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Identification and characterization of Noc2 as a potential Rab3B effector protein in epithelial cells

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Abstract

The Rab3 family small G proteins (Rab3A–D) are involved in the regulated secretory pathway of brain and secretory tissues. Among Rab3-interacting proteins, Rabphilin-3, Rim, and Noc2, all of which contain a conserved Rab3-binding domain (RBD3), are generally recognized Rab3 effector proteins in neurons and secretory cells. Although Rab3B was also detected in epithelial cells, its function remained unknown. We isolated cDNA sequences from human epithelial Caco2-cell mRNA by degenerate RT-PCR based on the conserved amino acid sequence of RBD3. Multiple cDNA clones were identified as encoding Noc2. Northern blot analysis revealed that Noc2 mRNA was expressed not only in secretory tissues but also in epithelial tissues and cell lines. A pull-down assay demonstrated that Noc2 bound to Rab3B in a GTP-dependent manner. When Noc2 was co-expressed with the GTP-bound form of Rab3B, it was recruited from the cytosol to perinuclear membranes. Furthermore, overexpression of Noc2 inhibited the cell-surface transport of basolateral vesicular stomatitis virus glycoprotein. These results suggest that Noc2 functions as a potential Rab3B effector protein in epithelial cells.

Keywords: Rab; Epithelial cells; RBD3; Noc2; VSV-G

The secretory pathway is used to deliver newly synthesized proteins to the cell-surface and the extracellular milieu. The *trans*-Golgi network (TGN), a primary station along this route, sorts proteins into distinct transport pathways. The subsequent movement of proteins to the plasma membrane (PM) can occur either directly or via the endocytic pathway [1,2]. Moreover, multiple and parallel pathways from the TGN to the PM appear to exist, not only in neurons and secretory cells, but also in non-secretory cells such as epithelia and fibroblasts [3–6].

The Rab family small G proteins, which comprise more than 60 family members in mammalian cells, are localized to the surfaces of distinct membrane compartments and regulate discrete transport steps along the secretory and endocytic pathways [7–9]. Rab proteins function as molecular switches, exploiting GTP hydrolysis and guanine nucleotide exchange to cycle continuously between structurally distinct GTP-bound active and GDP-bound inactive forms. By this mechanism, Rabs direct a number of vesicular transport steps including vesicle formation, motility, tethering/docking, and fusion. These events are mainly controlled by the cooperative action of the GTP-bound form of Rabs and their effector proteins. For example, Rab1 recruits its effector protein, the tethering factor p115, during COPII vesicle budding from the endoplasmic reticulum (ER) and Rab6 acts through Rabkinesin6, an effector protein that binds microtubules and has microtubule-activated ATPase activity [10,11]. The list of putative Rab effector proteins is continuously growing. It is clear now that a single Rab protein can interact with multiple effector proteins, as occurs in the Rab5 effector protein complex, and can regulate multiple biochemical reactions at distinct sites in a transport pathway [12].

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While many Rab proteins are ubiquitously expressed, Rab3 family proteins, which include Rab3A, Rab3B, Rab3C, and Rab3D, are enriched in brain and secretory tissues [13]. In agreement with this specific expression pattern, Rab3 family proteins are thought to be involved in the regulated secretory pathway of neurons and secretory cells [14]. Among the Rab3-binding proteins identified to date, Rabphilin-3, Rim (Rim1 and 2), and Noc2 are predominantly expressed in neurons and secretory cells, and are likely to mediate the function of Rab3 family proteins [15–17]. Interestingly, Rab3B is also expressed in epithelial cells and may be involved in the transcytosis of polymeric immunoglobulin receptor (pIgR) [18,19]. We have recently suggested that Rab3B directs the cell-surface transport of basolateral low-density lipoprotein receptor (LDLR) and may contribute to epithelial polarization [6]. However, a Rab3B effector protein operating in epithelial cells has yet to be identified.

The Rab3-binding domain (RBD3) originally identified as the N-terminal domain of Rabphilin-3 is highly conserved among Rab3 effector proteins, including Rim and Noc2. Sequence and structural analysis of the Rabphilin-3/Rab3A complex revealed that the SGAWFF motif found in Rabphilin-3 RBD3 directly contacts Rab3A [20]. In addition to RBD3, a Rab11-binding domain (RBD11) and a Rab27-binding domain (RBD27) were also identified and characterized in several Rab11 and Rab27 effector proteins [21–23]. Thus, we hypothesized that RBD3-containing proteins would be good candidates for putative Rab3B effector proteins in epithelial cells. To isolate cDNA encoding RBD3-containing proteins from epithelial cells, we performed degenerate RT-PCR using human epithelial Caco2-cell cDNA as a template. Multiple cDNA clones were found to encode Noc2, whose expression was thought to be restricted to secretory cells [17]. Surprisingly, Noc2 mRNA was also detected in epithelial tissues and cell lines by Northern blot analysis. Pull-down, in vivo recruitment, and vesicular stomatitis virus glycoprotein (VSV-G) transport assays all indicated that Noc2 functions as a potential Rab3B effector protein in epithelial cells.

Materials and methods

Materials and chemicals. BHK, Caco2, and MDCK cells were obtained from ATCC (Manassas, VA). Anti-Myc (9E10) and anti-HA (3F10) antibodies were purchased from Roche (Mannheim, Germany) and anti-Xpress was from Invitrogen (Carlsbad, CA). Anti-VSV-G (8G5) antibody and recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) were obtained as described previously [24].

DNA constructs. Bovine Rab3B cDNA was kindly supplied by Dr. Y. Takai (Osaka University, Osaka, Japan) [25]. Rat Noc2 cDNA was obtained as described previously [17]. Hamster Rab3B, canine Rab5, and human Noc2 cDNAs were isolated by RT-PCR performed on mRNA from BHK, MDCK, and Caco2 cells, respectively. Bovine Rab3B and canine Rab5 cDNAs were cloned into pRSET vectors. Full-length Noc2 (1–315 a.a.), its N-terminal half (1–156 a.a.), and its C-terminal half (157–315 a.a.) were amplified by PCR using human Noc2 cDNA as a template and cloned into a pCI-neo-Myc vector. pAR-G, pCI-neo-HA-Rab3B, pCI-neo-HA-Rab3B T36N, and pCI-neo-HA-Rab3B Q81L plasmids were generated as described previously [6,24]. All DNA constructs used in this study were sequenced using an automated DNA sequencer 377 (Applied Biosystems, Foster City, CA).

Degenerate RT-PCR. Six degenerate primers were designed to code for all possible amino acids present in the conserved RBD3 sequence of Rabphilin-3, Rim1, and Noc2 (Fig. 1A). Among them, the following primer pairs yielded PCR results: sense (5′-(CG)A(AG) (AC)GI (AG) (AT)I (GT)GI (AC)GI (CT)TI-3′, where I is inosine) and anti-sense (5′-(AT) (CG)I GGI GCI TGG TT(CT) T(AT) (CT)-3′). Total RNA isolated from human Caco2 cells was converted to cDNA and used as a template for degenerate RT-PCR. PCR amplification was performed for 35 cycles comprising a denaturation at 94 °C for 1.5 min, annealing at 40 °C for 2.5 min, and extension at 68 °C for 5 min using an Expand High-Fidelity PCR system (Roche, Mannheim, Germany). Reaction products of about 250 bp in size were isolated, cloned into a pGEM-T vector (Promega, Madison, WI), and sequenced.

Northern blot analysis. Two micrograms of poly(A)$^+$ RNA prepared from Caco2 and MDCK cells was denatured, electrophoresed on a 1% agarose gel containing 1.8% formaldehyde, and transferred by the capillary blot method in 10× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate) to a nylon membrane. This and a Rat Multiple Tissue Northern Blot membrane (Clontech, Palo Alto, CA) were prehybridized at 42 °C for 3 h in hybridization buffer containing 5× SSC, 5× Denhardt's solution, 1% SDS, 50% deionized formamide, and 100 μg/ml salmon sperm DNA, and they were then hybridized with a ^{32}P-labeled probe at 42 °C. Probes for Noc2 and Rab3B mRNA were prepared by labeling the full-length cDNA encoding rat Noc2 and hamster Rab3B with [α-^{32}P]dCTP using RediprimeII DNA Labeling kit (Amersham, Piscataway, NJ). After 16 h of hybridization, the membranes were washed in 2× SSC at room temperature for 5 min twice, in 2× SSC containing 0.1% SDS at 60 °C for 30 min twice, and finally in 0.1× SSC at room temperature for 30 min twice. The membranes were exposed to an Imaging Plate (Fuji Film, Tokyo, Japan) for 18 h and analyzed using a BAS2000 Image Analyzer (Fuji Film, Tokyo, Japan).

Pull-down assay. BHK cells were infected with vTF7-3 and transfected with the indicated combinations of expression plasmids encoding 6× His-/Xpress-tagged Rab and Myc-tagged Noc2 proteins. Six hours after transfection, cells were lysed in a lysis buffer [25 mM Tris/HCl (pH 7.5), 125 mM NaCl, 1 mM $MgCl_2$, 0.5% Chaps, and 10 μg/ml APMSF] containing either 100 μM GTPγS or GDP for 15 min at 4 °C. After taking a fraction of the lysates, the remaining Rab proteins were isolated with TALON beads (Clontech, Palo Alto, CA), washed three times with a wash buffer [25 mM Tris/HCl (pH 7.5), 300 mM NaCl, 1 mM $MgCl_2$, and 0.1% Chaps] containing either 20 μM GTPγS or GDP, and eluted with SDS–PAGE sample buffer. The samples were separated on SDS–PAGE, transferred to PVDF membranes, and immunoblotted with anti-Xpress or anti-Myc antibody. Blots were developed using an ECL-Plus kit (Amersham, Piscataway, NJ).

Immunofluorescence microscopy. BHK cells were transfected with the indicated combinations of expression plasmids encoding HA-tagged Rab13 and Myc-tagged Noc2 proteins and grown for 36–48 h. Cells were fixed with 2% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, blocked with 5% goat serum in PBS, incubated with anti-HA (3F10) or anti-Myc (9E10) antibody, and visualized with Alexa 488 anti-rat IgG or Alexa 594 anti-mouse IgG conjugates (Molecular Probe, Eugene, OR). Fluorescence images of the cells were acquired using a Radiance 2000 confocal laser scanning microscope (Bio-Rad, Hercules, CA).

Cell-surface transport assay. Cell-surface transport of VSV-G was assayed as described previously [26]. Briefly, BHK cells were infected with vTF7-3 and transfected with pAR-G in combination with pCI-neo (mock), -Myc-Noc2-F, -Myc-Noc2-N, -Myc-Noc2-C, or -HA-Rab3B Q81L. Six hours after transfection, cells were pulse-labeled with [^{35}S] EasyTag Express Protein Labeling Mix (Perkin–Elmer,

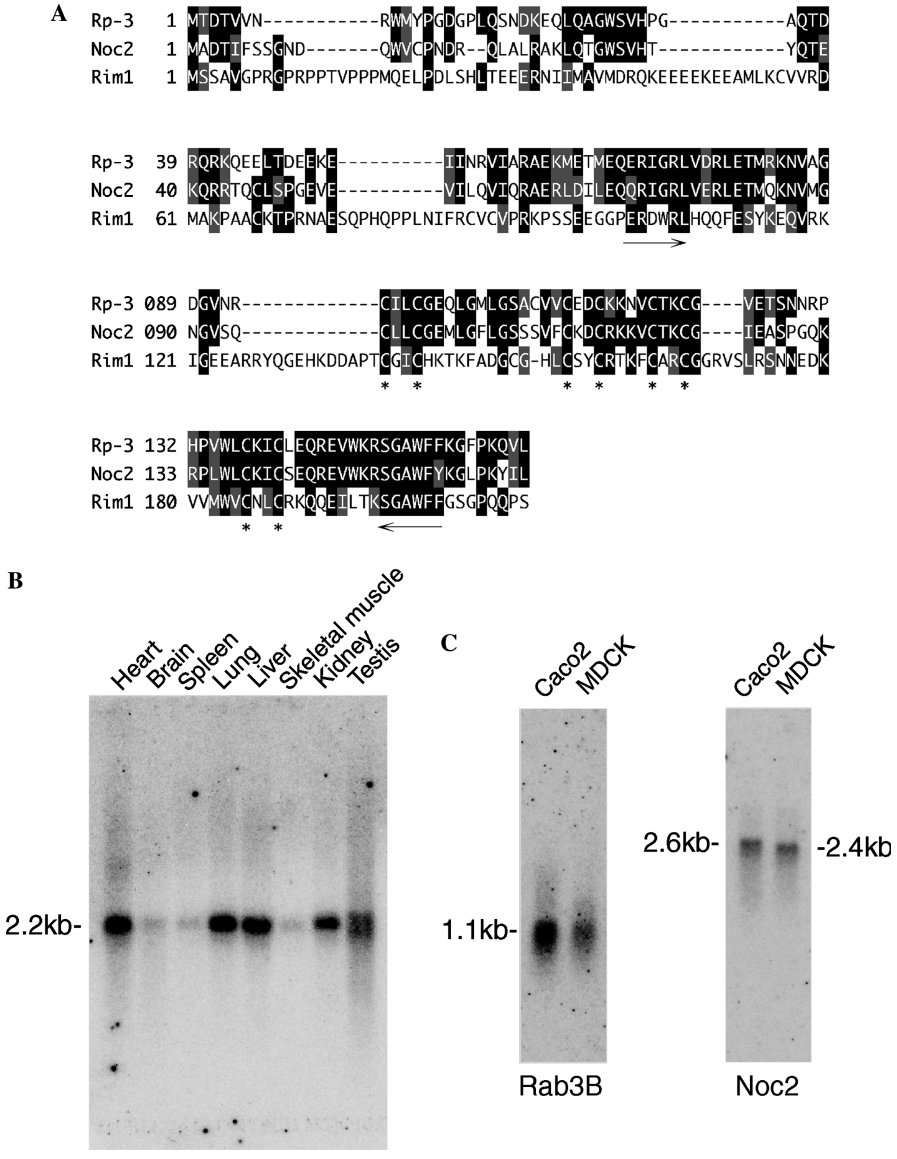

Fig. 1. Identification of Noc2 as a RBD3-containing protein in epithelial cells. (A) Sequence alignment of Rabphilin-3 (Rp-3), Noc2, and Rim1. Residues that are conserved and similar are shown against black and gray backgrounds, respectively. Zinc finger motifs are indicated by asterisks. Degenerate primers were designed based on the sequence marked by arrows. (B) Expression of Noc2 in rat tissues. A rat Multiple Tissue Northern Blot (Clontech #7764-1) was probed with rat Noc2 cDNA. (C) Expression of Rab3B and Noc2 in epithelial cell lines. Two micrograms of poly(A)$^+$ RNA prepared from Caco2 and MDCK cells was blotted onto a nylon membrane and probed with hamster Rab3B and rat Noc2 cDNA. The results shown in (B) and (C) are representative of three independent experiments.

Boston, MA) for 15 min and chased for the indicated periods of time. Cell-surface proteins were biotinylated with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in PBS containing 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$ (PBS/CM) at 4 °C for 60 min and quenched with 50 mM NH_4Cl in PBS/CM at 4 °C for 15 min. Following lysis in 20 mM Tris/HCl (pH 8.0) containing 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 0.2% BSA, and 10 μg/ml APMSF, total VSV-G was immunoprecipitated using 8G5 antibody bound to protein G–Sepharose (Amersham; Arlington Heights, IL), washed with 20 mM Tris/HCl (pH 8.0) containing 0.1% Triton X-100, 150 mM NaCl, and 5 mM EDTA, and eluted with 1% SDS in 50 mM Tris/HCl (pH 7.5) at 90 °C for 3 min. A fraction of the sample was saved and the remaining biotinylated VSV-G was subsequently isolated with UltraLink Immobilized NeutrAvidin Plus beads (Pierce, Rockford, IL). Total and surface fractions were separated on SDS–PAGE and analyzed using a BAS2000 Image analyzer (Fuji Film, Tokyo, Japan) and a NIH Image 1.62 program. The values for cell-surface biotinylated cargo proteins were normalized against the total amount of immunoprecipitated cargo proteins.

Results

Degenerate RT-PCR using human epithelial Caco2-cell cDNA as a template

In order to investigate the function of Rab3B, we attempted to isolate a putative Rab3B effector protein in

epithelial cells. For this purpose, we designed a series of degenerate oligonucleotides based on the conserved amino acid sequences of RBD3 of Rabphilin-3, Noc2, and Rim1 (Fig. 1A). We synthesized four sense and two antisense primers and tested all eight pairs by RT-PCR using human intestinal Caco2-cell cDNA as a template. Only one pair of primers produced RT-PCR products of the expected size. The resulting products were cloned and subjected to DNA sequencing. Out of 32 cDNA clones sequenced, 24 clones did not contain the corresponding sequence of the degenerate primer. The remaining eight clones were identified as sequences corresponding to human Noc2 cDNA. Neither Rabphilin-3 nor Rim cDNA was isolated in our degenerate RT-PCR. This prompted us to examine whether Noc2 can function as a Rab3B effector protein in epithelial cells.

Tissue distribution of Noc2

Since Noc2 was predominantly detected in endocrine tissues, we reexamined the tissue distribution of Noc2 by Northern blot analysis [17]. A single 2.2-kb transcript of Noc2 was detected at moderate levels in lung, liver, kidney, heart, and testis, and at low levels in brain, spleen, and skeletal muscle (Fig. 1B). Detection of Noc2 mRNA in epithelial tissues raises the possibility that Noc2 may function as a Rab3B effector protein in epithelial cells. If this were the case, both Rab3B and Noc2 mRNA should be detected in epithelial cell lines. Both Caco2 and MDCK cells expressed a single 1.1-kb transcript corresponding to Rab3B (Fig. 1C). The size of the Rab3B mRNA is consistent with that of a previous report [27]. In human Caco2 and canine MDCK cells, single bands of 2.6- and 2.4-kb, respectively, corresponding to Noc2 mRNA, were detected (Fig. 1C). This finding corroborates previous reports that human, mouse, and rat tissue express single 2.6-, 2.6-, and 2.2-kb Noc2 transcripts, respectively [17,28]. Taken together, these Northern blot analyses reveal that both Noc2 and Rab3B mRNA are expressed in epithelial cells.

Specific interaction between the RBD3 of Noc2 and the GTP-bound form of Rab3B

Although the interaction of Noc2 with Rab3A has been well characterized, its binding to Rab3B has not [17,29]. Thus, we systematically examined the interaction of Noc2 with Rab3B by co-transfection assay. We generated constructs encoding full-length Noc2 (Noc2-F), its N-terminal half (Noc2-N), and its C-terminal half (Noc2-C) (Fig. 2A) and used hamster fibroblastic BHK cells, in which both Rab3B and Noc2 mRNA were detected by Northern blot analysis (data not shown). The lysate of BHK cells co-expressing Rab3B and either Noc2-F, Noc2-N, or Noc2-C was incubated with TALON beads in the presence of GTPγS to pull down the GTP-bound form of His6-tagged Rab3B proteins. Then, Myc-tagged Noc2 proteins bound to the beads were detected by Western blot with an anti-Myc antibody. Rab3B interacted with Noc2-F and Noc2-N, but not with Noc2-C (Fig. 2B), suggesting that the N-terminal region of Noc2, which contains the RBD3, mediates Rab3B binding in our co-transfection assay. Next, we investigated the guanine nucleotide specificity of the interaction between Rab3B and Noc2. The binding of Noc2-F to Rab3B was analyzed in the presence of either GTPγS or GDP. Noc2-F preferentially interacted with Rab3B in the presence of GTPγS (Fig. 2B), confirming the GTP-dependence of the binding between Noc2 and Rab3B. Furthermore, no interaction between Noc2 and Rab5 was detected in our assay (Fig. 2B). Taken together, these results show that Noc2 binds specifically to the GTP-bound form of Rab3B, probably by direct interaction with its RBD3.

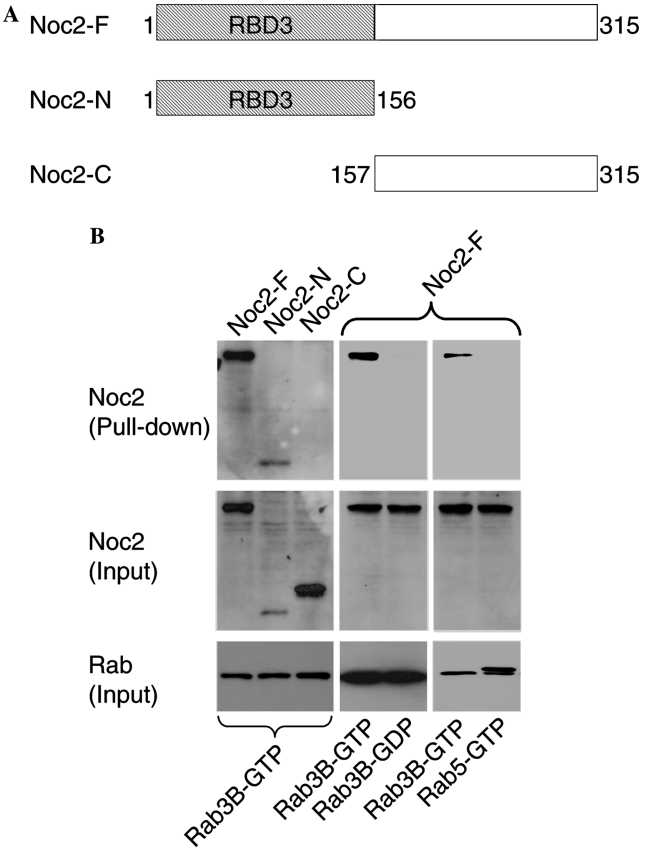

Fig. 2. GTP- and RBD3-dependent binding between Noc2 and Rab3B. (A) Schematic representation of Noc2-F, Noc2-N, and Noc2-C. (B) Rab and Noc2 proteins were co-expressed in BHK cells. After taking a fraction of the lysates, the remaining Rab proteins were incubated with TALON beads in the presence of either GTPγS (GTP) or GDP, washed, and eluted. The samples were subjected to Western blot analysis. Upper panel: Noc2 proteins bound to Rab3B. Middle panel: total Noc2 proteins expressed in BHK cells. Lower panel: total Rab proteins expressed in BHK cells. The results shown in (B) are representative of three independent experiments.

GTP- and RBD3-dependent recruitment of Noc2 from cytosol onto Rab3B-positive membranes

In order to express the GTP- or GDP-bound form of Rab3B in BHK cells, we constructed a Rab3B dominant-active mutant (Rab3B Q81L) that is defective in GTP hydrolysis, as well as a Rab3B dominant-negative mutant (Rab3B T36N) that has a lower affinity for GTP than for GDP. When Noc2-F, Noc2-N, or Noc2-C was singly expressed in BHK cells, it was diffusely distributed in the cytosol (Fig. 3A, not shown). Co-expression of wild-type Rab3B (Rab3B WT) or Rab3B Q81L with Noc2-F caused the recruitment of the cytosolic Noc2-F to the perinuclear membrane region, where Rab3B was localized (Fig. 3A, arrows). In contrast, co-expression of Rab3B T36N with Noc2-F did not change the cytosolic distribution of Noc2-F (Fig. 3A). Next, Rab3B Q81L was co-expressed with Noc2-F, Noc2-N, or Noc2-C in BHK cells. Whereas Noc2-F and Noc2-N were efficiently recruited to the Rab3B-positive perinuclear membrane

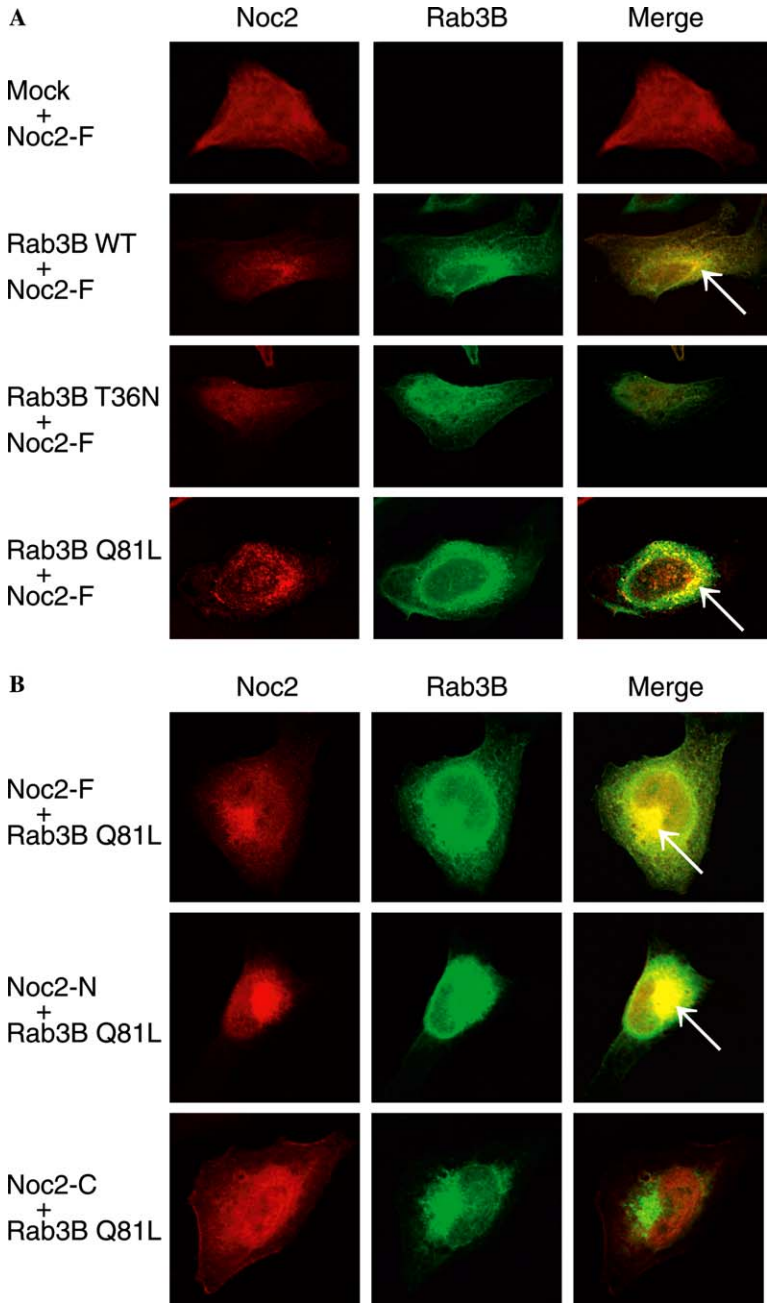

Fig. 3. Recruitment of Noc2 by the GTP-bound form of Rab3B in BHK cells. BHK cells were co-transfected either with Noc2-F and mock, Rab3B WT, Rab3B T36N, or Rab3B Q81L (A) or with Rab3B Q81L and Noc2-F, Noc2-N, or Noc2-C (B), incubated at 37 °C for 36–48 h, and processed for indirect immunofluorescence microscopy. The localization of Noc2 and Rab3B is shown in red and green, respectively. Arrows indicate the recruitment of Noc2. The results shown in (A,B) are representative of three independent experiments.

region, Noc2-C remained distributed diffusely throughout the cytosol (Fig. 3B). These immunofluorescence experiments together indicate that cytosolic Noc2 is recruited to Rab3B-positive membranes in a GTP- and RBD3-dependent manner.

Effect of Noc2 overexpression on the cell-surface transport of VSV-G

If Noc2 functions as a Rab3B effector protein in epithelial cells, it should play a role in regulating the secretory pathway. To test this possibility, we examined whether Noc2 was involved in regulating the cell-surface transport of a well-established basolateral marker protein, VSV-G, in BHK cells. As in polarized epithelial cells, VSV-G and apical proteins are sorted into different secretory pathways in BHK cells [3–5]. VSV-G was co-expressed with either an empty vector (mock), Noc2-F, Noc2-N, Noc2-C, or Rab3B Q81L in BHK cells, and the cell-surface transport of VSV-G was examined at 40 min time-point as described previously [6,26]. Whereas VSV-G was efficiently transported to the cell-surface in mock-transfected cells, its transport was considerably retarded in Rab3B Q81L-transfected cells (Figs. 4A and B). Overexpression of Noc2-F, but not of Noc2-N and Noc2-C, noticeably inhibited the cell-surface transport of VSV-G compared to mock (Figs. 4A and B). Importantly, the inhibitory ability of Noc2-F is comparable to that of Rab3B Q81L, indicating that Noc2 mediates the effect of Rab3B on the cell-surface transport of VSV-G in BHK cells. These data are consistent with a putative function of Noc2 in Rab3B-mediated vesicle transport in epithelial cells.

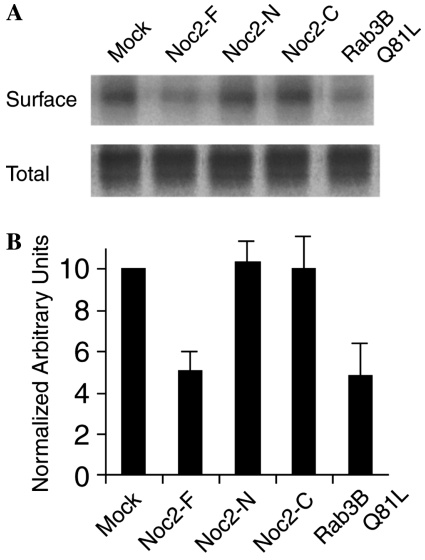

Fig. 4. Effect of Noc2 overexpression on the cell-surface transport of VSV-G. (A) BHK cells expressing VSV-G and mock, Noc2-F, Noc2-N, Noc2-C, or Rab3BQ81L were pulse-labeled and chased for 40 min. After cell-surface biotinylation, total and surface VSV-G were sequentially immunoprecipitated and isolated with avidin beads. Total and surface fractions were separated by SDS–PAGE and analyzed by autoradiography. The results shown in (A) are representative of three independent experiments. (B) Quantification of VSV-G transport. To compare the VSV-G transport between the experiments, the value for mock was set to 10 normalized arbitrary units. Data shown are means ± SEMs from three independent experiments.

Discussion

In the present study, we have performed degenerate RT-PCR using epithelial Caco2-cell cDNA as a template in order to identify a putative Rab3B effector protein in epithelial cells. We isolated Noc2 cDNA at an unexpectedly high frequency, but amplified neither Rabphilin-3 nor Rim cDNA. Although Noc2 expression was shown to be very specific to secretory tissues, we here detected Noc2 mRNA expression in epithelial tissues and cell lines by Northern blot analysis [17].

Although Noc2 is generally recognized as a Rab3A effector protein involved in the regulated secretory pathway, contradictory effects of Noc2 overexpression on regulated exocytosis in PC12 cells have been reported [17,30]. In pancreatic β-cells, silencing of the Noc2 gene by RNA interference impairs the regulated secretion of insulin [31]. Adding to the complexity of our knowledge of Noc2, it has been shown to bind to Rab27, which is also associated with secretory granules in secretory cells, as well as to Rab3 [29].

A number of distinct functions have been described for Rab3B. Inhibition of Rab3B expression in anterior pituitary cells by anti-sense oligonucleotides causes a severe reduction in regulated secretion of these cells, suggesting a positive role of Rab3B in regulated secretion [27]. In contrast, overexpression of Rab3B WT or Rab3B Q81L inhibits regulated hormone secretion in secretory cells, indicating an inhibitory role of Rab3B in regulated secretion [32,33]. In addition to this effect on regulated secretion, overexpression of Rab3B Q81L also inhibits dIgA-stimulated transcytosis of pIgR in epithelial cells [19]. We also have previously suggested that Rab3B directs the basolateral transport of LDLR in epithelial cells [6]. In this study, we have demonstrated that Rab3B regulates the basolateral transport of VSV-G through the action of its effector protein, Noc2, suggesting that Rab3B and Noc2 play a crucial role in controlling the basolateral secretory pathway in epithelial cells. Like Rab3 and Rab27 in secretory cells, Rab8, Rab11, and Rab3B are implicated in the basolateral secretory pathway in epithelial cells [6,29,34,35]. In addition, the adaptor protein (AP) complexes of AP-1B, AP-3, and AP-4 have been proposed as regulators of the same pathway [26,36,37]. As Rab8 is shown to specifically regulate the basolateral transport of AP-1B-dependent cargo proteins, further studies are necessary to determine the molecular mechanism linking Rab3B/

Noc2 and other regulators of the basolateral secretory pathway in epithelial cells [36].

Acknowledgments

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