Distinct role of Rab3A and Rab3B in secretory activity of rat melanotrophs

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Rupnik M, Kreft M, Nothias F, Grile S, Bohanovic LK, Johannes L, Kiauta T, Vernier P, Darchen F, Zorec R. Distinct role of Rab3A and Rab3B in secretory activity of rat melanotrophs. Am J Physiol Cell Physiol 292: C98–C105, 2007; doi:10.1152/ajpcell.00005.2006.—Members of the Rab3 (A–D) subfamily of small GTPases are believed to play a key role in regulated exocytosis. These proteins share ~80% identity at the amino acid level. The question of whether isoforms of Rab3 are functionally redundant was the subject of this study. We used RT-PCR analysis, in situ hybridization histochemistry, and confocal microscopy-based analysis of immunocytochemistry to show that rat melanotrophs contain about equal amounts of Rab3A and Rab3B transcripts as well as proteins. Therefore, these cells are a suitable model to study the subcellular distribution and the role of these paralogous isoforms in regulated exocytosis. Secretory activity of single cells was monitored with patch-clamp capacitance measurements, and the cytosol was dialyzed with oligodeoxyribonucleotides specific to Rab3A, but not to Rab3B, induced a specific blockage of calcium-dependent secretory responses, indicating an exclusive requirement for Rab3A in melanotroph cell-regulated secretion. Although the injection of purified Rab3B protein was ineffective, the injection of recombinant Rab3A proteins into rat melanotrophs revealed that regulated secretion was stimulated by a GTP-bound Rab3A with an intact COOH terminus and inhibited by Rab3AT36N, impaired in GTP binding. These results indicate that Rab3A, but not Rab3B, enhances secretory output from rat melanotrophs and that their function is not redundant.

There are at least two views of the role of Rab3 isoforms in regulated secretion. Early studies suggested that each of the different Rab3 isoforms may be distributed in specific tissues (2, 16) and that these isoforms may regulate cell type-specific secretory pathways. In contrast, later evidence pointed to the possibility that multiple isoforms of Rab3 were present in a single cell (2, 9, 37, 42). Therefore, it was asked whether more than one Rab3 is required to regulate different aspects of secretion in a single cell, or whether multiple isoforms of Rab3 are functionally redundant (37, 38, 48). It has been reported that Rab3 proteins play an inhibitory role in secretion (8, 11–14, 48). However, recent evidence suggests that these effects could be an indirect consequence of a secondary process. Schütler et al. (37) showed that overexpression of Rab3 proteins to stimulate constitutive exocytosis depleted the pool of dense-core vesicles. In contrast, results obtained in primary cells from the anterior pituitary showed that the presence of Rab3B is required for Ca2+–dependent secretory activity (22, 23). Weber et al. (48) addressed the question of functional redundancy; they transfected the Rab3B isoform into PC-12 cells, which are characterized by an exclusive Rab3A isoform background, and found that the isoforms have similar membrane targeting properties. However, overexpressed Rab3B stimulated, whereas Rab3A modestly inhibited, secretory activity in these cells. The different modes of action of these isoforms may be accounted for by the introduction of a heterologous protein and a “competition” for the endogenous Rab3 partners. An alternative system to study the function of Rab3A and Rab3B isoforms in regulated secretion would be a cell expressing both isoforms.

Here we have studied the function of Rab3A and Rab3B in the secretory activity of pituitary pars intermedia cells, the melanotrophs. Stettler et al. (43) reported that Rab3A and Rab3B isoforms are expressed in these cells. In support of these results, with RT-PCR analysis and in situ hybridization histochemistry we detected both Rab3A and Rab3B transcripts. Moreover, with confocal imaging we observed differential subcellular distribution of Rab3A and Rab3B immunolabeling. Whole cell patch-clamp membrane capacitance was used to assay Ca2+-dependent secretory activity in control cells and in cells where the amount and activity of Rab3A and Rab3B were

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manipulated. The results revealed that in melanotrophs only Rab3A is required for Ca\(^{2+}\)-dependent secretory activity. GTP-bound Rab3A with an intact COOH terminus stimulated, and GDP-bound Rab3A inhibited, regulated secretion in melanotrophs. We also propose that the GTP-bound Rab3A enhances the secretory output in a step prior to exocytosis, possibly by recruiting vesicles containing a specific hormone.

**MATERIALS AND METHODS**

**Cell cultures.** We prepared cells by standard methods (32, 33), plated them on poly-l-lysinated glass coverslips, and incubated them at 36°C, 95% humidity, and 5% CO\(_2\) for 1–10 days. For the experiment, we transferred the coverslips to the recording chamber on an inverted microscope (Opton IM 35). The recording medium in the chamber consisted of (in mM) 131.8 NaCl, 5 KCl, 2 MgCl\(_2\), 0.5 Na\(_2\)HPO\(_4\), 5 NaHCO\(_3\), 10 HEPES-NaOH, 10 t-glucose, and 1.8 CaCl\(_2\), pH 7.2.

**RT-PCR analysis.** We sedimented melanotrophs by centrifugation and isolated poly(A)\(^+\) RNA as described by Chirgwin et al. (5), using the commercially available QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech), and stored it at −80°C until RT-PCR.

First-strand cDNA was synthesized from poly(A)\(^+\) RNA (0.25–2.5 ng) in a mixture of the following reagents (final concentrations): 5 μM random hexamers (Pharmacia Biotech), dNTPs each at 0.5 mM (Pharmacia Biotech), 10 mM dithiothreitol (DTT; Sigma), 33 U of ribonuclease inhibitor (RNasin, Promega), 100 U of SuperScript II RNase H\(^−\) reverse transcriptase (Life Technologies), 50 mM Tris·HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl\(_2\). The reaction volume was 10 μl. Rab3A and Rab3B were identified with the upper primer 5′-CAG AAC TAC TTC AGT TTC [nucleotides 129–149 in the Rab3A sequence (GenBank accession no. X06889)] and nucleotides 157–178 in the Rab3B sequence (P. Vernier) and the lower primer 5′-AAG GAC TCC TCA TTG CCC ATG TC [nucleotides 409–387 in the Rab3A sequence and nucleotides 438–416 in the Rab3B sequence]. These primers yielded a product of 281 bp of both Rab3A and Rab3B. The PCR amplification of the respective fragments was performed with 1 μl of the first-strand cDNA template, adding 10 pmol of primers, dNTPs each at 0.1 mM (final), 10 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris·HCl, pH 8.4, 0.1% gelatin), 1.5 mM MgCl\(_2\) (final), and 5 U of Taq polymerase (AmpliTaq Gold DNA polymerase, Perkin Elmer) in a final volume of 100 μl overlaid with 40 μl of mineral oil (Sigma). A 9-min pre-PCR heating step at 95°C was performed, followed by 5 cycles of 60 s at 94°C, 60 s at 50°C, and 60 s at 72°C and 40 cycles of 60 s at 94°C, 45 s at 53°C, and 60 s at 72°C.

To harvest single-cell RNA, glass coverslips coated with single rat melanotrophs were bathed in a sterile solution (mM): 83 Tris\(\cdot\)HCl (pH 8.3), 125 KCl, and 5 MgCl\(_2\). For the patch pipette (1–2 M Ω) we used in the last part of the amplification procedure. In the second round of PCR amplification, 1 μl of the first PCR reaction was used to further increase the quantity of the DNA product. The PCR reaction mixture was the same as described above, except that the concentration of each of the dNTPs was 0.3 mM.

To discriminate between the DNA fragments arising from the Rab3A and Rab3B sequences, RT-PCR samples were digested with NciI and SacII (both from New England BioLabs), respectively. Restriction analysis was performed in a final volume of 40 μl, containing 25 μl of amplified DNA, 10 U of restriction enzymes, and 10× digestion buffer for 60 min at 37°C. Products were analyzed with 1.4% agarose (NuSieve GTG, FMC BioProducts) gels stained with ethidium bromide.

**In situ hybridization histochemistry.** Adult female Wistar rats (200–250 g) were killed by decapitation, the pituitaries were quickly frozen, and 15-μm sections were cut on a cryostat. The animals were euthanized in accordance with the Animal Protection Act (Official Gazette of the Republic of Slovenia, Nos. 98/99, 126/03, and 20/04) and in accordance with protocols approved by the Veterinary Administration of the Republic of Slovenia. RNA probes were prepared from Rab3A and Rab3B cDNA templates inserted into a p Bluescript II transcription vector (Stratagene). The Rab3A insert encompassed the complete coding sequence plus 20 nucleotides of noncoding sequence at the 3′ end of the rat mRNA, and the Rab3B insert corresponded to the complete coding and 3′ noncoding sequence of the rat mRNA. After plasmid linearization, Rab3A and Rab3B probes were transcribed by T7 (antisense probe) or T3 (sense probe) RNA polymerases, according to the manufacturer’s specifications. Probes were labeled in the presence of the transcription buffer mix of either 2.5 mM [\(^{35}\)S]UTP (1,000 Ci/mmol, Amersham) or 20 mM digoxigenin-11-UTP (Boehringer Mannheim) as described previously (43). Mounted sections were fixed in 3% paraformaldehyde, rinsed in 2× SSC (standard saline citrate) buffer (2× SSC contains 0.15 M NaCl-0.015 M sodium citrate), acetylated, treated with Tris-glycine, rinsed in 2× SSC buffer, dehydrated in ethanol, and air dried. The sections were hybridized in 30-μl aliquots of hybridization mixture (43) containing 2–3 ng of digoxigenin-labeled UTP-Rab3A and the [\(^{35}\)S]Rab3B riboprobes (1:1). Hybridization was carried out in humid chambers overnight at 50°C. After posthybridization procedures, which included washes in 50% formamide-2× SSC at 52°C, the slides were processed with RNase A, and rinses in buffer, the sections were processed for detection of digoxigenin-labeled hybrids with anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim). The enzymatic reaction was stopped in 10 mM Tris·HCl-1 mM EDTA (pH 8.0). The sections were then quickly rinsed in 75% ethanol, air dried, and processed for autoradiography with NTB2 emulsion (Kodak). The sections that were hybridized with only radiolabeled probes were counterstained with hematoxylin and eosin and examined with bright- and dark-field microscopy.

**Immunocytochemistry and confocal microscopy.** Melanotrophs were washed once with 1 ml of phosphate-buffered saline (PBS) and fixed in paraformaldehyde (4% in PBS) for 15 min. Cells were permeabilized with 4% paraformaldehyde-0.1% Triton X-100 for 10 min and washed four times with PBS. Subsequently, rabbit serum containing either anti-Rab3A (1:100; Ref. 7) or anti-Rab3B (1:100) antibody was applied to the melanotrophs, which were further incubated overnight at 4°C. The cells were then washed four times with PBS and exposed to the respective secondary antibody solution: Alexa Fluor 546-conjugated anti-rabbit IgG (1:500) and Alexa Fluor 488-conjugated anti-rabbit IgG. The cells were washed four times with PBS, and the preparation was finally supplied with the Slow Fade Light Antifade Kit (Molecular Probes, Leiden, Netherlands). Coverslips with double-labeled melanotrophs were examined with a Zeiss confocal microscope (LSM 510, Jena, Germany). Fluorescent images were acquired by a plan-apochromatic oil-immersion objective (63× magnification, 1.4 numerical aperture) using 488-nm Ar-Ion and 543-nm He-Ne laser excitation. Rab3A-associated fluorescence and fluorescence of labeled Rab3B were separated with BP 505–530 nm.
and LP 560-nm emission filters, respectively. The images were stored on an IBM-compatible PC (Siemens Nixdorf). Digitalized images were exported as TIFF files and analyzed with a custom-made program (19). Briefly, the program counted all the red, green, and colocalized pixels in the image. The degree of colocalization between the green and red pixels was expressed as a percentage of the colocalized pixels. Colocalization data were statistically analyzed by unpaired, two-tailed Student’s *t*-test.

Microinjection of oligodeoxyribonucleotides and recombinant proteins. Oligodeoxyribonucleotide probes and recombinant proteins were microinjected into single melanotrophs with a Transjector 4657 (Eppendorf) with a piezosteper (Luigs and Neumann). Pipettes (20–50 hPa, 0.3–1 s) were applied with a compensation pressure set to 1 hPa. Pipettes were prepared with a horizontal puller (P-87, Sutter Instruments), using borosilicate glass capillaries with filaments (GC150F, Clark Electromedical). Then, 45–90 h after the microinjection of oligodeoxyribonucleotide probes (1 μM, Operon) the secretory activity of the cells was measured (see below); cells microinjected with recombinant proteins (0.02 mg/ml) were electrophysiologically examined between 3 and 24 h after the microinjection. Microinjected cells were identified with coinjection of fluorescein-labeled dextran (5 mg/ml, Sigma Chemical) of molecular mass similar to that of the oligodeoxyribonucleotide probes (4 kDa) and proteins (25 kDa) and viewing in an epifluorescence inverted microscope and 2) by recording the position of the markers on Cellocate coverslips (Eppendorf). Injection of a dextran marker did not affect the secretory responses per se. Oligodeoxyribonucleotides of the following sequences were used (22): PT3 (antisense-Rab3A; 5′-TGG CTG AGG CCA TCT TGC CGG AT) dissolved in Millipore-grade water as a stock solution (50 μM), and N63 (antisense-Rab3B; 5′-AGT TAC TGA GGC CAT CTC CGG AT) dissolved in Millipore-grade water as a stock solution (50 μM) and stored at −80°C.

Electrophysiology. With the whole cell patch-clamp technique, the cells were voltage-clamped at a holding potential of −70 mV. Membrane capacitance ($C_m$) was recorded with a two-phase lock-in amplifier (1,600 Hz, 1 mV peak to peak) incorporated into a patch-clamp amplifier (SWAM II, Celica). A direct current (low pass, 1–10 Hz, −3 dB), holding potential, and real and imaginary admittance signals (low pass, 1 Hz, −3 dB) were used in the calculations (53). The reversal potential used in the calculation was −50 mV, which did not change during a recording. The plots of the passive cell parameters, access conductance, parallel combination of leak and membrane conductance, and $C_m$ were derived by a computer-aided reconstruction after analog-to-digital conversion (CED 1401, Cambridge, UK) with an IBM-compatible PC. The software was written by Dr J. Dempster (University of Strathclyde, Glasgow, UK). Recordings were made at room temperature with pipette resistances between 1 and 4 MΩ. The basic solution in the recording pipette contained (in mM) 2 MgCl₂, 10 HEPES-KOH, 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 150 KCl, and 2 Na₂ATP, pH 7.2. To this 0.5 mM EGTA and 3.5 mM Ca²⁺-EGTA were added in order to obtain Ca²⁺ activity of −1,000 nM, prepared as described by Neher (25). Intracellular Ca²⁺ concentration was calculated, assuming an apparent dissociation constant ($K_d$) for the Ca-EGTA complex of 150 nM (10). The total EGTA concentration was 4 mM, which exceeds the buffering capacity of melanotrophs (44). All salts were obtained from Sigma.

Secretory responses were measured 200 s after the start of the recordings as a change in $C_m$ ($C_m$) relative to the resting $C_m$. Unless stated otherwise, statistics are in the format of means ± SE and the differences between samples were tested by Student’s *t*-test, considering $P < 0.001$ and $P < 0.01$ to be statistically significant. Error bars in Figs. 4 and 6 show SE.

Purification of recombinant Rab3A and Rab3B mutant proteins. The cDNA encoding c-Myc-tagged human Rab3A, Rab3AQ81L, Rab3AT36N, Rab3AV55E, and Rab3AΔC (ΔC denotes that the last 3 amino acids at the COOH terminus are lacking), Rab3B, Rab3BQ81L, and Rab3BV55E were subcloned in pGEX-2T (Pharmacia) and expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins as described previously (12, 13). To create the mutants (Rab3AT36NΔC, Rab3AV55EΔC, and Rab3AQ81LΔC) the Nhel-SrI fragment of pGEX-TR3aT36N or pGEX-TR3aV55E or the Nhel-EcoRI fragment of pGEX-TR3aQ81L was exchanged for the corresponding fragment of pGEX-TR3aΔC.

**RESULTS**

**Rab3A and Rab3B mRNA in rat pituitary.** A number of previous studies have evaluated tissue distribution of Rab3 proteins at the mRNA level. This analysis turns out to be critical in tissues where obvious function was reported, e.g., testes (47, 51), but no proteins were detected with specific antibodies (37). Nevertheless, Rab3A and Rab3B gene transcripts were readily detected in rat melanotrophs (Fig. 1). For this purpose, cDNA samples were reverse transcribed from mRNA isolated from a population of cells (Fig. 1A) or from single melanotrophs (Fig. 1B, n = 4) and amplified with Rab3 generic primers (see MATERIALS AND METHODS). One amplified product of 281 bp was observed (Fig. 1, lanes 2 and 5), showing that Rab3-related mRNAs were expressed in rat melanotrophs. No amplified DNA fragments were observed when PCR was performed before reverse transcription or with no added cDNA sample (not shown). This showed that the amplified fragments were generated from Rab3 mRNA and did not originate from genomic DNA or result from contamination. The presence of Rab3A and Rab3B mRNAs was determined by digesting the PCR product with restriction enzymes. The product was separated into 229- and 51-bp fragments by NciI (identifying Rab3A transcript; Fig. 1, lanes 2 and 6) and into 231- and 50-bp fragments by SacII (identifying Rab3B transcript; Fig. 1, lanes 4 and 7).

To further verify that rat melanotrophs express mRNA for Rab3A and Rab3B, in situ hybridization was performed on...
sections of the pituitary. In agreement with Stettler et al. (43), the results showed more of the Rab3A gene transcript in pars intermedia compared with anterior or posterior pituitary (Fig. 2A). We found higher levels of Rab3B transcripts in the anterior pituitary and pars intermedia compared with the posterior pituitary (Fig. 2B). However, both transcripts significantly overlapped in the pars intermedia. Finally, to visualize the localization of both transcripts to a single cell we performed double in situ histochemistry. As seen in Fig. 2C, both Rab3A and Rab3B gene transcripts are present in most, if not all, of the rat melanotrophs, Rab3A again being more abundant than Rab3B.

These results (Figs. 1 and 2) strongly indicate that rat melanotrophs express Rab3A and Rab3B, which agrees well with the protein levels measured in the rat pituitary (37). Thus melanotrophs are a useful model to study the role of these Rab3 proteins in regulated secretion.

Subcellular distribution of Rab3A and Rab3B in rat melanotrophs. We then investigated the subcellular localization of Rab3A and Rab3B in melanotrophs. Confocal analysis of the primary culture of rat melanotrophs was performed by staining cells with anti-Rab3A and anti-Rab3B specific antibodies. Both proteins were readily detected in a single melanotroph (Fig. 3). Although the signals for the proteins largely overlap, there appeared to be subplasmalemmal regions with the Rab3A signal only (Fig. 3B). To quantify whether the signals related to the two proteins were colocalized, we measured the level of colocalization between the anti-Rab3A and the anti-Rab3B fluorescence signal, as described by Kreft et al. (19). The contrast of all images was uniformly set by linearly reassigning the value of the pixel intensity to use the full 8-bit range (0–255). Images were binarized at 50% threshold level (Fig. 3B). We analyzed a total of 77 confocal sections from 6 cells; 28 ± 2% of the total fluorescent area was stained with antibodies marking Rab3A only, and 55 ± 2% of the total fluorescent area was stained with antibodies marking Rab3B only. Just 17 ± 1% of the total fluorescent area was stained with both fluorescent markers, showing regions of Rab3A and Rab3B colocalization. Generally, Rab3A is localized more abundantly and closer to the plasma membrane than Rab3B.

Pretreatment by antisense-Rab3A, but not antisense-Rab3B, blocks Ca2+-dependent secretory responses. Overexpression studies brought interesting insights into GTPase activity and effector binding of Rab3 proteins but did not provide a complete description of the activity of endogenous proteins. In the present work we have used the same antisense approach as we used previously on anterior pituitary cells (12, 20, 22, 34) to knock out the expression of Rab3A and Rab3B at the single-cell level.

Forty-five hours after the microinjection of the Rab3A antisense probe, a complete inhibition of Ca2+-dependent secre-
Statistical significance at mRNA (preinjection with antisense oligodeoxyribonucleotide probes against Rab3A probe and an antisense probe specific for Rab3B (antisense responses were measured as relative changes in Cm/H11011,000 nM free Ca2+-dependent secretory responses of rat melanotrophs by preinjection with antisense oligodeoxyribonucleotide probes against Rab3A mRNA (●), antisense oligodeoxyribonucleotides against Rab3B mRNA (▲), or a sense oligodeoxyribonucleotide probe against Rab3A mRNA (●). Secretory responses were measured as relative changes in Cm 200 s after the establishment of a whole cell recording with a pipette-filling solution containing ~1,000 nM free Ca2+ (see MATERIALS AND METHODS). Note the recovery of responses 90 h after the injection of antisense probes specific for Rab3A mRNA. Numbers adjacent to symbols represent the number of cells tested. □ Statistical significance at P < 0.001.

Rab3B. Therefore, we next studied the effect of recombinant proteins on secretory responses in rat melanotrophs. GTP-bound form of Rab3A potentiates Ca2+-dependent secretory responses. To investigate the contribution of each step of the GTPase cycle to secretory activity and to test whether there is a role for Rab3B protein on the protein level in the secretory activity of melanotrophs, wild-type or mutated recombinant Rab3 proteins were microinjected into melanotrophs. All proteins were expressed as GST fusion proteins, purified on glutathione-Sepharose (12), microinjected, and incubated for 2 h before patch-clamp experiments were performed. Cells microinjected with GST only were taken as controls. Microinjection of wild-type Rab3A resulted in a response very similar to that obtained in control cells (Figs. 5 and 6). In contrast, microinjection of Rab3AQ81L, a mutant deficient in GTPase activity, promoted a threefold potentiation of Ca2+-dependent secretory responses compared with controls. Importantly, Rab3BQ81L had no significant effect on secretory responses (Fig. 6). The “split” version of Rab3A protein (without the GST moiety) also potentiated Ca2+-dependent secretory responses. In contrast, microinjection of Rab3AT36N, a mutant deficient in GTP binding, resulted in a complete inhibition of Ca2+-dependent secretory responses. This protein probably acts by titrating the guanine nucleotide exchange factor that catalyzes the activation of endogenous Rab3A. These results thus confirm the data obtained with antisense oligonucleotides and indicate that GTP-bound Rab3A but not Rab3B is required for Ca2+-dependent secretory activity of melanotrophs.

Ca2+-dependent secretory responses were also strongly inhibited by a Rab3AV55E protein, a dominant-negative form of the effector binding domain. Accordingly, the V55E mutation was found to abolish the effect of overexpressed Rab3AQ81L in PC-12 cells (40). As many effectors, e.g., Raphlin3A, RIM, and Noc2, were found to bind this region, it is possible that one of these is involved in secretory activity signaling, distal to Rab3A. This observation contrasts with reports from chromaffin and PC-12 cells, in which interaction with putative effectors had no apparent function in Ca2+-dependent secretory activity (37).
C5: Rab3AQ81L, cysteines (H9004/H9004C, Rab3AV55E in injection into cytoplasm, we prepared mutants with deleted of Rab proteins is required for their action (4, 24, 27).

Involvement of multiple steps including posttranslational modifications and recognition of targeting sequences by organelle-specific receptors. Studies on mutants suggest that isoprenylation of distinct cysteine motifs of an intact COOH-terminal end of Rab proteins with a given membrane compartment is a process involving multiple steps behind translocation and complete recognition of targeting sequences by organelle-specific receptors. Early studies on mutants suggested that isoprenylation of distinct cysteine motifs of an intact COOH-terminal end of Rab proteins is required for their action (4, 24, 27).

Deletion of cysteines at the COOH terminus abolishes the action of injected recombinant proteins. Association of Rab proteins with a given membrane compartment is a process involving multiple steps including posttranslational modifications and recognition of targeting sequences by organelle-specific receptors. Studies on mutants suggest that isoprenylation of distinct cysteine motifs of an intact COOH-terminal end of Rab proteins is required for their action (4, 24, 27).

To verify whether in our cells the recombinant proteins required a modification at their COOH termini after their injection into the cytoplasm, we prepared mutants with deleted cysteines (ΔC): Rab3AQ81LΔC, Rab3AV55EΔC, and Rab3AT36NΔC. Microinjection of Rab3A mutant proteins, carrying the cysteines, is modified in such a way that it mediates a functional response. In contrast, the injection of Rab3AV55EΔC or Rab3AT36NΔC resulted in secretory responses that were not significantly different from controls (Fig. 5), suggesting that after injection the COOH terminus of Rab3A protein, carrying the cysteines, is modified in such a way that it mediates a functional response. In contrast, the injection of Rab3AV55EΔC or Rab3AT36NΔC resulted in secretory responses that were not significantly different from those obtained with Rab3AV55E or Rab3AT36N (Fig. 5), respectively. This indicates that the inhibition of secretory responses by the effector domain mutant and by the GDP-bound Rab3A probably acts by titrating effectors or regulatory proteins, and that this does not require an intact COOH terminus.

DISCUSSION

Rab3 proteins constitute a family of GTP-binding proteins that are implicated in regulated exocytosis, but the exact function of these proteins in this process is associated with many contradictory attributes, which preclude the formulation of a synthetic hypothesis about the function of Rab3 proteins (6, 37). One potential reason for this is that the conventional assays used, whether genetically modified mice or transfected cells, have distinct limitations and are inherently indirect (37), since they allow compensatory mechanisms during ontogenesis or heterologous expression in an incompatible cytosolic milieu.

The aim of this work was to address the question of the respective roles of Rab3A and Rab3B in Ca2+-dependent secretory activity of neuroendocrine cells by using a single-cell approach. Therefore, we used cells that natively express both isoforms. Using in situ hybridization, RT-PCR, and confocal microscopy, we showed that single rat melanotrophs express Rab3A and Rab3B isoforms and, as demonstrated by RT-PCR, at a similar level (Fig. 1), confirming earlier results (37, 43). However, the confocal microscopy study revealed that Rab3A and Rab3B are not strictly colocalized at the subcellular level: a fraction of subplasmalemmal secretory vesicles appear to be exclusively decorated with Rab3A but not with Rab3B proteins (Fig. 3). Similar conclusions were reported previously (2, 21, 29).

As proposed by Schlüter et al. (37), incomplete overlay of localization of both Rab3 isoforms excludes complete functional redundancy, suggesting more specific roles of Rab3 proteins in regulated Ca2+-dependent secretory activity. Moreover, our results are consistent with the view that secretory vesicles may carry various sets of proteins, not always consisting of all known vesicle proteins associated with regulated exocytosis. For example, it was shown that vesicles carrying the CAPS protein in melanotrophs enter exocytosis at a lower calcium threshold, but with rapid kinetics (35). Furthermore, by studying the fusion pore kinetics, it was shown that vesicles carrying synaptotagmin I or synaptotagmin IV exhibit different functional properties (45, 46). Even if all the members of a protein family are localized to secretory vesicles, the fusion of an individual vesicle with the plasma membrane may depend on the actual proteins present on this vesicle. Hence it is difficult to assign the function of a protein when the nature of the proteins is not known for the particular vesicle. This possibly represents a problem that is inherent to many biochemical approaches in which the average composition of a vesicle is assumed from homogenates and cell fractionation experiments.

Here we monitored the secretory activity of melanotrophs with patch-clamp capacitance measurements in cells preinjected with antisense probes to Rab3A or Rab3B transcripts. The single-cell knockout approach in differentiated adult cells leaves significantly less time for compensatory mechanisms compared with whole organism knockouts. Previous studies demonstrated that this strategy could inhibit the expression of Rab3A or Rab3B proteins (12, 22). Using antisense probes identical to those used by Lledo et al. (22), we found that the preinjection of Rab3A but not Rab3B antisense probe completely abolishes secretory responses (Fig. 4), indicating a role for Rab3A, but not Rab3B, in regulated, Ca2+-dependent secretion of melanotrophs. We cannot rule out the possibility that the slow dialysis of Ca2+ into the cytosol selectively stimulates Rab3A-dependent secretory activity, while the putative secretory response involving Rab3B can only be activated with a faster delivery of Ca2+ (35), or that significantly higher Ca2+ levels could selectively work on Rab3B-dependent processes. Nonetheless, preinjection of GTPase-deficient Rab3A and Rab3B proteins confirmed the conclusion that Rab3A, but not Rab3B, is required for regulated secretion in melanotrophs (Fig. 6). These results are inconsistent with the view that Rab3 isoforms may be interchangeable in controlling a process related to regulated secretion (9, 37). Our results...
suggest rather that Rab3 isoforms may all participate in regulated secretion but with distinct functions (2, 48). Although the function of Rab3D, present in the pituitary (37), was not studied here, it is likely that Rab3D also plays a specific role in the secretory pathway of pituitary cells. It was shown that Rab3D is required to maintain normal size of secretory granules (30), possibly by affecting homotypic fusion of granules, which was also observed in pituitary cells (18).

In the present study we also used recombinant Rab3A proteins, mutated in several domains, to learn about the role of Rab3A in the secretory activity of melanotrophs. The injection of wild-type Rab3A resulted in responses not significantly different from those in controls (Fig. 6). Similar results were obtained with Rab5 mutant proteins in studies on endocytic fusion (42). In contrast, we found that the GTPase-deficient mutant (Rab3AQ81L) stimulates Ca\(^{2+}\)-dependent secretory activity (Figs. 5 and 6), whereas the preferentially GDP-bound mutant (Rab3AT36N), and a protein carrying an effector domain mutation (Rab3AV55E), strongly inhibited Ca\(^{2+}\)-dependent secretory activity. Our results differ from those reported for chromaffin cells (11, 12) and Aplysia neurons (8, 13), in which the GTPase-deficient Rab3A mutant was found inhibitory overall, whereas the mutant favoring the GDP-bound state had no effect on secretory activity. Moreover, in chromaffin cells the inhibition of Rab3A expression by antisense probes resulted in a potentiation of secretory activity (12, 15). Thus GTP-bound Rab3A may be inhibitory for exocytosis in chromaffin cells and in neurons, whereas in rat melanotrophs it may generally play a stimulatory role in regulated secretion. This proposal is based on the consideration that recombinant Rab3A proteins enter the functional cycle of endogenous Rab3. In support of this, we found that the effector domain mutant (Rab3AV55E) inhibits secretory responses (Fig. 6), as would be expected if the latter were competing with the endogenous Rab3A with a target in the cycle. Furthermore, in agreement with earlier studies demonstrating that the COOH-terminal domain is required for the association of Rab proteins with membranes of specific organelles (4, 27), the presence of COOH-terminal cysteine residues was required for the action of the GTPase-deficient mutant (Rab3AQ81L; Fig. 6). Finally, the injection of the wild-type Rab3A did not alter secretory activity (Fig. 6), which argues against a major titration of a limiting factor in the cycle of endogenous Rab3A protein.

Recently, Schlüter et al. (37) reported that overexpression of Rab3 proteins in PC-12 cells increased the release activity in the absence of stimulus and proposed that the inhibitory effect of Rab3 proteins on secretory responses may result from a depletion of the readily releasable pool of vesicles. Indeed, we found that Rab3AQ81L expression in PC-12 cells reduced the number of release-ready vesicles (39). However, our results in PC-12 cells did not support an effect of Rab3A on basal release activity (unpublished observations).

Rab proteins can act as molecular switches, flipping between two conformational states, the active GTP-bound and an inactive GDP-bound form (3, 26). To explain our results we consider two models. In the first model, the Rab3A switch may act in regulated exocytosis of melanotrophs. In this model, GTP hydrolysis is not rate limiting and may occur after fusion. GTP-bound Rab3A would be required for a predocking/docking process. Rab3A might recruit tethering factors or may displace N-sec1 from syntaxin, allowing subsequent formation of the syntaxin-SNAP25-VAMP2 complex (31, 49). The stimulation of melanotroph secretory activity by a GTPase-deficient Rab3A mutant (Fig. 6) supports this model and is consistent with the view that the GTPase activity provides timing for the exocytic fusion, as proposed for Rab3 in endocytosis (36). In the second model, GTP-bound Rab3A would promote the formation of the fusion machinery, as described in the first model, but would inhibit another downstream step. According to this model, GTP hydrolysis might be limiting and would therefore occur before fusion (12, 13).

It is interesting to note that Rab3A controls secretory activity differently in chromaffin cells (11, 12) and in melanotrophs. The mode of Rab3A action in chromaffin cells and cholinergic Aplysia neurons (13) may be similar to the model in which Rab3A activity also controls a step downstream of docking. It remains to be determined whether the discrepancy in the regulation of secretory activity by Rab3A in chromaffin cells and melanotrophs is due to differences in the partner molecules interacting with Rab3A.

To sum up, we have shown that Rab3A and Rab3B are expressed in single rat melanotrophs, but only Rab3A is required for Ca\(^{2+}\)-dependent secretory activity. The GTP-bound Rab3A is mandatory for this activity, but GTPase activity is not associated with exocytotic fusion. We propose that GTP-bound Rab3A enhances fusion competence and secretory output at a step prior to exocytosis (predocking/docking), as reported for Rab5 in endocytic fusion (36, 42). A similar role of Rab3A has been recently described in pancreatic beta cells (50).

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