Novel localization of Rab3D in rat intestinal goblet cells and Brunner’s gland acinar cells suggests a role in early Golgi trafficking

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Valentijn JA, van Weeren L, Ultee A, Koster AJ. Novel localization of Rab3D in rat intestinal goblet cells and Brunner’s gland acinar cells suggests a role in early Golgi trafficking. Am J Physiol Gastrointest Liver Physiol 293: G165–G177, 2007. First published March 29, 2007; doi:10.1152/ajpgi.00520.2006.—Rab3D is a small GTP-binding protein that associates with secretory granules of endocrine and exocrine cells. The physiological role of Rab3D remains unclear. While it has initially been implicated in the control of regulated exocytosis, recent deletion-mutation studies have suggested that Rab3D is involved in the biogenesis of secretory granules. Here, we report the unexpected finding that Rab3D also associates with early Golgi compartments in intestinal goblet cells and in Brunner’s gland acinar cells. Expression of Rab3D in the intestine was demonstrated by the rat duodenum and colon. Confocal laser scanning microscopy revealed Rab3D immunofluorescence in the Golgi area of goblet cells of the duodenum and colon and in Brunner’s gland acinar cells. There was no colocalization between Rab3D and a trans-Golgi network marker, TGN-38. In contrast, Rab3D colocalized partially with a cis-Golgi marker, GM-130, and with a marker of cis-Golgi and coat protein complex I vesicles, β-COP. Strong colocalization was observed between Rab3D and the lectins Griffonia simplicifolia agglutinin II and soybean agglutinin, which have been described as markers of the medial and cis-Golgi, respectively. Rabphilin, a putative effector of Rab3D, displayed an identical pattern of Golgi localization. Incubation of colon tissue with carbamylcholine or deoxycholate to stimulate exocytosis by goblet cells caused a partial redistribution of Rab3D to the cytoplasm and mucous granule field and a concomitant transformation of the Golgi architecture. Taken together, the present data suggest that Rab3D and rabphilin may regulate the secretory pathway at a much earlier stage than what has hitherto been assumed.

rabphilin; Golgi apparatus; intestinal goblet cells

Rab proteins are low-molecular-weight GTP-binding proteins belonging to the superfamily of Ras-like proteins (21). More than 60 different Rab proteins have been identified, and many are ubiquitously expressed. Rab proteins behave as molecular switches by cycling through an active, i.e., GTP bound and membrane associated, and an inactive, i.e., GDP bound and cytosolic, state. They are able to confer specificity to intracellular membrane compartments because each Rab isoform associates in its active state with a particular subset of membranes (55). This spatiotemporal organization enables Rab proteins to coordinate the dynamic transport and fusion of membrane compartments. To achieve such complex function, Rab proteins interact with numerous effector proteins including actin-and microtubule-based motor proteins and protein complexes involved in tethering, docking, and fusion of membrane vesicles (17).

Rab3D is a member of the Rab3 subfamily, which comprises four isoforms (Rab3A, Rab3B, Rab3C, and Rab3D) (1, 14). Rab3 proteins typically associate with secretory vesicles, including synaptic vesicles, and have been implicated in the control of regulated exocytosis (14). Rab3D is expressed prevalently in exocrine cells and, with a few exceptions, appears specialized for large secretory granules (diameter > 0.5 μm) (13, 24, 34, 40, 50, 52, 54). Numerous studies have dealt to various extents with Rab3D, yet its molecular role remains to be established. Nevertheless, it has been demonstrated that Rab3D can interact in vitro with the Rab effectors rabphilin, Rim, and Noc2 (15). Furthermore, indirect evidence has suggested an interplay between Rab3D and actin filaments that coat around secretory granules during the course of exocytosis (53).

The gastrointestinal tract and its accessory glands are rich in secretory cells that are mostly exocrine in nature. Rab3D appears to be a major Rab3 isoform in the digestive system, since it is present in gastric chief cells (34, 50), hepatocytes (25), and acinar cells of the pancreas (34, 52), parotid (34, 40), and von Ebner’s glands (13). However, up to now, surprisingly little is known about the distribution and function of Rab3 proteins, in particular Rab3D, in the intestine. The present study reports our effort to fill this information gap by characterizing the cells and subcellular structures to which Rab3D localizes in the rat duodenum and colon. Quite unexpectedly, we found a significant association of Rab3D with the cis- to medial Golgi compartments in goblet cells and Brunner’s gland acinar cells. These data challenge the current views of Rab3D function.

MATERIALS AND METHODS

Animals. Animal procedures were approved by the Animal Ethics Committee of the University of Utrecht. Adult male Wistar rats (Harlan, Horst, The Netherlands), weighing 100–150 g, were used as tissue donors. Animals were housed under standard conditions and for at least 1 wk after their arrival. They were killed through stunning followed by cervical dislocation. Tissues were quickly dissected and processed for biochemical or immunocytochemical procedures as described below.

Antibodies, lectins, and fluorescent probes. Two well-characterized rabbit polyclonal antisera were used to detect Rab3D (38, 53, 54). One
was raised against bacterially expressed GST-Rab3D, and the other was raised against the unique carboxy-terminal peptide sequence of Rab3D, SSSPGSNGGPALGDTTPQPS. In a number of pilot experiments (Western blot analysis and immunofluorescent labeling), both antisera gave identical results. However, the antisera raised against the holoprotein produced a consistently stronger signal at 10-fold higher dilution. Therefore, this antisera was used throughout the present study. A rabbit polyclonal antisem against a peptide sequence (WHQLQNEHVSSD) that is unique for rabphilin was acquired from Synaptic Systems (Göttingen, Germany). Mouse monoclonal antibodies against transit-Golgi network (TGN) marker TGN-38 and cis-Golgi marker GM-130 were purchased from BD Biosciences (Alphen aan den Rijn, The Netherlands), and mouse monoclonal anti-beta-coatomer protein (b-COP) was from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Secondary goat anti-mouse and goat anti-rabbit antibodies, conjugated with Alexa Fluor488 or Alexa Fluor568, were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands). Ultrasound gold-conjugated goat anti-rabbit and 15-nm colloidal gold-conjugated goat anti-biotin antibodies were purchased from Aurion (Wageningen, The Netherlands), Alexa Fluor568-phaloidin, Alexa Fluor47-labeled Soybean agglutinin (SBA), Alexa Fluor594-labeled wheat germ agglutinin (WGA), Alexa Fluor568-labeled Helix pomatia agglutinin (HPA), Alexa Fluor488-labeled Griffonia simplicifolia agglutinin (GSA)-II, Alexa Fluor594-labeled GSA-II, and 4,6-diamidino-2-phenylindole (DAPI) were also from Molecular Probes. Rhodamine-conjugated peanut agglutinin (PNA) and biotinylated GSA-II were obtained from Vector Laboratories (Brunschwig Chemie, Amsterdam, The Netherlands).

Biochemical procedures. All procedures for tissue homogenization, subcellular fractionation, SDS-PAGE, and Western blot analysis have been described previously (54).

HT-29-5M21 cells (26) were cultured in DMEM (Imperial Laboratories, Salisbury, UK) supplemented with heat-inactivated 10% FCS, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and nonessential amino acids (Sigma). Cells were grown at 37°C in an atmosphere of 5% CO2 and 95% air. Homogenates of HT-29 cells were prepared by brief ultrasonication of cells that were scraped and collected in homogenization buffer [0.3 M sucrose, 0.5 mM MgCl2, and 25 mM HEPES (pH 6.8)] supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany)].

Exocytosis of mucous granules was induced either by the acetylcholine receptor agonist carbamylcholine (37) or by the bile salt deoxycholate (DOC) (3). Freshly prepared transverse slices of the descending colon were rinsed in oxygenated Krebs-Ringer-HEPES medium (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgCl2, 2 mM CaCl2, 20 mM HEPES, and 14 mM glucose, adjusted to pH 7.4 with NaOH) supplemented with 0.1% BSA. Slices were incubated in the same medium for 30 min at 37°C with or without the addition of 100 μM carbamylcholine or 10 mM DOC. Immediately thereafter, slices were fixed and processed for immunofluorescence microscopy.

Immunofluorescence procedures. Slices of the intestine, cut transversely and ~1 mm thick, were immersion fixed overnight at 4°C with 4% paraformaldehyde in 0.3 M sucrose and 5 mM phosphate buffer, pH 7.4. They were processed for immunofluorescence as described previously (54).

Fluorescent signals were visualized using a Bio-Rad Radiance 2100MP confocal and multiphoton system (Bio-Rad, Hertfordshire, UK) equipped with a Nikon TE300 inverted microscope (Uvikon, Bunnik, The Netherlands). Excitation of DAPI was achieved by multiphoton radiation at 750 nm using a mode-locked titanium: sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA) pumped by a 10-W solid-state laser (Millennia Xs, Spectra-Physics), while all other fluorescent probes were excited by confocal lasers.

Immunoelectron microscopy. Ultrastructural localization of Rab3D was carried out using postembedding immunocytochemistry. Small pieces of the intestine (volume: ~1 mm3) were immersed overnight at 4°C in fixative (4% paraformaldehyde in 0.3 M sucrose and 5 mM phosphate buffer, pH 7.4). Subsequently, tissue fragments were washed two times for 5 min and cryoprotected overnight in 2.3 M sucrose, after which time they were snap frozen in liquid nitrogen. The fragments were then freeze substituted in methanol containing 1.5% uranyl acetate and UV polymerized at ~45°C in Lowicryl HM20 (Electron Microscopy Sciences, Arison, Wageningen, The Netherlands). Ultrathin sections of 70–100 nm were cut on a Reichert Ultracut S ultramicrotome and collected on 200-mesh nickel grids. Immunogold cytochemistry and silver enhancement (Arion) were performed according to the manufacturer’s protocols. Rab3D antisem was diluted 1:50 and incubated on the sections for 1 h. Double labeling was done in the following sequence: biotin-GSA-II for 30 min, anti-Rab3D for 1 h, nanogold-conjugated goat anti-rabbit mixed together with 15-nm gold-conjugated goat anti-biotin for 2 h, and silver enhancement. Sections were viewed and photographed with a Philips CM10 transmission electron microscope.

Image processing and colocalization measurements. Raw images acquired by the photomultipliers of the confocal and multiphoton systems were processed in Adobe Photoshop solely for the sake of presentation. The image processing consisted of minor adjustments to the dynamic range (levels settings) and cropping of areas of interest. Adobe Illustrator was used to make montages and to append scale bars and labels. For colocalization measurements, raw unprocessed images were first restored using Huygens Pro deconvolution software (Scientific Volume Imaging, Hilversum, The Netherlands). Colocalization coefficients were determined using NIH ImageJ with the “Colocalization Threshold” plug-in. A major advantage of this plug-in is that it employs an algorithm to determine automatically the threshold, i.e., background, of each channel, thus eliminating user bias (8). Golgi

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Fig. 1. Western blot detection of Rab3D in the rat intestine and human colon carcinoma-derived mucus-secreting HT-29-5M21 cells. A: homogenates prepared from the duodenum and colon and HT-29 cell lysate were resolved by 12% SDS-PAGE, electrotransferred to polyvinylidene difluoride membranes, and immunoblotted with a polyclonal Rab3D antiserum. In all samples, the antiserum detected a band of ~27 kDa, which corresponded to the molecular weight of Rab3D and which comigrated with Rab3D in the pancreas and lung homogenates that were used as positive controls. B: difference in subcellular distribution of Rab3D between the rat colon and HT-29 cells. Postnuclear supernatant (PNS) prepared from colon homogenate or HT-29-cell lysate was centrifuged for 1 h at 100,000 g to prepare a supernatant fraction containing the cytosol and a particulate fraction containing the membranes. The particulate fraction was washed in Na2CO3 (pH 11.5) to remove peripherally associated proteins. Note that in the colon most of the Rab3D signal associates with the particulate fraction, whereas in HT-29 cells Rab3D partitions mainly into the supernatant fraction.
areas were selected for colocalization measurements using the polygon tool in ImageJ. Correlation coefficients were calculated according to the algorithm of Manders et al. (28). The algorithm generates two coefficients per dual-channel image, indicating the degree of overlap, i.e., colocalization, between pixels above threshold in the first channel and those in the second channel, and vice versa. Differences between the colocalization coefficients were evaluated by means of two-way ANOVA using Graphpad Prism software (San Diego, CA).

Electron micrograph negatives were scanned at high resolution and bit depth on an Agfascan XY-15 flatbed scanner. The thus-obtained 16-bit images were adjusted for dynamic range and subsequently sampled down to 8-bit.

RESULTS

A transcriptome database (47) search for mRNA levels of Rab3D in miscellaneous tissues revealed a high level of expression in the large intestine. According to the transcriptome data, the level of expression in the large intestine was comparable to that in the pancreas, which is an abundant source of Rab3D protein. To determine whether colon tissue expressed Rab3D at the protein level, we prepared homogenates from the rat colon for analysis by SDS-PAGE and subsequent Western blot analysis using a well-characterized Rab3D antiserum. As shown in Fig. 1A, the Rab3D antiserum detected a protein band in colon homogenates that comigrated with the Rab3D band of 27 kDa in the pancreas and lung homogenates used as positive controls. The intensity of the Rab3D-like band in the colon was similar to the one in the pancreas when samples of equal protein content were compared. A Rab3D-like band was also detected, but at lower intensity, in homogenates from the small intestine (duodenum; Fig. 1A). Since Rab3D is found predominantly in secretory cells, the Rab3D immunoreactivity in the colon was likely to be associated with mucus-secreting goblet cells, which constitute the major secretory cell type in the colon. This was corroborated by the finding

Fig. 2. Immunofluorescence detection of Rab3D in the rat duodenum. Samples of duodenal tissue were fixed in 4% paraformaldehyde, cryostat sectioned, and immunolabeled with rabbit polyclonal Rab3D antiserum followed by Alexa Fluor488-conjugated goat anti-rabbit antibodies. To resolve tissue topology, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei and either Alexa Fluor568-conjugated phalloidin for F-actin or Alexa Fluor594-conjugated wheat germ agglutinin (WGA) for mucous granules and Golgi areas. A: two Paneth cells at the base of a crypt of Lieberkühn. Rab3D immunofluorescence outlines the secretory granules. B: Brunner’s gland acinar cells displaying Rab3D immunoreactivity in both the secretory granule field (arrow) and Golgi area (arrowheads). C: a villar goblet cell in which Rab3D immunofluorescence is restricted to the prominent Golgi area (arrowhead). D: two immature goblet cells at the base of a crypt of Lieberkühn showing Rab3D labeling in the Golgi area (arrowheads). Scale bar = 2 μm.
that cell lysates of the mucus-secreting clone HT-29-5M21 (derived from human colon carcinoma cell line HT-29) also displayed an intense band at 27 kDa on Western blots probed with Rab3D antiserum (Fig. 1A). However, following high-speed centrifugation of colon homogenates and HT-29-5M21 cell lysates, most of the Rab3D signal in the colon was associated with the particulate fraction containing the membranes, whereas in the HT-29-5M21 cells, Rab3D was present mostly in the supernatant fraction containing the cytosol (Fig. 1B). The latter finding was reminiscent of previous data showing that Rab3D resides predominantly in the cytosol of fetal pancreatic acinar cells that have not yet acquired a regulated secretory pathway (51). In the colon, most of the Rab3D signal detected in the particulate fraction remained associated with that fraction after removal of peripherally associated proteins by washing in Na2CO3 (pH 11.5), suggesting that this pool of Rab3D was tightly membrane bound.

To characterize the cell type(s) expressing Rab3D in the rat intestine, and to determine the subcellular distribution of Rab3D, we conducted a series of (immuno)fluorescent labeling experiments on cryostat sections of paraformaldehyde-fixed tissue samples from the duodenum and descending colon.

Using the same Rab3D antiserum as in the Western blot experiments, we detected three types of immunoreactive cells by means of confocal laser scanning microscopy (Fig. 2). In the duodenum, Rab3D immunofluorescence outlined the secretory granules of Paneth cells at the base of the crypts of Lieberkuhn (Fig. 2A). Also labeled was the secretory granule field in the apical portion of Brunner’s gland acinar cells found in the duodenal submucosa (Fig. 2B). Surprisingly, the prominent Golgi area of these cells exhibited considerable Rab3D labeling as well. The goblet cells in the epithelium of crypts and villi constituted the third cell type that displayed Rab3D immunofluorescence (Fig. 2, C and D). Here, and equally surprisingly, the Rab3D label was localized almost exclusively to the pronounced Golgi area.

In the descending colon, goblet cells appeared to be the only cell type showing Rab3D immunoreactivity (Fig. 3). At low magnification, it became evident that the Rab3D immunofluorescence was strongest in immature goblet cells of the deep crypt epithelium (Fig. 3A). The staining pattern consisted mainly of two elements: 1) a large supranuclear annulus surrounding 2) several smaller vesicular structures (Fig. 3B). We interpreted these elements as parts of the Golgi area and forming mucous granules, respectively. The immature goblet cells also contained small clusters of Rab3D-positive vesicles, presumably mucous granules, outside the Golgi area (Fig. 3B). These clusters were devoid of WGA staining, suggesting that they represented a class of immature granules. In mature goblet cells of the superficial epithelium in the upper crypts and villi, Rab3D staining was restricted to the Golgi area, which, depending on the orientation in the optical section, assumed an

Fig. 3. Immunofluorescence detection of Rab3D in the rat colon. Tissue was prepared as in Fig. 2. A: low-magnification overview displaying Rab3D immunoreactivity in the epithelium layer, with the strongest signal deep inside the crypts (arrowheads). B: detail of deep crypt epithelium with several Rab3D-positive Golgi areas (arrowheads) enclosing Rab3D-positive forming mucous granules in immature goblet cells. Some areas containing Rab3D-positive mucous granules are devoid of WGA stain. C: detail of the superficial epithelium on a villus containing a goblet cell with strong Rab3D immunofluorescence in the Golgi area (arrowhead) and faint Rab3D labeling in the mucous granule field. HPA, Helix pomatia agglutinin. Scale bars = 20 μm in A and 2 μm in B and C.
annular, oval, or V shape (Fig. 3C). While labeling of forming mucous granules was never observed in superficial goblet cells, mature granules were faintly outlined (Fig. 3C).

Hitherto, studies on the role of Rab3D have focused on the post-Golgi secretory pathway due to the predominant association of Rab3D with secretory granules (30). The considerable localization of Rab3D to the Golgi area of goblet cells and Brunner’s gland acinar cells demonstrated herein is a novel finding that merits further analysis as it may shine a different light on the function of Rab3D. To substantiate our finding and to pinpoint the distribution of Rab3D in the Golgi area, we performed a series of colocalization experiments with various markers of Golgi compartments. Intestinal goblet cells and Brunner’s gland acinar cells synthesize O-linked glycoproteins in abundance and, thus, are very suitable for lectin cytochemistry (16, 43, 48). It has been shown that in Brunner’s gland acinar cells, glycine max or SBA binds to cis-cisternae of the extensive Golgi apparatus, whereas GSA-II labels medial and trans-cisternae (49). In agreement with this, using the same combination of fluorescently labeled lectins, we could also discriminate two Golgi areas in Brunner’s gland acinar cells: an outer region stained with SBA alone, and an inner region stained with GSA-II alone (Fig. 4A). However, about half of the GSA-II signal overlapped with SBA, as indicated by the colocalization measurements, but SBA overlapped only weakly with GSA-II (Manders’ coefficients: 0.55 ± 0.05 for GSA-II and 0.29 ± 0.03 for SBA; Fig. 4B). This partial overlap was likely to represent the transition from cis- to medial Golgi cisternae. The Rab3D-immunofluorescence coincided rather weakly with GSA-II (Manders’ coefficients: 0.32 ± 0.02 for Rab3D and 0.36 ± 0.03 for GSA-II), whereas there was a strong colocalization with SBA (Manders’ coefficients: 0.75 ± 0.02 for Rab3D and 0.51 ± 0.03 for SBA). Thus, it appeared that Rab3D localized preferentially to SBA-positive cis-Golgi elements in Brunner’s gland acinar cells.

We next focused on the Golgi localization of Rab3D in superficial goblet cells of the descending colon. It has been shown by electron microscopy that in these cells, HPA binds to cis- and trans-Golgi cisternae but not medial Golgi cisternae and that HPA also labels forming and mature mucin granules (11, 43). By confocal microscopy, we observed strong reactivity of fluorescently labeled HPA with an uninterrupted field of mucin granules and contiguous Golgi elements. The HPA-positive Golgi area was adjoined by a thin, unstained border, presumably representing the medial Golgi, and subsequently by a thin, HPA-stained border, most likely representing the cis-Golgi. It was with the latter area that Rab3D immunofluorescence coincided (Manders’ coefficients: 0.69 ± 0.07 for Rab3D and 0.27 ± 0.05 for HPA; Fig. 5A). WGA displayed a similar staining pattern than HPA except that it also labeled the HPA-negative area corresponding to the medial Golgi (Manders’ coefficients: 0.60 ± 0.08 for Rab3D and 0.31 ± 0.02 for WGA).
0.03 for WGA; Fig. 3C). GSA-II and SBA both exclusively labeled elements of the supranuclear Golgi apparatus in goblet cells. In contrast with Brunner’s gland acinar cells, GSA-II and SBA stained for the most part the same Golgi compartment, which was indicated by the elevated correlation indexes (Mander’s coefficients: 0.91 ± 0.02 and 0.81 ± 0.02 for GSA-II vs. SBA). GSA-II and SBA also displayed a high degree of colocalization with Rab3D (Mander’s coefficients: 0.69 ± 0.02 and 0.76 ± 0.02 for Rab3D vs. GSA-II and 0.71 ± 0.02 and 0.68 ± 0.02 for Rab3D vs. SBA; Fig. 5, B and C). Labeling with *Arachis hypogaea* or peanut lectin (PNA) was restricted to the Golgi area as well, but there was significantly less overlap with Rab3D (Manders’ coefficients 0.45 ± 0.04 for Rab3D and 0.49 ± 0.06 for PNA; Fig. 5D). Comparable intermediate levels of colocalization were found between Rab3D and antibodies against the cis-Golgi marker GM-130 (Manders’ coefficients: 0.41 ± 0.04 for Rab3D and 0.46 ± 0.03 for GM-130; Fig. 6A) or antibodies against a marker of cis-Golgi and coat protein complex I (COPI) vesicles, β-COP (Manders’ coefficients: 0.57 ± 0.06 for Rab3D and 0.53 ± 0.06 for β-COP; Fig. 6B). Interestingly, the outermost layer of GM130 immunofluorescence appeared upon visual examination devoid of Rab3D, whereas for β-COP the innermost layer appeared devoid of Rab3D. These observations explained the
intermediate colocalization coefficients. Little or no colocalization was found between Rab3D and the TGN marker, TGN-38 (Manders' coefficients: 0.16 ± 0.04 for Rab3D and 0.16 ± 0.03 for TGN-38; Fig. 6C).

A histogram summarizing the colocalization coefficients measured in colon goblet cells for Rab3D and the various Golgi markers mentioned above is shown in Supplementary Fig. 1, and a table summarizing their statistical significance is shown in Supplementary Table 1.1 Taken together, these data indicated that Rab3D colocalized with cis- or medial Golgi compartments, but a more precise determination was not possible due to the apparent discrepancy between the colocalization coefficients of Rab3D and HPA on the one hand and Rab3D and GM-130 on the other hand. This, added to the finite resolution inherent to the light microscope, prompted us to seek corroboration by means of immunoelectron microscopy. For this purpose, we embedded paraformaldehyde-fixed tissue fragments of the descending colon in Lowicryl HM20 and immunolabeled ultrathin sections with Rab3D antiserum followed by silver-enhanced nanogold-conjugated secondary antibodies. In some experiments, double labeling was performed with biotinylated GSA-II and 15-nm colloidal gold-labeled goat anti-biotin antibodies. As illustrated by the typical micrograph shown in Fig. 7A, Rab3D immunogold-silver complexes coincided with GSA-II-immunogold at the level of the Golgi apparatus in goblet cells. The Golgi apparatus of these cells consists of a relatively high number of tightly stacked cis- to trans-cisternae [10–14 cisternae according to Roth (43) or 7–12 cisternae according to Neutra and Leblond (33)]. The Rab3D immunogold-silver label was concentrated in middle cisternae, suggesting that Rab3D localized preferentially to the medial Golgi (Fig. 7B). We found no evidence for an association of Rab3D with membrane vesicles, such as COPI vesicles, in the Golgi area. There was also no detectable label in the TGN region, whereas some sparse labeling was present in the mucous granule field and over the rough endoplasmic reticulum. Because of the overwhelming distribution of the Rab3D signal to the Golgi apparatus relative to the other areas, there was no incentive to quantify the immunogold-silver complexes.

In an attempt to address the question of the functional significance of Rab3D associating with early Golgi elements, we opted for two approaches. First, we investigated the localization of rabphilin, a putative effector of Rab3D, in the duodenum and ascending colon. For this purpose, we used a commercially available, well-characterized polyclonal antibody raised against a synthetic peptide corresponding to a

1 Supplemental data for this article is available online at the American Journal of Physiology-Gastro intestinal and Liver Physiology website.
Mucous discharge was elicited by incubating fragments of colon tissue for 30 min with either the acetylcholine receptor agonist carbamylcholine (100 μM) or the bile salt DOC (10 mM). After subsequent fixation, cryostat sectioning, and immunoelectron microscopy, goblet cells displayed characteristic cavitations that were indicative of the occurrence of massive exocytosis. This was accompanied by dramatic changes in the distribution of Rab3D, as demonstrated by the examples shown in Fig. 9. Compared with control tissue, where Rab3D was restricted to the early Golgi area, the overall Rab3D signal in cavitated goblet cells had a diffuse appearance throughout the cell. As a result of this diffuse staining pattern, the contours of the nucleus could often be discerned (Fig. 9, A and D). Thus, it appeared that a pool of Rab3D had relocated from Golgi membranes to the cytoplasm. In addition, the mucous granule field, or what was left of it, displayed a striking lacy pattern of Rab3D immunofluorescence (Fig. 9, A and B). The lacy pattern appeared to outline individual mucous granules (Fig. 9D). Because mucous granules are surrounded by very narrow cytoplasmic spaces, it was not possible to determine whether this pool of Rab3D was cytoplasmic or associated with the mucous granule membranes. In heavily cavitated goblet cells, the Rab3D signal was most pronounced at the level of a cup-shaped rim that surrounded the remainder of the mucous granule field (Fig. 9, C and D). This structure also reacted with fluorescent SBA, suggesting that it represented the SBA/Rab3D-positive early Golgi compartment, and, suggesting further, that the architecture of the Golgi apparatus had drastically changed.

**DISCUSSION**

Rab3D was characterized initially in adipocytes, but became known best for its association with secretory vesicles in exocrine and endocrine cells that exhibit a regulated secretory pathway. Hence, it was widely assumed that Rab3D plays a role in regulated exocytosis. In favor of this hypothesis, Chen et al. (6) showed that amylase release was inhibited when mouse pancreatic acini were virally transfected with dominant negative Rab3D. However, subsequent investigations demonstrated that cells and tissues from Rab3D knockout mice displayed normal secretory responses and that the animals did not display any overt phenotypic anomalies (42). The discrepancies between morphological, physiological, and molecular-biological data have been explained by functional redundancy of the four closely related Rab3 isoforms, Rab3A, Rab3B, Rab3C, and Rab3D. Indeed, quadruple Rab3 knockout mice died shortly after birth, whereas single, double, and some combinations of triple Rab3 knockout mice are viable and fertile. Nevertheless, of the four isoforms, Rab3D stands out as the least efficient one in rescuing the lethal phenotype of quadruple Rab3 knockout mice (45). This is probably due to the fact that Rab3D is expressed predominantly in peripheral tissues, whereas the other three isoforms codistribute primarily in the brain and pituitary gland (44). It thus appears that the aforementioned functional redundancy between Rab3 isoforms displayed normal secretory responses and that the animals did not display any overt phenotypic anomalies (42). The discrepancies between morphological, physiological, and molecular-biological data have been explained by functional redundancy of the four closely related Rab3 isoforms, Rab3A, Rab3B, Rab3C, and Rab3D. Indeed, quadruple Rab3 knockout mice died shortly after birth, whereas single, double, and some combinations of triple Rab3 knockout mice are viable and fertile. Nevertheless, of the four isoforms, Rab3D stands out as the least efficient one in rescuing the lethal phenotype of quadruple Rab3 knockout mice (45). This is probably due to the fact that Rab3D is expressed predominantly in peripheral tissues, whereas the other three isoforms codistribute primarily in the brain and pituitary gland (44). It thus appears that the aforementioned functional redundancy between Rab3 isoforms does not apply to Rab3D. In agreement with this, a subsequent analysis of Rab3D-deficient mice revealed that these mice are osteoclastogenic due to impaired activity of osteoclasts (36). It follows that alternative hypotheses need to be considered for the putative role of Rab3D.

**Fig. 7.** Electron microscopic localization of Rab3D in colon goblet cells. Postembedding immunoelectron microscopy was carried out on ultrathin sections of Lowicryl HM20 embedded colon tissue. A silver-enhanced nanogold procedure was used to detect Rab3D. A: typical Golgi localization of Rab3D. This section was double labeled with biotinylated GSA-II that was detected with 15-nm gold-conjugated goat anti-biotin antibodies. Note the V-shaped pattern of Rab3D immunogold-silver complexes reminiscent of the immunofluorescence data. Note also the codistribution of the Rab3D label (arrowheads) and the GSA-II immunogold (arrows). B: detail of a Golgi area showing that the preponderance of Rab3D label is over medial Golgi cisternae. The single 15-nm gold particle that can be observed over the rough endoplasmic reticulum (rER) is nonspecific, resulting from a failed attempt to double label this section with WGA. cis, cis-Golgi area; trans, trans-Golgi area. Scale bars = 0.5 μm.

**Fig. 8.** Rabphilin immunofluorescence was detected in all cell types of the duodenum and colon that displayed Rab3D immunoreactivity. Moreover, in each cell type, the pattern of rabphilin labeling was very similar to that of Rab3D. Thus, in duodenal Paneth cells (Fig. 8A), rabphilin immunofluorescence was punctate and outlined secretory granules, whereas in Brunner’s gland acinar cells (Fig. 8B), both the secretory granule field and the Golgi area were immunolabeled. In goblet cells of the duodenum (Fig. 8C) and colon (Fig. 8C), rabphilin immunofluorescence corresponded to the typical oval- to V-shaped Golgi pattern that was observed for Rab3D. In addition to that, in duodenal goblet cells, some rabphilin labeling was present either in the theca or plasma membrane surrounding the apical mass of mucous granules. Our second approach consisted of determining the effect of secretory activity on the localization of Rab3D in colon goblet cells.
The objective of the present study was to provide a careful morphological analysis of the subcellular localization of Rab3D in secretory cells of the rat intestine. As so often is the case in cell biology research, this is an important step in elucidating a protein’s function (23). While the preponderance of previous work has concentrated on cells where Rab3D localizes primarily to secretory granules, we here report two novel findings pertaining to the subcellular distribution of Rab3D. First, we found that Brunner’s gland acinar cells and intestinal goblet cells manifest a major association of Rab3D with cis- or medial cisternae of the Golgi apparatus, respectively. Second, we found considerable localization of Rab3D to structures resembling forming mucous granules of immature goblet cells in colonic crypts. These data expand the possible field of action of Rab3D by including pre-TGN Golgi elements and nascent secretory granules. In contrast with the present data, Ohnishi et al. (34) reported that little, if any, Rab3D was associated with goblet cells in intestinal crypts and villi. Because no data were shown to support their statement, it is difficult for us to explain the discrepancy with the present findings.

Recent advances in signal transduction research have yielded a new paradigm referred to as compartmentalized signaling, according to which signaling molecules can become modified or activated on, and signal from different membrane compartments, i.e., organelles or microdomains within membranes (46). The spatial component thus added to signaling pathways is thought to bestow a higher level of complexity by allowing regional variations in kinetics and interactions with downstream effectors. Compartmentalized signaling of Ras small GTPases has been inferred from a recent study (39) demonstrating that Ras proteins are able to translocate in a retrograde fashion from the plasma membrane to the Golgi apparatus, where they can undergo modifications. By analogy, Rab3D, which is a member of the Ras superfamily of low-molecular-weight GTP-binding proteins, might also shuttle between different organelles for the purpose of compartmentalized signaling. Evidence that Rab3D is able to translocate from one membrane compartment to another stems from a study (20) in the exocrine pancreas, where a Rab3-like protein, later identified as Rab3D, was found to migrate from secretory granules to the TGN following stimulation of regulated

Fig. 8. Localization of rabphilin in the rat intestine is nearly identical to Rab3D. Cryostat sections of the duodenum and colon were immunolabeled with rabbit polyclonal rabphilin antiserum followed by Alexa Fluor488-conjugated goat anti-rabbit antibodies (green channel). Counterstains (red channel) were anti-TGN-38 (A), WGA (B and C), or GSA-II (D). A: example of a Paneth cell at the base of a crypt of Lieberkühl displaying punctate rabphilin immunoreactivity that outlines secretory granules (arrowheads). B: Brunner’s gland acinar cells showing rabphilin immunofluorescence in the secretory granule field (arrows) and in the Golgi area (arrowheads). C: a duodenal goblet cell with punctate rabphilin labeling concentrated in the Golgi area (arrows) and, to a lesser extent, in the theca (arrowheads). D: detail of a colonic goblet cell displaying rabphilin immunoreactivity in the GSA-II-positive Golgi area. Scale bar = 2 μm.
exocytosis. In the present study, we found that the staining pattern of Rab3D changed considerably in goblet cells in which exocytosis was induced, suggesting that a pool of Rab3D redistributes from the Golgi apparatus during secretory activity.

What is the functional significance of Rab3D associating with the Golgi apparatus? Our data indicate that the Golgi area of intestinal goblet cells and Brunner’s gland acinar cells harbors not only Rab3D but also one of its putative effectors, rabphilin, which in itself is a novel finding. It leads us to hypothesize that Rab3D, through an interaction with rabphilin, plays a regulatory role within the Golgi apparatus. Rabphilin has, so far, only been localized to synaptic vesicles in neurons and secretory granules in endocrine cells (19, 32), where it has been implicated in regulated exocytosis (9). In the light of the present findings, there would be merit in evaluating the function of rabphilin in a wider context of membrane trafficking than exocytosis alone. As a matter of fact, there is evidence suggesting that rabphilin can dissociate from Rab3 to promote endocytosis (7). It is also interesting to note here that several groups have reported on the ability of rabphilin to bind to α-actinin (2, 7, 22) and to enhance α-actinin-mediated cross linking of actin filaments (2, 22). Yet another study (31) has demonstrated that rabphilin can bind to β-adducin, which induces actin bundling as well and promotes the formation of spectrin-actin complexes. Actin is involved in the budding and trafficking of Golgi vesicles and plays an important role in maintaining the architecture of the Golgi apparatus (10). Evidence is accumulating that Rab proteins control membrane traffic through interactions, by intermediates of effector and motor proteins, with microtubules and actin filaments (21). Others together with the first author of the present study have previously proposed that Rab3D is involved in the regulation of actin polymerization (53). On the basis of these pieces of information, it is tempting to suggest that Rab3D regulates, in concert with rabphilin, actin-mediated formation or trafficking of early Golgi vesicles. Additional studies will be required to substantiate this tentative hypothesis. Thus, there are currently no data available on the possible association of α-actinin and β-adducin with the Golgi apparatus. Furthermore, codistribution of Rab3D and rabphilin does not necessarily imply a functional interaction, especially since rabphilin can also bind to other Rab3 isoforms as well as Rab27 and Rab8 (15).

At present, we have no data to explain the difference in Golgi localization of Rab3D between Brunner’s gland acinar cells (cis-Golgi) and goblet cells (medial Golgi). Both cell types synthesize and secrete mucosubstances with complex carbohydrate moieties, and, for this purpose, they both have a prominent Golgi apparatus composed of somewhere between 7 and 14 cisternae (33, 41, 43, 48). However, we observed differences in the localization of SBA and GSA-II, suggesting that there is dissimilarity in the topology of glycosylation in the Golgi apparatus of these cells. If and how this relates to the localization of Rab3D is currently a matter of speculation. Nevertheless, it is interesting to mention that there is evidence indicating that a luminal cargo protein can drive the recruitment of Rab27a to the membrane of Weibel-Palade bodies (18).

The marked presence of Rab3D on nascent mucous granules in immature goblet cells suggests that it plays a role in the biogenesis of secretory granules. This is consistent with previous observations showing that the overexpression of a dominant negative Rab3D mutant leads to a reduction in the number of Weibel-Palade bodies in endothelial cells (24) and of secretory granules in PC12 cells (29). Moreover, Pavlos et al. (36) have recently demonstrated that dominant negative Rab3D localizes to the TGN and inhibits the biogenesis of Rab3D-bearing vesicles in osteoclasts. In further agreement, it has been shown that the diameter of zymogen granules in the pancreas and parotid gland is about one and a half times larger in Rab3D knockout mice than in wild-type littermates (42). An interesting parallel can be drawn here with a study on the developing rat pancreas showing that zymogen granules have a twofold larger diameter in newborn animals (12), whereas at the same age Rab3D appears inactive because it resides principally in the cytosol and possesses very little GTP-binding capacity (51). These data are all the more intriguing given that the neonatal period also coincides with the onset of stimulus-secretion coupling in the exocrine pancreas (5).

When considering, in this context, the current observation of preferential Rab3D localization to immature goblet cells in colonic crypts, the picture emerges that Rab3D plays a role in secretogenesis.

The following question now arises: What, if any, is the interrelationship between Rab3D localized to the Golgi apparatus and Rab3D associated with (immature) secretory granules? Assuming that there is a flux between both pools of Rab3D, evidence for which has been discussed above, it seems plausible that they participate in the same signal transduction pathway. The reduction of one pool and the concomitant increase of the other pool could potentially suffice to constitute a base signal triggering a physiological response, such as the replenishment of one of the membrane compartments involved. This line of reasoning raises another question: How does Rab3D migrate from early Golgi elements to secretory granules, and vice versa? At first consideration, it would appear logical that the Golgi-associated Rab3D accompanies the secretory cargo as it traverses the Golgi and TGN and becomes incorporated into secretory granules. But evidence for this is lacking, because we observed no association of Rab3D with either trans-Golgi cisternae or the TGN. On the other hand, Rab proteins, which are anchored into membranes via two posttranslationally added geranylgeranyl moieties, can be recruited into the cytosol by means of the chaperone protein GDP dissociation inhibitor (27). In addition, it has been shown that Rab3A can also be extracted from membranes by calmodulin.
It is thus conceivable that Rab3D shuttles between secretory granules and cis- or medial Golgi compartments by means of the cytosol. An alternative but less favorable hypothesis is that a direct trafficking pathway exists between the Rab3D-bearing Golgi compartment and secretory granules.

The general conclusion of the present study is that Rab3D, on the basis of its novel localization in intestinal epithelia, may operate earlier in the secretory pathway than hitherto postulated. Further studies will be needed to elucidate the potentially new role this implies for Rab3D. Of equal importance is to determine how universal the localization of Rab3D to early Golgi compartments is, as it may have escaped detection in cell types with less-proliferative Golgi stacks and an overshadowing Rab3D signal in the secretory granule field. Preliminary unpublished data from our laboratory have indicated that Rab3D also localizes to a SBA-positive Golgi compartment in rat prostate epithelial cells.

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