Regulated Exocytosis in the Pancreatic Acinar Cell

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The studies reported here will summarize the major events taking place during the synthesis, intracellular transport and discharge of secretory proteins from the pancreatic acinar cell. We will summarize the work that led to the definition of the regulated secretory pathway in the acinar cell followed by an update of the major steps in the pathway to incorporate new information on vesicular transport that has been gathered over the past 10 years from a number of laboratories. These studies arise from an amazing convergence of information derived from studies on the simpler eukaryote, \(S.\) cerevisiae, from biochemical analysis of neurotransmitter release, and from \textit{in vitro} membrane fusion systems that have allowed for the dissection of the proteins involved in membrane recognition and fusion. Taken together, these studies have shown that the major proteins involved in membrane targeting and fusion, and the accessory proteins that control these events, are highly conserved over vast periods of evolutionary time. Thus, information derived from each of these systems and approaches can be transferred directly to regulated exocytosis in the pancreatic acinar cell — a system that has superimposed on it the complexities of organization into a polarized epithelium and control from the extracellular milieu via neurohormones. The ensuing hypothesis that integrates this body of information is termed the SNARE hypothesis. According to this hypothesis, the core complex of NSF (N-ethylmaleimide sensitive fusion protein) and SNAPs (soluble NSF attachment proteins) pair with their cognate receptors, SNAREs, present on the vesicles (\(v\)-SNARE) and the target membrane (\(t\)-SNARE) to form a complex that can lead to specific docking and fusion of the vesicles with their target membranes. This process is believed to be controlled by a variety of accessory proteins including synaptotagmin, a \(Ca^{2+}\) binding clamp for exocytosis and members of the rab family of low molecular weight GTP-binding proteins. Several of these proteins have been found by us to be present in the pancreatic acinar cell and are likely involved in similar processes that have been worked out in simpler systems. For example, we have shown that rab3D is uniquely associated with the cytosolic side of zymogen granule membranes as an integral membrane protein and that peptides from the effector domain of the rab proteins are able to induce secretion from permeabilized acinar cells, suggesting a role for this process in regulated exocytosis. These types of approaches are being used to define the localization and function of members of the SNARE family of proteins and of proteins that control formation of the SNARE complex with a particular emphasis on their role in hormonally-elicited secretion. In our presentations, we will also discuss the acquisition of stimulus secretion coupling during the perinatal period in the developing rat pancreas since this system provides the possibility of defining, in a system that does not require exogenous transfection, the sequential expression of factors involved in membrane targeting and fusion. For example, during secretogenesis, rab3D is initially cytosolic at a time when the machinery of exocytosis is present but not functional, and only becomes associated with zymogen granule membranes after birth when stimulus-secretion coupling is acquired.

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\(\text{b}^{Abbreviations:} \ NSF, \ N\text{-ethylmaleimide sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, soluble NSF attachment protein receptor; } \text{v-SNARE, vesicle-SNARE; } \text{t-SNARE, target-SNARE; SB, synaptobrevins; TeTx, tetanus toxin; TGN, trans Golgi network; GDI, guanine nucleotide dissociation inhibitor; CCK, cholecystokinin; AP-2, clathrin adaptin protein-2; GAP, GTPase activating protein.\)
INTRODUCTION: REGULATED AND CONSTITUTIVE SECRETION

The pancreatic acinar cell continues to provide a model system for examination of the pathway involved in the synthesis, intracellular transport and storage of secretory proteins in a polarized secretory epithelial cell. It has also proven useful for studies on regulated exocytosis where stimulation by secretagogues such as cholecystokinin or acetylcholine culminates in the massive release of zymogen granule content at the apical plasma membrane [1, 2]. Although the events involved in interaction of secretagogues with their receptors at the basolateral plasma membrane are reasonably well understood, coupling of the stimulus to the fusion of secretory granules with the apical plasmalemma in this non-neuroendocrine cell remains poorly understood. Figure 1 indicates the overall pathway of membrane interactions involved in the intracellular transport and exocytosis of secretory proteins from this typical polarized secretory epithelial cell.

Figure 1. Summary of primary steps in the intracellular transport and exocytosis processing of secretory proteins. 1) Synthesis on polysomes and transfer into the RER; 2) protein modification in the RER; 3) transfer to the Golgi complex; 4) further modification and sorting in the trans Golgi network (TGN) and concentration (4') in condensing vacuoles (CV); 5) storage in the zymogen granules (ZG); 6) exocytosis. RER, rough endoplasmic reticulum; GC, Golgi cisternae; tr, transitional elements of the RER; LY, lysosome; Solid lines, secretory protein transport routes in the pancreatic acinar cell; dashed line, lysosomal protein transport from the Golgi complex to lysosomes. The putative pathway for constitutive-like secretion during maturation of condensing vacuoles is shown.
THE SNARE HYPOTHESIS

In most cells, including simpler systems such as yeast, secretion consists of the “constitutive” pathway where proteins are continually released; the regulated pathway (as occurs in pancreatic acinar cells) requires that proteins destined for release are temporarily stored in granules whose contents are discharged in response to secretagogues [3]. Most evidence indicates that all of the steps of intracellular transport from eukaryotic cells, including exocytosis from the pancreatic acinar cell [4-6], consists of specific vesicle/vacuole targeting and fusion. The results of studies on systems as diverse as S. cerevisiae and the mammalian brain have led to a remarkable convergence of information which indicates that the basic mechanisms of membrane fusion along the secretory pathway share many common features that have been highly conserved during evolution. This has led to the elucidation of the SNARE (Soluble NSF Attachment protein Receptor) hypothesis for membrane fusion [7, 8]. According to this hypothesis, each transport vesicle on the secretory pathway has a specific set of proteins (v-SNAREs) that pairs with its cognate receptor on the target membrane (t-SNAREs), providing the basis for specificity of membrane interaction. Following interaction between the v- and t-SNAREs, the shared fusion proteins, α-, β and γ-SNAP (Soluble NSF Attachment Proteins), combine with the SNAREs which facilitates association with NSF (N-ethylmaleimide Sensitive Factor). Hydrolysis of ATP by NSF is presumed to provide the driving force for membrane fusion.

The best characterized of these SNAREs are the synaptobrevins (v-SNAREs; [9, 10]), SNAP-25 (Synaptosome Associated Protein, Mr = 25; [11, 12]; different from the SNAP25s) and the syntaxins (t-SNAREs; [13, 14]). All these have been shown to be involved in the release of neurotransmitters from the synaptic vesicle in response to increase in intracellular level of calcium. Importantly, most of these proteins or isoforms of them have been identified by us or others as being present in the pancreatic acinar cell, and thus we argue will be found to be involved in each of the steps of intracellular transport and exocytosis.

Synaptobrevins (SB) are integral membrane proteins with a short cytoplasmic C-terminus, a single transmembrane domain, and an N-terminus exposed on the cytoplasmic face. Originally identified in the rat and human brain, they have since been found in transport vesicles from a wide variety of species and tissues [5, 15-18]. Three highly homologous isoforms of SB are known: SB 1 and 2 are 18kDa proteins identified initially in brain. SB 3 or cellubrevin [17, 18] is a more general smaller isoform (14kDa) found in all tissues examined. All SB isoforms are exquisitely sensitive to proteolysis by the clostridial neurotoxins botulinum D, F [19, 20] or to tetanus toxin (TeTx). The clostridial neurotoxins, which are potent inhibitors of neurotransmitter release, cleave these proteins at single sites near the centrally conserved region. The pathological effects of these toxins are mediated by cleavage of the SBs, thereby impairing fusion of synaptic vesicles with the plasma membrane.

SB isoforms have been identified in the pancreatic acinar cell [21]. Acinar cell SB 2 is associated with a zymogen granule membrane fraction, and it has been proposed that it may be involved in exocytosis since TeTx partly inhibits [22] Ca<sup>2+</sup>-induced release of amylase from Streptolysin O-permeabilized acinar cells. Our light and electron microscopic immunocytochemical studies [23] show that SB 3 (cellubrevin) is localized in acinar cells primarily to the trans Golgi network (TGN) including condensing vacuoles with a decreasing gradient of labeling of apical zymogen granules. From its association with the TGN and condensing vacuoles, we suggest that cellubrevin may be involved in the maturation of secretory granules at the TGN including the process of constitutive-like secretion (see Figure 1) where excess membrane is removed from the forming secretory granule and moved to the cell surface [24]. The function of other members of the SNARE family of proteins that we and other have detected in the acinar cell remains to be elucidated.
REGULATORY PROTEINS IN EXOCYTOSIS

Other proteins, including synaptotagmin [25-27] and rabphilin [28, 29], a protein that binds rab3, may play important roles in the release of neurotransmitter from synaptic vesicles and other regulated secretory cells. Synaptotagmin has two C2 domains similar to those found in protein kinase C, and binds Ca²⁺. Synaptotagmin is believed to be a negative regulator of exocytosis, acting as a clamp preventing the fusion of even properly docked vesicles in absence of relevant stimuli [25, 30] ensuring that secretion does not occur spontaneously when the two SNARE partners interact. Functional support for its role in exocytosis has come from microinjection studies, which show that antibodies against synaptotagmin or peptides encompassing its C2 domain (found also in protein kinase C and rabphilin-3A, a target protein for rab3A), inhibit secretion from PC12 cells or the giant squid axon [25, 31, 32]. These proteins may delineate the pathway of constitutive secretion from that of regulated secretion in that in the absence of the appropriate stimulation, synaptotagmin or rabphilin complexes may block interaction of the SNARE complex with SNAPs [25, 33] and prevent membrane fusion. Upon secretagogue stimulation, Ca²⁺ binding to synaptotagmin (among other factors) relieves the inhibition and allows SNAP/NSF interaction to lead to fusion. For constitutive secretion, fusion occurs spontaneously, and it is assumed that synaptotagmin or a functional equivalent is not involved.

LOW Mr GTP-BINDING PROTEINS AND EXOCYTOSIS

Finally, it is clear from a large body of information, that members of the rab family of low Mr GTP-binding proteins play important roles in vesicular transport and exocytosis [5, 34-36]. Initially recognized as essential for constitutive secretion from yeast, low Mr GTP-binding proteins have now been found to be associated with the majority of the exocytic and endocytic fusion events in eucaryotic cells. While low Mr GTP-proteins were initially proposed to determine docking specificity of transport vesicles, recent evidence suggests that they primarily function as molecular switches affecting the rate of membrane interaction and/or fusion, inhibitors of interaction between SNAREs and SNAPs [33], or facilitators of formation of SNAP/SNARE complexes [37]. In regard to regulated exocytosis, the function of rab3A is best understood from studies on synaptic vesicle release. In this system, rab3A in its GTP-bound form is initially associated with synaptic vesicles [37]. Upon completion of neurotransmitter exocytosis and hydrolysis of GTP by a GTPase (GAP), rab3A is believed to be removed from the transport vesicle/fusion complex by interaction with GDI (Guanine nucleotide Dissociation Inhibitor) such that the protein enters the cytoplasm for further rounds of exocytosis [38, 39]. Activity of all rab proteins is under the regulation of several cytosolic proteins that determine the state of binding of GTP [40-42].

In non-neuronal cells, it has been determined that rab3 homologues are involved in regulation of exocytosis. For example, our laboratory was among the first to show that the effector region of rab3, when introduced into Streptolysin O-permeabilized pancreatic cells [43, 44], is able to stimulate secretion, a finding now confirmed in several regulated secretory cell types [45-49]. The results of the peptide studies are, however, still open to interpretation [46]. Our immunocytochemical studies on the acinar cell have further shown that a rab3-like protein, now tentatively identified as rab16 [50], is associated with the zymogen granule membrane in resting cells but following initiation of massive zymogen granule discharge by secretagogues, it rapidly becomes associated with the TGN without an intervening phase of interaction with the apical plasmalemma or appearance in a cytosolic form [51]. More recently, we have shown using a monospecific antibody against rab3D, that this small GTP-binding protein localizes exclusively with zymogen
granules in resting acinar cells [50] and does not relocate to the Golgi during carbamylcholine stimulation (unpublished observations). (Rab3D has been implicated in exocytotic fusion of GLUT4 transporters in adipocytes [52] where it was initially discovered). Interestingly, rab3D and the putative rab16 proteins co-localize to zymogen granules. We suggest that each rab protein may play a separate role during exocytosis, one possibly being involved in the forward steps of exocytic membrane fusion and the other in retrograde membrane retrieval that accompanies internalization of the massive amounts of zymogen granule membrane that are temporarily contributed to the apical plasmalemma [53].

MEMBRANE RETRIEVAL FOLLOWING REGULATED EXOCYTOSIS

The culmination of regulated exocytosis is the insertion of massive amounts of intracellular membrane into the plasmalemma, which must be compensated for to maintain membrane homeostasis. In several systems, ranging from nerve terminals to cortical granule exocytosis from sea urchin oocytes, it has been observed that coated pits are assembled and likely function in the retrieval of exocytosed membrane [54-56]. The pancreatic acinar cell also is faced with a major problem of membrane retrieval during regulated exocytosis since we estimate that complete discharge of zymogen granules leads to ~30 fold expansion of the apical plasmalemma where granule discharge exclusively occurs [51, 53]. We previously reported that secretagogue-induced exocytosis is associated at early times of stimulation with a 2-4 fold expansion of the apical plasmalemma, but, thereafter, compensatory membrane retrieval keeps pace with continued secretory granule exocytosis, and this is correlated with an ~2-fold expansion of the surface area of the Golgi complex. Expansion of the Golgi complex is also associated with the premature concentration of secretory proteins in the TGN that results in the formation of small secretory granules. We have observed a large increase in the number of clathrin coated vesicles under the apical plasmalemma [53] and in the TGN during zymogen granule exocytosis. A number of electron microscope tracer studies has demonstrated the potential for continuity between the apical plasmalemma and elements of the Golgi complex during regulated exocytosis from glandular cells [57-59]. The properties of the retrieved membrane and its route back to the Golgi have not been completely defined but retrieved membrane may even be reutilized in formation of secretory granules. The factors controlling membrane retrieval and trafficking to the Golgi are also not known in detail.

Recently, an important study has presented evidence that helps clarify the coupling between exocytosis and coated vesicle-mediated membrane retrieval. In this study, it was shown that synaptotagmin I serves as a high affinity receptor for the clathrin AP-2 adaptor that initiates formation of plasmalemmal coated pits [60]. This has led to a model according to which synaptotagmin in the resting state inhibits membrane fusion by interacting with the SNARE complex, but upon initiation of regulated secretion, synaptotagmin dissociates from the SNARE complex, possibly upon binding Ca\(^{2+}\), allowing fusion to occur (Figures 2 and 3). At the same time, synaptotagmin is relocated to the plasma membrane where its binding sites for AP-2 are accessible. It now is able to initiate clathrin assembly into coated pits. In this way, exocytosis may be coupled to membrane retrieval. Subsequent to formation of coated pits and vesicles, other proteins may be involved in regulation of endocytosis/recycling to the Golgi complex. It is unlikely that SB 2 and cellubrevin are involved since fusion of endosomes [17] and a portion of receptor recycling [61] is insensitive to neurotoxins. The possible roles of rab4 and rab5, low Mr GTP-binding proteins implicated in early and late endosome function [62-64], remains to be explored. In any case, the acinar cell appears to possess the molecular machinery that couples exocytosis to endocytic membrane retrieval and we intend to examine this possibility in our proposal.
DEVELOPMENT OF REGULATED EXOCYTOSIS

Previous studies from our laboratory have shown that the pancreatic acinar cell undergoes a dramatic change during the perinatal period, whereby the cytodifferentiated prenatal pancreas, which is only capable of constitutive secretion, is converted into a regulated secretory system during the first 24-48 hr after birth. Specifically, morphogenesis, cytodifferentiation and biochemical maturation of the fetal rat pancreas [65-69] is completed by day 21 of gestation (gestation is 22 to 23 days) while structurally differentiated cells are first identifiable at ~day 17. At day 21, we [70, 71] and others [72, 73] have shown that lobules prepared from day 21 fetal pancreases do not release secretory proteins in response to CCK or carbachol despite the presence of hormone binding sites on the plasmaemalma. Stimulation of adult pancreatic acini by CCK results in a rise in intracellular Ca^{2+}, and 45Ca^{2+}-loaded day 21 fetal pancreatic lobules also show an elevation of intracellular Ca^{2+} in response to CCK [71]. This is consistent with the presence of CCK receptors on the day 21 fetal acinar cell surface [71, 74].
Figure 3. Final stages in regulated exocytosis including initiation of membrane internalization following exocytosis.

We have recently reexamined secretogenesis in the fetal rat acinar cell and have shown that one or more low Mr GTP-binding proteins of ~25 kD are missing or at low levels on zymogen granule membranes from day 21 glands. During the first day after birth and progressing through 6 days of postnatal life, these GTP-binding proteins increase to adult levels. Cell fractionation indicates that one of these low Mr GTP-binding proteins, rab3D, a specific marker for zymogen granules in adult rat acinar cells, is almost completely cytosolic in day 21 glands but rapidly becomes granule membrane-associated immediately after birth [75], suggesting that acquisition of stimulus-secretion coupling is closely tied to membrane association of the rab3D protein. Although we do not know the function of rab3D in the acinar cell, the results of these studies on the developing pancreas strongly implicate rab3D in the membrane recognition or fusion events that accompany regulated exocytosis. Studies of the developmental appearance of other proteins of the SNARE complex and modulators of this complex will, we hope, shed light on mammalian regulated exocytosis.
CONCLUSION

In summary, the acinar cell continues to provide a useful system for examining mechanisms of regulated exocytosis and coupled endocytosis. First, we now have evidence that the major players in SNARE-mediated exocytosis are present in the acinar cell and that some may be novel isoforms that are uniquely expressed in non-neuroendocrine cells. Second, the membrane fusion events are relatively slow and exaggerated compared to those in nerve terminals, which may allow us to explore the kinetics of exocytosis and coupled endocytosis more easily. Third, key steps in the secretory pathways can be studied using various probes introduced into live, permeabilized acinar cells, allowing correlations of biochemical events with those at the subcellular level.

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