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Report

Requirement for Galectin-3 in Apical Protein Sorting

Delphine Delacour,[1] Catharina I. Cramm-Behrens,[1] Hervé Drobecq,[2] Andre Le Bivic,[3] Hassan Y. Naim,[4] and Ralf Jacob[1,*]

[1] Department of Cell Biology and Cell Pathology
University of Marburg
D-35033 Marburg
Germany

[2] Centre National de la Recherche Scientifique
Unité Mixte de Recherche 8525
Institut de Biologie
Institut Pasteur de Lille
59045 Lille
France

[3] Laboratoire de Neurogenese et Morphogenese au cours du Developpement et chez l'Adulte (NMDA)
Institut de Biologie du Developpement de Marseille (IBDM)
Faculte des Sciences de Luminy
13288 Marseille
France

[4] Department of Physiological Chemistry
School of Veterinary Medicine
30559 Hannover
Germany

Summary

The central aspect of epithelial cells is their polarized structure, characterized by two distinct domains of the plasma membrane, the apical and the basolateral membrane. Apical protein sorting requires various signals and different intracellular routes to the cell surface. The first apical targeting motif identified is the membrane anchoring of a polypeptide by glycosylphosphatidyl-inositol (GPI) [1, 2]. A second group of apical signals involves N- and O-glycans [3], which are exposed to the luminal side of the sorting organelle. Sucrase-isomaltase (SI) and lactase-phlorizin hydrolase (LPH), which use separate transport platforms for trafficking, are two model proteins for the study of apical protein sorting. In contrast to LPH, SI associates with sphingolipid/cholesterol-enriched membrane microdomains or "lipid rafts" [4–6]. After exit form the *trans*-Golgi network (TGN), the two proteins travel in distinct vesicle populations, SAVs (SI-associated vesicles) and LAVs (LPH-associated vesicles) [7, 8]. Here, we report the identification of the lectin galectin-3 delivering non-raft-dependent glycoproteins in the lumen of LAVs in a carbohydrate-dependent manner. Depletion of galectin-3 from MDCK cells results in missorting of non-raft-dependent apical membrane proteins to the basolateral cell pole. This suggests a direct role of galectin-3 in apical sorting as a sorting receptor.

*Correspondence: jacob@staff.uni-marburg.de

Results and Discussion

For the identification of protein components in LAVs or SAVs, post-Golgi-enriched gradient fractions were separated by two-dimensional (2D) electrophoresis before or after immunoisolation. The identity of the LAV- or SAV-associated proteins was determined by MALDI-TOF-TOF analysis. Figure 1A shows that post-Golgi-enriched fractions of MDCK cells revealed the presence of the two polypeptides annexin-2 and galectin-3. Following immunoisolation, we could corroborate our previous observations that annexin-2 exclusively accumulates in SAVs [9]. On the other hand, an enrichment of the lectin galectin-3 was identified in immunoisolated LAVs. Furthermore, this lectin did not precipitate with SAVs, and we could confirm the exclusive presence of galectin-3 in LAVs by western blotting and immunoelectron microscopy (Figures 1B and 1C). On the basis of the finding that LAVs are characteristic for delivering the non-raft-associated hydrolase LPH to the apical cell surface [7], we also checked by floating analysis the association of galectin-3 with detergent-resistant membrane microdomains (DRMs), a characteristic biochemical feature of lipid rafts [10]. For comparison, the distribution of LPH, SI, and the raft-marker protein caveolin-1 was monitored by western-blot analysis. Figure 1D demonstrates that significant quantities of SI and caveolin-1 were isolated from the floating DRM fractions of the gradient, whereas galectin-3 and LPH did not float but remained in the bottom fractions. This indicates that galectin-3, like LPH, is not associated with the lipid-based apical-transport platforms.

We then examined the subcellular localization of galectin-3 by confocal fluorescence microscopy. For this, fluorescent fusion proteins of galectin-3 were generated. Expression of these constructs in COS-1 cells allows the analysis of intracellular organelles in a single focal plane by confocal microscopy. Here, Gal3-CFP labeling was predominantly found in some areas of the cytosol and vesicular or tubular structures (Figure 2A). Thus, the staining pattern of Gal3-CFP reflects the intracellular distribution described for endogenous galectin-3 [11]. Gal3-CFP-positive vesicles did not costain with *trans*-Golgi-galactosyltransferase (GT-CFP) or the lysosomal membrane protein LAMP-2, but galectin-3 labeling overlapped in part with rab11-positive recycling endosomes, which are involved in apical membrane trafficking [12]. Gal3-CFP/LPH-YFP- or SI-YFP-expressing COS-1 cells costained galectin-3 with LPH- or SI-carrying vesicles in the perinuclear area (Figure 2B). Following TGN release for 20 min, vesicles labeled by Gal3-CFP and LPH-YFP appeared in the cell periphery. To determine whether costaining between Gal3-CFP and LPH-YFP reflects a close contact between the two partners, we performed fluorescence resonance energy transfer (FRET) analysis. In 30 vesicles analyzed, 20.11% ± 2.35% FRET efficiency was measured for galectin-3 and LPH. However, no FRET (0.1% ± 0.02% efficiency) could be detected between galectin-3 and SI, thus

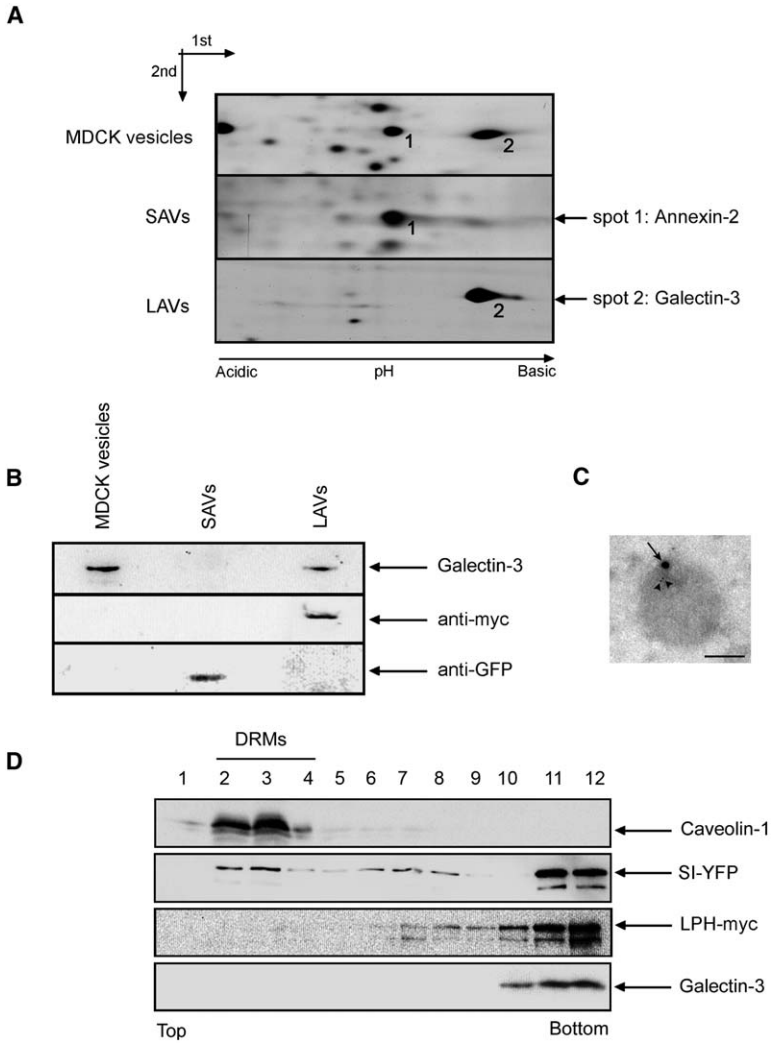

Figure 1. Galectin-3 Is Specifically Associated with LAVs

(A) MDCK, MDCK-LPH$_{myc}$, and MDCK-SI-YFP cells were incubated at 20ºC for 4 hr followed by TGN-release at 37ºC for 20 min. The cell homogenates were separated on a step sucrose gradient, and vesicles of TGN-38 positive fractions were pelleted at 100,000 × g (MDCK vesicles) or immunoprecipitated with mAb anti-GFP (SAVs) or anti-myc (LAVs). The precipitates were separated by 2D electrophoresis and silver-stained. Spots identified by MALDI-TOF-TOF are indicated.

(B) Vesicles were isolated as indicated above, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and stained with pAb anti-galectin-3, mAb anti-myc, or mAb anti-GFP.

(C) Immunogold labeling of immunoisolated LAVs. Galectin-3 (arrow) was labeled with 20 nm gold particles and LPH (arrowhead) by 8 nm gold. The bar represents 100 nm.

(D) Membrane fractions of MDCK-SI-YFP or MDCK-LPH$_{myc}$ cells were solubilized with Triton X-100 at 4ºC, and the detergent extracts were loaded onto a step sucrose gradient. Each gradient was divided into 12 fractions. Following TCA-precipitation, each fraction was subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and immunostained with mAb anti-caveolin-1, anti-GFP or anti-myc, or pAb anti-galectin-3. Floating fractions containing DRMs are indicated.

demonstrating vicinity between galectin-3 and LPH in the nm-range but not with SI.

Next, the intracellular distribution of galectin-3 in polarized MDCK-cells was studied. Figure 2C demonstrates that costaining between endogenous galectin-3 and apically directed LAVs was observed in the subapical area of epithelial cell sheets. Again, only minor amounts of vesicles were costained for SAVs and galectin-3, a result that corroborates our 2D-electrophoresis data and the observations in COS-1 cells. We then determined the exact time interval of galectin-3 accumulation within isolated LAVs or SAVs. Therefore, a cohort of transported material was accumulated in the TGN followed by TGN release at 37ºC for increasing time intervals. Western-blot analysis of immunoisolated vesicles revealed the appearance of galectin-3 in LAVs 5 min after TGN release. A maximum level of this lectin in these vesicles was reached 10 min after TGN release. This level remained high for 35 min until it declined at the end of the experiment (Figure 3A). For SAVs, no significant amounts of galectin 3 could be detected, indicating that galectin-3 accumulates in the non-raft-dependent apical route after exit of the TGN. Next, we wanted to identify whether galectin-3 localizes on the cytosolic or the luminal side of vesicular carriers. Therefore, LAVs were lysed or not lysed with 1% Triton X-100, followed by proteinase K treatment and western-blot analysis with pAb anti-galectin-3. As depicted in Figure 3B, galectin-3 isolated by vesicle precipitation was resistant to proteinase K digestion unless LAVs were solubilized with Triton X-100. This demonstrates that galectin-3 accumulates on the luminal side of post-Golgi vesicles.

Another question based on the FRET experiments in COS-1 cells was whether in MDCK cells galectin-3 and LPH directly associate with each other in the vesicle lumen. For this purpose, LPH or SI was immunoprecipitated from MDCK cell lysates, and this was followed by immunoblot analysis of the coprecipitated galectin-3 in the presence or absence of glucose, galactose, or lactose (Figure 3C). In the control experiment and in the presence of glucose, galectin-3 was isolated in association with LPH. Galactose and lactose on the other hand inhibited this association, thus suggesting that in contrast to glucose, these two sugars compete with the binding of galectin-3 for LPH. Because the disaccharide lactose comprises glucose as well as galactose, our observations point to a galactose-dependent interaction of galectin-3 with LPH. In conjunction with the FRET data in COS-1 cells, no coprecipitation of galectin-3 with SI could be detected in MDCK cells.

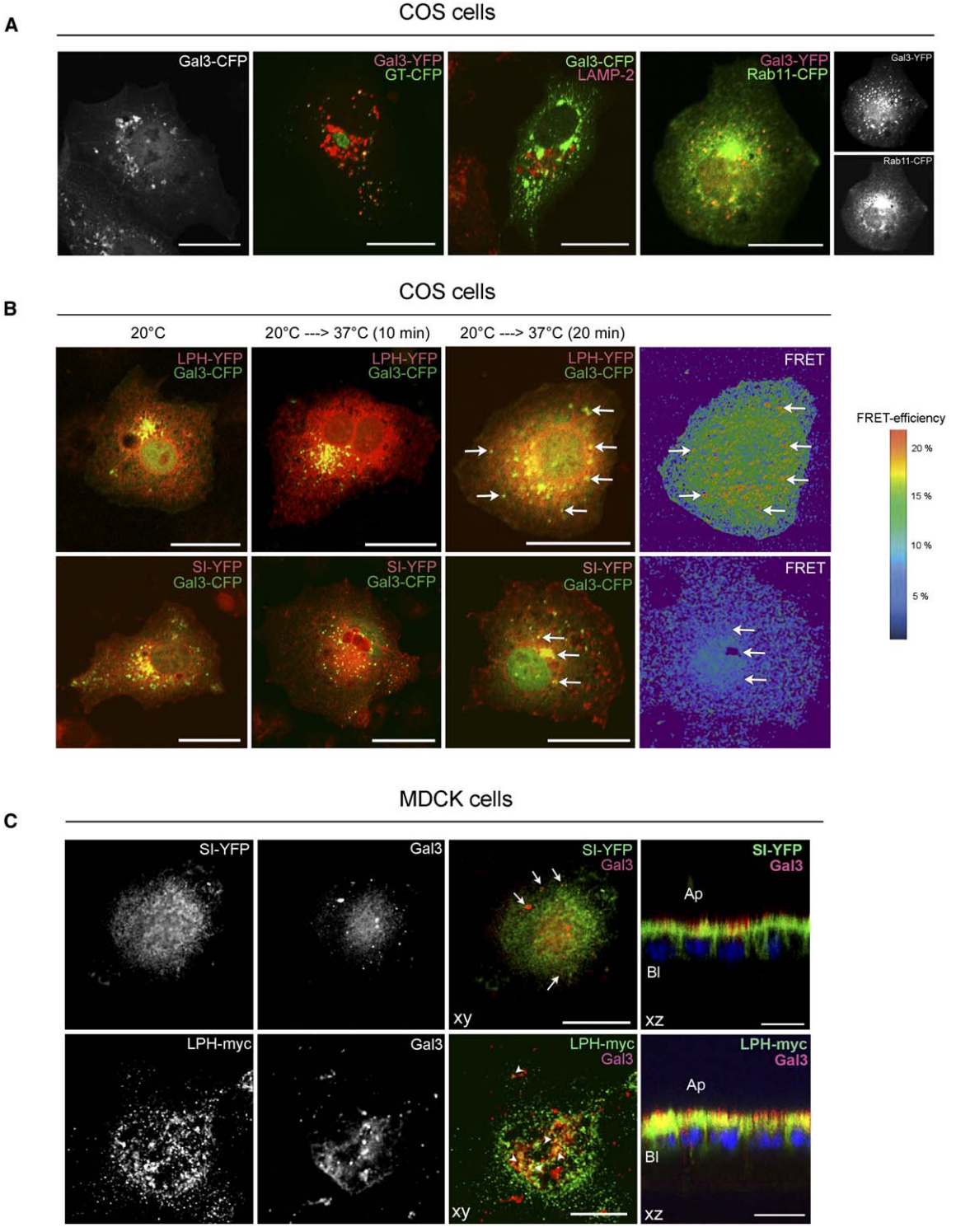

Figure 2. Galectin-3 Is Colocalized with LPH in COS-1 and MDCK Cells

(A) COS-1 cells were transfected with Gal3-CFP or combinations of Gal3-CFP/-YFP and GT-CFP or rab11-CFP. Forty-eights hours after transfection, the cells were fixed, immunostained with anti-LAMP-2/anti-mouse Alexa 633 when indicated, and analyzed by confocal microscopy.

(B) COS-1 cells transfected with combinations of LPH-YFP/Gal3-CFP or SI-YFP/Gal3-CFP were fixed after accumulation of the transported material in the TGN at 20ºC or subsequent TGN release at 37ºC for 10 or 20 min. The corresponding FRET efficiency patterns of cells analyzed 20 min after TGN release are depicted on the right. Vesicular structures indicated by arrows showed a FRET efficiency of 15%–20% between Gal3-CFP and LPH-YFP. For SI-YFP, no significant FRET efficiency could be detected in vesicles with Gal3-CFP as depicted by arrows.

(C) Endogenous galectin-3 of fixed filter-grown MDCK-LPH_{myc} or MDCK-SI-YFP cells was immunostained with pAb anti-galectin-3 and anti-rabbit Alexa 633. For LPH_{myc}-staining, mAb anti-myc in combination with anti-mouse Alexa 488 were applied. Cells were scanned in the

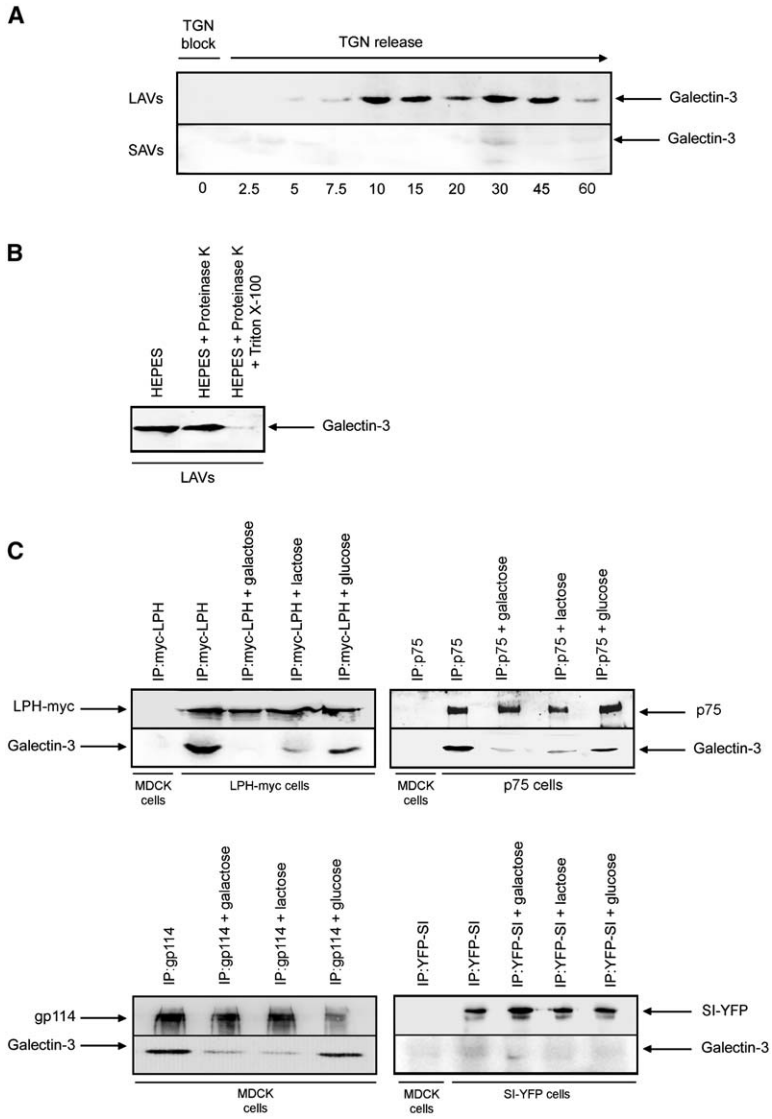

Figure 3. Galectin-3 Interacts with Glycoproteins in the Lumen of LAVs

(A) For a TGN accumulation of newly synthesized material, MDCK-LPH$_{myc}$ and MDCK-SI-YFP cells were incubated at 20°C for 4 hr followed by TGN release at 37°C for the indicated time points. The galectin-3 content in immunoisolated LAVs or SAVs was detected by immunoblot.

(B) LAVs were treated or not treated with proteinase K in the presence or absence of Triton X-100. The samples were loaded on SDS-PAGE and processed for immunoblot with pAb anti-galectin-3.

(C) The coprecipitation efficiency of galectin-3 with LPH, SI, p75, or gp114 was assessed by immunoprecipitation (IP) of LPH$_{myc}$, SI-YFP, p75, or gp114 from lysates of MDCK-LPH$_{myc}$, MDCK-SI-YFP, MDCK-p75, or MDCK cells. Lysates from MDCK cells were used as negative control. Precipitation was performed in the presence or absence of 0.3 M galactose, 0.1 M lactose, or 0.3 M glucose solutions followed by western-blot analysis of the immunoprecipitates.

On the basis of the successful coprecipitation of galectin-3 with LPH, we tested the general character of galectin-3 as a receptor in apical protein sorting. Here, we used two other apical non-raft-associated membrane proteins, the neurotrophin receptor (p75) [13] and gp114, an endogenous apical glycoprotein of MDCK cells [14, 15]. At first, p75 or gp114 was precipitated from cell lysates, and galectin-3 was detected on immunoblots as indicated above. Figure 3C depicts coprecipitation of galectin-3 with p75 and gp114 in the presence of glucose as well as under control conditions. In analogy to LPH, a significant decline in the interaction of p75 or gp114 with the lectin could be observed in the presence of galactose or lactose. As a conclusion, galectin-3 binds to the apical glycoproteins LPH, p75, and gp114 in a galactose-dependent fashion, whereas raft-associated SI travels independently of this lectin to the cell surface.

We wondered whether apical sorting in MDCK cells depends on the presence of galectin-3 and studied a putative role of this lectin in the sorting process by RNA interference with two specific siRNA duplexes. The level of galectin-3 was reduced to less than 20% according to western-blot analysis 48 hr after transfection (Figure 4A). Moreover, 72 hr after transfection of a combination of both siRNA duplexes, an even more dramatic decrease of galectin-3 expression could be observed by western-blot and quantitative RT-PCR (Figures 4A and 4B). We employed these conditions for galectin-3 knockdown to monitor the polarized delivery of LPH, p75, gp114, or SI in polarized MDCK cells by confocal imaging. Interestingly, galectin-3 depletion caused a shift of LPH, p75, and gp114 from the apical membrane to a predominant quantity in the basolateral plasma membrane (Figure 4C). Apart from their appearance in the basolateral membrane, LPH and gp114 also

subapical area (xy) or in x/z view (xz). Vesicular carriers exclusively labeled with galectin-3 (white arrows) are depicted. Vesicular structures costained by galectin-3 and LPHmyc are indicated by arrowheads. Hoechst nuclear counterstaining is indicated in blue. Ap denotes apical; Bl denotes basolateral. The scale bars represent 10 μm.

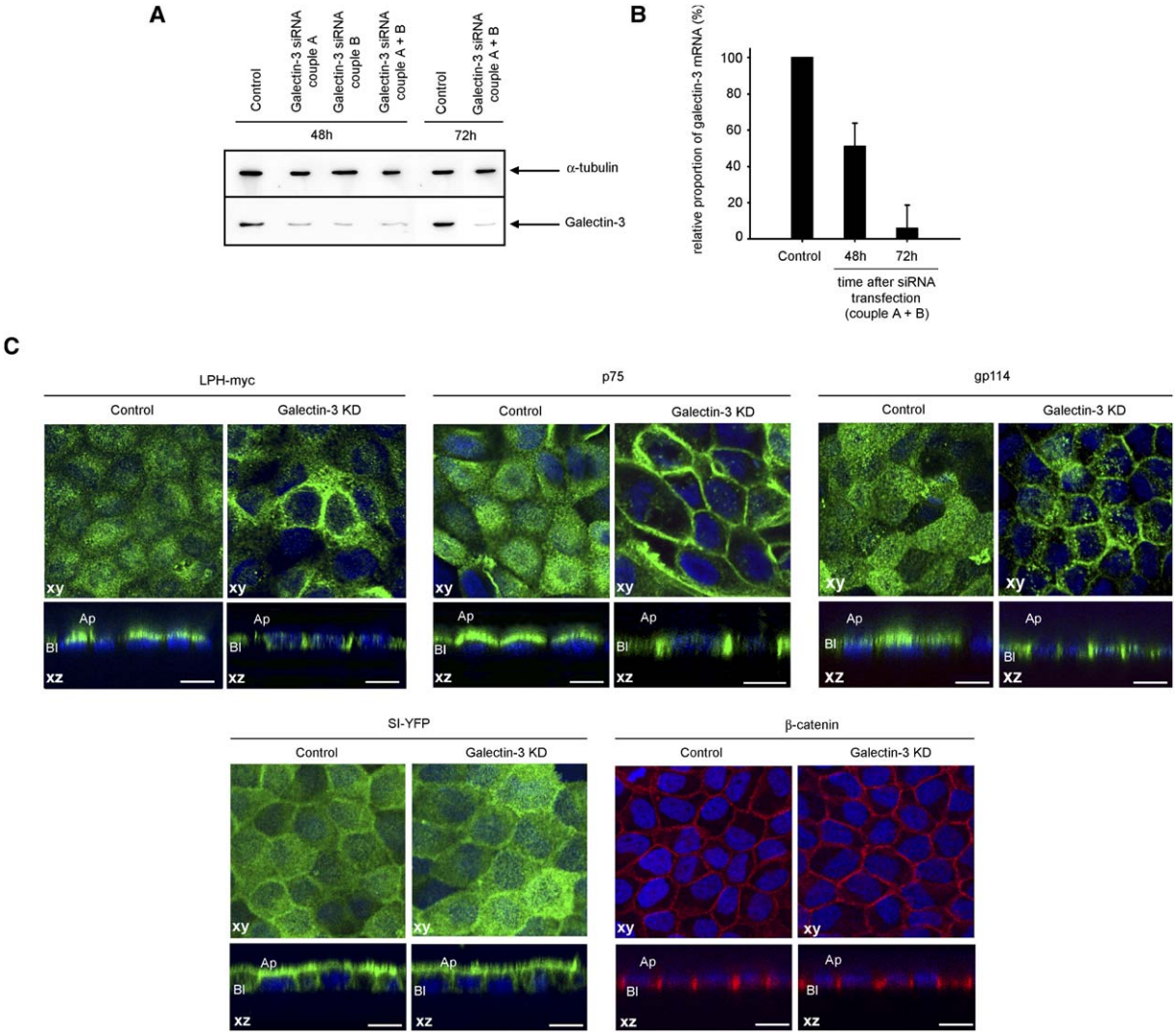

Figure 4. Disturbed Apical Targeting of Non-Raft-Associated Proteins after Galectin-3 Knockdown

(A and B) MDCK cells were transfected with galectin-3-specific siRNA-duplexes or nonspecific luciferase siRNA as control. Forty-eight or seventy-two hours after transfection, the galectin-3 knockdown (KD) was analyzed by western blot with pAb anti-galectin-3 and mAb anti-α-tubulin as internal control (A) or quantitative RT-PCR (B).

(C) Filter-grown MDCK-LPH$_{myc}$, MDCK-SI-YFP, MDCK-p75, or MDCK cells were transiently transfected with galectin-3 or luciferase siRNA as control. Seventy-two hours after transfection, the cells were fixed and immunostained with mAb anti-LPH, anti-p75, anti-gp114, or anti-β-catenin followed by anti-mouse Alexa 488 or 633 as secondary antibody. Monolayers of cells were studied in xy or xz direction as indicated. The cell nuclei are depicted in blue. Ap denotes apical; Bl denotes basolateral. The scale bars represent 10 μm.

accumulated intracellularly after galectin-3 depletion; this accumulation might be due to an inefficient entry into alternative transport routes. Basolateral delivery of p75 can be explained by sorting signals in its cytosolic domain [16]. However, apical SI was correctly sorted in the presence or absence of galectin-3. No influence of galectin-3 depletion could be observed on the basolateral localization of β-catenin. In a second approach, the polypeptides were precipitated from the apical or basolateral surface of biosynthetically labeled MDCK cells. Again, apical sorting of the non-raft-associated proteins was significantly perturbed by galectin-3 knockdown to an average apical to basolateral ratio of about 52:48 (LPH) or 38:62 (p75), whereas no effect on the polarized targeting of raft-associated SI molecules could be detected (Figures 5A and 5B).

Taken together, both techniques we employed to determine an influence of galectin-3 knockdown on the sorting of non-raft-associated apical glycoproteins have shown that they are missorted to the basolateral membrane in the absence of galectin-3. This effect on polarized trafficking supports the notion that galectin-3 is required for the high fidelity of non-raft-dependent apical sorting in MDCK cells. Moreover, in view of the observations that galectin-3 accumulates in post-Golgi carriers and interacts with LPH, p75, and gp114, this lectin fulfils the requirements of an apical sorting receptor.

Already ten years ago, different groups came to the conclusion that sugar binding receptors might be involved in apical transport by direct interaction with sugar moieties of glycoproteins [4, 13, 17]. A candidate lectin, which was first identified in exocytic carriers,

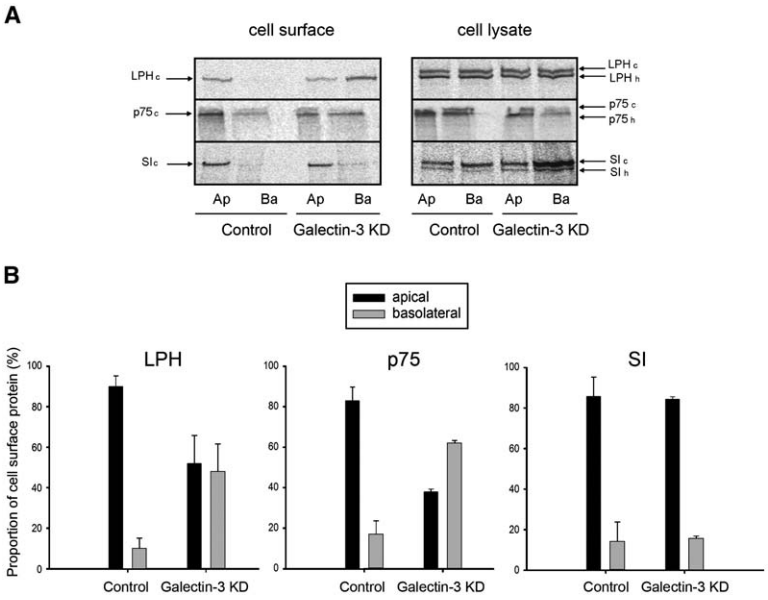

Figure 5. Galectin-3 Depletion Increases Basolateral Delivery of Non-Raft-Associated Apical Proteins

(A) MDCK-LPH_{myc}, MDCK-SI-YFP, or MDCK-p75 cells were grown on transmembrane filters and biosynthetically labeled with [^{35}S]methionine for 5 hr in the presence or absence of RNAi. Cell-surface immunoprecipitation of LPH_{myc}, SI-YFP, or p75 from the apical or basolateral membranes was performed with the corresponding antibodies. LPH_{myc}, SI-YFP, and p75 were precipitated from the remaining cell lysates for comparison. The immunoprecipitates were subjected to SDS-PAGE followed by phosphoimaging analysis. High-mannose (LPH_h, SI_h) or complex glycosylated forms (LPH_c, SI_c) of LPH and SI are indicated. Ap denotes apical; Ba denotes basolateral.

(B) The quantification (± standard errors) of three independent experiments is depicted.

was VIP36 [18]. However, this localization was caused by overexpression of VIP36, whereas the endogeneous pool accumulates in the *cis*-Golgi [19]. Thus, direct involvement of this lectin in apical sorting at the *trans* side of the Golgi appears to be unlikely. Recently, galectin-4 was identified in post-Golgi carriers [20]. Depletion of this lectin induces mistargeting of apical proteins. However, no clear interaction of galectin-4 with glycoproteins could be observed. Instead, it associates with sulfatides, which are highly enriched in lipid rafts. This suggests that galectin-4 is engaged in the process of raft formation or stabilization instead of sorting glycosylated polypeptides.

It is well known that galectins provide the ability to form cross-linked lattices with specific multivalent carbohydrates. Galectin-3 differs from other galectins in having one carbohydrate recognition domain and an additional dimerization domain [21]. This dimer formation enables galectin-3 to form multimeric complexes with the extracellular matrix protein hensin in epithelial cells [22]. How galectin-3 is secreted via a "nonclassical" secretory pathway is unclear [11]. It might be translocated through the plasma membrane and taken up by endocytosis or directly transported into exocytic carriers. Because we observed accumulation of galectin-3 in post-Golgi carriers 10 min after TGN release, this interaction most likely intensifies in an endosomal compartment. Evidence for the involvement of endosomes in exocytic polarized trafficking comes from studies on the polymeric IgA receptor [23], the VSVG protein [24], the transferring receptor [25], the asialoglycoprotein receptor [26], and E-cadherin [27]. In addition, the observation that apical and basolateral cargo pass a population of recycling endosomes further suggests that these endosomes serve not merely as an intermediate compartment between TGN and plasma membrane but also as a common site for polarized sorting [24].

Apical sorting of raft-associated as well as of non-raft-associated polypeptides involves the presence of N- or O-glycans [3]. Because galectins recognize β-galactose, which is present in N- and O-glycans, they represent putative candidates for binding receptors. Furthermore, our data demonstrate that galectin-3 depletion disturbs apical segregation of the non-raft-associated proteins LPH and p75, whereas polarized trafficking of SI remains unaffected. Two conclusions can be drawn from this observation. First, galectin-3 binding underlies one variant of carbohydrate-dependent apical sorting. Second, galectin-3 is not involved in protein translocation to the cell surface but represents a central element in the apical sorting process of non-raft-associated glycoproteins.

Moreover, our data indicate that the arrangement of clusters consisting of glycoproteins and galectin-3 plays a central role in apical sorting. This observation of networks between apical proteins corresponds to a model that is based on the oligomerization of polypeptides into clusters that drive apical sorting and assist in the generation of apical vesicles [28]. Thus, it is tempting to conclude that galectin-3 fulfils the role of a receptor that recognizes apical glycoproteins, drives the oligomerization process, and stabilizes the network for vesicle formation.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/4/408/DC1/.

Acknowledgments

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