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Heterogeneous modes of insulin granule exocytosis: molecular determinants

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TABLE OF CONTENTS

1. ABSTRACT

Glucose-induced insulin secretion is biphasic: the first phase forms a transient peak of secretion lasting a few minutes after the stimulation, whereas the second maintains a lower but persistent secretion rate. It was suggested that two different exocytic mechanisms operate during the two phases: the first phase exocytosis is derived from docked granules, whereas the second derives from newly recruited granules. However, total internal reflection fluorescence microscopy has revealed that, while the exocytosis in the first few minutes is derived from granules located close to the plasma membrane, it is also significantly effected by deeper granules, whereas that in the second phase is mainly derived from the deeper granules. Consistently, pancreatic beta cells deficient in the Rab27a effector, granuphilin, which is indispensable for the stable attachment (docking) of insulin granules to the plasma membrane, exhibit no delay or reduction of secretagogue-induced insulin secretion, and instead exhibit increased secretion. Future studies should explore the mechanism for time-dependent differences in the exocytic behaviors, namely the molecular determinants of the rate-limiting exocytic steps for docked and undocked granules.

2. INTRODUCTION: HETEROGENEOUS PREFUSION BEHAVIORS

Time-lapse imaging recordings under total internal reflection fluorescence (TIRF) microscopy in living cells permit direct observation of the fusion events of secretory vesicles as well as analysis of the prefusion behavior of fused vesicles. In the case of pancreatic beta cells, secretory granules are not uniformly processed prior to their fusion (1-3). For example, in our study in mouse pancreatic beta cells (3), fused insulin granules are categorized into three classes depending on the prefused behavior: the granules visible before stimulation, *residents*; those that have become visible during the stimulation, *visitors*; and those without stable visualization (in less than 56 msec) before fusion, *passengers*. During the first 1 min of 25 mM glucose stimulation, about half of the fusion is derived from *residents*, whereas another half derives from *visitors* and *passengers*, which are not located close to the plasma membrane prior to the stimulation. Although *residents* and *visitors* stay near the plasma membrane or closely attached to it (docking) for a while before fusion, *passengers* appear to bypass stable docking to the plasma membrane. Therefore, TIRF microscopic recordings at a

very short interval have revealed heterogeneous prefusion steps among fused granules. Moreover, these findings challenge the generally assumed sequential model for regulated exocytosis which proposes that stable docking is a necessary first step before a vesicle can acquire fusion competence (priming) and that a subset of stably docked secretory vesicles constitutes a readily releasable pool (4, 5). In fact, pancreatic beta cells devoid of the Rab27 effector granuphilin exhibit no delay or reduction of insulin secretion compared with control beta cells (3), despite the lack of docked granules established by electron microscopy (6). In this review, I discuss the molecular mechanisms of multiple exocytic steps of insulin granules, with reference to findings from other secretory cells such as neurons and chromaffin cells.

3. FUSION MACHINERY

It is generally believed that any physiological membrane fusion is mediated by the assembly of so-called *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins on donor vesicles (v-SNARE) and on the target membrane (t-SNARE). There are about 35 such SNARE proteins in humans (7), each member of which is thought to function specifically in a distinct traffic route. There is, however, evidence that some members can act redundantly and/or differentially in the same route. In regulated exocytic pathways in neuroendocrine cells, the canonical SNARE members are synaptobrevin2 (also called VAMP2) on the secretory vesicles and syntaxin-1 and SNAP-25 on the plasma membrane. Cultured hippocampal neurons derived from knockout mice of the v-SNARE synaptobrevin2 show a more than 100-fold decrease in fast Ca^{2+}-triggered fusion and a 10-fold decrease in spontaneous fusion (8). By contrast, embryonic chromaffin cells from the same knockout mice exhibit only moderately diminished Ca^{2+}-triggered secretion (9). However, chromaffin cells deficient in both synaptobrevin2 and cellubrevin, another v-SNARE ubiquitously expressed, exhibit almost no evoked secretion, at least in the short period after Ca^{2+}-stimulation, although lack of cellubrevin alone has no profound effect on Ca^{2+}-triggered secretion. These findings indicate that synaptobrevin2 is sufficient to mediate the full range of Ca^{2+}-evoked secretion, although cellubrevin can partially compensate for the lack of synaptobrevin2. Pancreatic beta cells of synaptobrevin2 knockout mice have not been investigated because homozygous mutant mice die immediately after birth. However, pretreatment of permeabilized beta-cell lines with tetanus or botulinum B neurotoxin that selectively cleaves synaptobrevin2 and cellubrevin abolishes Ca^{2+}-induced insulin secretion (10). Thus, synaptobrevin2 likely plays a pivotal role in insulin granule exocytosis, as in the case of chromaffin granule exocytosis.

In studies of t-SNARE proteins, analyses in SNAP-25-deficient mice demonstrate that SNAP-25 is essential for evoked synaptic transmission both at neuromuscular junctions and in cultured hippocampal neurons, although it is not required for stimulus-independent neurotransmitter release (11). Embryonic chromaffin cells from SNAP-25-null mice also show an absence of fast Ca^{2+}-triggered release (12). Again, the phenotype of beta cells from those knockout mice has not been examined because they die at birth. However, pretreatment of the permeabilized beta cell line with botulinum toxin E, which selectively cleaves SNAP-25, inhibits Ca^{2+}-induced insulin secretion by ~60% (13). Knockout mice of another t-SNARE, syntaxin-la, are viable and show apparently normal basic synaptic transmission, although some abnormal behaviors are detected (14). Pancreatic beta cells of the knockout mice exhibit a ~50% decrease of insulin secretion in the first 4 min of glucose stimulation, but no alterations thereafter (15). Electron microscopy reveals loss of granules stably docked to the plasma membrane. TIRF microscopy shows selective abolishment of the exocytosis from granules closely located to the plasma membrane in the first several minutes, although significant exocytosis of undocked granules continuously occurs in this relatively early period. These findings suggest that syntaxin-1a specifically functions on stably docked granules. It is unknown whether other syntaxin isoforms or completely different molecules function in the remaining exocytosis from undocked granules. Pancreatic beta cells of the mice heterozygous for the syntaxin-4 mutation show a 50% decrease in the first 7 min but no significant decrease in the subsequent 28 min during the islet perifusion assay (16). Therefore, syntaxin-4, like syntaxin-1a, functions in a relatively early period after glucose stimulation. Because syntaxin-4-null mice are not viable, it is unknown whether syntaxin-4 is essential for insulin exocytosis in any phase. However, a dominant-negative type of experiment suggests its partial involvement in the exocytosis of stably docked granules, but no involvement in that of undocked granules (15).

4. STABLE DOCKING TO THE PLASMA MEMBRANE

Fusion can be detected in several ways, such as by increased membrane capacitance, release of cargo molecules, or post-synaptic response in neurons. By contrast, docking is morphologically defined by electron microscopy as stable attachment of secretory vesicles to the plasma membrane in fixed cells, although its functional meaning is unknown (4). Although the presence of secretory vesicles near the plasma membrane can be detected in living cells by TIRF microscopy, it has not been established that those visible vesicles are directly attached to the plasma membrane. In contrast to the general involvement of SNARE proteins in fusion reactions, molecular machinery for the docking step has not universally been defined. Because beta cells deficient in the Rab27a effector granuphilin exhibit loss of docked granules in the electron micrograph (6), granuphilin is considered to be essential for the stable attachment of insulin granules to the plasma membrane. Granuphilin shows affinities to both syntaxin-la and one of the Sec1/Munc18 proteins, Munc18-1 (17-20), and is thought to mediate granule docking by linking Rab27a on the granule membrane (21) with the syntaxin-la/Munc18-1 complex on the plasma membrane. If this model is correct, loss of docked granules should also be seen in cells deficient in Rab27a, syntaxin-la, or Munc18-1. Although Rab27a-deficient beta cells do not

exhibit the docking defect (3, 22), which will be discussed in a later section, syntaxin-la-null beta cells do show it (15). Granule docking has not been investigated in Munc18-1-deficient beta cells because the null mice are not viable at birth. However, Munc18-1-null embryonic chromaffin cells show a defect in granule docking (23). Thus, all those granule-docking defects can be ascribed to the failure of a granuphilin-Munc18-1-syntaxin-la complex formation on the plasma membrane.

Despite the docking defect, granuphilin-null beta cells exhibit enhanced insulin secretion (3, 6). Granuphilin is thought to inhibit fusion of docked granules through its interaction with the closed form of syntaxin-la (17). The closed form of syntaxin-la strongly interacts with Munc18-1 but fails to form a core complex with other SNARE proteins to execute a fusion reaction. In addition to this inhibitory effect on the exocytosis of docked granules, granuphilin may indirectly decrease the fusion rate of incoming undocked granules simply by occupying limited fusion sites and/or machinery with granuphilin-mediated docked granules. In contrast to a loss of granuphilin, deficiency of either syntaxin-1a or Munc18-1 causes decreased fusion because those two molecules are involved in post-docking reactions such as the priming and/or fusion steps. Thus, granuphilin is the first and, so far, the sole molecule that has differential effects on docking and fusion steps.

In any intracellular pathway, the donor membrane is first tethered to the target membrane to appose closely enough prior to fusion. This tethering step is generally executed by effector proteins of the small GTPase Rab superfamily (24). In intracellular pathways other than regulated exocytosis, the attachment of the vesicular membrane to the target membrane instantly leads to constitutive fusion, and therefore, vesicles stably docked to the target membrane can barely be discerned in static electron micrographs. By contrast, in regulated exocytosis where secretion occurs only in response to proper stimulation, a subset of secretory vesicles is found to attach stably to the plasma membrane. Although it is generally postulated that such stable docking is a prerequisite for subsequent fusion in regulated secretion, we previously proposed the opposite model based on findings from granuphilin-null beta cells. Namely, although docked vesicles at the plasma membrane seem to be poised for release upon secretagogue sensing, the stable docking uniquely found in regulated exocytosis results from the fusion-inhibitory nature of the docking process (4).

Although regulated exocytosis must have an inhibitory step to prevent spontaneous fusion of vesicles, it is generally thought that such a gating step lies just prior to the final fusion reaction. The best candidate molecule for triggering fusion is Ca^{2+}-sensing synaptotagmin-1. However, apart from its widely accepted role as a Ca^{2+}-sensor for the final fusion reaction, Verhage's group has recently shown that deficiency of synaptotagmin-l causes a docking defect in chromaffin cells (25). Thus, any members of the synaptotagmin family may impose a docking constraint for fusion and relieves this inhibition in response to elevated $[Ca^{2+}]_i$ after secretagogue stimulation. Although it is currently unknown whether granuphilin is involved in the docking of chromaffin granules, synaptotagmins and granuphilin share several properties. First, both proteins associate with secretory vesicles through the N-terminal transmembrane and Rab27-binding domain, respectively. Second, they commonly bear C-terminal tandem C2 domains and have affinities to both t-SNARE proteins and the plasma membrane phospholipids.

Contrary to the original morphological concept, some have proposed that docking involves the pairing of SNARE proteins between two opposing membranes. Indeed, syntaxin-la-null beta cells do show granule-docking defects (15), and chromaffin cells that express botulinum neurotoxin C, which cleaves syntaxin-1, -2, and -3, have been shown to exhibit impaired granule docking (26), although this toxin also cleaves SNAP-25 (27). It has also been shown that chromaffin cells of SNAP-25 knockout mice exhibit a docking defect (25), although another study reports no docking defect in chromaffin cells derived from the same knockout mice (12). However, the docking process is not necessarily correlated with the SNARE protein assembly. The lack of the v-SNARE synaptobrevin2 causes no docking defect in chromaffin cells (9). Instead of synaptbrevin2, synaptotagmin-l or other isoforms may connect granules with syntaxin-l/SNAP-25 accepter complexes on the plasma membrane during the docking process. It is curious that chromaffin cells deficient in either SNAP-25 or synaptotagmin-l exhibit less severe docking defects than those deficient of Munc18-l or syntaxin-l (25). Docking may actually comprise multiple steps and the docking defects described in the literature may simply represent dysfunction of different steps in the process.

5. PRIMING OF DOCKED GRANULES

Priming is even less well understood, in both conceptual and molecular terms. It was originally proposed in the work on permeabilized cells as an Mg-ATP-dependent process(es) to increase the extent of exocytosis by a Ca^{2+} trigger (28, 29). Priming (or maturation) was also described differently in electrophysiological studies as the process by which the pool of readily releasable vesicles is refilled (30). For either concept, there is not much evidence to directly support the assumption that priming is downstream of docking (4, 5). Nevertheless, it has been thought that docked vesicles undergo a priming step after docking to become releasable because only a small population of docked vesicles is able to undergo exocytosis in response to Ca^{2+} elevation. As previously mentioned, however, TIRF microscopic analyses have detected a significant fraction of fusing granules that appear in the evanescent field only within 50~300 msec of the fusion events (1-3). Similar fast fusion events without pausing at the plasma membrane have also been reported in chromaffin cells (31). This type of fusion, which is later referred to as *crash fusion* (5), seems to bypass the stably docked state and subsequent priming reactions.

As stated earlier, granuphilin mediates stable docking of insulin granules and simultaneously interacts

with Munc18-l and the closed form of syntaxin-1a, which cannot form a SNARE complex with SNAP-25 and synaptobrevin2 to execute fusion. If the priming is restrictedly used as the process necessary for stably docked granules to become competent for fusion, it can be viewed as the means by which granuphilin relieves the fusion constraint on the syntaxin-1a/Munc18-l complex, which would eventually proceed to full SNARE assembly. It is possible that a local signal such as increased $[Ca^{2+}]_i$ after stimulation may dissociate the granuphilin-Munc18-1-syntaxin-la complex and allow SNARE assembly. Although some investigators speculate granuphilin mediates nonfunctional, dead-end docking that cannot be primed for fusion (5), the inhibition can be temporal and reversible. Otherwise, futile docked granules would eventually accumulate at and occupy the fusion sites. Furthermore, granuphilin-deficient cells exhibit a modest, but not massive, increase in spontaneous and evoked fusion compared with wild-type cells (3, 6). Future studies should explore the molecular nature of priming in relationship to the docking machinery.

6. RECRUITMENT OF GRANULES AT FUSION SITES

While TIRF microscopy has shown that fusion can occur without the stable docking of secretory vesicle to the plasma membrane, transient attachment to the plasma membrane must still occur prior to any fusion. Granuphilin is not involved in this sort of crash fusion, which has a very short docking period (less than 100 msec), because such fusion actually increases in granuphilin-null beta cells (3). It is uncertain whether the SNARE protein assembly can occur within such a short period to execute the fusion reaction. However, very rapid fusion could also be mediated by SNARE proteins. It has been shown, at least in a cell-free system, that virtually all of the endogenous syntaxin-l and SNAP-25 are highly reactive and readily form SNARE complexes with exogenously added synaptobrevin2 (32).

The precise mechanism by which granules are drawn towards the plasma membrane before fusion is not fully understood. Live cell imaging in neuroendocrine PC12 cells has revealed that immature secretory granules formed at the *trans*-Golgi network are transported in a microtubule-dependent manner within a few seconds to the cell periphery (33). Kinesin-1 localized on secretory granules is thought to act as a motor protein on the microtubules because exogenous expression of its motor domain carrying a mutation at the ATP-binding motif blocks fast and long-ranged granule movements in the pancreatic beta-cell line INS-1 (34). Secretory cells such as chromaffin cells have a continuous ring of actin filaments underneath the plasma membrane and many granules are apparently retained within the cortical actin network at a distance of ~250 nm from the plasma membrane (35). An actin-based motor protein, myosin Va, may play a role in granule binding to F-actin because expression of its C-terminal tail fragment lacking the motor domain inhibits cortical localization of granules and induces their cluster formation in PC12 cells and in the beta-cell line MIN6 (36, 37). A secretagogue-induced Ca^{2+} influx regulates many actin-binding proteins and causes dissolution of the cortical F-actin, which results in granule detachment and gives the granules free access to exocytic sites on the plasma membrane. Secretagogue-induced depolymerization of cortical F-actin has also been observed in pancreatic beta and MIN6 cells (38). However, cortical actin may act not only as a physical barrier but also may play a role in vectorial granule transport. Myosin Va might mediate the recruitment of secretory granules near the plasma membrane. It has been shown that myosin Va interacts with syntaxin-la at a submicromolar (~0.3 microM) Ca^{2+} level, within the range of $[Ca^{2+}]_i$ occurring after secretagogue stimulation, and that inhibition of the complex formation by anti-myosin-Va antibody in chromaffin cells reduces the late sustained phase of exocytosis, in which releasable vesicles could be supplied (39). Therefore, this interaction may mediate recruitment of granules from the cortical actin area to the plasma membrane during the stimulation. However, the cortical actin filaments run parallel to the plasma membrane and it has not been established that myosin Va serves as a point-to-point granule transporter along F-actin tracks. On the contrary, TIRF microscopy has revealed that myosin Va inhibition does not decrease granule transport toward release sites but reduces the time that granules spend near the plasma membrane in the enterochromaffin-cell line BON (40). This suggests that myosin Va plays no role in powering the motion of granules as a motor protein and indicates a role in trapping granules within the cortical actin network as a tethering protein.

It has been shown that the exocytosis of insulin granules in response to a high glucose concentration, but not to a high potassium concentration, is specifically affected in Rab27a-deficient beta cells (22). Subsequent analysis indicates that the main effect is on the fusion of granules located outside an evanescent field prior to stimulation (3). These findings suggest that Rab27a is involved in the recruitment of insulin granules close to the plasma membrane. In contrast to potassium stimulation, glucose stimulation induces ATP production via its metabolism and resultant $[Ca^{2+}]_i$ oscillations. Thus, the impaired secretion specific to glucose stimulation in Rab27a-deficient beta cells might reflect defects in an energy and/or $[Ca^{2+}]_i$ oscillation-dependent recruitment process. Granuphilin-null beta cells do not show the glucose-specific impairment of insulin secretion. Thus, other Rab27 effectors (41), such as melanophilin and MyRIP, which have affinities to myosin Va and VIIa, might mediate granule recruitment, although there is no direct evidence to support such roles. Obviously, the mechanism by which granules are recruited to the plasma membrane is a significant, yet poorly characterized, step in granule exocytosis that merits further investigation.

7. ENDOCYTOSIS AND RECYCLING

The Rab27 subfamily, Rab27a and Rab27b, associate with many kinds of secretory vesicles and regulate their exocytic processes such as recruitment and docking to the plasma membrane (42, 43). Upon the dense

granule secretion in platelets, the GTP-bound Rab27a level drastically decreases due to enhanced GTP hydrolysis activity (44). GTP hydrolysis of Rab27a has also been observed in a pancreatic beta cell line after glucose stimulation (45). Thus, it is likely that secretagogue stimulation can activate the GTPase-activating protein activity for Rab27a and that the GTP-bound Rab27 associated with secretory vesicles is generally converted to the GDP-bound form at some exocytic step. However, the GTP hydrolysis of Rab27a may be unnecessary for the induction of granule secretion because non-hydrolyzable GTP analogue GppNHp-loaded permeabilized platelets exhibit a significant increase in Rab27a-dependent granule secretion upon Ca^{2+} stimulation (44). GTP hydrolysis of Rab27a may be mediated by EPI64, which has a GTPase-activating protein activity toward Rab27a in vitro and induces melanosome aggregation in melanocytes (46).

Recently, coronin 3 has been observed to directly bind the GDP-bound form of Rab27a (45). This interaction seems to be involved in the endocytosis of insulin granules because its disruption inhibits the internalization of the granule-associated protein, phogrin, and the uptake of FM4-64 dye added in the medium. If Rab27a indeed regulates both exocytosis and endocytosis, and thus the recycling of insulin granules, its deficiency should affect the long-lasting glucose-induced insulin secretion more than the depolarization-induced secretion, as observed in Rab27a-deficient beta cells (22). The absence of docking defects in Rab27a-deficient beta cells (3, 22) may also reflect defects in endocytosis, which could inhibit fused granules from detaching from the plasma membrane and thus mask docking defects.

8. BIPHASIC MODES OF INSULIN SECRETION

Pancreatic beta cells show biphasic insulin secretion in response to glucose stimulation. Although the reported period of each secretory phase is inconsistent across studies, the first phase generally corresponds to the first 1~5 min after stimulation, and the second phase starts thereafter. The boundary between the two phases is roughly or ambiguously defined to a resolution of about 1 minute, in contrast to the millisecond resolution employed by electrophysiological measurements using patch-clamp or amperometry techniques. Nevertheless, the findings obtained by electrophysiological studies in beta cells have often been inappropriately extrapolated to secretory events occurring at the 1 minute resolution. According to TIRF microscopic analyses, a single fusion event occurs, on average, every few seconds, even during the most active first phase, although actual fusion events of insulin granules never occur evenly, but rather proceed in a focal and concentrated manner. With this relatively long time scale defined to the resolution of 1 minute, differences in the time course of exocytic events cannot simply be explained by the original location of the granules. In other words, the distance required for granules to move to the fusion site, or the distance for locally elevated Ca^{2+} to diffuse to the granule site, cannot account for the biphasic behaviors. Nevertheless, it is often claimed that transient first-phase secretion is derived exclusively from the exocytosis of previously docked granules, whereas the second sustained phase is attributed to newly recruited granules (newcomers). However, if we carefully look at the data from TIRF microscopic analyses from any laboratories, the exocytosis of granules originally located close to the plasma membrane is largely restricted in the first few minutes, while that of the newcomers is also marked during this early period (1-3). Thus, the secretion during the first few minutes represents the sum of exocytosis from granules located both close to and relatively remote from the plasma membrane.

Glucose induces a peak of Ca^{2+} concentration within the first 1~2 minutes and maintains an elevated plateau or oscillation of Ca^{2+} concentration thereafter. It has recently been proposed that newcomer granules residing far from the Ca^{2+} channels constitute a highly Ca^{2+}-sensitive pool compared with the readily releasable pool that has lower Ca^{2+} sensitivity (47). This model explains why exocytosis from highly Ca^{2+}-sensitive newcomer granules occurs during both the first and the second phases of insulin secretion. Granuphilin is known to mediate stable docking and simultaneously imposes a fusion constraint. Docked granules might require a higher local Ca^{2+} concentration to relieve this inhibition. On the other hand, undocked granules without such a constraint may be able to fuse with a lower Ca^{2+} concentration. With this assumption, exocytosis from both docked and undocked granules occurs in parallel in the first phase, whereas only undocked granules can persistently fuse during the second phase under the lower local Ca^{2+} concentration (4). In the case of artificial high potassium stimulation, in which the increased Ca^{2+} concentration lasts for a relatively short period, monophasic secretion occurs from both docked and undocked granules (2, 3). Glucose augments insulin secretion by the so-called amplifying pathway, which works only in the condition of elevated $[Ca^{2+}]_i$, although via an unknown mechanism other than an increase in $[Ca^{2+}]_i$ (48). Based on the phenotype of Rab27a-deficient beta cells (22), glucose-specific amplification may be mediated by a Rab27a-dependent mechanism.

9. SUMMARY AND PERSPECTIVE

In this review, I make several new proposals about glucose-induced biphasic insulin secretion (Figure 1): 1) the first phase secretion derives from both docked and undocked granules in parallel, whereas the second phase secretion derives from newly recruited undocked granules; 2) the Rab27a effector granuphilin mediates docking of insulin granules and simultaneously imposes a constraint for fusion on them, which should somehow be relieved by secretory stimulation; 3) undocked granules can be fused without significant docking in the presence of a relatively low Ca^{2+} concentration; and 4) during the stimulation, granules are recruited to a fusion-competent area near the plasma membrane in a Rab27a- and energy-dependent manner.

There are many questions requiring further investigation. For example, how can granuphilin-mediated stably docked granules be competent for fusion? Are those

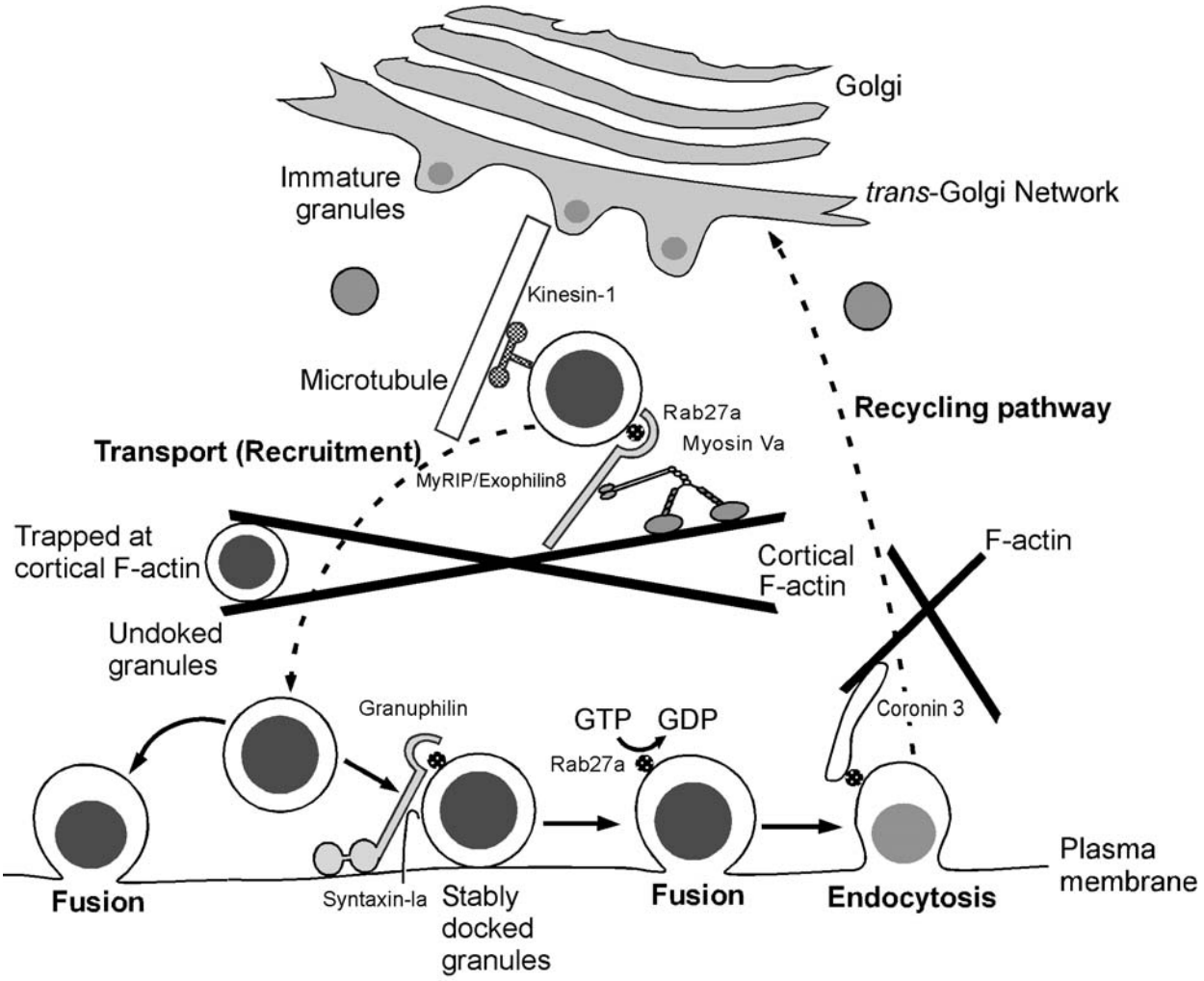

Figure 1. A schematic model of secretory granule recycling. Granules generated at the *trans*-Golgi network are transported along microtubules and transferred at the cortical actin network, which lies at a distance of ~250 nm from the plasma membrane. Although the molecular mechanism for the transfer of secretory granules from microtubules to actin filaments is unknown, the involvement of the Rab27a effector, MyRIP/exophilin8, is tentatively depicted, based on findings in pigment melanosomes. Secretory granules untrapped or trapped within the actin network are somehow recruited close to the plasma membrane upon secretagogue stimulation. Some granules are stably attached (docked) to the plasma membrane by the action of the Rab27a effector, granuphilin, which links Rab27a on the granule membrane with the Munc18-1/syntaxin-la complex and phospholipids on plasma membrane. Both docked and undocked granules can fuse in a relatively early period (within a few minutes) after glucose stimulation. In the later period, undocked granules are continuously recruited via an unknown mechanism and fuse with or without stable docking to the plasma membrane. After exocytosis, the GTP-bound Rab27 is converted to the GDP-bound form by the activity of a Rab27 GTPase-activating protein such as EPI64. Fused granules are then endocytosed and recycled by the action of an actin-binding protein such as coronin 3, although the precise mechanism remains unknown.

granules unable to fuse as nonfunctionally dead-end docked granules? What kind of mechanism recruits granules near the membrane for fusion? Is the docking constraint relieved by a high Ca^{2+} concentration, and does granule recruitment require a lower, but still elevated, Ca^{2+} concentration? If Rab27 effector proteins other than granuphilin function, do both Rab27a effectors act in series or in parallel? Do synaptotagmins function in the docking process of insulin granules? If so, how do granuphilin and synaptotagmins work together in the docking step? Finally, does the rate-limiting step of exocytosis differ between docked and undocked granules? These are just some of many questions that will guide further research in the field.

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