Cell. Mol. Life Sci. (2013) 70:2099–2121
DOI 10.1007/s00018-012-1156-5

Cellular and Molecular Life Sciences

REVIEW

Multiple roles for the actin cytoskeleton during regulated exocytosis

Natalie Porat-Shliom · Oleg Milberg · Andrius Masedunskas · Roberto Weigert

Received: 30 May 2012 / Revised: 28 August 2012 / Accepted: 30 August 2012 / Published online: 18 September 2012

Abstract Regulated exocytosis is the main mechanism utilized by specialized secretory cells to deliver molecules to the cell surface by virtue of membranous containers (i.e., secretory vesicles). The process involves a series of highly coordinated and sequential steps, which include the biogenesis of the vesicles, their delivery to the cell periphery, their fusion with the plasma membrane, and the release of their content into the extracellular space. Each of these steps is regulated by the actin cytoskeleton. In this review, we summarize the current knowledge regarding the involvement of actin and its associated molecules during each of the exocytic steps in vertebrates, and suggest that the overall role of the actin cytoskeleton during regulated exocytosis is linked to the architecture and the physiology of the secretory cells under examination. Specifically, in neurons, neuroendocrine, endocrine, and hematopoietic cells, which contain small secretory vesicles that undergo rapid exocytosis (on the order of milliseconds), the actin cytoskeleton plays a role in pre-fusion events, where it acts primarily as a functional barrier and facilitates docking. In exocrine and other secretory cells, which contain large secretory vesicles that undergo slow exocytosis (seconds to minutes), the actin cytoskeleton plays a role in post-fusion events, where it regulates the dynamics of the fusion pore, facilitates the integration of the vesicles into the plasma membrane, provides structural support, and promotes the expulsion of large cargo molecules.

Keywords Regulated exocytosis · Actin cytoskeleton · Myosin · Secretion

N. Porat-Shliom · O. Milberg · A. Masedunskas · R. Weigert (✉)
Intracellular Membrane Trafficking Unit, Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, 30 Convent Dr. 303A, Bethesda, MD 20892-4340, USA
e-mail: weigertr@mail.nih.gov

O. Milberg
Department of Chemical and Biochemical Engineering, Rutgers University, Piscataway, NJ 08854, USA

O. Milberg
Department of Biomedical Engineering, Rutgers University, Piscataway, NJ 08854, USA

A. Masedunskas
Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, USA

Introduction

Exocytosis is a fundamental process that every cell utilizes to deliver membranes, lipids, and soluble molecules (here referred to as cargo molecules or cargo) to the cell surface and the extracellular space [1]. This process is mediated by membranous carriers of different sizes and shapes (e.g., vesicular, tubular, or pleomorphic; for simplicity here referred to as secretory vesicles), which originate from the biosynthetic or the endocytic pathways [2–4]. Exocytosis can occur either constitutively, when secretory vesicles fuse with the plasma membrane without any stimulation, or in a regulated fashion [1, 5, 6]. Constitutive exocytosis is a constant, on-going process that occurs in every cell type and is necessary for the maintenance of the plasma membrane and several other basic cellular functions. On the other hand, during regulated exocytosis, secretory vesicles are delivered to the cell periphery where they reside until an extracellular stimulus triggers their fusion with the plasma membrane. This process occurs in specialized secretory cells in order to satisfy specific physiological

tasks, such as neurotransmission, respiration, digestion, reproduction, the immune response, and many others.

The secretory tissues contain different types of specialized secretory cells that undergo regulated exocytosis and can be divided into four major categories: neuronal, endocrine, exocrine, and hematopoietic [1]. In neurons, neurotransmitters contained in vesicles are secreted from the axon terminals, which are specialized areas of the plasma membrane, and into synaptic clefts where they reach their targets on the juxtaposed dendrites of the following neurons [1, 5, 6]. However, recent evidence suggests that neurotransmitters are exocytosed from dendrites as well [7]. In endocrine and neuroendocrine glands, hormones are secreted into the extracellular space and from there diffuse into the circulatory system to ultimately reach their targets [8–10]. In exocrine glands, macromolecules are contained in large secretory vesicles (or granules) that are secreted from specialized domains of the plasma membrane that are in direct communication with the external environment (e.g., ducts and canaliculi) [11–13]. In hematopoietic cells, such as natural killers (NK) or cytotoxic T-lymphocytes (CTL), molecules are released into the extracellular space from a specific plasma membrane domain (termed the "immunological synapse") that is formed upon their interaction with target cells [4, 14]. Furthermore, different kinds of secretory cells may coexist within the same organ as occurs in the pancreas in which 1–2 % of its mass is occupied by the islets of Langherans, which are formed by endocrine cells (α, β, δ, PP, and ε cells), and the rest is occupied by exocrine acinar and ductal cells. Finally, there are a few secretory systems such as sperm [15], endothelial cells [16], melanocytes [17], mammary glands [18], and eggs [19] that posses distinct features that will be discussed later.

In the last two decades, the molecular machinery controlling regulated exocytosis has been the subject of extensive investigations, and several factors have been identified as key players in the process, including G proteins-coupled receptors, ion channels, intracellular Ca^{++} levels, small GTPases, lipid modifying enzymes, and the actin cytoskeleton [20–25]. The actin cytoskeleton, in particular, plays a fundamental role in various membrane trafficking pathways [26–29]. Its remodeling at the surface of intracellular organelles and at the plasma membrane regulates short-range membrane transport and facilitates various processes such as membrane budding, fusion, and fission [27, 30, 31]. In regulated exocytosis, the actin cytoskeleton has been proposed to act either as a positive or negative regulator, depending on the secretory system under examination [9, 19, 28, 31, 32]. These models are based on measuring the amounts of secreted proteins under conditions in which the dynamics of the actin cytoskeleton was impaired by specific pharmacological agents or bacterial toxins. Recently, the use of more specific molecular tools combined with either high-resolution electron microscopy or time-lapse light microscopy have revealed a more complex involvement of the actin cytoskeleton in regulated exocytosis.

The goal of this review is to discuss our current understanding of how actin remodeling controls each step of regulated exocytosis, while comparing the differences and commonalities among the various secretory cells. Although we have tried to cover the subject as extensively as possible, we will not discuss regulated exocytosis in invertebrates and suggest other reviews to the readers interested in this aspect [33–36].

Regulated exocytosis

Secretory vesicles biogenesis and maturation

In most of the secretory cells, newly synthesized molecules destined for regulated exocytosis are transported from the endoplasmic reticulum (ER) to the Golgi apparatus, and are constitutively packaged into secretory vesicles in the trans-Golgi network (TGN) [11–15]. Since the machinery required for the formation of regulated secretory vesicles is present only in specialized tissues, it has been proposed that their biogenesis is regulated by a single "master" regulatory gene. Initial evidence pointed to chromogranin A, a protein expressed in several secretory tissues [37]. Indeed, anti-sense mediated depletion of chromogranin A in both pheochromocytoma cells (PC12) [38] and in mice [39] resulted in a reduction in secretory vesicles that has been attributed to an increased degradation of granular proteins [40]. Further support came from complementary experiments, which showed that over-expression of chromogranin A in non-secretory cells resulted in the formation of secretory vesicles-like structures [38]. On the other hand, deletion of the chromogranin A gene did not affect the synthesis of proteins targeted to the secretory granules or their biogenesis [41] and, in other cell types, such as pancreatic β-cells, insulin vesicle biogenesis was completely independent from both chromogranin A and B [42, 43]. Moreover, chromogranin A ablation resulted in the compensatory expression of other granins. Taken together, this evidence suggests that there are a series of switches that control the overall process of secretory vesicle biogenesis, rather than a single one [44, 45].

Another aspect that has been extensively investigated is the machinery controlling the sorting of regulated cargo molecules away from the constitutive pathway. Two main mechanisms have been proposed (Fig. 1a). The first, known as "sorting by entry," [11, 46] involves the binding of cargo molecules to specific receptors that are either

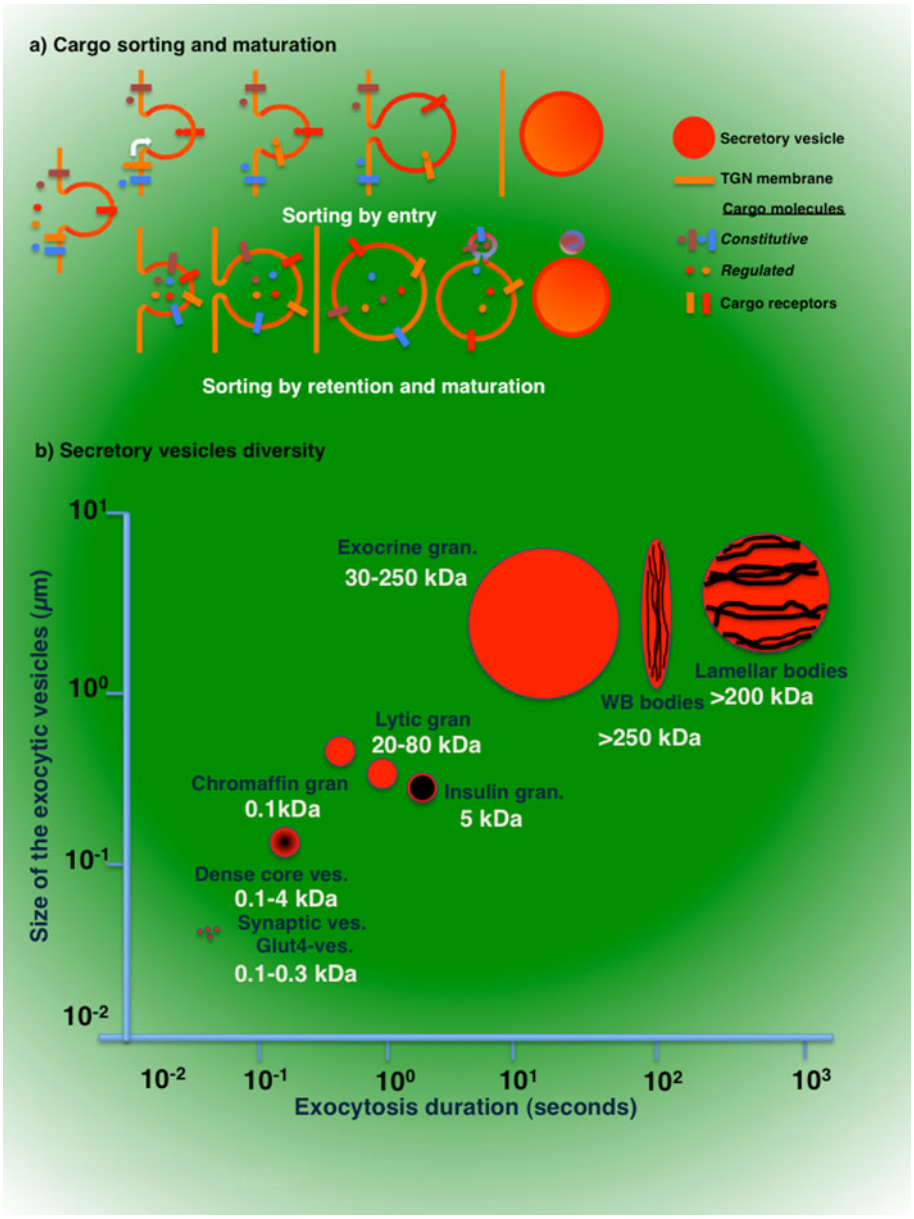

Fig. 1 Cargo sorting into secretory vesicles and their morphological diversity. **a** Cargo sorting at the TGN. Molecules destined for secretion are sorted into regulated secretory vesicles at the TGN by two main mechanisms. According to the "sorting by entry" model, cargo molecules bind to specific receptors and are sorted into the nascent secretory vesicles, which detach from the TGN and are transported towards the plasma membrane. According to the "sorting by retention" model, cargo molecules freely enter newly formed vesicles that are released into the cytoplasm. These "immature" vesicles undergo an extensive "maturation" step, which is mediated by the removal of specific molecules and membranes via clathrin-coated vesicles. **b** Correlation between the sizes of the secretory vesicles and the duration of exocytosis. The sizes of the secretory vesicles ranges from 50 nm up to 2–3 μm in diameter and roughly correlate with the molecular weights of the cargo molecules (from 0.1 kDa up to >200 kDa). The duration of exocytosis, as measured from the opening of the fusion pore to the moment in which the secretory vesicle is completely integrated into the plasma membrane, inversely correlates with vesicles size. Small synaptic vesicles complete exocytosis in a time scale ranging from a microsecond to a few milliseconds. Dense core vesicles, lytic and insulin granules complete exocytosis within a few seconds, whereas large secretory granules in exocrine glands last for several seconds up to a minute, and Weibel–Palade bodies, lamellar bodies, and cortical granules last for several minutes

proteinaceous [47–50] or lipidic in nature [51–53], and localize or segregate into the nascent secretory vesicles. The second, known as "sorting by retention," is based on the fact that cargo molecules freely enter newly formed vesicles that are released into the cytoplasm. These "immature" vesicles undergo an extensive "maturation" step, which is mediated by homotypic fusion with other newly formed carriers and/or the removal of specific

molecules and membranes via clathrin-coated vesicles [11, 46, 54, 55] (Fig. 1a). The "maturation" step has been proposed for several secretory systems, such as insulin-secreting [10], neuroendocrine [56], salivary [57, 58], endothelial [59], and pancreatic acinar cells [58].

Secretory vesicles can also originate from the endocytic and the degradative pathways, or from the contribution of multiple organelles. For example, in cytotoxic T-lymphocyte, mature secretory lysosomes are formed from late endosomal compartments upon stimulation with of the T cell receptor. The late endosomes then transition to multivesicular bodies and become progressively enriched in a dense core formed by newly synthesized lytic proteins that are delivered from the TGN [4]. In adipocytes, GLUT4-containing vesicles originate from both the TGN and the recycling endosomal compartment in a process that requires GGA (Golgi-localizing Gamma-adaptin ear domain homology ARF-binding proteins), clathrin, phosphatidylinositol 4-phosphate (PI4P) [60], and GLUT4 ubiquitination [61]. In alveolar type II cells, surfactant phospholipids are arranged in tightly packed membrane sheets (lamellae), and are transported by multivesicular body-derived organelles, called lamellar bodies. [62]. This process is mediated by the hydrophobic SP-B peptide, which is transported via the Golgi apparatus to the lamellar bodies, whereas phospholipids are transported directly from the ER [62]. Finally, in mammary glands, lipid droplets are synthesized in the ER and are transported directly to the plasma membrane where they are secreted by an unusual mechanism [63].

Morphological and content diversity of the secretory vesicles

The diversity in the modality of vesicle biogenesis is reflected in the astonishing diversity of their sizes (ranging from 50 nm up to 2–3 μm in diameter) and in their heterogeneity within the same secretory cell (Fig. 1b), which can be correlated with the molecular nature of the transported molecules, as shown by the following examples. In neurons, synaptic vesicles contain primarily small molecular weight neurotransmitters (such as acetylcholine, epinephrine, GABA, dopamine, glutamine, etc.) and have diameters between 50 and 60 nm, while dense-core vesicles contain slightly larger neuropeptides (such as NPY, Galanin, etc.) and have a diameter of around 100 nm [19]. In neuroendocrine and endocrine glands, the nature of the cargo molecules vary from catecholamines to various hormones (molecular weight up to 5–10 kDa) that are transported either in small vesicles or granules that range from 60 to 300 nm. In addition, their cargo molecules can be loosely associated, form electron-dense aggregates (i.e., dense core vesicles), or assemble in crystalline structures, as shown for insulin [64]. Hematopoietic cells appear to have the most diversified set of secretory vesicles. Neutrophils, for example, contain up to four different kinds of secretory vesicles (azurophil, specific, gelatinase, and non-granular), which contain molecules such as myeloperoxidase (140 kDa) and lactoferrin (80 kDa) and have diameters of 200–250 μm. In CTL and NK cells, lytic granules contain large molecules, such as perforins or granzymes (60 and 85 kDa, respectively) and have diameters of 250–300 μm [65]. Moreover, multiple cargo molecules can be packaged within the same vesicle, as in mast cells where small molecules such as histamine and serotonin are co-transported with large cargo such as heparin and gelatinase. In exocrine glands, zymogens (50–100 kDa) or heavy glycosylated mucins (>200 kDa) are transported in large vesicles (or granules) that have diameters of 1–2 μm [66]. In endothelial or alveolar type II cells, molecules such as the von-Willebrand factor (50–20,000 kDa) and the surfactant are densely packed in cigar shaped Weibel–Palade bodies (0.5 × 1.5 μm) and lamellar bodies, respectively (diameter 1.5–2 μm). Finally, a peculiar case is sperm where a complex mixture of proteins needed for fertilization such as hyaluronidase and acrosin, are released from the acrosome, a large, single, cup-shaped organelle that can reach a few microns in size [67].

In preparation for fusion: reaching the cell periphery, docking, and priming

Once assembled, the newly formed secretory vesicles are transported toward the cell periphery. This process is facilitated by microtubules and requires kinesin or dynein motors that are recruited onto the vesicles during their biogenesis [68, 69]. Several studies suggest that microtubules support long-range transport from the sites of biogenesis to the plasma membrane, where the secretory vesicles ultimately associate with the actin cytoskeleton until a stimulus triggers the fusion event [70–72] (Fig. 2a). In neurons, where synaptic vesicles travel for long distances, the disruption of microtubules or interfering with specific microtubule motors result in severe impairment in exocytosis [73–75]. On the other hand, in cells where the secretory vesicles travel for shorter distances, disruption of microtubules leads to delays or slight impairments in exocytosis [68, 76–82].

Once at the cell periphery, secretory vesicles fuse with the plasma membrane and this process requires the proper assembly of the SNARE (SNAP-Receptor) complex, the core component of the fusion machinery that is conserved among several organisms [83, 84]. SNAREs are small proteins (18–42 kDa) containing cytoplasmic amphipathic helices, referred to as SNARE motifs, which engage in coiled-coil interactions with other SNAREs. SNAREs are

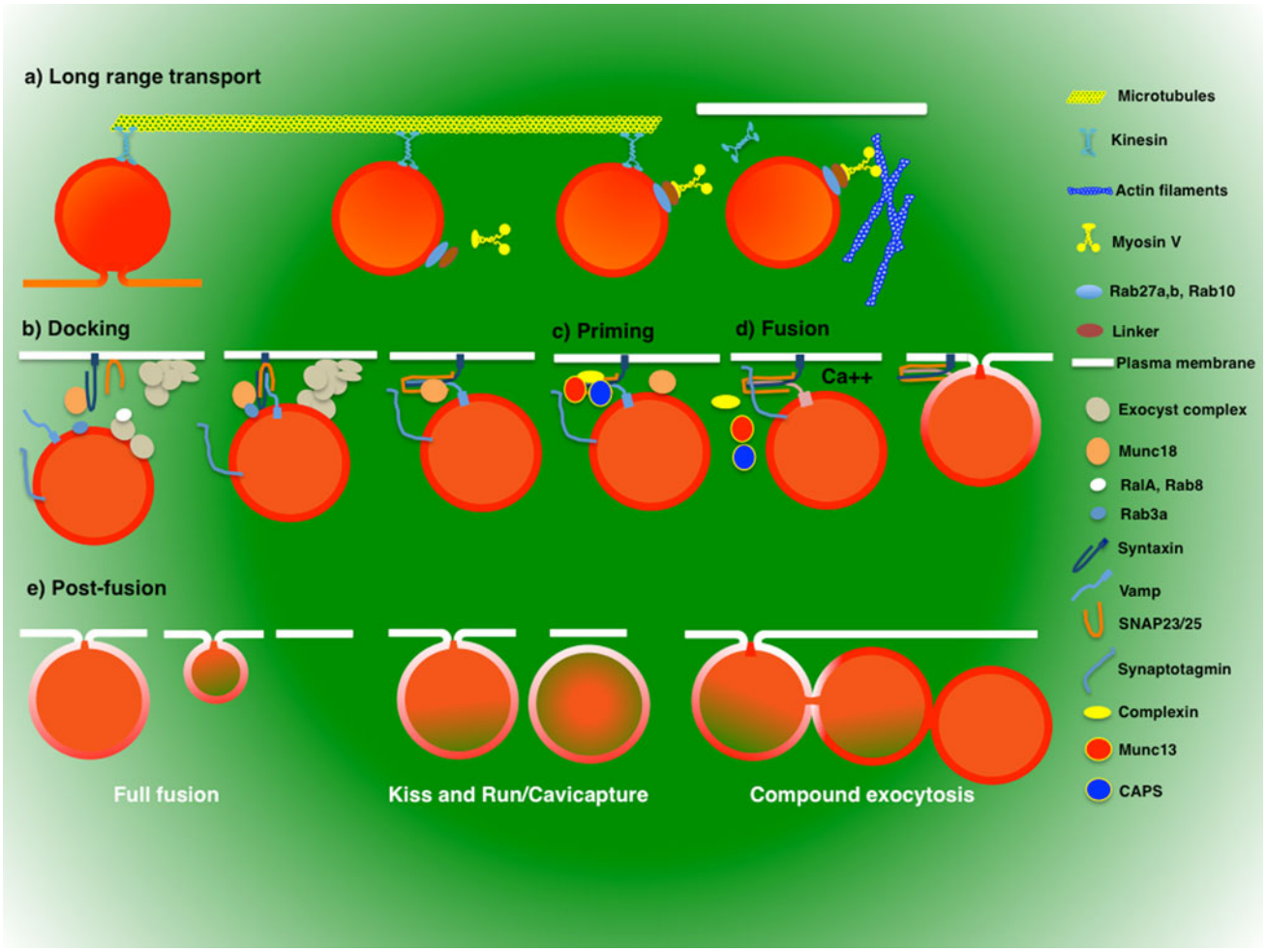

Fig. 2 Steps in regulated exocytosis. Newly formed secretory vesicles are transported toward the cell periphery using microtubules and their associated motors kinesin and dynein (**a**). At the cell periphery, the secretory vesicles associate with the actin cytoskeleton by recruiting and activating myosin motors. Vesicles at the plasma membrane may dock using multiple mechanisms (**b**). The initial tether may be initiated by the exocyst complex, whereas the docking may be achieved by the interactions between the SNARE complex and Munc18. Both processes are coordinated by small GTPases, such as Rab3a, Rab8, and RalA (**b**). In order to become fusion-competent, docked secretory vesicles have to undergo priming that is mediated by proteins such as Mun13 and CAPS, which displaces Mun18 from the SNARE complex and facilitates the binding of complexin (**c**). Upon increase of Ca^{++} levels of, synaptotagmin, binds to the phospholipid at the plasma membrane, displaces complexin enabling the SNARE complex to promote the fusion of the bilayers (**d**). After fusion, vesicles can undergo full fusion, kiss and run/cavicapture or compound exocytosis

divided into three families: syntaxins, SNAPs, and VAMPs [85]. VAMPs are present on secretory vesicles (v-SNAREs) whereas syntaxins and SNAPs are present at the plasma membrane (t-SNAREs). Typically, three SNAREs are required to promote fusion, with one SNARE providing two helices (typically a SNAP protein) [85–87]. Individual SNAREs are unfolded, but they can assemble into a stable four-helix bundle, called the "trans-SNARE complex" or "SNARE pin." The assembly begins from the N-terminus of the SNAREs and zippers them up. This mechanical process forces the membranes to come into close apposition with each other and enables them to overcome the activation energy barrier required for lipid bilayers fusion [84]. In order for the N-termini of the SNAREs to interact, secretory vesicles have to be in close proximity (i.e., docked) to the plasma membrane (Fig. 2b).

Although there are no rigorous criteria to define docked vesicles, they have been operationally identified by either morphological or functional criteria [88]. "Morphological" docking, as defined by using electron microscopy, refers to those vesicles, which appear either apposed to or within less than 10–30 nm from the plasma membrane [88, 89]. "Functional" docking, as defined by using total internal reflection (TIRF) microscopy, refers to those vesicles that exhibit restricted mobility in the evanescent field [3, 88–90]. Docking is controlled by a series of molecules, and among them Munc18 isoforms play a fundamental role [88, 91–93].

In chromaffin cells, modulation of the expression levels of Munc18-1 results in severe disruption of vesicles docking at the plasma membrane, as shown by electron microscopy [94, 95]. In other cells, although docking has not been specifically assessed, manipulation of Munc proteins affects exocytosis. For example, in pancreatic β-cells, overexpression of both Mun18-1 and Munc18-2 increases glucose-stimulated exocytosis of insulin granules [96]. In hematopoietic cells, Munc18-2 seems to be the major regulator of exocytosis. Indeed, in mast cells, increased expression of either Munc18-2 or derived peptides containing an interfering effector loop inhibits IgE-triggered exocytosis [97]. Moreover, in patients affected by familial hemophagocytic lymphohistiocytosis (FHL5), a mutation in Munc18-2 determines the impairment in the release of cytotoxic granules [98]. Finally, PKC-induced phosphorylation of Munc18-3 has been implicated in exocytosis of parotid granules [99].

Although the exact mechanism by which Munc18 proteins promote docking has not been fully elucidated, evidence suggests that their interactions with syntaxins, but not with other SNAREs, play a major role (Fig. 2b) [99–101]. Small GTPases of the Rab family appear to regulate these interactions, as shown in chromaffin cells, where the activation of Rab3a promotes the interaction between Munc18-1 and syntaxin 1 [102, 103]. In addition, Rab27a and Rab27b have been shown to mediate docking in several secretory cells through some of their effectors, such as rabphilin in chromaffin cells, PC12 cells, and neurons [104–106], granuphilin in pancreatic β-cells [107], and exophilin 4 in pancreatic α-cells [108] (Fig. 2b). Interestingly, recent data support the idea that Rab proteins may have intrinsic tethering activity forming interactions in *trans* [109].

Another set of molecules that regulates docking is the exocyst, an octameric complex initially discovered in a screening for mutants of *S. Cerevisiae* defective in secretion [110]. Two of the exocyst subunits are associated with the secretory vesicles, whereas six are associated with the plasma membrane (Boyd et al. 2000). The exocyst has been characterized in mammalian cells and shown to be regulated by GTPases, such as RhoA, cdc42, TC10, RalA [111–114], and the scaffolding protein IQGAP1 (Fig. 2b) [115]. Its role in regulated exocytosis has just started to be evaluated. For example, in adipocytes, the Exo70 subunit has been shown to be recruited to the plasma membrane in a TC10-dependent fashion and to regulate the insulin-stimulated exocytosis of Glut4 [111, 116]. In salivary gland cells, antibodies directed against the exocyst subunits sec6 and sec8 inhibited the isoproterenol-stimulated release of amylase [117], and in hippocampal neurons the IGF1-activated release of plasmalemma precursor vesicles was affected by silencing Exo70 and TC10 [118].

In order to become fusion-competent, docked secretory vesicles have to undergo another step called priming (Fig. 2c). The concept of priming was formulated to describe an ATP-dependent process that precedes the fusion step [119]. The first two molecules described to prime secretory vesicles were NSF (*N*-ethylmaleimide Sensitive Factor) and α-SNAP, whose function is to disassemble the SNARE complex (see below and [120, 121]). The SNARE complex is exceptionally stable and the energy required for its disassembly is provided by the ATPase activity of NSF [122, 123]. In addition, other molecules implicated in ATP-independent priming have been described, and include Munc13 and CAPS proteins [88, 89, 92].

Munc13-1 has been proposed to prime secretory vesicles by binding to syntaxin 1 and displacing Mun18-1, thus preparing for the assembly of the SNARE complex (Fig. 2c) [124]. Although this model has been recently challenged [125, 126], several studies show that down-regulation of Munc13-1 inhibits exocytosis without altering the number of docked vesicles. Munc13-1 regulates priming in neurons [127], chromaffin cells [128], and β-cells [129, 130], and other isoforms have been recently shown to regulate priming in other secretory systems such as mucin granules in airway goblet cells (Munc13-2 [131]), platelets, mast cells, and in LPS-stimulated azurophilic granules in neutrophils (Munc13-4, [132, 133]). A similar function to Munc13 may be performed by CAPS proteins, which contain a Munc13-homology domain. Deletion of CAPS1 and CAPS2 in mice [134, 135] and cell cultures [136] severely impair catecholamine and glucose-stimulated-insulin release. However, the redundancy of CAPS1 and CAPS2 in tissue expression [137, 138] has not allowed researchers to precisely pinpoint their mechanism of action with the exception of the fact that both CAPS proteins bind to phosphatidylinositol 4,5-bisphosphate (PIP2), a phosphoinositide that has been shown to be required for priming [139]. Once the secretory vesicles are primed, a trigger is the only requirement to promote the fusion between the lipid bilayers of the vesicles and the plasma membrane.

Triggering fusion

Generally, fusion is initiated by an extracellular stimulus that is transduced intracellularly through one of several different types of plasma membrane proteins, such as G protein-coupled receptors, tyrosine kinase receptors, or voltage-dependent calcium channels (VDCC) [1, 5, 6]. This stimulus induces the influx or synthesis of second messengers, such as cytosolic Ca^{++} or cAMP. These second messengers initiate several signaling cascades, which ultimately trigger fusion by affecting the conformation of the SNARE complex.

Increase in cytosolic Ca^{++} levels above 0.1–1 μM is considered to be the main factor responsible for fusion. The intracellular Ca^{++} increase can originate from a number of

sources, including an extracellular influx that is mediated by a variety of ion channels at the plasma membrane, release from intracellular calcium stores in the endoplasmic reticulum (ER), or both [24, 140]. The most thorough evidence for how an increase in intracellular Ca^{++} promotes fusion comes from neurons and adrenal chromaffin cells. In neurons, the Ca^{++} sensor synaptotagmin I (sytI) and its cofactor, complexin, are responsible for vesicle fusion at the presynaptic active zone [5]. SytI is a small transmembrane protein that is localized to the secretory vesicles and possesses two C2 domains that bind Ca^{++}. Complexin is a protein that is bound to the primed SNARE complex in the absence of Ca^{++}, and whose proposed function is to act as a clamp preventing fusion. Upon Ca^{++} influx, SytI displaces complexin and binds to the phospholipids and to the SNARE complex [141]. By doing so, SytI pulls the vesicle closer to the plasma membrane and into a position favorable for fusion (Fig. 2d) [142]. Furthermore, there is evidence that this mechanism may be universally conserved, since four isoforms have been identified for complexin and sytI belongs to a family of 16 members that are expressed in several secretory tissues [5]. Indeed, SytI has been shown to play a role in the exocytosis of zymogen granules in the pancreas and dense core vesicles in the adrenal medulla [143], whereas sytII, V, VII, and IX have been reported to regulate the exocytosis of Glut 4 vesicles [144, 145] and secretory granules in mast and NK cells [146, 147].

In some cells, an increase in cAMP has been shown to regulate fusion events by either modulating the Ca^{++} signaling or by acting alone. Indeed, cAMP modulates and enhances Ca^{++}-dependent exocytosis in synapses, adrenal chromaffin cells, melanotrophs, pancreatic β- and acinar cells, and CTL [148]. However, in these cells, elevation of cAMP alone is not sufficient to trigger exocytosis. Interestingly, in the submandibular and parotid salivary glands, cAMP alone is sufficient to elicit exocytosis, even in conditions of low Ca^{++} concentrations, suggesting that either cAMP reduces Ca^{++} sensitivity or that in these systems regulated exocytosis is Ca^{++}-independent [149]. Although the precise mechanisms are not well understood, cAMP may act through at least two major pathways: protein kinase A (PKA) and the exchange protein activated by cAMP (EPAC) [148]. Both pathways can modulate calcium signaling through a variety of effectors [148]. Recently, components of the exocytic machinery, including molecules that interact with and regulate SNAREs, such as Syt12 [150], cystein string protein [151], and syntaphilin [152], have been identified as substrates of PKA. In addition, PKA anchoring proteins, such as AKAP5 in the parotid glands and the linker Myrip, have also been shown to participate in the process [153]. As for EPAC, its role in exocytosis has been linked to a few molecules, such as the small GTPases Rap1 and Rab3a [154–156], Rim2, and phospholipase C-ε [157].

The fusion pore and the fate of the secretory vesicles after fusion

The fusion between the lipid bilayers of secretory vesicles and plasma membrane occurs within a few milliseconds leading to the formation of the "fusion pore" [158]. The successive behavior of the fusion pore controls the release of the cargo into the extracellular space and dictates the fate of the secretory vesicles for which multiple scenarios have been described [158, 159]. In the first, the fusion pore progressively expands and leads to the absorption and the integration of the vesicle into the plasma membrane (Fig. 2e). This process, known as "full fusion", occurs in most of the secretory cells [19, 160–164]. A second possibility is that the fusion pore closes after a fraction of the cargo is secreted, and the vesicle detaches from the plasma membrane (Fig. 2e) [165–168]. This process can be extremely rapid, as observed in neurons (where it is termed "kiss and run"), or it may last for several seconds as shown for neuroendocrine cells (where it is termed "cavicapture") [169–171]. Finally, the prolonged opening of the fusion pore makes secretory vesicles at the plasma membrane competent to fuse with other secretory vesicles in the cytoplasm, thus generating strings of fused vesicles whose lumens are in continuity with the extracellular space (Fig. 2e). This process, termed compound exocytosis, occurs primarily in exocrine cells but also in insulin-secreting and neuroendocrine cells [165, 172–175].

If we focus on full fusion, the time to complete exocytosis, as measured from the opening of the fusion pore to the moment in which the secretory vesicle is completely integrated into the plasma membrane, varies considerably between secretory cells (Fig. 1b). Small synaptic vesicles complete full fusion in a time scale ranging from μsec to a few milliseconds, as determined by amperometric measurements [176, 177]. On the other hand, dense core vesicles and insulin granules complete exocytosis within a few seconds, whereas large secretory granules in exocrine glands last for several seconds up to a minute, and Weibel–Palade bodies, lamellar bodies, and cortical granules last for several minutes [19] (Fig. 1b). These differences correlate with the kinetics of the fusion pore, which depends on the secretory cell and the size of the secretory vesicle [158, 176, 177]. Indeed, smaller vesicles exhibit a faster pore closure [176, 177], whereas larger vesicles maintain the pore open for longer times, as shown by using imaging approaches such as TIRF in cell cultures [77, 90, 178], two-photon microscopy in explanted organs [179], and confocal microscopy in live animals [164]. At a molecular level, the maintenance of the pore is regulated by several factors,

such as syt1 [180] dynamin1, a GTPase regulating fusion and fission of endocytic vesicles [181, 182], and SNAREs [183]. Recently, an intriguing model has been proposed in which two of the SNAREs in the complex promote the fusion of the bilayers, whereas the third one prevents the pore from collapsing [183]. Another family of SNARE interactors, the secretory carrier membrane proteins (SCAMPs), has provided novel insights into this process. In PC12 cells, both SCAMP1 and SCAMP2 regulate the closure of the fusion pore [171, 184], with the latter operating through its interactions with the small GTPase Arf6 and phospholipase D1 (PLD1) [184]. Interestingly, inhibition of PLD1 activity blocked the expansion of the fusion pore during the exocytosis of GLUT4 vesicles in adipocytes [178], thus suggesting a fundamental regulatory role for the lipid composition of the pore [185].

Another factor that may affect the kinetics of full fusion events is the biophysical properties of both vesicles and plasma membrane (e.g., lipid organization, membrane tension, or membrane mobility). Indeed, it has been proposed that upon the establishment of a physical continuity between the two bilayers, membranes spontaneously flow toward the area with the lowest membrane tension [186–189]. According to this model, full fusion would be more energetically favorable when secretory vesicles have a higher membrane tension than the plasma membrane. Based on geometrical considerations, the membrane tension of a secretory vesicle is inversely proportional to its diameter, suggesting that full fusion is a more favorable process for smaller vesicles. Although a precise determination of the membrane tension for the plasma membrane is not so straightforward, it is reasonable to assume that neurons and endocrine cells may have a lower membrane tension than in exocrine cells, where exocytosis occurs on curved surfaces such as the acinar canaliculi (diameters ranging from 0.3–0.4 μm in salivary glands to 1–1.5 μm in the pancreas). As a consequence, a prediction would be that exocytosis in neuron, endocrine, and hematopoietic cells may be more energetically favorable than in exocrine cells or other systems harboring large vesicles. Although this simplistic model does not take in account the nature of the cargo molecules (molecular weight and formation of aggregates) and the fact that fusion may occur in specialized structures such as the porosomes (plasma membrane domains with very specific membrane compositions and morphology) [190–193], it nicely correlates with the experimental data on the kinetics of exocytosis (Fig. 1b).

Role of the actin cytoskeleton in exocytosis

The actin cytoskeleton has been proposed to play multiple and often opposing roles during regulated exocytosis [9, 19, 28, 31, 32, 194]. The two main ideas are that (1) it acts as a physical/functional barrier that is removed during exocytosis, thus allowing the secretory vesicles to access the plasma membrane [9, 28, 31], and (2) it plays a more active role by directing exocytic vesicles to the fusion site, regulating the fusion pore, and providing the driving force to complete fusion [19, 32, 195].

Notably, many studies have provided contradictory findings for the role of the actin cytoskeleton in regulated exocytosis. This is largely due to the use of a single experimental approach and the use of drugs or toxin that induce global changes in the actin cytoskeleton. More recently, specific molecular tools were developed and multiple tools have been integrated. Approaches such as biochemistry, electrophysiology, electron microscopy, and time-lapse imaging have contributed to better define the role of the actin cytoskeleton during exocytosis supporting the view that multiple roles for the actin cytoskeleton are not necessarily mutually exclusive and may coexist within the same secretory cell.

Role of the actin cytoskeleton in pre-fusion steps

Anchoring site for the secretory vesicles and barrier function

During pre-fusion events, the actin cytoskeleton has been suggested to negatively affect regulated exocytosis in several secretory systems, and at least two models have been proposed so far for this type of regulation. The first model suggests that under resting conditions the actin cytoskeleton provides a scaffold for anchoring secretory vesicles in close proximity to the plasma membrane (Fig. 3a). The second model treats it as a barrier, blocking exocytosis from occurring (Fig. 3b).

The idea behind the first model is based on the observations that secretory vesicles are associated with actin and other cytoskeletal elements and that upon stimulation they detach from the cytoskeleton, increasing their mobility. In neuromuscular and cerebellar synapses, for instance, synaptic vesicles are associated with an intricate network of actin filaments, as shown by using quick-freeze deep-etch electron microscopy [196, 197]. In live pre-synaptic terminal of rat neurons, synaptic vesicles are associated with actin filaments labeled by expression of EGFP-actin [198]. Furthermore, upon stimulation, synaptic vesicles are released from the cytoskeleton through the CAM kinaseII-mediated phosphorylation of the presynaptic marker synapsin I [199, 200]. The mechanism involves Synapsin I binding to actin, and this interaction appears to be modulated by the small GTPase Rab3a [201].

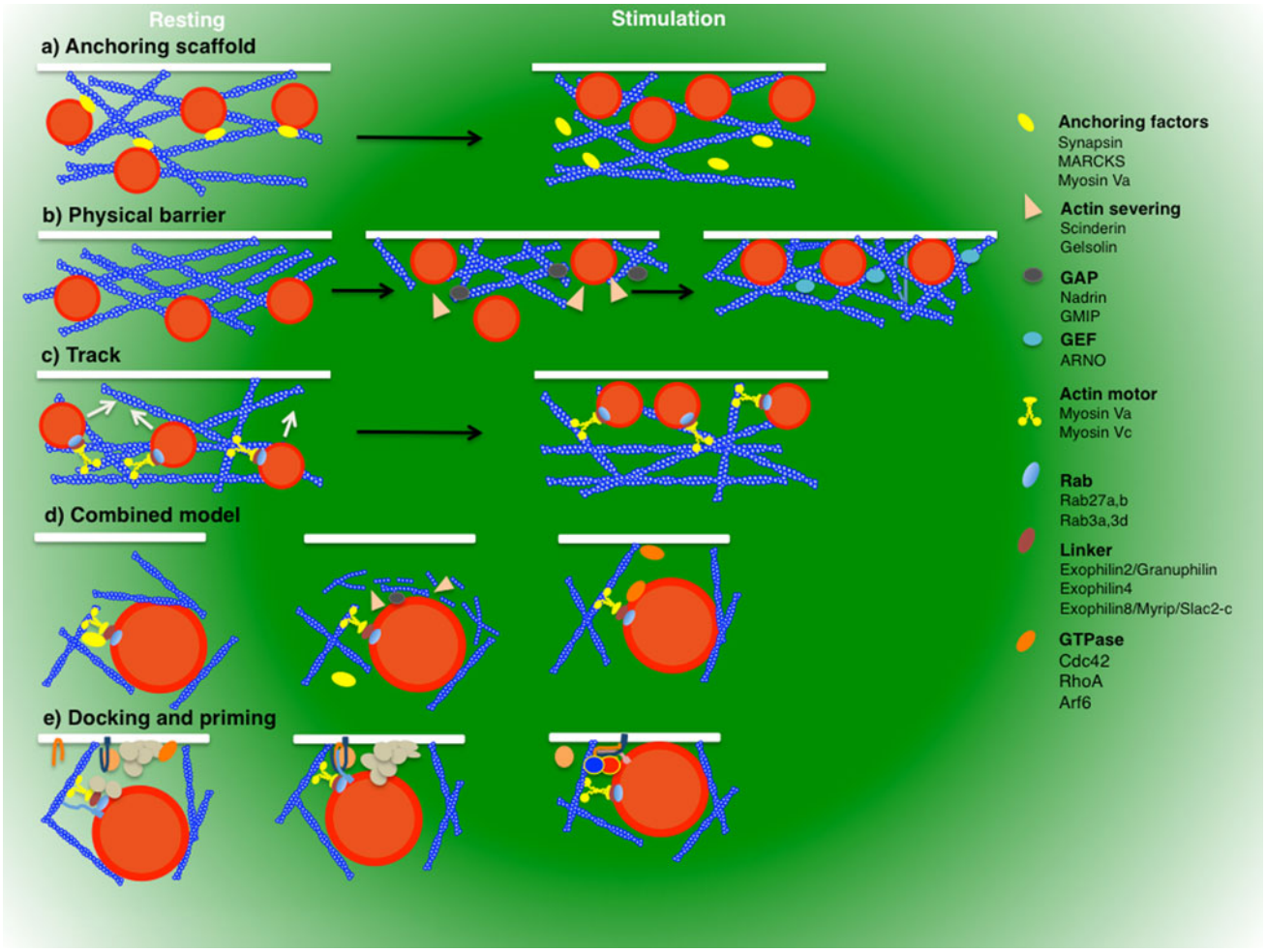

Fig. 3 The actin cytoskeleton in pre-fusion steps. Before fusion, the actin cytoskeleton may provide a scaffold for anchoring secretory vesicles in close proximity to the plasma membrane (**a**), act as a physical barrier (**b**), or provide a track to transport the vesicles to the fusion site (**c**). These models are not mutually exclusive (**d**). A possible scenario envisions that under resting conditions, secretory vesicles may bind to the cortical actin cytoskeleton via multiple molecules (which include SynapsinI, myosin Va, and MARCKS) and be "functionally" entrapped by actin filaments. Upon stimulation, the small GTPase-mediated disassembly and reassembly of the actin cytoskeleton would transport the vesicles into a position suitable for tethering. Localized actin disassembly permits the vesicles to access the plasma membrane and requires the Ca^{++}-mediated activation of actin severing proteins and the activation of GAPs for specific GTPases. Actin reassembly enables the activation of myosin motors and requires the GEF-mediated activation of selected GTPases and their effectors (d). Myosins and the actin cytoskeleton interact with the exocyst, facilitating tethering, and with the munc18/SNARE complex, regulating docking and priming (e)

In the pancreas, photonic force microscopy has revealed that secretory vesicles are tethered to filamentous structures in a cytochalasin-dependent manner [202]. Moreover, insulin granules are anchored to actin filaments in resting state via machinery that involves Rab27a, its effector molecule exophilin8 (also known as MyRip or Slac2-c) and the actin motor myosin Va [203], a molecule that plays multiple roles during exocytosis. Myosin Va forms homodimers, through the N-terminal head domain, which contains the ATP binding site and the motor domain. The neck domain contains six calmodulin repeats whereas the C-terminus globular domain contains the organelle binding site [204–206]. Although myosin Va is a processive motor, recent studies have suggested that in resting conditions, it may work as a "dynamic tether" mediating a slow transport along actin cables [207]. Evidence for this was seen in hippocampal neurons, where the expression of a dominant negative tail domain of myosin Va resulted in the release of dense core vesicles from actin and induced their exocytosis [207].

Finally, actin-disrupting agents have been used in some studies in support of the first model. However, although enhanced exocytosis has been consistently observed with the controlled disruption of the actin cytoskeleton with the controlled disruption of the actin cytoskeleton, some groups also reported an increased mobility of secretory

vesicles [207, 208], whereas others did not [198, 209]. These contradictory findings lead to the idea that the actin cytoskeleton may act as a storage compartment for molecules that regulate exocytosis [198].

According to the second model, actin acts as a physical barrier between the secretory carriers and the plasma membrane (Fig. 3b). Cortical actin is indeed organized as a thick meshwork of filaments that occasionally present some openings that may allow small secretory vesicles to access the plasma membrane [210, 211]. This model has been first proposed for insulin secretion and is based on the observation that cytochalasin D induces exocytosis of insulin-containing granules in resting pancreatic β-cells [212]. In addition, time-lapse microscopy in neuroendocrine cells has shown that stimulation of exocytosis results in the selective disruption of cortical actin with the creation of actin-free areas that allow the vesicles to dock and fuse at the plasma membrane [213]. Recently, in NK cells, structural illumination microscopy revealed the organization of the immunological synapse, which is characterized by an actin ring delimiting the area of granules fusion. During exocytosis, actin de-polymerizes, forming clear areas of 250 nm where lytic granules dock and fuse with the plasma membrane [214].

A few molecules have been implicated in actin de-polymerization during the stimulation of exocytosis and the most extensively characterized is scinderin (or adseverin), a Ca^{++}-dependent actin severing protein [215, 216]. The N-terminus of scinderin binds actin and PIP2 and both interactions are required during agonist stimulation in chromaffin cells [217, 218]. Although scinderin plays a role in other secretory events, such as mucin secretion in airway cells [219] or insulin secretion in β-cells [220], its expression is limited to few tissues [221], suggesting the involvement of other actin-severing proteins. Indeed, gelsolin has been reported to affect GLUT4 exocytosis in adipocytes, possibly through its interaction with the SNARE protein syntaxin4 [222], enhance the release of amylase in pancreatic acinar cells [194], and control both the acrosomal reaction in the sperm [223] and insulin secretion in β-cells [224]. Interestingly, although other actin de-polymerizing proteins, such as cofilin, are activated during stimulation, they do not have any effect on exocytosis, suggesting that actin de-polymerization has to be spatially targeted [225, 226].

Another molecule implicated in remodeling the actin cytoskeleton during exocytosis is protein kinase C (PKC), which affects the cytoskeleton through myristoylated alanine-rich C-kinase substrate (MARCKS), which is an actin crosslinker whose activity is inhibited by phosphorylation [227]. MARCKS seems to be implicated in controlling several secretory systems, from neurons to airway cells [227–235]. However, PKC can also regulate the activity of phospholipase A2 that has been involved in acrosomal exocytosis [236] most likely by controlling actin dynamics, as suggested by others [237]. Lastly, de-polymerization of the actin cytoskeleton during exocytosis can also be achieved through inhibition of the GTPase-mediated actin assembly via specific GTPase-activating proteins (GAPs) or guanine nucleotide exchange factors (GEF). For example, the expression of Nadrin, a GAP for Rho, Rac, and cdc42, enhances exocytosis in PC12 cells [238] whereas the activation of the Rho-GAP GMIP on the azurophilic granules in neutrophils has been shown to progressively disrupt the cortical actin barrier [239].

Actin cytoskeleton as a track to deliver secretory vesicles to the fusion site

Although the evidence described above suggests that actin performs either a barrier or an anchoring function, a series of other studies indicate that actin plays an active role facilitating the transport of the secretory vesicles toward the fusion sites (Fig. 3c). This movement along actin filaments requires actin-based motors such as members of the myosin V family [205, 240]. Myosin Va is associated with several secretory vesicles and among them are dense core granules [240], melanosomes [241], chromaffin granules [242], insulin granules [243], and synaptic vesicles [244]. Interestingly, its motor activity is activated by increased Ca^{++} levels, consistent with its role in exocytosis [245]. In chromaffin granules, myosin Va is recruited by Rab27a and the linker myrip [246]. Similar machinery has been proposed for the parotid salivary glands where myosin Va is recruited via Rab27b [99]. Recently, this complex has been shown to be required for the delivery and exocytosis of the Weibel–Palade bodies in endothelial cells, as well [247].

Another myosin motor, myosin II, has been proposed to play a role in vesicular movement along the cytoskeleton. Inhibition of myosin II motor activity with BDM or blebbistatin led to reduction of vesicular mobility in the cortical area [248]. Moreover, a non-phosphorylatable mutant of myosin IIa reduces granule mobility in chromaffin cells [249]. Although non-muscle myosin II is a non-processive motor, its ability to cross-link actin filaments induces contractions that can sustain the movement of small colloidal beads [250].

The apparent discrepancies between the anchoring/physical barrier and the track model are primarily due to the use of pharmacological agents that have different effects depending on their mode of action and dosage. One important point that has been raised is the extent of the disruption of the actin cytoskeleton. Indeed, its partial disassembly stimulates exocytosis and increases the motility of the secretory vesicles [212]. On the other hand, its complete disruption results in a total block of secretion

and vesicle movement [194]. This suggests that regulated exocytosis requires the coordinated disassembly and assembly of actin (Fig. 3d). Secretory vesicles may bind to the cortical actin cytoskeleton via multiple molecules and be "functionally" entrapped by actin filaments. In the absence of stimulation (i.e., low Ca^{++}), Myosin Va may serve as an anchor rather than a motor interacting with actin either directly or through MARCKS, as recently reported [251]. Upon stimulation, sequential events may take place including PKC-mediated phosphorylation of MARCKS, which inhibits actin-crosslinking, activation of scinderin (or other actin severing proteins), and GAPs for specific GTPases, which de-polymerize actin (Fig. 3d). These steps are rapidly followed by activation of GTPases, which promote actin assembly, and Myosin Va, which pulls the vesicles close to the plasma membrane (Fig. 3d). This hypothesis is supported by observations that actin initially de-polymerizes and successively re-polymerizes during stimulation of chromaffin cells [210]. This sequential regulation is achieved by coordinating the activities of the two GTPases RhoaA and cdc42. Indeed, activation of RhoA leads to an increase in actin polymerization at the plasma membrane, which impairs exocytosis [252], whereas activation of cdc42 leads to the N-Wasp- and Arp2/3-mediated increase of actin polymerization, which stimulates regulated exocytosis [253]. Other GTPases have been implicated in regulated exocytosis. Arf6 activates both PIP5 K and PLD that leads to the generation of PIP2 and phosphatidic acid, respectively [254]. The important role of these lipids and Arf6 in exocytosis was recently demonstrated in neuroendocrine cells [255, 256]. Furthermore, RalA was shown to interact with Arf6-activated PLD1, suggesting a sequential activation of multiple GTPases in the process [257]. Additional evidence for the role of RalA in exocytosis comes from studies done in pancreatic cells and exocytosis of Weibel–Palade bodies in endothelial cells [258, 259]. Finally, Rap1 and EPAC were linked to regulated exocytosis of Weibel–Palade bodies in endothelial cells [260] and insulin secretion [261].

Actin and regulation of docking and priming

Actin and its motors have been shown to interact or to be functionally linked to some of the molecules involved in vesicle docking and priming (Fig. 3e). Downregulation of munc18-1 affects docking and severely impairs the organization of cortical actin [91, 93]. The PIP2- and cdc42-dependent polymerization of actin promotes docking of secretory vesicles [262] in a process that is also dependent on intersectin-1L, an actin binding protein that binds to SNAREs [263]. Furthermore, F-actin has been shown to directly interact with SNAREs. In primary rat β-cells and in MIN6 β-cells, F-actin interacts directly with the first two N-terminal alpha helical coiled-coil domains of syntaxin 4 and the impairment of this interaction enhances glucose-stimulated insulin release [264]. Defective actin remodeling impairs insulin-dependent fusion of GLUT4 vesicles whereas docking is not affected, suggesting a possible involvement for actin in priming [265]. Among the molecules that may link F-actin, SNAREs, and the lipids at the plasma membrane, we have to mention the members of the Annexin family [266]. Annexins affect membrane domain organization and exocytosis by: (1) binding different phospholipids both on the plasma membrane and on vesicles, in a calcium-dependent manner [267], (2) regulating the delivery of cholesterol and other lipids to plasma membrane microdomains (such as lipid rafts) that may facilitate the assembly of the SNARE complex [268–270], and (3) binding to F-actin [271].

Myosin Va has been found to interact with both syntaxin 1a and VAMP2 and this complex is likely to facilitate tethering at the plasma membrane (Fig. 3e) [244, 272]. The expression of the MyoVa tail in enterochromaffin cells reduces the number of docked granules as assessed by TIRF microscopy [246]. Interestingly, it was recently reported that myo2, the yeast homologue of Myosin Va, interacts with the exocyst complex, suggesting a possible mechanism for the role of myosin Va during docking [273]. Whether Myosin Va plays a role as a processive motor or as a tethering factor may also depend on the linker. Indeed, in β cells, the adaptor exophilin8 regulates the delivery of insulin granules to an actin-rich region, whereas the adaptor granulophilin mediates their docking [203]. The role of myosin Va may also extend to priming since an increase in intracellular Ca^{++} releases myosin Va from the SNARE complex, facilitating fusion [272]. Myosin Vc is another myosin motor that has been shown to regulate exocytosis primarily in exocrine glands. Myosin Vc has been shown to regulate exocytosis of secretory granules in lacrimal glands [68, 274] and it is expressed in salivary glands, exocrine pancreas [275], and in mucin-containing granules in airway goblet cells [276]. Although the precise step where myosin Vc operates has not been identified, the fact that it does not behave as a processive motor points to a role in docking [277, 278].

Role of the actin cytoskeleton after fusion

Controlling the fusion pore and the dynamics of the membranes after fusion

Although there is no evidence for the recruitment of the cytoskeleton onto the limiting membranes of small secretory vesicles (<500 nm), it has been known for a few decades that F-actin and myosin II coat large secretory

vesicles in close proximity to the plasma membrane [279]. However, only recently has it been shown that the recruitment occurs after their fusion with the plasma membrane [164, 280–283]. The observed recruitment of the actomyosin complex occurs with a delay of a few seconds from the opening of the fusion pore, indicating that the fusion step does not require the cytoskeleton [164]. In addition, pharmacological impairment of either actin assembly or myosin motor activity does not affect the fusion of the secretory vesicles with the plasma membrane [164, 281], thus raising several other possibilities about the role of the actomyosin complex.

First, it has been proposed that actin regulates the maintenance and closure of the fusion pore, as shown in pancreatic acinar cells, where latrunculin B-mediated actin disruption promotes pore closure [284] (Fig. 4a). In the same system, non-muscle myosin IIa has been localized at the apical plasma membrane and inhibition of its motor activity resulted in closure of the fusion pore [285]. Although myosin II has been localized onto the membranes of large fused secretory vesicles [164, 281], its role in regulating the fusion pore has been mainly characterized in chromaffin cells [249, 286–289]. Inhibitors of myosin light chain kinase [290] and expression of a non-phosphorylatable form of the regulatory light chain of myosin II [249] slows the fusion kinetics of chromaffin granules. Detailed analysis using either amperometry [287, 289] or TIRF microscopy [286] revealed that myosin II contributes to the expansion of the fusion pore. Interestingly, a series of elegant studies have shown that myosin II activity determines whether exocytosis occurs via full collapse or by a "kiss-and-run" mechanism [182, 288, 291]. Indeed, activation of myosin II by phosphorylation results in the complete expansion of the fusion pore leading to the full collapse of dense core vesicles and complete release of catecholamines. On the other hand, impairment of myosin II activity leads to the early closure of the fusion pore, a partial release of the cargo molecules, and the scission of the vesicles from the plasma membrane [288, 291]. At the molecular level, the recruitment of myosin II and other regulators of the pore expansion are controlled by the phosphorylation of dynamin I, revealing a complex coordination between fusion and fission machineries [182].

A second function for the actin cytoskeleton is to provide the force to drive exocytosis to completion via the contractile activity of the actomyosin complex [19, 32, 195] (Fig. 4b). The force generated by the complex can be used to compress the secretory vesicles to facilitate the extrusion of cargo molecules that form large aggregates such as the von-Willebrand factor [281], surfactant [280, 292], or lectin [293–295]. Strikingly, in endothelial cells, actin forms a polarized ring around the fused Weibel–Palade bodies that is perfectly oriented to perform the mechanical squeezing of the cargo molecules [281]. The kinetics of recruitment of both actin and myosin mirror the opening of the fusion pore and the complete emptying of the secretory vesicles. Moreover, disruption of both actin and myosin assembly around the vesicles results in the impairment of cargo release [280, 281, 292]. In other systems in which cargo molecules are more loosely associated, such as salivary glands, pancreas, and lacrimal glands, the contractile activity of the actomyosin complex is required to facilitate the integration of vesicles membranes into the cell surface [164, 248, 296, 297], although with different modalities. In particular, in pancreatic and salivary acini, actin and myosin coat individual granules [164, 297], whereas in lacrimal glands, actin encloses multiple granules [248]. As discussed earlier, in these systems the full collapse of the vesicles is not energetically favorable, and thus the actomyosin complex may be required to provide the necessary energy for the absorption of the membranes. We speculate that the actomyosin complex may act in two different ways by applying: (1) a force tangential to the surface of the vesicles that pull the membranes toward the plasma membrane, and (2) a force parallel to the plasma membrane that expands the fusion pore (Fig. 4b) [296, 298]. In pancreas and lacrimal glands, both forces may be generated by myosin IIa, which is the only isoform of myosin II that is localized onto the secretory vesicles [248, 285]. In salivary glands, in which both myosin IIa an IIb are localized onto the secretory vesicles, it is tempting to speculate that each of the myosins regulates one of the two processes.

The actomyosin complex has also been proposed to stabilize the limiting membranes of the secretory granules during exocytosis in order to prevent changes in their shape or size that may affect either the release of the cargo or their gradual collapse (Fig. 4c). In exocrine pancreas, where compound exocytosis is the main modality for vesicle secretion, impairment in the assembly of the actin cytoskeleton, by using cytochalasin D or agonist overstimulation, increases the rate of compound exocytosis fusion events leading to the formation of large secretory vesicles at the plasma membrane [299]. In lacrimal glands, impairment of F-actin assembly and inhibition of myosin II motor activity resulted in the enhancement of compound fusion revealed by a significant increase in the size of the granules [248]. In explanted salivary glands, cytochalasin D induced the formation of vacuolar structures containing amylase [78], whereas in live animals, the same treatments led to an increase in the size of the secretory vesicles fused with the plasma membrane [164]. A more detailed analysis revealed that this effect is due initially to the hydrostatic pressure generated by the concomitant fluid secretion that occurs in the live animal, suggesting that the actomyosin complex exerts a structural function around

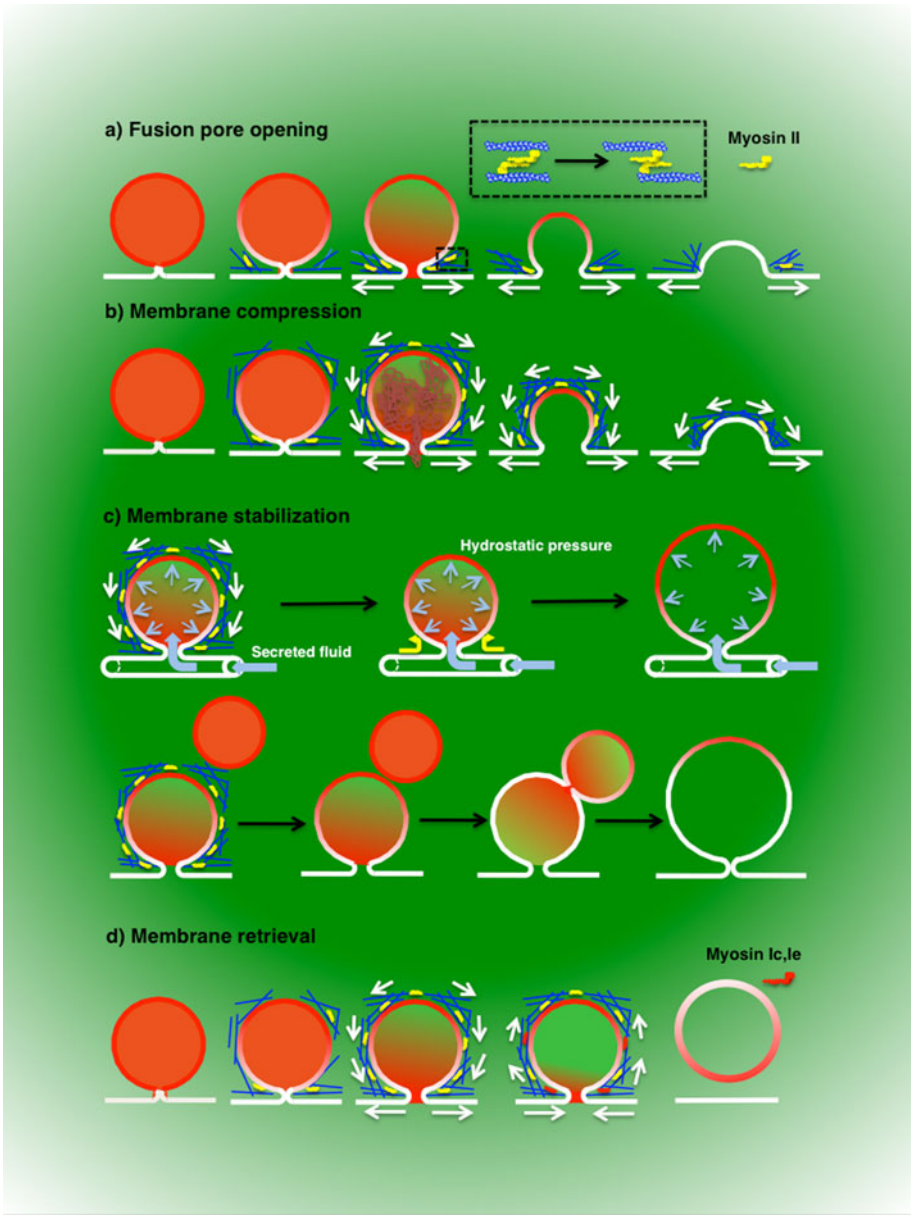

Fig. 4 The actin cytoskeleton in post-fusion steps. The contractile activity of the actomyosin complex is required to expand the fusion pore by exerting a force parallel to the plasma membrane (**a**) and/or to pull the membranes of the secretory vesicles toward the plasma membrane by exerting a force tangential to the surface of the vesicles (**b**). Both processes favor the expulsion of cargo molecules (**a**, **b**). In exocrine glands, the actomyosin complex may also serve as a scaffold to stabilize the secretory vesicles that are fused with the plasma membrane (**c**). This would prevent the uncontrolled expansion of the vesicles that is caused by either an increase in hydrostatic pressures, as shown to occur in exocrine glands in live animals, or fusion with adjacent secretory vesicles (**c**). The main myosin motor used for these functions is the non-muscle myosin II. The actomyosin complex may also control the retrieval of the vesicular membranes in order to maintain plasma membrane homeostasis. This process requires myosin II, Ie, and Ic (**d**)

the granules (Fig. 4c). However, at later stages, the increase in size was due to compound exocytosis. Especially for secretory systems with slow post-fusion kinetics, the diffusion of proteins and lipid from the plasma membrane into the membranes of the granules make them competent to fuse with other granules [300, 301]. Interestingly, although compound exocytosis has been extensively reported in ex vivo salivary glands [302–305], in live animals it is observed only when actin assembly is impaired [164].

The last function that has been proposed for the actomyosin complex is to control the retrieval of the vesicular membranes (Fig. 4d). Indeed, in order to maintain cell homeostasis, the membranes added to the plasma membrane are retrieved by compensatory endocytosis [166, 298] either via clathrin-mediated [165, 166, 306] or clathrin-independent pathways [307–309]. Both exocytosis and compensatory endocytosis are strictly interdependent, as shown by the fact that they share common machineries [181, 310]. In case of full-fusion events, actin would serve to regulate a de novo endocytic process [165, 307] involving specific motors such as myosin VI [204]. In eggs, where cortical granules are not fully absorbed into the plasma membrane, the retrieval process requires de novo actin polymerization around the granular membranes (a process termed Kiss and Coat [19]) and the motor activity of different myosins such as myosin II, myosin 1c, and 1e [294, 295] (Fig. 4d).

The mechanism of actin recruitment onto the secretory granules has not been fully elucidated. In cortical granules, actin assembly is dependent on the actin nucleation factors Arp2/3 and the small GTPase cdc42 [19, 282, 294, 295]. This process requires the synthesis of PIP2 suggesting a role for a PIP5 kinase. Furthermore, a mixing compartment model has been proposed in which the machinery regulating actin nucleation is split between the secretory vesicles and the plasma membrane and only comes into contact after fusion. This model is consistent with the lag time in actin polymerization detected after fusion of the bilayers [164, 281]. In addition, PI4 kinase, an enzyme upstream to the production of PIP2, has been localized onto the secretory granules and is supposedly involved in the activation of RhoA [311]. In pancreatic acini stimulated by CCK, RhoA and Rac1 activation lead to the recruitment of actin on zymogen granules [12, 312–314], whereas inhibition of RhoA by C3 toxin induces the impairment in actin recruitment and deregulation of compound exocytosis [299].

RhoA activation has also been implicated in regulating actin polymerization during exocytosis of lamellar bodies in a process that requires the actin nucleation factor formin, rather than Arp2/3 [280]. Actin recruitment is also temporally correlated with the loss of Rab3d from the zymogen granules, although the link with actin polymerization is not clear [297]. Finally, other pathways may be implicated in regulating actin. In salivary glands for example, Rap1 is activated by beta-adrenergic stimulation [315, 316] via the cAMP/EPAC pathway [153, 317]. Interestingly, Rap1 has been reported to activate the exchange factors for Rac1, which triggers actin polymerization at the plasma membrane [318].

Conclusions and perspectives

The actin cytoskeleton is clearly implicated in every step of regulated exocytosis in vertebrate cells. The large number of regulatory molecules that temporally and spatially control its dynamic nature and the diversity in the architecture and physiology of the various secretory systems have made it challenging to fully pinpoint its precise role. However, based on our current knowledge, it is reasonable to assign at least two main functions to the actin cytoskeleton in regulated exocytosis: (1) the tight control and coordination of the delivery of the secretory vesicles to their fusion sites at the plasma membrane, and (2) the regulation of the vesicle membranes dynamics after fusion.

The first function appears to be prevalent in cells in which the secretory vesicles have relatively small diameters, exhibit fast rates of fusion, and release small molecular weight cargo (Fig. 5). Typically, these morphological characteristics are associated with neuronal, endocrine, neuroendocrine, and hematopoietic cells, which are designed to elicit fast and highly controlled responses in order to carry out their physiological tasks. From a biophysical point of view, the integration of these small vesicles with the plasma membrane is energetically favorable (Fig. 5) and proceeds very rapidly (milliseconds to seconds). Hence, the actin cytoskeleton may provide an additional layer of regulation to prevent the uncontrolled delivery of secretory vesicles to a fusion competent area of the plasma membrane that may result in severe pathological conditions.

The second function seems to be prevalent in cells in which secretory vesicles have large diameters and the post-fusion process is kinetically slow. These characteristics are associated with exocrine systems and some specialized secretory cells, which are designed to generate slower and more sustained responses. Since the integration of large vesicles that fuse with curved membranes, such as canaliculi and ducts, is not energetically favorable (Fig. 5) and proceeds slowly (seconds to minutes), the recruitment of a contractile actomyosin complex on the membrane of the secretory vesicles may provide the energy to facilitate this process. This can be achieved by enlarging the fusion pore and/or pulling the membranes (Fig. 5). The contractile activity may also facilitate the expulsion of very large cargo that would normally require a longer time to be released into the extracellular space, and the retrieval of membranes by compensatory endocytosis in order to maintain cell homeostasis. Moreover, since the large fused vesicles are held at the plasma membrane for long periods of time, the actomyosin complex may also serve as a scaffold to stabilize them and prevent their disruption, which can be caused by either an increase in hydrostatic pressures, as shown to occur in exocrine glands in live animals, or fusion with adjacent secretory vesicles (Fig. 5). Interestingly, one notable exception to this model is the small vesicles in neuroendocrine cells. Although, the actomyosin complex has not been detected onto the membrane of the secretory vesicles, it regulates closure of the fusion pore dictating the fate of the vesicles after fusion (full fusion vs. kiss and run).

In conclusion, continuous efforts have to be devoted toward elucidating the role of the actin cytoskeleton in regulated exocytosis. The direction to follow is clearly the use of a multi-disciplinary approach that will increase our understanding of these processes. Particularly, more sophisticated imaging technologies, such as TIRF, super-resolution microscopy, and intravital microscopy are very promising, enabling simultaneous imaging of single exocytic events and actin cytoskeleton dynamics. This, in combination with the use of more sophisticated molecular

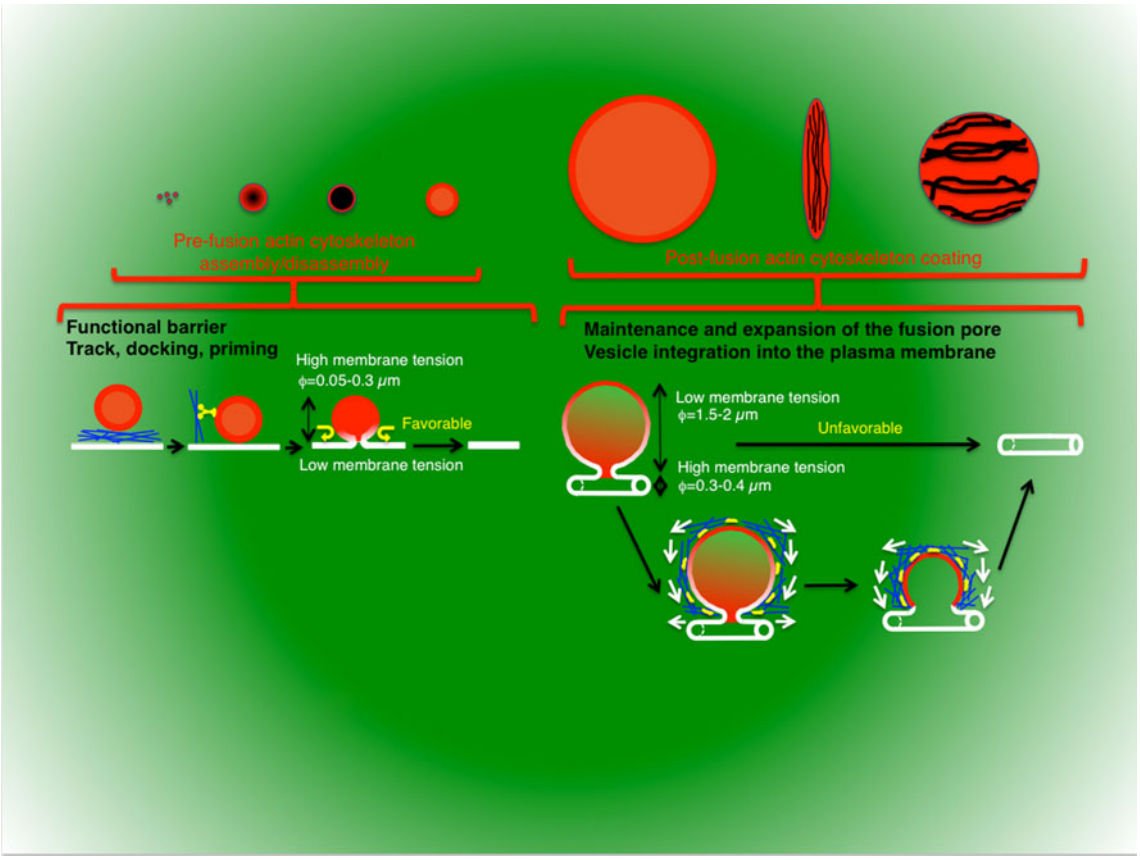

Fig. 5 Role of the actin cytoskeleton and the morphology of the secretory apparatus. The actin cytoskeleton performs two main functions in regulated exocytosis: (1) the tight control and coordination of the delivery of the secretory vesicles to their fusion sites at the plasma membrane, and (2) the regulation of the vesicle membranes dynamics after fusion. The first function is prevalent in cells in which the secretory vesicles have relatively small diameters, exhibit fast rates of fusion, and release small molecular weight cargo (synaptic vesicles, dense core vesicles, insulin, and lytic granules). From a biophysical point of view, the integration of these small vesicles into the plasma membrane is energetically favorable and proceeds very rapidly. In these systems, the actin cytoskeleton may provide an additional layer of regulation to prevent the uncontrolled delivery of secretory vesicles to fusogenic areas of the plasma membrane that may result in severe pathological conditions. The second function is more prevalent in cells in which secretory vesicles have large diameters and the post-fusion process is kinetically slow. Since the integration of large vesicles that fuse with curved membranes, such as canaliculi and ducts, is not energetically favorable and proceeds slowly (seconds to minutes), the recruitment of a contractile actomyosin complex onto the membranes of the secretory vesicles may provide the energy to facilitate this process. The contractile activity may also facilitate the expulsion of very large cargo, the retrieval of membranes by compensatory endocytosis, and to serve as a scaffold to stabilize them and prevent their disruption, which can be caused by either an increase in hydrostatic pressures fusion with adjacent secretory vesicles. For some small vesicles, such as in neuroendocrine cells, the actomyosin complex regulates the fusion pore and the fate of the vesicles after fusion (full fusion vs. kiss and run) without being recruited onto the membrane of the vesicles

tools and the advancement of structural biology, has the potential to provide novel insights into this process and to define novel paradigms.

Acknowledgments This research was supported by the Intramural Research Program of the NIH, National Institute of Dental and Craniofacial Research. We sincerely apologize to those whose work could not be cited due to space limitations.

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