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Transport of influenza virus envelope proteins from the Golgi complex to the apical plasma membrane in MDCK cells: pH-controlled interaction with a cycling receptor is not involved

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In influenza virus-infected monolayers of the epithelial cell line MDCK the viral envelope proteins, haemagglutinin and neuraminidase, are targetted specifically to the apical surface. In this study we have tested the hypothesis that the polarized delivery of these proteins to the plasma membrane involves the operation of a receptor that cycles between the *trans* Golgi network and the plasma membrane, binding the proteins at low pH in the former compartment and releasing them at normal extracellular pH in the latter. The hypothesis predicts that apical, but not basolateral, low pH would eventually delay or block delivery of the proteins to the plasma membrane. We found that basolateral low pH in fact had the more profound effect, in line with its greater effect on intracellular pH. We conclude that the hypothesis is not valid, and that low extracellular pH causes its effect on protein transport by changing intracellular pH.

Influenza; Envelope protein; Epithelial polarity

1. INTRODUCTION

Despite intense interest, the mechanisms underlying transport of proteins between the Golgi complex and the plasma membrane remain largely obscure. It does appear, however, that a compartment on the *trans* side of the Golgi complex, the *trans* Golgi network (TGN) may be the site at which plasma membrane proteins are segregated from proteins that are resident in the Golgi complex or are destined for other locations, such as secretory granules or endosomes [1]. In epithelial cells, proteins, including the envelope proteins of some viruses [2], are targetted to one of two plasma membrane domains. The available evidence suggests that targetting to both domains involves a specific recognition event in the TGN and does not occur by default. For example, in several experiments in which sorting has been disturbed, the result has been lack of sorting, and not targetting to a new destination [3–7].

The lumen of the TGN is mildly acidic (pH about 6.0) [1,8], and the maintenance of this low pH appears to be crucial to its normal functioning in epithelia. For example, treatment of MDCK cell monolayers with ammonium chloride delays the transport of influenza virus haemagglutinin from the TGN to the apical cell surface [9], while in the same cell line chloroquine causes mis-sorting of the basolaterally directed secretory proteins laminin and heparan sulphate proteoglycan to both surfaces [5].

The best-characterised intracellular transport steps are those involved in the delivery of proteins to the endosome from the TGN [10] and from the cell surface [11]. In both cases, the protein to be transported is bound by a receptor at a high pH and released at a low pH (about 5.0) of the target compartment. The receptor then recycles to bind another ligand. We set out to test the hypothesis that a similar system operates for transport between the TGN and the plasma membrane in

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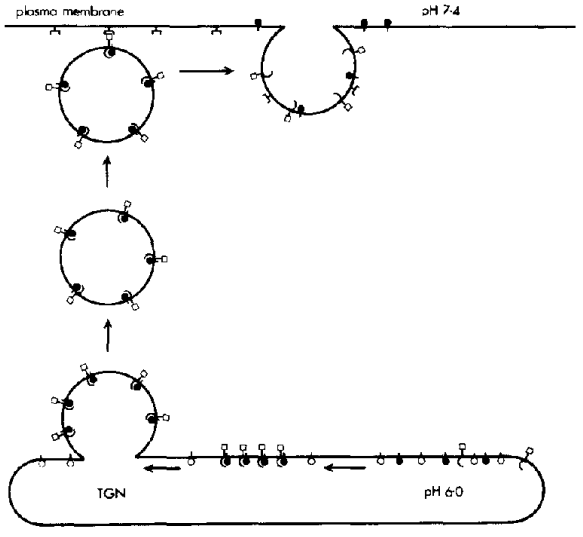

Fig.1. Hypothetical mechanism for transport of a membrane protein from the TGN to the plasma membrane. (♀) Protein to be transported; (♀) receptor; (♀) resident TGN protein; (♀) vesicle marker; (♀) vesicle 'docking receptor' on the inside of the plasma membrane. The protein binds to its receptor at pH 6.0 in the TGN and dissociates at normal extracellular pH. The receptor is then free to recycle to the TGN. If extracellular pH is lowered to 6.0, the protein should no longer dissociate from its receptor, which would eventually become saturated. Once saturation has occurred, newly synthesized protein should be transported as far as the TGN but no further.

epithelial cells. For this transfer, receptor–ligand affinity would be high at pH 6.0 and low at pH 7.4 (fig.1). One prediction of this hypothesis is that raising the pH inside the TGN should delay or block transport of proteins to the plasma membrane. As discussed above, such an effect has been seen. Another prediction of the hypothesis is that low extracellular pH should have the same effect, by preventing receptor–ligand dissociation and so saturating the receptors. Furthermore, in epithelia, receptors should cycle through only a single plasma membrane domain, and therefore the effect of extracellular pH should be asymmetric. We have tested this prediction by examining the effect of asymmetrically lowered extracellular pH on the delivery of the influenza envelope proteins, haemagglutinin and neuraminidase, to the apical surface of MDCK cell monolayers.

2. MATERIALS AND METHODS

MDCK cells grown as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 10% newborn calf serum, non-essential amino acids and 50 IU/ml penicillin/50 μg per ml streptomycin at 37°C in an atmosphere of 5% CO_2 in air. Media and supplements were supplied by Flow Laboratories, Rickmansworth, England. MDCK cells used for the experiments on haemagglutinin transport were obtained from Flow Laboratories. Experiments on neuraminidase transport were carried out on a high-passage strain that was originally obtained from Dr K. Simons (EMBL, Heidelberg, FRG).

The X-31 strain of influenza virus, and a monoclonal antibody to the X-31 haemagglutinin, were obtained from Dr A. Hay (NIMR, London). The WSN strain of influenza virus was obtained from Dr S.C. Inglis (Department of Pathology, University of Cambridge). Semliki Forest virus (SFV) was obtained from Dr T.R. Hesketh (Department of Biochemistry, University of Cambridge). Viruses were grown, purified and assayed as described previously [12].

MDCK cells were grown until just confluent on either 2.5 cm nitrocellulose filters (0.45 μm pore size; Millipore, Harrow, England) in perspex chambers or 3 cm culture dishes. Monolayers were washed twice with 2 ml EMEM containing 0.2% BSA and infected with >10 plaque-forming units of influenza virus in the same medium for 1 h at 37°C. The medium was then replaced with bicarbonate-free EMEM of appropriate pH containing 20 mM Hepes, 25 mM Mes, 10 mM Pipes and 2% newborn calf serum.

Delivery of haemagglutinin to the plasma membrane was followed by radioimmunoassay on paraformaldehyde-fixed monolayers, using anti-haemagglutinin ascites fluid (10^3 × dilution) and ^{125}I-labelled sheep anti-mouse immunoglobulin (specific activity 10 μCi/μg; Amersham International, England).

Delivery of neuraminidase to the cell surface was assayed as described previously [12,13].

Intracellular pH (pH_i) was measured through the partitioning of [^{14}C]benzoic acid (120 mCi/mmol; Amersham International) across the plasma membrane, as described by Davoust et al. [14]. To measure pH_i in monolayers growing on nitrocellulose filters and exposed to different pHs on its two surfaces, [^{14}C]benzoic acid was added to both sides at the equilibrium concentration ratio predicted by the equation

$$pH_{(ap)} - pH_{(bl)} = \log\left(\frac{\text{conc [}^{14}\text{C]benzoic acid (ap)}}{\text{conc [}^{14}\text{C]benzoic acid (bl)}}\right)$$

Specifically, the concentration of tracer in medium of pH 7.4 was 25-times that in medium of pH 6.0 (1.925:0.077 μCi/ml). pH_i was calculated from the mean value obtained by considering partitioning across both apical and basolateral membranes.

Intracellular ATP levels were determined using a luciferin-luciferase assay (Boehringer, Mannheim, FRG), as described by Davoust et al. [14].

3. RESULTS AND DISCUSSION

pH_i in MDCK cell monolayers grown on filters was determined for cells exposed to medium of pH 7.4 and 6.0, either asymmetrically or symmetrical-

ly. The results obtained for both strains of MDCK cell are given in table 1. It can be seen that exposure of the cells to pH 6.0 on the basolateral surface causes a more profound fall in intracellular pH than exposure to pH 6.0 apically. Table 1 also shows the values of pH_e for cells grown on dishes that produce the same values of pH_i as those obtained for cells grown on filters.

Since ATP is required for various steps in the biosynthetic transport pathway [15,16], the possibility was considered that variation in pH_i might produce effects as a result of a depletion of cytosolic ATP. However, ATP levels, in both strains of cell, were found not to fall significantly below the control value of 26 nmol/mg protein, even when pH_e was lowered as far as 5.4.

Exposure of monolayers to pH_e 6.0 apically from 3 h post-infection did not delay the arrival of haemagglutinin at the cell surface; however, exposure to pH_e 6.0 basolaterally did cause a delay (fig.2a). Since pH_i falls further when basolateral pH_e is lowered than when apical pH_e is lowered, the most likely explanation of these results is that the delivery of haemagglutinin to the cell surface is delayed progressively as pH_i falls. To confirm this, monolayers grown on dishes were exposed to media of pH_e that would produce pH_i values identical to those found in the asymmetric conditions. The results obtained (fig.2b) were very similar to those shown in fig.2a.

Neuraminidase activity at the cell surface was assayed through its ability to cleave free [^{3}H]sialic acid from SFV containing envelope proteins with ^{3}H-labelled sialic acid residues. The time-points chosen were 3 h post-infection, when activity is just detectable, and 6 h post-infection, which is just before activity reaches a plateau [13]. When MDCK cells were grown on filters, the effect of lowering apical pH_e to 6.0 on neuraminidase

Table 1

Dependence of pH_i on pH_e

Apical pH	7.4	6.0	7.4	6.0
Basolateral pH	7.4	7.4	6.0	6.0
pH_i, MDCK (Flow)	7.4	7.2	6.9	6.5
pH_e on dishes	7.4	7.0	6.4	5.7
pH_i, MDCK (EMBL)	7.4	7.2	7.0	6.4
pH_e on dishes	7.4	7.0	6.5	5.5

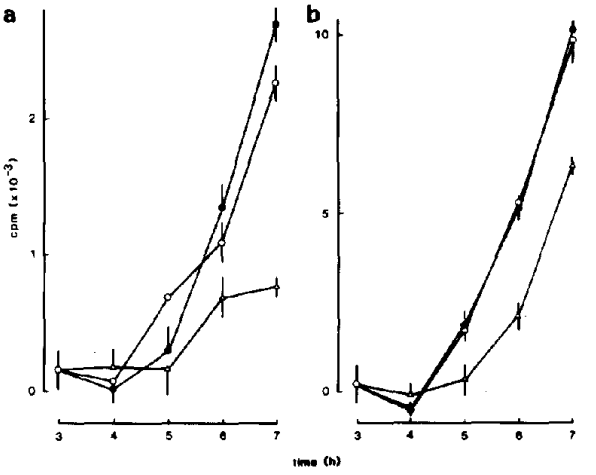

Fig.2. Delivery of haemagglutinin to the plasma membrane. (a) Cells grown on filters. (○) Control; (●) pH_e 6.0 apically (pH_i 7.2); (△) pH_e 6.0 basolaterally (pH_i 6.9). (b) Cells grown on dishes. (○) Control; (●) pH_e 7.0 (pH_i 7.2); (△) pH_e 6.4 (pH_i 6.9). Values are means ± SE (n = 3). Error bars are not shown where they lie within the dimensions of the symbol.

delivery to the cell surface was not as severe as that of lowering basolateral pH_e (fig.3a). Lowering pH_e on both sides of the monolayers produced a pro-

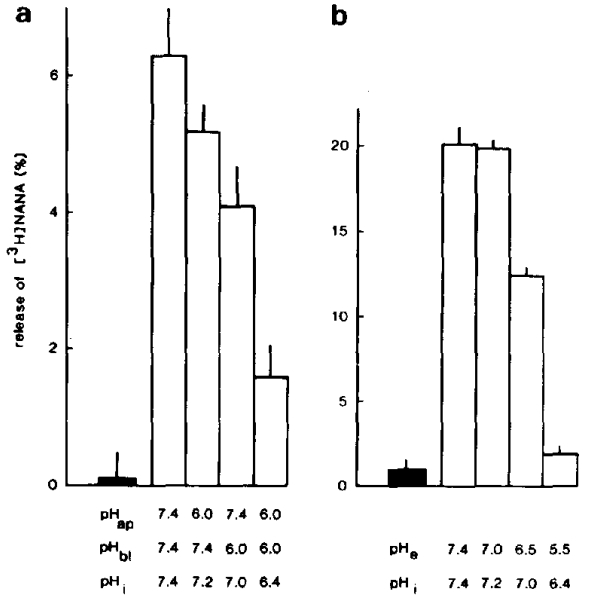

Fig.3. Delivery of neuraminidase to the plasma membrane. (a) Cells grown on filters. (b) Cells grown on dishes. Filled bars, 3 h post-infection; open bars, 6 h post-infection. Values are means ± SE (n = 3).

found effect. A similar pattern was seen when the cells were grown on dishes and pH_e was lowered to produce pH_i values identical to those found in cells grown on filters (fig.3b).

Our results show that the transport of the two envelope proteins of influenza virus from the TGN to the plasma membrane does not involve pH-mediated binding and release of the proteins by a receptor that cycles between the two membrane compartments. The evidence presented points instead to an effect of pH_e on intracellular protein transport through its effect on pH_i. Such an effect is probably not surprising, since it has been demonstrated that other steps in intracellular protein transport, such as early stages in endocytosis [14,17], are sensitive to acidification of the cytosol.

The possibility remains that both a receptor and the low pH are involved in sorting, but not in the way envisaged here. For example, the low pH may be necessary to cause the protein to adopt a conformation that is recognised by the receptor and the receptor may then be responsible for the packaging of proteins into vesicles, without itself being transported to the plasma membrane. Alternatively, the receptor may travel to the cell surface with the protein, but protein–receptor dissociation may occur en route.

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