Rab3A Effector Domain Peptides Induce Insulin Exocytosis via a Specific Interaction with a Cytosolic Protein Doublet*

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A key protein involved in the regulated exocytotic mechanism in neuroendocrine cells is the GTP-binding protein, Rab3A. Rab3A is thought to mediate exocytosis by an interaction of its effector domain with a putative effector protein. We demonstrate here that Rab3A effector domain peptides specifically stimulated insulin exocytosis in electroporated insulin-secreting cells (K+ activation, 6-8 μM) in a Ca2+-independent manner, although in the presence of Ca2+ insulin exocytosis was further potentiated. By using a 125I-radiolabeled photoactivated cross-linking Rab3A effector domain peptide, we identified a cytosolic protein doublet (REEP-1 and REEP-2) which specifically interacted with the Rab3A effector domain. Competitive inhibition studies revealed this protein-protein interaction to be at a concentration of 6-8 μM. Exocytosis was further potentiated. By using a %radiolabeled photoactivated cross-linking Rab3A effector domain peptide, we identified a cytosolic protein doublet (REEP-1 and REEP-2), which specifically interacted with the Rab3A effector domain. Competitive inhibition studies revealed this protein-protein interaction to be at a concentration equivalent to that required for Rab3A effector domain peptides to trigger insulin exocytosis (K+, 6-8 μM). Furthermore, under basal secretory conditions (REEP-1 and -2 were membrane-associated, but upon stimulation of exocytosis they were released into a cytosolic fraction. Our results suggest that REEP-1 and -2 are part of the regulated exocytotic machinery, and their dissociation upon stimulation of hormone release (likely from a protein complex) may be essential to the mechanism that triggers regulated exocytosis in pancreatic β-cells.

Insulin release from pancreatic β-cells is regulated by a variety of nutrients, pharmacological, hormonal, and neuronal signals, the most physiologically relevant of which is glucose (1). The signaling mechanism by which a rise in extracellular glucose induces insulin exocytosis is complex but involves accelerated glucose metabolism that then instigates changes in ionic fluxes across the β-cell plasma membrane, leading to a rapid rise in cytosolic Ca2+ (see Refs. 1–3 for reviews). A rise in [Ca2+]i has long been thought to be the intracellular trigger to induce insulin exocytosis. However, the actual molecular mechanism whereby [Ca2+]i promotes association and then fusion of an insulin secretory granule membrane with the β-cell's plasma membrane for insulin exocytosis is poorly understood. While Ca2+ can evoke insulin exocytosis in semi-permeabilized cells (4), it has been suggested that signaling factors other than Ca2+[Ca2+], are necessary to mediate glucose-stimulated insulin release (5). Among these additional factors, protein kinases (3), GTP, and low molecular weight GTP-binding proteins (4, 8–8) have been implicated, but the molecular mechanism as to how these factors promote insulin exocytosis is not known.

Guanine nucleotide-binding proteins of the Ras superfamily function as molecular switches for a wide variety of cellular functions (9). A sub-branch of the Ras family, the Rab class of GTP-binding proteins, is implicated in directing vesicular transport in eukaryotic cells (10). One member, Rab3A, has been specifically implicated in controlling mammalian regulated exocytosis. It has significant homology to the yeast Sec4 protein, which is required for vesicular transport from the trans-Golgi network to the plasma membrane (10). Rab3A is specifically expressed in neuroendocrine cells (11) and is a cytosolic protein of 25 kDa that is mostly located on the cytoplasmic face of secretory granules (12) or synaptic vesicles (13). Peptides that mimic the effector domain of Rab3A induce regulated exocytosis in cells (14–17) including pancreatic β-cells (8). This has led to the proposal that Rab3A mediates neuroendocrine exocytosis via an interaction of its effector domain with a putative effector protein (15, 16), but such a "Rab3A effector protein" has not yet been identified. However, in this study we have used a synthetic photoactivatable cross-linking Rab3A effector domain peptide to identify a protein doublet that specifically associates with the Rab3A effector domain. This protein-protein interaction could play a role in the regulation of insulin exocytosis.

EXPERIMENTAL PROCEDURES

Materials—Na125[I]I was from Amersham Corp. t-Butyoxycarbonyl benzyl-protected amino acids for peptide synthesis were from Applied Biosystems, except t-butyloxycarbonyl benzylphenylalanine (Bpa),† which was purchased from Bachem Inc. NycodenzTM was from Nycomed Pharma (Oslo, Norway). Unless otherwise indicated, all other chemicals were purchased from Sigma or Fisher and were of the highest grade/purity available.

Peptide Synthesis—Synthetic peptides that mimicked the effector domain of Rab proteins (16) were synthesized as described previously (18). The Rab3A effector domain peptide sequence mimicked the wild type effector domain of Rab3A (13), protein (19), VSTGVDFKVKTYRNYR, and the Rab3AL effector domain peptide sequence was VSLALID-FKVKTYRNYR. The photoactivatable cross-linking Rab3AL-X effector domain peptide sequence was VSLALID-Bpa-KVKTYRNYR, where the phenylalanine in the Rab3AL peptide has been replaced with Bpa. Rab2 peptide (DTITGVEGARMITID), Rab4 peptide (NHTIGVEFSGK) and Rab5 peptide (EPTIGAVFLQTVVLD) sequences mimicked the effector domains of Rab2, Rab4, and Rab5, respectively (16). A

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§ This abbreviation was used: Bpa, benzylphenylalanine; REEP, Rab3A exocytotic effector protein; HP/LC, high pressure liquid chromatography; BSA, bovine serum albumin; GTP-7, guanosine 3'-5'-O(thio)triphosphate; GTPase-accelerating protein; GRF, guanine nucleotide-releasing factor; EC50, half-effective concentration.
hexadecapeptide of the sequence DTVGEPDTDPKFPQQWY that had little similarity to the Rab3A effector domain, named “nonsense,” was used as an additional control peptide. All synthetic peptides were HPLC-purified as described previously (18), and amino acid analyses were as predicted.

Iodination—The Rab3A-L-X peptide was iodinated on Tyr34 and Tyr37 by HPLC as described previously (18). A post-purification specific radioactivity of 1.8-2.0 mCi/μg of peptide was obtained.

Electroporation and Incubation of Semi-permeabilized HITT15 Cells—A 7:25 flask of confluent insulin producing HIT-T15 cells was harvested from the flask with trypsin, washed in phosphate-buffered saline, and then washed twice in a buffer consisting of 370 mM mannitol, 10 mM KCl, 10 mM glutamate, 0.4 mM EGTA, and 5 mM Hepes (pH 7.0). The cell suspension was divided into two equal portions and then washed in the same buffer consisting of either 10 mM EGTA (−Ca2+ buffer) or 1.5 mM CaCl2 + 2 mM EDTA (+Ca2+ buffer) to give an approximate free Ca2+ of between 10 and 50 μM that would maximally induce insulin exocytosis (4). The two HIT cell suspensions in 0.8 ml of the +Ca2+ or −Ca2+ buffer were then electroporated (using a Bio-Rad electroporation apparatus) with 3 x 46-ms pulses at 250 V/500 μF forora. The electroporated cells were then washed in an incubation buffer consisting of 50 mM KHCO3, 20 mM KHPO4, 1 mM MgCl2, and 10 mM Hepes (pH 7.4), 0.05% (w/v) BSA, 4 mM KH2PO4, 1 mM MgCl2, and an ATP-regenerating system consisting of creatine phosphate (5 mM), MgATP (4 mM), and creatine kinase (50 μg/ml) (unless otherwise stated), with either 10 mM EGTA (for −Ca2+ buffer) or 1.5 mM CaCl2 + 2 mM EDTA (for +Ca2+ buffer) present. HIT cells in +Ca2+ or −Ca2+ buffers were divided into 10 samples of ice. Each sample was washed and then resuspended in 200 μl of the same +Ca2+ or −Ca2+ incubation buffer containing either no addition, or GTP·S (50 μM), or Rab effector domain peptide (1-100 μM). The cells were then incubated on ice for 10 min, followed by 15 min at 37 °C. The incubation was stopped by placing the cells on ice-water. The cells were pelleted by centrifugation (1000 x g, 5 min, 4 °C), where the supernatant was retained for assessment of insulin release and the pellet for assessment of insulin content by radioimmunoassay (20). The semi-permeabilization of HITT15 cells by electroporation was assayed by significant 125I-Rab3A-L-X peptide and trypan blue uptake (4) compared with non-electroporated cells. In addition, significant radioactive insulin exocytosis was detected from electroporated HIT-T15 cells during a 15-min incubation at 37 °C, suggesting that there is minimal leakage of HIT cell cytosolic proteins.

Photoactivated Cross-linking of Bpa-containing Peptide—A 5-μg total protein sample of a rat transplacental insulinoma tissue lysate was suspended in "cross-link buffer" consisting of 50 mM Tris/HCl (pH 7.4), 0.1% (v/v) Triton X-100, 0.25% (v/v) Tween 20 buffer, protease inhibitors of 1 mM phenylmethylsulfonyl fluoride, 0.1 mM tosylphenylalanyl chloromethyl ketone, 20 mM E64 (trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane), 20 μM pepstatin A, 1 mM N-ethylmaleimide, 1 mM leupeptin, 1 μM leupeptin, and 1 mM EDTA. 131I-Rab3A-L-X peptide (2 x 106 cpm) was added, and the sample was subjected to photoactivated cross-linking by incubating at 4 °C directly in front of an ultraviolet lamp (300-nm emission wavelength) for 10 min (21). 10 μl of 0.05% (w/v) BSA was added, followed by 500 μl of ice-cold 10% (w/v) trichloroacetic acid, mixed, and incubated on ice for 10 min. The precipitated protein was pelleted by microcentrifuge centrifugation (20,000 x g, 5 min) and washed in 1 ml of 5% (w/v) trichloroacetic acid, followed by a 1-mL ether wash. The pellet was air-dried and resuspended in Laemml sample buffer. Samples were then analyzed by SDS-15% polyacrylamide gel electrophoresis, autoradiography, and densitometric scanning (18).

The insulinoma tissue (22) propagated in NEDH (New England Deaconess Hospital strain) rats was used as an abundant source of pancreatic β-cell tissue. Rat insulinoma subcellular fractions, enriched in plasma membrane, insulin secretory granules, and cytosol, were prepared by differential and Nycodenz density gradient centrifugation and characterized by marker enzyme analysis as described previously (23, 24).

**Rat Pancreatic Islet Incubation and Fractionation**—Rat pancreatic islets were isolated as described (25). Samples of 150 islets were pre-incubated at 37 °C for 50 min in 200 μl of Krebs-Ringer Heps buffer pH 7.4 containing 0.5% (w/v) BSA and a basal 2.8 mM glucose as previously outlined (25). The islets were washed once in the same buffer and then incubated for a further 30 min at 37 °C in the same buffer containing either basal 2.8 mM glucose or a stimulating mixture of 16.7 mM glucose, 10 μM forskolin, and 30 mM KCl that was intended to achieve a maximal rate of insulin exocytosis (1). Islets were pelleted by centrifugation (2 min at 800 x g), and the media were removed for radioimmunoassay of insulin release (20). Islets were resuspended in 1 ml of ice-cold 10 mM ammonium bicarbonate (pH 9.0), disrupted with 3-4 strokes of a Potter homogenizer at 400 rpm, and incubated on ice for 30 min. The osmotically lysed islet homogenate was centrifuged at 1500 x g for 5 min to remove unlysed cells, and the supernatant was removed and then centrifuged at 200,000 x g for 30 min at 4 °C (Beckman SW 55 rotor). The supernatant was removed as a soluble “cytosolic” fraction from the “membrane” pellet, lyophilized, and then resuspended in 50 μl of cross-link buffer described above. The membrane pellet was directly resuspended in 50 μl of cross-link buffer. Proteins that associate with the Rab3A effector domain peptides were detected in these islet subcellular fractions by photoactivated cross-linking of an excess of 131I-Rab3A-L-X (2 x 106 cpm) as described above.

**Rab3A Detection**—Analysis of Rab3A present in rat pancreatic islet fractions was by immunoprecipitation with specific Rab3A mAbs (non-immune mAbs was used as a control), followed by GTP·S binding to the immunoprecipitate as described previously (26).

**Other Procedures**—Protein was determined by using the BCA (bicinchoninic acid) method (Pierce) with BSA as standard. Free Ca2+ concentration was estimated from reference stability constants as described (27).

**RESULTS AND DISCUSSION**

Insulin exocytosis is an ATP-dependent process (4). Without ATP only a low basal rate of insulin release was apparent from semi-permeabilized HIT-T15 cells (Fig. 1A). On addition of ATP, significant Ca2+-stimulated insulin release was instigated and was potentiated in the presence of GTP·S (Fig. 1A), as previously observed (4). Rab2 directly vesicular transport from the endoplasmic reticulum to the cis-Golgi apparatus (10), rather than regulated exocytosis, and a Rab2 effector domain peptide

![Fig. 1. Rab3A effector domain peptides specifically stimulate insulin release from electroporated semi-permeabilized HIT-T15 cells. Panel A, effect of Ca2+, GTP·S, and synthetic Rab effector domain peptides on insulin release from electroporated HIT-T15 cells in the presence of absence of Ca2+. All incubations, except for the control incubation, were carried out in the presence of an ATP-regenerating system. Where indicated, 50 μM Rab effector domain peptide or 50 μM GTP·S was used. Results are presented as a mean ± S.E. of five individual experiments. Panel B, concentration dependence of Rab3A effector domain peptide-stimulated insulin release from electroporated HIT-T15 cells in the presence or absence of Ca2+. Results are presented as a mean ± S.E. of three individual experiments. Panel C, concentration dependence of Rab3A effector domain peptide-stimulated insulin release from electroporated HIT-T15 cells in the presence or absence of Ca2+. Results are presented as a mean ± S.E. of four individual experiments.](image-url)
had no additional effect of Ca\textsuperscript{2+} on insulin release (Fig. 1A). In addition, the nonsense hexadecapeptide control also had no effect on insulin release (Fig. 1A). However, Rab3A effector domain peptides, Rab3A and Rab3AL peptides, markedly stimulated insulin release in a Ca\textsuperscript{2+}-independent manner that was further potentiated in the presence of Ca\textsuperscript{2+} (Fig. 1A). Synthetic Rab3A and Rab3AL peptide induction of insulin release was dose-dependent (Fig. 1, B and C). The EC\texttextsubscript{50} were calculated as follows (mean ± S.E. (n ≥ 3)): Rab3A Ca\textsuperscript{2+}-independent, EC\texttextsubscript{50} = 7.9 ± 0.9 μM; Rab3A Ca\textsuperscript{2+}-dependent, EC\texttextsubscript{50} = 7.2 ± 0.7 μM; Rab3AL Ca\textsuperscript{2+}-independent, EC\texttextsubscript{50} = 6.7 ± 0.8 μM; and Rab3AL Ca\textsuperscript{2+}-dependent, EC\texttextsubscript{50} = 5.9 ± 0.7 μM. Ca\textsuperscript{2+} potentiated insulin exocytosis irrespective of Rab3A or Rab3AL concentration (Fig. 1, B and C). Specific Ca\textsuperscript{2+}-independent Rab3A and Rab3AL peptide-induced exocytosis has been previously observed (8, 14). The Ca\textsuperscript{2+}-independent manner of insulin release induced by Rab3A effector domain peptide suggested that a Ca\textsuperscript{2+}-dependent step in insulin exocytosis was before a Rab3A-dependent step. This supports the notion that intracellular signals other than a rise in cytosolic [Ca\textsuperscript{2+}]\textsubscript{i} are necessary to trigger insulin exocytosis from β-cells (5).

An analog of the Rab3A effector domain peptide (Rab3AL-X) was synthesized with the photoactivatable amino acid analog, Bpa, substituted for phenylalanine at position −8. The Rab3AL-X peptide induced insulin exocytosis (Fig. 1A), indicating that the Bpa for phenylalanine substitution did not adversely affect its biological activity. When exposed to ultraviolet radiation, Bpa-containing peptides cross-link covalently to the specific proteins with which they interact (21). The 125I-Rab3AL peptide was used as a probe to identify specific β-cell proteins that interact with the Rab3A effector domain. In a rat insulinoma homogenate, two proteins were found to specifically cross-link to 125I-Rab3AL-X that we have tentatively named REEP-1 and REEP-2 (Fig. 2A, lane A). The electrophoretic mobility of REEP-1 and -2 suggested apparent molecular masses of 20 and 17 kDa. However, these proteins are covalently cross-linked to the 2.8-kDa 125I-Rab3AL-X so that representative molecular masses of REEP-1 and -2 were more likely 17 and 14 kDa, respectively. In the presence of 30 μM control peptides, nonsense, Rab2, Rab4, and Rab5, no competitive inhibition of 125I-Rab3AL-X cross-linking to REEP-1 and -2 was observed (Fig. 2A, lanes B, C, F, and G, respectively). In contrast, in the presence of 30 μM Rab3A or Rab3AL peptides, cross-linking of 125I-Rab3AL-X to REEP-1 and -2 was inhibited (Fig. 2A, lanes C and D). The Rab effector domain peptides are hydrophobic and may be prone to nonspecific interaction with proteins. However, REEP-1 and -2 cross-linking to 125I-Rab3AL-X was not competed by Rab2, Rab4, or Rab5 peptides, which have equivalent hydrophobicity to Rab3A and Rab3AL peptides. Thus, REEP-1 and -2 appear to specifically interact with the Rab3A effector domain. Other detectable proteins that cross-linked to the 125I-Rab3AL-X were considered nonspecific, since cross-linking was not blocked by Rab3A or Rab3AL peptides. The protein of around 66–68 kDa that nonspecifically cross-links to 125I-Rab3AL-X was likely to be BSA, which was used as a “carrier” for trichloroacetic acid precipitation after the cross-linking reaction. Specific inhibition of 125I-Rab3AL-X cross-linking to REEP-1 and -2 by Rab3AL peptide was dose-independent (Fig. 2B). The calculated mean ± S.E. (n ≥ 4) K\textsubscript{i} for REEP-1 = 8.2 ± 0.9 μM and for REEP-2 = 6.1 ± 0.8 μM (Fig. 2B). Thus, the specific interaction of REEP-1 and -2 with Rab3A effector domain peptides (Fig. 2B) was at a concentration of these peptide equivalent to that which specifically evoked insulin exocytosis (Fig. 1, B and C).

Cross-linking of 125I-Rab3AL-X to rat insulinoma subcellular fractions suggested REEP-1 and -2 to be cytosolic proteins in pancreatic β-cells (Fig. 3). However, in isolated rat islets the intracellular location of REEP-1 and -2 was dependent upon the regulatory state of β-cell exocytosis. At basal insulin exocytosis, about 70% of REEP-1 and -2 were membrane-associated and 30% were cytosolic (Fig. 4, A and B). However, upon a maximal stimulation of insulin release (Fig. 4D), REEP-1 and -2 redistributed so that about 25% were membrane-associated and 75% were cytosolic (Fig. 4, A and B). An excess of 125I-Rab3AL-X was used in these experiments to ensure that all of REEP-1 and -2 present in the islet fractions could be accounted for. In addition, it can be noted that cytosolic REEP-1 and -2 redistributes around an apparent lower molecular weight than “membrane-associated” REEP-1 and -2. However, this was not due to a modification of membrane-associated REEP-1 and -2 per se; rather it was due to composition of the samples that gave the cytosolic preparation a greater electrophoretic mobility. In parallel islet experiments, using specific Rab3A antisera as de-
mained in the membrane fraction during stimulation of insulin exocytosis. However, it should be realized that Rab3A associates with most secretory granules in a cell (12) and that only a minor proportion of the insulin secretory granule population undergoes exocytosis from islet β-cells, even under stimulatory circumstances (<5% of insulin content released in 30 min (28)). Translocation of Rab3A upon exocytosis would be difficult to observe against a massive background of Rab3A associated with an insulin secretory granule storage pool. Thus, local redistribution of Rab3A at the site of exocytosis should not be ruled out (29).

Several proteins associate with Rab3A that are generally related to GTP/GDP cycling. GDP dissociation inhibitor binds to the C terminus of Rab3A (30), so REEP-1 and -2 are not GDP dissociation inhibitors as they associate with the Rab3A effector domain. Rab3A-specific GAP (GTPase-accelerating protein) has a high molecular weight (31), and point mutations to the effector domain of Rab3A do not generally affect GAP sensitivity (19). Thus, it is unlikely that REEP-1 and -2 are GAPs. In contrast to GAP, mutations to the effector domain of Rab3A affect GRF (guanine nucleotide-releasing factor) sensitivity (19), but Rab3A GRF has a high molecular mass (31), which implies that either REEP-1 and -2 are subunits of a Rab3A-GRF complex or not necessarily GRFs. However, in yeast a Sec4-specific GRF of 17 kDa (named Dss4-1) has been isolated (32) that has a mammalian equivalent (named Mss4) of 14 kDa (33). Although Mss4 was rather effective at promoting GDP release from yeast Sec4 protein it was relatively poor at releasing GDP from Rab3A. Because of their similar molecular weight, however, it may be possible that REEP-1 and -2 are members of a Mss4-related GRF family that is specific for Rab3A (33). Soluble cross-linking techniques have unveiled an 85-kDa protein, named rabphilin-3A, that associates with the GTP-bound form of Rab3A (34, 35). The molecular weight difference between REEP-1 and -2 and rabphilin-3A suggests these proteins are not related. Furthermore, because the Rab3AL-X peptide did not specifically detect an 85-kDa protein, it suggests that rabphilin-3A interaction with Rab3A is not via Rab3A's effector domain. Although the identity of

![Fig. 3. Localization of REEP-1 and REEP-2 in subcellular fractions of rat insulinoma tissue (5 μg of total protein each). An example autoradiograph analysis of 125I-Rab3AL-X cross-linking to REEP-1 and -2 in insulinoma subcellular fractions is shown. Lane A, insulinoma tissue homogenate; lane B, plasma membrane-enriched fraction; lane C, secretory granule-enriched fraction; lane D, cytosolic-enriched fraction.](image-url)

![Fig. 4. Dissociation of REEP-1 and REEP-2 from a membrane associated to a soluble fraction upon stimulation of insulin exocytosis in isolated rat pancreatic islets. Panel A, an example autoradiograph of photoactivated cross-linking of 125I-Rab3AL-X to REEP-1 and -2 in separated crude membrane and soluble cytosolic fractions from 150 isolated rat pancreatic islet samples (between 30 and 40 μg, total protein) that were either incubated under basal or stimulated conditions for insulin exocytosis (17). Panel B, percentage distribution of REEP-1 and -2 in membrane or cytosolic fractions in islets either incubated for 30 min at 37 °C under basal or stimulated conditions for insulin exocytosis. Results are mean ± S.E. of at least six independent determinations. Panel C, distribution of Rab3A in crude membrane and soluble cytosolic fractions of islets either incubated under basal or stimulated conditions for insulin exocytosis (17). Results are mean ± S.E. (n = 3). Panel D, insulin release from the same islets as depicted in panel B incubated under basal (2.8 mM glucose) or maximum stimulated conditions (16.7 mM glucose, 10 μM forskolin, and 30 mM KCl) for insulin exocytosis (19). Results are mean ± S.E. of at least six independent determinations. REEP-1 and -2 has yet to be established, for the moment they are likely novel candidates involved in regulated exocytotic mechanism, if not novel proteins themselves. Our results also raise the question as to the role of REEP-1 and -2 in regulated exocytosis. Experimental evidence suggests that a pre-exocytotic protein complex is formed (36, 37) among other proteins and Rab3A.
secretory granule (or synaptic vesicle), cytosolic membrane, and plasma membrane proteins (of which Rab3A is a likely member (10, 37, 38)). Recently, Rab3A has been implicated in recruiting synaptic vesicles for docking with the plasma membrane, in a step prior to triggering exocytosis (39), and may therefore play a role in the formation of pre-exocytotic protein complexes. It has been proposed that dissociation of this pre-exocytotic complex is key to promoting fusion between the secretory granule and plasma membranes for regulated exocytosis (38, 40). Rab3A has been demonstrated as an inhibitor of regulated hormone release, and it is alleviation of the Rab3A inhibition that may, in part, be key to triggering exocytosis (41, 42). It is possible that REEP-1 and -2 are inhibitory proteins that are released from the Rab3A effector domain upon stimulation of exocytosis. The membrane association of REEP-1 and -2 with the Rab3A effector domain in the basal state may be consistent with these proteins also being members of a pre-exocytotic protein complex. Redistribution of REEP-1 and -2 to a cytosolic fraction upon stimulation of exocytosis could be symptomatic of a dissociation of the pre-exocytotic protein complex (38, 40, 41). It follows that the introduction of Rab3A effector domain peptides into semi-permeabilized cells would shift the equilibrium of REEP-1 and -2 associating with endogenous Rab3A effector domain (presumably on the secretory granule surface in a pre-exocytotic fusion complex) toward REEP-1 and -2 associating with Rab3A effector domain peptides in the cytosol. Hence, REEP-1 and -2 come out of the pre-exocytotic protein complex promoting its dissociation that, in turn, initiates exocytosis in a Ca²⁺-independent fashion. It follows that further detailed characterization of specific protein-protein interactions within the pre-exocytotic complex could identify the key regulatory events that trigger exocytosis in vivo.

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