

Rab3a controls exocytosis in cholecystokinin-secreting cells

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Abstract The expression of rab3A and rab3D isoforms in the enteroendocrine, cholecystokinin-secreting, cell lines STC-1 and GLUTag is here demonstrated. In contrast, rab3B is undetectable in these two cell lines, and rab3C is only slightly expressed in GLUTag cells. Using a transient co-transfection system with human growth hormone as reporter protein, we show that overexpression of the GTPase-deficient mutant rab3AQ81L, but not rab3DQ81L, significantly decreases human growth hormone secretory responses to various agonists in STC-1 cells. These results indicate that endocrine cell lines of intestinal origin express rab3A and rab3D proteins, but the GTP-bound form of rab3A only acts as a negative modulator in the control of cholecystokinin secretion from STC-1 cells. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Exocytosis; rab3; Cholecystokinin; Enteroendocrine cell

1. Introduction

Cholecystokinin (CCK) exerts important actions in the digestive tract [1]. Agonist-induced stimulation of CCK release from intestinal endocrine I-cells is achieved through activation of protein kinase A and C, and of calcium channels [2,3]. This was observed as well in the enteroendocrine cell line STC-1 [4,5], in which the role of the extracellular signal-regulated kinase (ERK)1/ERK2 transduction pathway and of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins in the control of CCK release was demonstrated [6,7]. Further information on exocytosis processes in intestinal-type endocrine cells is presently lacking.

Small GTP-binding proteins of the rab3 family appear to play a central role in vesicle trafficking prior to SNARE-mediated docking in neurons and endocrine cells, under the control of several regulatory proteins [8]. Rab3A has been shown to modulate exocytosis in several systems through overexpression of GTPase-deficient mutants of rab3A in adrenal chromaffin cells [9], PC12 cells [10], rat basophilic leukemia cells

[11], insulinoma cells [12,13], or by using antisense oligonucleotides directed to rab3A mRNA in chromaffin cells [10,14], or through knockout of rab3A gene for the study of hippocampal neuronal stimulation [15]. Insulin-secreting cells also express rab3B, rab3C and rab3D, and transient overexpression of GTPase-deficient mutants inhibited insulin secretion [12,13].

The expression and potential role of rab3 proteins have not been investigated in intestinal-type endocrine cells. This was here studied in the enteroendocrine, CCK-secreting STC-1 cell line using a co-transfection system with human growth hormone (hGH) as reporter protein [16].

2. Materials and methods

2.1. Antibodies and plasmids

Anti-rab3A and anti-rab3C antisera were generated by immunizing rabbits with synthetic C-terminal peptides of rat rab3A (residues 204–217) and rab3C (residues 211–224) conjugated with keyhole limpet hemeocyanin, and the IgGs were purified on protein A Sepharose. Rabbit polyclonal anti-rab3D [17] was obtained from Dr. G. Baldini (Columbia University, New York, USA). Monoclonal anti-rab3A (clone 42.2, used for immunocytochemistry) and polyclonal anti-chromogranin A antibodies were obtained from Synaptic System (Göttingen, Germany) and DAKO Diagnostics (Zug, Switzerland), respectively. Polyclonal anti-rab3B antiserum and monoclonal anti-human-c-myc antibody (clone 9E10) were from Santa Cruz Biotechnology (Tebu, le Perray-en-Yvelines, France) and from Invitrogen (Leek, The Netherlands), respectively. hGH was detected with rabbit anti-human pituitary growth hormone polyclonal antibody (National Hormone and Pituitary Program, NIDDK, National Institute of Health, Bethesda, MD, USA). The Q81L mutant of rab3D, containing the N-terminal myc epitope, was subcloned in the mammalian expression vector pcDNA3 (Invitrogen). The generation of Q81L mutant of human myc-tagged rab3A has been previously described [10]. The two mutants are deficient for GTP hydrolysis. hGH was expressed using pXGH5 vector (Nicholas Institute, San Juan Capistrano, PR, USA).

2.2. Cell lines and culture conditions

Two intestinal CCK-expressing cell lines were used in the present study. The STC-1 cell line was derived from an intestinal endocrine tumor in a double-transgenic mouse carrying the rat insulin promoter linked to the simian virus 40 large T antigen and to the polyoma virus small t antigen [4]. The GLUTag cell line was derived from a large bowel tumor in a transgenic mouse carrying the glucagon/simian virus 40 large T antigen transgene [18]. The other cell lines used in this study were RINm5F (rat β cell line [19]), CA-77 (rat medullary thyroid carcinoma [20]), IEC-17 (rat intestinal epithelial crypt cell line [21]), and the human colon adenocarcinoma cell lines Caco-2 (ATCC HTB 37), HT29 (ATCC HTB 38) and HT29-16E [22]. The following cell culture conditions were used: STC-1 cells in RPMI with 5% fetal calf serum (FCS); RINm5F cells in RPMI with 10% FCS; GLUTag, HT-29, HT29-16E and Caco-2 cells in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS; CA-77 in Ham-F-10/DMEM (1:1) with 10% FCS. All culture media were supplemented with

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Abbreviations: CCK, cholecystokinin; hGH, human growth hormone; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor

2 mM glutamine and antibiotics (100 IU/ml penicillin and 50 μ M streptomycin) and cells were grown in a humidified CO₂:air (5:95%) incubator at 37°C.

2.3. Western blotting

STC-1 cells (3×10^5) were seeded into 6-well culture plates and either untreated or transfected as described below. Two days later, cells were homogenized in 320 mM sucrose, 10 mM HEPES (pH 7.4), 0.2 mg/ml phenylmethylsulfonyl fluoride (PMSF), 20 μ M leupeptin and 100 U/ml aprotinin, before centrifugation ($900 \times g$, 10 min, 4°C) to remove nuclei and cell debris. To prepare cytosolic and membrane fractions, the postnuclear supernatant was centrifuged ($100\,000 \times g$, 60 min, 4°C). The resulting supernatant represented the soluble fraction. The membrane pellet was solubilized in Laemmli buffer, and represented the particulate fraction. To prepare whole-cell extracts, cells were lysed in cold solubilization buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM Na₃VO₄, 100 mM NaF, 100 U/ml aprotinin, 20 μ M leupeptin and 0.2 mg/ml PMSF. Lysates were separated on 12% SDS–polyacrylamide gels. Western blotting was performed as described previously [7]. The antigen–antibody complex was detected by chemiluminescence using appropriate secondary antibody coupled to horseradish peroxidase.

2.4. Immunocytochemistry

Endogenous and mutant rab3 proteins, as well as chromogranin A (secretory granule marker), were detected by indirect immunofluorescence. Untreated or transfected STC-1 cells were grown on glass coverslips coated with 0.2 mg/ml poly-L-lysine (Sigma, Saint Quentin Fallavier, France) and 17 μ g/ml laminin (Boehringer-Mannheim, Germany). Cells were fixed with 4% formaldehyde for 20 min and incubated for 2 h at room temperature with the appropriate antibodies, i.e. anti-chromogranin A (1:1000), anti-rab3A (1:1000) or anti-c-myc (1:30) diluted in phosphate-buffered saline with 0.3% Triton X-100, 2% bovine serum albumin (BSA) and 10% goat serum. The coverslips were then washed and exposed for 30 min to anti-rabbit and anti-mouse antibodies conjugated to Oregon green and Cy3, respectively. Fluorescence was visualized by confocal microscopy (Leica model TCS NT, Lasertechnik, Heidelberg, Germany). Single-section images of 512×512 pixels were taken with a $40 \times$ objective, NA 1.32.

2.5. Transfection of STC-1 cells

Cells were transiently co-transfected with 1 μ g pXGH5 together with either 5 μ g pcDNA3, 5 μ g pcDNA3/rab3AQ81L or 5 μ g pcDNA3/rab3DQ81L using the ESCORT[®] transfection reagent (Sigma) according to the manufacturer's instructions. STC-1 cells were incubated with the Escort-plasmid DNA mixture for 6 h at 37°C, then replaced in fresh complete RPMI medium for an additional 48-h period before secretion experiments. In these conditions, 3–5% of the STC-1 cells had been transfected, and 70–80% of the hGH-expressing cells also expressed the myc-tagged protein (not shown).

2.6. Secretion experiments and peptide determination

On the day of the experiment, culture medium was removed and plates were incubated at 37°C with Krebs–Ringer bicarbonate buffer (118 mM NaCl, 20 mM HEPES, 12 mM NaHCO₃, 10 mM glucose, 4.6 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 0.2% (w/v) BSA, pH 7.4) and the tested agents for 1 h. The incubation was stopped by cooling the cell culture plates on ice. The medium was collected, immediately centrifuged at 4°C for 5 min at $100 \times g$ to remove any detached cells, and the supernatants were frozen at -20°C for subsequent radioimmunoassays (RIAs). For determination of peptide cell content, cells were homogenized in 2 ml extraction medium (2 M acetic acid, 20 mM HCl). The homogenate was sonicated, boiled at 100°C for 10 min, neutralized with ammonia, and stored at -20°C . hGH was determined by RIA using the manufacturer's instructions. Labeling of hGH was performed with ¹²⁵Ia using chloramine-T, and tracer was purified on a G-10 Sephadex column. The final dilution of the anti-serum was 1:1 500 000. The detection limit and the ID₅₀ were 60 pM and 450 pM, respectively. CCK immunoreactivity was measured with a previously described RIA [6,23].

Secretion was expressed as the percentage of the total cellular hGH or CCK that was released into the medium. Control values were referred as 100 to standardize results with the same plasmids from repeated experiments. In these conditions, there was usually 10–15 ng hGH and 6–8 ng CCK contained by one million cells. All values were

expressed as means $\pm$ S.E.M. Results were analyzed by one-way analysis of variance followed by post-hoc comparison of Fisher. Differences between two means with a *P* value < 0.05 were regarded as significant.

3. Results

3.1. Expression of rab3 proteins in enteroendocrine cells

rab3A and rab3B were expressed in the CCK-producing cell lines STC-1 and GLUTag (Fig. 1A). As expected, the presence of rab3A, but not rab3D, was revealed in rat brain extracts and in bovine adrenal chromaffin cells [9]. rab3C was absent in STC-1 cells, and only slightly expressed in GLUTag cells (Fig. 1A). rab3B was undetectable by Western blotting in the two enteroendocrine cell lines (not shown).

To assess the selectivity of rab3A and rab3D expression in enteroendocrine cells, different intestinal non-endocrine cell lines were tested, comparatively to non-intestinal endocrine cell lines (Fig. 1B). rab3A and rab3D were not detected in the colon adenocarcinoma cell lines Caco-2, HT29 and HT29-16E, but were expressed in the β cell line RINm5F [24], as well as in CA-77 medullary thyroid carcinoma cells that transcribe the CCK gene [20]. Rab3D alone was expressed in the non-transformed intestinal epithelial cell line IEC-17.

3.2. Cytosol/membrane distribution of rab3A and rab3D in STC-1 cells

The cytosol/membrane distribution of rab3A and rab3D in the enteroendocrine cell line STC-1 was investigated by Western blotting and indirect immunofluorescence experiments. After cell lysis and separation of the two fractions by centrifugation, rab3A immunoreactivity was largely associated with

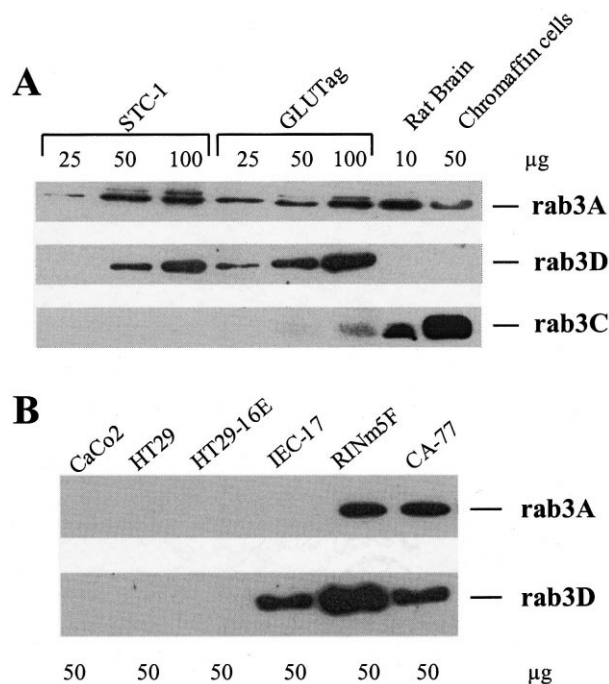


Fig. 1. Expression of rab3 proteins in cells and tissues of different origin. Indicated amounts of solubilized extracts from enteroendocrine cells (A) and non-enteroendocrine cells (B) were subjected to SDS–PAGE, and rab3 proteins were detected by Western blotting. Results are representative of three independent experiments.

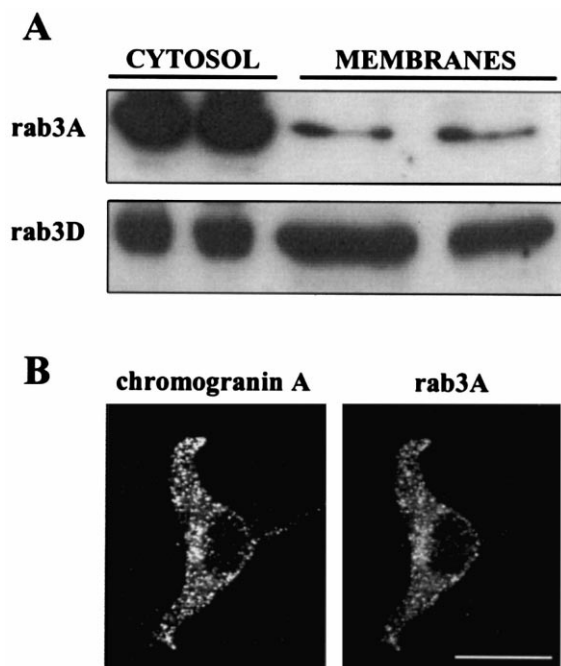


Fig. 2. Expression of rab3A and rab3D in membrane and cytosol fractions in STC-1 cells. A: After cell lysis and elimination of nuclei and cell debris, membranes and cytosol were separated by centrifugation as described in Section 2, and 50 μ g of each fraction (in duplicate) were subjected to SDS-PAGE. rab3A and rab3D were detected by Western blotting. The results are representative of three independent experiments. B: STC-1 cells were analyzed by indirect immunofluorescence followed by confocal microscopy using a polyclonal antibody directed against the secretory granule marker chromogranin A and a monoclonal antibody directed against rab3A. The scale bar corresponds to 10 μ m.

the cytosolic fraction (Fig. 2A), although there was a weak immunoreactive band in the membrane fraction. Immunofluorescence experiments followed by confocal microscopy revealed that the membrane-bound pool of rab3A co-localizes with chromogranin A, indicating that at least a fraction of the GTPase associates with secretory granules (Fig. 2B). rab3D was more evenly distributed between soluble and particulate compartments, with comparable amounts of immunoreactive protein detected in cytosolic and membrane fractions (Fig. 2A). Because of the lack of appropriate antibodies, we were unable to assess the precise localization of membrane-bound rab3D by immunofluorescence. However, membrane-bound rab3D is most probably also localized on secretory granules, because myc-tagged wild-type rab3D transfected in STC-1 cells co-localizes with chromogranin A (not shown).

3.3. Transient expression of hGH and mutant rab3 proteins in STC-1 cells

Myc-tagged rab3A and rab3D mutant proteins were detected in transfected cells by Western blotting and indirect immunofluorescence experiments (Fig. 3). Although the transfection conditions were identical for the two constructs, rab3DQ81L was more efficiently expressed than rab3AQ81L (Fig. 3A). Cytosol/membrane fractionation showed that rab3 mutant proteins had a relatively similar distribution as endogenous proteins (Fig. 3B). By Western blotting, rab3AQ81L was found in the cytosolic fraction, but remained undetectable in the particulate fraction. This was probably due to a lack of

assay sensitivity, because confocal microscopy revealed that at least part of myc-tagged rab3AQ81L co-localizes with secretory granules (Fig. 3C, upper panel). rab3DQ81L was predominantly found in the cytosol, but was detectable both in the soluble and in the particulate fractions (Fig. 3B). Analysis by immunofluorescence and confocal microscopy of STC-1 cells expressing myc-tagged rab3DQ81L demonstrated that, as was already the case for rab3AQ81L, the rab3D mutant co-localizes with chromogranin A (Fig. 3C, lower panel). We also evaluated the ratio between transiently expressed rab3 mutants and their endogenous counterparts. As shown in Fig. 4, rab3AQ81L (upper panel) and rab3DQ81L (lower pan-

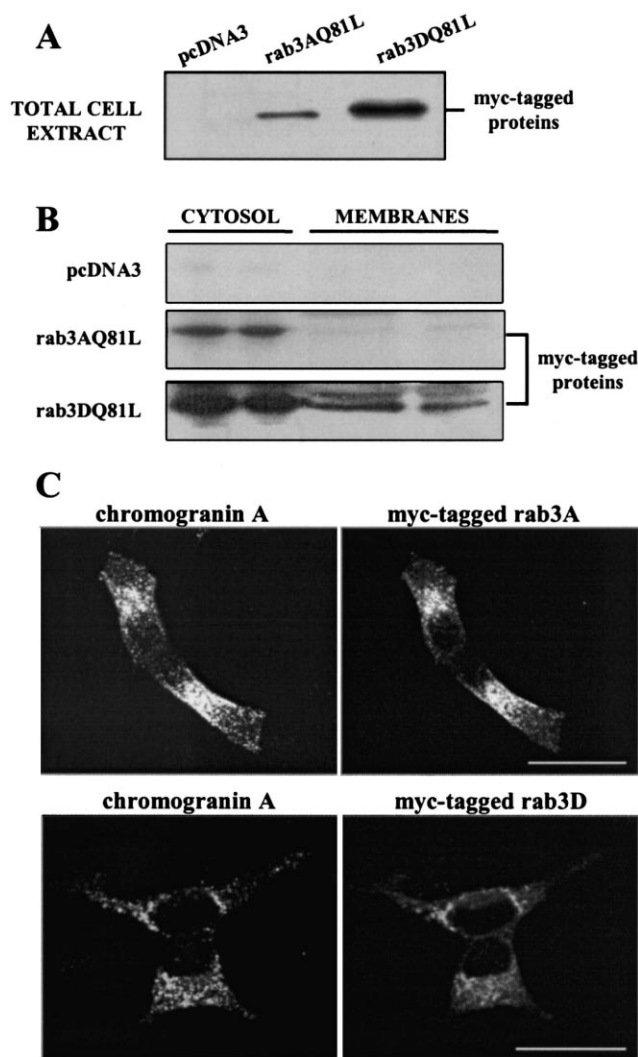


Fig. 3. Expression of rab3A and rab3D mutants in transfected STC-1 cells. Cells were transiently co-transfected with a plasmid encoding hGH (pXGH5), and with either the empty vector pcDNA3, or plasmids encoding myc-tagged rab3AQ81L or myc-tagged rab3DQ81L. Cells were lysed and (A) 100 μ g of total cell extracts or (B) cytosol and membrane fractions (75 μ g, in duplicate) were subjected to Western blot using an anti-c-myc antibody. The results are representative of three independent experiments. C: The subcellular distribution of myc-tagged rab3AQ81L and rab3DQ81L was determined by double-immunofluorescence staining followed by confocal microscopy. The rab3 mutants were localized using a monoclonal anti-c-myc antibody. The position of secretory granules was visualized with a polyclonal anti-chromogranin A antibody. The scale bar corresponds to 10 μ m.

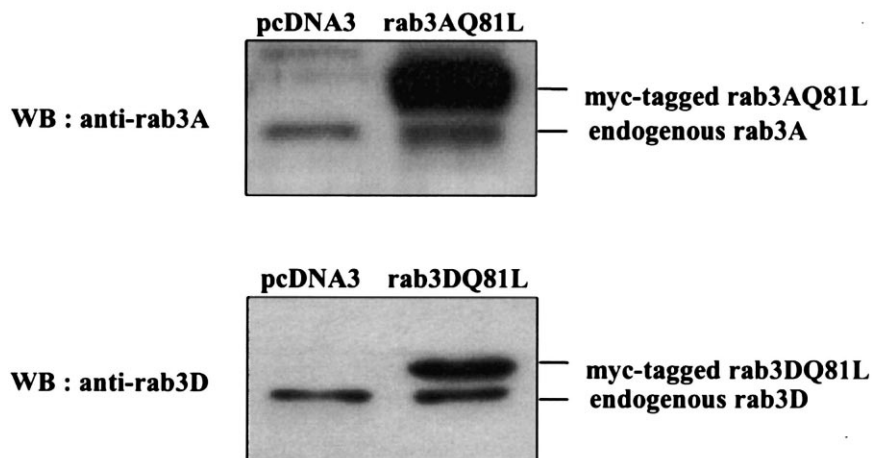


Fig. 4. Comparative expression of mutant and endogenous rab3A (upper panel) and rab3D (lower panel) proteins in transfected STC-1 cells. Cells were transiently co-transfected with pXGH5, and with either pcDNA3 or the plasmid encoding myc-tagged rab3AQ81L or rab3DQ81L. Total cell extracts (100 μ g) were subjected to SDS-PAGE, blotted onto nitrocellulose, and expression of both mutant and native proteins was detected using specific antibodies against rab3A and rab3D. Results are representative of three independent experiments.

el) were both overexpressed compared to the respective endogenous proteins.

Since under our experimental conditions only 3–5% of STC-1 cells are transfected, we used a hGH transient co-transfection assay in which hGH acts as a reporter protein for CCK release only in transfected cells. To determine whether hGH

was released with CCK, STC-1 cells were transfected with pXGH5 and the empty vector pcDNA3. Two days after transfection, a series of stimulants previously studied in non-transfected STC-1 cells [5,6,23] were applied for 1 h. Forskolin/IBMX, PMA, bombesin, meat or albumin egg hydrolysates (MH, AEH) significantly increased both the secretions of CCK (Fig. 5A) and of hGH (Fig. 5B), thus suggesting that expression of pXGH5 did not alter the native secretory mechanisms of STC-1 cells. The percentage of hGH secretion was similar to the percentage of CCK secretion in response to the various agonists, except for the secretion of hGH induced by forskolin/IBMX (145 ± 10) which was less than that of CCK (238 ± 2).

3.4. Inhibition of agonist-induced hGH release by rab3AQ81L but not rab3DQ81L

Transfection of STC-1 cells with mutant rab3AQ81L (Fig. 6A) resulted in a major reduction of hGH release in response to forskolin/IBMX ($78 \pm 11\%$ vs. $145 \pm 10\%$), bombesin ($126 \pm 17\%$ vs. $176 \pm 9\%$), MH ($117 \pm 26\%$ vs. $189 \pm 18\%$) and AEH ($114 \pm 17\%$ vs. $180 \pm 31\%$), as compared to control release rates. The effect of hGH secretion induced by PMA ($186 \pm 23\%$ vs. $237 \pm 20\%$) was less pronounced. Basal hGH release was not affected. In contrast, overexpression of mutant rab3DQ81L did not alter agonist-stimulated hGH release (Fig. 6B).

4. Discussion

Information on the exocytosis processes in gut endocrine cells remains scarce. By immunocytochemistry, the v-SNARE synaptobrevin, and the t-SNAREs syntaxin-1 and SNAP-25 were detected in human intestine [25]. These SNARE proteins were found to be involved in Ca^{2+} -induced CCK secretion in the enteroendocrine cell line STC-1 [7]. These data suggest the possible implication of proteins of the rab3 GTPase family, since its members regulate the formation and the stabilization of SNARE complexes in secretory organelles of neurons and non-intestinal endocrine cells [26]. The present study shows that two enteroendocrine, CCK-secreting cell lines, GLUTag and STC-1, express rab3A and rab3D.

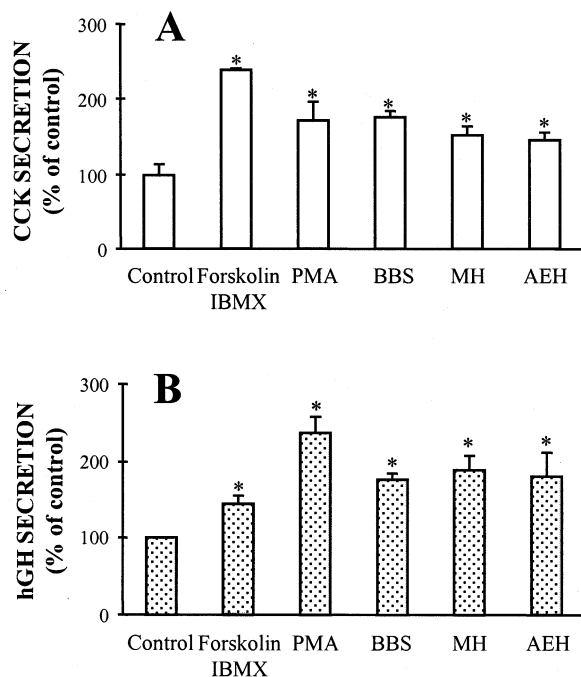


Fig. 5. Secretion of endogenous CCK (A) and ectopically expressed hGH (B) from transfected STC-1 cells. Cells were co-transfected with pXGH5 and pcDNA3. After two days of culture, cells were washed and incubated at 37°C for 1 h in Krebs–Ringer bicarbonate buffer alone (control), or supplemented with forskolin/IBMX (10^{-4} M/ 10^{-3} M), 10^{-7} M PMA, 10^{-7} M bombesin (BBS), 2% MH or 2% AEH. CCK and hGH released into the medium and contained in the cells were determined by RIA, and values were calculated in percent of total cell content for each well. Data are the mean $\pm$ S.E.M. of five independent experiments, each performed in triplicate. * $P < 0.05$, as compared to the respective control secretion.

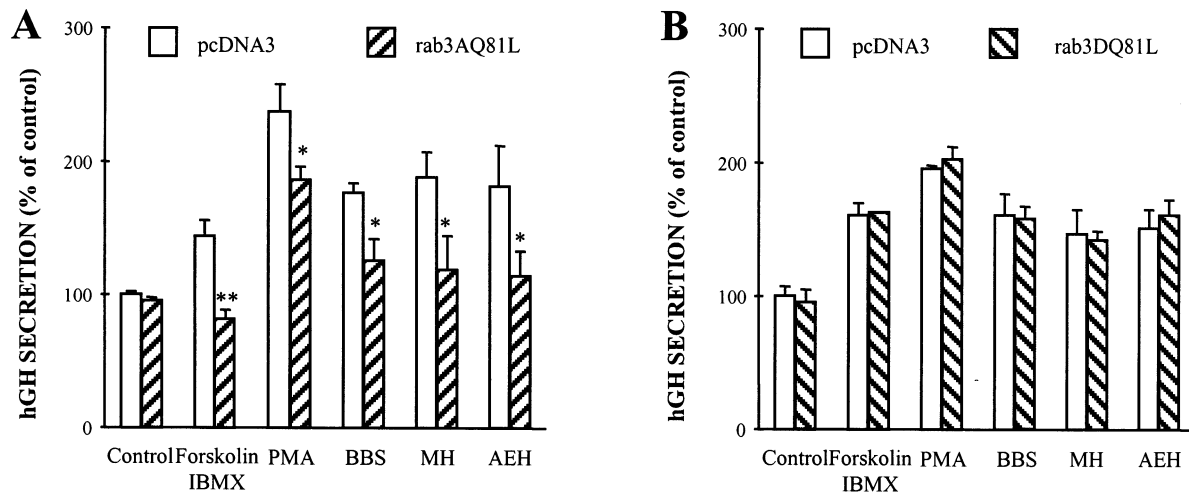


Fig. 6. Effect of rab3 mutants expression of hGH secretion from transfected STC-1 cells. Cells were co-transfected with pXGH5, and with either pcDNA3 or plasmids encoding rab3AQ81L (A) or rab3DQ81L (B). Two days later, cells were treated with forskolin/IBMX (10^{-4} M/ 10^{-3} M), PMA (10^{-7} M), bombesin (BBS, 10^{-7} M), MH (2%) or AEH (2%) for 1 h. hGH released into the medium and contained in the cells were measured by RIA, and values were calculated in percent of total cell content for each well. Data are the mean $\pm$ S.E.M. of five independent experiments, each performed in triplicate. * $P < 0.05$, ** $P < 0.01$, as compared to the corresponding stimulated condition with the empty vector.

Our data extend the list of rab3A-expressing endocrine cells beyond insulin-secreting cells [12,13], chromaffin cells and rat PC12 cells [27]. rab3D is expressed in adipocytes [28], pancreatic and parotid acinar cells [29], gastric chief cells [29], insulin-secreting cells [12] and, as reported here, in intestinal CCK-secreting cells, suggesting that rab3D could be able to regulate exocrine or endocrine secretions according to the cell type. rab3B and rab3C were not detected in the present immunoblots, at variance with observations in insulinoma cells, chromaffin cells and PC12 cells [12,13]. Low-level expression in enteroendocrine cells, undetectable by immunoblotting, cannot be ruled out.

Numerous studies have focused on rab3 proteins subcellular localization, with divergent results depending on the cell type. For example, rab3A was found exclusively in the cytosolic fraction of rat basophilic leukemia cells [30], mainly in membranes of large dense core vesicles from PC12 cells [31] or divided between these two fractions in AtT-20 cells [32]. Possibly, the main cytosolic localization of rab3A in STC-1 cells may be explained by a high level of rab3A associated with a GDP-dissociation inhibitor and then confined to a cytosolic pool [8]. Similarly, rab3D has been detected in both soluble and particulate fractions in different cell types including rat parotid acini [33] and guinea pig gastric chief cells [34]. Thus, our results with enteroendocrine STC-1 cells provide an additional example of complex distribution of rab3 proteins between cytosol and membrane compartments.

The implication of rab3A and rab3D in exocytosis was tested in STC-1 cells using a transient co-transfection system with a plasmid encoding hGH, as reported by others for chromaffin cells [9], PC12 cells [10] and insulinomas [35,36]. The fact that the GTPase-deficient mutant rab3AQ81L inhibits several agonist-induced hGH responses points to a significant contribution of rab3A in the regulation of exocytosis of STC-1 cells. The data presented in this study are consistent with results obtained in chromaffin cells [9] and in insulin-secreting cell lines [12], suggesting that rab3A is involved in the exocytotic process in a number of endocrine cells. In con-

trast, overexpression of rab3DQ81L was ineffective in STC-1 cells, whereas the same mutant of rab3D inhibited exocytosis in insulin-secreting cells [13]. Interestingly, a mutant of rab3D thought to be predominantly in the GTP conformation also impaired peptide secretion in the neuroendocrine AtT-20 cell line [17]. Possibly, the effect of rab3D on exocytosis is critically dependent on cell type. The lack of effect of rab3DQ81L in our system is not due to differential targeting of rab3A and rab3D mutants. In fact, in STC-1 cells both rab3AQ81L and rab3DQ81L associate with secretory granules. Our data can also not be explained by a difference in the absolute amount of exogenous rab3 expressed in transfected cells. In fact, the rab3D mutant was expressed even better than rab3AQ81L. It should be noted that STC-1 cells contain much more rab3D than rab3A. For this reason, the ratio achieved after transfection between rab3AQ81L and endogenous rab3A is higher than the ratio between rab3DQ81L and native rab3D. Provided that the molecular targets of rab3A and rab3D can discriminate between the two isoforms, this could eventually explain the absence of effect of rab3DQ81L in our system. At present, however, none of the putative targets of rab3 proteins display a selective interaction with rab3A or rab3D.

In neurons and in endocrine cells outside the gut, rab3A could GTP-dependently associate to various proteins, including rabphilin3A [37], rim (rab3A interacting molecules) proteins [38] and rabin [39]. Whether these proteins are involved in rab3A effects on enteroendocrine cells remains to be studied. In addition to these putative effectors, it was recently shown that rab3 interacts in a Ca^{2+} -dependent manner with calmodulin to regulate exocytosis [35,40]. As bombesin and protein hydrolysates, potent stimulants of CCK release in I-cells [2] and in STC-1 cells [23] require intracellular calcium to exert their effect. An attractive hypothesis could be the formation of a rab3A- Ca^{2+} /calmodulin complex, which could contribute to a fine tuning of CCK exocytosis. Additional work is needed to identify rab3A effectors in intestinal-type endocrine cells, and to clarify the mechanisms that mediate

the inhibitory effect of rab3A on agonist-induced CCK secretion.

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