Primaquine Blocks Transport by Inhibiting the Formation of Functional Transport Vesicles

STUDIES IN A CELL-FREE ASSAY OF PROTEIN TRANSPORT THROUGH THE GOLGI APPARATUS*

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The lysosomotropic amine primaquine has previously been shown to inhibit both secretory and recycling processes of cells in culture. We have used a cell-free assay that reconstitutes glycoprotein transport through the Golgi apparatus to investigate the mechanism of action of primaquine. In this assay, primaquine inhibits protein transport at a half-maximal concentration of 50 μM, similar to the concentration previously reported to disrupt protein secretion in cultured cells. Kinetic analysis of primaquine inhibition indicates that its point of action is at an early step in the vesicular transport mechanism. Primaquine does not inhibit the fusion of vesicles already attached to their target membranes. Primaquine irreversibly inactivates the membranes that form transport vesicles (donor), but not the membranes that are the destination of those vesicles (acceptor). Morphological data indicate that primaquine inhibits the budding of vesicles from the donor membranes. Once formed, the vesicles are refractile to primaquine action, and their attachment to and fusion with acceptor membranes proceeds unimpaired. In addition to illuminating the mechanism of action of primaquine, this study suggests that the selective action of this agent will make it a useful tool in the study of the formation of transport vesicles.

Lysosomotropic amines disrupt intracellular transport processes such as secretion and receptor recycling (1-5). For example, the antimalarial primaquine (PQ) inhibits the secretion of albumen, orosomucoid, and transferrin (4). Whereas it does not affect endocytic uptake directly, PQ does prevent the recycling of the asialoglycoprotein receptor from an intracellular compartment back to the cell surface (3, 19). Interestingly, PQ is more effective than its congener chloroquine in inhibiting protein secretion (5), whereas the two agents have comparable efficiencies in inhibiting receptor recycling (19). The effects of lysosomotropic amines are generally considered to be due to the ability of these agents to increase the pH of acidic intracellular compartments. However, the details of how transport is inhibited by this alteration are not known, and it is not known whether other secondary effects of these agents may be important.

Recently, the use of systems that reconstitute transport using either isolated organelles or semi-intact cells has contributed to a more detailed understanding of transport processes. One such system measures the recycling of the cation-independent mannose 6-phosphate receptor from a post Golgi compartment back to the trans Golgi apparatus (7). When PQ was tested in this system, it was found to have no effect. In contrast, it was noted that transport through Golgi apparatus of the membrane glycoprotein of vesicular stomatitis virus (VSV) was strongly inhibited by PQ when examined in a similar reconstituted system. This was a striking result as it had been previously found that the ionophores monensin and nigericin, which collapse pH gradients, are without effect on protein transport through the Golgi apparatus when measured in a cell-free assay (12). These experiments indicate that PQ may have a selective effect on transport and spurred us to examine the mechanism by which PQ acts.

We have utilized the well-characterized assay for transport through the Golgi apparatus (9). This assay reconstitutes the transport of glycoproteins from cis to medial Golgi compartments. Two populations of Golgi membranes are prepared. “Donor” Golgi fractions are prepared from a VSV-infected mutant of CHO cells. This mutant cell line is deficient in the enzyme N-acetylglucosamine transferase I, a resident Golgi enzyme that catalyzes addition of a GlcNac to asparaginelinked oligosaccharide side chains of glycoproteins as they are transported through the Golgi apparatus. “Acceptor” Golgi fractions are prepared from uninfected wild-type CHO cells. When these two Golgi populations are mixed in the presence of ATP and a cytosolic protein fraction, transport vesicles containing the underglycosylated VSV glycoprotein bud from the donor population and fuse with the medial Golgi cisternae of the acceptor population. The fusion of transport vesicles with the acceptor Golgi fraction is marked by the addition of radiolabeled N-acetylglucosamine to the protein and is quantitated by immunoprecipitation with a monoclonal antibody to the glycoprotein.

Biochemical, kinetic, and morphological studies have led to a preliminary model of the series of events that occur in the formation, attachment, and fusion of transport vesicles in this system (Fig. 1). The first event that is detected is termed priming (Fig. 1, Event 1). Morphologically, this corresponds to the formation of buds on the surface of the Golgi apparatus. These buds are coated with an amorphous material that is distinct from the well-characterized coat of clathrin-coated vesicles (20), but which may be structurally related to clathrin (28, 33). Elements of this coat have recently been purified (18, 27). Priming is detected kinetically by a reduction in the
Primaquine Blocks Secretory Vesicle Formation

**RESULTS**

**Primaquine Blocks Early Event in Transport**—Using the cell-free Golgi transport system, it has previously been demonstrated that there are biochemically distinct kinetic intermediates in the vesicular transport of proteins through the Golgi apparatus (see Introduction). A schematic representation of the transport process and the defined intermediates are shown in Fig. 1. “Priming” defines a cytosol-dependent process thought to correspond to budding of nascent transport vesicles from the donor membrane (Event 1). These buds are

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**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[6-3H]GlcNAc (26.8 Ci/mmol) was obtained from Du Pont-New England Nuclear, PQ, creatine phosphokinase, ATP, UTP, and HEPES were purchased from Sigma. Monoclonal antibody to VSV glycoprotein (clone 8G5) was a gift from Dr. Leo LeFrancois of The Upjohn Co. (11). Polyclonal rabbit anti-mouse serum was obtained from Cappel. Tannic acid (electron microscopy-grade) was from Polysciences, Inc. (Warrington, PA) as was Epon (Poly/Bed).

**Cells and Culture**—Wild-type CHO cells and CHO mutant clone 15B (13) were obtained from Dr. Stuart Kornfeld (Washington University, St. Louis, MO). Alternatively, lecl cells (American Type Culture Collection) (14), a line with a genetically identical lesion to 15B cells, were used. Results from the two were indistinguishable. Cells were maintained in monolayer culture in α-minimal essential medium containing 10% fetal calf serum. Stock of VSV was grown in baby hamster kidney cells as described by Balch et al. (9). Infection of 15B cells, the preparation of donor and acceptor Golgi fractions by sucrose density gradient centrifugation, and the preparation of cytosol from CHO cells were done as described (8).

In Vitro Assay Conditions—In a “standard assay,” donor membranes, acceptor membranes, and cytosol were incubated in a 50-μl volume containing 25 mM HEPEs, pH 7.5, 2.5 mM MgCl2, 2.5 mM Mg2(C2H3O2)2, 50 μM ATP, 250 μM UTP, 2 mM creatine phosphate, 7.3 units/ml creatine phosphokinase, and 0.4 μM (0.5 μCi) UDP-[3H] GlcNAc. The assay was initiated by transfer to 37 °C for 60 min. The assay was terminated by addition of 50 μl of detergent solution containing 50 mM Tris, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% sodium cholate. VSV glycoprotein was precipitated by addition of 0.6 μl of monoclonal antibody in 1.25 ml of rabbit anti-mouse serum. After incubation at 37 °C for 15 min, the immunoprecipitate was collected by retention on Millipore filters (type HA, 0.45 μm). After washing, filters were dried and counted using Beckman Ready-Safe.

Assays for late stage activity were performed essentially as previously described (10). Donor membranes, acceptor membranes, and excess CHO cytosol were incubated for 25 min in the reaction mixture described above, but lacking UDP-[3H]GlcNAc, resulting in the formation of a prefusion complex termed the LCRI. Membranes were then pelleted in a microcentrifuge at 13,000 × g for 3 min through a cushion of 0.5 M sucrose. Membranes were resuspended in a similar reaction mixture, but containing 15 mM NaCl versus 25 mM KCl and 1.5 versus 2.5 mM Mg2(C2H3O2)2. Fractionated cytosol was added back, and incubations were continued for an additional 90 min. Reactions were terminated, immunoprecipitated, and quantitated as described (16).

Electron Microscopy—Membranes were fixed for 1 h at 4 °C with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, containing 0.5 mM CaCl2. After washing, membranes were post-fixed for 1 h at 25 °C with cacodylate-buffered 1% (w/v) OsO4 followed by contrast staining using the method of Simionescu and Simionescu (21) as described by Orci et al. (20). Briefly, the membranes were stained for 30 min with 1% (w/v) tannic acid in 0.05 M sodium cacodylate, pH 7.4; rinsed; and washed for 5 min in cacodylate-buffered 1% sodium sulfate. After washing, the membranes were stained en bloc for 30 min in 0.5 M uranyl acetate in 0.05 M sodium cacodylate, pH 7.4; washed; dehydrated through a graded series of ethanol; and embedded in Epon (22). Thin sections were picked up onto 200-mesh grids, post-stained with 2.5% (w/v) uranyl acetate and lead citrate (23), and viewed with a JEOL 1200 electron microscope operated at 80 kV.

Quantitation of Buds and Vesicles in Golgi Areas—Random fields of each sample were photographed at a constant magnification (×42,000), and areas containing identifiable Golgi profiles were outlined. The area circled was determined using Sigma Scan® (Jandel Scientific, Corte Madera, CA). The number of buds or vesicles was counted for each area by an observer unaware of which group each photograph represented. This number was then normalized to the area counted. The data were treated by the Wilcoxon rank sum test for statistical analysis (see Ref. 25).

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![Fig. 1. Summary of events thought to occur during disso- ciative transport of proteins between successive Golgi com- partments.](image-url)
coated with a non-clathrin coat protein and have been demonstrated to contain proteins in transit from cis to medial Golgi compartments (9, 20). Presumably these buds pinch off to form transport vesicles (Event 2), which subsequently target to the acceptor membrane. After attachment to the acceptor membranes (Event 3), transport vesicles must be "processed" prior to their fusion into the acceptor membrane. One consequence of this extensive processing is the formation of a prefusion intermediate termed the LCRI. The attached vesicles that have accumulated in the LCRI undergo further reactions that allow fusion to occur, thus completing the process.

We have tested the effect of PQ in this cell-free assay system. As can be seen in Fig. 2, when PQ is added coincident with the initiation of the assay (standard assay), it inhibits incorporation of [3H]GlcNAc into the VSV glycoprotein in a concentration-dependent manner with half-maximal inhibition at 50 μM. This concentration is similar to that required for PQ inhibition of protein secretion and receptor-mediated endocytosis in HepG2 cells (3). This observation is in agreement with results previously reported by Goda and Pfeffer (7).

If donor membranes, acceptor membranes, and CHO cytosol are incubated at 37°C for 25 min, the majority of the transport vesicles can be accumulated in the late prefusion intermediate termed the LCRI (15). As seen in Fig. 2, if PQ is added after formation of the LCRI, it has little effect on the subsequent processing and fusion of transport vesicles. This would suggest that PQ is acting to inhibit some event prior to the formation of this prefusion complex between transport vesicles and target membranes.

Analysis of protein traffic in tissue culture models has indicated that protein secretion and targeting as well as receptor recycling are inhibited by agents that disrupt intracellular pH gradients (1-6). To test the possibility that PQ was inhibiting protein transport by virtue of its disruption of pH gradients in our cell-free assay, we compared the effect of PQ to that of the basic amine chloroquine and the ionophore monensin. Fig. 3 illustrates that only PQ has any effect on transport in our cell-free system at concentrations that have been demonstrated to inhibit transport in vivo. Chloroquine does inhibit protein transport, but only at a 20-fold higher concentration than typically required to inhibit protein secretion in tissue culture cells (4). In agreement with the findings of Balch and Rothman (12), monensin has no effect on transport in our cell-free assay at any concentration tested. It would therefore appear that the inhibitory effect of PQ is not simply due to the disruption of a pH gradient within the Golgi cisternae.

*Primaquine Blocks Priming of Donor Membranes*—Further data supporting PQ inhibition at an early transport step was obtained by treating donor or acceptor membranes separately with PQ prior to their addition to the assay. This would indicate whether donor- or acceptor-specific functions were being inhibited by PQ or if there was a more general disruption of membrane function. Donor or acceptor membranes were incubated with or without 500 μM PQ for 5 min at 37°C. Membranes were then pelleted through a cushion of 0.3 M sucrose to remove PQ. Membranes were gently resuspended and tested in the standard transport assay against their untreated counterparts. G protein, VSV glycoprotein.

![Fig. 2. Effect of PQ on transport of VSV glycoprotein in the cell-free system.](image-url) Various concentrations of PQ were added to the standard reaction mixture coincident with the initiation of the assay (standard) or after formation of the LCRI (late stage). Reactions were incubated at 37°C for 60 min and then terminated and quantitated as described under "Experimental Procedures." G protein, VSV glycoprotein.

![Fig. 3. Effect of other pH-disrupting agents on transport of VSV glycoprotein in the cell-free system.](image-url) Various concentrations of chloroquine, monensin, or PQ were added to standard reaction mixtures. Reactions were incubated at 37°C for 60 min and then terminated and quantitated as described under "Experimental Procedures." G protein, VSV glycoprotein.

![Fig. 4. PQ effects on donor and acceptor membrane function.](image-url) Donor or acceptor membranes were incubated separately at 37°C with or without 500 μM PQ for 15 min. Membranes were then pelleted through a 0.3 M sucrose cushion to remove PQ. Membranes were gently resuspended and tested in the standard transport assay against their untreated counterparts. G protein, VSV glycoprotein.
donor membranes with PQ greatly reduces protein transport activity, whereas treatment of acceptor membranes has little effect. Clearly, the effect of PQ is primarily on a donor-specific function, and this effect is independent of the presence of acceptor membranes. Vesicle formation is a function of the donor membranes and therefore may be the process affected by PQ. From these data, it appears that the effect of PQ is not readily reversible when PQ is removed, thus implying that PQ is modifying or inactivating some crucial membrane component or strongly partitioning into the membrane.

Balch et al. (10) have demonstrated that in this cell-free assay, there is a distinct lag time of 7–10 min in the rate of incorporation of [3H]GlcNAc into the VSV glycoprotein before transport proceeds at a linear rate. This lag time appears to be the time required to populate transport intermediates in the transport process. The lag time can be decreased by preincubating donor membranes in the presence of cytosol (10). This reduction in lag time is referred to as priming and is coincident with the appearance of glycoprotein-containing buds on the membrane surface (Fig. 1, Event 1). Priming is therefore thought to represent the formation of buds, the precursors of transport vesicles.

To test the effect of PQ on the formation of these buds, donor membranes were incubated with cytosol at either 37 °C (primed) or 0 °C (unprimed) for 15 min. Acceptor membranes were then added with various concentrations of PQ, and the incubation was continued at 37 °C for 20 min. Reactions were terminated and quantitated as described under "Experimental Procedures." G protein, VSV glycoprotein.

FIG. 5. Effect of PQ on priming. Donor membranes were incubated with ATP and cytosol for 15 min at 37 °C (primed) or held at 0 °C (unprimed). Acceptor membranes were then added with various amounts of PQ, and incubations were continued at 37 °C for 20 min. Reactions were terminated and quantitated as described under "Experimental Procedures." G protein, VSV glycoprotein.

FIG. 6. Time course of PQ action, LCRI formation, and glycoprotein transport in the cell-free assay. Standard reaction mixtures were prepared and incubated at 37 °C. For the time course of VSV glycoprotein (G protein) in the assay, 50-μl reactions were terminated by quick-freezing on dry ice at time t. For the time course of PQ action, PQ was added to a final concentration of 300 μM at time t, and incubations were continued for [90-t] min. LCRI formation was measured by 5-fold dilution of cytosol concentration in the assays at time t, and incubations were continued for [90-t] min. All reactions were terminated and quantitated as described under "Experimental Procedures."

FIG. 7. Morphological consequence of PQ on Golgi priming. Donor membranes were incubated under standard transport conditions for 20 min either on ice (A and C) or at 37 °C (B and D). The incubations were performed either without treatment (A and B) or with the inclusion of 300 μM PQ (C and D). The membranes were then pelleted and processed for electron microscopy as outlined under "Experimental Procedures." The bar in A represents 0.5 μM and is applicable also for B–D. Enlargements to show the vesicle coat are shown in the insets in B and D. The bar in the inset in B represents 100 nm and is applicable also for the inset in D.

donor membranes were incubated with cytosol at either 37 °C (primed) or 0 °C (unprimed) for 15 min. Acceptor membranes were then added with various concentrations of PQ, and the incubation was continued at 37 °C for 20 min to allow consumption of the primed membranes. The results of this experiment are shown in Fig. 5. Preincubation of donor membranes at 37 °C for 15 min prior to addition of acceptor resulted in an increased incorporation of [3H]GlcNAc into the glycoprotein VSV of ~2000 cpm over donor membranes kept at 0°C; this increase in counts represents the priming event. Although PQ inhibits the overall incorporation of counts with both primed and unprimed membranes, the difference between the two, i.e. priming, is maintained at ~2000 cpm at PQ concentrations up to 300 μM. This would imply that the budded vesicles, once formed, are refractile to the effects of PQ and continue to pinch off, target, and fuse normally. PQ must therefore be inhibiting the formation of the buds at the donor membrane surface.

The block of vesicle budding, but not subsequent transport steps, by PQ should allow measurement of the formation of the first stable post-priming transport intermediate. A kinetic experiment was designed to measure the time course of the entry of transport vesicles into PQ-resistant intermediates. This was compared to the time course of the formation of a prefusion intermediate (the LCRI) and the time required for the glycoprotein to transit the entire transport pathway from donor to acceptor Golgi compartments. To measure acquisition of PQ resistance, standard reactions were incubated at 37 °C; and at time t, PQ was added to a final concentration of 300 μM. The reaction was then continued for 90 minus t min to allow all PQ-resistant glycoprotein to progress through the remaining transport steps. The entry into the LCRI was measured by diluting the cytosol concentration in the assay 5-fold at time t and continuing the incubation for 90 minus t min. It has previously been shown that steps leading to the formation of the LCRI require higher concentrations of cytosol than the reactions that consume that intermediate (14, 15). The cytosol dilution assay is therefore diagnostic for the formation of the LCRI. The rate of glycoprotein transport through the entire pathway was measured by simply freezing incubations at time t.

The results of these kinetic experiments are presented in...
**FIG. 8. Quantitation of bud-vesicle formation during priming with and without PQ.** Random fields from sections of the priming experiment depicted in Fig. 7 were quantitated for the appearance of buds and/or vesicles on individual Golgi complexes as described under "Experimental Procedures." Histograms were constructed placing each Golgi area counted into a class according to its bud-vesicle density. The results are reported as the percentage of total Golgi complexes counted that fall into each class. A and C, incubations on ice (unprimed); B and D, incubations for 20 min at 37°C (primed); A and B, untreated; and C and D, treated with 300 μM PQ. 47 Golgi areas were measured in the untreated primed sample, 57 in the unprimed primed sample, 40 in the PQ-treated unprimed sample, and 46 in the PQ-treated primed sample. The number of buds/vesicles per Golgi membrane in the untreated primed sample differs significantly from that of the PQ-treated primed sample (p < 0.013) when analyzed by the Wilcoxon rank sum test (see Ref. 26).

Fig. 6. Comparison of these curves show that the glycoprotein passes the point of PQ inhibition at a time very close to the time of the formation of the LCRI. The half-times of formation of the LCRI and the acquisition of PQ resistance are both 10–15 min. The time course of acquisition of PQ resistance does not reach a stable plateau as does that for the formation of the LCRI. This is presumably because at this concentration PQ is also slightly inhibiting some later event(s) in the transport or fusion pathway. The time required for the glycoprotein to traverse the entire pathway is much longer, with a half-time of 30–35 min.

This result indicates that the steady-state levels of all kinetic intermediates preceding the prefusion complex (LCRI) are low and that their lifetimes are short. Because of this, it is not until transport vesicles have reached the first stable long-lived intermediate (the LCRI) that PQ resistance can be measured. This is consistent with earlier experiments, which suggested that the free pool of transport vesicles is small and that their uptake by acceptor membranes is extremely rapid (10).

**Morphological Consequences of Primaquine Treatment**—
The same conditions that lead to the kinetic priming outlined above also lead to a morphological change in the donor membranes. Before priming, Golgi membranes appear relatively quiescent, with few forming vesicles associated with Golgi stacks. After incubation at 37°C, under conditions that promote protein transport, the Golgi membranes exhibit coated buds and vesicles decorating their surface (10, 20). Therefore, it was of interest to determine what effect PQ had on this morphological transition. Fig. 7 shows examples of four incubations: two at 0°C (unprimed) and two at 37°C (primed), each condition with and without PQ. In unprimed samples, whether treated with PQ or not, few budding profiles are observed. In control incubations at 37°C, donor Golgi membranes exhibit the characteristic formation of coated buds on their surface. When treated with PQ and incubated at 37°C, some formation of buds does take place, but is diminished compared to the untreated sample. The overall morphology of Golgi regions is unaffected by PQ treatment. This rules out that PQ is exerting its effect by gross disruption of Golgi structure.

Quantitation of the number of Golgi membrane-associated buds/vesicles in random fields of Golgi compartments is presented in Fig. 8. Whereas the PQ-treated primed sample shows an increase in the density of buds formed when compared to the unprimed sample, the level of morphological priming is clearly diminished relative to the untreated sample. Statistical analysis of the distributions reveals that the difference between untreated and PQ-treated samples is significant at p < 0.013.

The buds that are formed during priming are coated with an amorphous protein complex that differs from the clathrin coat characteristic of endocytic vesicles (20). This coat does not appear to be significantly altered by PQ treatment (Fig. 7, B and D, insets).

**DISCUSSION**

The data presented here indicate that the lysosomotrophic amine primaquine selectively inhibits the formation of functional transport vesicles. This conclusion is based on an analysis of vesicle budding and fusion intermediates previously defined in the cell-free system reconstituting transport through the Golgi apparatus. It was found that PQ acts prior to the formation of a prefusion complex between transport vesicles and their target membranes. PQ was then shown to inactivate the membranes that form transport vesicles (donor), but not the membranes that are the destination of the vesicles (acceptor). Finally, PQ was found to inhibit a donor-specific process known as priming.

Priming refers to the phenomenon in which the preincubation of donor membranes reduces the lag time that is characteristic of transport (10). Morphologically, priming corresponds to the formation of coated buds on the surface of untreated Golgi membranes (10, 20). The biochemical measurement of priming can only detect the formation of functional nascent transport vesicles. If vesicles form but are not released from donor membranes or if inactive vesicles are formed, this would not be detected as a priming event. Here,
we find that by a morphological criteria, the formation of these buds is inhibited by PQ, albeit not completely. Biochemically detected priming is totally inhibited. Since the morphological consequence of PQ treatment is not absolute, we cannot state unequivocally that PQ is inhibiting priming solely by its effect on transport vesicle formation. It may also lead to the formation of some inactive transport vesicles. PQ is known to intercalate into the lipid bilayer and affect membrane deformation. The best studied example is the formation of shape change in erythrocytes by PQ and other amphiphiles (26). It is thought that by incorporating into the inner leaflet of the bilayer, these agents cause an asymmetric expansion of the bilayer and thereby cause an invagination of the membrane. This has been theoretically formalized as the bilayer couple hypothesis (24). PQ may be exerting its effects by incorporating into the luminal leaflet of Golgi membranes and therefore counteracting the forces that drive vesicle budding and/or pinching off.

The levels of PQ utilized here are consistent with those that are efficacious in intact cells. Consequently, our results bear directly on the action of PQ in vivo. One action of PQ and other amines is to neutralize acidic compartments (1). The effect of such agents is at the level of endosomes in receptor recycling and the trans Golgi compartment in secretion. Both endosomes and the trans Golgi compartment are acidic compartments. It is reasonable to conclude that the action of the amines is therefore mediated through their pH-neutralizing effect. However, the acidic nature of these compartments concentrates the amines, which may allow them to exert membrane-active effects. Consistent with our findings, it has been shown that PQ inhibits the formation of transport vesicles in intact cells (3). In that study, this was attributed not to the inhibition of vesicle budding, as we have found here, but to the inability of those buds to pinch off to form functional transport vesicles. It is striking that PQ is relatively more active in inhibiting secretion (5) than receptor recycling (19) when compared to the related amine chloroquine. It may be that the inhibition of transport vesicle formation reported here is reflected in that disparity.

The biochemistry of the formation of transport vesicles has been difficult to study. Because PQ selectively inhibits priming, it has become an important tool in understanding the underlying biochemical process. It has indeed been successfully constructed and used to identify and isolate a protein that acts in the priming process (2). It may also be possible to functionally assess the role of the recently described "coatamer" proteins, proposed to be components of the non-clathrin coat of Golgi transport vesicles (27). Primaquine may become as important a tool in the study of transport in vitro as it has proved to be in vivo.

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