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ADP-ribosylation Factor 1-independent Protein Sorting and Export from the *trans*-Golgi Network*

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Polarized epithelial cells efficiently sort newly synthesized apical and basolateral proteins into distinct transport carriers that emerge from the *trans*-Golgi network (TGN), and this sorting is recapitulated in nonpolarized cells. While the targeting signals of basolaterally destined proteins are generally cytoplasmically disposed, apical sorting signals are not typically accessible to the cytosol, and the transport machinery required for segregation and export of apical cargo remains largely unknown. Here we investigated the molecular requirements for TGN export of the apical marker influenza hemagglutinin (HA) in HeLa cells using an *in vitro* reconstitution assay. HA was released from the TGN in intact membrane-bound compartments, and export was dependent on addition of an ATP-regenerating system and exogenous cytosol. HA release was inhibited by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) as well as under conditions known to negatively regulate apical transport *in vivo*, including expression of the acid-activated proton channel influenza M2. Interestingly, release of HA was unaffected by depletion of ADP-ribosylation factor 1, a small GTPase that has been implicated in the recruitment of all known adaptors and coat proteins to the Golgi complex. Furthermore, regulation of HA release by GTPγS or M2 expression was unaffected by cytosolic depletion of ADP-ribosylation factor 1, suggesting that HA sorting remains functionally intact in the absence of the small GTPase. These data suggest that TGN sorting and export of influenza HA does not require classical adaptors involved in the formation of other classes of exocytic carriers and thus appears to proceed via a novel mechanism.

Newly synthesized transmembrane and secretory proteins in eukaryotic cells traverse a complex route to their final destinations. The biosynthetic pathway begins when newly synthesized proteins are translocated into the endoplasmic reticulum and ends at the *trans*-Golgi network (TGN),[1] a Golgi-associated tubular network where proteins are sorted into distinct membranous carriers. The TGN serves as a sorting station to segregate proteins destined for endosomes, lysosomes, and the plasma membrane. To add to this complexity, proteins destined for the cell surface can be sorted into distinct types of transport carriers. This additional level of sorting is most obvious in polarized epithelial cells where the plasma membrane is differentiated into discrete apical and basolateral domains. In these cells, delivery of newly synthesized apically and basolaterally destined proteins from the TGN to the cell surface is critical for the maintenance of the different protein and lipid compositions at either cell surface domain (for reviews, see Refs. 1 and 2). Interestingly, the distinct sorting of "apical" and "basolateral" proteins into separate carriers that bud from the TGN is recapitulated in nonpolarized epithelial cells as well as in several non-differentiated cell types including fibroblasts and Vero cells (3–7). Nonpolarized cells thus provide a useful model in which to investigate the regulation of TGN sorting and export of different classes of proteins, including markers of the apical and basolateral biosynthetic pathways.

While considerable research has been directed toward dissecting the signals on cargo molecules that mediate their polarized delivery, the mechanisms by which these signals are interpreted and the machinery involved in sorting apical and basolateral proteins remain largely unknown. Basolateral targeting signals are generally localized to discrete amino acid sequences in the cytoplasmic domains of proteins. Cytosolic adaptor protein (AP) complexes recognize these motifs and link the cargo to a coat protein such as clathrin (8). Various AP complexes have been implicated in targeting proteins to the basolateral but not the apical surface (9–11). However, TGN-derived vesicles containing basolaterally destined proteins are not clathrin-coated (12). A role for coatomer in basolateral protein export has also been suggested (13); however, a recent report has questioned the requirement for any coats in the formation of TGN-derived basolateral carriers enriched in basolateral proteins (14).

In contrast to basolateral proteins, sorting of apically targeted proteins generally depends on motifs within the luminal or transmembrane domains, although some examples of cytoplasmically disposed apical targeting signals have also been described (15, 16). *N*- and *O*-linked carbohydrates are required for apical targeting of a subset of proteins (17–21). The association of some proteins with sphingolipid-enriched microdomains or rafts in the TGN has also been proposed to direct their apical delivery (22).

Influenza hemagglutinin (HA) has been used extensively as a marker for the apical pathway in polarized cells. Sorting information resides within the transmembrane domain of HA, although no transferable amino acid sequence has been identified (23, 24). HA is efficiently incorporated into lipid rafts, although the correlation between lipid raft association and polarity is unclear (23–25). While little is known about how this

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[1] The abbreviations used are: TGN, *trans*-Golgi network; ARF, ADP-ribosylation factor; AP, adaptor protein; COP, coatomer; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GMP-PNP, guanosine 5′-(β,γ-imido)triphosphate; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; VSV G, vesicular stomatitis virus glycoprotein; YFP, yellow fluorescent protein; GalT, β1,4-galactosyltransferase.

protein is packaged into apically destined vesicles at the TGN, cell surface delivery of HA is disrupted by expression of influenza M2, an acid-activated proton channel that elevates the pH of acidified intracellular compartments, including the TGN (26–30).

To begin to identify the cytosolic requirements for the formation of apically destined vesicles from the TGN, we reconstituted ATP- and cytosol-dependent TGN export of prestaged HA in perforated HeLa cells. Our data suggest that this transport assay faithfully recapitulated *in vivo* requirements for efficient HA release from the TGN. Moreover we found that ADP-ribosylation factor 1 (ARF1), a small GTP-binding protein required for recruitment of adaptor and coat proteins to the Golgi complex and the TGN, was not required for efficient HA release. Additionally HA release under ARF-depleted conditions remained sensitive to selective inhibitors that disrupt HA export in the presence of ARF1, suggesting that ARF depletion does not cause rerouting of HA into alternative transport carriers. Together our data suggest that packaging and export of apical proteins does not require the assembly of known coat protein complexes.

MATERIALS AND METHODS

Antibodies—Monoclonal antibody Fc125 directed against influenza HA was a gift from Dr. T Braciale (University of Virginia, Charlottesville, VA). Monoclonal antibody mAD against β-COP was from Sigma. Anti-ARF monoclonal antibody 1D9 was from AbCam. Polyclonal antibody against the AP-1 γ-subunit was a gift from Dr. Linton Traub (University of Pittsburgh, Pittsburgh, PA). Anti-giantin and -GM130 were gifts from Dr. Adam Linstedt (Carnegie Mellon University, Pittsburgh, PA). Anti-TGN46 antibody (AHP500) was obtained from Serotec. Anti-green fluorescent protein polyclonal antibody was obtained from Molecular Probes.

Cell Lines, Adenoviral Infection, and Transfection—HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. The generation and propagation of replication-defective recombinant adenoviruses encoding tetracycline-repressible influenza HA Japan serotype, influenza M2 Rostock serotype, a control virus (encoding influenza M2 in the reverse orientation), and constitutively expressed tetracycline transactivator are described in Ref. 31. Cells were infected using replication-defective recombinant adenoviruses (multiplicities of infection of 50 for HA, 200 for M2 or M2 in the reverse orientation, and 100 for tetracycline transactivator) as described previously (27, 31), and experiments were performed the following day. cDNA constructs encoding the amino terminus of human β1,4-galactosyltransferase linked to yellow fluorescent protein (GalT-YFP) and the endoplasmic reticulum-Golgi-intermediated compartment cycling protein p58 linked to yellow fluorescent protein (p58-YFP) were provided by Dr. Jennifer Lippincott-Schwartz and transiently expressed in HeLa cells using LipofectAMINE 2000 (Invitrogen).

Preparation of Cytosol—Rabbit liver cytosol was prepared from one fresh rabbit liver using the protocol described for rat liver cytosol (32). Rat brain cytosol was prepared from 50 frozen unstripped rat brains (Pelfreeze Biologicals) as described previously (33). ARF-depleted cytosol was prepared from rat brain cytosol using a procedure described previously (34). The resultant cytosols ranged in concentration from ~36 mg/ml (rabbit liver cytosol) to ~12 mg/ml (rat brain cytosol) and were used in reconstitution assays at a concentration of 2 mg/ml unless otherwise stated.

Immunofluorescence and Immunogold Electron Microscopy—Indirect immunofluorescence detection of giantin, AP-1, ARF1, and β-COP was performed using the method described in Ref. 35. Images were acquired using a Nikon Optiphot microscope connected to a Hamamatsu C5985 chilled CCD camera (8 bit, 756 × 483 pixels) (for epifluorescence) or a Leica DMRXE microscope (for confocal sections). Immunogold electron microscopy was performed by centrifuging the supernatants from reconstituted reactions onto Formvar-coated nickel grids at 100,000 × *g* for 2 min in a Beckman Airfuge centrifuge. Samples were fixed in 8% paraformaldehyde, permeabilized for 2 min in 0.05% Triton X-100, and then treated as described in Ref. 36. Fc125 antibody was used as the primary antibody, and 10-nm colloidal gold-conjugated rabbit anti-mouse (Amersham Biosciences) was used as the secondary antibody. Phosphotungstic acid (2%) or uranyl acetate (4%) was used as a negative stain.

Protein Staging in the TGN—HeLa cells infected with replication-defective recombinant adenoviruses were incubated for 30 min at 37 °C (unless otherwise stated) in bicarbonate-free, cysteine-free, methionine-free Dulbecco's modified Eagle's medium and then metabolically radiolabeled for 10 min in the same medium supplemented with 50 μCi/ml Tran^{35}S Label (MP Biomedicals). The medium was then replaced with cold bicarbonate-free minimal essential medium, and the cells were incubated at 19 °C for 2 h to stage newly synthesized proteins in the TGN. The dishes were then transferred to an ice-cold aluminum plate, and the cells were perforated as described below prior to reconstitution of protein export.

Reconstitution of Vesicle Release from Perforated Cells—Protein export from the TGN was reconstituted using a modification of the assays described in Refs. 37 and 38. HeLa cells in 10-cm dishes were incubated for 10 min on ice in 10 mM HEPES, pH 7.2, 15 mM KCl, and the cells were then scraped into break buffer (BB; 50 mM HEPES, pH 7.2, 90 mM KCl) using a rubber policeman. The cells were then transferred to a 15-ml conical tube, and the buffer was adjusted to 500 mM KCl by addition of an equal volume of 50 mM HEPES, pH 7.2, 1 M KCl. The suspension was centrifuged at 800 × *g* in a Beckman GS-6R centrifuge, and the cell pellet was washed with BB and then resuspended in GGA buffer (25 mM HEPES, pH 7.4, 38 mM potassium glutamate, 38 mM potassium aspartate, 38 mM potassium gluconate, 2.5 mM $MgCl_2$, 2 mM EGTA free acid, 1 mM dithiothreitol). During the perforation procedure, Eppendorf tubes were preloaded with GGA buffer with or without an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 50 μg/ml creatine phosphokinase (all based on final concentrations in a total assay volume of 50 μl)), cytosol (2 mg/ml final concentration), and other reagents as described in individual experiments in a final volume of 25 μl. Aliquots of the perforated cell suspension (25 μl) were distributed into these tubes, and the samples were incubated at 37 °C for 1 h unless otherwise specified and then centrifuged in a tabletop microcentrifuge at 12,000 rpm for 2 min to pellet the cells. The supernatant (containing released vesicles) and pellet (cells) were collected separately. HA was immunoprecipitated from radioactive samples and quantitated by PhosphorImager analysis. Export of endogenous proteins from non-radiolabeled cells was analyzed by immunoblotting. Western blots were quantitated after densitometric scanning using QuantityOne software. Data were analyzed by Student's *t* test using SigmaStat software (Systat) unless otherwise indicated. $p < 0.05$ was considered to be statistically significant.

Protease Protection Assay—Supernatants from perforated cells reconstituted with an ATP-regenerating system and cytosol were incubated with 100 μg/ml trypsin (bovine pancreas, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, Sigma) in GGA buffer on ice in the presence or absence of 0.05% Triton X-100. After 10 min, soybean trypsin inhibitor was added to a final concentration of 200 μg/ml. The samples were then centrifuged at 100,000 × *g* for 30 min in a Sorvall RC M120EX centrifuge. Pelleted vesicles were solubilized, and HA was immunoprecipitated from the supernatants as described in Ref. 26. The samples were run on 12% SDS-polyacrylamide gels, dried, and quantified as described above.

β-COP and ARF1 Recruitment to Permeabilized Cells—Coatomer and ARF recruitment was assayed to confirm depletion of functional ARF from rat brain cytosol using the method described in Ref. 39. HeLa cells grown on coverslips were washed with GGA buffer and then permeabilized in GGA buffer with 40 μg/ml digitonin (Sigma) for 10 min at ambient temperature. The cells were then washed with 50 mM HEPES, pH 7.2, 500 mM KCl for 15 min on ice, then washed again with GGA buffer, and incubated with or without control or ARF-depleted cytosol and 500 μM GTPγS for 15 min at 37 °C. Samples were fixed with 4% paraformaldehyde and processed for indirect immunofluorescence using antibodies against β-COP, ARF1, AP-1γ, and giantin.

RESULTS

Reconstitution of HA Release from Perforated Cells—As a prelude to identifying cellular factors that regulate the TGN sorting and export of apical proteins, we tested the ability of several previously described *in vitro* transport assays to reconstitute the release of TGN-staged HA in epithelial cells. Our goal was to develop an assay that was reproducible, that was relatively robust, and that recapitulated *in vivo* sorting in the TGN. Although we could reconstitute pH-sensitive HA release from mechanically perforated polarized MDCK cells (27), ves-

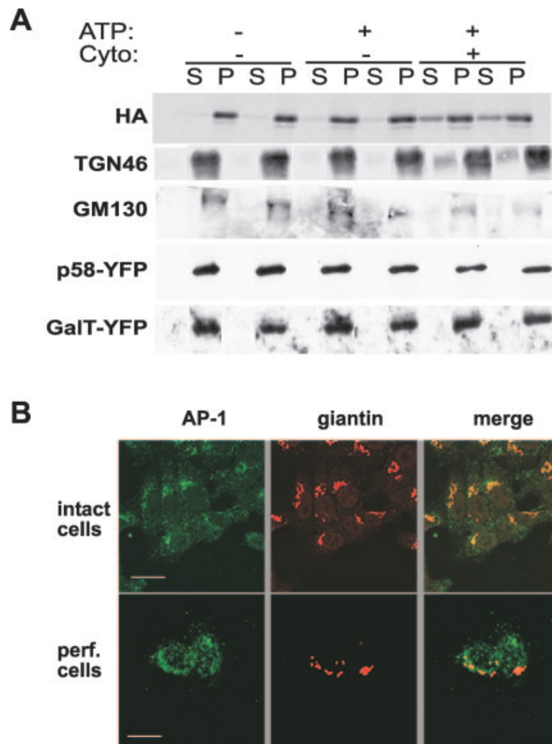

FIG. 1. **ATP- and cytosol-dependent release of TGN-staged HA from perforated cells.** *A*, HeLa cells expressing radiolabeled, TGN-staged HA were perforated as described under "Materials and Methods," and vesicle release was reconstituted in the presence or absence of an ATP-regenerating system (*ATP*) and cytosol (*Cyto*, 2 mg/ml). Released HA (*top panel*) was recovered in the supernatant (*S*) and compared with cell-associated HA in the pellet (*P*). The endogenous protein TGN46 was also released in an ATP- and cytosol-dependent manner as assessed by immunoblotting; however, the peripheral Golgi protein GM130, the *trans*-Golgi marker GalT-YFP, and p58-YFP, which is localized to the intermediate compartment and Golgi complex at steady state, were not released under these conditions. *B*, the cellular distribution of Golgi markers AP-1γ and giantin was examined by indirect immunofluorescence labeling of intact cells and perforated (*perf.*) HeLa cells after reconstitution of vesicle release. *Scale bar*, 15 μm.

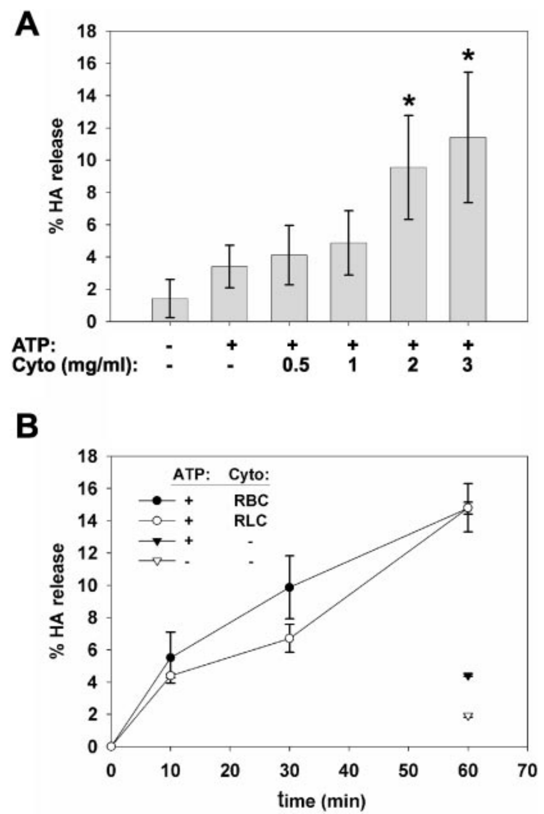

FIG. 2. **Kinetics and cytosol dose dependence of HA release.** *A*, HA release from permeabilized HeLa cells was reconstituted for 1 h in the presence of increasing concentrations of rat brain cytosol. *, p = 0.002 compared with ATP alone; n = 9. *B*, the kinetics of HA release were monitored in perforated HeLa cells incubated with 2 mg/ml rat brain cytosol (*RBC*) or rabbit liver cytosol (*RLC*). *Cyto*, cytosol.

icle release in this assay was inefficient, and the degree of perforation obtained was inconsistent. Reconstitution of HA release from isolated Golgi preparations purified from polarized MDCK cells was both efficient and reproducible but did not recapitulate physiological properties of sorting observed *in vivo*.[2] However, we found that release of TGN-staged HA from hypo-osmotically swelled, perforated HeLa cells was relatively robust, reproducible, and sensitive to conditions that inhibit HA transport *in vivo*. HeLa cells expressing HA were metabolically radiolabeled for 10 min, and newly synthesized HA was staged in the TGN by incubation for 2 h at 19 °C. Subsequently the cells were perforated by swelling and scraping, and vesicle release was reconstituted in the presence or absence of an ATP-regenerating system and cytosol. As shown in Fig. 1*A*, release of HA was robust only when both ATP and cytosol were present. The efficiency of HA release was dependent on the concentration of cytosol with maximal release observed at 2 mg/ml (Fig. 2*A*). Moreover, using this assay, we observed similar kinetics of HA release when transport was reconstituted using either rat brain or rabbit liver cytosol (Fig. 2*B*).

To confirm that HA release in this assay was not due to nonselective fragmentation of the Golgi/TGN upon reconstitution, we examined the release of Golgi residents in this assay. Reconstitution of vesicle release did not dislodge the Golgi-associated peripheral membrane protein GM130 (Fig. 1*A*). In addition, we did not observe measurable release of p58-YFP or the *trans*-Golgi marker GalT-YFP. However, release of TGN46, a TGN protein that constitutively cycles via the TGN and the basolateral cell surface of polarized MDCK cells (40), was very efficiently reconstituted in the presence of ATP and cytosol (Fig. 1*A*). Efficient release of this protein from purified rat liver Golgi membranes has been reported previously by another group (41). The release of TGN46 in our assay likely represents release of this protein from the TGN as opposed to the budding of endocytic vesicles from the cell surface because 1) this protein localizes to the TGN in HeLa cells at steady state and after incubation at 19 °C and 2) the small population of TGN46 that can be biotinylated at the plasma membrane at steady state was not released into the medium upon cell perforation and reconstitution.[2]

We also used immunofluorescence microscopy to examine the effect of perforation and reconstitution on the morphology and localization of the Golgi complex/TGN. Staining with antibodies directed against the Golgi-resident protein giantin and the TGN-localized AP-1 complex demonstrated that these compartments remain concentrated in the perinuclear region of perforated cells after reconstitution, similar to their localization in intact cells (Fig. 1*B*).

A prediction of our assay is that TGN-staged HA should be released in intact membrane-bound carriers. To test this, we first combined immunoelectron microscopy with negative staining to visualize the membranes of released carriers containing HA. Released transport carriers were fixed and processed for visualization of HA-positive structures. Anti-HA antibodies localized to membrane-limited, spherical vesicles with

[2] M. A. Ellis and O. A. Weisz, unpublished observations.

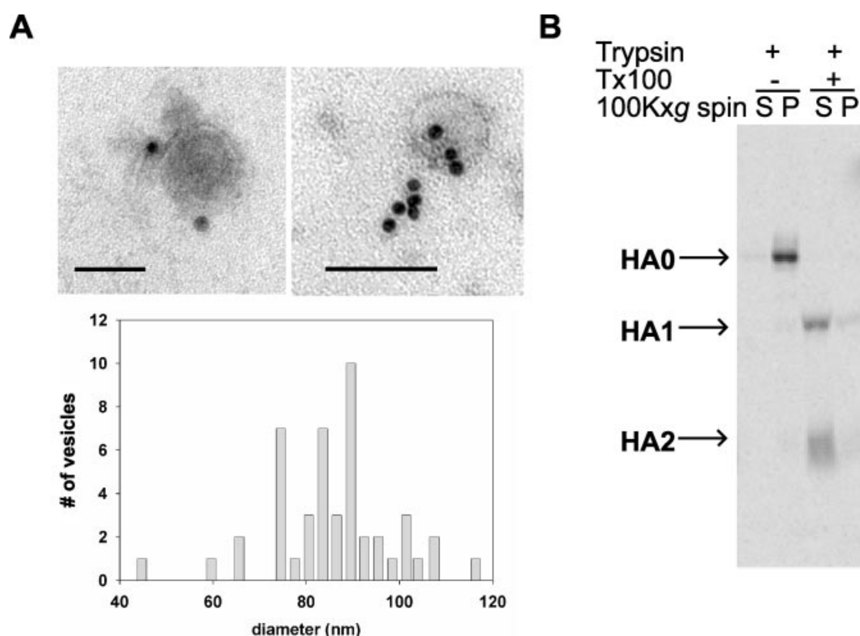

FIG. 3. **Released HA is recovered in membrane-bound vesicles.** *A*, newly synthesized HA was staged in the TGN of HeLa cells by incubation for 2 h at 19 °C, and vesicle release was reconstituted after perforation. HA-containing vesicles released into the supernatant were visualized by immunogold labeling followed by negative staining. Two representative vesicles are shown. *Scale bars*, 100 nm. The *lower panel* shows the histogram of vesicle diameter (nm) measured from 47 vesicles (mean diameter ± S.D. = 86 ± 13 nm). *B*, released HA is protected from luminal proteolytic cleavage. Radiolabeled HA released from perforated, reconstituted cells was treated with trypsin in the absence or presence of detergent (Triton X-100 (*Tx100*)). After addition of soybean trypsin inhibitor to quench the reaction, the sample was centrifuged at 100,000 × *g*, and HA was immunoprecipitated from the resulting supernatant (*S*) and pellet (*P*). The mobilities of intact (*HA0*) and the two HA trypsin cleavage products (*HA1* and *HA2*) on the SDS-polyacrylamide gel are marked.

an average diameter of 86 ± 13 nm (mean ± S.D., $n = 47$; Fig. 3*A*). To examine the topology of released HA, we examined whether the protease-sensitive luminal domain was protected from trypsin cleavage. Supernatants from a transport reconstitution experiment were incubated with trypsin in the presence or absence of Triton X-100 and then centrifuged at high speed to pellet membrane-bound vesicles (Fig. 3*B*). In the absence of detergent, cleavage of HA was virtually undetectable, and nearly all of the protein was recovered intact in the pelleted vesicle fraction. By contrast, when Triton X-100 was included, HA was efficiently cleaved and was recovered primarily in the supernatant.

Nucleotide Dependence of HA Release—A number of GTPases have been implicated in the delivery of HA from the TGN to the plasma membrane, including ARF1 and $G_s\alpha$ (42, 43). To determine whether there is a requirement for GTP or GTP hydrolysis in HA release, we tested the effect of poorly hydrolyzable GTP analogs in our assay (44). Inclusion of GTPγS in the reconstitution reaction decreased HA release in a dose-dependent manner with complete inhibition of ATP-dependent release observed at 100 μM concentration (Fig. 4*A*). However, the more poorly hydrolyzable GTP analog GMP-PNP did not inhibit HA release even at high concentration (Fig. 4*C*). Neither GTPγS nor GMP-PNP affected TGN46 release at 100 μM concentration, indicating that GTP hydrolysis is not required for efficient release of TGN46 from the TGN (Fig. 4, *B* and *D*).

HA Release Is Sensitive to in Vivo Perturbants of Biosynthetic Traffic—We have observed previously that disruption of TGN pH by expression of the acid-activated ion channel influenza M2 selectively inhibits efficient release of apical proteins including HA from the TGN in intact MDCK and HeLa cells (26, 27). To examine whether this requirement for acidic TGN luminal pH is preserved in our perforated cell assay system, we examined HA release in HeLa cells co-infected with adenovirus encoding either M2 or as a control M2 in the reverse orientation. After radiolabeling and incubation at 19 °C, cells were perforated, and transport was reconstituted. One set of M2-containing samples was reconstituted in the presence of the M2 channel blocker amantadine. HA release in perforated cells expressing active M2 was decreased by roughly 50% relative to HA release observed in control cells (Fig. 5*A*). Moreover the effect of M2 on HA release was abrogated by inclusion of amantadine in the reconstitution mixture. The effect of M2 on HA release was reproducible and statistically significant ($p <$ 0.001 *versus* control cells and $p = 0.04$ *versus* M2-expressing cells reconstituted in the presence of amantadine by paired *t* test analysis of nine independent experiments). By contrast, release of the basolaterally targeted protein TGN46 was unaffected by expression of active M2 (Fig. 5*B*). In the experiment shown, a slight inhibition was observed in the presence of amantadine; however, this was neither statistically significant nor reproducible.

ARF-independent Release of HA—A role for ARF1 has been suggested in numerous transport steps through and from the Golgi complex, but whether it functions in the release of apical proteins from the TGN is not known. Although ARF1 is dissociated from the TGN in intact MDCK cells by brefeldin A, this drug does not function *in vitro* (45). Therefore, to address the role of ARF1 in HA release *in vitro*, we used anion-exchange chromatography to deplete rat brain cytosol of ARF1. Immunoblotting of the resulting cytosol routinely demonstrated removal of >87% of ARF1 with no loss of β-COP (Fig. 6*A*).

β-COP is readily recruited to the Golgi complex of digitonin-permeabilized cells incubated with exogenous cytosol in the presence of ARF1 and GTPγS. Whereas we observed robust binding of β-COP to the Golgi complex in permeabilized cells incubated with control cytosol, only background levels of binding were observed in cells incubated with ARF-depleted cytosol (Fig. 6*B*). Furthermore recruitment of clathrin to the Golgi complex/TGN was also decreased by depletion of ARF1 from cytosol, confirming that recruitment of these coats in this system is still dependent on ARF1 function.[2]

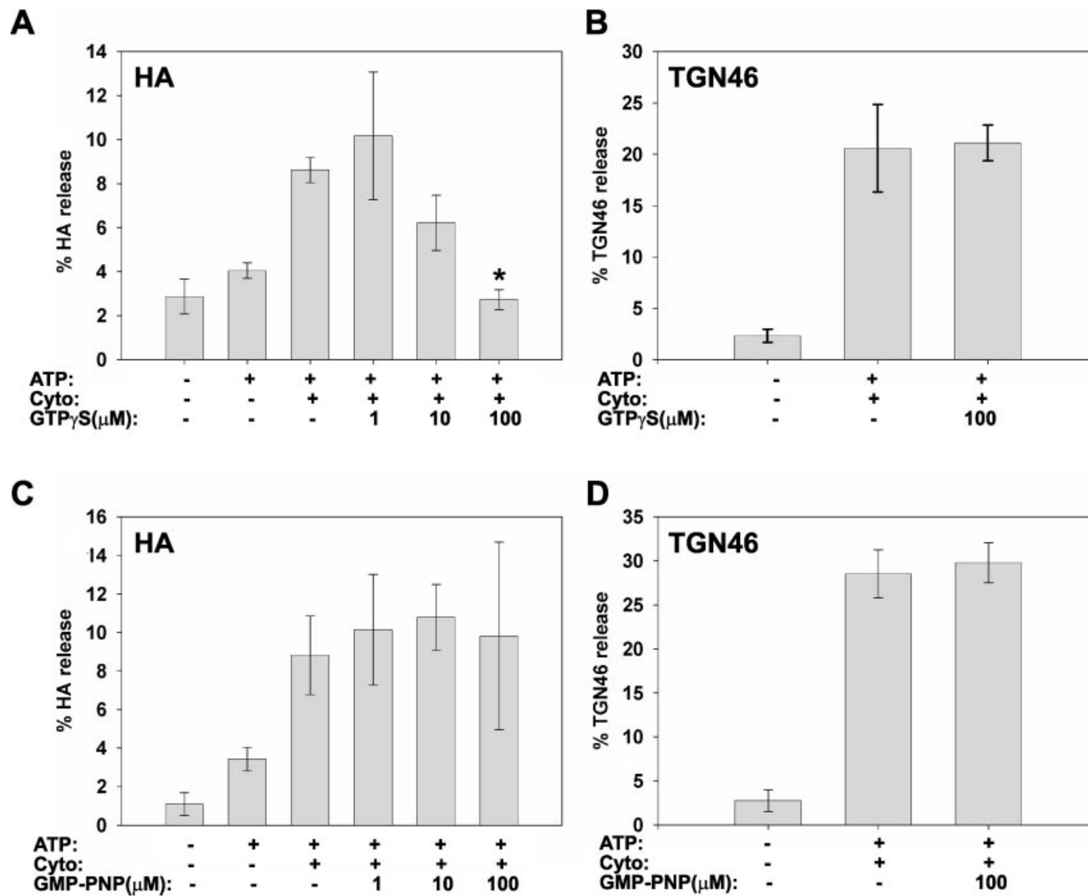

FIG. 4. **Effect of GTPγS and GMP-PNP on release of HA and TGN46 from the TGN.** The release of TGN-staged HA (*A* and *C*) and TGN46 (*B* and *D*) was quantitated in the presence of increasing concentrations of GTPγS (*A* and *B*) or GMP-PNP (*C* and *D*). The mean ± S.D. of triplicate samples is plotted in each case. Similar results were obtained in three independent experiments (*, $p < 0.001$ *versus* 0 μM GTPγS). *Cyto*, cytosol.

We next examined the effect of ARF-depleted cytosol on the release of endogenous proteins in HeLa cells in our *in vitro* assay. Uninfected HeLa cells were radiolabeled for 15 min and then incubated in chase medium at 20 °C for 2 h to accumulate newly synthesized proteins in the TGN. After perforation, vesicle release was reconstituted using ATP and control or ARF-depleted cytosol. Released vesicles were centrifuged in high salt-containing buffer to remove peripheral proteins whose association with membranes might be ARF-dependent, and the protein profile in the pelleted vesicles was analyzed by SDS-PAGE and densitometry (Fig. 6*C*). While the overall profile of proteins released in the presence of control and ARF-depleted cytosol was similar, we reproducibly observed a decrease in the release of a ~58-kDa protein (p58) when transport was reconstituted using ARF-depleted cytosol (Fig. 6*C*). Together these data suggest that ARF depletion effectively inhibits ARF1 function in perforated cells.

We next examined the effect of ARF-depleted cytosol on release of HA and TGN46 from perforated cells. By contrast with p58, whose release was inhibited by ~50% (Fig. 6*D*, *upper panel*), depletion of ARF1 had no effect on the release of TGN46 (Fig. 6*D*, *middle panel*) or HA (Fig. 6*D*, *bottom panel*).

Others have shown that when ARF is depleted from cytosol, release of proteins can still occur *in vitro* but is no longer sensitive to inhibition by poorly hydrolyzable GTP analogs (34, 45). We therefore tested whether HA release in the presence of ARF-depleted cytosol was affected by GTPγS. Release of HA was inhibited under these conditions at the same concentrations of GTPγS that blocked release in control cytosol (Fig. 7*A*). Moreover expression of active M2 inhibited HA release reconstituted in the presence of ARF-depleted cytosol, suggesting that HA is not shunted into alternative transport carriers in the absence of ARF1 (Fig. 7*B*). Together these results suggest that the release of TGN-derived transport carriers containing HA is not regulated by ARF1 and by inference does not require the function of known adaptors and coat proteins.

DISCUSSION

Identifying the molecular requirements for specific steps in protein trafficking has proven difficult due to the numerous compartments within the biosynthetic pathway. To circumvent this issue, many studies have utilized *in vitro* assays to reconstitute a specific step in membrane transport. Here we used a perforated cell assay to examine the requirements for TGN export of influenza hemagglutinin, a raft-associated protein that has apical sorting information when expressed in polarized epithelial cells. Release of TGN-staged HA from perforated, salt-washed cells was dependent on addition of an ATP-regenerating system and cytosol and was not due to nonspecific fragmentation of the Golgi complex as no release of peripheral or integral Golgi proteins was observed. HA was released in the correct topology in roughly spherical intact transport carriers with an average diameter of 86 ± 13 nm. Importantly, the assay allows rapid removal of cytosolic components prior to reconstitution and appears to preserve physiological pH gradients within organelles as expression of the acid-activated proton channel influenza M2 inhibited the release of HA in our assay. By contrast, while we were also able to efficiently reconstitute HA release from TGN-containing Golgi membranes isolated by subcellular fractionation, release in this assay was insensitive to M2 activity.[2] Interestingly, HA release from perforated cells was profoundly inhibited by GTPγS but was un-

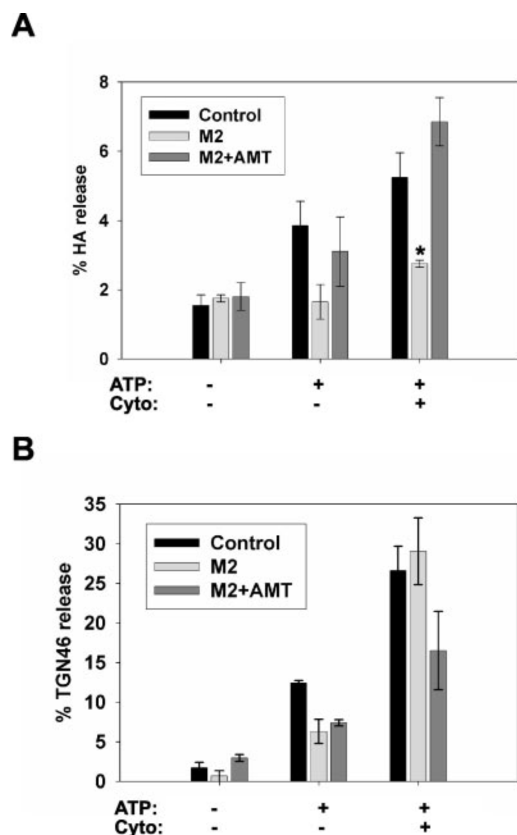

FIG. 5. **Disruption of TGN luminal pH inhibits HA but not TGN46 release from perforated cells.** *A*, HA release was measured from permeabilized cells co-infected with adenovirus encoding M2 or a control virus (encoding the M2 gene in the reverse orientation). Amantadine (*AMT*, 5 μM) was included during reconstitution of the indicated M2-expressing samples to inhibit M2 activity. The mean release ± range of duplicate samples is plotted. A representative experiment is shown; statistical significance was calculated by paired *t* test of nine independent experiments (*, $p < 0.001$ relative to control; **, $p = 0.04$ relative to amantadine-treated samples). *B*, TGN46 release from perforated M2-expressing or control cells was quantitated by immunoblotting. The mean release ± range of duplicate samples is plotted. This experiment was performed four times with similar results. The slight inhibition observed in amantadine-treated cells in this experiment is due to an aberrant point in this experiment and is not statistically significant. *Cyto*, cytosol.

affected by depletion of clathrin[2] or ARF1, a GTPase that regulates the binding of all known coats and adaptor proteins to the TGN. Thus, the formation of HA-containing transport carriers appears to proceed via a novel mechanism.

We also reconstituted ATP- and cytosol-dependent release of TGN46, a protein localized to the TGN at steady state but that cycles constitutively through the basolateral cell surface of polarized epithelial cells (40, 46). The release of TGN46 in our assay system was remarkably robust (typically ~40% within 30 min), consistent with a previous report that also noted very efficient release of the rat homolog of TGN46 (TGN38) from isolated Golgi membranes (41). TGN46 colocalizes with green fluorescent protein-tagged vesicular stomatitis virus glycoprotein (VSV G) to transport carriers emanating from the TGN, suggesting that this protein is an appropriate marker for the basolateral biosynthetic pathway (12, 14). However, release of TGN-staged VSV G in our assay system was consistently less efficient than release of TGN46.[3] The difference in TGN export efficiency may reflect distribution of TGN46 into alternate export pathways; for example, previous studies have demonstrated rapid cycling of this protein between the TGN and endosomes (47, 48).

In contrast to the release of HA, export of TGN46 from the TGN was insensitive to expression of active influenza M2. TGN-staged VSV G release was also insensitive to M2 in our assay system.[4] These results are consistent with our previously published studies demonstrating a selective effect of M2 expression on the TGN export of apically sorted proteins in polarized MDCK cells (27) and add further support to the hypothesis that segregation of apical and basolateral proteins into distinct transport carriers also occurs in nonpolarized cells.

Cytosol prepared from a number of sources, including rat brain, rabbit liver, and MDCK cells, was able to support HA release with similar dose dependence. The ability to use various cytosol sources allows the potential modification of cytosolic components by using either genetically altered animals or modified cells. The kinetics of HA release could be monitored in our assay, and similar rates of HA release were observed when TGN export was reconstituted using either rat brain or rabbit liver cytosol. Previous studies have implicated specific cytosolic proteins, including members of the annexin family (49, 50), a heterotrimeric G_s protein (42), dynamin (51), and Cdc42 (52) in the TGN export of apical proteins. The ability to measure the rate of protein release from the TGN should prove useful in experiments designed to determine the order in which different cytoplasmic components function in the formation of HA-containing transport carriers.

Guanine nucleotide analogs have been used extensively to mimic GTP in systems where the nucleotide would be rapidly hydrolyzed. We found that GTPγS but not GMP-PNP inhibited HA release from the TGN, while TGN46 release was not affected by either GTP analog. Our result is consistent with the previous demonstration that HA traffic from the TGN to the apical plasma membrane in streptolysin O-permeabilized MDCK cells was inhibited by GTPγS (43). GMP-PNP and GTPγS are structurally very different, and binding of these nucleotides differentially alters the conformation of the small G protein H-Ras (53). Differences between the effects of GTP analogs on membrane transport have been previously reported: for example, ARF1 recruitment to Golgi membranes is enhanced by GTPγS but not by GMP-PNP (54).

Morphology of TGN-derived Transport Carriers—Although numerous adaptor and coat proteins are known to bind TGN membranes, the requirement for these proteins in polarized biosynthetic transport remains unclear. Simon *et al.* (13, 55) demonstrated that release of sialylated VSV G from Golgi-enriched MDCK membranes requires ARF and occurs via COPI-coated vesicles, whereas another group has suggested a requirement for AP-3 in the TGN export of VSV G (10). Vesicular and tubular structures coated with p230, a peripheral protein of unknown function, have also been observed emanating from the TGN; however, whether biosynthetic cargo molecules are enriched in these carriers is not known (56). Gα-interacting protein has been reported to regulate the fission of VSV-G-containing TGN-derived tubules that are devoid of clathrin or coatomer (57), and a similar redistribution of VSV G and TGN46 was observed upon expression of a dominant-negative mutant of protein kinase D (12). Finally Polishchuk *et al.* (14) recently reported that export of VSV G from the TGN is independent of coat formation and occurs via the protrusion and detachment of large sections of TGN membrane that also contain TGN46. These differences in transport requirements may reflect a physiological redundancy in the routes that VSV

[3] M. A. Ellis, M. T. Miedel, and O. A. Weisz, unpublished observation.

[4] M. T. Miedel and O. A. Weisz, unpublished observation.

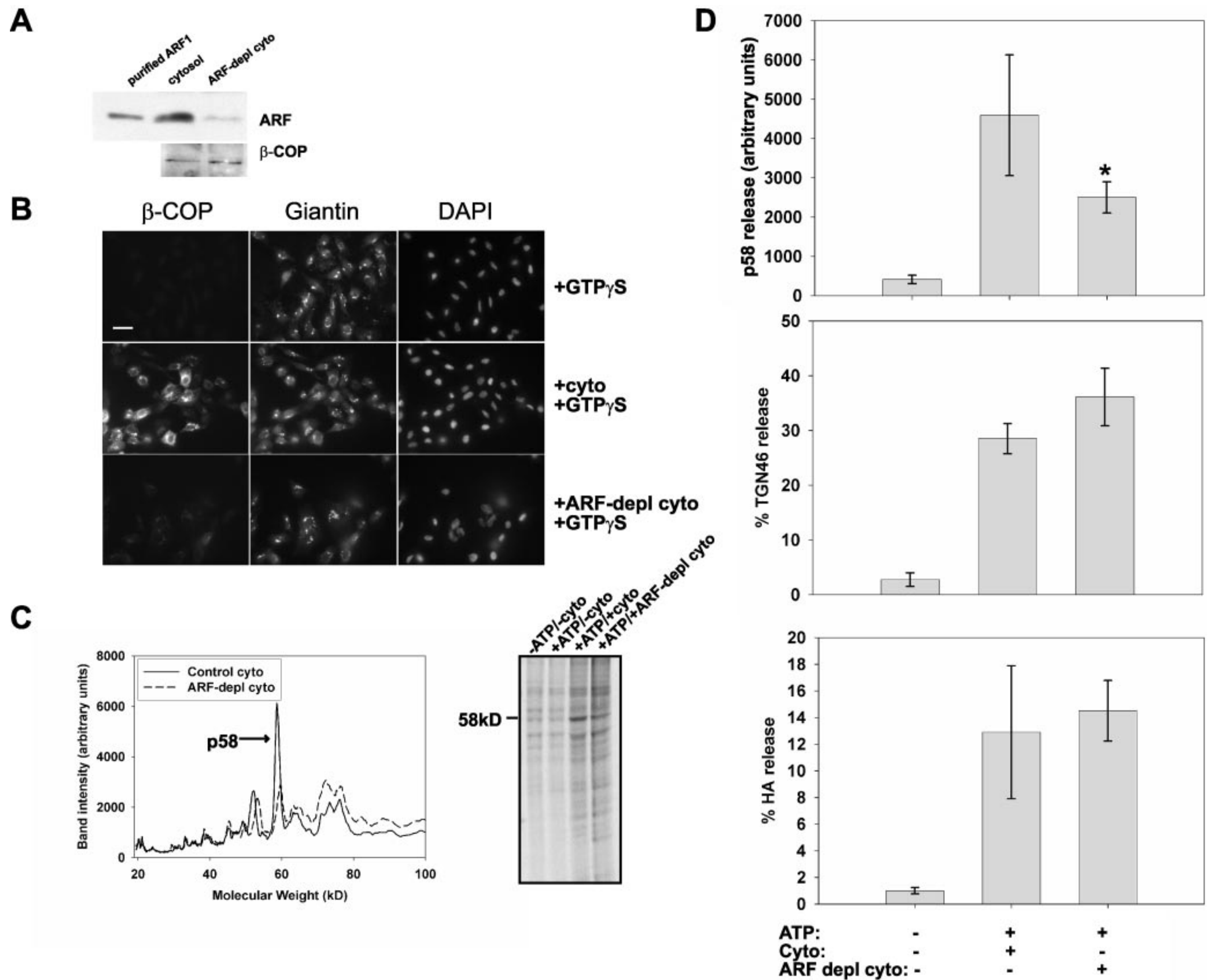

FIG. 6. **HA release from the TGN is ARF-independent.** *A*, immunoblotting of ARF-depleted *versus* control cytosol using antibodies against ARF (*upper panel*) and β-COP (*lower panel*). Purified bovine ARF1 was included as a positive control. In this preparation, the resulting cytosol was depleted of ARF relative to control cytosol by 87%. *B*, digitonin-permeabilized HeLa cells were incubated with 500 μM GTPγS and 2 mg/ml control or ARF-depleted cytosol for 10 min at 37 °C prior to fixation and processing for indirect immunofluorescence to detect β-COP or giantin. Cell nuclei were stained using 4,6-diamidino-2-phenylindole (*DAPI*). *Scale bar*, 25 μm. *C*, uninfected HeLa cells were briefly radiolabeled and then incubated for 2 h at 19 °C to stage newly synthesized endogenous proteins in the TGN. Vesicle release was reconstituted after cell perforation in the presence or absence of an ATP-regenerating system (*ATP*) and with control or ARF-depleted cytosol. Released vesicles were centrifuged at 100,000 × *g* in the presence of 1 M KCl to remove peripheral proteins, and the pelleted vesicles were solubilized and analyzed by SDS-PAGE. The release of a 58-kDa protein (p58, *arrow*) was reduced upon reconstitution with ARF-depleted cytosol compared with control. The corresponding trace shows the intensity of bands in the gel (*right two lanes*); the band corresponding to p58 is marked. *D*, the effect of ARF-depleted cytosol on the release of p58 (*top panel*; mean ± S.D. of four experiments; *, $p = 0.03$ relative to control cytosol), TGN46 (*middle panel*; mean ± S.D. of triplicate samples), and HA (*lower panel*; mean ± S.D. of triplicate samples) is plotted. *Cyto*, cytosol; *depl*, depleted.

G can use to reach the cell surface; alternatively at high levels of expression or upon perturbation of the regulatory mechanisms that control cargo loading and export from the TGN, VSV G may be rerouted to alternative pathways that are not normally prominent.

How the sorting and TGN export of proteins that are targeted apically in polarized cells is regulated is no better understood. Apical targeting signals on many proteins, including HA, are localized to the transmembrane or luminal domains of these molecules and are thus unavailable to bind directly to cytoplasmic adaptors (24, 58). Thus, it is not surprising that the regulation of apically destined protein export would be distinct from basolateral transport as we observed. Indeed the molecular requirements for *fusion* of TGN-derived carriers with the apical plasma membrane of MDCK cells are known to be distinct from those involved in basolateral delivery (59).

Several studies have used live cell imaging to characterize Golgi export of fluorescent apical marker proteins in both nonpolarized and polarized cells. Kreitzer *et al.* (51) described a mixture of vesicular and very long tubular carriers enriched in a non-raft-associated apical cargo molecule that emanated from the TGN of MDCK cells and fused with the plasma membrane. Other groups have reported that a fluorescent apically targeted glycosylphosphatidylinositol-anchored (raft-associated) protein is transported from the TGN primarily in "small" spherical carriers in both nonpolarized and polarized cells compared with transport carriers enriched in basolateral cargo (5, 7). In contrast, Jacob and Naim (6) described the release of large globular structures from the TGN that contained both raft-associated and raft-independent apically targeted cargoes; these large structures resolved into distinct carriers later during transport. Most recently, Polishchuk *et al.* (60) reported

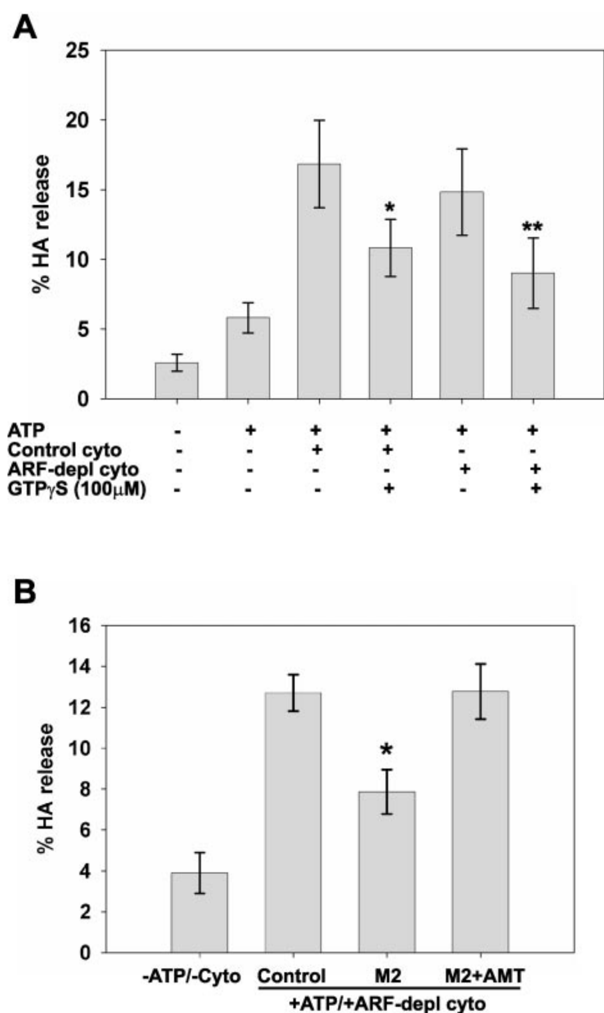

FIG. 7. **ARF-independent HA release from the TGN is inhibited by GTPγS and by expression of influenza M2.** *A*, HA release from the TGN of cells reconstituted in the presence of ARF-depleted or control cytosol with or without 100 μM GTPγS. A representative experiment (mean ± S.D. of triplicate samples) is shown; statistical significance was calculated by *t* test analysis of four experiments (*, $p = 0.013$ *versus* control; **, $p = 0.015$ *versus* ARF-depleted cytosol). *B*, HA release from the TGN of cells expressing M2 was reconstituted in the presence of ARF-depleted or control cytosol. Amantadine (*AMT*) was included in the indicated samples. The graph represents the mean ± S.E. of nine samples (*, $p = 0.003$ *versus* control and $p = 0.012$ *versus* M2 + amantadine). *Cyto*, cytosol; *depl*, depleted.

that raft-associated glycosylphosphatidylinositol-anchored proteins are sorted from the TGN to the basolateral membrane of MDCK cells in the same carriers that contain VSV G and then selectively transcytosed to the apical membrane.

In contrast to the wide variety of morphologies observed in live cells, the size and structure of transport carriers we isolated upon *in vitro* reconstitution of HA export was relatively uniform (86 ± 13-nm diameter). Our data are very consistent with a previous study by Wandinger-Ness *et al.* (61), who reported a diameter of 78 ± 15 nm for TGN-derived, HA-containing vesicles immunoisolated from perforated MDCK cells. Although the vesicles we identified were devoid of a noticeable coat, they were comparable in size to COPI- (50 ± 16-nm diameter (62)), COPII- (70 ± 20-nm diameter (63)), and clathrin-coated (95.9 ± 10.5-nm diameter (64)) vesicles. It is possible that tubule formation is inefficient in our *in vitro* reconstitution assay; alternatively large globular or tubular transport carriers may not be efficiently released or separated from perforated cells.

HA Export from the TGN Is ARF1-independent—ARF1 is required for the binding of all adaptor and coat proteins that have been localized to the TGN. As such, we were somewhat surprised to find no requirement for ARF1 in the release of either HA or TGN46. Depletion of ARF1 from the cytosol used to reconstitute TGN export inhibited the association of coatomer and clathrin with the Golgi complex, but an effect on the release of only a single protein could be detected. Happe and Weidman (34) have previously observed that intra-Golgi transport was also efficiently reconstituted in the absence of ARF1 but was no longer sensitive to inhibition by GTPγS. However, we found that HA release in the absence of ARF1 was still inhibited by GTPγS. We conclude that another GTP-binding protein(s) is required for the formation of HA-containing transport carriers.

Previous studies have suggested a role for ARF1 in apical biosynthetic transport. Brefeldin A, a drug that inhibits ARF1 function, has been reported to cause rerouting of apical proteins, including HA, to the basolateral cell surface of MDCK cells (65–67). While the drug has no effect on the morphology of the Golgi complex of MDCK cells, brefeldin A treatment resulted in the redistribution of both HA and a chimeric protein containing TGN38-derived sorting information into a network of tubules emanating from the TGN (67). However, cell surface delivery of basolateral markers in MDCK cells was unaffected even at high concentrations of brefeldin A (65, 66). These results are most consistent with a role for ARF1 in sorting of apical cargo as opposed to the formation of apically destined vesicles. To test this idea, we asked whether TGN export of HA upon reconstitution with ARF-depleted cytosol was sensitive to inhibition by influenza M2. We found that HA release in ARF-depleted cytosol was still inhibited by expression of active M2. This suggests that the fidelity of HA sorting into transport carriers devoid of basolaterally destined proteins remained intact in the absence of ARF1 function.

Although we found no evidence for a direct role for ARF1 in HA export from the TGN, it is possible that ARF1 is important for other steps in TGN-to-cell surface transport. For example, ARF1 could mediate retrograde trafficking of proteins whose function is required for sorting. It has been hypothesized that lectin-like sorting receptors may be responsible for recognition of glycan sorting signals on some apical proteins in the TGN (20). An alternative hypothesis is that ARF1 mediates the recruitment of molecular motors that drive surface delivery of TGN-derived vesicles. ARF1 has recently been implicated in recruitment of the Arp2/3 complex to the Golgi (68, 69). In conjunction with N-WASP, the Arp2/3 complex can drive actin polymerization that propels HA-containing transport carriers from the Golgi region to the cell periphery (70). Thus ARF1 may function in post-Golgi delivery of newly synthesized apical proteins to the plasma membrane *in vivo*, but this function may not be observed in our *in vitro* assay.

In summary, we used an *in vitro* reconstitution assay that faithfully preserves *in vivo* requirements for polarized protein sorting to demonstrate that the formation of TGN-derived transport carriers enriched in a raft-associated apical protein requires cytosolic factors but occurs independently of ARF1 function. The existence of apical sorting mechanisms independent of ARF1 at the TGN is a novel finding. Future experiments will be directed toward identifying the cytosolic machinery involved in this transport step. In addition, it will be important to determine whether similar factors are involved in TGN release of raft-independent apical cargo molecules that contain different sorting information.

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REFERENCES

1. Nelson, W. J., and Yeaman, C. (2001) *Trends Cell Biol.* **11,** 483–486
2. Rodriguez-Boulan, E., Müsch, A., and Le Bivic, A. (2004) *Curr. Opin. Cell Biol.* **16,** 436–442
3. Müsch, A., Xu, H., Shields, D., and Rodriguez-Boulan, E. (1996) *J. Cell Biol.* **133,** 543–558
4. Yoshimori, T., Keller, P., Roth, M. G., and Simons, K. (1996) *J. Cell Biol.* **133,** 247–256
5. Keller, P., Toomre, D., Diaz, E., White, J., and Simons, K. (2001) *Nat. Cell Biol.* **3,** 140–149
6. Jacob, R., and Naim, H. Y. (2001) *Curr. Biol.* **11,** 1444–1450
7. Rustom, A., Bajohrs, M., Kaether, C., Keller, P., Toomre, D., Corbeil, D., and Gerdes, H. H. (2002) *Traffic* **3,** 279–288
8. Bonifacino, J. S., and Traub, L. M. (2003) *Annu. Rev. Biochem.* **72,** 395–447
9. Simmen, T., Honing, S., Icking, A., Tikkanen, R., and Hunziker, W. (2002) *Nat. Cell Biol.* **4,** 154–159
10. Nishimura, N., Plutner, H., Hahn, K., and Balch, W. E. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 6755–6760
11. Folsch, H., Pypaert, M., Maday, S., Pelletier, L., and Mellman, I. (2003) *J. Cell Biol.* **163,** 351–362
12. Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., Van Lint, J., and Malhotra, V. (2001) *Cell* **104,** 409–420
13. Simon, J. P., Shen, T. H., Ivanov, I. E., Gravotta, D., Morimoto, T., Adesnik, M., and Sabatini, D. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1073–1078
14. Polishchuk, E. V., Di Pentima, A., Luini, A., and Polishchuk, R. S. (2003) *Mol. Biol. Cell* **14,** 4470–4485
15. Chuang, J. Z., and Sung, C. H. (1998) *J. Cell Biol.* **142,** 1245–1256
16. Takeda, T., Yamazaki, H., and Farquhar, M. G. (2003) *Am. J. Physiol.* **284,** C1105–C1113
17. Potter, B. A., Ihrke, G., Bruns, J. R., Weixel, K. M., and Weisz, O. A. (2004) *Mol. Biol. Cell* **15,** 1407–1416
18. Yeaman, C., Le Gall, A. H., Baldwin, A. N., Monlauzeur, L., Le Bivic, A., and Rodriguez-Boulan, E. (1997) *J. Cell Biol.* **139,** 929–940
19. Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H. P., and Matter, K. (1998) *EMBO J.* **17,** 1919–1929
20. Scheiffele, P., and Fullekrug, J. (2000) *Essays Biochem.* **36,** 27–35
21. Alfalah, M., Jacob, R., Preuss, U., Zimmer, K. P., Naim, H., and Naim, H. Y. (1999) *Curr. Biol.* **9,** 593–596
22. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
23. Tall, R. D., Alonso, M. A., and Roth, M. G. (2003) *Traffic* **4,** 838–849
24. Lin, S., Naim, H. Y., Rodriguez, A. C., and Roth, M. G. (1998) *J. Cell Biol.* **142,** 51–57
25. Keller, P., and Simons, K. (1998) *J. Cell Biol.* **140,** 1357–1367
26. Henkel, J. R., and Weisz, O. A. (1998) *J. Biol. Chem.* **273,** 6518–6524
27. Henkel, J. R., Gibson, G. A., Poland, P. A., Ellis, M. A., Hughey, R. P., and Weisz, O. A. (2000) *J. Cell Biol.* **148,** 495–504
28. Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992) *Cell* **69,** 517–528
29. Ciampor, F., Bayley, P. M., Nermut, M. V., Hirst, E. M., Sugrue, R. J., and Hay, A. J. (1992) *Virology* **188,** 14–24
30. Grambas, S., and Hay, A. J. (1992) *Virology* **190,** 11–18
31. Henkel, J. R., Apodaca, G., Altschuler, Y., Hardy, S., and Weisz, O. A. (1998) *Mol. Biol. Cell* **9,** 2477–2490
32. Apodaca, G., Cardone, M. H., Whiteheart, S. W., DasGupta, B. R., and Mostov, K. E. (1996) *EMBO J.* **15,** 1471–1481
33. Traub, L. M., Bannykh, S. I., Rodel, J. E., Aridor, M., Balch, W. E., and Kornfeld, S. (1996) *J. Cell Biol.* **135,** 1801–1814
34. Happe, S., and Weidman, P. (1998) *J. Cell Biol.* **140,** 511–523
35. Bruns, J. R., Ellis, M. A., Jeromin, A., and Weisz, O. A. (2002) *J. Biol. Chem.* **277,** 2012–2018
36. Altschuler, Y., Kinlough, C. L., Poland, P. A., Bruns, J. B., Apodaca, G., Weisz, O. A., and Hughey, R. P. (2000) *Mol. Biol. Cell* **11,** 819–831
37. Ling, W. L., Siddhanta, A., and Shields, D. (1998) *Methods* **16,** 141–149
38. Beckers, C. J., Keller, D. S., and Balch, W. E. (1987) *Cell* **50,** 523–534
39. Acharya, U., Mallabiabarrena, A., Acharya, J. K., and Malhotra, V. (1998) *Cell* **92,** 183–192
40. Rajasekaran, A. K., Humphrey, J. S., Wagner, M., Miesenbock, G., Le Bivic, A., Bonifacino, J. S., and Rodriguez-Boulan, E. (1994) *Mol. Biol. Cell* **5,** 1093–1103
41. McLauchlan, H. J., James, J., Lucocq, J. M., and Ponnambalam, S. (2001) *Cell Biol. Int.* **25,** 705–713
42. Pimplikar, S. W., and Simons, K. (1993) *Nature* **362,** 456–458
43. Gravotta, D., Adesnik, M., and Sabatini, D. D. (1990) *J. Cell Biol.* **111,** 2893–2908
44. Stumber, M., Herrmann, C., Wohlgemuth, S., Kalbitzer, H. R., Jahn, W., and Geyer, M. (2002) *Eur. J. Biochem.* **269,** 3270–3278
45. Spiro, D. J., Taylor, T. C., Melancon, P., and Wessling-Resnick, M. (1995) *J. Biol. Chem.* **270,** 13693–13697
46. Reaves, B. J., Banting, G., and Luzio, J. P. (1998) *Mol. Biol. Cell* **9,** 1107–1122
47. Reaves, B., and Banting, G. (1994) *FEBS Lett.* **345,** 61–66
48. Banting, G., Maile, R., and Roquemore, E. P. (1998) *J. Cell Sci.* **111,** 3451–3458
49. Lafont, F., Lecat, S., Verkade, P., and Simons, K. (1998) *J. Cell Biol.* **142,** 1413–1427
50. Jacob, R., Heine, M., Eikemeyer, J., Frerker, N., Zimmer, K. P., Rescher, U., Gerke, V., and Naim, H. Y. (2004) *J. Biol. Chem.* **279,** 3680–3684
51. Kreitzer, G., Marmorstein, A., Okamoto, P., Vallee, R., and Rodriguez-Boulan, E. (2000) *Nat. Cell Biol.* **2,** 125–127
52. Müsch, A., Cohen, D., Kreitzer, G., and Rodriguez-Boulan, E. (2001) *EMBO J.* **20,** 2171–2179
53. Hu, J. S., and Redfield, A. G. (1997) *Biochemistry* **36,** 5045–5052
54. Traub, L. M., Ostrom, J. A., and Kornfeld, S. (1993) *J. Cell Biol.* **123,** 561–573
55. Simon, J. P., Ivanov, I. E., Shopsin, B., Hersh, D., Adesnik, M., and Sabatini, D. D. (1996) *J. Biol. Chem.* **271,** 16952–16961
56. Gleeson, P. A., Anderson, T. J., Stow, J. L., Griffiths, G., Toh, B. H., and Matheson, F. (1996) *J. Cell Sci.* **109,** 2811–2821
57. Wylie, F. G., Lock, J. G., Jamriska, L., Khromykh, T., Brown, D. L., and Stow, J. L. (2003) *Traffic* **4,** 175–189
58. Altschuler, Y., Hodson, C., and Milgram, S. L. (2003) *Curr. Opin. Cell Biol.* **15,** 423–429
59. Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C., and Simons, K. (1995) *Cell* **81,** 571–580
60. Polishchuk, R., Di Pentima, A., and Lippincott-Schwartz, J. (2004) *Nat. Cell Biol.* **6,** 297–307
61. Wandinger-Ness, A., Bennett, M. K., Antony, C., and Simons, K. (1990) *J. Cell Biol.* **111,** 987–1000
62. Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R., and Orci, L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11199–11204
63. Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Schekman, R., and Yeung, T. (1998) *Cell* **93,** 263–275
64. Pauloin, A., Tooze, S. A., Michelutti, I., Delpal, S., and Ollivier-Bousquet, M. (1999) *J. Cell Sci.* **112,** 4089–4100
65. Low, S. H., Wong, S. H., Tang, B. L., Tan, P., Subramaniam, V. N., and Hong, W. (1991) *J. Biol. Chem.* **266,** 17729–17732
66. Low, S. H., Tang, B. L., Wong, S. H., and Hong, W. (1992) *J. Cell Biol.* **118,** 51–62
67. Wagner, M., Rajasekaran, A. K., Hanzel, D. K., Mayor, S., and Rodriguez-Boulan, E. (1994) *J. Cell Sci.* **107,** 933–943
68. Fucini, R. V., Chen, J. L., Sharma, C., Kessels, M. M., and Stamnes, M. (2002) *Mol. Biol. Cell* **13,** 621–631
69. Chen, J. L., Lacomis, L., Erdjument-Bromage, H., Tempst, P., and Stamnes, M. (2004) *FEBS Lett.* **566,** 281–286
70. Rozelle, A. L., Machesky, L. M., Yamamoto, M., Driessens, M. H., Insall, R. H., Roth, M. G., Luby-Phelps, K., Marriott, G., Hall, A., and Yin, H. L. (2000) *Curr. Biol.* **10,** 311–320