Althought epithelial cells are known to exhibit a polarized distribution of membrane components, the pathways responsible for delivering membrane proteins to their appropriate domains remain unclear. Using an optimized approach to three-dimensional live cell imaging, we have visualized the transport of newly synthesized apical and basolateral membrane proteins in fully polarized filter-grown Madin–Darby canine kidney cells. We performed a detailed quantitative kinetic analysis of trans-Golgi network (TGN) exit, passage through transport intermediates, and arrival at the plasma membrane using cyan/yellow fluorescent protein–tagged glycosylphosphatidylinositol-anchored protein and vesicular stomatitis virus glycoprotein as apical and basolateral reporters, respectively. For both pathways, exit from the TGN was rate limiting. Furthermore, apical and basolateral proteins were targeted directly to their respective membranes, resolving current confusion as to whether sorting occurs on the secretory pathway or only after endocytosis. However, a transcytotic protein did reach the apical surface after a prior appearance basolaterally. Finally, newly synthesized proteins appeared to be delivered to the entire lateral or apical surface, suggesting—contrary to expectations—that there is not a restricted site for vesicle docking or fusion adjacent to the junctional complex.

Introduction

Epithelial cells are characterized by the asymmetric distribution of plasma membrane proteins to form apical and basolateral domains. Many membrane proteins are thought to reach their respective domains by intracellular sorting events mediated by distinctive targeting elements (Matter and Mellman, 1994; Mostov et al., 2003; Rodriguez-Boulan et al., 2005). For example, many apical proteins, including those with glycosylphosphatidylinositol (GPI) membrane anchors, are sorted into clustered lipid rafts and traffic to the apical surface (Lisanti et al., 1989; Paladino et al., 2004; Schuck and Simons, 2004). In contrast, many basolateral proteins, such as low density lipoprotein receptor and vesicular stomatitis virus glycoprotein (VSVG), are sorted as a result of the recognition of signals localized in the cytoplasmic tail (Matter and Mellman, 1994). The protein components responsible for at least some basolateral sorting events, such as the AP–1B clathrin adaptor complex, are beginning to be discovered (Folsch et al., 1999; Simmen et al., 2002; Folsch, 2005). However, the actual pathways of polarized membrane protein trafficking, including the identity or location of sorting sites, the itinerary taken by individual proteins, and the spatial distribution of plasma membrane delivery sites, remain largely unresolved.

Polarized membrane protein sorting on the secretory pathway has long been presumed to occur at the TGN (Griffiths et al., 1985; Keller et al., 2001), although the closely associated recycling endosomes also appear to play an important role (Gan et al., 2002; Ang et al., 2003, 2004; Folsch et al., 2003). Recently, however, questions have emerged concerning the extent to which polarized sorting even occurs intracellularly (Nelson and Rodriguez-Boulan, 2004). In one study, the transport of polarized membrane proteins was assayed in MDCK cells whose apical or basolateral surface had been selectively inactivated by tannic acid fixation. Striking images suggested that a well-characterized reporter of apical proteins, a GPI-anchored GFP (GPI-GL-YFP), was first inserted into the basolateral surface and transcytosed across the cell to reach the apical domain (Polishchuk et al., 2004). Although inconsistent with some classic biochemical studies (Matlin and Simons, 1984; Lisanti et al., 1989; Paladino et al., 2004; Schuck and Simons, 2004). In contrast, many basolateral proteins, such as low density lipoprotein receptor and vesicular stomatitis virus glycoprotein (VSVG), are sorted as a result of the recognition of signals localized in the cytoplasmic tail (Matter and Mellman, 1994). The protein components responsible for at least some basolateral sorting events, such as the AP–1B clathrin adaptor complex, are beginning to be discovered (Folsch et al., 1999; Simmen et al., 2002; Folsch, 2005). However, the actual pathways of polarized membrane protein trafficking, including the identity or location of sorting sites, the itinerary taken by individual proteins, and the spatial distribution of plasma membrane delivery sites, remain largely unresolved.

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et al., 1990; Matter et al., 1990), this indirect pathway is reminiscent of what is thought to occur for many apical proteins in hepatocytes (Bartles et al., 1987). In MDCK cells, however, apical proteins bearing conventional membrane anchors were suggested to take the classic direct route (Lipardi et al., 2000; Polishchuk et al., 2004; Schuck and Simons, 2004; Anderson et al., 2005).

It remains similarly unclear whether membrane proteins insert at specific sites on the apical or basolateral surfaces. Junctional complexes have been suggested to define a spatially restricted insertion site for basolateral traffic based on the distribution of tethering complexes (Sec6–Sec8) involved in vesicle docking at the basolateral membrane (Grindstaff et al., 1998). However, the only direct evidence from live cell imaging experiments thus far is that basolateral proteins do not first appear at the adherent surface of coverslip-grown MDCK cells (Kreitzer et al., 2003). Some transport vesicles were seen to enter the junctional complex region, but definitive evidence for vesicle fusion to the plasma membrane or a quantitative assessment of vesicle traffic to this domain of the lateral surface was not achieved. Such considerations have emphasized the need for visualizing the biosynthetic pathway of membrane proteins in filter-grown and fully polarized epithelial cells. Although this approach has thus far proved challenging because of the cells’ z-axis height (~10 μm; Keller et al., 2001), we have now been able to image and quantify the features of secretory membrane traffic in live, unperturbed MDCK cells.

Results

By combining laser scanning confocal microscopy with rapid data acquisition and computation, we were able to visualize and quantitate polarized membrane protein traffic in filter-grown MDCK cells for a period of ~30 min. As a test, we first studied the intracellular sorting and insertion of a basolateral membrane protein, ts045 VSVG-YFP (Keller et al., 2001). VSVG-YFP was quantified from the 3D renderings. Global fit (solid lines) according to model 2 (Fig. 2), with $k_3$ associated region if VSVG-YFP was first inserted at this site, the dashed line is the predicted fluorescence in the junction- lateral domain that is apical to the junctional complex. The fluorescence in the apical-most segment (yellow dots) was lowest, but this region also contains a portion of the lateral membrane was determined at each time point from the 3D renderings. The Xs demarcate the approximate location of the tight junction. Each data point represents the percentage of VSVG-YFP present in the section of the diagrammed membrane and was color coded accordingly. The fluorescence in the apical-most segment (yellow dots) was lowest, but this region also contains a portion of the lateral domain that is apical to the junctional complex. The dashed line is the predicted fluorescence in the junction-associated region if VSVG-YFP was first inserted at this site, according to model 2 (Fig. 2), with $k_3 = 0.12 \text{ min}^{-1}$, the fastest sorting rate cited in polarized MDCK cells (Sheff et al., 2002). The yellow dots, which correspond to a portion of the apical region, deviate significantly from this prediction. Open cyan circles represent the amount of VSVG-YFP fluorescence at each time point that could not be accounted for either at the lateral membrane or in the TGN. Solid cyan line represents the fit to model 1 (Fig. 2). The dimensions of the cell image shown (x, y, and z) are ~12, 14, and 8 μm.

Figure 1. 3D time-lapse confocal imaging and quantification of the transport of VSVG-YFP to the basolateral surface in single fully polarized MDCK cells. (A) Illustration of the MDCK cell domains and orientation for 3D live cell imaging. MDCK cells were grown on polycarbonate filters and transfected as described in Materials and methods. A piece of filter was excised and inverted on a coverslip in a medium-filled dish. The dish was placed in a temperature-controlled chamber mounted on a laser scanning confocal microscope. (B) 3D transport of newly synthesized VSVG-YFP from the TGN to the basolateral membrane. Transport from the TGN [marked by TGN38-CFP, green) was monitored at 31°C, with complete z-axis stacks taken every minute. Individual stacks were assembled as videos. Here, the same cell is viewed from two different orientations (looking down from the apical side and from the lateral side), as illustrated by the 3D cell diagram, at three time points of chase: 0, 17, and 34 min. VSVG-YFP (red) at 0 min largely colocalized with the TGN marker and gradually exited from the TGN to appear at the lateral surface (Video 1, available at http://www.jcb.org/cgi/content/ full/jcb.200512012/DC1). (C) Kinetics of VSVG-YFP transport from the TGN to the lateral surface (same cell as in B.). The presence of VSVG-YFP in the TGN (TGN38-CFP–positive volumes, green dots) or at the lateral surface (including ~1.5 μm of adjacent cytoplasmic volume, red dots) was quantified from the 3D renderings. Global fit (solid lines) of the two-step pathway (Fig. 2, model 1) to the two curves yielded $k_1 = 0.02 \text{ min}^{-1}$, $k_2 = 0.11 \text{ min}^{-1}$, and $R^2 = 0.9857$. The data shown are collected from a single cell, and the rate constants are representative of measurements from 12 different cells as summarized in Fig. 2. (D) Spatial distribution of VSVG-YFP (same cell as in B and C). The distribution of VSVG-YFP on three equal vertical sections of the lateral membrane was determined at each time point from the 3D renderings. The Xs demarcate the approximate location of the tight junction. Each data point represents the percentage of VSVG-YFP present in the section of the diagrammed membrane and was color coded accordingly. The fluorescence in the apical-most segment (yellow dots) was lowest, but this region also contains a portion of the lateral domain that is apical to the junctional complex. The dashed line is the predicted fluorescence in the junction-associated region if VSVG-YFP was first inserted at this site, according to model 2 (Fig. 2), with $k_3 = 0.12 \text{ min}^{-1}$, the fastest sorting rate cited in polarized MDCK cells (Sheff et al., 2002). The yellow dots, which correspond to a portion of the apical region, deviate significantly from this prediction. Open cyan circles represent the amount of VSVG-YFP fluorescence at each time point that could not be accounted for either at the lateral membrane or in the TGN. Solid cyan line represents the fit to model 1 (Fig. 2). The dimensions of the cell image shown (x, y, and z) are ~12, 14, and 8 μm.
was transfected into MDCK cells expressing CFP-tagged TGN38 (TGN38-CFP; Keller et al., 2001) as markers for the TGN. VSVG-YFP was accumulated in the ER by incubation at 40°C overnight and was released and chased to the TGN by a 1-h incubation at 20°C (Griffiths et al., 1985). Synchronous cell surface transport was imaged by transferring the filters to the permissive temperature (31°C) on a temperature-controlled stage, mounted as shown in Fig. 1 A. The preparations remained viable for >12 h.

YFP and CFP fluorescent signals in single MDCK cells were collected in multiple z stacks over a 30–40-min time course and combined to produce three-dimensional (3D) videos (Video 1; available at http://www.jcb.org/cgi/content/full/jcb.200512012/DC1). 3D stacks were acquired at 1-min intervals to minimize photobleaching. At the start of the 31°C chase, most of the VSVG-YFP (Fig. 1 B, red) colocalized with the lateral membrane (Fig. 1 C). TGN exit could be approximated by a linear decay curve, suggesting that the exit rate was independent of VSVG concentration. The rate of VSVG appearance at the lateral membrane increased after a brief lag (Fig. 1 C, red), suggesting at least one intermediate step before VSVG’s plasma membrane arrival. To compute the kinetics of arrival at the lateral domain, we constructed a simple three-state model that described the localization of VSVG in its three definable compartments: TGN, the lateral plasma membrane, and transport carriers between the TGN and plasma membrane, the latter representing cytoplasmic volume that was not associated with the TGN or the lateral membrane (Fig. 1 D, open circles; the line is nonlinear because of its multicomponent nature). As illustrated in Fig. 2, two forward rate constants were used to fit the data (model 1): k1, the rate of exit from the TGN, and k2, the rate of exit from the transit compartment. k1 and k2 were determined by measuring the rate of exit from the TGN and arrival at the lateral domain, which was defined to include the cell margin and an intracellular volume ∼1.5 μm from the cell margin. Thus, k2 describes the duration of VSVG in the transit compartment; i.e., the time required to transfer from the TGN to the plasma membrane but not docking and fusion to the membrane. The kinetic analysis indicated that exit from the TGN was rate limiting (k1 = 0.019 min−1), with 1.9% of the VSVG-YFP exiting the TGN per minute, which is approximately equal to the first-order rate measured using a linear fit to the TGN exit curve. k2 was ∼10 times faster than TGN exit (Fig. 2; n = 12 cells), indicating that once in transit, VSVG was rapidly transferred to the lateral domain. The Golgi exit rate was comparable with that measured in nonpolarized COS cells by Hirschberg et al. (1998).

Previous studies have suggested that basolateral traffic is initially targeted to a site near the junctional complex and possibly even subjected to sorting from apical proteins upon delivery (Louvard, 1980; Grindstaff et al., 1998; Kreitzer et al., 2003). Therefore, we analyzed our dataset to determine whether there was evidence for a transient accumulation of VSVG-YFP at the junction-associated region or any other subdomain of the lateral membrane. We modified the kinetic model to include a new rate k3 that would describe possible processes restricted to the junctional complex region (Fig. 2, model 2). Such events might include transport vesicle docking, vesicle fusion, sorting of apical versus basolateral proteins, or exit from the junction-associated

![Model 1](https://example.com/model1.png)

**Model 1**

| TGN | $\rightarrow$ | Intermediate Transit | $\rightarrow$ | Plasma Membrane |

<table>
<thead>
<tr>
<th>VSVG-YFP (n = 12)</th>
<th>GPI-GL-YFP (n = 9)</th>
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<tbody>
<tr>
<td>Exit rate from TGN; $k_1$ (min$^{-1}$)$^a$</td>
<td>0.019 ± 0.003</td>
</tr>
<tr>
<td>Rate of delivery; $k_2$ (min$^{-1}$)$^a$</td>
<td>0.23 ± 0.06</td>
</tr>
</tbody>
</table>

$^a$Mean ± SEM.

**Model 2**

| TGN | $\rightarrow$ | Intermediate Transit | $\rightarrow$ | Junction-Associated Region | $\rightarrow$ | Plasma Membrane |

**Model 3**

| TGN | $\rightarrow$ | Intermediate Transit | $\rightarrow$ | Basolateral | $\rightarrow$ | Apical Membrane |

Figure 2. The rate of exit from the TGN and delivery to the destined plasma membrane measured by live cell imaging.
region (via diffusion or vesicular transport). At steady state, the amount of VSVG that accumulated in the junction-associated region would thus depend exclusively on $k_2$ and $k_3$. If this junctional region was the unique site to which post-TGN carriers were targeted, we might expect a transient accumulation of VSVG in that segment of the lateral domain.

To test this hypothesis, we measured the appearance of VSVG-YFP in three equal vertical, circumferential segments of the lateral membrane. The most apical section contained the junctional complex, as confirmed by staining with the tight junction marker ZO-1 (unpublished data). Contrary to expectations, however, VSVG-YFP did not appear preferentially in this upper segment. Fluorescence instead appeared in the bottom two lateral segments at equivalent rates (Fig. 1 D, red and blue dots). The same trend was observed for the apical-most segment (Fig. 1 D, yellow dots), although the amount of signal was reduced because this segment likely also contained a portion of the apical domain (above the junctional complex). Nevertheless, if all of the VSVG represented by the yellow dots (Fig. 1 D) reflected accumulation in the junction-associated region, the quantity was far less than predicted for preferential targeting (Fig. 1 D, dashed line). To fit the actual data to model 2 (Fig. 2), $k_3$ would need to be $\geq 1.6 \text{ min}^{-1}$ (corresponding to $t_{1/2}$ of $\sim 0.6 \text{ min}$). $k_2$ is unlikely to be that fast because our previous study using glass-grown cells found that the docking of post-TGN VSVG-containing transport vesicles is considerably slower than $1.6 \text{ min}^{-1}$ ($k = 1.1 \text{ min}^{-1}$; $t_{1/2}$ of 39 s; Toomre et al., 2000). Thus, despite the possible restriction of vesicle-tethering complexes close to the junctional complex (Grinstead et al., 1998), VSVG-YFP is delivered to a broad area of the lateral domain or, alternatively, the docking and fusion of vesicles at a restricted region is far faster than observed for other closely related events.

Next, we studied the intracellular transport of a newly synthesized apical membrane protein, GPI-GL-YFP, which is the same construct also used by Polishchuk et al. (2004). Newly synthesized GPI-GL-YFP was accumulated in the TGN by a 1-h temperature block at 20°C (Polishchuk et al., 2004), released at 31°C, and imaged at 1-min intervals in three dimensions (Videos 2 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200512012/DC1). GPI-GL-YFP (Fig. 3 A, red) migrated out from the TGN region (Fig. 3 A, green) and appeared at the apical surface, which is defined as red pixels that were within $\sim 2 \mu\text{m}$ of the apical cell margin (Fig. 3 A; 0, 9, and 18 min of chase; see Image and data processing). Transport was largely complete within 20 min. Importantly, no GPI-GL-YFP was observed visually at the lateral membrane at any time point. There was, however, an intermediate time when GPI-GL-YFP appeared as a region or compartment juxtaposed apically to the TGN (Videos 4 [~12 min] and 5). This region could correspond to a subdomain of the TGN or a subapical compartment containing presorted GPI-anchored molecules (Hannan et al., 1993). In any event, the video data strongly suggested a simple model in which GPI-GL-YFP migrates upward out of the TGN through an intermediate subapical domain and reaches the apical surface without ever appearing basolaterally.

We then quantified the kinetics of GPI-GL-YFP transport from the TGN to the apical regions. As shown in Fig. 3 B, the amount of GPI-GL-YFP in the TGN region decreased in accordance with the increase in the amount of GPI-GL-YFP at the apical membrane (green and red dots, respectively). The simplest three-state model (Fig. 2, model 1) accurately represented the
transport data (Fig. 3 B, solid green and red lines). Again, the rate-limiting step along the apical pathway was exit from the TGN. At 0.023 min\(^{-1}\) (2.3% GPI-GL-YFP transferred out of the TGN per minute), this rate was similar to that derived for the TGN exit of VSVG-YFP (Fig. 2; \(n = 9\) cells).

To test quantitatively whether GPI-GL-YFP was delivered to the junctional region or the lateral domain before appearing apically (Fig. 3 C, inset; models 2 and 3), we determined the distribution of GPI-GL-YFP at each time point in the reconstructed 3D datasets. We first compared the appearance of GPI-GL-YFP in the apical membrane (choosing a segment \(\sim 2.5 \mu m\) from the cell lateral margin to avoid any contribution of the lateral domain; Fig. 3 C, inset; red) versus a perimeter segment including the edge of the apical membrane, the junctional region, and the apical-most segment of the lateral membrane (Fig. 3 C, inset; yellow). GPI-GL-YFP accumulated in both regions with similar kinetics (Fig. 3 C, red and yellow dots), but far more GPI-GL-YFP accumulated in the delimited apical region despite being a smaller volume. As with our analysis of VSVG-YFP transport, the only way model 2 could be consistent with the data is if the junctional residence of GPI-GL-YFP was so short as to be experimentally undetectable (\(k_3\) of \(>4\) min\(^{-1}\)). This is also unlikely because the newly delivered GPI proteins are immobile on the apical surface (Hannan et al., 1993) and should diffuse from a lateral insertion site slowly, enabling their detection. Thus, it seems unlikely that GPI-GL-YFP is inserted at the apical margin or the junction-associated region.

Although not visually obvious, a small fraction of GPI-GL-YFP was quantified as residing at the lower two segments of the lateral membrane at the start of the 31°C chase; this amount decreased with the time of chase (Fig. 3 C, blue and pink dots). However, this behavior was not consistent with a major indirect route to the apical membrane (Fig. 2 and Fig. 3 C, inset; model 3). Such a mechanism was calculated to require a flat or slightly increasing curve for the amount of lateral GPI-GL-YFP during the 20-min time course used here, which is inconsistent with the data. We suspect that the lateral GPI-GL-YFP reflected a partial missorting as expected (Keller et al., 2001; Paladino et al., 2004). Although it is possible that the lateral GPI-GL-YFP proceeded by transcytosis to the apical side, which is consistent with previous results (Polishchuk et al., 2004), the kinetic analysis is inconsistent with it being an obligatory intermediate on the apical pathway for the entire newly synthesized cohort.

At all times of chase, \(\sim 20\%\) of GPI-GL-YFP fluorescence was localized neither to the TGN region nor to the apical (or lateral) membrane. Instead, it was distributed intracellularly underneath the apical membrane (Fig. 3 C, open circles). This signal may represent GPI-GL-YFP in equilibrium with endosomes in the apical cytoplasm. Therefore, it was important to determine whether the GPI-GL-YFP that appeared in the apical region had actually fused with the apical membrane. We repeated the experiment with fluorescent anti-GFP antibody in the medium (accessible to both apical and basolateral membranes). The insertion of GPI-GL-YFP will expose the extracellular YFP domain to the medium, enabling concentration of anti-GFP at the plasma membrane. Indeed, as GPI-GL-YFP was sorted away from TGN38-CFP and delivered toward the apical domain, it was accompanied by a steadily increasing amount of anti-GFP labeling on the apical membrane (Fig. 4 A). No significant anti-GFP labeling in the basolateral domain was detected, again indicating that little GPI-GL-YFP was inserted in the basolateral membrane. The total amount of antibody on the apical membrane increased with time, and the rate of insertion in the apical domain was in agreement with the kinetic data derived from quantifying GPI-GL-YFP fluorescence (Fig. 4 B).
In MDCK cells, the TGN may exhibit an overall expansion in surface area as a result of the 20°C block (Griffiths et al., 1985; Ladinsky et al., 2002). Because of this, upon shifting to 31°C, the TGN returns to its original size, perhaps slowing the initial rates of exit. Therefore, we measured the TGN exit rate of VSVG-YFP in the absence of a 20°C block. VSVG-YFP was accumulated in the ER at 40°C and was released by shifting directly to 31°C. The colocalization of VSVG-YFP with TGN38-CFP was used to estimate entry and exit from the TGN. Indeed, the rate of exit under these conditions (0.008 ± 0.002 min⁻¹; n = 2) was somewhat slower as compared with the exit rate after the 20°C block (Fig. 2). This suggests that after the 20°C block, VSVG exit from TGN may occur normally, with the faster rate perhaps reflecting the accumulation of VSVG in the TGN (Griffiths et al., 1985). To further characterize the effect of the 20°C block on VSVG-YFP exit from the TGN, rates were calculated from the first and last 10 min of our complete dataset (n = 12). On average, the rate calculated for the last 10 min was 17% slower, which is also inconsistent with the possibility that VSVG exit is slowed after 20°C block. When this analysis was performed for GPI-GL-YFP exit, we found that the initial rate was somewhat slower in three of nine cells (e.g., the initial lag in Fig. 3 B; green line), perhaps reflecting the time required for partitioning of GPI-anchored proteins into lipid microdomains before TGN exit (Paladino et al., 2004; Schuck and Simons, 2004).

To avoid the issue of temperature blocks entirely, we developed an assay to monitor transport kinetics at steady state and to assess independently the contribution of basolateral to apical transcytosis to GPI-GL-YFP targeting. For this purpose, we determined the binding and transport of anti-GFP antibody that was vectorially added to the basolateral or apical media of filter-grown cells. GPI-GL-YFP at both surfaces was first blocked with nonfluorescent anti-GFP IgG for 2 h. Cells were then incubated with anti–GFP-AlexaFluor647 in the basolateral medium for 30 min at 37°C (Fig. 5 A, diagram; blue antibody). Blue antibody detected by 3D imaging at the apical surface would, therefore, reflect GPI-GL-YFP transcytosis. At the end of the chase (at 0°C), anti–GFP-AlexaFluor594 (Fig. 5 A, diagram; red antibody) was added to the apical medium to detect GPI-GL-YFP molecules that had failed to bind the blue antibody (i.e., the population of GPI-GL-YFP that had not been exposed to the basolateral medium). As expected, the apical membrane was heavily labeled with AlexaFluor594 (red) antibody and was only slightly labeled with AlexaFluor647 (blue) antibody (Fig. 5 A). Thus, the majority of GPI-GL-YFP was apparently transferred directly from the TGN to the apical surface, as it did not bind anti-GFP in the basolateral medium. For reference, the TGN was indicated by TGN38-CFP (Fig. 5 A, green).

The digital reconstructions were used to quantify the relative contributions of direct versus transcytotic transport by determining the ratio of the two antibodies at the apical surface. To eliminate any bias caused by the differences in detection or binding of the two antibodies, we also collected datasets in which the antibodies were reversed. In either event, <5% of GPI-GL-YFP on the apical membrane came via the transcytotic pathway (i.e., had been tagged by the basolateral antibody; Fig. 5 C). The result was independent of the antibody concentration, indicating that anti-GFP saturated the membrane-bound GPI-GL-YFP. The same result was obtained if the cells were blocked for 1 h at 20°C, as in the imaging experiments (Fig. 3).

As a positive control to show that established transcytosis markers could be labeled on the basolateral surface while en route to their final destination (the apical surface), we tested cells expressing NgCAM-GFP (Anderson et al., 2005). Cells were incubated with anti–NgCAM-AlexaFluor647 (Fig. 5 B, blue antibody) in the basolateral medium for up to 90 min at 37°C (transcytosis of NgCAM takes ~90 min, which is slower than the apical appearance of GPI-GL-YFP; Anderson et al., 2005). The cells were cooled to 0°C, and anti–NgCAM-AlexaFluor594 (Fig. 5 B, red antibody) was then added to the apical medium to detect any NgCAM that had been directly inserted apically. The blue antibody labeled both the apical and lateral surfaces as well as a variety of intracellular endosomal compartments, which is consistent with transcytosis (Fig. 5 B). Only a small amount of red antibody bound to the apical surface. Quantitation of red versus blue revealed that >95% of the apical NgCAM-GFP had captured antibody in the basolateral medium (Fig. 5 C).

Discussion

3D imaging of living filter-grown MDCK cells has finally permitted a quantitative analysis of the rate and spatial restriction of domain-specific membrane protein transport in polarized epithelial cells. To image entire pathways, however, it was necessary to collect data over an ~50-min time course, requiring that frames be taken at relatively long (1 min) intervals to minimize photobleaching. Although interstack time intervals were too long to monitor individual transport vesicles, we did demonstrate unambiguously that at least two well-described apical and basolateral proteins were sorted intracellularly and inserted with a high degree of fidelity directly into their respective domains. Our visual and computed results confirm several early biochemical studies on traffic in polarized MDCK cells (Matlin and Simons, 1984; Griffiths et al., 1985; Pfeiffer et al., 1985; Lisanti et al., 1989; Casanova et al., 1999; Vieira et al., 2005), conclusions that were recently questioned based on immunofluorescence studies of vectorially fixed (and therefore highly perturbed) cells, using the same constructs as used here (Nelson and Rodriguez-Boulan, 2004; Polichok et al., 2004).

Some interesting differences were revealed between the apical and basolateral pathways. Although VSVG-YFP and GPI-GL-YFP had comparable exit rates from the TGN, the overall rate of exit from the transit compartment and apical delivery was demonstrably slower in the case of GPI-GL-YFP. One reason for this may be the appearance of a subapical intermediate compartment that accumulated GPI-GL-YFP at equilibrium. Similar compartments have been previously noted and termed apical endosomes or vacuolar-apical compartments, and they do appear to play a role in the endocytosis and recycling of apical components or as a biosynthetic transport intermediate (Vega-Salas et al., 1987; Casanova et al., 1999; Sheff et al., 1999; Brown et al., 2000). No such kinetic intermediate was observed on the basolateral pathway. Given the limited time resolution...
used in this study, we would not have expected to detect the transit of VSVG or GPI-GL-YFP through recycling endosomes (Ang et al., 2004) unless this step was similarly rate limiting (i.e., $<0.23 \text{ min}^{-1}$). It apparently was not. The apical endosomal compartment may accumulate GPI-GL-YFP because of its ability to partition into relatively immobile lipid microdomains that may be abundant in these compartments (Gagescu et al., 2000; Paladino et al., 2004; Schuck and Simons, 2004).

The processes of vesicle docking and fusion were not studied in detail. For VSVG, we could not distinguish whether VSVG transporters were docked versus fused with the lateral membrane. Increased time resolution alone would certainly permit the tracking of individual vesicles, as accomplished by Kreitzer et al. (2003). However, from the point of view of establishing kinetics, such an analysis would not help. At present, there is no way to judge definitively that fusion of a single vesicle has occurred at the lateral surface, which cannot yet be studied by total internal reflectance microscopy. In our dataset, appearance at the lateral membrane represents the aggregate rate of docking and fusion followed by rapid diffusion of VSVG in the lipid bilayer.

As a result, we cannot definitively rule out the possibility that laterally targeted vesicles dock and fuse at a site close to the junctional complex. Because of the high mobility of VSVG on the lateral membrane, even if insertion is strictly limited to the junctional region, VSVG-GFP would appear evenly distributed on the lateral membrane in our experiments. However, the data do predict that if insertion at the junctional region is obligatory, the mean residence time at the junction (including docking, fusion, and diffusional exit) must be 26 s or less. Longer residence times, such as those measured for post-TGN VSVG-containing vesicles docking at the plasma membrane of glass-grown cells ($t_{1/2}$ of 39 s; Toomre et al., 2000), would have been detected.

Although our approach is certainly just a methodological beginning to the problem of elucidating the kinetic and spatial control of membrane traffic in complex cells, it has permitted a definitive resolution of the vectorial nature of biosynthetic protein transport in MDCK cells, which is one of the oldest and most fundamental problems in the field. Clearly, the approach will lend itself to other cargos and cell types, especially cells that have been manipulated to exhibit the reduced expression of candidate genes involved in polarity or transport.

**Materials and methods**

**Microscopy**

A confocal laser scanning inverted microscope (LSM-510; Carl Zeiss MicroImaging, Inc.) with a temperature-controlled stage set at 31°C was used for image acquisition. To minimize refractive index mismatch, we used...
a 40× NA 1.2 water immersion lens (Carl Zeiss MicroImaging, Inc.) with temperature and coverslip thickness correction. To achieve quantitative 3D live cell imaging of the entire sorting pathway that takes ~30 min, with minimum photobleaching of the fluorescence, the excitation laser power was adjusted to instrument minimum (0.1%), and the maximum laser scanning speed was used. It took 2–3 s (typically 1.5 s) to complete 6–10 z stacks of a single cell at ~1 μm per image, depending on the number of fluorescent channels. Typically, the size of the scanning areas was approximately 50–100 μm, and the number of scanning repeats (typically two). Pinhole size was increased up to 4 Airy units to increase light collection without significant background noise from stray light. Image resolution is independent of pinhole size under these conditions. Total photobleaching (typically 50–100 fluorescent channels to scan (typically four), the size of the scanning areas stacks of a single cell at was adjusted to instrument minimum (0.1%), and the maximum laser scan-

were proportional to the photon input (LSM-510; Carl Zeiss MicroImaging, in independent of pinhole size under these conditions. Total photobleaching of our CFP and YFP probes (see next section) under these conditions was ~40% (see Fig. S1). The detectors (photomultiplier tubes) were adjusted to maximally amplify but not saturate the signal. The collected digital signals were proportional to the photon input (LSM-510; Carl Zeiss MicroImaging, Inc.), which, was, to the first order, proportional to the amount of fluorescent proteins being illuminated in a given area. To compensate for the slow z drift during the course of recording, the Zeiss software package was used to automatically correct the focus every 3 min according to the z position of the filter on which the MDCK cells were grown.

Tissue cell culture, protein expression, and antibodies

Standard MDCK II cells or MDCK II that stably expressed human transferrin receptor (Sheff et al., 1999) were cultured under standard procedures (Hunziker et al., 1991); i.e., incubated at 5% CO2 in DME with 10% FCS, 1× glutamine. TGN38-CFP, VSVG-SP-YFP (abbreviated here as VSVG-YFP), GPI-GL-YFP, NgCAM-GFP constructs, and anti-NgCAM antibody were described previously (Toomre et al., 2000; Keller et al., 2001; Ang et al., 2003; Anderson et al., 2005). Anti-GFP antibody labeled with Alexafluor594 and 647 was obtained from Invitrogen. Anti-NgCAM was labeled with Alexafluor568 and 647 with the Zenon Antibody Labeling Kit (Invitrogen). For microscopy experiments, MDCK cells were seeded at 2 × 10⁵ cells/well on polycarbonate transwell filters (clear type; Corning) in six-well plates. Six h later, cells were transfected with 2–3 μg DNA per well using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions and incubated at 40°C (except for transcytosis assays, which were performed at 37°C). The 40°C overnight incubation blocked the temperature-sensitive (Huh7) VSVG-YFP in the ER but not GPI-GL-YFP or NgCAM-GFP. Imaging was performed ∼16 h after transfection.

Time-series microscope experiment

MEM medium without phenol red (Invitrogen) was supplemented with 10 mM Hepes and used for all microscope experiments (MEM-H). A 35-mm petri dish (Matsunami) containing a coverslip bottom was precleaned with ethanol. A piece of filter was cut off (together with a patch of cells grown on top) and placed on the coverslip with cells facing down the coverslip. A custom-made three prop weight was put on top of the filter–cell complex for sample stabilization while enabling free medium exchange for the cells through the space between the cells and the coverslip (Fig. 1 A). Cells were viable for ~2 h. All of the cells recorded (three nuclei in the cases described in Fig. 2) showed behavior and kinetics similar to those shown in Figs. 1 and 3. For all assays (except the transcytosis assay), the incubation medium was MEM-H. When needed, the medium was supplemented with 33 μg/ml transferrin-Alexafluor568 and 250 μg/ml dextran-Alexafluor647 or 3 μg/ml anti-GFP antibodies. Transferrin and dextran were used to label the recycling endosomes and lysosomes, respectively, and VSVG transport rates in MDCK cells expressing human transferrin receptor is indistin-
guishable to normal MDCK cells. Cells were incubated at 40°C with MEM-H for 1 h and blocked at 20°C for 1 h to accumulate cargo (VSVG-YFP and GPI-GL-YFP) in the TGN. GPI-GL-YFP that was already on the apical surface was removed by 0.8 μg/ml phosphatidylinositol-specific PLC (Sigma- Aldrich) treatment for 30 min at 20°C in PBS supplemented with 100 μg/ml CoCl₂ and Mg²⁺. The time from the placement of the cells in 31°C medium to the start of the image acquisition was recorded (t₀) and was typically between 3–30 min. Live cell microscope experiments (except for the transcytosis assay) were performed at 31°C with 20 μg/ml cycloheximide to inhibit new protein synthesis (Anderson et al., 2003).

Transcytosis assay

Cells were incubated with 50 μg/ml of unlabeled antibody in both the apical and basolateral medium for 2 h at 37°C, washed, and chased with 10 μg/ml Alexafluor594 (or Alexafluor568) labeled antibody in basolateral medium. The chase time was 30 min for the GPI-GL-YFP experiment and 90 min for the NgCAM-GFP experiment (the transcytosis of NgCAM (Anderson et al., 2005). Cells were washed, and a 10× dilution of the Zenon Kit (Invitrogen) blocker reagent was added to the apical medium and incubated for 10 min. Cells were washed, and 10 μg/ml Alexafluor647-labeled antibody was added to the apical medium and incubated for 5 min. Cells were then incubated in antibody-free medium for 10 min at 0°C, and the filter–cell complex was cut off and imaged at <20°C without fixation. The duration of the microscope experiment was <10 min, and the pattern of the antibody labeling was unchanged during the time. Similar results were obtained with 4% PFA-fixed cells except that the cells slightly deformed in the axial direction as a result of fixation, and the auto-fluorescent background was higher. For the GPI-GL-YFP transcytosis assay, the antibody concentration described was referred to as 3X in the text. The experiment with the Alexafluor647 antibody used in the basolateral medium and Alexafluor594 (or Alexafluor568) in the apical medium were performed simultaneously under otherwise identical conditions.

Image and data processing

Images were processed and quantified with LSM-510 software (Carl Zeiss Microimaging, Inc.), and Volocity with the visualization and classification modules (Improvision). Slight submicrometer spatial spectral shifts were sometimes observed and corrected using the software shift function. Background noise (e.g., the fluorescence signal in neighboring cells not expressing GFP or its color variant) was minimal when optimal gain/offset settings for the detectors were used. No nonlinear algorithms were used to alter the quantification of the fluorescent signals, so the final signals were proportional to the amount of fluorescent proteins. In some cases, the reflection of the laser from the filter creates wide-spectrum noise at the z sections close to the filter, and the affected sections were removed from further processing. The definition of cellular structures are as follows: the TGN region was defined by the 3D TGN38-CFP image through the classification process in Volocity. Specifically, an intensity threshold was first applied to eliminate background fluorescence followed by a medium spatial filter to smooth the image. Background signal was further rejected using particle size threshold to exclude particles that were ∼0.06 μm³. The resulting fluorescent volume was used to represent the TGN structure; its fluctuation was <20% over 20 min. The boundary of the cell of interest (that expresses both CFP and YFP) was determined by the increased background fluorescence level in the target cell. The apical domain was defined as the top 25% of the cell imaged. For the transcytosis experiments, if there was any overlap. The lateral domain was defined as the volume from the cell’s lateral edge to approximately two thirds of the distance from the cell’s center vertical axis minus the TGN and apical regions. For kinetic measurements, at every time point, the total fluo-

rescent signal of the transport cargo proteins (GPI-GL-YFP and VSFG-YFP) in the defined structures (such as the TGN) was quantified, and the final signal was calculated by the fluorescent signal in that structure divided by total fluorescent signal in the whole cell. In the transcytosis assay, we measured total fluorescence on the apical domain because of antibody added to the apical (Fₐ) or basolateral medium (Fₐ) and calculated the ratio k’ = Fₐ/Fₐ. Two sets of the experiments—one with the Alexafluor647 antibody in the basolateral medium and Alexafluor594 (or Alexafluor568) in the apical medium and the other with the antibody color swapped—were performed side-by-side. The geometry of the k’ measurement performed on two sets of experiments was calculated [k(f)]. The fraction of new apical proteins that captured antibody at the basolateral surface was expressed by k'(1+k).

Kinetic measurements

The protein transport steps that we observed fit well with a simple two-step process (Figs. 2, model 1) in which the intermediate transit of proteins was detected as significant intracellular fluorescence outside of the TGN. Protein quantity in the TGN under the experimental condition PTGN was P₀ × (1 − k₁ × (t + t₀)), where P₀ is the initial protein quantity and t₀ is the dura-

tion between the start of the 31°C chase and the start of the recording, was defined in Time-series microscopes. In the experiment described in Time-series microscope experiment the destination plasma membrane is PPM = P₀ × k₁/(k₁ + k₂ + k₃) × k₃/k₁ × [1 − e⁽−k₁(t+t₀)⁾(1 + [e⁽−k₃(t+t₀)⁾]⁻¹)] and in the intermediate transit is PINT = P₀ × k₁/(k₁ + k₂ + k₃) × [1 − e⁽−k₃(t+t₀)⁾] × D, where D is the fixed amount of intracellular labeling as a result of various background noise. Thus, the total fluorescence is PT = P₀ × KGN + P₁N + P₁P + P₂N/P₁P + P₂P/P₁P and P₁P/P₁P were globally fitted to the experimental curves such as those in Figs. 1 C and 3 B (SigmaPlot software; Systat). D was set to 0.5. All experiments (typically four), I, 2, and 3, three steps with rates k₁, k₂, and k₃ are used. The last two states were first combined, and such a two-step process was fitted to the data to obtain k₁ and k₂ as described above. For model 2, P₂N = k₂/k₃ = [k₂P₁N − P₀ × k₁/k₃] × [1 − e⁽−k₁(t+t₀)⁾] and P₁N = P₀ × k₁/(k₁ + t + t₀) − P₂N, with the accuracy of (1 + g) and P₁N = P₁N × g, where g is the proportion of the lateral membrane included in the yellow section [Figs. 1 D and 3 C], were fitted to fluorescence in the blue + purple lateral background.
References


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