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Abstract
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Subcellular Distribution and Function of Rab3A, B, C, and D Isoforms in Insulin-Secreting Cells

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Insulin-secreting cells express four GTPases of the Rab3 family. After separation of extracts of INS-1 cells on a sucrose density gradient, the bulk of the A, B, and C isoforms was recovered in the fractions enriched in insulin-containing secretory granules. Rab3D was also mainly associated with secretory granules, but a fraction of this isoform was localized on lighter organelles. Analyses by confocal microscopy of immunostained HIT-T15 cells transfected with epitope-tagged constructs confirmed the distribution of the Rab3 isoforms. Transfection of HIT-T15 cells with GTPase-deficient mutants of the Rab3 isoforms decreased nutrient-induced insulin release to different degrees (D > B > A > C), while overexpression of Rab3 wild types had minor or no effects. Expression of the same Rab3 mutants in PC12 cells provoked an inhibition of K⁺-stimulated secretion of dense core vesicles, indicating that, in β-cells and neuroendocrine cells, the four Rab3 isoforms play a similar role in exocytosis. A Rab3A/C chimera in which the carboxyterminal domain of A was replaced with the corresponding region of C inhibited insulin secretion as Rab3A. In contrast, a Rab3C/A chimera containing the amino-terminal domain of C was less potent and reduced exocytosis as Rab3C. This suggests that the degree of inhibition obtained after transfection of the Rab3 isoforms is determined by differences in the variable amino-terminal region. (Molecular Endocrinology 13: 202–212, 1999)

INTRODUCTION

Pancreatic hormones released by the cells of the islets of Langerhans play a pivotal role in the regulation of nutrient disposal and metabolism. In fact, insulin secretion from pancreatic β-cells is an essential requirement for the achievement of blood glucose homeostasis. Although the molecular details of the process of insulin exocytosis remain to be fully clarified, during the last few years several important components of the machinery permitting the targeting of secretory vesicles to the plasma membrane have been identified (1).

According to current models, the basic components controlling the targeting and fusion of secretory vesicles with the plasma membrane are largely conserved between cell types and between species (2, 3). The docking of transport vesicles to the appropriate membrane is thought to be specified by pairing of proteins located on the vesicle membrane termed v-SNAREs (vesicular SNAP receptors) with their specific partners on the acceptor membrane termed t-SNAREs (target SNAP receptors) (3). Pancreatic β-cells and clonal insulin-secreting cell lines express the v-SNAREs, VAMP-2 and cellubrevin, and the t-SNAREs, SNAP-25.
and syntaxin-1 (4–7). Each of these proteins has been demonstrated to play a role in insulin exocytosis using clostridial neurotoxins or with inactivating antibodies (5–8).

Rab GTPases represent a large family of homologous Ras-like GTP-binding proteins that direct the vectorial movement of secretory vesicles. These regulatory proteins act as molecular switches that flip between two conformational states, the active GTP-bound and an inactive GDP-bound form (9). The activated form of Rab GTPases has been proposed to catalyze the formation of the v-SNARE/t-SNARE complex. Under resting conditions, t-SNAREs are unable to interact efficiently with v-SNAREs as they are bound to members of the Sec-1 protein family (10, 11). Studies performed in yeast suggest that activated Ras-like GTPases located on transport vesicles disrupt the association between t-SNAREs and Sec-1-like proteins on the acceptor membrane (12). The displacement of Sec-1-like proteins would result in the assembly of t-SNAREs and v-SNAREs, leading to the docking of the vesicle to the target membrane (12).

The members of the Rab3 subfamily are the best candidates for controlling docking and fusion of Golgi-derived secretory vesicles with the plasma membrane (9, 13). Four isoforms of Rab3 (Rab3A, -B, -C, and -D) have been identified so far. At the protein level Rab3 isoforms display about 80% amino acid identity and differ almost exclusively in two short variable domains located at the amino- and at the carboxy terminus. Rab3A and -C are primarily expressed in neuronal and neuroendocrine cells, while Rab3B and -D are more abundant outside the nervous system (9, 13). The precise function of the different Rab3 isoforms remains to be established. Differences in the subcellular localization of Rab3 isoforms (14–16) and in their functional involvement in the exocytic process (17) have been reported. On the other hand, the relatively mild phenotype observed in Rab3A-deficient mice (18) and the ability of distinct isoforms to interact with the same effectors (17, 19, 20) suggest at least some degree of redundancy between Rab3 proteins.

Initial evidence for the possible implication of Rab3 proteins in insulin secretion came from studies performed in permeabilized cells. In this cell preparation, the introduction of peptides mimicking the putative effector-binding domain of Rab3 proteins stimulates insulin release (21–23). Subsequently, it was found that Rab3A is associated with the membrane of secretory granules and that the overexpression of a GTPase-deficient mutant of this isoform inhibits nutrient-stimulated insulin secretion (24). Insulin-secreting cells also express Rab3B, -C, and -D (24). Consequently, fine tuning of insulin secretion may result from the interplay between different isoforms.

In this study, we compared the subcellular distribution and the functional role of the four Rab3 isoforms in exocytosis. Our results indicate that a fraction of each Rab3 isoform is associated with dense-core insulin-containing secretory granules and that all of them, if kept in a GTP-bound form, inhibit exocytosis.

RESULTS

We have previously demonstrated that the β-cell line INS-1 expresses four Rab3 isoforms and that Rab3A is associated with dense-core insulin-containing secretory granules (24). In a first attempt to evaluate the localization of Rab3B, -C, and -D in insulin-secreting cells, we determined the subcellular distribution of each Rab3 isoform using a continuous sucrose density gradient (0.45–2 M) (24, 25). INS-1 cells contain a large number of secretory granules (26) and are, therefore, well suited for this type of biochemical characterization. As demonstrated in the top panel of Fig. 1, when homogenates of INS-1 cells are centrifuged at equilibrium, dense-core insulin-containing secretory granules are recovered in the fractions corresponding to 1.3–1.8 M sucrose, while synaptophysin, a marker of γ-aminobutyric acid-containing synaptic-like mi-

![Fig. 1. Separation of INS-1 Organelles on a Sucrose Density Gradient](image)
microvesicles, is recovered at 0.8–1 M sucrose. The distribution of plasma membranes, endoplasmic reticulum, and the Golgi complex is shown in the lower panel of Fig. 1. The plasma membrane marker Na⁺/K⁺-ATPase was recovered in the fractions containing 0.9–1.2 M sucrose; BHKp23, a protein associated with the cis-side of the Golgi complex (27), was concentrated in the fractions containing 1.2–1.3 M sucrose; calreticulin, a resident protein of the endoplasmic reticulum (28) was found in the fractions containing 1.3–1.4 M sucrose.

The distribution of the four Rab3 isoforms in the sucrose gradient is given in Fig. 2. A fraction of Rab3A, -B, and -C was recovered between 1.3 and 1.8 M sucrose consistent with an association with insulin-containing granules. These GTPases were also detected in lighter fractions (0.5–0.6 M sucrose), most likely reflecting the presence of a soluble pool (29). These Rab3 isoforms were barely detectable in fractions enriched in synaptic-like microvesicles and plasma membrane (Fig. 2). The distribution of Rab3D was, in part, different from that of the other Rab3 isoforms. As was the case for Rab3A, -B, and -C, Rab3D was mainly associated with organelles displaying the same density as insulin-containing secretory granules (Fig. 2). However, in contrast to the other isoforms, a significant portion of Rab3D was recovered in fractions containing 0.8–1.1 M sucrose (Fig. 2). In addition, Rab3D was almost absent from light fractions, suggesting that the soluble pool of this GTPase is very small.

The subcellular localization of Rab3A, -B, -C, and -D was also investigated after transient transfection of the hamster β-cell line HIT-T15. To follow the expression of the transfected proteins, a myc epitope was inserted at the amino terminus of each Rab3 isoform. Immunostaining followed by conventional fluorescence microscopy revealed that 70–75% of the transfected HIT-T15 cells contained detectable levels of insulin in the four series of experiments (Table 1). A minority of these cells expressed levels of Rab3 isoform that were clearly detectable at the immunofluorescence level (Table 1). In virtually all cells transfected with myc-tagged Rab3A, Rab3B, and Rab3C, the GTPases were found in the cellular compartment containing immunoreactive insulin (Table 1). In most of the cells transfected with myc-tagged Rab3D, the GTPase colocalized with insulin. However, Rab3D was also observed within cytoplasmic compartments of insulin-positive cells that did not contain detectable levels of the hormone or in cells in which insulin was not detectable (Table 1). Similar results were obtained after transfection of INS-1 cells with Rab3A, -B, -C, and -D (not shown). These results confirm the biochemical data obtained with the sucrose gradient and suggest that Rab3D has a subcellular distribution different, at least in part, from the other isoforms of Rab3.

The subcellular localization of Rab3 isoforms in HIT-T15 cells was analyzed by high-resolution confocal microscopy. Using this technique the myc-tagged Rab3 isoforms (Fig. 3, A–D) as well as insulin (Fig. 3, E–H) were localized on punctate structures in the cytoplasmic compartment. Mathematical comparison of the images obtained in the fluorescein and the rhodamine channels demonstrates that myc-tagged Rab3 proteins colocalize with insulin-containing secretory granules (Fig. 3, I–L). As a negative control, HIT-T15 cells were also transfected with myc-tagged Rab5, another member of the Rab family that is normally associated with early endosomes (30). As expected, myc-tagged Rab5 did not colocalize with insulin (data not shown). In contrast to the other isoforms and in agreement with the results in Table 1, in some cells myc-tagged Rab3D was also detected in cellular compartments that did not contain detectable levels of insulin (Fig. 4).

We have previously determined the role of Rab3A in insulin exocytosis by transiently coexpressing this GTPase and human proinsulin in the hamster β-cell line HIT-T15 (24). This line displays a higher transfection efficacy than INS-1 cells, which makes it more suitable for transient transfection experiments. The release of human C-peptide by the transfected hamster cells was used as a reporter for exocytosis. Using this system we found that the overexpression of a GTPase-deficient mutant of Rab3A, in which Glu81 is replaced by Leu (Q81L), leads to the inhibition of nutrient-induced secretion (24). To assess the involvement of Rab3B, -C, and -D in exocytosis, HIT-T15 cells were cotransfected with human proinsulin and with the wild-type or with the Q81L mutants of the four Rab3 isoforms. As shown in the upper panel of Fig. 5, the isoforms were expressed at comparable levels. None of the constructs affected the content or the basal release of human C peptide (not shown). The overexpression of wild-type Rab3A, -B, and -C had no sig-
nificant effect on stimulated human C peptide release, while exocytosis triggered by nutrients and bombesin was slightly decreased by the overexpression of Rab3D (Fig. 5, lower panel). In contrast to the wild type, the mutants of the four Rab3 isoforms deficient in GTPase activity (Q81L) inhibited exocytosis elicited by secretagogues (Fig. 5, lower panel). Despite the fact that all Rab3 Q81L constructs were expressed at a similar level, the Rab3 isoforms inhibited exocytosis with different efficacy. On average the most potent isoform was Rab3D followed in order by Rab3B, and -A. C Peptide secretion in cells transfected with Rab3C Q81L was also significantly different from the secretion measured in control cells ($p < 0.05; n = 20$; paired t test). However, the inhibitory effect of Rab3C Q81L was significantly smaller ($p < 0.05; n = 9$; paired t test) than the effect of the other Rab3 isoforms. A similar variation in the degree of inhibition was observed if secretion was triggered by depolarization potassium concentrations (not shown). In HIT-T15 cells, differences exist between the level of expression of endogenous Rab3 isoforms (24). Thus, we examined whether the efficacy with which the four isoforms inhibit exocytosis correlates with the ratio between endogenous and transfected proteins. As estimated by immunoblotting compared with the endogenous protein, Rab3A was overexpressed about 100-fold (Rab3A is barely detectable in HIT-T15 cells), while Rab3B, -C, and -D were overexpressed about 5- to 7-fold. Thus, the ratio between the amount of endogenous and transfected proteins cannot explain the differences between the four Rab3 isoforms.

We also tested for the effect on exocytosis of another mutant of Rab3 that is deficient in guanine nucleotide binding. This mutant, in which Asn$^{135}$ is replaced by leu(N135I), displays a very high dissociation rate for both GDP and GTP (31). Rab3A N135I and Rab3D N135I were well expressed in HIT-T15 cells (Fig. 6, upper panel) and inhibited exocytosis triggered by nutrients and bombesin with an efficacy similar to Rab3A Q81L and Rab3D Q81L, respectively (Fig. 6, lower panel). We also attempted to investigate the effect on exocytosis of Rab3B N135I and Rab3C N135I. However, the level of expression of these two mutants achieved in transfected HIT-T15 cells was too low to permit the interpretation of the results (data not shown).

Permeabilized cells have been used as a model system to measure effects on exocytosis that are independent of membrane depolarization, channel activity, and soluble second messengers (32). We found that the release of C peptide, resulting from the incubation of Streptolysin-O-permeabilized HIT-T15 cells with 100 mM GTPyS or with 10 mM Ca$^{2+}$, was strongly reduced by the overexpression of the Q81L and N135I mutants of Rab3A (Fig. 7). Thus, the inhibition caused by the overexpression of Rab3 Q81L and Rab3 N135I is likely to reflect an effect of the mutants on the exocytotic process rather than an effect on the signaling pathway that triggers hormone secretion.

Stable expression of wild-type Rab3B or of Rab3B N135I has been reported to enhance Ca$^{2+}$-evoked secretion in the neuroendocrine cell line PC12 (17). Differences between neuroendocrine cells and HIT-T15 cells could potentially explain the discrepancy between our data and the results obtained with PC12 cells. To assess whether this was the case, we analyzed exocytosis in PC12 cells transiently cotransfected with the Q81L mutants of the four Rab3 isoforms and with human GH (hGH). Exocytosis from the fraction of cells expressing the Rab3 constructs was determined by measuring hGH release (33). As was the case in HIT-T15 cells, the GTPase-deficient mutants of the four Rab3 isoforms inhibited stimulus-induced secretion (Fig. 8).

The GTPase-deficient mutant of Rab3C is less efficient than the corresponding mutants of Rab3A, -B, and -D. Rab3 isoforms contain two short variable domains at the amino- and at the carboxy terminus of the protein. To address whether either one of these domains is responsible for the differences observed between isoforms, we generated chimeric constructs in which the amino- or the carboxy terminus of Rab3A Q81L was replaced by the corresponding region of Rab3C (Fig. 9). After transfection, all the chimeric proteins were expressed at comparable levels (Fig. 10, upper panel). The Rab3A chimera containing the carboxy terminus of Rab3C (Rab3A/C) inhibited exocytosis...
Fig. 3. Immunolabeling of Transfected Clones for Insulin and Rab3 Isoforms

HIT-T15 cells were transiently transfected with myc-tagged Rab3A (panels A, E, and I), Rab3B (panels B, F, and J), Rab3C (panels C, G, and K), and Rab3D (panels D, H, and L). The cells were analyzed by confocal microscopy after double immunofluorescence with a monoclonal antibody against the myc epitope (revealed using fluorescein-conjugated antibodies) (panels A–D) and with a polyclonal antibody against insulin (detected using rhodamine-conjugated antibodies) (panels E–H). Panels I–L show the regions of colocalization between transfected Rab3 proteins and insulin granules. The colocalization images were obtained as described in Materials and Methods by plotting all pixels whose intensities in both channels were simultaneously above the 40% level (representing <10% of the total pixel count).
sis to the same extent as Rab3A Q81L (Fig. 10, lower panel). Similar results were obtained with two Rab3A chimeric constructs containing, respectively, the carboxy terminus of Rab3B and Rab3D (data not shown). In contrast, the Rab3A chimera with the amino terminus of Rab3C (Rab3C/A) was much less efficient, decreasing stimulated secretion like Rab3C. As a control, we also tested the corresponding Rab3A chimeric constructs containing the amino terminus of Rab3B and Rab3D. The inhibition of exocytosis obtained with these constructs was not significantly different from that of Rab3A, -B, or -D (data not shown). The localization of all the chimeric constructs analyzed by immunofluorescence was identical to that of Rab3A (data not shown).

**DISCUSSION**

The members of the Rab3 family are thought to control the targeting and/or the fusion of Golgi-derived vesicles with the plasma membrane (9, 13). However, the precise subcellular distribution and the respective role of the different Rab3 isoforms in exocytosis are still unclear. In bovine chromaffin cells, antibodies against Rab3A and Rab3C were found to stain vesicle structures, whereas an antibody against Rab3B stained the plasma membrane (16). In polarized epithelial cells, Rab3B was localized to the apical pole of the cells near the tight junctions (34). However, the same investigators observed that transfected epitope-tagged Rab3B colocalized with dense-core granules of PC12 cells (17). We have previously demonstrated that Rab3A is associated with insulin-containing secretory granules of pancreatic β-cells (24). In this study, using double immunofluorescence experiments analyzed by confocal microscopy, we demonstrate that myc-tagged Rab3B, -C, and -D are also associated with insulin-containing granules and that, clearly, none of the isoforms are localized on the plasma membrane. It is very unlikely that our morphological observations are biased by the overexpression of the proteins. In fact, first, these findings are corroborated by subcellular fractionation studies indicating that the endogenous Rab3 isoforms display a similar distribution. Second,
overexpression of about 100-fold of Rab3A (that is expressed at low level in HIT-T15 cells) does not affect the targeting of the protein to the secretory granules. In addition, we found the same localization in cells expressing low levels of the exogenous protein and in cells producing high levels of the transfected Rab3 isoforms. Third, when Rab5, another member of the Rab family known to be associated with early endosomes, was overexpressed in HIT-T15 cells, the protein was not associated with secretory granules.

In contrast to the other isoforms, Rab3D was also found in cells containing undetectable levels of insulin or within cytoplasmic compartments of insulin-positive cells not containing detectable levels of the hormone. It has been previously reported that Rab3A and Rab3D are associated with different vesicular compartments (14, 15, 35). The present data confirm a partial difference in the localization between the two isoforms but indicate that a considerable fraction of Rab3D is associated with insulin-containing granules. At present, the precise nature of the vesicular structures containing Rab3D but devoid of insulin is unknown. In CHO cells, Rab3D cofractionates with a population of post-Golgi storage vesicles slightly larger than synaptic vesicles that have been identified with labeled glycosaminoglycan chains (36). When combined with our results, it is tempting to speculate that the fraction of Rab3D not associated with insulin-containing secretory granules may be located on a similar type of storage vesicles.

The presence of more than one isoform within the same cell raises the question whether Rab3 proteins are redundant or whether they play a specific role in the exocytotic process. Transient overexpression of a constitutively active mutant of Rab3A reduces stimulus-induced secretion in neuroendocrine (33, 37) and in insulin-secreting cells (24). These observations have been taken as an indication that Rab3A is a negative

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Fig. 6. Effect of the Rab3 Mutants Deficient in Guanine Nucleotide Binding on Insulin Secretion

HIT-T15 cells were transiently cotransfected with human proinsulin and with the N135I mutants of Rab3A and Rab3D. Two days after transfection, the cells were incubated as described in Fig. 5. At the end of the incubation the cells were collected and analyzed by Western blotting using an antibody against the myc epitope (top panel). The amount of human C peptide secreted by the cells in response to the stimuli was measured by ELISA. The lower panel shows the mean ± se of three representative experiments. In control cells the mixture of nutrients and bombesin elicited a 4- to 6-fold increase in C-peptide release.

Fig. 7. Effect of Rab3A Q81L and Rab3A N135I on Exocytosis in Streptolysin-O Permeabilized HIT-T15 Cells

HIT-T15 cells were transiently cotransfected with human proproinsulin and with the vector alone (C), with Rab3A Q81L (Q81L), or with Rab3A N135I (N135I). Two days after transfection the cells were permeabilized with Streptolysin-O. The medium was then removed and the cells were incubated for 7 min in the presence of 0.1 μM free Ca²⁺, 0.1 μM Ca²⁺ plus 100 μM GTPγS, or 10 μM Ca²⁺. The amount of human C peptide secreted by the cells was measured by ELISA. The results represent the mean ± se of three independent experiments.

Fig. 8. Effect of the GTPase-Deficient Mutants of Rab3 Isoforms on Exocytosis in PC12 Cells

PC12 cells were transfected by electroporation with hGH and with the Q81L mutants of Rab3A (A), Rab3B (B), Rab3C (C), or Rab3D (D). Four days after transfection the cells were stimulated with depolarizing concentrations of K⁺ for 2 min. The figure shows the amount of hGH released in response to the stimulus. The results correspond to the mean ± se of three independent experiments. K⁺ depolarization of control cells caused an increase of 4- to 6-fold in hGH release.
modulator of exocytosis (33, 37). In agreement with this model, Rab3A-deficient mice display an increased number of synaptic vesicle fusion events shortly after the arrival of the nerve impulse (38). An alternative interpretation of the results would be that the hydrolysis of GTP is a prerequisite for the fusion of secretory vesicles with the plasma membrane. In this case, the GTPase-deficient mutant of Rab3A associated with secretory vesicles would have a dominant-negative effect and would inhibit exocytosis. The latter view is favored by the results obtained in yeast with the corresponding GTPase-deficient mutant of Sec4 (39).

Rab3B has been suggested to have functional properties distinct from Rab3A. Thus, in pituitary cells, inhibition of Rab3B expression was found to attenuate Ca\(^{2+}\)-dependent exocytosis (40) and in PC12 cells stable expression of wild-type Rab3B or of Rab3B N135I has been reported to potentiate the efficiency of Ca\(^{2+}\)-evoked secretion and to markedly increase the accumulation of norepinephrine in secretory granules (17). Here we show that transient overexpression of wild-type Rab3B in HIT-T15 cells has no significant effect on stimulated secretion, and that Rab3B Q81L strongly inhibits exocytosis. Thus, the data are consistent with a similar role for Rab3A and Rab3B in insulin-secreting cells. Our findings cannot be attributed to differences between insulin-secreting cells and neuroendocrine cells, since we demonstrate that transient expression of Rab3B Q81L diminishes stimulated secretion also in PC12 cells. Unfortunately, in HIT-T15 cells, Rab3B N135I was poorly expressed and it was, therefore, impossible to evaluate its effect on insulin release. A possible explanation for the low level of Rab3B N135I and Rab3C N135I detected in transfected cells is that the high dissociation rate for guanine nucleotides is affecting the turnover rate of the protein.

We have analyzed the effect of Rab3B mutants on exocytosis after short-term expression (2 days). In contrast, Weber \textit{et al.} (17) analyzed the impact of Rab3B expression on secretion using stable cell lines. After long-term expression of Rab3B, the stability of Rabphilin, a putative Rab3 effector, was found to decrease (17). Thus, it is possible that long-term expression of Rab3B leads to secondary alterations in the secretory phenotype that are not evident in transient transfection experiments.

The overexpression of a GTPase-deficient mutant of each of the four Rab3 isoforms tested in this study inhibited exocytosis. However, the decrease in nutrient-induced secretion observed in the presence of Rab3C was much less pronounced than the decrease caused by the other isoforms. Exchange of the carboxy-terminal domain of Rab3A with those of any of the other Rab3 isoforms did not alter the extent of inhibition measured after overexpression of the chimeric constructs. Substitution of the amino terminus of Rab3A with the corresponding domain of Rab3B or Rab3D was also without effect, but replacement of the amino terminus of Rab3A with the residues of Rab3C reduced the effect of the GTPase on exocytosis. The amino termini of Rab5 and of Rab2 have been shown to be required for the function of these GTPases and for the interaction with components of the transport machinery (41, 42). The variable domain at the carboxy terminus of Rab proteins appears to dictate the localization of the GTPases to specific cellular compart-
ments (43, 44). Our results suggest that the amino terminus of the Rab3 isoforms may be involved in the interaction with an, as yet unidentified, component of the secretory machinery. Differences in the amino-terminal domain of the Rab3 isoforms may affect the affinity for this putative component and could determine the efficacy with which they regulate insulin secretion.

In conclusion, we have demonstrated that the secretory granules of pancreatic β-cells contain four Rab3 isoforms with similar roles in the regulation of insulin secretion. Further experiments are needed to determine the step(s) and the precise mechanism by which the GTPases of the Rab3 family control the exocytotic process.

**MATERIALS AND METHODS**

**Materials**

The cDNA coding for human wild-type Rab3B was kindly provided by Dr. K. Kirk (University of Alabama at Birmingham, AL). Bovine wild-type Rab3C cDNA and polyclonal antibodies directed against Rab3A, Rab3B, and Rab3C were generously provided by Dr. A. Zahraroui (Curie Institute, UMR 144 CNRS, Paris, France). Plasmid pBPhins, derived from pBR322 and containing the human preproinsulin gene under the control of the cytomegalovirus promoter), was kindly provided by Dr. J.-C. Irminger (University of Geneva). The generation of rabbit polyclonal antibodies against the amino-terminal region of the Rab3 isoforms may affect the secretory machinery. Differences in the amino-terminal domain of the Rab3 isoforms may be involved in the exocytotic process.

**Cell Culture**

The insulin-secreting cell lines HIT-T15 and INS-1 were cultured in RPMI 1640 medium supplemented with 10% FCS and other additions as described for each cell line (26, 45). PC12 cells were cultured in DMEM supplemented with 6% FCS and 6% horse serum.

**Subcellular Fractionation**

Subcellular fractionation of approximately 10⁸ INS-1 cells was performed as described (24). Briefly, a postnuclear supernatant obtained after disruption of the cells by nitrogen cavitation was loaded on a continuous sucrose density gradient (8 ml; 0.45–2 M sucrose). After centrifugation for 18 h at 110,000 × g, 16 fractions of 0.5 ml were collected from the top of the tube. The concentration of sucrose in the fractions was determined by measuring the refractive index of the solution. The amount of insulin present in the fractions was measured by RIA. The distribution throughout the gradient of BHKp23, calreticulin, Na⁺/K⁺ ATPase, synaptophysin, and Rab3A/B/C and D was assessed by Western blotting (24) followed by densitometric scanning of the autoradiographic films.

**Generation of DNA Constructs**

The generation of myc-tagged human wild-type Rab3A and of the mutant at positions 81 (Q81L) and 135 (N135I) has been described previously (37). Myc-tagged wild-type Rab3A and the corresponding Q81L and N135I mutants were subcloned in the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). Wild-type Rab3B, Rab3C, Rab3D, and the Q81L mutant of Rab3D were subcloned into pcDNA3 containing the N-terminal myc epitope. To construct the Rab3A/C chimera containing the carboxy terminus of Rab3C, we generated a XhoI restriction site in Rab3A and Rab3C at the level of amino acid 178; the fragment of Rab3D downstream to the XhoI site was then excised and inserted in the corresponding XhoI site of Rab3A/Q81L. A similar approach was used to produce the amino-terminal Rab3C/A chimera. In this case, a XhoI site was introduced in the sequence of Rab3A and Rab3C at the level of amino acid 19; the fragment downstream to the XhoI site of Rab3A/Q81L was inserted in the corresponding site of Rab3C.

**Site-Directed Mutagenesis**

The mutants of Rab3B and Rab3C were generated by site-directed mutagenesis according to the method of Kunkel (46). The mutations generated were confirmed by DNA sequencing of the plasmids.

**Transfection**

For transient transfection experiments, HIT-T15 cells were seeded in 24-multiwell plates (4 × 10⁵ cells per well). After 3 days of culture, the cells were cotransfected using the lipopolymamine Transfectam (Promega) with 2.5 μg of the vector encoding human preproinsulin and with 5 μg of the plasmids containing the cDNAs under study (24, 47). Transient transfection of PC12 cells was performed by electroporation; 4 × 10⁵ cells were resuspended and electroporated in 400 μl of serum-free DMEM in the presence of 40 μg of a plasmid vector encoding hGH (Nichols Institute, San Juan Capistrano, CA) and 40 μg of pcDNA3 encoding the Rab3 proteins. Immediately after transfection the cells were diluted in culture medium and seeded in 24-multiwell plates.

**Secretion from Transfected HIT-T15 Cells**

Forty-eight hours after transfection the cells were preincubated for 30 min in modified Krebs-Ringer bicarbonate buffer (24). They were then incubated for 30 min in Krebs-Ringer bicarbonate buffer in the presence or in the absence of 10 mM glucose, 5 mM leucine, 5 mM glutamine, and 100 μM bombesin, a mixture known to strongly stimulate insulin secretion in these cells. Secretion from transfected cells was assessed by measuring the amount of human C peptide released into the medium during the incubation period by enzyme-linked immunosorbent assay (ELISA) (Dako Corp., Carpinteria, CA). Secretion experiments with permeabilized cells were performed as described (48). Forty-eight hours after transfection the cells were permeabilized with Streptolysin-O for 8 min. The medium was then removed, and exocytosis was triggered by adding 100 μM GTPγS or by increasing the free Ca²⁺ concentration from 0.1 μM (basal) to 10 μM.

**Secretion from Transfected PC12 Cells**

Four days after transfection the cells were preincubated during 30 min in 20 mM HEPES, pH 7.4, 128 mM NaCl, 5 mM KCl,
cultures were simultaneously exposed for 2 min with the same buffer but containing 53 mM NaCl and 80 mM KCl. Exocytosis from the subpopulation of transfected cells was determined by measuring ELISA (Boeringer Mannheim, Indianapolis, IN) the amount of hGH secreted into the medium during the incubation period.

**Immunofluorescence**

For immunofluorescence labeling, the cells were grown on glass coverslips coated with extracellular matrix produced by A431 epidermoid cells (49, 50). Subconfluent monolayers were fixed 30 min in a 4% paraformaldehyde-0.1 M phosphate buffer solution, pH 7.4. After rinsing for 10 min in PBS supplemented with 0.5% BSA (PBS) and 10 mM NH₄Cl, the cultures were simultaneously exposed for 2 h at room temperature to a guinea pig polyclonal serum against porcine insulin (diluted 1:200) (51) and to a purified mouse monoclonal antibody against human c-myc (clone 9E10; diluted 1:600). After rinsing in PBS, the cultures were incubated again for 1 h at room temperature in the presence of both a rhodamine-labeled goat serum against guinea pig Ig (Cappel, Organon Teknika AG, Switzerland; diluted 1:200) and a fluorescein-conjugated sheep antimouse Ig (Biosys, Compiegne, France; diluted 1:1200). After careful rinsing, the coverslips were mounted with 0.02% para-phenylenediamine in PBS-glycerol (1:2, vol/vol).

Two independent experiments were run for quantitative analysis. In each experiment and for each of the four Rab3 isoforms, 10 fields were randomly photographed at the fixed magnification of 100×. Counts were performed on these photographs, which were projected on a screen at the final magnification of 370×. The total number of cells was evaluated by scoring the number of nuclei present per field. The number of insulin-containing cells was established by scoring those cells that showed a granular, rhodamine labeling of cytoplasm clearly above background level. The number of Rab3-transfected cells was established by scoring those cells that showed a fluorescein labeling of cytoplasm clearly above background level. Colocalization of the two immunoreactivities was assessed by evaluating the insulin labeling of all Rab3-positive cells.

Two separate experiments were made for confocal microscopy (Leica Lasertechnik, Heidelberg, Germany; TCS NT) analysis, to discriminate intracellular granular staining from diffuse cytoplasmic or membrane staining. Excitation was obtained with an Argon-Krypton laser, with line set at 488 nm for fluorescein excitation and 568 nm for rhodamine excitation, and the emitted light was filtered through appropriate filters (BP 530/30 for fluorescein, LP 590 for rhodamine). Images of 512 × 512 pixels (RabA, -B, and -C) and 1024 × 1024 pixels (RabD) were taken with a 63× objective, NA 1.32. A typical pixel size was 35 nm (RabD, with a zoom of 2.3). For each field, digitized series of optical sections at different planes of focus were collected on the host computer; care was taken to use the full dynamic range of the photomultiplier by using a special look up table (glowover-glowunder, Leica); the sections were processed using Imaris software (Bitplane AG, Zurich, Switzerland) on a Silicon Graphics computer. No filters were applied, but background noise was reduced. To calculate colocalization between transfected Rabs and insulin granules, a correlogram of all intensities was generated, normalizing the intensities from 0 to 100%; all pixels whose intensities in both channels were simultaneously above the 40% level (representing <10% of the total pixel number) were then plotted.

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