**Distinct Functional Properties of Rab3A and Rab3B in PC12 Neuroendocrine Cells***

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Rab3A and Rab3B are highly homologous monomeric GTPases that are putative regulators of exocytosis in those tissues in which they are expressed. We have characterized and directly compared the targeting and functional properties of these isoforms in PC12 neuroendocrine cells. Rab3A and Rab3B both targeted to norepinephrine (NE)-containing large dense core vesicles (LDCVs) when stably expressed in PC12 cells, as determined by immunofluorescence and membrane fractionation. Both Rab3 isoforms also bound to recombinant rabphilin-3A in a GTP-dependent manner. The membrane association of rabphilin-3A was modestly enhanced in Rab3A-expressing PC12 cells relative to Rab3A-overexpressing cells. In addition, overexpression of Rab3A modestly inhibited Ca\(^{2+}\)-evoked NE release, whereas Rab3B and a GTP binding mutant (Rab3B N135I) markedly stimulated the efficiency of \(^{3}H\)NE secretion by PC12 cells (i.e. secretion normalized to total cell radioactivity). Expression of Rab3B and Rab3B N135I increased not only the efficiency of NE secretion but also the accumulation of \(^{3}H\)NE into LDCVs (i.e. the secretory cargo available for secretion). Neither of these effects was attributable to changes in the numbers of LDCVs nor the docking of LDCVs at the plasma membrane. Our results indicate that Rab3A and Rab3B have similar membrane targeting properties and are capable of interacting with the same putative downstream effector; i.e. rabphilin-3A. However, these isoforms are functionally distinct monomeric GTPases with Rab3B stimulating a late step in Ca\(^{2+}\)-evoked secretion when expressed in PC12 cells.

Members of the Rab family of Ras-related monomeric GTPases are major candidates for controlling membrane docking and/or fusion in a wide variety of cell types. According to current models, the docking and fusion of donor and acceptor membranes is specified by interactions between proteins unique to transport vesicles, termed v-SNAREs, with their cognate t-SNAREs located on the intended target membrane (1, 2). Rab3 are not found in purified SNARE complexes (3); however, genetic studies in yeast indicate that additional proteins, including members of the Rab and Sec1 families, serve to control SNARE assembly by proofreading the fidelity of SNARE interactions and/or by imposing an additional layer of specificity (4, 5).

The Rab family represents a large number (>30) of homologous proteins, most of which are expressed in a wide variety of tissues where they probably regulate protein traffic pathways that are common to all cells (e.g. endocytosis in the case of Rab5) (6). However, members of the Rab3 subfamily (Rab3A–Rab3D), which are 77–85% identical at the amino acid level, are tissue-specific proteins that may regulate cell type-specific secretory pathways. For example, Rab3A is a brain-specific monomeric GTPase that binds to small synaptic vesicles, from which it dissociates during neurotransmitter release (7). Rab3A also reportedly associates with the large secretory granules characteristic of adrenal chromaffin cells and PC12 neuroendocrine cells (8). Rab3B and Rab3D are primarily expressed outside of the nervous system, with Rab3B being expressed in a wide variety of epithelial tissues (9) and Rab3D enriched in adipocytes (10). Rab3C shares many properties with Rab3A including expression in brain, targeting to synaptic vesicles, and transient dissociation from synaptic vesicles during neurotransmitter release (11). Rab3A and Rab3C both bind in a GTP-dependent manner to rabphilin-3A, a Ca\(^{2+}\) and phospholipid binding protein that is a putative downstream effector for Rab3A in neurons (12–14).

All Rab3 isoforms have been proposed to regulate exocytosis in their respective tissues; however, in most cases direct evidence for such regulation is lacking. Moreover, there is evidence to suggest that these structurally related proteins may in fact be functionally distinct. For example, on the basis of the results of antisense RNA experiments performed on anterior pituitary cells, Lledo et al. (15) concluded that Rab3B is a positive regulator of Ca\(^{2+}\)-evoked secretion from these cells. Conversely, Holz et al. (16) have reported that transient overexpression of wild type Rab3A and certain mutants inhibited Ca\(^{2+}\)-dependent secretion from bovine chromaffin cells. A similar conclusion was drawn by J channes et al. (17), who examined the effects of Rab3A mutants that were microinjected or transiently expressed in PC12 neuroendocrine cells. Such data have led to the proposal that Rab3A participates in the formation of a multimeric prematrix complex that must be dissociated prior to membrane fusion and secretion. This proposal seems consistent with the phenotype exhibited by Rab3A-negative transgenic mice, which display no obvious decrement in synaptic transmission except during repetitive stimulation, i.e. when synaptic vesicle recruitment to the presynaptic membrane becomes rate-limiting (18).

The preceding results imply that Rab3 isoforms, in particular Rab3A and Rab3B, may not be functionally interchangeable molecules, despite the fact that they are highly homologous proteins (~80% amino acid identity). The goal of the present study was to test this notion by characterizing the targeting and functional properties of Rab3A and Rab3B in PC12 neu-
roendocrine cells. PC12 cells were utilized because they have a well-characterized, regulated secretory pathway (i.e. the nor-
epinephrine-containing LDCVs) and because they normally
express Rab3A but not Rab3B. Our results indicate that both
of these monomeric GTPases target to LDCVs when stably
expressed in PC12 cells and that both are capable of binding
to rabphilin-3A, a putative downstream effector for Rab3A.
Moreover, our functional data confirm that Rab3A overexpression
inhibits secretion, albeit modestly and to varying degrees.
Conservatively, we observed that wild type Rab3B and a GTP-binding
mutant (i.e. Rab3B N135I) are potent stimulators of cate-
cholamine release. Interestingly, Rab3B stimulated secretion
both by increasing the efficiency of radiolabeled catecholamine
release (i.e. release normalized to cell-associated radioactivity)
and by increasing the uptake of exogenous catecholamine into
LDCVs (i.e. increasing the cargo available for secretion). Thus,
Rab3A and Rab3B are functionally distinct molecules, with
Rab3B enhancing secretion by PC12 cells at multiple levels of
regulation.

MATERIALS AND METHODS

Reagents and Antibodies—RPMI 1640, poly-L-lysine (P3199), reser-
pine, GTP-y-S, and the anti-synaptophysin monoclonal antibody (Clone
SVP-38) were purchased from Sigma, fetal bovine serum and equine
(horse) serum from Hyclone (Logan, UT), and minimal essential me-
dium (MEM), lipofectamine, and Opti-MEM media from Life Technol-
gies, Inc. Hygromycin B and ionomycin were obtained from Calbio-
chem, [3H]norepinephrine ([3H]NE) from DuPont NEN, norepinephrine
from Research Biochemical International (Natwick, MA), and cell culture
dishes from Costar (Cambridge, MA). PC12 cells were purchased from
ATCC (catalog number ATCC CRL 1721). The generation and charac-
terization of a Rab3B polyclonal antibody was described previously (9).
The monoclonal antibodies specific for Rab3A (C142.2) and for Rab3A,
Rab3B, and Rab3C (C142.1) were generously provided by Dr. R. J.
Jahn (Department of Cell Biology, Yale University Medical School, New
Haven, CT). The polyclonal rabphilin-3A antibody (14) and the
coding regions for bovine Rab3A and rat rabphilin-3A (14) were kind
gifts of Dr. T. C. Südhof (University of Texas Southwestern Medical
Center, Dallas, TX). The secretogranin II polyclonal antibody was
kindly provided by Dr. J. D. Neill (Department of Physiology and
Biophysics, University of Alabama at Birmingham, Birmingham, AL).

Cell Culture—PC12 cells were cultured in RPMI 1640, 10% heat
inactivated horse serum, 5% fetal bovine serum at 37 °C in a humidified
5% CO2 incubator as described (21). Stably transfected PC12 cells
(passage numbers 1–24) and untransfected cells were cultured and
collected on poly-L-lysine (50 μg/ml)-coated dishes for NE secretion assays
or on glass coverslips for immunofluorescent staining. All studies were
performed in the absence of nerve growth factor.

cDNA Cloning, Mutagenesis, and Expression Vector—The coding
region of human Rab3B was generated by reverse transcription-poly-
merase chain reaction as described (9). Sequence analysis of this
done showed two differences when compared with the human Rab3B
sequence originally reported by Zahraoui et al. (22) and submitted to
the GenBank®/EMBL Data Bank (accession number M28214). These
differences were as follows: AC instead of CA at nucleotide positions 25
and 26, which results in Thr versus His at amino acid position 9, and
G instead of C at nucleotide position 120, which results in Phe versus Leu
at amino acid position 40. To rule out the possibility of polymerease
chain reaction error in the generation of our Rab3B clone, we also
sequenced the human Rab3B clone originally isolated by Zahraoui et al.
(22) from a human pheochromocytoma cDNA library. (kindly provided
by A. Zahraoui, INSERM U-248, Paris, France). The two clones are
distinct, at the genetic level, with two differences (single-stranded M13 templates using the dideoxy chain


1 The abbreviations used are: LDCV, large dense core vesicle; MEM, minimal essential medium; GST, glutathione S-transferase; NE, norepinephrine; PBS, phosphate-buffered saline; GTP-y-S, guanosine 5'-3-O(thio)triphosphate.

2 E. Weber, T. Jilling, and K. L. Kirk, unpublished observations.
were also labeled with 40 μCi of [3H]norepinephrine/dish for 1 h at 37 °C and then chased for 1 h at 37 °C prior to fractionation (details described below for NE release assay). All gradients contained the protease inhibitor mixture described above. Equilibrium sedimentation was achieved by centrifugation in a SW40 rotor (Beckman) at 30,000 rpm for 6 h at 4 °C. Fractions were collected from the bottom of the tube (450–500 μl fraction; 20 or 21 fraction). The amount of Rab protein from the 20 fraction from unlabelled cells was determined with the micro-BCA assay kit. The sucrose concentration of each fraction was determined by measuring the refractive index. The radioactivity of each fraction from cells preloaded with [3H]NE was measured by scintillation counting (as determined by counting [3H]NE standards in 0.6–1.8 h sucrose). For immunoblot analysis fractions were diluted 1:20 in water, and proteins were precipitated by incubation with 10% trichloroacetic acid and 0.1 M sodium deoxycholate overnight at 4 °C. Protein pellets were washed twice with acetone and then sublimated in 5 × SDS sample buffer prior to immunoblot analysis.

Production of GST Fusion Proteins and Immunoblot Analysis of Binding Proteins—GST fusion proteins containing full-length Rab3A, Rab3B, and rabphilin-3A were expressed in bacteria using the pGEX-2T prokaryotic expression vector (Pharmacia Biotech Inc.). Transformed Escherichia coli were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h (for GST-Rab3A and GST-Rab3B) or with 50 μM isopropyl-1-thio-β-D-galactopyranoside at room temperature for 5 h (GST-rabphilin-3A). GST-fusion proteins were affinity-purified from bacterial cell lysates with glutathione-agarose, as described (28). For binding experiments cells were lysed in 1% Nonidet P-40 lysis buffer (see immunoblotting protocol under “Materials and Methods”) or homogenized (see membrane preparation protocol under “Materials and Methods”) in the presence or absence of 50 μM GTPγS. Clarified cell lysates or high speed supernatants (i.e. cytosol) were incubated with GST fusion proteins (GST-3A, GST-3B, GST-rabphilin-3A) or GST alone immobilized on agarose beads for 2 h at 4 °C. Beads were washed four times with lysis or homogenization buffer. Bound proteins were eluted in similar volumes of 5 × sample buffer, and equal volumes of eluates were analyzed by immunoblotting.

[3H]Norepinephrine Release Assay—PC12 cells were grown in 35-mm poly-L-lysine-coated Petri dishes for 2–4 days until they reached 40–70% confluency. Cells were washed in RPMI and loaded at 37 °C for 1 h in 10% horse serum, 5% fetal bovine serum containing 1–2 μCi of [3H]NE, rinsed with PBS, and then chased in complete media for an additional 1 h in a 5% CO2 humidified incubator. Release assays were performed at 37 °C in 10 mM Hepes-buffered MEM (pH 7.4) or low K+ high K+ medium (10 mM Hepes, 5 or 55 mM KCl, 145 or 95 mM NaCl, 2 mM CaCl2, 10 mM glucose, pH 7.4) (29). The low K+ medium was used for assays where dependence of secretion on extracellular Ca2+ was examined, with Ca2+ concentrations that varied from 1 μM to 1.8 mM. PC12 cells were incubated in MEM or low K+ medium for 10 min at 37 °C prior to inducing secretion with 1 μM ionomycin or 55 mM K+. Except where noted, release was assessed in time course experiments in which the media was collected and replaced every 2 min. At the end of the experiment the cells were solubilized twice in 1 ml of 0.25% NaOH, and the lysates were carefully collected. Media cpm were determined by liquid scintillation counting and normalized to total cell cpm. Total cell cpm were determined by summing all media counts and the radioactivity remaining in the cells at the end of the experiment, the latter of which was determined by counting the cell lysates.

Transmission Electron Microscopy and Morphometry—PC12 cells were grown in 35-mm poly-L-lysine-coated Petri dishes for 2–4 days until they reached 40–70% confluence, rinsed twice in PBS, and incubated in MEM supplemented with 10 mM Hepes (pH 7.4) for 10 min. Monolayers were fixed in 1% buffered gluteraldehyde, postfixed in 1% buffered OsO4, dehydrated and embedded in Polybed, sectioned, and stained with uranyl acetate and lead citrate. The ultrathin sections were stained with uranyl acetate and lead citrate. The ultrathin sections were then photographed with a Hitachi-7000 transmission electron microscope. Numbers of dense core granules/cell and the numbers of granules within 100 nm of the plasma membrane were counted by six investigators in a double blind fashion.

RESULTS

Expression and Membrane Association of Rab3A and Rab3B in PC12 Cells—In order to directly compare the targeting and functional properties of Rab3A and Rab3B, we have generated PC12 cell lines that stably express recombinant Rab3A, Rab3B, or the Rab3B mutant, Rab3B N135I. Rab3B N135I corresponds to an oncogenic Ras mutant (H-Ras N116I) that has a substantially elevated dissociation rate for both GTP and GDP. Fig. 1A confirms for selected cell lines the expression of wild type Rab3B and Rab3B N135I protein, and the overexpression of Rab3A protein, as compared with nontransfected PC12 cells (nPC12) and mock-transfected cell lines. No expression of Rab3B protein was detected in nontransfected or in mock-transfected PC12 cells. Endogenous Rab3A and recombinant Rab3A and Rab3B all migrated as a ~25-kDa band in Western blots of total cell lysates, whereas Rab3B N135I migrated as a doublet with slightly faster mobility. Fig. 1B compares the efficiency of membrane association for Rab3A, Rab3B, and Rab3B N135I. The efficiency of Rab3B association with membranes was not substantially altered by the expression of recombinant Rab3B or Rab3B N135I. Interestingly, Rab3B N135I associated with PC12 membranes much less efficiently than either wild type Rab3B or wild type Rab3A; i.e. the great majority of mutant protein was present in the cytosol. Four out of five Rab3B N135I clones exhibited detectable but modest membrane association of the mutant protein (e.g. see clone 3B N135I 18 in Fig. 1B), whereas for one clone (3B N135I 32; see Fig. 1B) little or no Rab3B N135I protein was present on the membranes. This latter clone (mutant 32) also exhibits a different secretory phenotype than the other mutant Rab3B-expressing clones, as will be discussed below. Cytosolic Rab 3B N135I typically migrated as a doublet in which the dominant band had a higher mobility than that of wild type Rab3B, as noted above for cell lysates (Fig. 1A). Rab3B N135I also exhibits its higher mobility by SDS-polyacrylamide gel electrophoresis and poorly associates with membranes when expressed in Madin-Darby canine kidney renal epithelial cells.2

Rab3A and Rab3B Target to Secretory Granules—The targeting of Rab3A and Rab3B in PC12 cells was initially characterized by immunofluorescence staining (Fig. 2). Native Rab3A in untransfected cells and mock-transfected cells, as well as recombinant Rab3A in overexpressing cells, targeted at or very near the plasma membrane; in particular, at regions of cell-cell contact. Neither expression of recombinant Rab3B (Fig. 2) nor of Rab3B N135I (data not shown) changed this pattern of Rab3A targeting. Recombinant Rab3B protein was also detected predominantly in the region of the plasma membrane. Rab3B N135I protein was not detectable in any clone by im-
Immunochemical staining when the cells were permeabilized by our standard procedures (i.e. 0.5% saponin, see “Materials and Methods”). However, if the Rab3B N135I clones were permeabilized in 0.1% saponin, faint immunoreactivity was detected near the plasma membrane (except for mutant clone 32), along with increased cytoplasmic staining.2

To further characterize the subcellular locations of Rab3A and Rab3B in PC12 cells, we fractionated selected PC12 clones by equilibrium sedimentation through sucrose density gradients (Figs. 3 and 4). Homogenates of stably transfected PC12 cells were layered onto 0.6–1.8 M sucrose gradients and centrifuged to equilibrium. Individual fractions were collected and immunoblotted with antibodies against Rab3A, Rab3B, secretogranin II (i.e. a marker of dense secretory granules (30)) and synaptophysin (i.e. a marker of endosomes and small secretory vesicles (26)). The dense core granule fraction was also identified by preloading the cells with [3H]norepinephrine prior to fractionation.3

Fig. 3. Rab3B cofractionates with LDCVs as determined by equilibrium density gradient centrifugation. A, equal volumes of fractions of wild type Rab3B-expressing PC12 clone (Rab3B-WT10) were blotted with monoclonal anti-synaptophysin (1:2,000) and polyclonal anti-Rab3B antibodies (10 μg/ml). Total cell lysates of nontransfected PC12 cells (nPC12) and of clone WT10 were also blotted as controls (far right). B, top panel, plot of sucrose concentration, as determined by refractive index measurements and plot of protein concentration for each fraction. Bottom panel, plots of [3H]NE radioactivity and of Rab3B, synaptophysin, and secretogranin II immunoreactivity (see panel A) for each fraction. Immunoreactivity for each fraction was determined by densitometry and normalized to total immunoreactivity summed over all fractions. Fractions were collected from the bottom of the tube, with the first fraction representing the heaviest sucrose fraction. Rab3B immunoreactivity in the lightest fractions presumably represents cytosolic Rab3B.

lysates (i.e. cells that express Rab3B but not Rab3A (9)) in a GTPase-independent manner. In addition, immobilized GST-Rab3B bound rabphilin-3A from PC12 supernatants (i.e. cytosol) as well as a protein from HT29-C119A supernatants that has the same electrophoretic mobility (80 kDa) and that is recognized by the same rabphilin antibody. This 80-kDa band was also recognized in Western blots of colonic epithelial cell lysates using the N-terminal rabphilin antibody (data not shown). These results support two conclusions: (i) rabphilin-3A interacts with Rab3B as well as with Rab3A and Rab3C (14), and (ii) colonic epithelial cells express a Rab3-binding protein that appears to be related to brain rabphilin-3A.

On the basis of the results of these in vitro binding experiments, we examined the levels and membrane association of rabphilin-3A in Rab3A- and Rab3B-transfected PC12 cell lines. This analysis was motivated in part by the proposal that Rab3A potentiates the stability and/or membrane targeting of rabphilin-3A in brain (14). We found no apparent differences between mock-transfected, Rab3A-transfected, and Rab3B-transfected clones in the total amount of rabphilin-3A protein present in cell lysates or homogenates (data not shown). However, two potentially interesting differences between the
Rab3A- or Rab3B-overexpressing cells. This band presumably represents a proteolytic fragment of rabphilin-3A, which is highly sensitive to proteases (14). Second, the relative amount of rabphilin-3A that was membrane-associated was higher in these clones expressing recombinant Rab3A or Rab3B as compared with Rab3A-transfected or mock-transfected cells. Shown here are the results obtained for Rab3A-overexpressing clone (clone 3A 17). Levels of endogenous Rab3A were insufficient for such an analysis of untransfected or mock-transfected cells (data not shown).

**Fig. 4.** Rab3A cofractionates with catecholamine-containing secretory granules as determined by equilibrium density centrifugation. Top panel, plots of sucrose concentration and protein concentration for each fraction. Bottom panel, plots of [3H]NE radioactivity and of Rab3A and synaptophysin immunoreactivity for each fraction. Shown are the results for a Rab3A-overexpressing clone (clone 3A 17). Levels of endogenous Rab3A were insufficient for such an analysis of untransfected or mock-transfected cells (data not shown).

Rab3A- or Rab3B-overexpressing cells and all other clones did emerge when we analyzed the relative distribution of rabphilin-3A between membranes and cytosol (see Fig. 6). First, both Rab3B-expressing clones that were examined (WT8 and WT9) exhibited a 40-kDa immunoreactive band in the cytosol that was much more prominent than in the nontransfected, mock-transfected, or Rab3A-overexpressing cells. This band presumably represents a proteolytic fragment of rabphilin-3A, which is highly sensitive to proteases (14). Second, the relative amount of rabphilin-3A that was membrane-associated was higher in these Rab3B-expressing clones as compared with Rab3A-transfected clones (e.g. in clone Rab3B-WT8, 50% of the total rabphilin signal (i.e. the sum of the 80- and 40-kDa signals in the membrane pellet and supernatant) was membrane-associated, as compared with 24, 30, and 39% for the Rab3A-transfected, mock-transfected, and untransfected cells, respectively). Thus, the stable expression of Rab3B in PC12 cells has consequences on the membrane association and stability of rabphilin-3A that are not evident in mock-transfected or Rab3A-transfected cells.

**Fig. 5.** Rab3B as well as Rab3A binds to rabphilin-3A. A, binding of Rab3A and Rab3B to recombinant rabphilin-3A. Bacterially expressed GST or a GST-rabphilin fusion protein containing full-length rabphilin-3A (GST-rabphilin-3A) were immobilized on glutathione-agarose beads and incubated with PC12 or H129 C119A lysates in the presence or absence of 50 \( \mu \)M GTP-\( \gamma \)S. Bound proteins were resolved and immunoblotted with a monoclonal antibody recognizing multiple Rab3 isoforms. Note that the GST control beads contained 10 times more recombinant protein (i.e. GST) than the corresponding GST-rabphilin beads, which accounts for the diffuse, nonspecific band in the GST samples. Note also that the binding of either Rab3A or Rab3B to GST-rabphilin-3A was GTP-dependent.

**Fig. 6.** Immunoblot analysis of rabphilin-3A protein in membranes and cytosol of Rab3B- and Rab3A-transfected PC12 clones. All lanes were loaded with 50 \( \mu \)g of protein. Samples were blotted with polyclonal rabphilin-3A antibody (1:2,000). P, high speed pellete (membrane); S, supernatant (cytosol).
were normalized to total cell counts. Media were collected and replaced every second minute. Media counts and Rab3A-transfected PC12 cells. Secretion was induced at time 0. (nPC12) and selected clones of mock-transfected, Rab3B-transfected, Rab3B. We also assayed the secretory responses of two additional Rab3A-expressing clones displayed dramatically different secretory responses than those clones that were expressing wild type Rab3B. In general, the Rab3A-overexpressing clones exhibited lower secretory responses than mock-transfected or nontransfected cells, although this inhibitory effect was more subtle and variable than the stimulatory effect of Rab3B. This relatively small inhibitory effect of Rab3A overexpression on secretory efficiency is partially obscured when data from experiments that were performed on different days and different seedings are averaged together (as for the data reported in Fig. 7C).

Fig. 8 shows that the expression of Rab3B and Rab3B N135I had effects, not only on the efficiency of secretion (i.e. release normalized to total cell radioactivity) but also on the accumulation of [3H]NE (i.e. secretory cargo) by PC12 cells. Shown is the amount of [3H]NE that was accumulated from the media by mock-transfected cells and cells that were expressing Rab3A, 3B, and Rab3B N135I. Most striking are the markedly higher levels of [3H]NE uptake exhibited by the Rab3B and Rab3B N135I-expressing clones, which were on average approximately 8-fold (wild type Rab3B) and 50-fold (Rab3B N135I) greater than the uptake exhibited by mock-transfected cells. The uptake of NE by these clones was saturable and reserpine-sensitive (Table I), as expected if the majority of the [3H]NE had accumulated within secretory granules (see also the results of our density gradient analysis shown in Fig. 3). These dramatic increases in granule uptake of [3H]NE cannot be explained by differences in cell number or cell density, nor can they be explained by increased numbers of secretory granules in Rab3B-expressing and Rab3B N135I-expressing cells (see below). Interestingly, Rab3B N135I clone 32, which exhibits virtually no detectable membrane association of Rab3B N135I (Fig. 1B) and no alteration in secretory efficiency as compared with mock-transfected cells (Fig. 7C), does exhibit a markedly increased capacity for [3H]NE uptake (range: 1.4 × 10^5 to 3.7 × 10^5 cpm/35-mm Petri dish). Thus, the effect of Rab3B N135I on [3H]NE uptake appears to be independent of its ability to stimulate the efficiency of Ca^{2+}-induced NE release and requires little or no membrane association of the mutant protein.

The Effects of Rab3B and Rab3B N135I on NE Secretion and Uptake Are Not Due to Changes in Secretory Granule Number or Distribution—Given the marked effects of Rab3B and Rab3B N135I on catecholamine uptake and release by PC12 cells, we...
characterized the numbers and distributions of secretory granules (i.e. LDCVs) in selected clones. Table II shows the results of a double blind morphometric analysis in which we determined the average number per section of LDCVs in nontransfected cells, a Rab3B-expressing clone (3B 8) and a Rab3B N135I-expressing clone (3B N135I 17). We found no significant differences among clones in the numbers of LDCVs per cell. Also, we found no differences between the Rab3B-expressing clone and nontransfected cells in the numbers of LDCVs within 100 nm of the plasma membrane, despite the fact that the Rab3B clone exhibits an approximately 3-fold higher secretory efficiency (i.e. release normalized to total cell radioactivity; see Fig. 7C). Thus, the dramatic effects of Rab3B and Rab3B N135I on the secretion and uptake of \([^{3}H]NE\) cannot be explained by changes in the number or distribution of LDCVs.

**DISCUSSION**

Our results indicate that two highly homologous Rab3 isoforms (i.e. Rab3A and Rab3B) have similar targeting properties but opposite effects on regulated catecholamine secretion in PC12 cells. Heterologous expression of Rab3B markedly increased, whereas Rab3A overexpression modestly inhibited, the efficiency of Ca^{2+}-triggered NE secretion by PC12 cells. Rab3B N135I also potentiated NE release; provided that it was detected at the plasma membrane, decreased membrane association as compared with wild type Rab3B when expressed either in PC12 cells (present study) or in Madin-Darby canine kidney epithelial cells. The corresponding mutant of Rab1B (i.e. 1B N121I) is also less efficient at associating with membranes than wild type protein when transiently expressed in HeLa cells. Such Rab mutants, which correspond to an oncogenic Ras mutant (Ras N12I), have higher dissociation rates for both GDP and GTP (35). Given that the association of Rab proteins with membranes involves dissociation from guanine nucleotide dissociation inhibitor (GD1) and GDP/GTP exchange (36), it is possible that the altered nucleotide binding properties of Rab3B N135I are responsible for its less efficient membrane association (37). Another possibility is that Rab3B N135I is not as efficiently prenylated as wild type Rab3B in PC12 cells (as shown in vitro for the corresponding Rab5 mutant N133I (38), which could also reduce membrane binding. However, nonprenylated monomeric GTPases typically have lower apparent mobilities on SDS-polyacrylamide gel electrophoresis (39), whereas Rab3B N135I exhibits a higher mobility than wild type protein, as does Rab3A N135I when expressed in chromaffin cells (16). Interestingly, in preliminary experiments we have observed that phosphatase treatment of cell homogenates eliminates this difference in mobility, which implies that Rab3B and Rab3B N135I are phosphorylated to different degrees. It will be of interest to determine if such posttranslational modifications modulate the membrane targeting and functions of Rab3 isoforms.

In our studies Rab3A-overexpressing clones showed modest but variable reductions in the efficiency of secretion as compared with mock-transfected and nontransfected PC12 cells. These results are consistent with the inhibitory effect of Rab3A on secretion when transiently overexpressed in chromaffin cells (16), and the results of the Rab3A antisense and microinjection studies reported by Johannes et al. (17). This negative regulation by Rab3A also seems to be consistent with the phenotype exhibited by Rab3A-minus transgenic mice (18), which has been interpreted to indicate that Rab3A participates in the formation and/or maintenance of a prefusion docking complex (40). Overexpression of Rab3A may inhibit the dissociation of such a complex and thereby inhibit Ca^{2+}-evoked fusion and secretion.

In contrast to Rab3A, Rab3B is capable of functioning as a positive regulator of secretion when stably expressed in PC12 cells. It seems highly unlikely that the stimulatory effect of Rab3B on regulated secretion by PC12 cells is due to some
nonspecific effect on the availability of accessory proteins such as guanine nucleotide dissociation inhibitor and guanine nucleotide exchange factor for other Rabs, in particular Rab3A. The activities of such accessory proteins are not typically limited to conditions of moderate Rab overexpression (16, 41). Moreover, we noted no effect of Rab3B expression on the membrane targeting of endogenous Rab3A, which might otherwise be expected if Rab3B were competing with Rab3A for accessory proteins such as guanine nucleotide dissociation factor and guanine nucleotide exchange factor. On the other hand, we cannot rule out the possibility that long-term Rab3B expression leads to secondary alterations in the secretory phenotypes of PC12 neuroendocrine cells that might not be evident in transient transfection experiments (e.g. changes in rabphilin stability, see below).

It seems plausible that the stimulatory effect of Rab3B on secretion in PC12 cells is related to its interaction with rabphilin-3A, a putative downstream effector for Rab3A (31, 42). Our results indicate that rabphilin-3A is also capable of interacting with Rab3B in a GTP-dependent fashion. Chung et al. (43) have recently reported that rabphilin-3A is a positive regulator of secretion in chromaffin cells, possibly by regulating interactions between secretory granules and the actin cytoskeleton. Rab3B could enhance secretion through rabphilin-3A, by competing with endogenous Rab3A for rabphilin-3A binding (which may release rabphilin-3A from an inactive state) and/or by activating rabphilin-3A in a way that Rab3A cannot. The apparently lower stability of rabphilin in Rab3B-expressing PC12 cells could be related to a disruption of Rab3A-rabphilin binding, similar to the reduced rabphilin stability that was reported for the Rab3A knockout mouse (14). In this case, the effects of heterologous Rab3B expression in PC12 cells could be considered to be a nonphysiological maneuver to disrupt Rab3A function in these cells. However, competitive interactions between Rab3A and Rab3B for such downstream effectors also could be physiologically relevant, given a report that some neural cells express both isoforms (44). We also think it is likely that Rab3B can regulate exocytosis independently of any such competitive interactions it may have with Rab3A. For example, inhibition of Rab3B expression in anterior pituitary cells (which do not express Rab3A) reportedly leads to an inhibition of Ca2+-dependent exocytosis (15). In addition, our identification of a rabphilin-like protein in colonic epithelial cells (i.e. cells that express Rab3B but not Rab3A) indicates that Rab3B-rabphilin interactions may be functionally relevant in a Rab3A-negative setting. Rabphilin may be an important component of the exocytic machinery in a variety of cell types where its functional activity may be regulated differentially by various Rab3 isoforms.

We found that Rab3B N135I also stimulates the efficiency of NE secretion in PC12 cells, provided that it is membrane-associated. On the basis of the relative rates of GTP and GDP dissociation for Rab3A N135I, it has been argued that this mutant would likely be preferentially GTP-bound in vivo (35). Our results are consistent with this notion, given that the GTP-bound form of a Rab protein is presumably the functionally active form. However, we note that apparently conflicting results have been obtained in studies examining the functional properties of this corresponding mutation in Rab3B (41). Moreover, we observed that Rab3B and Rab3B N135I have qualitatively similar effects on Ca2+-induced regulated secretion in PC12 cells. Jahnnes et al. (17) have reported a similar finding for Rab3A and Rab3A N135I; namely, both inhibit Ca2+-evoked secretion. In contrast, the corresponding mutant of Rab1B and Rab5 function as dominant negative mutants; e.g. Rab5 N1331 inhibits endocytosis, whereas the overexpression of wild type Rab5 stimulates endocytosis. As argued by Holz et al. (16), this disparity may be attributable to the different dynamics of regulated secretion (e.g. Ca2+-evoked catecholamine release) and constitutive membrane traffic (e.g. endocytosis). For example, in the context of a constitutive membrane traffic pathway where the Rabs must continuously cycle on and off the relevant donor membrane, such mutants may sequester certain accessory proteins (e.g. guanine nucleotide dissociation inhibitor or guanine nucleotide exchange factor) in nonproductive conformations. However, the corresponding Rab3B mutants, once targeted to a secretory granule where they may remain until secretion is evoked, appear fully capable of mimicking the corresponding wild type protein during a round of stimulated secretion.

Perhaps the most surprising result of the present study was the dramatic stimulation by Rab3B and Rab3B N135I of the accumulation of [3H]NE into PC12 secretory granules. The approximately 8-fold (wild type Rab3B) and 5-fold (Rab3B N135I) greater uptake cannot be explained by increased numbers of secretory granules in Rab3B-expressing and Rab3B N135I-expressing cells. Moreover, the effects of Rab3B and Rab3B N135I on NE secretory efficiency and on NE uptake into granules may be separable; specifically, whereas the stimulation of secretory efficiency appears to require membrane association, catecholamine uptake was stimulated in the virtual absence of any membrane association of Rab3B N135I (see Figs. 1B and 7; clone Rab3B N135I 32). The implication of these results is that Rab3B isoforms are capable of regulating the secretory pathway at multiple levels including the amount of cargo available for secretion. We presently do not understand the mechanism by which Rab3B and Rab3B N135I stimulate NE uptake into granules, nor do we know if these Rab3B isoforms (including Rab3A) regulate other aspects of catecholamine metabolism. In this regard we note that rabphilin-3A binds GTP cyclohydrolase I, the rate-limiting enzyme for catecholamine synthesis (45). This observation, in combination with the present results, argues for a detailed examination of the roles of Rab3 isoforms in regulating the various steps in catecholamine metabolism (i.e. biosynthesis, transporter activity, etc.) in neuroendocrine cells. The various stably transfected PC12 clones that we have generated should be useful for such an analysis.

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