

## Expression, localization and functional role of small GTPases of the Rab3 family in insulin-secreting cells

Romano Regazzi<sup>1,\*</sup>, Mariella Ravazzola<sup>2</sup>, Mariella Iezzi<sup>1</sup>, Jochen Lang<sup>1,3</sup>, Ahmed Zahraoui<sup>3</sup>, Elisabeth Anderegg<sup>4</sup>, Philippe Morel<sup>4</sup>, Yoshimi Takai<sup>5</sup> and Claes B. Wollheim<sup>1</sup>

<sup>1</sup>Division of Clinical Biochemistry, Department of Medicine, University of Geneva, Switzerland (Member of the Geneva Diabetes Group)

<sup>2</sup>Department of Morphology, University of Geneva, Switzerland

<sup>3</sup>Curie Institute, UMR 144 CNRS, Paris, France

<sup>4</sup>Transplant Unit, Department of Surgery, University of Geneva, Switzerland

<sup>5</sup>Department of Molecular Biology and Biochemistry, Osaka University Medical School, Suita 565 Japan

\*Author for correspondence

### SUMMARY

We examined the presence of small molecular mass GTP-binding proteins of the Rab3 family in different insulin-secreting cells. Rab3B and Rab3C were identified by western blotting in rat and in human pancreatic islets, in two rat insulin-secreting cell lines, RINm5F and INS-1, as well as in the hamster cell line HIT-T15. In contrast, Rab3A was detected in rat pancreatic islets as well as in the two insulin-secreting rat cell lines but not in human pancreatic islets and was only barely discernible in HIT-T15 cells. These findings were confirmed by two-dimensional gel electrophoresis followed by GTP-overlay of homogenates of pancreatic islets and of the purified protein. Northern blotting analysis revealed that Rab3D is expressed in the same insulin-secreting cells as Rab3A. Separation of the cells of the rat islets by fluorescence-activated cell sorting demonstrated that Rab3A was exclusively expressed in  $\beta$ -cells. Rab3A was found to be associated with insulin-containing secretory granules both by immunoflu-

orescence, immunoelectron microscopy and after sucrose density gradient. Overexpression in HIT-T15 cells of a Rab3A mutant deficient in GTP hydrolysis inhibited insulin secretion stimulated by a mixture of nutrients and bombesin. Insulin release triggered by these secretagogues was also slightly decreased by the overexpression of wild-type Rab3A but not by the overexpression of wild-type Rab5A and of a Rab5A mutant deficient in GTP hydrolysis. Finally, we studied the expression in insulin-secreting cells of rabphilin-3A, a putative effector protein that associates with the GTP-bound form of Rab3A. This Rab3A effector was not detectable in any of the cells investigated in the present study. Taken together these results indicate an involvement of Rab3A in the control of insulin release in rat and hamster. In human  $\beta$ -cells, a different Rab3 isoform but with homologous function may replace Rab3A.

Key words: Exocytosis, Pancreas, Islets of Langerhans

### INTRODUCTION

The involvement of small molecular mass GTP-binding proteins (small G-proteins) in the regulation of intracellular vesicular trafficking has been demonstrated using both genetic and biochemical approaches (Takai et al., 1992; Ferro-Novick and Jahn, 1994; Fischer von Mollard et al., 1994a). These proteins control the vesicular movement between ER and Golgi and within the Golgi complex (Takai et al., 1992; Ferro-Novick and Jahn, 1994; Fischer von Mollard et al., 1994a). A role for small G-proteins in exocytosis has also been proposed since, in yeast, one of these proteins (SEC4) is implicated in the targeting of post-Golgi secretory vesicles to the plasma membrane (Goud et al., 1988) and, in mammalian cells, small G-proteins have been localized on secretory granules (Darchen et al., 1990, 1995; Fischer von Mollard et al., 1990; Matteoli et al., 1991; Jena et al., 1994). Several lines of evidence make the members of the Rab3 family attractive candidates for the

regulation of exocytosis in mammalian cells. First, Rab3A and Rab3C have been found on synaptic vesicles from which they have been shown to dissociate during exocytosis in a synaptosomal preparation (Fischer von Mollard et al., 1991, 1994b). Second, inhibition of Rab3B expression by anti-sense oligonucleotides resulted in a decrease in  $Ca^{2+}$ -stimulated secretion from pituitary cells (Ledo et al., 1993). Third, overexpression of Rab3A mutant proteins defective in GTP hydrolysis or in guanine nucleotide binding inhibited exocytosis from neuroendocrine cells (Holz et al., 1994; Johannes et al., 1994). Fourth, a synthetic peptide mimicking the putative effector binding domain of Rab3 proteins has been shown to affect exocytosis from different secretory cell systems (Oberhauser et al., 1992; Padfield et al., 1992; Senyshyn et al., 1992), including insulin-secreting cells (Li et al., 1993; Olszewski et al., 1994; Regazzi et al., 1995a).

In yeast, a small G-protein of the Rab family has been shown to control the assembly and the activation of a complex of

proteins implicated in the fusion of Golgi vesicles with their target membrane (Sogaard et al., 1994; Lian et al. 1994). Whether this is the mechanism by which Rab3 proteins control exocytosis remains to be established. Small G-proteins in their GDP-bound state are found in the cytosol associated with the regulatory protein GDP-dissociation inhibitor (GDI) (Regazzi et al., 1992a; Ullrich et al., 1993; Soldati et al., 1994). GDI is delivering the protein to the appropriate membrane where GDP is exchanged for GTP (Pfeffer et al., 1995). It has been postulated that this permits the correct targeting of the vesicles to the appropriate acceptor membrane (Bourne, 1988). The process is thought to necessitate the interaction with specific effectors. Rabphilin-3A has been proposed to represent such an effector (Shirataki et al., 1992; Li et al., 1994). This protein interacts preferentially with the GTP-bound form of Rab3A (Shirataki et al., 1992). The sequencing of rabphilin-3A revealed homologies with the vesicle associated protein synaptotagmin. Rabphilin-3A contains C2 domains homologous to protein kinase C (Shirataki et al., 1993; Li et al., 1994) that confers the ability to bind phospholipids in a  $Ca^{2+}$ -dependent manner (Yamaguchi et al., 1993).

The involvement of small G-proteins of the Rab3 family in the regulation of insulin secretion remains to be clarified, since Rab3A is almost exclusively expressed in neuronal cells and is believed to play a role in neurotransmitter release. This particular small G-protein has, however, also been detected in some insulin-secreting cell lines both by western and by northern blotting (Regazzi et al., 1992b; Lankat-Buttgereit et al., 1992, 1994; Kowluru et al., 1994; Baldini et al., 1995). However, the role of Rab3A in insulin secretion is still a matter of debate. In particular, there is no direct functional evidence available for the involvement of Rab3A in the control of insulin release. Moreover, Rab3A has been proposed to control GABA release rather than insulin secretion (Lankat-Buttgereit et al., 1992). Indeed, insulin-secreting cells possess synaptic-like vesicles containing GABA (Reetz et al., 1991; Sorenson et al., 1991; Thomas-Reetz et al., 1993) and the secretion of this neurotransmitter may exert a paracrine effect within the islet (Rorsman et al., 1989).

In this study, we analysed the expression of the members of the Rab3 (Rab3A, B, C and D) family in different insulin-secreting cells and the possible association of Rab3A with synaptic-like microvesicles and with insulin-containing secretory granules. In addition, we examined the functional implication of this small G-protein in the regulation of insulin secretion by overexpressing wild-type Rab3A and a mutant deficient in GTPase activity. Our results provide functional evidence that Rab3A is involved in the regulation of insulin secretion. In human pancreatic  $\beta$ -cells that do not express Rab3A, this GTPase may be substituted by another protein with similar function.

## MATERIALS AND METHODS

### Antibodies

The polyclonal antibody directed against Rab3A was generated as described (Moya et al., 1992). Rab3B and Rab3C antisera were raised against synthetic peptides covalently coupled to ovalbumin (Neosystem, Strasbourg, France). The peptide sequences were derived from human Rab3B C-terminal end: PSMLGSSKN-

TRLSDT (position 195-209) and from bovine Rab3C C-terminal end: PAITAAKQNTRLKET (position 195-209). The coupled peptides were emulsified in Freund's complete adjuvant and injected subcutaneously into New Zealand white rabbits. The rabbits were reinjected with the same antigens in Freund's incomplete adjuvant at 4 week intervals and bled 10 days after each booster. Rabphilin-3A, Rab3A and the rabbit polyclonal antibody generated against the peptide corresponding to the sequence PARAPTRGDTEDR-RGPGQ of rabphilin-3A were produced as described (Shirataki et al., 1993; Kikuchi et al., 1988). The monoclonal antibody against synaptophysin (clone SVP-38) was purchased from Sigma (St Louis, USA). The cDNA of Myc-tagged human wild-type Rab3A and of the mutant at position 81 (Q81L) was kindly provided by Dr F. Darchen, CNRS 1112 Paris, France. The cDNA of Myc-tagged wild-type Rab5A and of the mutant at position 79 (Q79L) was a generous gift from Dr M. Zerial, EMBL, Heidelberg, Germany. The cDNA of Rab3D (Rab16) was obtained from Dr R. H. Scheller, Stanford University, USA.

### Cells

RINm5F (Vallar et al., 1987), INS-1 (Asfari et al., 1992) and HIT-T15 (Regazzi et al., 1990) cells were cultured as described in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. The culture medium of HIT-T15 cells also contained 32.5  $\mu$ M glutathione and 0.1  $\mu$ M selenium. INS-1 cells were cultured in the presence of 1 mM pyruvate and 50  $\mu$ M  $\beta$ -mercaptoethanol.

### Pancreatic islets

Rat pancreatic islets were obtained by collagenase digestion as described (Pralong et al., 1990). Human pancreatic islets were prepared essentially according to the method of Ricordi et al. (1988). After collagenase digestion the islets were purified on a Ficoll gradient. The viability of human pancreatic islets was verified *in vitro* by their ability to secrete insulin in a glucose-dependent fashion.

### Preparation of cell homogenates

The cells of the insulin-secreting lines or the pancreatic islets were washed twice with ice-cold homogenization buffer (HB): 20 mM Tris-HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 6 mM  $\beta$ -mercaptoethanol, 10  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin and then disrupted by brief sonication ( $3 \times 1$  second). The homogenates were stored at  $-20^\circ\text{C}$  until use.

### Sucrose gradient

Synaptic-like vesicles and insulin-containing secretory granules were separated essentially according to the method of Reetz et al. (1991). Briefly, INS-1 cells were homogenized by nitrogen cavitation (9 bars, 30 minutes) in: 5 mM HEPES, pH 7.4, 1 mM EGTA, 10  $\mu$ g/ml leupeptin and 2  $\mu$ g/ml aprotinin. The cell debris and the nuclei were eliminated by centrifuging the homogenate for 10 minutes at 3,000 *g*. The supernatant obtained was loaded onto a continuous sucrose gradient (0.45 M-2 M, 8 ml) and centrifuged for 18 hours at 110,000 *g*; 16 fractions of 0.5 ml each were collected from the top of the tube.

### Western blotting

Western blotting was performed as previously described (Regazzi et al., 1992b) except that the primary antibody was detected by chemiluminescence using horseradish peroxidase coupled secondary antibody.

### Northern blotting

Total RNA was isolated after cell lysis in guanidinium thiocyanate (Chomczynski and Sacchi, 1987). Rab3D mRNA sequences were detected by northern blot hybridization with a fragment corresponding to the carboxyl-terminal domain of the protein. The cDNA probes were radioactively labeled using the random priming technique (Boehringer, Mannheim, Germany).

### GTP-overlay

Binding of [ $\alpha$ - $^{32}$ ]GTP to proteins blotted on nitrocellulose sheets was carried out as described previously in detail (Regazzi et al., 1991).

### Sorting of $\beta$ and non- $\beta$ cells by fluorescence activated cell sorting

Rat pancreatic islets were trypsinized and  $\beta$  and non- $\beta$  cells were separated according to the size and FAD autofluorescence using the fluorescence activated cell sorter (FACS) (Rouiller et al., 1990).

### Immunocytochemistry

Monolayer cultures of pancreatic endocrine cells (Orci et al., 1973) were fixed with Bouin's fluid and analysed by the indirect immunofluorescence method using anti-Rab3A antibodies (1:50). Isolated rat islets fixed with 1% glutaraldehyde were processed for cryo-ultramicrotomy (Tokuyasu, 1980) and immunolabeled for Rab3A by the Protein A-gold method (Roth et al., 1978).

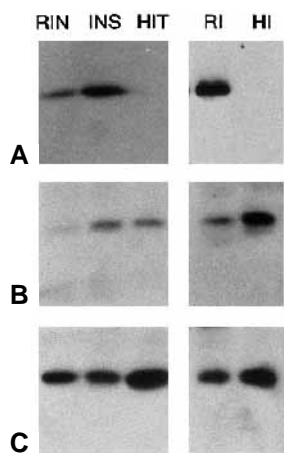
To determine the coexpression of Myc-tagged proteins with human proinsulin, HIT-T15 cells were grown on glass coverslips and cotransfected as described below with Rab3A and with human proinsulin. After two days of culture the cells were washed twice in PBS and fixed with 4% paraformaldehyde for 20 minutes. After one wash with PBS and two washes in PBS containing 0.38% glycine and 0.27%  $\text{NH}_4\text{Cl}$  the cells were permeabilized with 0.1% saponin in the presence of 0.5% bovine serum albumin for 30 minutes. The cells were incubated with anti-cMyc antibody (1:100) and anti-human C-peptide antibody (1:500) for 1 hour. After five washes the cells were incubated for 45 minutes with rhodamine-conjugated anti-mouse antibodies and with fluorescein isothiocyanate (FITC)-conjugated anti-rat antibodies.

### Transient transfection of HIT-T15 cells

Wild-type Rab3A and Rab5A or variants bearing a point mutation at position 81 (Rab3A Q81L) or at position 79 (Rab5A Q79L) were subcloned into the expression vector pcDNA 1 (Invitrogen). HIT-T15 cells, seeded at  $0.5 \times 10^6$  cells/well, were transiently cotransfected as previously described (Lang et al., 1995) with 2.5  $\mu\text{g}$  of a plasmid containing the human preproinsulin cDNA and with 12.5  $\mu\text{g}$  of the vector alone or containing the cDNA of the constructs under study. After 48 hours the cells were washed twice with a modified Krebs-Ringer bicarbonate buffer containing 1 mM  $\text{CaCl}_2$ , 5 mM  $\text{NaHCO}_3$  and 25 mM HEPES and preincubated for 30 minutes at 37°C. Thereafter, the medium was replaced with Krebs-Ringer buffer alone or supplemented with 10 mM glucose, 5 mM leucine, 5 mM glutamine and 100 nM bombesin. Exocytosis from transfected cells was monitored by assessing the release of human insulin C-peptide by radioimmunoassay (Novo-Nordisk).

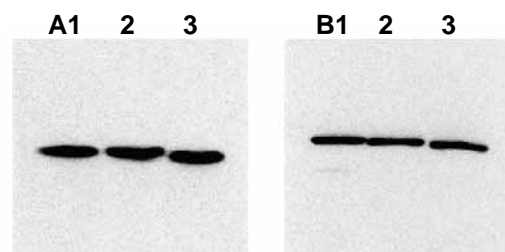
## RESULTS

The small molecular mass GTP-binding proteins of the Rab3 family have been implicated in the control of exocytosis (Takai et al., 1992; Ferro-Novick and Jahn, 1994; Fischer von Mollard et al., 1994a). In order to investigate a possible role for these proteins in the regulation of insulin secretion we analysed by western blotting the expression of Rab3A, Rab3B and Rab3C in homogenates of rat and human pancreatic islets and of the insulin-secreting cell lines RINm5F, INS-1 and HIT-T15. Both Rab3B and Rab3C were present in all of the cell extracts analysed (Fig. 1B,C). The level of expression of Rab3C was about equal among the different preparations (Fig. 1C). Similar results were obtained for Rab3B, except that, compared to the other cells, this protein was less abundant in RINm5F cells (Fig. 1B). We then analysed the expression of Rab3A using an

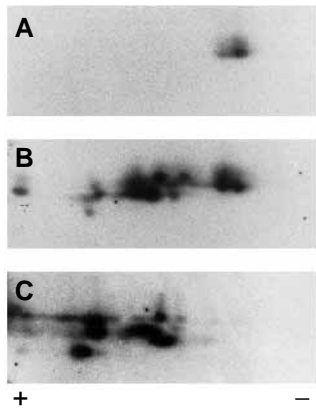


**Fig. 1.** Expression of (A) Rab3A, (B) Rab3B and (C) Rab3C in insulin-secreting cells. Homogenates (100  $\mu\text{g}$ ) of RINm5F (RIN), INS-1 (INS), HIT-T15 (HIT) cells and of rat (RI) and human (HI) pancreatic islets were analysed by PAGE and blotted on nitrocellulose membranes. Rab3A, Rab3B and Rab3C were detected by incubating the nitrocellulose with specific polyclonal antibodies. The antigen-antibody complex was detected by chemiluminescence using an anti-rabbit antibody coupled to horseradish peroxidase.

isoform specific antibody that does not cross-react with recombinant Rab3B and Rab3C (A. Zahraoui, unpublished observation). We found that Rab3A was present in rat pancreatic islets and in the two rat insulin-secreting cell lines RINm5F and INS-1 but was only barely detectable in the hamster cell line HIT-T15 and undetectable in human islets (Fig. 1A). These results are not due to the difference in the amino acid sequence between species because the antibody recognized Rab3A equally well in brain homogenates of rat, human and hamster origin (Fig. 2). In addition, Rab3A could also be detected in hamster  $\beta$ -cells (not shown) and the ability of the Rab3A antibody to recognize human Rab3A by western blotting analysis could also be demonstrated by overexpressing the human isoform of this small G-protein in HIT-T15 cells (not shown). We confirmed the absence of Rab3A in human islets by two-dimensional gel electrophoresis followed by the GTP-overlay technique (Regazzi et al., 1991). This method enabled us to visualize several small G-proteins (Fig. 3). Most of them were found in both rat and human islets, however, the doublet migrating at 27 kDa/pI 5.2-5.4 was detected exclusively in rat pancreatic islets. The migration of this doublet exactly matched that of Rab3A purified from bovine brain (Fig. 3A,B). Two different antibodies raised against rat Rab3A and one antibody raised against bovine Rab3A reacted with this doublet (not shown). The 27 kDa/5.2-5.4 spots were also present in RINm5F and INS-1 that express Rab3A but not in rat pancre-



**Fig. 2.** Detection of Rab3A and of rabphilin-3A in homogenates of human, rat and hamster brain. Homogenates (100  $\mu\text{g}$ ) of (lanes 1) human, (lanes 2) rat and (lanes 3) hamster brain were separated on PAGE and blotted on nitrocellulose membranes. Rab3A (A) and rabphilin-3A (B) were detected using polyclonal antibodies directed against each of these proteins. The antigen-antibody complex was detected by chemiluminescence using an anti-rabbit antibody coupled to horseradish peroxidase.

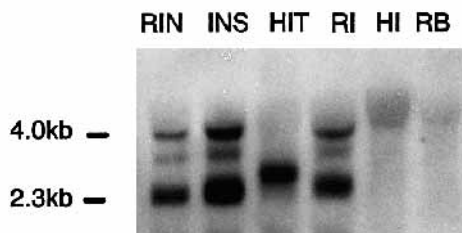


**Fig. 3.** Comparison between small G-proteins expressed in rat and human pancreatic islets. Rab3A purified from bovine brain (400 ng) (A) and homogenates of rat (B) and human (C) pancreatic islets (300 µg) were resolved by two-dimensional PAGE and blotted on nitrocellulose membranes. Rab3A and other small G-proteins were visualized by autoradiography after incubation of the nitrocellulose in the presence of radioactive GTP.

atic acini and rat liver that do not contain this small G-protein (not shown). We also determined the expression in insulin-secreting cells of Rab3D, an additional member of the Rab3 family. Northern blotting analysis indicated that Rab3D mRNAs with the expected size (2.3 and 4.0 kb) can be found in INS-1 cells, RINm5F cells and in rat pancreatic islets but not in human islets (Fig. 4). An additional transcript of about 3.2 kb was also observed after longer exposure of the films (Fig. 4). In contrast, in HIT-T15 cells a single mRNA species of approximately 2.6 kb could be observed. Similar results were obtained using a different probe that included the cDNA coding for the C-terminal portion of the protein and part of the untranslated region immediately after the stop codon (not shown).

Rab3A has been localized almost exclusively in neuroendocrine cells (Takai et al., 1992; Ferro-Novick and Jahn, 1994; Fischer von Mollard et al., 1994a). Pancreatic islets are composed of several cell types with different functions. It was felt important to investigate whether Rab3A is indeed expressed in  $\beta$ -cells. As depicted in Fig. 5, when rat islet cells were separated into  $\beta$ - and non- $\beta$ -cells by FACS, Rab3A was detectable only in  $\beta$ -cells.

The localization of Rab3A on synaptic vesicles and its involvement in the control of neurotransmitter release has been demonstrated by different studies (Fischer von Mollard et al., 1994a). The presence of Rab3A on dense core secretory



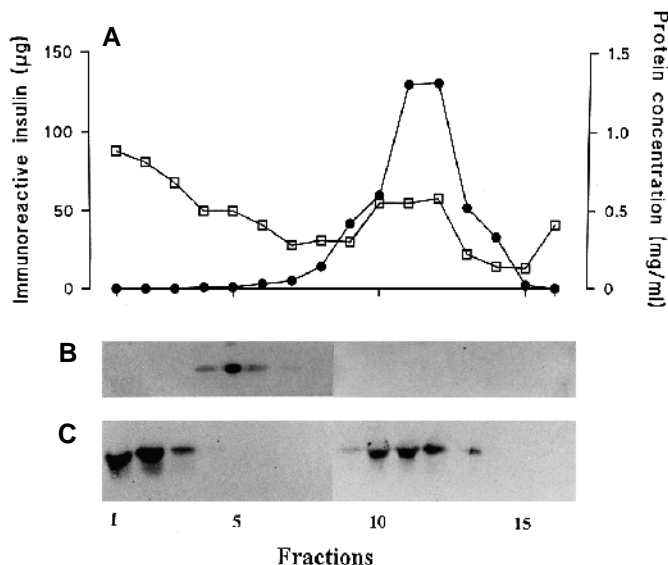
**Fig. 4.** Expression of Rab3D mRNA in insulin-secreting cells. Total RNA (12 µg) from RINm5F, INS-1, HIT-T15, rat pancreatic islets (RI) and human pancreatic islets (HI) and whole rat brain (RB) was analysed by northern blotting (1% agarose gels) using a radiolabeled cDNA fragment of Rab3D. Before hybridization, the filters were reversibly stained with methylene blue to detect the presence of equal amounts of intact 28 S and 18 S ribosomal RNAs. The film was exposed for a long period to demonstrate the absence of Rab3D mRNA in human pancreatic islets.



**Fig. 5.** Cell specific expression of Rab3A in rat pancreatic islets. The cells from rat pancreatic islets were isolated by trypsinization and separated into two populations, non- $\beta$  (NB) and  $\beta$ -cells (B) by fluorescence-activated cell sorting as described (Rouiller et al., 1990). After homogenization 50 µg of the cell extract was separated on a 13% polyacrylamide gel and the expression of Rab3A was assessed by western blotting as described in Fig. 1.

granules is, however, more controversial (Darchen et al., 1990, 1995; Fischer von Mollard et al., 1990). Apart from secretory granules containing insulin,  $\beta$ -cells have been shown to possess synaptic-like vesicles containing the neurotransmitter GABA (Reetz et al., 1991; Sorenson et al., 1991; Thomas-Reetz et al., 1993). We examined whether Rab3A is present on synaptic-like vesicles and/or on insulin-containing granules by loading INS-1 extracts on a sucrose gradient permitting the separation of these two organelles (Reetz et al., 1991). As shown in Fig. 6 synaptophysin, a marker of synaptic-like vesicles, was recovered in fractions 4-6 while insulin was found in fractions 10-12. Rab3A was detected in the first 2-3 fractions, containing cytosolic proteins, and in the fractions corresponding to the insulin-containing granules but was undetectable in the fractions containing synaptic-like vesicles. Thus, in insulin-secreting cells, Rab3A is mainly sedimenting with secretory granules.

To further demonstrate the association of Rab3A with insulin-containing granules the distribution of this small G-protein in rat pancreatic islets was analysed by immunofluorescence and by immunoelectron microscopy. Inspection of



**Fig. 6.** Subcellular distribution of Rab3A in INS-1 cells. INS-1 cells were homogenized by nitrogen cavitation. After elimination of nuclei and cell debris synaptic-like microvesicles and insulin-containing secretory granules were separated on a continuous sucrose gradient (0.45-2 M). (A) The amount of insulin detected by radioimmunoassay and the protein concentration in the different fractions of the gradient. Samples (80 µl) of the gradient fractions were analysed by western blotting with antibodies against synaptophysin (B) and Rab3A (C).

monolayer cultures of pancreatic endocrine cells by immunofluorescence showed a number of cells containing Rab3A immunoreactivity. Fluorescence was located either along peripheral rims or on the cytoplasmic tip of the cell (Fig. 7). Note that no staining was observed in the perinuclear region. By immunoelectron microscopy Rab3A could be localized to the limiting membrane of the insulin secretory granules (Fig. 8). Very scattered gold particles were observed on the clear halo and the dense core of the granules or on cytoplasmic organelles such as mitochondria and crinophagic bodies (Fig. 8).

Overexpression of a Rab3A mutant deficient in GTP hydrolysis (Q81L) was shown to inhibit exocytosis in chromaffin cells and PC12 cells (Holz et al., 1994; Johannes et al., 1994). The involvement of Rab3A in the control of insulin secretion was assessed by transiently cotransfecting HIT-T15 cells with human proinsulin cDNA and with Rab3A wild-type or with Rab3A mutated at position 81. Immunofluorescence studies revealed that under our transfection conditions virtually all the transfected cells (10–15%) coexpress human proinsulin and Rab3A (Fig. 9). No interference between the fluorescein isothiocyanate (FITC) and the rhodamine channel was observed (not shown). Since human insulin C-peptide is coexpressed with the transfected Rab3A and the C-peptide is secreted together with insulin, we were able to monitor exocytosis of the subpopulation of transfected HIT-T15 cells. We have previously demonstrated that human C-peptide is sufficiently different from the endogenous hamster C-peptide to be measured selectively (Lang et al., 1995). Using this approach we found that the overexpression of mutant Rab3A did not significantly affect basal secretion (Fig. 10). In contrast, exocytosis triggered with a mixture of nutrients and the phospholipase C activator bombesin was inhibited by more than 60% (Fig. 10). Transfection of wild-type Rab3A resulted in a similar degree of overexpression as the Q81L mutant, as assessed by western blotting (not shown), but resulted only in a small decrease in stimulated C-peptide secretion (Fig. 10). Rab5A is a small GTPase involved in early endosome fusion (Gorvel et al., 1991; Bucci et al., 1992). Overexpression of wild-type Rab5A and of a GTPase deficient Rab5A mutant (Rab5A Q79L) neither affected basal nor stimulated C-peptide secretion (Fig. 10).

Next we turned to the putative target protein for the GTP-bound form of Rab3A, rabphilin-3A (Shirataki et al., 1993; Li et al., 1994). Because of its characteristics, this protein could play a role in exocytosis, in particular in insulin-secreting cells expressing Rab3A. Consequently, we performed western blotting to search for the presence of rabphilin-3A in pancreatic islets and in the three cell lines. The antibody recognized equally well rat, human and hamster rabphilin-3A (Fig. 2). Despite this, rabphilin-3A was found neither in homogenates of the insulin-secreting cells not expressing Rab3A nor in the extracts of the cells containing this small G-protein (Fig. 11). Similar negative results were obtained when purified synaptic like vesicles or secretory granules from INS-1 cells were analysed (not shown). Nonetheless, in the same blot control lanes show that the antibody recognizes rabphilin-3A in rat brain homogenates as well as the recombinant protein. The latter migrates slightly differently from the endogenous rabphilin-3A (Shirataki et al., 1993) (Fig. 11). These results suggest that the Rab3A effector in rat pancreatic  $\beta$ -cells could be different from that of rat brain.

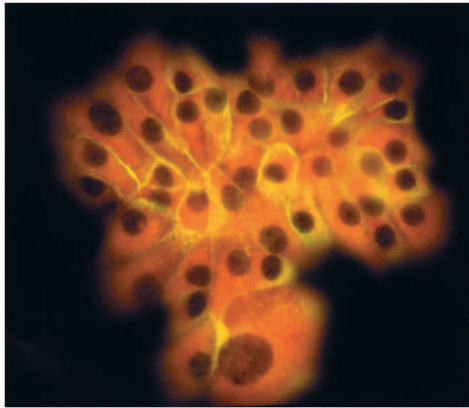
## DISCUSSION

Small G-proteins have been suggested to be involved in the control of exocytosis (Takai et al., 1992; Ferro-Novick and Jahn, 1994; Fischer von Mollard et al., 1994a). Rab3A appears to be the favoured candidate for the regulation of secretion in neurons and in neuroendocrine cells. Thus, this protein has been shown to be associated with synaptic vesicles from which it dissociates during or after the fusion of the vesicles with the plasma membrane (Fischer von Mollard et al., 1991, 1994b). Moreover, in neuroendocrine cells overexpression of wild-type or of mutated Rab3A proteins defective in GTP hydrolysis or in guanine nucleotide binding caused inhibition of exocytosis (Holz et al., 1994; Johannes et al., 1994). Rab3A is almost exclusively expressed in neural and neuroendocrine cells and can, therefore, not function as a regulator of exocytosis in all cell types.

Insulin-secreting cells exhibit some characteristics of neuronal tissue. Thus,  $\beta$ -cells possess synaptic-like vesicles containing the neurotransmitter GABA (Reetz et al., 1991; Sorenson et al., 1991; Thomas-Reetz et al., 1993). In addition, we have shown that VAMP-2, a protein implicated in neurotransmitter release, is also involved in the control of insulin secretion from pancreatic  $\beta$ -cells (Regazzi et al., 1995b). In this study, we demonstrate that rat  $\beta$ -cells and rat  $\beta$ -cell lines express Rab3A. The expression of Rab3A in primary  $\beta$ -cells rules out the possibility that the presence of this small G-protein in RINm5F and INS-1 cells is due to the transformed phenotype of the cell lines. Our results are in agreement with the results obtained by Kowluru et al. (1994). In contrast, based on northern blot analysis, a previous report had concluded that Rab3A is involved in the secretion of synaptic-like vesicles but not of insulin secretory granules as RINm5F but not HIT-T15 cells contained Rab3A mRNA (Lankat-Buttgereit et al., 1992). Here we show using different methods that Rab3A is mainly localized on secretory granules. Rab3A could not be detected in the fractions enriched in synaptic-like vesicles after subcellular fractionation of the cells on a sucrose gradient and no labelling was observed in the perinuclear region of the cells where GABA-containing vesicles are localized (Reetz et al., 1991).

Overexpression of a Rab3A mutant deficient in GTP hydrolysis decreases stimulated insulin secretion from HIT-T15 cells. These results are in agreement with those obtained in chromaffin cells (Holz et al., 1994; Johannes et al., 1994) and are consistent with a role for Rab3A in the control of insulin release. As in the case of chromaffin cells the overexpression of wild-type Rab3A had only small effects on exocytosis. The overexpression of wild-type Rab5A and of a GTPase deficient mutant of this G-protein had no effect on insulin secretion indicating that the results obtained with the Rab3A mutant are not due to interference with general regulatory components such as RabGDI.

Human pancreatic islets do not express Rab3A as assessed both by western blotting and by the GTP-overlay technique. These results are in agreement with those recently obtained by others by northern blotting (Lankat-Buttgereit et al., 1994; Inagaki et al., 1994). As shown here, at least two other members of the Rab3 family, namely Rab3B and Rab3C are expressed in human pancreatic islets. We have previously demonstrated that, in insulin-secreting cells, a large proportion



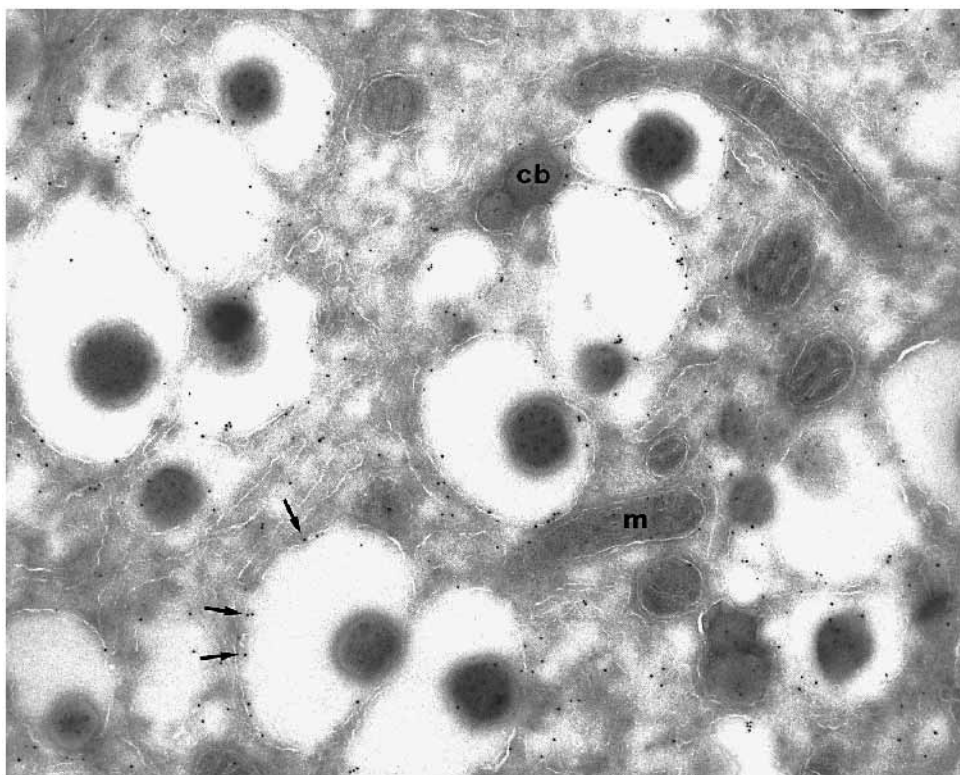
**Fig. 7.** Immunolocalization of Rab3A in the endocrine pancreas by light microscopy. Monolayer cultures of endocrine pancreas were fixed and immunolabeled with anti-Rab3A antibody as described in Materials and Methods. The localization of Rab3A was revealed using an FITC-coupled anti-rabbit antibody.  $\times 185$ .

of Rab3B and Rab3C is found in the cytosol and only a small amount is associated with secretory granules (Regazzi et al., 1992b). However, the possibility cannot be excluded that only a small amount of these Rab3 proteins bound to the granules could suffice to control insulin exocytosis. The respective roles of the members of the Rab3 family in exocytosis remain to be established. Intracellular injection of antisense oligonucleotides targeted to Rab3A mRNA enhances the responsiveness during repetitive stimulations of chromaffin cells (Johannes et al., 1994). Rab3C has recently been shown to copurify with Rab3A on synaptic vesicles and to dissociate

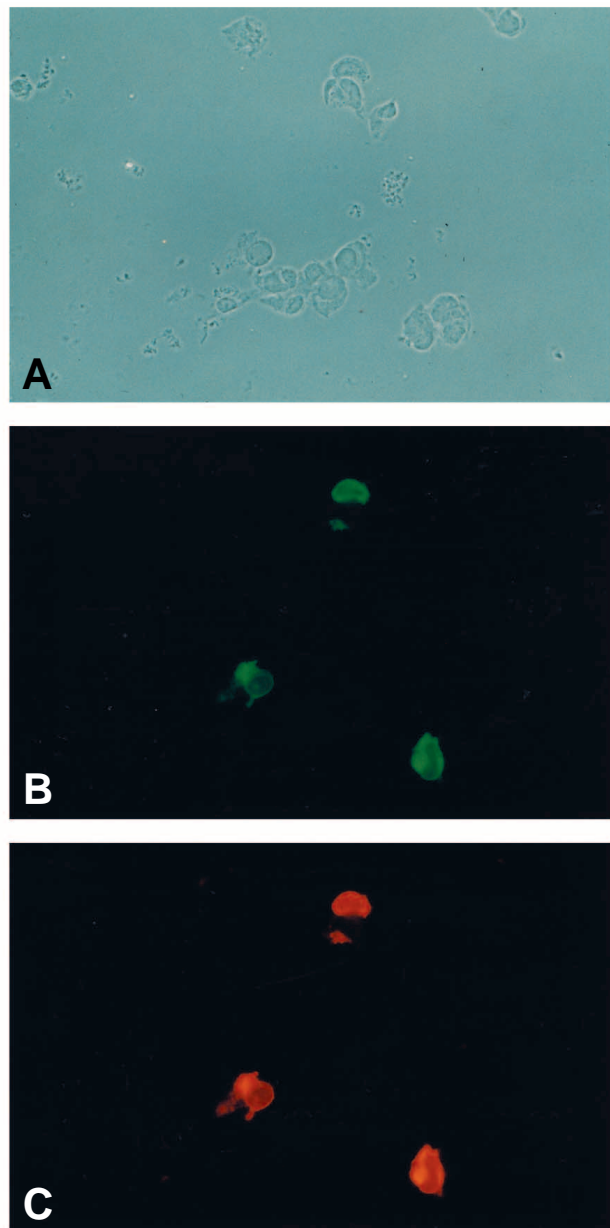
from the vesicles during exocytosis (Fischer von Mollard et al., 1994b). Thus, Rab3C may play a similar role to that of Rab3A preventing exocytosis to occur unless secretion is triggered. On the other hand, in anterior pituitary cells inhibition of Rab3B expression attenuates  $\text{Ca}^{2+}$ -stimulated secretion (Lledo et al., 1993). These results suggest that Rab3A and Rab3C may exert opposite effects on exocytosis from Rab3B. Taken together these observations indicate that in cells containing more than one member of the Rab3 family the regulation of secretion could result from the interplay between several Rab3 isoforms. Thus, in humans it is conceivable that Rab3B and Rab3C may substitute for Rab3A in the control of insulin exocytosis.

In addition to Rab3A, Rab3B and Rab3C, rat pancreatic islets as well as RINm5F and INS-1 cells also contain Rab3D mRNAs. The sizes of the two major transcripts detected by northern blotting (2.3 and 4.0 kb) correspond to those described for other tissues (Baldini et al., 1992, 1995; Elferink et al., 1992). HIT-T15 cells were found to express a different message with an intermediate size ( $\sim 2.6$  kb). In contrast, no Rab3D message could be detected in human pancreatic islets. The sequence of human Rab3D is unknown but it is likely to be very similar, at least in the coding region, to rat Rab3D. Since the probe that was used in this study corresponds to about 300 nucleotides coding for the carboxyl-terminal domain of the protein it is unlikely that our results are due to the difference in the sequence of rat and human Rab3D. Recently, Rab3A and Rab3D have been shown to be localized on different vesicles in adipocytes and in AtT-20 cells (Baldini et al., 1995; Martelli et al., 1995). Thus, at least in these cells Rab3A and Rab3D may be involved in different pathways of regulated exocytosis.

Overexpression of rabphilin-3A, a putative effector protein interacting with the GTP-bound form of Rab3A enhances cat-

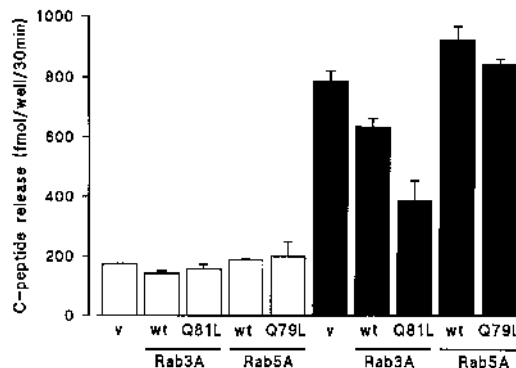


**Fig. 8.** Subcellular localization of Rab3A on ultrathin cryosections of rat pancreatic  $\beta$ -cells immunolabeled by the Protein A-gold method. The micrograph shows a cytoplasmic region containing insulin secretory granules. Gold particles appear selectively associated to the limiting membrane of the secretory granules (arrows). Very scattered gold particles can also be observed on the clear halo and the dense core of the granules or on cytoplasmic organelles such as mitochondria (m) and crinophagic bodies (cb).  $\times 36,000$ .

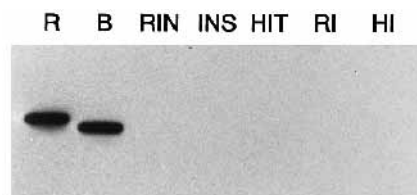


**Fig. 9.** Coexpression of human insulin and Rab3A Q81L in transfected HIT-T15 cells. HIT-T15 cells were transiently cotransfected with human proinsulin cDNA and with the cDNA of cMyc-tagged Rab3A mutated at position 81 (Q81L). After two days of culture the cells were fixed and incubated with an antibody directed to human insulin C-peptide and with an antibody against c-Myc. The expression of human C-peptide was detected with an FITC-conjugated anti-rat antibody while Rab3A was localized with a rhodamine-conjugated anti-mouse antibody. (A) Phase contrast; (B) human insulin C-peptide; (C) human c-Myc.

echolamine secretion from chromaffin cells (Chung et al., 1995). Rabphilin-3A mRNA has been detected by northern blotting in the two insulin-secreting cell lines MIN6 and HIT-T15, whereas RINm5F cells and rat pancreatic islets were revealed to be negative (Inagaki et al., 1994). In this study, we were unable to detect rabphilin-3A in  $\beta$ -cells. Since the signal in HIT-T15 cells was several orders of magnitude lower than



**Fig. 10.** Effect of the overexpression of wild-type Rab3A and Rab5A and their respective GTPase deficient mutants on insulin secretion. HIT-T15 cells were transiently cotransfected with human insulin cDNA and with the vector alone (v) or containing the cDNA coding for wild-type Rab3A, Rab5A or the mutants Rab3A Q81L and Rab5A Q79L. After two days of culture the cells were preincubated for 30 minutes in Krebs-Ringer solution and incubated for another 30 minute period in Krebs-Ringer solution alone (open bars) or supplemented with 10 mM glucose, 5 mM leucine, 5 mM glutamine and 100 nM bombesin (filled bars). Exocytosis from transfected cells was assessed by measuring human C-peptide release. The results are given as mean  $\pm$  s.e.m. of three independent experiments.



**Fig. 11.** Rabphilin-3A expression in insulin-secreting cells. Homogenates (100  $\mu$ g) of RINm5F (RIN), INS-1 (INS), HIT-T15 (HIT) cells and of rat (RI) and human (HI) pancreatic islets were resolved by PAGE, blotted on nitrocellulose membranes and analysed using a specific polyclonal antibody. Recombinant rabphilin-3A (R) and a rat brain homogenate (B) were also included as positive control.

that obtained in brain, the possibility cannot be excluded that the protein is expressed at very low levels in this particular cell line. Mice, in which the Rab3A gene was mutated by homologous recombination, do not express Rab3A and show a decreased level of rabphilin-3A in the brain (Geppert et al., 1994). Thus, at least in the brain, the expression of Rab3A influences the turnover rate of rabphilin-3A. However, in pancreatic  $\beta$ -cells, despite the presence of large amounts of Rab3A (close to those found in whole brain homogenates) rabphilin-3A cannot be detected. This may indicate that the role of rabphilin-3A is restricted to synaptic transmission or that a different protein but with homologous functions is present in pancreatic  $\beta$ -cells. Along this line a spliced variant of rabphilin-3A has been identified in bovine chromaffin cells (Chung et al., 1995). At present we do not know whether our polyclonal antibody would recognize this rabphilin-3A isoform since this splicing variant has a six amino acid insert in the region of the protein that was used to immunize the animals (Shirataki et al., 1993).

In conclusion, we have shown that Rab3A is expressed in rat

islet  $\beta$ -cells, where it is localized on secretory granules, but not in islet non- $\beta$ -cells. We have also demonstrated the functional implication of this small G-protein in the control of insulin secretion in a hamster cell line. Species specific variation in the expression of the members of the Rab3 family has become apparent. Future work should clarify the exact role of each of the different Rab3 proteins in the control of exocytosis.

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