Cellular/Molecular

Divergent Functions of Neuronal Rab11b in Ca^{2+}-Regulated versus Constitutive Exocytosis

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Using PC12 cells that express transfected human growth hormone (hGH) as a secreted reporter protein, we have searched for Rab proteins that function in exocytosis. Among the Rab proteins tested, we found that besides the previously described Rab3 proteins, only members of the Rab11 family (Rab11a, 11b, and 25) impaired Ca^{2+}-induced exocytosis. Rab11b, which is enriched in brain, had the strongest effect. Consistent with a role in exocytosis, Rab11 and Rab3 proteins were colocalized with other vesicle proteins on secretory vesicles in PC12 cells and on mature synaptic vesicles in brain. Rab11b mutants that fix Rab11b in the GTP- or GDP-bound state both effectively inhibited Ca^{2+}-induced exocytosis but seemed to act by distinct mechanisms: whereas GDP-bound Rab11b greatly stimulated constitutive secretion of hGH and depleted hGH stores in secretory vesicles, GTP-bound Rab11b only had a moderate effect on constitutive secretion and no effect on vesicular hGH stores. These results suggest that, consistent with a GTP-dependent regulation of Rab function, GDP-bound Rab11b indirectly inhibits Ca^{2+}-triggered exocytosis by causing the loss of hGH from the PC12 cells, whereas GTP-bound Rab11b directly impairs Ca^{2+}-triggered exocytosis. In contrast to neuroendocrine PC12 cells in which GTP- and GDP-bound Rab11b inhibited Ca^{2+}-induced, but not constitutive, exocytosis, in non-neuronal cells GTP- and GDP-bound Rab11b inhibited constitutive exocytosis and caused an accumulation of cellular hGH. Viewed together, our data suggest that, in addition to other functions, Rab11 has a specific role in neuronal and neuroendocrine but not in non-neuronal cells as a GTP-dependent switch between regulated and constitutive secretory pathways.

Key words: **neurotransmitter release; constitutive exocytosis; protein sorting; membrane fusion; subcellular distribution; PC12 cells; synaptic vesicles**

Introduction

Rab proteins are small Ras-like GTPases that function in membrane traffic. More than 60 Rab proteins are expressed in mammalian cells (Pereira-Leal and Seabra, 2001). Most Rabs participate in the directed transport and tethering of vesicles to their target membranes (Jahn et al., 2003; Pfeffer, 2003). These functions are executed via GTP-dependent, spatially and temporally restricted interactions of Rabs with effector proteins (Segev, 2001; Zerial and McBride, 2001). In yeast, elimination of a compartment-specific Rab often blocks downstream membrane fusion reactions and leads to the accumulation of transport vesicles (Segev et al., 1988; Haas et al., 1995; Benli et al., 1996). This has been particularly well studied for yeast exocytosis, in which the Rab protein Sec4p is essential (Salminen and Novick, 1987). Deletion of Sec4p is lethal and causes accumulation of small transport vesicles in the cytosol (Goud et al., 1988). Mammalian cells execute regulated and constitutive types of exocytosis, but no Rab protein was identified that could serve as a mammalian Sec4p-equivalent in either type of exocytosis. Rab3 proteins, abundant components of synaptic and secretory vesicles, have been implicated in regulated exocytosis (Geppert and Südhof, 1998). However, genetic studies in mice and *Caenorhabditis elegans* revealed that Rab3 proteins are not required for regulated or constitutive exocytosis, as such, but perform an important regulatory role (Castillo et al., 1997; Geppert et al., 1997; Nonet et al., 1997), suggesting that other Rab proteins must be involved.

Rab11a, Rab11b, and Rab25 are closely related, evolutionary conserved Rab proteins that are differentially expressed. Rab11a is ubiquitously synthesized (Sakurada et al., 1991), Rab11b is enriched in brain and heart (Lai et al., 1994) and Rab25 is only found in epithelia (Goldenring et al., 1993). Rab11/25 proteins seem to regulate recycling pathways from endosomes to the plasma membrane and to the *trans*-Golgi network (Chen et al., 1998; Ren et al., 1998; Wilcke et al., 2000). Furthermore, Rab11a is thought to function in the histamine-induced fusion of tubulovesicles containing H^+, K^+-ATPase with the plasma membrane in gastric parietal cells (Calhoun et al., 1998; Duman et al., 1999) and in insulin-stimulated insertion of GLUT4 in the plasma membrane of cardiomyocytes (Kessler et al., 2000). However, the precise roles of Rab11/25 proteins in regulated and constitutive exocytosis remain incompletely understood.

Ca^{2+}-regulated exocytosis of dense core vesicles in PC12 cells

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exhibits fundamental properties that are similar to synaptic vesicle exocytosis, making PC12 cells a useful biochemical model system to analyze regulated exocytosis (Hay and Martin, 1993; Darchen et al., 1995; Sugita et al., 1999). In the present study, we used PC12 cells to examine the function of Rabs in Ca^{2+}-dependent and constitutive exocytosis of human growth hormone (hGH) from dense core vesicles. A screen of Rab proteins identified Rab11b as a potent inhibitor of Ca^{2+}-regulated exocytosis in transfected PC12 cells, suggesting a role in regulated secretion in these neuroendocrine cells. In support of such a role, we show that Rab11 proteins are present on secretory vesicles in PC12 cells and on synaptic vesicles in brain. Structure/function studies revealed that GTP- and GDP-bound forms of Rab11b were equally effective in inhibiting exocytosis but exhibited distinct properties. Our data provide evidence that Rab11b participates in Ca^{2+}-regulated exocytosis of dense core and synaptic vesicles and integrates regulated and constitutive exocytosis in neurons and neuroendocrine cells.

Materials and Methods

Expression vectors. Expression vectors for wild-type and mutant Rab proteins were constructed by subcloning their cDNAs into pcDNA3 or pCMV5 mammalian expression vectors. Rab15 expression vector was kindly provided by Dr. L. Elferink (Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX). The hGH expression vector pHGHCMV5 was described previously (Sugita et al., 1999). To generate N-terminal green fluorescent protein (GFP) fusion constructs, wild-type and mutant Rab11a, Rab11b, and Rab25 cDNAs were amplified by PCR and subcloned into pEYFP-N1 expression vector (Clontech, Palo Alto, CA). All expression constructs were verified by sequencing.

Antibodies. Glutathione *S*-transferase (GST)-fused recombinant Rab11b was expressed in bacteria, purified on a glutathione agarose column, and digested with thrombin to remove GST part. Purified recombinant protein was then used as an antigen to produce polyclonal rabbit antibodies against Rab11b (T3746). Antibodies were immunopurified from the serum using standard procedures. Monoclonal antibody against Rab11 was from Transduction Laboratories (San Diego, CA). All other antibodies were described previously (Janz et al., 1999).

Measurements of regulated exocytosis in transfected PC12 cells. PC12 cells were maintained in RPMI 1640 media containing 10% heat-inactivated horse serum, 5% fetal bovine serum, and penicillin and streptomycin (50 U/ml each) at 37°C in a 5% CO_2-humidified atmosphere. PC12 cells were split from a confluent flask and seeded after vigorous trituration onto six-well plates (35 mm diameter) to achieve 50–60% confluence at the day of transfection. In most experiments, pHGHCMV5 and test plasmids were cotransfected at 1 μg of DNA/well each using Fugene 6 transfection reagent (Roche, Indianapolis, IN) at a w/v ratio of 1:3, according to the manufacturer's specifications. Two to 3 d after transfection, PC12 cells were collected and replated onto collagen-coated 12-well plates (22 mm diameter), with the cells from one 35 mm well distributed into four 22 mm wells. On the next day, cells were rinsed briefly with Krebs bicarbonate buffer (KBB) (in mM: 118 NaCl, 25 $NaHCO_3$, 3.5 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 11.5 glucose, and 5 HEPES-NaOH, pH 7.5) and incubated for 15 min at 37°C in 5% CO_2 atmosphere in KBB or stimulating media (high K^+ KBB containing 56 mM KCl and 59.5 mM NaCl, or 0.5 nM α-latrotoxin in KBB). Assay media were cleared by centrifugation at 1000 × *g* for 4–5 min, and EDTA was added to 4–5 mM. PC12 cells were lysed in PBS containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride by three cycles of rapid freezing and thawing, and resulting cell extracts were cleared by high-speed centrifugation. The amounts of hGH in the medium and the cell extracts were quantified by RIA (Nichols Institute Diagnostics, San Clemente, CA).

Imaging of PC12 cells. PC12 cells were plated onto polylysine-coated coverslips and transfected with GFP–Rab fusion proteins as described above. Three days after transfection, cells were fixed *in situ* for 10 min with 2% paraformaldehyde/PBS and imaged on a Leica TCS-SP2 confocal microscope using 100× (numerical aperture, 1.3) objective.

Subcellular fractionation of PC12 cells. PC12 cells were cotransfected with hGH expression vector and control empty vector or various Rab11b expression vectors as described above. Two to 3 d after transfection, PC12 cells were labeled overnight with [^{3}H]-norepinephrine (6 μCi, 30–50 Ci/mmol per six-well plate or 100 mm dish; Amersham, Piscataway, NJ) in the presence of 0.5 mM ascorbic acid. PC12 cells were washed with KBB buffer, resuspended in 0.25 ml of ice-cold water, and incubated 5 min on ice. Cell suspension was diluted with an equal volume of 2× TNE buffer (1× 150 mM NaCl, 2 mM EGTA, and 20 mM Tris-HCl, pH 7.5) and passed 10 times through the 28.5 gauge needle. Cell homogenate was centrifuged twice at 800 × *g* for 10 min to obtain postnuclear supernatant (PNS). PNS (0.4 ml) was loaded onto 10 ml discontinuous sucrose gradients made of 2 ml steps of 0.5, 1.0, 1.25, 1.5, and 2.0 M sucrose in TNE buffer and centrifuged at 35,000 rpm for 2.5 hr (SW41, ~200,000 × *g*). Fractions of 0.5–0.6 ml were collected from the top of gradients. The amounts of norepinephrine in fraction aliquots were determined by liquid scintillation counting. To measure hGH, fractions were diluted with PBS, subjected to three cycles of rapid freezing and thawing, and analyzed by RIA. For analysis of protein markers, fractions were diluted 10 times with TNE buffer and centrifuged overnight at 200,000 × *g*. Membranes were resuspended in SDS-PAGE loading buffer and analyzed by immunoblotting.

Measurements of constitutive exocytosis in transfected PC12, HeLa, and HEK293 cells. PC12 cell transfections were performed as described above. HeLa and HEK293 cells were cotransfected with pHGHCMV5 and test plasmids at a 1:20 ratio (typically 0.05 μg of pHGHCMV5 and 1 μg of a test plasmid) to ensure efficient cotransfection. Aliquots of the extracellular media (50 μl) were collected on days 2–4 after transfections for PC12 cells and on day 2 for HeLa and HEK293 cells, diluted 10-fold with PBS containing 5 mM EDTA, and assayed for hGH. PC12 cells on day 4 after transfections and HeLa and HEK293 cells on day 2 were collected, washed with PBS, and lysed by four cycles of freeze-thawing to determine intracellular hGH. The amounts of hGH secreted into the culture media were normalized to the amount of hGH remaining in transfected cells.

Miscellaneous procedures. Purification of synaptic vesicles was performed by controlled pore-glass chromatography as described (Nagy et al., 1976). Purity of fractions was evaluated using well established synaptic vesicle marker proteins (synaptophysin 1, synaptotagmin 1, Rab3a). COS cells were transfected using standard procedures. SDS-PAGE and immunoblotting were performed as described (Laemmli, 1970; Towbin et al., 1979).

Results

Rab11b inhibits regulated exocytosis in PC12 cells

We cotransfected PC12 cells with a test vector encoding a Rab protein and a reporter vector encoding hGH and measured secretion of hGH from the transfected PC12 cells under various conditions. Because in such cotransfection experiments most transfected cells coexpress both proteins, the secretion of hGH can be used as a marker for transfected cells (Schweitzer and Kelly, 1985). We induced Ca^{2+}-regulated secretion of hGH from PC12 cells with high K^+ that depolarizes the membrane and triggers exocytosis by gating Ca^{2+}-influx via Ca^{2+} channels and with α-latrotoxin that induces exocytosis in PC12 cells by a Ca^{2+}-dependent mechanism that bypasses Ca^{2+} channels (Südhof, 2001). Afterward, we quantified secreted hGH in the medium and cellular hGH by RIA and calculated hGH secretion as a function of the total hGH (Holz et al., 1994; Schluter et al., 2002). In a typical 15 min stimulation experiment, 4–6% of the hGH were recovered in the medium under basal conditions without secretagogues, and high K^+ induced a 4- to 5-fold stimulation of secretion above baseline (to 20–30% of total cellular hGH), whereas the more potent α-latrotoxin caused a 8- to 10-fold stimulation of secretion (to 35–60%). When we cotransfected eight different Rab proteins with hGH, we found that only Rab3a and

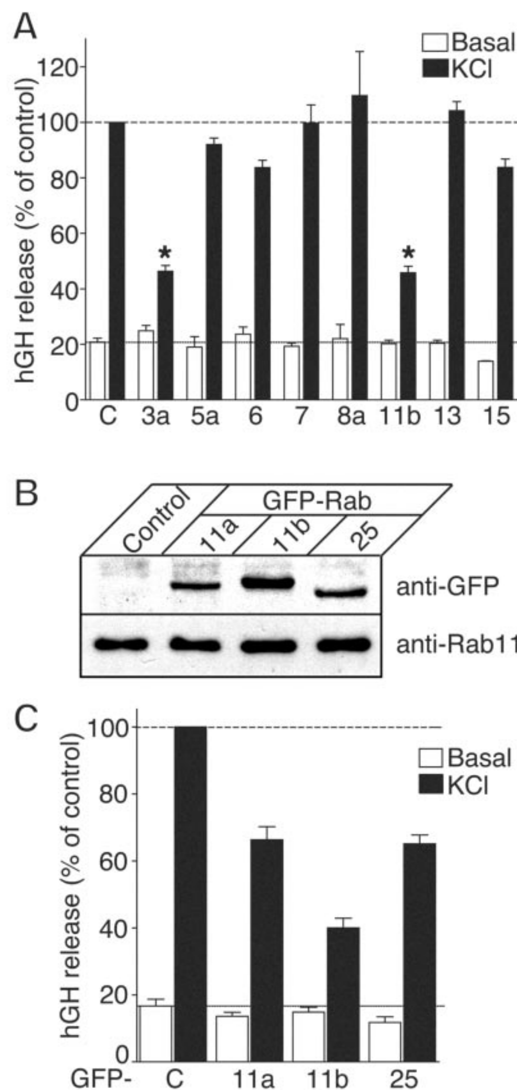

Figure 1. Rab11b inhibits regulated exocytosis in PC12 cells. *A*, Specificity of the inhibitory effect of Rab11b on regulated exocytosis. PC12 cells were cotransfected with an hGH reporter vector and various expression vectors: a control vector encoding no protein or vectors encoding the Rab proteins that are identified by their number below the bar diagrams. Seventy-two hours after transfection, cells were stimulated with control medium (□) or with depolarizing medium containing 56 mM KCl (■) for 15 min, and the amount of hGH released into the medium and retained in the cells was determined by RIA. hGH release was calculated as the fraction of total hGH and normalized for the K^+-induced secretion obtained under control conditions (100%). Results shown are means ± SEMs from four independent experiments performed in duplicates. *B*, Expression of GFP-fused Rab11/25 proteins in PC12 cells. PC12 cells were cotransfected with an hGH expression vector and either an expression vector encoding GFP (control) or GFP-fused wild-type Rab11a, Rab11b, and Rab25 proteins. The expression of the recombinant and endogenous Rab11/25 proteins was verified by immunoblotting. *C*, Effects of transfected GFP-Rab11/25 proteins on regulated exocytosis tested with the hGH cotransfection assay. The results were normalized for the K^+-induced secretion obtained under control conditions (100%). Data are from three independent experiments performed in duplicates.

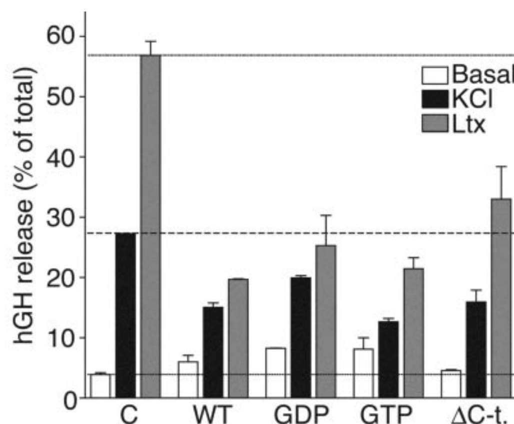

Figure 2. Inhibition of regulated exocytosis by Rab11b is independent of the secretagogue and the GTP/GDP-binding or prenylation status of Rab11b. PC12 cells were cotransfected with an hGH expression vector and either a control expression vector (C) or expression vectors encoding wild-type (WT) and mutant Rab11b proteins (see Results). Cells were treated under control conditions (□) or stimulated by K^+ depolarization (KCl; ■) or 0.5 nM α-latrotoxin (Ltx; ▒). Secretion was calculated as described above, except that the results were not normalized to the control condition. Data were derived from a single representative experiment performed in duplicates and repeated three times.

Rab11b appreciably inhibited hGH secretion in PC12 cells stimulated by K^+ depolarization (Fig. 1*A*). Rab11b potently impaired Ca^{2+}-dependent exocytosis induced by either K^+ depolarization or α-latrotoxin (Fig. 2) (data not shown). Similar to Rab3a, Rab11b inhibited Ca^{2+}-stimulated hGH secretion without a significant effect on basal secretion as measured over a 15 min period. The effect of Rab3a is well documented because the role of this protein in exocytosis has been studied extensively (Darchen et al., 1995; Chung et al., 1999; Schluter et al., 2002), but the strong inhibition of exocytosis by Rab11b was unexpected.

Rab11b is a member of a Rab subfamily that includes the closely related Rab11a and Rab25. To test whether Rab11a and Rab25 also inhibit regulated exocytosis in PC12 cells, we used N-terminal GFP fusion proteins of Rab11a, Rab11b, and Rab25. GFP fusion proteins were used because the common GFP moiety can be used to localize the proteins in cells and to compare their expression levels. In transfected PC12 cells, all three Rab GFP fusion proteins were expressed well, with higher levels for Rab11b than for Rab11a or Rab25, and exhibited similar localization patterns (Fig. 1*B*) (data not shown). All three Rab11 GFP fusion proteins depressed Ca^{2+}-induced hGH exocytosis, with less inhibition by Rab11a and Rab25 (35%) than by Rab11b (~60%) (Fig. 1*C*). The apparent difference in inhibition may be because of the higher expression levels of GFP-fused Rab11b in the transfected PC12 cells or to a preferential function of Rab11b in regulated exocytosis.

Rab proteins are thought to regulate membrane traffic in a GTP-dependent manner (Segev, 2001; Zerial and McBride, 2001). For many Rab proteins, only mutants that fix the Rab protein into a GDP-bound state are potent inhibitors of a trafficking reaction in a dominant negative manner, presumably because the exogenous GDP-Rab protein blocks recycling of the corresponding endogenous Rab to its cognate organelles. However, there are notable exceptions to this rule. In the case of Rab1 and 2 (Tisdale et al., 1992), Rab3a (Holz et al., 1994; Johannes et al., 1994), and Rab15 (Zuk and Elferink, 1999), the GTP-bound forms act as dominant negatives. To examine whether the nucleotide binding state influences the inhibitory action of Rab11 in exocytosis, we investigated Rab11b mutants that are preferentially bound to GDP (S25N) or GTP (S20V and Q70L) (Ren et al., 1998; Wang et al., 2000). Surprisingly, Rab11b potently impaired Ca^{2+}-dependent exocytosis independent of its nucleotide binding state (Fig. 2). The inhibitory effects of two different GTP-bound mutants of Rab11b (S20V and Q70L) were identical (data not shown), and in most experiments, the S20V mutant was used. In addition, we studied a Rab11b mutant from which the last five amino acids were removed (ΔC-t.). This truncation mutant lacks

the geranylgeranylation site required for attaching Rab11b to membranes but, nevertheless, also inhibited Ca^{2+}-dependent exocytosis (Fig. 2).

Inhibition of exocytosis by both GDP- and GTP-bound Rab11b is unexpected in view of the GTP-dependent regulation of Rab proteins (Segev, 2001; Zerial and McBride, 2001). To ensure that the different Rab11b mutants were not expressed at very different levels (which could result in apparent inhibition by a partially inactive GTP-bound form if the GTP-bound form was expressed at 10 times higher levels than the GDP-bound form) and that the different mutations, in fact, altered the properties of Rab11b, we again used N-terminal GFP fusion proteins as described above for the comparison of Rab11a, Rab11b, and Rab25 (Fig. 1*B*,*C*). Immunoblotting confirmed that the transfected GFP fusion proteins of wild-type, GDP-mutant, GTP-mutant, and C-terminally truncated Rab11b were expressed at similar levels in PC12 cells (Fig. 3*A*). We then used secretion assays to demonstrate that the GFP fusion proteins inhibited hGH secretion similarly to Rab11b proteins that were not fused to GFP (Fig. 3*B*); thus, both GTP- and GDP-bound mutants as well as the truncation mutant are comparably effective inhibitors. However, confocal microscopy revealed that the different mutants exhibited distinct subcellular distributions (Fig. 3*C*): wild-type and GTP-bound, membrane-anchored Rab11b exhibited a vesicular localization pattern with no staining of the nucleus, whereas the GDP form of Rab11b induced larger labeled subcellular structures that were absent from cells expressing GTP and wild-type Rab11b and may correspond to stabilized fused endosomes previously described in similarly transfected cells (Vitale et al., 1998). Finally, the GFP fusion protein of C-terminally truncated Rab11b was diffusely distributed in the cytoplasm and the nucleus, as expected for a small soluble protein (Fig. 3*C*). Overall, the observed localizations are consistent with previous results from transfected non-neuronal cells (Ren et al., 1998; Schlierf et al., 2000) and, thus, confirm that the different Rab11b mutants exhibit distinct properties as expected for different nucleotide-binding states.

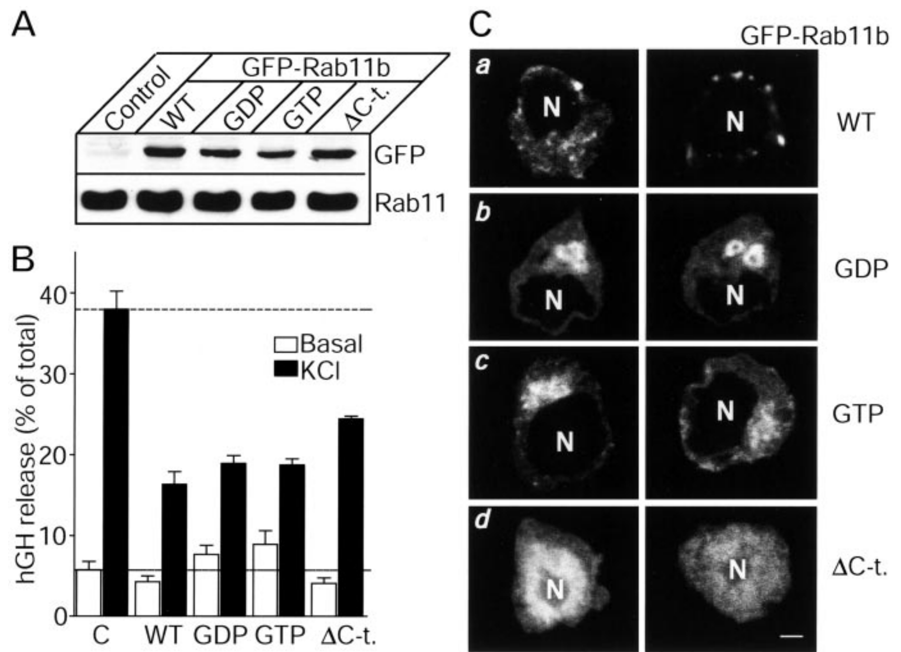

Figure 3. GFP-fused Rab11b proteins are potent inhibitors of K^+-induced exocytosis in PC12 cells. *A*, PC12 cells were cotransfected with an hGH expression vector and either an expression vector encoding GFP (control) or GFP-fused wild-type and mutant Rab11b proteins. The expression of the recombinant and endogenous Rab11b proteins was verified by immunoblotting. *B*, Effects of transfected GFP-Rab11b proteins on regulated exocytosis tested with the hGH cotransfection assay. Data were derived from a single representative experiment performed in duplicates and repeated three times. *C*, Localization of GFP-fused Rab11b proteins (*a*–*d*) determined by confocal laser microscopy. The position of nucleus is indicated by N. Scale bar, 2 μm.

Endogenous Rab11 proteins are localized to secretory and synaptic vesicles

In transfected PC12 cells, hGH is packaged into dense core vesicles that undergo Ca^{2+}-dependent fusion with the plasma membrane. Inhibition of Ca^{2+}-triggered hGH secretion by Rab11 proteins suggests a role for these Rab proteins in regulated exocytosis. Is Rab11 actually localized to the corresponding secretory organelles? To test this, we raised a polyclonal antibody to recombinant Rab11b and determined its specificity using immunoblots of transfected COS cells that express various Rab proteins. These experiments demonstrated that the newly generated antibody does not recognize Rab proteins that are not part of the Rab11 subfamily (Fig. 4*A*). However, the polyclonal Rab11b antibody does cross-react with Rab11a and Rab25, a not surprising result given the sequence similarity between Rab11b to Rab11a and

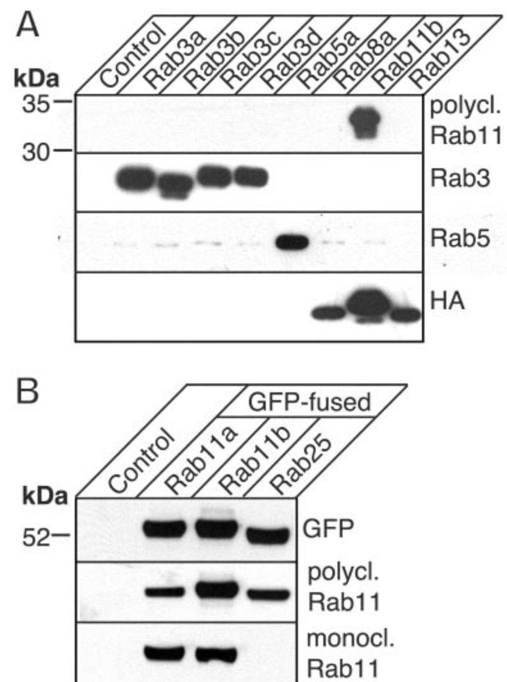

Figure 4. Characterization of Rab11 antibodies. *A*, COS cells were transfected with an empty vector (control) or Rab3a-d, Rab5a, Rab8a, Rab11b, or Rab13 expression vectors. Two days after transfection, cellular proteins were analyzed by immunoblotting using a newly generated polyclonal antibody against Rab11b or monoclonal antibodies against Rab3, Rab5, or the hemagglutinin antigen (HA) tag that was fused to Rab8a, 11b, and 13. Additional lower bands are attributable to incomplete geranylgeranylation of Rab proteins in transfected COS cells (Johnston et al., 1991). *B*, COS cells were transfected with an expression vector encoding GFP (control) or GFP-fused Rab11a, Rab11b, and Rab25. Two days after transfection, cellular proteins were analyzed by immunoblotting using antibody against GFP and polyclonal and monoclonal antibodies against Rab11 proteins.

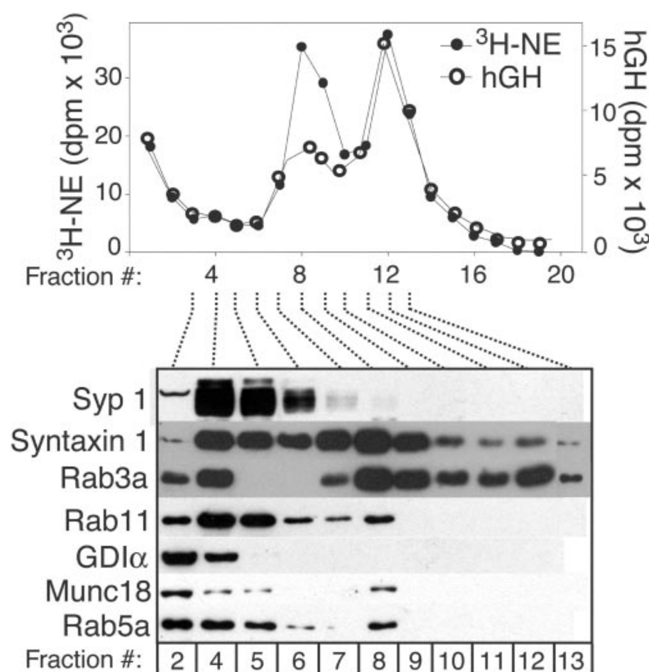

Figure 5. Subcellular fractionation of PC12 cells: identification of secretory compartments. PC12 cells were transfected with hGH as described above. Two days after transfection, PC12 cells were labeled overnight with ^{3}H-norepinephrine and lysed, and organelles in the postnuclear supernatants were separated on discontinuous sucrose gradients. Gradient fractions were analyzed by liquid scintillation counting to measure ^{3}H-norepinephrine (●, top), by RIA to determine hGH (○, top) and by immunoblotting of membranes for synaptophysin 1 (Syp 1), syntaxin 1, Rab3a, Rab11, GDIα, Munc18, and Rab5a (bottom).

Rab25 (Fig. 4*B*). We also examined the specificity of a commercially available monoclonal Rab11 antibody that only recognized Rab11a and Rab11b but not Rab25 (Fig. 4*B*).

We next transfected PC12 cells with hGH and labeled them with ^{3}H-norepinephrine that is taken up into secretory vesicles. Postnuclear supernatants prepared from the labeled PC12 cells were then fractionated by equilibrium centrifugation on a discontinuous sucrose gradient. We analyzed the gradient fractions for ^{3}H-norepinephrine by scintillation counting (to localize the secretory vesicle peaks), for hGH by RIA, and for marker proteins by immunoblotting (Fig. 5). Norepinephrine and hGH cofractionated at the top of the gradient in the cytosol fraction (fraction 2), and in two peaks in the middle of the gradient (fractions 8 and 12) that probably contain dense core vesicles. Consistent with this conclusion, secretory vesicle marker proteins were also detected in these two peaks and additionally present in a light fraction (Fig. 5, fraction 4). Interestingly, the ratios of the various secretory proteins differed between the three peaks. The lightest peak contained high concentrations of synaptophysin 1 and Rab3a, identifying it as the synaptic-like microvesicle fraction (Thomas-Reetz and De Camilli, 1994), although other light membranes, such as endosomes, trans-Golgi network (TGN) vesicles, and small transport vesicles, are also likely to be present in this fraction. The second peak (fraction 8) coincided with a high concentration of syntaxin 1, Rab3a, Rab5a, and Munc18, whereas the third peak (fraction 12) included relatively low concentrations of exocytic proteins. Only Rab3a and synaptotagmin 1 (data not shown) were enriched in this fraction, and synaptophysin 1, Munc18, and Rab5a were not detectable. Endogenous Rab11 was mostly found in the light fraction that contained the synaptic-like microvesicles and other light membranes, and in the first of the two dense core vesicle peaks (Fig. 5). The distribution of Rab11 was similar to that of Rab5a, Munc18, and synaptophysin 1, which is one of the best markers for synaptic and secretory vesicles, except that in dense core vesicle fractions, the relative concentration of Rab11 was higher than that of synaptophysin 1. Similar results were obtained with the monoclonal Rab11 antibody (data not shown), confirming that the detected immunoreactive band corresponds to Rab11a or Rab11b but not Rab25.

The subcellular fractionation shown in Figure 5 demonstrates that the distribution of Rab11 mirrors that of the secretory vesicle marker synaptophysin 1, suggesting that Rab11 may be present on mature dense core vesicles at low levels similar to synaptophysin 1, which is highly enriched on synaptic-like microvesicles but also present only at low levels on dense core vesicles (Fournier et al., 1989; Walch-Solimena et al., 1993). These results, however, are also consistent with previous results localizing Rab11 to other intracellular membranes (Goldenring et al., 1996; Ullrich et al., 1996; Casanova et al., 1999; Wilcke et al., 2000) and do not definitively demonstrate that Rab11 is actually present on secretory vesicles because of the limited purity of subcellular fractions isolated by gradient centrifugation. The secretory vesicles that can be prepared at the highest purity are synaptic vesicles. Therefore, we prepared synaptosomes (P2) from rat brain, isolated crude synaptic vesicles (LP2) by differential centrifugation from these synaptosomes, and further purified the vesicles by controlled pore-glass chromatography. The chromatography produced two major fractions: peak 1, which contains myelin and other small membrane fragments, and peak 2, which contains pure synaptic vesicles (Nagy et al., 1976). Immunoblotting failed to uncover significant enrichment of Rab11 in crude synaptic vesicles (Fig. 6). However, the additional purification step of controlled pore-glass chromatography revealed that pure vesicles do, in fact, contain appreciable amounts of Rab11 proteins (Fig. 6). The degree of enrichment of Rab11 in synaptic vesicles was comparable with that of Rab5a, which is known to be on synaptic vesicles (Fischer von Mollard et al., 1994), but not as high as that of the well characterized synaptic vesicle proteins synaptophysin 1 and synaptotagmin 1 (Fig. 6). These data indicate that, similar to Rab5a, Rab11 is a synaptic vesicle protein that is also localized to other organelles.

Distinct inhibitory mechanisms of GTP- and GDP-mutant Rab11b

It is surprising that both GTP- and GDP-bound Rab11b inhibited Ca^{2+}-induced exocytosis in PC12 cells. One possibility is that Rab11b, as a known *trans*-Golgi trafficking protein, simply interferes with the exit of secretory proteins from the *trans*-Golgi network by a nonspecific effect and that their presence on secretory vesicles is functionally insignificant. To examine this possibility, we measured the effects of transfected wild-type or mutant Rab11b proteins on constitutive hGH secretion in PC12 cells. A general *trans*-Golgi block should also inhibit constitutive secretion, and such a change would not be readily apparent in measurements of basal release during the Ca^{2+}-induced exocytosis experiments (Fig. 1), because constitutive secretion operates on a longer time scale than the short interval examined in the regulated secretion assays, and most "basal" release observed in the regulated secretion assays likely reflects cell lysis.

Using PC12 cells transfected with hGH alone or in combination with wild-type or mutant Rab11b, we measured the total amount of hGH secreted into the medium on days 2–4 after transfection (Fig. 7). We observed a dramatic increase (400–500%) in constitutive secretion from PC12 cells that ex-

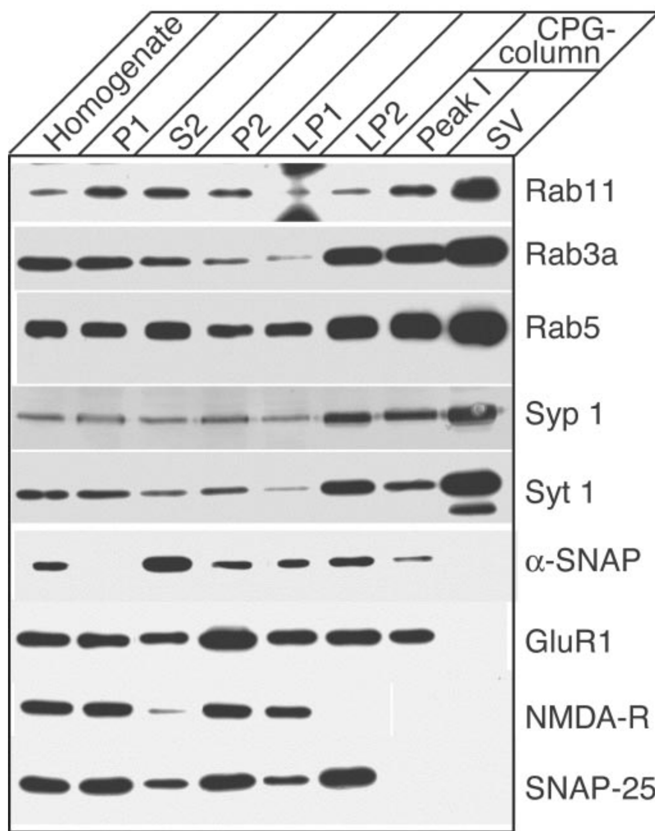

Figure 6. Rab11 is enriched on purified synaptic vesicles. Immunoblot analysis of fractions obtained during the purification of synaptic vesicles by differential centrifugation and controlled pore-glass chromatography. Note that Rab11 exhibits co-enrichment with synaptic vesicle markers such as synaptophysin 1 (Syp 1), synaptotagmin 1 (Syt 1), and Rab3a but not with plasma membrane proteins SNAP-25, glutamate receptors GluR1and NMDA-R, or with soluble cytosolic protein α-SNAP. The purification procedure was repeated two times with identical results.

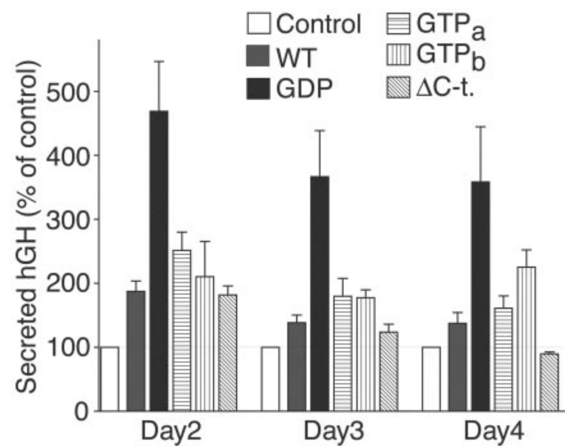

Figure 7. Effect of Rab11b proteins on constitutive exocytosis in PC12 cells. PC12 cells were co-transfected with expression vectors encoding hGH and wild-type or mutant Rab11b. Two GTPase-deficient Rab11b mutants were used: S20V (GTP_a) and Q70L (GTP_b). hGH secreted into the extracellular medium was measured by RIA on days 2–4 after transfection and is calculated as a fraction of the hGH remained in the cells and normalized to the value for control transfected cells. Data shown are means ± SEMs from five independent experiments performed in triplicates.

pressed GDP-bound Rab11b and a moderate increase (150–200%) from PC12 cells that expressed wild-type Rab11b, GTP-bound Rab11b, or Rab11b that lacked the C terminus (Fig. 7). In these experiments, we confirmed the effect of the GTP-bound forms with two different mutants of Rab11b (S20V (GTP_a) and Q70L (GTP_b)). The increase in constitutive secretion induced by the Rab11b mutants rules out the notion of a general *trans*-Golgi block. The differential potency of GTP-bound versus GDP-bound Rab11b suggests that the GTP- and GDP-bound forms of Rab11b have different actions.

Rab3a also inhibits Ca^{2+}-induced exocytosis and stimulates constitutive exocytosis, although in contrast to Rab11b only the GTP-bound form of Rab3a is effective (Table 1) (Schlüter et al., 2002). This suggests the possibility that a block in Ca^{2+}-induced exocytosis may generally cause an "overflow" of hGH into the constitutive pathway and that the relatively more potent action of the GDP-bound form of Rab11b may reflect a higher specific activity. To investigate this possibility, we tested whether the light chain of tetanus toxin, a potent inhibitor of Ca^{2+}-triggered exocytosis, also causes an increase in constitutive exocytosis. However, tetanus toxin light chain, when cotransfected with hGH, had no effect on constitutive exocytosis different from Rab11b (data not shown), demonstrating that blocking regulated exocytosis does not by itself induce a constitutive overflow.

The results of Figure 7 suggest that at least part of the inhibition mediated by GDP-bound Rab11b is caused by an activation of constitutive secretion and a depletion of hGH from the transfected cells. To confirm that GDP-bound versus GTP-bound Rab11b lead to a differential depletion of hGH from the transfected PC12 cells because it activates constitutive exocytosis, we subfractionated organelles present in the postnuclear supernatants from PC12 cells that were transfected with wild-type or GDP-bound Rab11b (Fig. 8). Fractionations were obtained by equilibrium centrifugation on sucrose gradients as described above (Fig. 5). When we measured hGH across the gradients by RIA, we found that wild-type Rab11b had no significant effect on the subcellular storage of hGH, whereas GDP-mutant Rab11b caused a loss of hGH from all gradient fractions (Fig. 8). This loss was particularly striking in the two peak fractions in the middle of the gradient that presumably contain dense core secretory vesicles. The loss of these two hGH peaks on the gradients was not a separation artifact because norepinephrine measurements performed in parallel revealed that the norepinephrine peaks representing the nontransfected majority of the PC12 cells were still present and colocalized with the hGH peaks (data not shown). Thus, GTP- and GDP-bound Rab11b have distinct effects in PC12 cells, consistent with the notion that, as GTP-dependent regulatory proteins, GDP- and GTP-bound Rab11b perform distinct actions despite the fact that these actions lead to the similar inhibition of Ca^{2+}-induced exocytosis.

Table 1. Comparison of the effects of transfected Rab3 and Rab11 proteins and tetanus toxin light chain on regulated and constitutive exocytosis

	Neuroendocrine PC12 cells		Non-neuronal cells
	Ca^{2+}-triggered exocytosis	Constitutive exocytosis	Constitutive exocytosis
Rab3 WT	↓ ↓	↑ ↑	↓ ↓
Rab3 GTP-fixed (Q81L)	↓ ↓	↑ ↑	n.d.
Rab3 GDP-fixed (T36N)	↔	↔	n.d.
Rab1 1b WT	↓ ↓	↑	↓ ↓
Rab1 1b GTP-fixed (S20V)	↓ ↓	↑	↓ ↓
Rab1 1b GDP-fixed (S25N)	↓ ↓	↑ ↑	↓
Tetanus toxin light chain	↓ ↓	↔	↔

The effects of Rab3 proteins on exocytosis in PC12 cells have been described (Schluter et al., 2002). Inhibition of regulated exocytosis in PC12 cells by tetanus toxin light chain has been described (Sugita et al., 1998). Data on Rab3 proteins in non-neuronal constitutive exocytosis and on tetanus toxin light chain in constitutive exocytosis are from our unpublished observations.

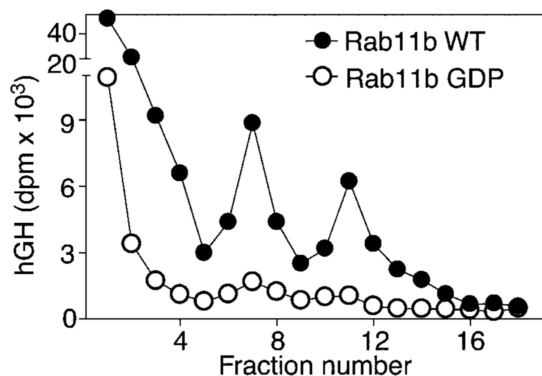

Figure 8. Large dense core vesicles storage is depleted in PC12 cells transfected with GDP-bound Rab11b. PC12 cells were cotransfected expression vectors encoding hGH and wild-type Rab11b or GDP-bound mutant Rab11b. Subcellular fractionation was performed as described in Figure 5. Gradient fractions were analyzed for hGH by RIA.

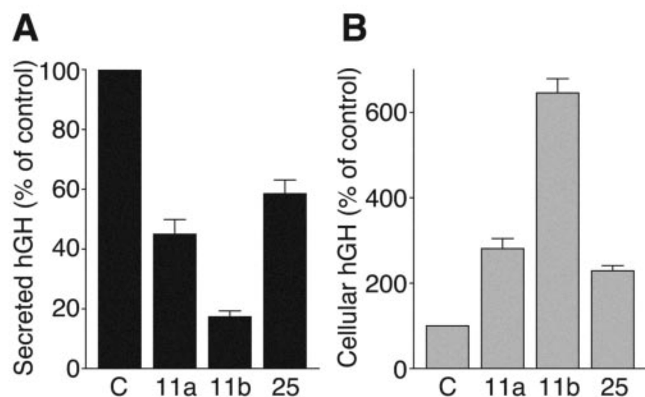

Figure 9. Effect of Rab11/25 proteins on constitutive exocytosis in HEK293 cells. HEK293 cells were cotransfected with expression vectors encoding hGH and GFP alone (control) or GFP-fused Rab11a, Rab11b, and Rab25 proteins. hGH was measured in the cells and aliquots of the medium 2 days after transfection. Secreted hGH (*A*, ■) and remaining cellular hGH (B, ▦) were calculated as described for Figure 7. Data shown are means ± SEMs from three independent experiments performed in duplicates.

Wild-type and mutant Rab11/25 proteins inhibit constitutive secretion in non-neuronal cells

The common, although differential, effects of wild-type and mutant Rab11b on constitutive and regulated exocytosis in PC12 cells implies that Rab11 may have a general role in exocytosis. To test this, we examined the effect of GFP-fused Rab11/25 proteins on constitutive hGH secretion in non-neuronal HEK293 cells using a protocol similar to that described above for neuroendocrine PC12 cells. Surprisingly, we found that all Rab11/25 proteins inhibited instead of stimulated constitutive secretion in HEK293 cells (Fig. 9*A*) and caused a dramatic increase in the cellular levels of hGH (up to 700% in the case of Rab11b) (Fig. 9*B*). Again, the effect of Rab11b was the strongest of the three Rab proteins.

The results shown in Figure 9 suggest that Rab11 proteins have differential effects on exocytosis in neuroendocrine versus non-neuronal cells. To gain insight into the mechanism and generality of this observation, we tested the effects of the various mutant Rab11b proteins on constitutive exocytosis in both HEK293 and HeLa cells. We found that all mutants led to an inhibition of constitutive secretion in both cell lines similar to wild-type Rab11b and caused an intracellular retention of hGH (Fig. 10). However, as in PC12 cells, constitutive secretion was not inhibited by cotransfection of tetanus toxin light chain (data not shown). These data indicate that constitutive secretion can be differentially altered by Rab11b in neuroendocrine PC12 cells and non-neuronal cells HEK293 and HeLa cells.

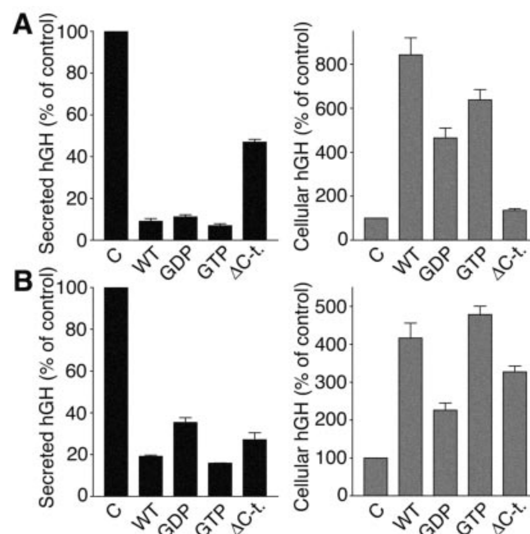

Figure 10. Effect of wild-type and mutant Rab11b proteins on constitutive exocytosis in HEK293 and HeLa cells. HEK293 (*A*) and HeLa (*B*) cells were cotransfected with expression vectors encoding hGH and no insert (control) or various Rab11b proteins. hGH was measured in the cells and aliquots of the medium 2 days after transfection. Secreted hGH (left, ■) and remaining cellular hGH (right, [gbox) were calculated as described for Figure 7. Data shown are means ± SEMs from three independent experiments performed in triplicates.

Discussion

Our study identifies Rab11 as a component of the exocytic machinery in Ca^{2+}-triggered and constitutive secretion and demonstrates that Rab11 alters exocytosis differently in neuroendocrine versus non-neuronal cells. These data suggest that the organization of the secretory pathway differs fundamentally between cells in which regulated and constitutive exocytosis reactions are performed in parallel (such as neurons and neuroendocrine cells) and cells that only execute constitutive exocytosis. Extensive previous studies have documented a function for Rab11 in the recycling of endosome-derived vesicles to the plasma membrane (Ullrich et al., 1996; Chen et al., 1998; Ren et al., 1998; Wilcke et al., 2000). In addition, Rab11 proteins were implicated in the fusion of tubulovesicles with the plasma membrane of gastric parietal cells (Calhoun et al., 1998; Duman et al., 1999). Our study provides the first direct evidence that Rab11 is intimately involved in the differential organization of Ca^{2+}-regulated and constitutive exocytosis and does not simply act by maintaining a normal recycling pathway between the *trans*-Golgi network, plasma membrane, and endosomes. This conclusion is supported by the following evidence:

(1) Transfected Rab11b blocked Ca^{2+}-dependent secretion in PC12 cells as strongly as Rab3a, a well established regulator of Ca^{2+}-dependent exocytosis, whereas closely related Rab11a and Rab25 were less potent (Figs. 1 and 2). The inhibition was specific and was not observed when other Rab proteins were transfected into PC12 cells [e.g., overexpression of Rab5a that is enriched on synaptic vesicles (Fischer von Mollard et al., 1994) did not inhibit exocytosis].

(2) Rab11 is present on secretory vesicles in PC12 cells and on mature synaptic vesicles in brain (Figs. 5 and 6). They, thus, remain associated with secretory vesicles after their biogenesis

from the *trans*-Golgi network, consistent with a function for Rab11 in exocytosis. These observations agree with previous reports of Rab11 localization on secretory vesicles in PC12 cells (Urbe et al., 1993) and on synaptic vesicles in electric ray (Volknandt et al., 1993).

(3) In contrast to Ca^{2+}-independent exocytosis, Rab11b did not inhibit but stimulated constitutive exocytosis in PC12 cells (Fig. 7). Thus, Rab11 does not simply "mess up" the *trans*-Golgi network but exerts a specific effect on the regulated secretory pathway. Tetanus toxin light chain inhibited Ca^{2+}-induced exocytosis but did not alter constitutive secretion in PC12 cells, suggesting that the stimulation of constitutive exocytosis by Rab11b is not attributable to an "overflow" of accumulated hGH.

(4) Although different mutants that fix Rab11b in either the GTP- or the GDP-bound state similarly inhibited Ca^{2+}-induced exocytosis of hGH in PC12 cells, they had distinct effects on constitutive exocytosis in PC12 cells: the GDP-bound form massively stimulated constitutive secretion and depleted the secretory stores of hGH, whereas the GTP-bound form only moderately stimulated secretion and did not deplete secretory hGH stores (Figs. 7 and 8). Thus, consistent with a general function of Rabs as GTP-dependent switches, GTP- and GDP-bound Rab11b seem to inhibit Ca^{2+}-induced secretion by different mechanisms: the former directly by blocking exocytosis, and the latter indirectly by depleting hGH from the cells.

(5) Although Rab11b activated constitutive secretion in PC12 cells, it inhibited constitutive exocytosis in HeLa and HEK293 cells and caused accumulation of intracellular hGH (Figs. 9 and 10). Thus, Rab11 does not act by a uniform effect on the biogenesis of exocytic transport vesicles in the *trans*-Golgi network (e.g., by simply catalyzing formation of constitutive secretory vesicles), consistent with an action of Rab11 downstream of vesicle biogenesis.

Overall, our data raise the interesting possibility that, in neuronal and neuroendocrine cells, Rab11 may have a special function as a GTP-dependent switch that determines the secretory fate of a transport vesicle. The effects of Rab11 observed here strongly resemble those of Rab3 that are not more related to Rab11 than are any other Rab proteins. In PC12 cells, both Rab3 and Rab11 decrease Ca^{2+}-induced exocytosis by stimulating constitutive exocytosis, and both Rab3 and Rab11 inhibit constitutive exocytosis in non-neuronal cells (Table 1). The difference, however, is that in the case of Rab3, the GDP-bound form is inactive, whereas in the case of Rab11b, it is the most active in altering exocytosis in PC12 cells.

Rab proteins execute diverse activities that include a role in the tethering of membranes as observed for Ypt7 in homotypic vacuole fusion (Haas et al., 1995; Wickner and Haas, 2000), a role in the actual fusion process in which Rab3a seems to act (Geppert et al., 1997), and a role in the budding of transport vesicles as shown for Ypt51p/52p and Rab5 (Jedd et al., 1997; Nielsen et al., 1999). The presence of $>$60 *Rab* genes in mammals suggests that a set of Rab proteins, rather than a single Rab protein, functions in one particular trafficking reaction and provides, at least partly, specificity to that reaction. Specificity seems to be mediated by combinations of Rab proteins because Rab proteins often participate in multiple trafficking steps in the cell. Rab11 proteins are also promiscuous and have been linked to multiple trafficking steps, including the regulation of trafficking at the Golgi, in the TGN, and in recycling endosomes (Chen et al., 1998; Ren et al., 1998; Wilcke et al., 2000). The mediation of multiple actions of Rab11 in membrane traffic is also suggested by the description of a large number of potential effectors (e.g., Rab11BP/Rabphilin 11, myosin Vb, and the Rab11-FIP family that includes Rip11 and Rab coupling protein) (Mammoto et al., 1999; Zeng et al., 1999; Prekeris et al., 2000; Hales et al., 2001). Our data are consistent with multiple points of action of Rab11 proteins; effects of Rab11 mutants on endosomal traffic, for example, would not have been detected because of the assay systems we used. The inhibition of regulated exocytosis and the differential effects of GTP-bound versus GDP-bound Rab11 on secretory vesicles and constitutive exocytosis that we observed are presumably mediated by the interaction of Rab11 with effector proteins and components of the Rab11 GTPase or GTP/GDP exchange complexes. This hypothesis is supported by the finding that unprenylated truncation mutants of Rab11 are also potent inhibitors of exocytosis in PC12 cells because these unprenylated mutants are probably still capable of interacting with effectors or GTP/GDP exchangers and, thus, as capable as prenylated proteins to interfere with their function. Identification of the putative Rab11 interaction partners in exocytosis will be of considerable interest for future studies.

The effect of Rab11 on regulated exocytosis observed in PC12 cells, however, does not mean that Rab11 functions in membrane fusion directly. Besides controlling fusion directly, our results are also consistent with a role for Rab11 in specifying the identity of secretory vesicles (regulated vs constitutive) in neuroendocrine/neuronal cells, or in the differential transport of these vesicles. Despite the role in regulated exocytosis for Rab11 demonstrated here, Rab11 is unlikely to be the vertebrate Sec4p equivalent. Ca^{2+}-regulated exocytosis is a complex process that requires the concerted action of multiple Rab proteins and possibly other small GTPases, at least some of which may perform part of the functions executed by Sec4p in yeast. One line of evidence indicating that Rab11 is not the mammalian Sec4p equivalent is the fact that yeast contains a separate set of Rab proteins (Ypt31/32) that are highly homologous to Rab11 (61% identity) and have a function related to the Golgi membrane traffic and exit from the Golgi apparatus (Benli et al., 1996; Jedd et al., 1997). Therefore, the role of Rab11 in regulated exocytosis suggested here is likely an extension of existing housekeeping functions of Rab11/25 proteins that was developed specifically for the integration of regulated secretory pathways.

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