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Notes & Tips

In vitro assays differentially recapitulate protein export from the *trans*-Golgi network

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In vitro assays have been broadly applied to reconstitute trafficking events along the biosynthetic and endocytic pathways of eukaryotic cells [1]. Cell-based assays employ mild detergents or mechanical means to perforate the plasma membrane and release cytosolic components while maintaining the integrity of intracellular membrane compartments (Supplementary Fig. 1). In contrast, cell-free assays are performed using isolated membrane fractions enriched in a particular organelle. Cell-based assays can allow analysis of multiple steps along a trafficking pathway, while cell-free assays are generally designed to reconstitute an individual membrane fission or fusion step. Both cell-based and cell-free assays have proven useful for dissecting the roles of various components in intracellular trafficking events. In many instances, these assays have been utilized to dissect the requirements for polarized biosynthetic sorting of cargo molecules from the *trans*-Golgi network (TGN)[1][2–5]. Interestingly, segregation of apical and basolateral marker proteins into distinct transport carriers that bud from the TGN appears to occur even in nonpolarized cells, enabling the use of nondifferentiated cell types for such assays [6–8]. However, the extent to which these assays recapitulate *in vivo* sorting and regulation of membrane traffic has not been systematically examined.

Here we have compared cell-based and a cell-free *in vitro* assays for their ability to reconstitute polarized protein sorting from the TGN. We previously found that efficient apical delivery of the marker protein influenza hemagglutinin (HA) in polarized Madin–Darby canine kidney (MDCK) cells requires TGN acidification [9]. In particular, expression of the acid-activated proton channel influenza M2 selectively inhibited TGN to apical surface delivery of HA but had no effect on delivery of a basolaterally destined protein [9]. Moreover, expression of a dominant-negative inhibitor of the GTPase dynamin has been shown to inhibit TGN export of apical proteins [10], suggesting a requirement for GTP hydrolysis in apical membrane delivery. Therefore, we asked whether cell-based and cell-free assays accurately reconstitute GTP- and acid pH-dependent export of HA from the TGN. Surprisingly, only the cell-based assay accurately recapitulated both of these *in vivo* requirements for HA sorting.

In the cell-based assay, HeLa cells grown to 70% confluence on 10 cm dishes were infected with replication-defective recombinant adenovirus encoding influenza HA. Cells were starved for 30 min in methionine-free medium, pulsed for 15 min in medium supplemented with 150 μCi/ml Tran-[^{35}S]-label (MP Biomedicals), and chased for 2–3 h at 19 °C to stage newly synthesized HA in the TGN. Cells were incubated on ice with swell buffer (10 mM Hepes, pH 7.2, 15 mM KCl), scraped into break buffer (50 mM Hepes, pH 7.2, 110 mM KCl), and washed with break buffer by centrifugation at 800*g* for 5 min at 4 °C. Cells were then brought to volume in GGA buffer (25 mM Hepes, pH 7.4, 38 mM K-aspartate, 38 mM K-glutamate, 38 mM K-gluconate, 2 mM EGTA, 1 mM dithiothrectol, and 2.5 mM $MgCl_2$). Release was reconstituted at 37 °C for 1 h in the presence of 1 mM ATP, 8 μM phosphocreatine, 5 μg/ml creatine phosphokinase, and 100 μg rat brain cytosol in a total volume of 50 μl. Released vesicles were separated from cells by centrifugation for 5 min at 12,500 rpm in an Epindorff microcentrifuge. Supernatants and pellets were solubilized and HA was immunoprecipitated using monoclonal anti-HA antibody. Samples were analyzed by SDS–PAGE and the efficiency of HA release was quantitated upon phosphorimager analysis as previously described [11].

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[1] *Abbreviations used:* Baf, bafilomycin A_1; HA, influenza hemagglutinin; MDCK, Madin–Darby canine kidney; TGN, *trans*-Golgi network; PBS, phosphate-buffered saline.

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We also used a cell-free assay to reconstitute the export of radiolabeled, TGN-staged HA from isolated Golgi membranes. Ten 15-cm plastic dishes containing confluent MDCK cells infected with replication-defective recombinant adenovirus were incubated with calcium-free PBS containing 1 mM magnesium chloride for 10 min at 37 °C to disrupt tight junctions and facilitate subsequent access of methionine to basolateral transporters, radiolabeled, and chased as described above. The cells were then scraped from the dish in PBS, pelleted, and resuspended in 2.5 ml homogenization buffer (20 mM Tris–HCl, pH 7.8, 1 mM EDTA, 0.25% sucrose, 1× protease inhibitor cocktail (Calbiochem)). The resuspended cells were passed through a ball-bearing homogenizer with a 0.001-inch clearance 15 times and then centrifuged at 1000*g* at 4 °C to obtain a postnuclear supernatant. This was mixed with an equal volume of 50% Nycodenz in TE buffer (20 mM Tris, pH 7.8, 1 mM EDTA; final volume 6 ml), overlaid sequentially with 4 ml 17.5% Nycodenz, and 2 ml 10% Nycodenz, and balanced with TE buffer. The gradient was centrifuged at 56,000*g* in a Sorvall TH641 rotor for 3 h to float Golgi membranes to the 17.5%/10% interface. The 10%/17.5% interface and much of the 10% fraction were collected (visible as a solid white band), diluted three-fold with homogenization buffer, and centrifuged for 1 h at 100,000*g* to pellet the membranes. Aliquots were flash frozen and stored at −80 °C. To reconstitute export, 50 µg of membranes was thawed rapidly and then placed on ice. The Golgi membranes were mixed with two-fold concentrated ATP regenerating system, 1 mM GTP, and 2 mg/ml cytosol in a final volume of 100 µl. Tubes were mixed gently by tapping and placed at 37 °C for 1 h. Golgi membranes were separated from released vesicles by centrifugation for 10 min at 12,500 rpm in a microcentrifuge, and HA was immunoprecipitated and analyzed as described above to quantitate release.

We routinely observed cytosol- and ATP-dependent release from both assays (Fig. 1). The release of TGN-staged HA in either assay typically ranged 5–20% of total and represented a 2- to 10-fold increase over background release measured in the absence of ATP and cytosol. To examine whether this release was sensitive to physiologically relevant regulators of apical membrane traffic, we tested the effect on these assays of including the nonhydrolyzable nucleotide GTPγS and the vacuolar ATPase inhibitor bafilomycin A_1 (Baf). Both of these treatments disrupt

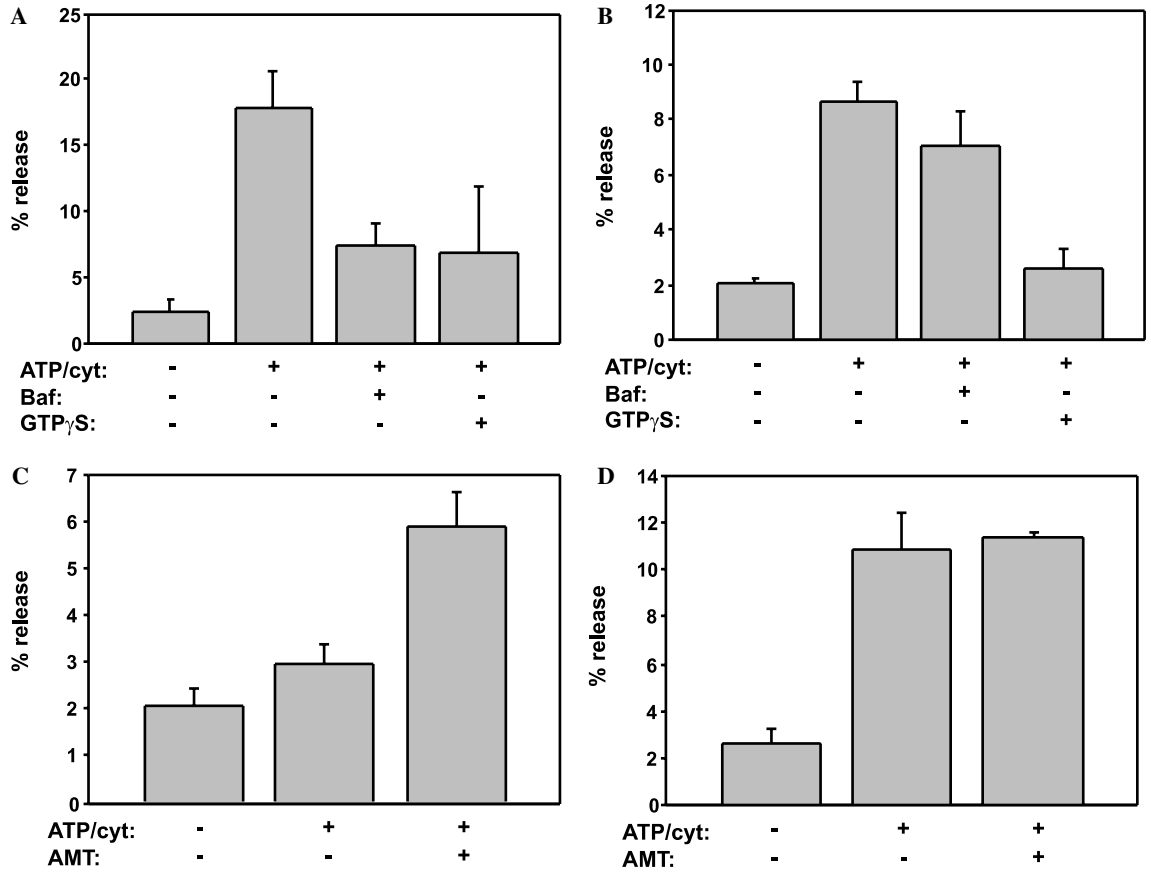

Fig. 1. Comparison of GTP and pH requirements in cell-based versus cell-free assays. Cells expressing HA were radiolabeled, chased at 19 °C and either perforated (A) or used as a source for Golgi membrane isolation (B). The effect of bafilomycin A_1 (5 µM) and GTPγS (10–100 µM) on TGN export was evaluated as described in the text. The mean ± SD of triplicate samples is plotted. Export of TGN-staged HA in the cell-based assay is inhibited in cells expressing influenza M2 but restored by addition of the proton channel blocker amantadine (AMT) (C), whereas export from Golgi membranes prepared from influenza- M2-expressing cells is robust and unaffected by inclusion of amantadine (D) The mean ± range of duplicate samples is plotted.

cell surface delivery of HA *in vivo* in both HeLa and MDCK cells ([12]; and our unpublished results). Surprisingly, both GTPγS and Baf inhibited HA release from the cell-based assay (Fig. 1A) whereas only GTPγS inhibited HA release from isolated Golgi membranes (Fig. 1B).

To further examine the requirement for TGN acidification in these assays, we evaluated the effect of expressing influenza M2, an acid-activated proton channel that effectively neutralizes the pH of the TGN. We previously found that expression of M2 inhibits apical delivery of HA *in vivo* and that the effect of M2 expression is completely rescued by addition of the M2 channel blocker amantadine to cells [9]. HA export in our *in vitro* cell-based assay is significantly inhibited in cells expressing M2 and restored to control levels by inclusion of amantadine in the reconstitution reaction [11] (Fig. 1C). However, HA export from Golgi membranes prepared from M2-expressing cells was unaffected by amantadine (Fig. 1D), confirming that release from isolated Golgi fractions occurs via a pH-independent mechanism.

In summary, comparison of a cell-based assay and a cell-free assay for pH-dependent export of the apical cargo molecule influenza hemagglutinin demonstrates that the former more closely reconstitutes regulation and sorting events that occur *in vivo*. While the cell-based assay maintained the physiological dependence of HA release from the TGN on luminal pH, export in the cell-free Golgi assay was not affected by Baf or by expression of active M2. Together, these data suggest that isolated Golgi membranes do not maintain physiological ion gradients that enable acidification of the TGN. Alternatively, HA sorting in these isolated membranes may occur via a different mechanism that is less sensitive to changes in pH. One might speculate that HA is missorted into basolaterally destined vesicles in the cell-free assay; however, neither M2 nor Baf affect the ultimate polarized distribution of HA in intact cells but rather they slow the rate of apical protein export from the TGN. Moreover, in contrast with the export of HA that we observed in our assays, basolateral protein export from the TGN is fairly insensitive to GTPγS [11,14].

Recent studies have found that some newly synthesized basolaterally destined proteins traverse endosomes *en route* to the plasma membrane (reviewed in [13]). It is not yet known whether newly synthesized HA traffics via this indirect route. Importantly, however, under conditions where we observed inhibition of HA biosynthetic delivery in intact HeLa cells, we did not see pH-dependent effects of M2 expression on recycling through endosomal compartments [14]. Thus, it is unlikely that this possibility accounts for the differences in pH requirement that we observed in the two assays.

Interestingly, whereas neutralization of TGN pH in permeabilized cells severely compromised the export of HA, we observed comparably efficient release of TGN-staged HA from Golgi membranes in the cell-free assay, even though the TGN is apparently not acidified under these assay conditions. Therefore, release of HA in the cell free assay does not appear to accurately reconstitute physiological sorting of HA, and TGN export in this assay may reflect redirection of HA into alternative transport carriers that mediate pH-independent sorting pathways.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.04.033.

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