Distinct roles of Rab3B and Rab13 in the polarized transport of apical, basolateral, and tight junctional membrane proteins to the plasma membrane

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Abstract

Regulated transport of proteins to distinct plasma membrane domains is essential for the establishment and maintenance of cell polarity in all eukaryotic cells. The Rab family small G proteins play a crucial role in determining the specificity of vesicular transport pathways. Rab3B and Rab13 localize to tight junction in polarized epithelial cells and cytoplasmic vesicular structures in non-polarized fibroblasts, but their functions are poorly understood. Here we examined their roles in regulating the cell-surface transport of apical p75 neurotrophin receptor (p75NTR), basolateral low-density lipoprotein receptor (LDLR), and tight junctional Claudin-1 using transport assay in non-polarized fibroblasts. Overexpression of Rab3B mutants inhibited the cell-surface transport of LDLR, but not p75NTR and Claudin-1. In contrast, overexpression of Rab13 mutants impaired the transport of Claudin-1, but not LDLR and p75NTR. These results suggest that Rab3B and Rab13 direct the cell-surface transport of LDLR and Claudin-1, respectively, and may contribute to epithelial polarization.

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Polarized vesicular transport is critically important for the correct physiological function not only of polarized epithelial cells but also of non-polarized fibroblasts [1]. The apical and basolateral domains are in contact with the lumen and basement membrane in polarized epithelial tissues. These domains have distinct functional properties and contain unique subsets of proteins. Sorting of proteins into the transport vesicles destined for the apical and basolateral domains occurs both in the trans-Golgi network (TGN) and, after internalization, in endosomes [2,3]. The transmembrane proteins p75 neurotrophin receptor (p75NTR) and low-density lipoprotein receptor (LDLR) have been used extensively as markers for apical and basolateral polarized transport pathways [4–6]. In fibroblasts, the specific transport of a subset of proteins to the leading edge of the plasma membrane (PM) is essential for directed cell locomotion [7]. Polarized vesicular transport is crucial for initiating and/or maintaining PM polarity in all eukaryotic cells.

Tight junctions (TJs) delineate a boundary between apical and basolateral domains and consist of at least three transmembrane proteins, Claudins, Occludin, and junctional adhesion molecules (JAMs) [8]. To date, more than 20 members of the Claudins family have been characterized and the majority of these proteins comprise the backbone of TJ strands. When expressed in the non-polarized fibroblasts, Claudin-1 is transported to the PM and forms TJ-like structures between adjacent cells [9]. The factors regulating Claudin-1 transport have yet to be identified and it is predicted that they will play a crucial role in polarized vesicular transport.

Vesicular transport consists of four steps: formation of transport vesicles, movement towards the target compartment, tethering/docking with the target compartment, and fusion with the target compartment.
Polarized vesicular transport requires the specific pairing of transport vesicles with the target compartment. It has yet to be resolved how the necessary accuracy of this pairing is achieved. The SNAP receptor (SNARE) proteins are central to the process of membrane fusion and encode some aspects of its specificity [10]. However, the first point of contact between a transport vesicle and its target compartment is defined by a poorly understood tethering process. Vesicle tethering complexes consist of several protein subunits that are recruited from the cytosol to the membrane by specific lipid–protein and protein–protein interactions [11]. Accumulating evidences indicate that the GTP-dependent interaction of tethering complexes with the Rab family small G proteins (Rabs) is important for the tethering process.

The Rab family contains more than 60 family members in mammalian cells, and Rab family members coordinate multiple stages of vesicular transport [12–14]. Intracellular compartments in both the exocytic and endocytic pathways contain distinct subsets of Rab family members. Rabs cycle between a GTP-bound active form and GDP-bound inactive form, and function as molecular switches at the site where they are localized. Of the over 60 Rab family members, Rab3B and Rab13 preferentially accumulate at the TJ complex in mammalian epithelial cells [15,16]. However, the roles of these Rabs in polarized vesicular transport have yet to be determined.

Here we have examined the regulatory roles of Rab3B and Rab13 in the transport of apical, basolateral, and TJ membrane proteins in non-polarized baby hamster kidney (BHK) cells, in which apical and basolateral membrane proteins are sorted into different transport vesicles in the TGN [17]. Through the use of p75NTR, LDLR, and Claudin-1 as markers for specific polarized transport pathways, we demonstrate that Rab3B and Rab13 are regulators of the polarized transport of basolateral and TJ membrane proteins in BHK cells.

**Materials and methods**

**Materials and chemicals.** BHK, Caco2, and HeLa cells were obtained from the ATCC (Manassas, VA). Anti-HA (3F10), anti-FLAG (M2), and anti-transfer receptor (TR) (H68.4) antibodies were purchased from Roche (Mannheim, Germany), Sigma (St. Louis, MO), and Zymed (San Francisco, CA), respectively. Recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) has been obtained from the ATCC (Manassas, VA). Anti-HA (3F10), anti-FLAG were pulse-labeled with [35S] EasyTag Express Protein Labeling Mix (Perkin–Elmer, 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 CaCl2 and 0.33 mM MgCl2 (PBS/CM) at 4°C for 60 min and quenched with 50 mM NH4Cl 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 cargo protein was immunoprecipitated with M2 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 cargo proteins were 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 to those of total immunoprecipitated cargo proteins.

**Endocytosis assay.** Endocytosis assay was performed as described previously [22]. Briefly, BHK cells transfected with pCR3.1-TR or pBS-Claudin-1-FLAG were biotinylated with 0.5 mg/ml Sulfo–NHS–SS–Biotin (Pierce, Rockford, IL), quenched at 4°C for 15 min, and incubated at 18°C for the indicated periods of time to induce endocytosis. The remaining cell-surface biotin was stripped with 50 mM MESNA in 100 mM Tris/HCl (pH 8.0) containing 100 mM NaCl and 2.5 mM CaCl2 at 4°C for 30 min, and quenched with 5 mg/ml indo-aceatoamide in PBS/CM at 4°C for 15 min. After lysis with 50 mM Tris/HCl (pH 8.0) containing 1.25% Triton X-100, 0.25% SDS, 150 mM NaCl, 5 mM EDTA, and 10 μg/ml APMSF, an aliquot was taken to determine the total amount of cargo proteins expressed in BHK cells. Biotinylated cargo proteins were then isolated with UltraLink Immobilized NeutrAvidin Plus beads (Pierce, Rockford, IL). The samples were separated on SDS–PAGE, transferred to PVDF membranes, and immunoblotted with M2 or H68.4 antibody. Blots were developed and quantitated using an ECL-Plus kit (Amersham, Piscataway, NJ) and NIH Image 1.62 program. The values for cell-surface biotinylated cargo proteins protected from MESNA treatment were normalized to those of total immunoprecipitated cargo proteins.

**Degradation assay.** BHK cells transfected with pBS-Claudin-1-FLAG were pulse-labeled with [35S] EasyTag Express Protein Labeling Mix for 15 min, chased for 0, 90, 180, and 360 min, and lysed with 50 mM Tris/HCl (pH 8.0) containing 1% Nonidet P-40, 0.25% SDS, 150 mM NaCl, 5 mM EDTA, and 10 μg/ml APMSF. An aliquot was taken to determine the total amount of cargo proteins expressed in BHK cells. Biotinylated cargo proteins were then isolated with UltraLink Immobilized NeutrAvidin Plus beads (Pierce, Rockford, IL). The samples were separated on SDS–PAGE, transferred to PVDF membranes, and immunoblotted with M2 or H68.4 antibody. Blots were developed and quantitated using an ECL-Plus kit (Amersham, Piscataway, NJ) and NIH Image 1.62 program. The values for cell-surface biotinylated cargo proteins protected from MESNA treatment were normalized to those of total cargo proteins expressed in BHK cells.

**Biochemical cell-surface transport assay.** Biochemical cell-surface transport assay was performed as described previously [21]. Briefly, BHK cells were infected with vTF7-3 and transfected with either pBS-LDLR-FLAG, -p75NTR-FLAG, or -Claudin-1-FLAG alone or in combination with pBS-EGFP (mock), -EGFP-Rab3B T36N, -EGFP-Rab3B Q81L, -EGFP-Rab13 T22N, or -EGFP-Rab13 Q67L. Six hours after transfection, cells were pulse-labeled with [35S] EasyTag Express Protein Labeling Mix (Perkin–Elmer, Boston, MA) for 15 min, and quantitated using an ECL-Plus kit (Amersham, Piscataway, NJ) and a NIH Image 1.62 program. The values for cell-surface biotinylated cargo proteins were normalized to those of total immunoprecipitated cargo proteins.

**Morphological cell-surface transport assay.** Morphological cell-surface transport assay was performed as described previously [23]. Briefly, BHK cells were transfected with either pCR259-LDLR-FLAG in combination with pCI-neo-HA (mock), -HA-Rab3B T36N, kit (Stratagene, La Jolla, CA). All cDNAs used in this study were cloned into vectors with N-terminal EGFP- or HA-tags (pBS-EGFP or pCI-neo-HA), a C-terminal FLAG-tag (pBS-FLAG, pCR259-FLAG, or pCI-neo-FLAG), or pCR3.1. The resulting expression plasmids were all sequenced using an automated DNA sequencer 377 (Applied Biosystems, Foster City, CA).
or -HA-Rab3B Q81L, or with pcI-neo-Claudin-1-FLAG in combination with pcI-neo-HA (mock). -HA-Rab13 T22N, or -HA-Rab13 Q67L using FuGENE (Roche, Mannheim, Germany) and incubated at 37°C for 14h. Cells were sequentially incubated at 20°C for 24h to restrict newly synthesized cargo proteins in the TGN. Cargo proteins were transported from the TGN to the PM by incubating at 37°C for 30min. Cells were then fixed in 2% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, blocked with 5% goat serum, and incubated with M2 and 3F10 antibody, and visualized with Alexa 488 anti-mouse IgG and Alexa 594 anti-rat IgG conjugates (Molecular Probe, Eugene, OR). Fluorescent images of the cells were acquired using a Radiance 2000 confocal laser-scanning microscope (Bio-Rad, Hercules, CA).

### Results

In order to examine the polarized transport of basolateral and apical membrane proteins in BHK cells, we utilized a biochemical transport assay based on cell-surface biotinylolation in conjunction with a recombinant T7 vaccinia virus system as previously described [21]. We chose the well-characterized LDLR and p75NTR as basolateral and apical cargo proteins in BHK cells. Consistent with previous reports [4–6], the apparent molecular masses of LDLR and p75NTR shifted from a precursor form found in the endoplasmic reticulum (ER) to a mature form modified by O-linked carbohydrates in the Golgi (Figs. 1A and B, total 0, 40, and 80 min). Cell-surface LDLR and p75NTR increased in a time-dependent manner (Figs. 1A and B, surface 0, 40, and 80 min).

In order to investigate the functions of Rab3B and Rab13 in the transport of LDLR and p75NTR from the ER to the PM, we generated dominant active mutants (Rab3B Q81L and Rab13 Q67L) that are defective in GTP hydrolysis as well as dominant negative mutants (Rab3B T36N and Rab13 T22N) that have lower affinity for GTP than GDP. LDLR or p75NTR was co-transfected with Rab3B or Rab13 mutants into BHK cells and cell-surface transport of LDLR or p75NTR was examined at 40 min. Rab3B T36N and Rab3B Q81L, but not Rab13 T22N and Rab13 Q67L, significantly inhibited the cell-surface transport of LDLR compared to empty vector (mock)-transfected cells (Figs. 1C and E). In contrast to the results seen for LDLR, no effects were seen in the cell-surface transport of p75NTR in Rab3B T36N-, Rab3B Q81L-, Rab13 T22N-, or Rab13 Q67L-transfected cells in comparison with mock-transfected cells (Figs. 1D and E). These results suggest that Rab3B may be involved in directing basolateral transport in BHK cells and that neither Rab3B nor Rab13 affects apical transport.

In contrast to the well-defined basolateral and apical trafficking of LDLR and p75NTR, the intracellular vesicular transport of tight junctional Claudin-1 has been poorly characterized. If Claudin-1 is more efficiently endocytosed from the PM than transported to the PM, it may not be quantitatively biotinylated on the cell-surface in our assay. Therefore, we have first characterized the endocytosis and degradation of Claudin-1 to analyze its polarized transport in BHK cells. Incubation of cells at 18°C blocks the recycling of endocytosed proteins and leads to their accumulation in early/sorting endosomes [24]. The endocytosed fraction of TfR, a well-characterized recycling marker, increases in a linear fashion up to 120 min following 18°C incubation (Figs. 2A and B). In contrast, endocytosis of Claudin-1 was not detected up to 120 min at 18°C (Figs. 2A and B). If the endocytosed Claudin-1, however, was rapidly degraded, its endocytosis may not be detected in our assay. We next examined the degradation of Claudin-1 by pulse-chase analysis, but failed to detect protein degradation up to 6 h following synthesis (Fig. 2C). Thus, Claudin-1 expressed in BHK cells using a recombinant

![Fig. 1. Cell-surface transport of basolateral LDLR and apical p75NTR examined by biochemical transport assay. (A) Time course of cell-surface transport of LDLR. (B) Time course of cell-surface transport of p75NTR. BHK cells expressing LDLR (A) or p75NTR (B) were pulse-labeled and chased for 0, 40, and 80 min. After cell-surface biotinylation, total and surface LDLR (A) or p75NTR (B) was sequentially immunoprecipitated and isolated with avidin beads. Total and surface fractions were separated by SDS-PAGE and analyzed by autoradiography. (C) Effect of Rab3B and Rab13 mutants on cell-surface transport of LDLR. (D) Effect of Rab3B and Rab13 mutants on cell-surface transport of p75NTR. BHK cells co-transfected with LDLR (C) or p75NTR (D) and mock, Rab3B T36N, Rab3B Q81L, Rab13 T22N, or Rab13 Q67L were pulse-labeled and chased for 40 min. Total and surface fractions of LDLR (C) or p75NTR (D) were prepared as described above. (a) mock; (b) Rab3B T36N; (c) Rab3B Q81L; (d) Rab13 T22N; and (e) Rab13 Q67L. (E) Quantitation of effects of Rab3B and Rab13 mutants on cell-surface transport of LDLR and p75NTR. (a)–(e) same as in (C) and (D). The results shown in (A)–(E) are representative of three independent experiments.](image-url)
T7 vaccinia virus system is neither endocytosed nor degraded under our experimental conditions.

We analyzed the cell-surface transport of Claudin-1 using the same biochemical cell-surface transport assay as LDLR and p75NTR. In contrast to LDLR and p75NTR, newly synthesized Claudin-1 exhibited a constant molecular mass of 24 kDa during the 80 min chase period (Fig. 2D, total 0, 40, and 80 min). Its transport to the PM was detected in a time-dependent manner (Fig. 2D, surface 0, 40, and 80 min). The cell-surface transport of Claudin-1 was analyzed at 40 min in BHK cells co-transfected with Claudin-1 and mock, Rab3B T36N, Rab3B Q81L, and Rab13 T22N, or Rab13 Q67L (Figs. 2E and F). Rab13 T22N and Rab13 Q67L significantly inhibited the transport of Claudin-1 to the PM compared to mock-, Rab3B T36N-, and Rab3B Q81L-transfected cells (Figs. 2E and F). To our knowledge, this is the first demonstration that Rab13 regulates the intracellular transport of a TJ membrane protein, Claudin-1.

Rab3B and Rab13 localize to the Golgi region and cytoplasmic vesicular structures in fibroblasts [15,16], suggesting that they potentially direct both early and post-Golgi biosynthetic transport pathways. Since our initial biochemical analysis examined the transport of cargo proteins from the ER to the PM, we elected to...
specifically investigate protein transport from the TGN to the PM through the use of a morphological transport assay. Incubation of cells at 20 °C blocks vesicular transport from the TGN and leads to protein accumulation [25], thus we treated cells at this temperature to synchronize LDLR and p75NTR in the TGN. When LDLR was co-expressed with mock, Rab3B T36N, or Rab3B Q81L in BHK cells at 20 °C. LDLR localized to the perinuclear TGN region (Fig. 3A, mock 0 min, Rab3B T36N 0 min, and Rab3B Q81L 0 min). While LDLR was clearly detected at the PM in the mock-transfected cells incubated at 37 °C for 30 min, it was barely detected at the PM of the Rab3B T36N- or Rab3B Q81L-transfected cells (Fig. 3A, mock 30 min, Rab3B T36N 30 min, and Rab3B Q81L 30 min).

As seen with other cargo proteins, Claudin-1 accumulated at the perinuclear TGN region in BHK cells cotransfected with Claudin-1 and mock, Rab13 T22N, or Rab13 Q67L incubated at 20 °C (Fig. 3B, mock 0 min, Rab13 T22N 0 min, and Rab13 Q67L 0 min). Whereas Claudin-1 was transported to the PM in the mock-cotransfected cells incubated at 37 °C for 30 min, it did not reach the PM in Rab13 T22N- or Rab13 Q67L-cotransfected cells (Fig. 3B, mock 30 min, Rab13 T22N 30 min, and Rab13 Q67L 30 min). Taken together with the biochemical data, these morphological data suggest that Rab3B and Rab13 regulate the polarized transport of LDLR and Claudin-1, respectively, from the TGN to the PM in BHK cells.

Discussion

The segregation of TJ membrane proteins from those destined for basolateral and apical domains could potentially occur in the TGN during biosynthetic transport, in the PM by means of lateral movement and endocytosis, and/or in endosomes after endocytosis in polarized epithelial cells [2,3]. However, the precise mechanism by which this process is regulated is unclear. Here we have identified Rab3B and Rab13, which localize to TJ in epithelial cells [15,16], as specific regulators of polarized transport of basolateral and TJ membrane proteins using biochemical and morphological transport assays in non-polarized cells. From the TGN, newly synthesized PM membrane proteins are sorted into at least two distinct basolateral and apical transport routes to the PM in both polarized and non-polarized cells [17,26,27]. In addition to Rab3B identified in this study, Rab8 and Rab11 have been shown to be regulators of polarized transport of basolateral membrane proteins from the TGN to the PM [28,29]. Adding more complexity, polarized transport to the basolateral PM has been shown to utilize multiple adaptor protein (AP) complexes including AP–1B, AP–3, and AP–4; AP–1B complex contains the polarized epithelial cell specific μ1B isoform [21,30,31]. Further studies are needed to elucidate the molecular mechanisms linking Rab3B and other regulators of the basolateral polarized vesicular transport.

Two general mechanisms could explain the polarized transport of TJ membrane proteins [2,3]. One current model favors the selective retention of TJ proteins. This model predicts that TJ-destined proteins are transported to apical and/or basolateral domains of the PM but subsequently moved laterally and/or endocytosed throughout the membrane and selectively retained in the TJ. Alternatively, TJ membrane proteins could be segregated into distinct transport vesicles in the TGN and delivered to the PM.

Although the current results obtained in non-polarized BHK cells do not address this issue, the existence of a selective Rab13-dependent pathway for Claudin-1 in non-polarized BHK cells would support the selective delivery mechanism of Claudin-1 in polarized epithelial cells. Indeed Zahraoui and co-workers [32] recently reported that stable expression of the Rab13 Q67L mutant in epithelial cells altered the distribution of Claudin-1. Since Claudin-1 is necessary and sufficient for the construction of TJs and TJ-like junctions preventing membrane proteins and lipids from mixing between different PM domains of epithelial cells and fibroblasts, Rab13 would play a crucial role in establishing and maintaining cell polarity. Further studies are necessary for a greater understanding of the physiological functions of Rab13 in non-polarized and polarized cells.

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