 120 rSec8-TagRFP traces to the moment of fusion (based on the TfRc-pH spike; Fig. 3 C, dashed line) and then averaged them. The resulting Sec8 intensity profile showed three features: (1) a rising phase before fusion, (2) a peak just before vesicle fusion, and (3) a decay phase with kinetics virtually identical to the diffusional loss of TfRc-pH. These distinct results strongly suggest that an exocyst component is associated with the vesicle until fusion is complete. Thus, our dynamic molecular imaging of Sec8 indicates that vesicle tethering is coordinated not only with the initial moment of fusion, but also with the later stage of fusion pore dilation.

Exocyst vesicles emanate from recycling endosomes

The origin of the vesicles that use the exocyst is unclear as exocyst components have been reported to regulate the delivery of both post-Golgi vesicles (Grindstaff et al., 1998; Grosshans et al., 2006) and recycling endocytic vesicles to the PM (Prigent et al., 2003; Langevin et al., 2005; Zárský and Potocký, 2010). In epithelial cells, the exo- and endocytic circuits may cross, as post-Golgi cargo can traverse exocyst-positive recycling endosomes and Fig. S1 B, trace) a few seconds before its exocytic release, which may correspond to either the opening of the fusion pore (as luminal GFP is slightly acid quenched) or an axial movement of the vesicle before fusion. As this assignment was difficult, we later turned to the highly pH-sensitive GFP variant pHluorin to better monitor vesicle fusion. Nonetheless, these results provide direct evidence that the components of the exocyst are present on vesicles that arrive and tether at the PM. It should be noted that our findings are incongruent with a mammalian model in which Sec8 resides on a PM subcomplex (Moskalenko et al., 2003; Wu et al., 2008); rather, they agree with yeast studies indicating that Sec8 is on the vesicle (Boyd et al., 2004).

Sec8 remains localized until full expansion of the fusion pore

To better address the kinetics of Sec8 relative to vesicle fusion and identify the origin of Sec8-positive vesicles we used pHluorin-tagged cargo. As shown in the maximum-intensity projection (Fig. 3 A) and in Video 3, about half of the Vamp2-pHluorin fusion events had rSec8-TagRFP associated with them (Fig. 3 A, yellow arrowheads and circles). In several cells the colocalization was pronounced in the cell periphery (Fig. 3 A, white arrows), consistent with the view that the exocyst promotes fusion at specific PM sites (Rossé et al., 2006; Letinic et al., 2009).

Using pHluorin, we were able to unambiguously identify the initial opening of the fusion pore, as Vamp2-pHluorin rapidly (within a frame) brightened because of de-acidification of the vesicle with the neutral extracellular milieu (Fig. 3 B, asterisk). Strikingly, the Sec8 signal did not disappear at the opening of the fusion pore, but instead persisted until the vesicle fully fused, as assayed by measuring the lateral diffusion of Vamp2 into the PM (Xu et al., 2011). This indicates that Sec8 remains at fusion sites until the cargo disperses into the PM. We next imaged the release of pHluorin-tagged transferrin receptor (TIRc-pH; Fig. 3 B, second panel). To obtain a quantitative temporal profile of Sec8 recruitment, we temporally aligned 120 rSec8-TagRFP traces to the moment of fusion (based on the TIRc-pH spike; Fig. 3 C, dashed line) and then averaged them. The resulting Sec8 intensity profile showed three features: (1) a rising phase before fusion, (2) a peak just before vesicle fusion, and (3) a decay phase with kinetics virtually identical to the diffusional loss of TIRc-pH. These distinct results strongly suggest that an exocyst component is associated with the vesicle until fusion is complete. Thus, our dynamic molecular imaging of Sec8 indicates that vesicle tethering is coordinated not only with the initial moment of fusion, but also with the later stage of fusion pore dilation.

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en route to the basolateral surface (Fölsch et al., 2003; Ang et al., 2004). Our results in Fig. 2 D show that the majority of Rab11-containing vesicles (∼65%) colocalized with the exocyst, unlike post-Golgi cargo (∼20%). To directly determine to which extent prototypic recycling and post-Golgi cargo use the exocyst, we compared the colocalization of TIRc (a bona fide recycling marker) and a pulsed-released post-Golgi exocytic cargo with rSec8-TagRFP during vesicle fusion; both cargos were tagged with pHluorin on their extracellular side. As shown earlier, many TIRc (and Vamp2)-containing vesicles that underwent fusion colocalized with Sec8, but some did not (Fig. 3 A, green arrowheads). Quantification of 260 TIRc-pH fusion events revealed that ∼50% of them were associated with a Sec8 signal (Fig. 3 D). As transferrin receptor can also recycle through a “short” route via sorting endosomes, exocyst-negative vesicles may originate from these compartments. As a post-Golgi cargo we generated a secreted protein and TIRc containing four FM aggregation domains and a lumenal pHluorin (ssFM4-pH and TIRcFM4-pH); FM4 retains cargo in the ER until AP21988 (2 µM) is added, after which time the cargo can come out as a wave to the surface (Rivera et al., 2000; see Fig. S2 A). After 30-min incubation with the ligand, only ∼20% of ssFM4-pH or TIRcFM4-pH fusion events colocalized with Sec8, indicating that most post-Golgi carriers in nonpolarized cells do not use the exocyst. This minor colocalization may be reconciled if a portion of the post-Golgi carriers passed en route to the surface through the recycling compartment, as shown in epithelial cells (Fölsch et al., 2003; Ang et al., 2004). In contrast, after 120-min treatment with the AP21988, which allowed TIRcFM4-pH to recycle through endosomes to the cell surface, the colocalization with Sec8 increased to ∼50%.

Consistent with a major role of the exocyst in the endocytic recycling pathway, and as a third functional assay for rSec8-TagRFP, we observed that Sec8KD caused an approximately fourfold increase in the perinuclear accumulation of transferrin-Alexa568 (Fig. S2 C, normalized to transferrin receptor) relative to control KD cells (Fig. S2 D, asterisk). This recycling block was fully rescued by expressing rSec8-tagRFP (Fig. S2 D, double asterisk). Together with the biochemical and live-cell imaging assays, these results support that rSec8-tagRFP is functional and that vesicles emanating from recycling endosomes use the
Kymograph (Fig. 4 A, bottom). FRAP analysis revealed that the recovery, after correcting for minor photobleaching, could be fitted by a single exponential with a half-time of 40 s (Fig. 4 B; n = 5 cells). Notably, about one third (32.5%) of Sec8 in cell protrusions was in a nonrecoverable immobile fraction. In yeast, however, it was reported that there was no immobile fraction because 40% recovery could be observed after bleaching 66% of total exocyst at the bud tip (Boyd et al., 2004). In our case the rSec8-TagRFP signal in the box shown in Fig. 4 A before FRAP only accounted for 6% of the total fluorescence; thus even after correcting for the bleached fraction, 26% of Sec8 was immobile. A potential ramification is that an immobile pool of exocyst may act as a local signaling hub and/or spatial cue as postulated previously (He and Guo, 2009).
protein that is a component of the HOPS tethering complex, regulates vacuolar fusion pore expansion (Pieren et al., 2010). Our data offer tantalizing spatiotemporal evidence that a multisubunit tether might promote vesicle fusion; causal experiments are underway to validate this concept.

**Materials and methods**

**Tissue cell culture, lentivirus generation, and reagents**

HeLa and EA hy926 cells were cultured in DMEM (Invitrogen) with 10% FBS (Sigma-Aldrich) for selection of shRNA and with 3 µg/ml puromycin (Sigma-Aldrich) for selection of rSec8-TagrFP stables. HEK293FT cells (Invitrogen) were cultured in DMEM and used for lentivirus production. In brief, HEK293FT cells were transfected with 2 µg of shRNA or cDNA vectors, plus 1 µg psPAX2 (Addgene) and 1 µg pMD2.G (Addgene) using Lipofectamine 2000 (Invitrogen). After overnight incubation, the medium was replaced and cells were grown for 48 h. Medium was recovered and centrifuged for 15 min at 1,000 g to remove cells debris. Supernatant was mixed at a 3:1 ratio with Lenticoncentrator (Takara Bio Inc.) to precipitate and concentrate the virus particles. Particles were resuspended with 500 µl PBS and 100–150 µl were used to infect cells in the presence of 10 µg/ml polybrene. The next day, medium was replaced and cells were incubated for 24 h before adding medium with hygromycin B (invitrogen) for selection of Sec8KD/rSec8-TagrFP double infected cells, the Sec8 shRNA stable cells were generated first, followed by SEC8-TagrFP infection.

Antibodies used were as follow: Sec8 (mouse monoclonal; M. Caplan, Yale University, New Haven, CT), GAPDH (rabbit polyclonal; New England Biolabs, Inc.), TagRFP (rabbit polyclonal; Evrogen), Exo70 (mouse monoclonal; S. Hsu, Rutgers University, New Brunswick, NJ), Sec5 (mouse monoclonal; A. Saltiel, University of Michigan, Ann Arbor, MI), and Sec6 (mouse monoclonal; M. Caplan).

To monitor the dynamics of Sec8 relative to vesicle traffic and polarized membrane expansion we imaged rSec8-TagRFP in migrating human endothelial cells (EA hy926); supplemental experiments validated the KD of Sec8 and replacement with rSec8-TagRFP (Fig. S3 A). Similar to HeLa cells, rSec8-TagRFP was found in protrusions and colocalized with TIRc-pH fusion events (Fig. S3 B). Remarkably, in the course of 30 min, as the cell turned and repositioned itself toward the bottom of the field of view, fluorescent exocyst punctae were continuously seen near the leading edge, where membrane expansion occurred (Fig. 4 C and Video 5). Together with our FRAP analysis, these results suggest that the exocyst may act as a local hub that can dynamically respond during early stages of cell polarization (Liu and Guo, 2012).

In summary, our combined genetic replacement and imaging approach indicates that an exocyst component (Sec8) is in the right place at the right time to contribute to the tethering of recycling endocytic vesicles. Moreover, this tether remains until SNARE-mediated fusion is completed. Because many multisubunit tethering complexes can interact with SNARES, they may serve to proofread SNARE assembly or promote fusion (He and Guo, 2009; Yu and Hughson, 2010; Heider and Munson, 2012). For the yeast exocyst, Sec6 interacts with both Sec9 (a SNAP-25 orthologue; Sivaram et al., 2005) and Sec1 (a Munc18 orthologue or SM protein; Morgera et al., 2012) but not both simultaneously. Intriguingly, SM proteins are known to play a key role in fusion pore expansion (Vardjan et al., 2013). Moreover VPS33, an SM protein that is a component of the HOPS tethering complex, regulates vacuolar fusion pore expansion (Pieren et al., 2010). Our data offer tantalizing spatiotemporal evidence that a multisubunit tether might promote vesicle fusion; causal experiments are underway to validate this concept.

Figure 4. Exocyst rSec8-TagRFP is highly dynamic on cell protrusions and accumulates in clusters at the leading edge during cell migration. (A) Images of rSec8-TagRFP dynamics at cell protrusions by TIRFM/FRAP (see Video 4). Images of rSec8-TagRFP in stable Sec8-replaced HeLa cells in which a ROI is photobleached at time 0 s; kymographs of the ROI are shown beneath. Arrowheads show the appearance and disappearance of rSec8-TagRFP tracks. (B) ROI signal corrected for photobleaching (n = 5; error bars = SEM) fits to a single exponential (black line) and was used to calculate the halftime of recovery (t1/2), mobile fraction (Fm), and immobile fraction (Fi). (C) rSec8-TagRFP dynamics at the leading edge of migrating cells by dual color TIRFM. (left) Images of a stable EA hy926 cell (Sec8KD/rSec8-TagRFP) transfected with TIRc-pH show the cell position at time 0 (green) and at 30 min (yellow). A gallery of the ROI is on the right. rSec8-TagRFP accumulated where the cell subsequently moved (see Video 5).
Transient KD and replacement experiments for Sec8 were done using a 19-nucleotide RNAi sequence: 5′-CCUUAGAUACCCUCACUAUUC-3′ (Invitrogen). Transient transfection of HeLa cells with 100 nM RNAi was done with Lipofectamine RNAiMAX reagent using the reverse transfection protocol (Invitrogen) with Medium GC scramble RNAi control sequence as a negative control. The next day, cells were washed with DMEM and transiently transfected with 0.6–1 μg of plasmids expressing Sec8-tagRFP or other cargos (e.g., Vamp2-GFP or Vamp2-pHluorin) using Eugene HD transfection reagent (Promega). Cells were incubated for 36 h to account for 60 h of RNAi treatment before imaging.

Cloning of shRNA and plasmids constructs

Lentiviruses production plasmids pLKO-scrambled (scram), pLKO-hyg, psFAZ, and p2A were obtained from Addgene (42905). The Addgene plasmids for plK0-hyg plK0-hyg plasmid was cloned into pLVX-shRNA plK0-hyg plasmid as described in brief. Sec8 shRNA primers 5′-CCGGATATGAGGATCAGGACTCGAGCTTGTAGCTACCCACTTTTCGTT-3′ and 5′-AATTCAGAAAAATGTAGGATGATTATGCTACAGGTTATGAAAGGCCTCCCTGTTACACTTCTATTTTG-3′ were annealed and cloned into pLKO-hyg, which was cut with EcoRI and Agel restriction enzymes. Positive clones were analyzed by sequencing to validate Sec8 shRNA cloning. To generate pLVX-puro-Sec8-tagRFP lentivirus vector, first the rat Sec8 cDNA was cloned into pMAGFP-TNF1 vector (provided by M. Davidson, Florida State University, Tallahassee, FL) as a Xhol-KpnI fragment to generate Sec8-tagRFP. After sequencing and expression validation the Sec8-tagRFP fragment was cut as a Xhol-Ncol and subcloned into pLVX-puro plasmid in which a NotI site was added to the 3′ end of the multiple cloning site (Tabak Bio Inc.). The sfM4-H2ORFP construct was generated by replacing the 5′ SpeI-Fcs-H3-BamHI fragment on pCAs1-FM4-Fcs6-HGH (Jaiswal et al., 2009) with 5′ SpeI-phluorin-3′BamHI PCR fragment amplified from Vamp2-pHluorin plasmid (J. Rothman, Yale University). To generate TIRFM-phluorin, the signal sequence from sfM4-phluorin was replaced by 5′ EcoRI-TIR3′XbaI PCR fragment amplified from TIRFc-phluorin plasmid.

Exocytosis IP

Stable HeLa Scram and Sec8KD/Sec8-tagRFP were used for these experiments. For each cell line, two 10-cm dishes were prepared at 60–80% confluence. Dishes were transferred to a cold-room and cells were washed twice with 25 mM Tris-HCl, pH 6.8, 20 mM NaNO3, and 20 mM NaF. Cell lysis was done by treatment for 5 min with IP lysis buffer (25 mM Tris–HCl, pH 6.8, 150 mM NaCl, 1 mM EDTA, 1% Tween 20, 20 mM NaNO3, 20 mM NaF, 100 mM PMSF, and protease inhibitor cocktail [Roche]). Cells were scraped from the dishes and transferred to 1.5 ml eppendorf tubes. The lysates were passed through a 25-gauge syringe 10 times. To clear the lysates the samples were centrifuged for 10 min at 10,000 g at 4°C. Supernatants were recovered and 750–800 µl were used for IP. The IP samples were pre-cleared for 1 h at 4°C with 30 µl of 50% Protein A agarose beads that were prewashed with lysis buffer. After centrifugation at 600 g the supernatants were incubated with 3 µg of rabbit polyclonal against TagRFP (Evrogen) overnight with constant rotation at 4°C. To isolate the complex, 40 µl of 50% Protein A agarose beads were added and incubated for 1 h. Samples were centrifuged at 600 g for 5 min and beads were washed four times with 750 µl of cold lysis buffer. The beads were then resuspended with 30 µl of lysis buffer and blotting buffer and boiled for 5 min to elute the proteins from the beads. For Western blot analysis, 2% of total lysate and 20% of the IP samples were run side by side on a 10% SDS-PAGE and visualized using a west-pico chemiluminescent substrate kit (Thermo Fisher Scientific) as per the manufacturer’s recommendations.

Live-cell imaging and analysis

TIRFM was done as described previously (Xu et al., 2011) using a microscope (IX-70; Olympus) equipped with argon (488 nm) and argon/krypton (568 nm) laser lines, a TIRFM condenser (Olympus or custom condenser), a 60× 1.45 NA TIRF objective (Olympus), and an EMCCD camera (iXon887; Andor Technology) and controlled using iQ software (Andor Technology). All live-cell microscopy was done at 37°C using a custom incubation chamber. A Dual Head Dual Cooled EM camera (with 10% CFS and 25% FCS) and 25× 0.95 NA TIRF objective was used to generate Quicktime movies the raw TIFF files were compressed sixfold. To generate TIRFM movies the raw TIFF files were compressed sixfold (this introduces some high frequency pattern noise not present in the original data). Only linear adjustments were made to the brightness and contrast.

Online supplemental material

Fig. S1 shows colocalization of Sec8-tagRFP puncta with Vamp2-GFP in curvilinear tracks around the perinuclear region of cells and the shows changes in Sec8-tagRFP intensity relative to Vamp2-GFP vesicles. Fig. S2 shows images and quantification of the observed increase in accumulation of TIRFM and the increase of rSec8 tagged vesicle fusion was done as described in Xu et al. (2011), and the intensity of Sec8-tagRFP object “tracks” were selected based on their intensities; the duration of the tracks was measured by determining the length of the selected tracks in pixels and multiplying it by 500 ms, which represents the two frames per second acquisition rate used.

For improved presentation, in all figures and supplemental movies the raw microscopy data were Gaussian blurred (0.75 pixels) in ImageJ. To generate Quicktime movies the raw TIFF files were compressed sixfold (this introduces some high frequency pattern noise not present in the original data). Only linear adjustments were made to the brightness and contrast.

References


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Figure S1. *Localization of rSec8-TagRFP with Vamp2-GFP, VSVG-GFP, or GFP-Rab11 vesicles.* (A) HeLa cells that were transiently knocked down for Sec8 and cotransfected with rSec8-TagRFP and Vamp2-GFP were imaged by TIRFM at a deeper penetration depth (>300 nm) for 3 min at 2 Hz. Bar, 10 µm. A merged maximum projection is shown (left), and individual maximum projections are shown for the regions enclosed in box a and b (right). Red and green lines indicate the curvilinear tracks of puncta that contained rSec8-TagRFP and Vamp2-GFP. Bar, 2 µm. (B) Graphs showing the intensity changes in rSec8-TagRFP and Vamp2-GFP (from Fig. 2 E) signals show a clear rapid brightening (asterisk) and full fusion in the left panel, but fusion is less clear in the right panel (these data are from a single representative experiment of >50 vesicles that was repeated twice). (C) Stable Sec8KD/rSec8-TagRFP HeLa cells were transiently transfected with VSVG-sp-GFP and imaged by TIRFM for 4 min at 2 Hz. Kymographs for the white line are shown for each protein plus the merged image (right). Examples of vesicles containing only VSVG-GFP appearance and disappearance are indicated by open and closed green arrowheads, respectively. Examples of vesicles containing only rSec8-TagRFP are indicated with red arrowheads. (D) Stable Sec8KD/rSec8-TagRFP HeLa cells were transiently transfected with GFP-Rab11 and imaged by TIRFM for 2.5 min at 2 Hz. Kymographs for the white line are shown for each protein plus the merged (right). Examples of vesicles containing both GFP-Rab11 and rSec8-TagRFP are indicated with yellow arrowheads.
Figure S2. **Kinetic and colocalization of TfrcFM4-pH with rSec8-TagRFP and Tf-Alexa568 recycling defect in Sec8KD cells.** (A) TIRFM images of stable HeLa Sec8KD/rSec8-TagRFP cells transfected with TfrcFM4-pH with no AP21988 ligand or at different time points after addition of 2 µM Ap21988. Images represent different cells showing the release of TfrcFM4-pH from the ER aggregates (no ligand) to post-Golgi and PM (30–120 min). Bar, 10 µm. (B) Maximum projection images of TfrcFM4-pH fusion events and rSec8-TagRFP at two different time points (30 and 120 min) to illustrate the increase in colocalization of TfrcFM4-pH fusion events with rSec8-TagRFP. TffrcFM4-pH accumulates at the PM, TfrcFM4-pH fusion events that colocalize with rSec8-TagRFP are shown as yellow circles; noncolocalizing events are in green circles. (C) Stable HeLa cells for Scrambled (Scram), Sec8KD, and Sec8KD/rSec8-TagRFP (Sec8 replaced) were loaded for 1 h with 10 µg/ml Tf-Alexa-568. After washing, the cells were incubated with unlabeled Tf for 10 min at 37°C, fixed, and labeled by immunofluorescence with an antibody against Tfrc to identify the perinuclear region. Images were captured and the ratio of Tf-Alexa568 signal was divided by the Tfrc-Cy5 in the perinuclear region (white lines). Bars, 10 µm. (D) Analysis of Tf-Alexa568/Tfrc-Cy5 signal was performed on 64 Scram, 100 Sec8KD, and 130 Sec8KD-recued cells. A bar histogram (left) and cumulative probability line graph (right) show the increased number of cells with a high level of Tf-Alexa568/Tfrc (>0.5) in Sec8KD but not in Scram or Sec8-replaced cells. P-value results for the K-S test between Scram and Sec8KD (*) and Scram and Sec8KD-recued cells (**) are included in the cumulative probability graph to show the significant difference between the samples.
Figure S3. Validation of Sec8kd and rSec8-TagRFP expression in stable EA hy926 cells and colocalization with TfRc-pH. (A) EA hy926 cells were infected and selected for Scram and Sec8 shRNA. The Sec8KD cells were later infected with rSec8-TagRFP to generate EA hy926 Sec8KD/rSec8-TagRFP stables. Total protein lysates [6 µg] from the stable cells were run in duplicate on an SDS-PAGE for Western blot analysis against Sec8 and GAPDH. Densitometry analysis of the Sec8 signal was done and normalized relative to the signal in Scram. The Sec8KD was ~80% and the rSec8-TagRFP was overexpressed approximately threefold relative to endogenous Sec8. (B) TIRFM images of stable EA hy926 Sec8KD/rSec8-TagRFP cells transiently transfected with TfRc-pH. Maximum projection images of TfRc-pH fusion events and rSec8-TagRFP to show the colocalization of TfRc-pH fusion events with rSec8-TagRFP. TfRcFM4-pH fusion events that colocalize with rSec8-TagRFP are shown as yellow circles; noncolocalizing events are shown as green circles. A mean of 64.7 ± 5.5% of TfRc-pH fusion events colocalized with rSec8-TagRFP events in EA hy926 cells (n = 2 cells; 400 events/cell).
Video 1. **Localization of rSec8-TagRFP after knockdown of endogenous Sec8.** HeLa cells with a transient Sec8KD were transfected with rSec8-TagRFP. Images were acquired on a custom-built objective type TIRF microscope. Frames were taken every 0.5 s for 5 min and are shown at 30 fps.

Video 2. **Dual color TIRFM of rSec8-TagRFP and Vamp2-GFP.** HeLa cells with a transient Sec8KD were transfected with rSec8-TagRFP (red) and Vamp2-GFP (green). Images were acquired on a custom-built objective type TIRF microscope. Frames were taken every 0.5 s for 5 min and are shown at 30 fps. Left shows rSec8-TagRFP signal and right shows the merged signal between rSec8-TagRFP and Vamp2-GFP.

Video 3. **Dual color TIRFM of rSec8-TagRFP and Vamp2-pHfluorin.** HeLa cells with a transient Sec8KD were transfected with rSec8-TagRFP (red) and Vamp2-pHfluorin (green). Images were acquired on a custom-built objective type TIRF microscope. Frames were taken every 0.5 s for 5 min and are shown at 15 fps. Left shows rSec8-TagRFP signal and right shows the merged signal between rSec8-TagRFP and Vamp2-pHfluorin. Representative red circles were drawn on the rSec8-TagRFP and yellow in the merged to show examples of Vamp2-pHfluorin events with rSec8-TagRFP.

Video 4. **TIRFM/FRAP analysis of rSec8-TagRFP in membrane protrusion.** HeLa cells stable for Sec8KD/rSec8TagRFP were imaged on a custom-built objective type TIRF/FRAP microscope. Frames were taken every 1 s for 2 min before photobleaching and 5 min after and are shown at 30 fps.

Video 5. **Dual color TIRFM of rSec8-TagRFP and TIRc-pH during cell migration.** EA hy926 stables for Sec8kd/rSec8-TagRFP (red) transiently transfected with TIRc-pH (green) were imaged on a custom-built objective type TIRF microscope. Images were collected every 0.5 s for 30 min and are shown at 15 fps. Bars, 10 μm.