Rab11a-dependent exocytosis of discoidal/fusiform vesicles in bladder umbrella cells

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The discoidal/fusiform vesicles (DFV) of bladder umbrella cells undergo regulated exocytosis in response to stretch, but little is known about their biogenesis or the molecular machinery that modulates this process. We observed that Rab11a was expressed in umbrella cells (but not Rab11b or Rab25) and was associated with DFV. Using adenovirus-mediated delivery we transduced umbrella cells *in situ* with either dominant active (DA) or dominant negative (DN) mutants of Rab11a. DA-Rab11a stimulated an increase in apical surface area in the absence of stretch, whereas DN-Rab11a inhibited stretch-induced changes. Endocytosed fluid and membrane markers had little access to Rab11a-positive DFV, but virally expressed human growth hormone (hGH), a secretory protein, was packaged into DFV. Whereas expression of DA-Rab11a stimulated release of hGH into the bladder lumen, expression of DN-Rab11a had the opposite effect. Our results indicate that DFV may be biosynthetic in nature and that their exocytosis depends on the activity of the Rab11a GTPase.

uroepithelium | urothelium | GTPases

The outermost layer of the uroepithelium is lined by a single layer of polarized umbrella cells, which directly interface with the urine and form an impermeable barrier that must adjust to large variations in mechanical stress during cycles of bladder filling and voiding (1). During filling, the umbrella cell accommodates these changes by unfolding its apical plasma membrane and by exocytosis of a subapical population of discoidal/fusiform vesicles (DFV), which increase apical plasma membrane surface area (1–3). Upon voiding, the apical surface of the umbrella cell refolds, and added membrane is apparently retrieved by endocytosis (1, 4). The biogenesis of DFV is poorly understood, but morphological studies in rat bladders indicate that discoidal-shaped vesicles initially form in the trans-Golgi network (TGN), and then mature into a fusiform shape as they accumulate in the apical cytoplasm (5). Others have proposed that DFV are derived, in part, from an endocytic recycling pathway that regenerates the DFV population after voiding (1, 6). Regardless of their mechanism of formation, DFV undergo regulated exocytosis in a process that is modulated by cAMP, Ca^{2+}, extracellular ATP, adenosine, the epidermal growth factor receptor, and the actin cytoskeleton (1–4). However, the molecular machinery that governs DFV exocytosis is largely unknown.

The Rab GTPase family contains more than 60 member proteins that are key regulators of the transport, docking, and fusion of vesicular carriers within the exocytic and endocytic pathways (7). At present there is limited information about which Rabs are expressed in the uroepithelium, the subcellular localization of these Rabs, or information about which trafficking steps they may regulate. Chen *et al.* (8) recently observed the expression of nine different Rab isoforms (Rab4, Rab5, Rab8, Rab11, Rab13, Rab15, Rab27b, Rab28, and Rab32) in the uroepithelium and at least one of them, Rab27b, is associated with DFV; however, which Rabs regulate DFV exocytosis remains an open question. Although Rab27b is one possibility, an additional candidate includes Rab11 (a and b isoforms), which regulates trafficking pathways along both the endocytic and biosynthetic routes of polarized epithelial and neuroendocrine cells (9–18). However, the function of Rab11 in modulating the regulated secretory pathways of polarized epithelial cells has not been explored and nothing is known about the role of Rab11 in mechanotransduction or DFV exocytosis.

Results

Rab11a Is Expressed in the Bladder Uroepithelium. We used RT-PCR to determine which members of the Rab11 family (Rab11a, Rab11b, and Rab25) were expressed in bladder uroepithelium. PCR products of the expected size were obtained for Rab11a and Rab25 in all species tested (Fig. 1*A*). However, we were unable to successfully detect Rab11b from these tissues (data not shown). Immunoblot analysis confirmed expression of Rab11a and Rab25 in uroepithelial lysates, but not Rab11b (Fig. 1*B*). We confirmed the Rab11b antibody was functional by showing that it detected this GTPase in lysates of stomach tissue (Fig. 1*B*), which expresses both Rab11 isoforms (19).

Rab11a Is Associated with DFV. Rab11a was found concentrated underneath the apical plasma membrane of the umbrella cells, where it was associated with small-punctate vesicular structures (Fig. 1*C*). Rab11a expression was also observed in the apical-most intermediate cell layer in the rat and rabbit uroepithelium, but not in mouse tissue (Fig. 1*C*). In contrast, Rab25 was not expressed in the umbrella cell layer but was found concentrated in vesicular structures in the underlying intermediate cell layers [supporting information (SI) Fig. S1*A*]. We detected no Rab11b-positive staining in the uroepithelum (Fig. S1*B*); however, Rab11b staining was observed in rat stomach epithelium (data not shown). The distribution of Rab11a was reminiscent of that observed for uroplakin (UP)3a, an apical plasma membrane protein that is also found in DFV (2). In fact, we observed a high degree of colocalization between Rab11a and UP3a (colocalization coefficient of ≈0.9; Fig. 1*D* and Table S1). To confirm this finding we double-labeled ultrathin cryo-sections with antibodies to Rab11a and UP3a. We observed both markers on DFV, including those in close proximity to the apical plasma membrane (Fig. 1*E*) and deeper in the cell cytoplasm (data not shown). These results indicate that Rab11a, but not Rab11b or Rab25, is expressed in umbrella cells and is associated with DFV.

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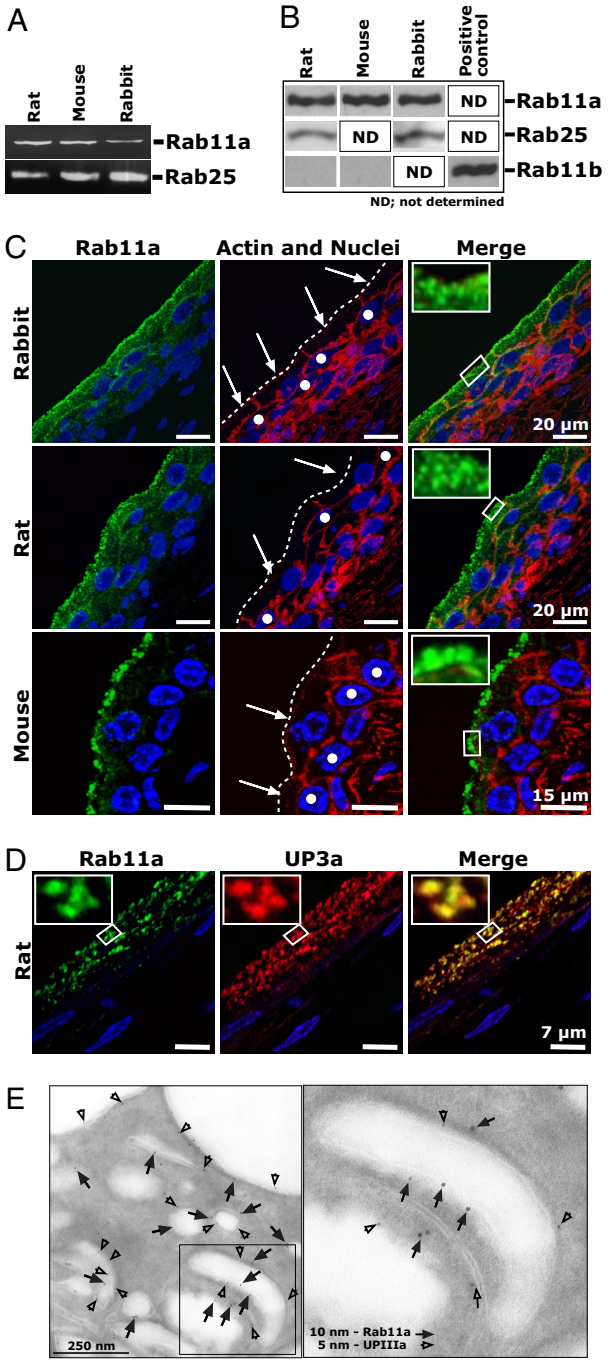

Fig. 1. Expression of endogenous Rab11a in the uroepithelium and localization to DFV. (*A*) Expression of Rab11a and Rab25 as determined by RT-PCR. (*B*) Expression of Rab11a, Rab25, and Rab11b in lysates of uroepithelium or stomach mucosa. ND, not determined. (*C*) Distribution of Rab11a (green), actin (red), and nuclei (blue) in uroepithelium. The dashed lines show the position of the apical surfaces of the umbrella cell layer. (*D*) Distribution of Rab11a (green), UP3a (red), and nuclei (blue) in rat uroepithelium. Images are projections of confocal optical sections; the boxed regions in the merged panels are magnified in the *Insets*. Arrows indicate umbrella cells and solid circles indicate the first layer of intermediate cells. (*E*) Immunolocalization of Rab11a (green) and UP3a (red) on ultrathin cryosections of rat umbrella cells. The boxed area in *Left* is magnified in *Right*.

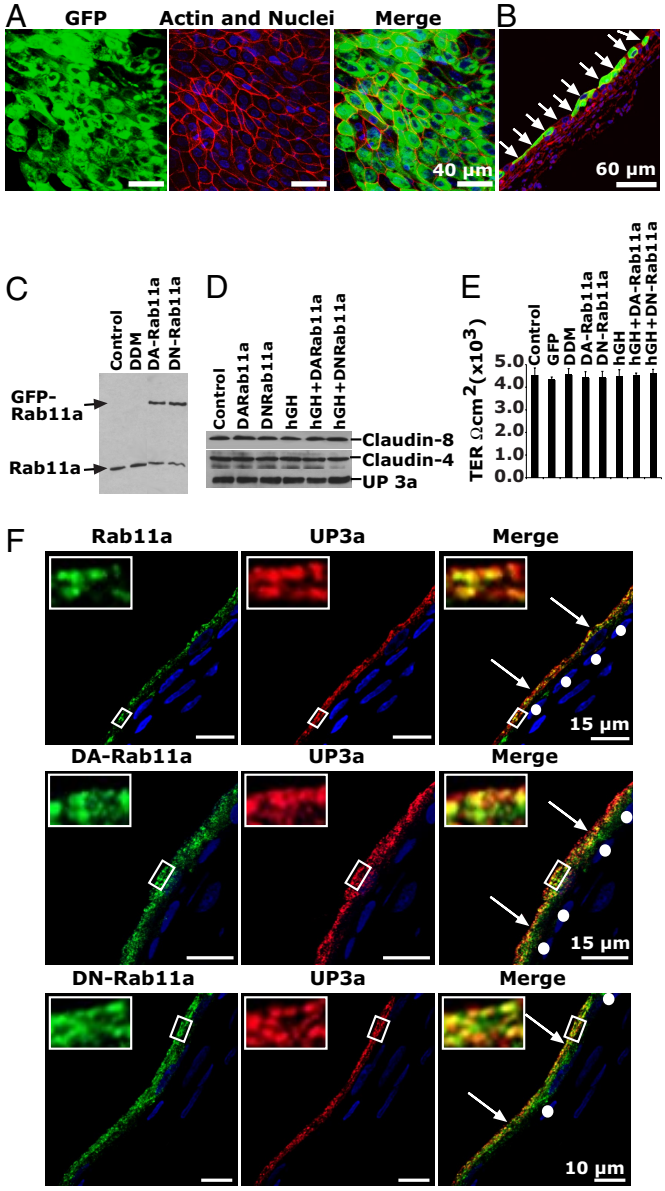

Fig. 2. Adenovirus-mediated expression of GFP or GFP-tagged DA-Rab11a or DN-Rab11a in umbrella cells. (*A* and *B*) *In situ* expression of GFP in umbrella cells. An *en face* projection is shown in *A* and a cross section is shown in *B*. GFP is shown in green, actin in red, and nuclei in blue. (*C*) Western blot analysis of Rab11a expression in untreated control umbrella cells, those treated with n-dodecyl-β-D-maltoside (DDM), or those expressing DA-Rab11a or DN-Rab11a. (*D*) Western blot analysis of claudin-8, claudin-4, or UP3a expression in control cells or those expressing DA- or DN-Rab11a ± hGH. (*E*) Measurement of TER in control or transfected uroepithelium. Data are mean ± SEM ($n = 4$). (*F*) Distribution of endogenous or mutant Rab11a (green), UP3a (red), and nuclei (blue) in uroepithelium. Images are projections and the boxed regions in *Right* are magnified in the *Insets*.

Rab11a Regulates Stretch-Induced Exocytosis. To assess the functional relevance of Rab11a association with DFV we infected rat umbrella cells *in situ* with adenoviruses encoding the following proteins: GFP alone or GFP-tagged versions of constitutively GTP-bound Rab11aS20V, which is a dominant active (DA) form of Rab11a (DA-Rab11a), or constitutively GDP-bound Rab11aS25N, which is a dominant negative DN form of Rab11a (DN-Rab11a). Using this technique, we selectively expressed the exogenous proteins in the umbrella cell layer with an efficiency of ≈70% (Fig. 2 *A* and *B*). Infection of the underlying intermediate cell layer was rare and when observed was limited to one or two individual cells. By adjusting the multiplicity of infection we were able to express the exogenous proteins at levels ≈2- to

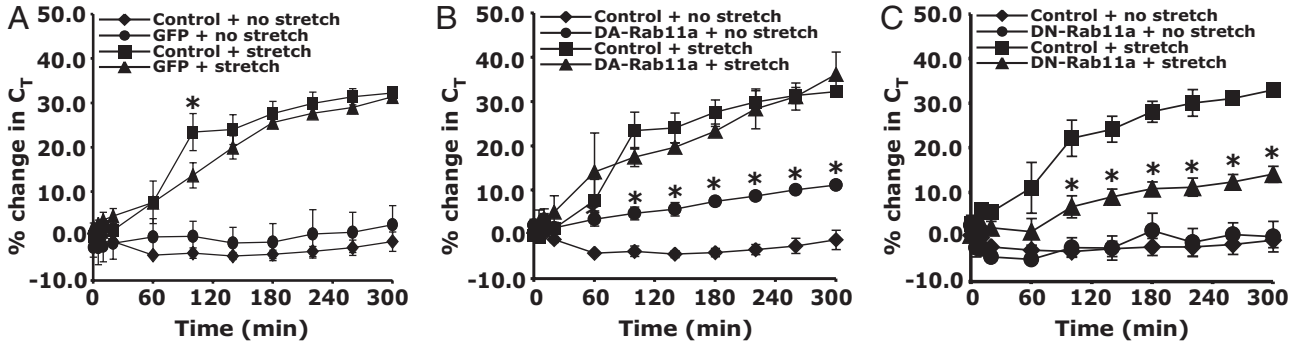

Fig. 3. Effect of GFP, DA-Rab11a, or DN-Rab11a expression on C_T in quiescent or stretched umbrella cells. Control uroepithelium or that infected with viruses encoding GFP (*A*), GFP-tagged DA-Rab11a (*B*), or GFP-tagged DN-Rab11a (*C*) was either left unstretched or stretched, and changes in C_T were monitored over time. Data are mean ± SEM ($n \geq 10$). * denotes a value that is significantly different from that of the appropriate control ($P < 0.05$).

3-fold above that of the endogenous Rab11a (Fig. 2*C*). At these levels there were no observed effects on the expression profile of UP3a or the tight junction-associated proteins claudin-4 or claudin-8 (Fig. 2*D*). Furthermore, the tissue remained intact and retained its barrier function as determined by measurements of transepithelial resistance (TER) (Fig. 2*E*). Grossly, the bladders looked normal, there was no change in their size, and the ultrastructure of the infected cells looked similar to that of untreated control tissue (Fig. S2). Like endogenous Rab11a, both DA- and DN-Rab11a were associated with vesicular elements in the apical cytoplasm of the umbrella cells and colocalized with UP3a (colocalization coefficients of >0.85; Fig. 2*F* and Table S1).

To examine whether Rab11a regulated DFV exocytosis we excised control or adenovirus-infected bladders, mounted them in Ussing stretch chambers, and measured increases in membrane capacitance (C_T; where 1 μF is ≈1 cm^2 of surface area). In the absence of stretch no significant change in C_T was observed over 5 h. However in cells expressing GFP alone or control uninfected rat bladders, stretch induced an ≈32% increase in C_T over the same period (Fig. 3*A*). The expression of DA-Rab11a resulted in a small, but significant, ≈11% increase in C_T in the absence of stretch, but had no significant effect on stretch-induced exocytosis over a 5-h period (Fig. 3*B*). Conversely, expression of DN-Rab11a resulted in a >50% decrease in C_T in stretched tissue, but had no significant effect in the absence of stretch (Fig. 3*C*). The magnitude of the effects mediated by expression of DA-Rab11 or DN-Rab11a is a lower estimate, as only ≈70% of cells were transfected by our techniques. Taken together, these results indicate that Rab11a may modulate DFV vesicle exocytosis in umbrella cells.

Modulation of DFV Exocytosis by Rab11a May Be Independent of Its Role in Apical Recycling. In other polarized epithelial cell types Rab11a has a well defined role in regulating apical membrane recycling (9–13). However, the relationship of apically internalized membrane/fluid markers and the pool of Rab11a-positive DFV is not known. One possibility is that all or some of the Rab11a-positive DFV are endocytic in nature. If so, the DFV should be accessible to apically internalized markers. Alternatively, if the Rab11a-positive DFV are formed in the TGN and primarily associated with a regulated biosynthetic pathway then few may be labeled with endocytosed tracer.

To distinguish between these possibilities we instilled a fluid-phase marker, FITC-dextran, or one of two membrane markers, FITC-wheat germ agglutinin (WGA) or FITC-Con A, into the bladder. FITC-WGA binds to glycolipids and glycoproteins on the apical surface of the umbrella cells (20), whereas FITC-Con A binds to the apical membrane protein UP1a (21). We previously showed that apical endocytosis is stimulated by stretch in response to bladder filling (2) and is apparently enhanced upon bladder voiding (4). To ensure maximal stimulation of endocytosis we filled bladders with tracer and after a 30-min incubation the bladders were voided. The bladders were then excised and in some cases incubated for up to 45 min to allow endocytosed tracer additional time to be delivered to DFV. In all cases, the endocytosed markers were internalized and appeared in numerous vesicular elements scattered across the apical cytoplasm of the umbrella cell (Fig. 4 *Center*). However, no association of internalized markers with Rab11a-positive DFV was observed (Fig. 4 *Right*). Furthermore, there was no colocalization of endocytosed marker and DN-Rab11a (data not shown), which has previously been shown to block exit of cargo from early

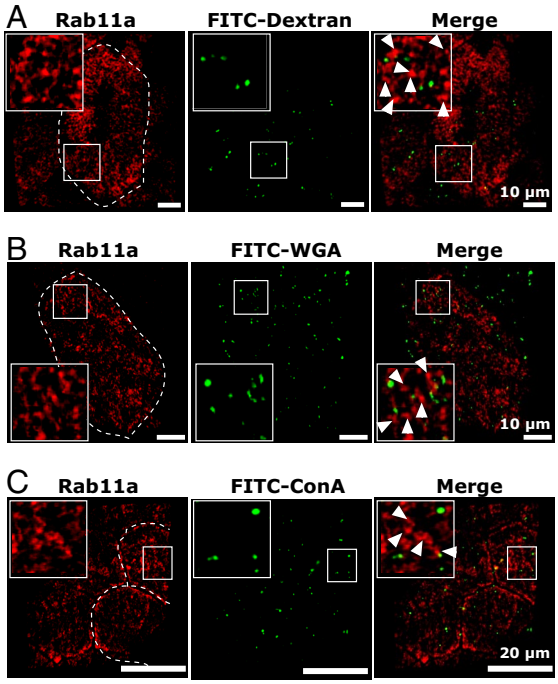

Fig. 4. Uptake of FITC-labeled dextran (*A*), WGA (*B*), or Con A (*C*) in umbrella cells. Bladders were filled with the indicated marker, and after 30 min the tracer was released and the distribution of endocytosed fluorescent marker and Rab11a-positive DFV was assessed by immunofluorescence. Projected images of whole-mounted uroepithelium are shown. The borders of representative umbrella cells are marked with hashed lines. Because of differences in cell height the Rab11a staining is not apparent in all adjacent umbrella cells. The boxed regions are magnified in the *Insets*.

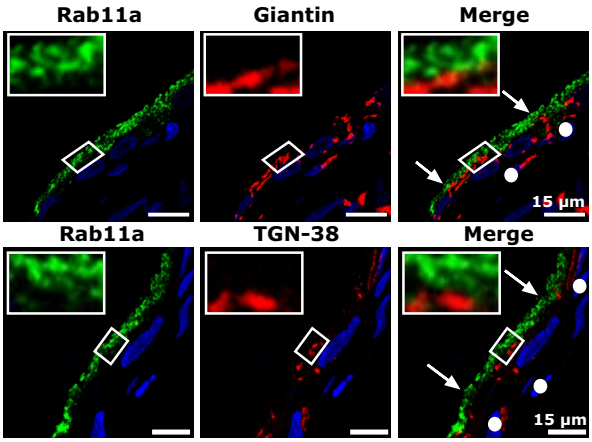

Fig. 5. Localization of Rab11a-positive DFV and the Golgi or TGN. Rab11a is shown in green, the Golgi/TGN in red, and nuclei in blue. Images are projections, and the boxed regions are magnified in the *Insets*.

endosomes and recycling endosomes (22). In contrast, we did observe that the FITC-dextran-positive punctae colocalized with UP3a (Fig. S3), which is consistent with previous observations that the UP3a-rich asymmetric unit membrane is also found in multivesicular endosomes and lysosomes (23, 24). Taken together, these results indicate that the Rab11a-positive pool of DFV may be primarily associated with the biosynthetic pathway.

In yeast the Rab11 homologs ypt31p/ypt32p are localized to the TGN, and a similar localization has been reported in nonpolarized cells (14, 25). We previously showed that long-term changes in C_T depend, in part, on new protein synthesis (4). Thus DN-Rab11a may exert some of its effects by altering the exit of secretory vesicles from TGN. However, we observed little colocalization between Rab11a and the Golgi marker giantin or the TGN marker TGN-38 in umbrella cells (Fig. 5).

Rab11a Regulates DFV Exocytosis Along the Biosynthetic Route. To further examine the possibility that Rab11a regulates DFV exocytosis along the biosynthetic route, we expressed human growth hormone (hGH) in the bladder umbrella cell layer. hGH is packaged into newly synthesized secretory vesicles and is routinely used to follow regulated secretion (17). Furthermore, previous studies have shown that in transgenic mice hGH is present in DFV and is released into the urine (26). Unlike membrane proteins, secreted proteins are not recycled, and thus provide a method for detection of exocytosis, independent of plasma membrane recycling. Exogenously expressed hGH was detected in lysates of uroepithelium (Fig. 6*A*) and when its distribution was assessed by immunofluorescence it was found to be associated with punctate vesicular structures in the apical cytoplasm of the umbrella cells (Fig. 6*B*). hGH colocalized with UP3a and Rab11a, confirming its presence in DFV (colocalization coefficients of >0.94; Fig. 6*C* and Table S1), and further indicated that Rab11a-positive DFV are likely to be biosynthetic in nature.

Next, we assessed whether the Rab11a mutants affected hGH secretion. Coexpression of hGH with DA-Rab11a or DN-Rab11a had no effect on the amount of hGH expression (Fig. 6*A*). In control rats, or those expressing GFP alone, hGH secretion was observed after 15 min of bladder filling and increased during each time interval (Fig. 6*D*). The total amount of hGH expression was modest and may reflect that impact of expressing large amounts of a secretory protein that forms dense cores within the lumens of DFV (26), which normally lack such structures. It has been previously shown that nonglycosylated hGH can be released from the basolateral poles of Madin–Darby

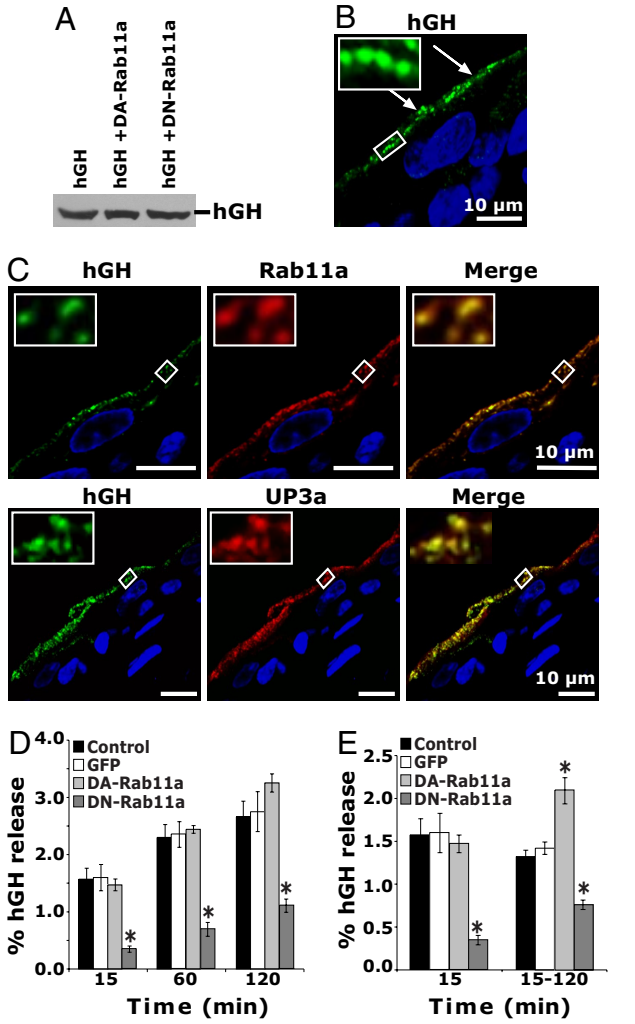

Fig. 6. Rab11a-mediated regulation of hGH secretion into the bladder lumen. (*A*) Western blot of cells expressing hGH alone or hGH and DA- or DN-Rab11a. (*B* and *C*) Distribution of hGH (green) and Rab11a (red) or UP3a (red) in umbrella cells. Images are projections, and the boxed regions in *Right* are magnified in the *Insets*. (*D* and *E*) Secretion of hGH in cells expressing GFP, GFP-tagged DA-Rab11a, or GFP-tagged DN-Rab11a. Data are mean ± SEM ($n = 8$). * denotes a value that is significantly different from cells expressing GFP alone ($P < 0.05$).

canine kidney cells (27). However, because of technical limitations we were unable to measure release of hGH from the serosal surface of the uroepithelium *in situ*. In contrast, expression of DN-Rab11a resulted in a significant ≈65% reduction in hGH secretion, and decreased release was evident at each time interval examined (Fig. 6*D*). hGH secretion tended to increase over time in cells expressing DA-Rab11a, but the increase was not significant under these conditions (Fig. 6*D*). However, if the secreted hGH was collected in two intervals, from 0–15 min and 15–120 min, expression of DA-Rab11a caused a significant increase in hGH secretion during the second time window (Fig. 6*E*). DN-Rab11a exhibited its negative effects on secretion during both time windows (Fig. 6*E*). These data indicate that Rab11a may regulate the kinetics of DFV exocytosis in a GTP-dependent manner.

Discussion

Rab11 has a well defined function in endosomal recycling at the apical surface of polarized epithelial cells (9–13). However,

information about its role in the biosynthetic traffic of these cells is limited, and there is almost no information about its role in regulated secretion in these cells. In the WIF-B9 hepatocyte cell line, Rab11a and its effector protein myosin Vb modulate the apical (canalicular) delivery of ATP-binding-cassette proteins, which unlike most hepatocyte membrane proteins use a direct (nontranscytotic) route to the apical cell surface (12). Apical delivery in fly photoreceptor cells may also involve Rab11 (15), and recently we showed that trafficking of newly synthesized endolyn to the apical surface of Madin–Darby canine kidney cells requires two Rab11a effectors, myosin Vb and Sec15, whereas the raft-associated protein hemagglutinin does not (28). Surprisingly, basolateral delivery of E-cadherin in mammalian and *Drosophila* cells may also require Rab11 (29, 30).

A common theme in the above studies is that delivery of newly synthesized membrane proteins involves passage through a Rab11-positive endosomal intermediate before delivery to the cell surface. In contrast, our analysis in umbrella cells indicates that Rab11a is associated primarily with apically targeted DFV and these Rab11a-positive carriers are largely inaccessible to endocytosed tracers. As further evidence of the biosynthetic nature of Rab11a-positive DFV, we observed that newly synthesized secretory protein hGH is packaged into the majority of these vesicles. These findings confirm previous studies that demonstrated the presence of hGH in the DFV of mouse umbrella cells (26). In some ways umbrella cells are more like neuroendocrine cells, where Rab11b is associated with secretory granules and is required for their regulated exocytosis (17). Although we cannot rule out that other endocytic proteins may access DFV, or that that there is a regulated recycling pathway similar to that observed in gastric parietal cells (13, 19), our findings are consistent with electron microscopic studies that show little uptake of apically internalized markers into DFV (23, 24).

In the classical model DFV are envisioned to exocytose during bladder filling and then reform upon voiding when the added apical membrane is endocytosed (6, 31). However, based on the currently available data it appears more likely that DFV are synthesized in the TGN (5) and undergo exocytosis in a Rab11a-dependent manner. Upon voiding the apical membrane added during bladder filling is likely endocytosed and may then be degraded in lysosomes, the previously described fate of apically internalized membrane and fluid markers in umbrella cells (2, 23, 24).

The lack of association of Rab11a with the TGN/Golgi of umbrella cells, and the observation that the majority of DFV appear to be UP3a, hGH, and Rab11a positive, indicates that Rab11a associates with DFV sometime after their release from the TGN. Although this association likely depends on the formation of active Rab11a-GTP, there must be a clamping mechanism that prevents Rab11a from promoting exocytosis until an appropriate extracellular signal (e.g., stretch) is sensed by the cell. One possibility is that under conditions where there is little stretch (e.g., after voiding) there is rapid cycling between Rab11a-GTP and Rab11a-GDP. Presumably, such rapid cycling may prevent Rab11a-GTP from forming stable interactions with its effectors. Stretch would shift the equilibrium in favor of Rab11a-GTP, which would efficiently recruit effectors such as the Rab11 family-interacting proteins 1–5, myosin Vb, or Sec15 to the surface of DFV. In turn, these effectors would promote vesicle movement, docking, and/or fusion. An alternative model is that Rab11a-GDP associates with DFV, and that stretch promotes nucleotide exchange, generating Rab11a-GTP and recruiting its downstream effectors. Regardless of the model, Rab11a-GTP is likely to undergo hydrolysis either before vesicle fusion or after DFV exocytosis, releasing Rab11a for additional rounds of DFV binding.

An open question is why both Rab11a and Rab27b are present on DFV (8). One possibility is that these GTPases function in a sequential fashion to regulate DFV maturation and exocytosis. For example, Rab5 on early endosomes recruits numerous effectors including the HOPs complex, which acts as an exchange factor to recruit and activate Rab7 on the maturing endosome before its fusion with late endosomes (32). In an analogous manner, Rab27b (or Rab11a) may associate with disk-shaped vesicles as they emerge from the TGN and as the vesicles undergo maturation Rab27b would recruit Rab11a (or vice versa), which would ultimately promote vesicle docking and fusion. In cytotoxic T cells, Rab11 on recycling endosomes and Rab27a on late endosomes fuse with each other before fusion with cytotoxic granules to form a hybrid organelle that can release cargo that destroys target cells (33). It is unknown whether umbrella cells have multiple populations of apically targeted vesicles, but if they do it is possible that fusion of distinct populations of Rab11a and Rab27b DFV are necessary to achieve stretch-mediated exocytosis. Although there remains much to learn about the process, the tools and observations we have made provide insight into the molecular machinery that regulates DFV exocytosis and further suggests that Rab11a is an important modulator of regulated exocytosis in polarized umbrella cells.

Materials and Methods

Reagents and Antibodies. Unless specified otherwise, all chemicals were obtained from Sigma. Polyclonal antibodies to Rab11a and Rab11b and mouse mAbs against Rab11a (8H10) and Rab25 (12C3) have been described (19, 34). Other antibodies included rabbit anti-hGH (National Hormone and Peptide Program, Torrance, CA), mouse monoclonal K8B12 antibody against UP3a (35), rabbit anti-TGN-38 (Sigma), mouse anti-giantin (Adam Linstedt, Carnegie Mellon University, Pittsburgh), mouse mAb to claudin-4 (Zymed), and rabbit anti-claudin-8 (Zymed). Fluorophore or HRP-labeled affinity-purified and minimal cross-reacting goat anti-mouse, anti-rat, and anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch. Gold-conjugated secondary antibodies were obtained from Sigma. Rhodamine phalloidin and TO-PRO3 were obtained from Molecular Probes.

Animals. Urinary bladders were obtained from female New Zealand white rabbits (3–4 kg), female C56BL/6J mice (3–4 months old), or female Sprague–Dawley rats (250–300 g). Rabbits were euthanized by injecting 300 mg of sodium pentobarbital into their ear vein, and mice and rats were euthanized by inhalation of 100% CO_2. All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

RT-PCR. The uroepithelium was recovered, and PCR was performed as described (4). The primers used were as follows: Rab11a, GGCTGAAAGAACTGAGAGATCATGC and GATGTTCTGACAGCACTGCACC; Rab25, GACCTRACCAAGCACCAGACC and CTGCTTGGACACCTTYGCAAAG.

Western Blot Analysis. Uroepithelial cell lysates were prepared as described (2). Samples (containing 10 μg of total protein) were resolved by SDS/PAGE and Western blots were performed as described (35).

Immunofluorescence Labeling and Image Acquisition. Bladder tissue was fixed, processed, and imaged as described (2, 4). Measures of colocalization are described in *SI Methods*.

Immunoelectron Microscopy. Bladder tissue was isolated and then fixed with 0.05% (vol/vol) glutaraldehyde and 4% (wt/vol) paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4 containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ for 60 min. The tissue was then cut into small pieces, incubated in 3% (wt/vol) gelatin for 30 min at 37°C, and then placed overnight at 4°C in 1.8 M sucrose and 20% (wt/vol) polyvinylpyrrolidone. The tissue was cryosectioned, immunolabeled, and imaged as described (2).

Preparation of Adenoviruses and Infection of Umbrella Cells *in Situ*. Adenoviruses expressing GFP, GFP-tagged DN-Rab11aS25N, or hGH (kindly provided by B. Baum, National Institutes of Health, Bethesda) were produced and purified as described (36). To generate adenoviruses expressing GFP-tagged DA-Rab11a, the serine residue at position 20 in plasmid pAdTet-GFP-HA-

Rab11a was mutated into valine by site-directed mutagenesis. Bladders were infected *in situ* with viruses expressing GFP, DA-Rab11a, DN-Rab11a, or hGH using a previously described technique to express exogenous proteins in umbrella cells (37). Details are provided in *SI Methods*.

Mounting of Rat Bladder Uroepithelium in Ussing Stretch Chambers and Measurement of C_T and TER. Modifications to our previously described techniques to mount tissue and measure C_T and TER (2) are provided in *SI Methods*.

Uptake of FITC-WGA, FITC-Con A, or FITC-Dextran. Internalization of fluorescent markers was performed as described (2), with modifications described in *SI Methods*.

Estimation of hGH Secretion. Rat bladders were infected *in situ* with adenoviruses expressing hGH alone or in combination with DA-Rab11a or DN-Rab11a. The bladders were filled and hGH released into the bladder lumen was collected and quantified as detailed in *SI Methods*.

Statistical Analysis. Statistically significant differences between means were determined by two-tailed Student's *t* test; $P < 0.05$ was considered statistically significant.

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