Sorting of an Apical Plasma Membrane Glycoprotein Occurs Before It Reaches the Cell Surface in Cultured Epithelial Cells

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ABSTRACT   In Madin-Darby canine kidney (MDCK) cells (a polarized epithelial cell line) infected with influenza virus, the hemagglutinin behaves as an apical plasma membrane glycoprotein. To determine biochemically the domain on the plasma membrane, apical or basolateral, where newly synthesized hemagglutinin first appears, cells were cultured on Millipore filters to make both cell surface domains independently accessible. Hemagglutinin in virus-infected cells was pulse-labeled, chased, and detected on the plasma membrane with a sensitive trypsin assay. Under all conditions tested, newly made hemagglutinin appeared simultaneously on both domains, with the bulk found in the apical membrane. When trypsin was continuously present on the basolateral surface during the chase, little hemagglutinin was cleaved relative to the amount transported apically. In addition, specific antibodies against the hemagglutinin placed basolaterally had no effect on transport to the apical domain. These observations suggested that most newly synthesized hemagglutinin does not transiently appear on the basolateral surface but rather is delivered directly to the apical surface in amounts that account for its final polarized distribution.

The plasma membrane of epithelial cells is divided into apical and basolateral domains that have unique protein and lipid compositions (12, 27, 33). A major question is how apical and basolateral plasma membrane proteins synthesized in the rough endoplasmic reticulum are sorted to their final destinations. Existing data suggest that the sorting occurs as a late step on the transport pathway (30, 34). Viral membrane glycoproteins destined for either the apical or basolateral domains have been shown by immunocytochemistry to be intermixed as late in transport as the Golgi complex (30). Whether sorting occurs before or after insertion into the plasma membrane is not yet known.

If plasma membrane proteins are sorted prior to reaching the epithelial cell surface, then they would be distributed asymmetrically as soon as they reach the plasma membrane. If, on the other hand, sorting occurs after insertion in the plasma membrane, then newly made proteins need not have initially the correct polar distribution. Apical membrane proteins, for example, could be inserted at first either in the basolateral domain (9, 23, 24) or in both domains, and only later achieve the proper apical distribution. Sorting in this instance might be a function of the endocytic-recycling machinery (15, 21).

In this paper a biochemical approach has been used to determine the sites of insertion of an apical plasma membrane glycoprotein into the plasma membrane of Madin-Darby canine kidney (MDCK)[1] cells. MDCK cells, which form a polarized epithelal monolayer in vitro (11, 17), are grown on Millipore filters to permit independent assay of the apical and basolateral domains, (19, 29). Previous studies have shown that the influenza virus hemagglutinin behaves as an apical membrane protein in these cells (16, 31, 32, 34, 36). The appearance of hemagglutinin on the cell surface of virus-infected cells can be very sensitively assayed by trypsinization of the cells in the cold (16), conditions which do not open the tight junctions (13). Using these techniques, it is shown that most newly synthesized hemagglutinin is sorted directly to the apical surface before reaching the plasma membrane.

MATERIALS AND METHODS

The MDCK cells used in this study were the typical low resistance line cloned by Louvard to exhibit high blistering activity (12, 16).

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[1] *Abbreviations used in this paper:* EMEM, Earle's minimum essential medium containing 0.2% BSA, 10 mM HEPES pH 7.3, and penicillin-streptomycin; MDCK cells, Madin-Darby canine kidney cells; MEM, Earle's minimum essential medium.

Cell Cultures on Plastic Substrata: Flasks and plastic Petri dishes of MDCK cells were grown as described earlier (14, 16).

Cell Cultures on Filters: For growth of cells on filters, Millipore (Molsheim, France) filters (type HA) with a diameter of 2.5 cm and a pore size of 0.45 μm were brought to a boil three times in glass distilled water to remove the surfactant wetting agent. In some cases Triton-free filters (type HATF) were used in which case one cycle of boiling was sufficient. The wet filters were mounted in mini-Marbrook chambers (Hendley Engineering, Essex, Great Britain) (see Fig. 1) using a metal tool (designed and built by the European Molecular Biology Laboratory workshop) to insure proper alignment of the filter and chamber. The chambers have no floor and consist essentially of two plastic rings that snap together (Fig. 1). As a preliminary test for tightness, the chambers were filled with water; those that rapidly leaked were discarded. The remaining chambers were then placed in an 11- or 13.5-cm diam glass Petri dish with a height of 3 cm, and the Petri dish placed in a disposable autoclave bag. This package was then sterilized for 15 min at 120°C and 15 psi. Harsher autoclaving tended to break many filters.

For every ten filters, one confluent flask (75 cm^2) of MDCK cells was rinsed with phosphate buffered saline (PBS, Dulbecco formulation) lacking calcium and magnesium and the cells brought into suspension by incubation with 5 ml trypsin-EDTA solution. Excess trypsin was removed either by pouring off ~4 ml of the trypsin solution before the cells detached from the plastic, or by pelleting the cells after suspension. The cell suspension was diluted to 10 ml with Earle's minimal essential medium (MEM) containing 10% fetal calf serum, 10 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid) pH 7.3, and penicillin-streptomycin-fungizone to a final cell density of ~2 × 10^6 cells/ml.

Growth medium (as above) was added to the bottom of the Petri dish containing the filter chambers until the chambers just began to float. Air bubbles trapped under the filters were removed by gripping each chamber with sterile forceps and agitating it horizontally on the surface of the medium. Any filters that rapidly took up medium at this time were discarded. Cells (1 ml/ filter chamber) were added to the inside of the chamber and the Petri dish incubated at 37°C in 5% CO$_2$ for 3–5 h to permit the cells to attach. The inner medium was then carefully aspirated and replaced with 1 ml fresh growth medium. The cultures were then returned to the incubator and normally used after 3 d. A representative growth curve is shown in Fig. 2.

Cell Counts on Filters: Because cells on filters were difficult to release quantitatively with trypsin-EDTA, cell nuclei were counted in situ after staining with Hoechst dye (no. 33258) (6). Filters were removed from the chambers, placed in individual plastic Petri dishes and, with multiple intermediate washes in PBS, the cells fixed in 3% formaldehyde in PBS for 20 min, the formaldehyde quenched with 50 mM NH$_4$Cl in PBS for 40 min, and the cells permeabilized with 0.2% Triton × 100 in PBS for 15 min. After three washes in PBS containing 0.2% gelatin, the nuclei were stained with 1 ml Hoechst dye (2.5 μg/ml) for 5–10 min, dehydrated in ethanol containing 25 mM acetic acid, and mounted between a coverslip and slide in a drop of Eukitt. All steps were at room temperature. Nuclei were observed in a Zeiss photomicroscope III (Oberkochen, Federal Republic of Germany) using a 63 × Planapo objective and a 390–440-nm band pass filter after excitation with the ultraviolet lamp, and were photographed at 250 × magnification and counted on contact prints.

Resistance Measurements: Electrical resistance across the monolayer was measured in PBS containing calcium and magnesium at room temperature using a device similar to that developed by R. Steele (20) which permitted measurements directly in the mini-Marbrook chambers. A matched pair of Ag/AgCl electrodes were used to deliver a 100 μA current. The voltage was measured on a Keithly multimeter (Cleveland, OH) using Hg/HgCl electrodes. Contact between the electrodes and the solution bathing the cells was made via saturated KCl/3% agar bridges.

Virus Stocks: Stocks of influenza virus strain N (A/chick/Germany/ 49, Hav2 Ne 1) were grown in embryonated chicken eggs, concentrated, and titered on MDCK cells as described earlier (14, 16).

Pulse-chase Experiments: Filter cultures 3 d old were washed by dipping sequentially in two changes (~200 ml each) warm Earle's MEM containing 0.2% BSA, 10 mM HEPES pH 7.3, and penicillin-streptomycin (EMEM), and placed in 60–80 ml fresh EMEM in a new glass Petri dish. After washing the upper (apical) surface of the cells once more with EMEM, a virus suspension was added at a concentration of ~20 pfu/cell in 250 μl and the virus allowed to adsorb for 1 h at 37°C and 5% CO$_2$. The inoculum was then replaced with 1 ml fresh EMEM and incubation continued for three additional hours.

Pulse labeling and chase incubations were performed at various temperatures in open water baths. Infected filter cultures were washed in two changes warm Hank's balanced salt solution and placed upside down in individual small plastic cups (capacity ~35–40 ml) in either Hank's balanced salt solution or Earle's MEM lacking methionine and containing 0.2% dialyzed (methione free)
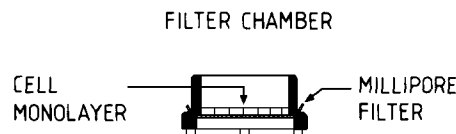
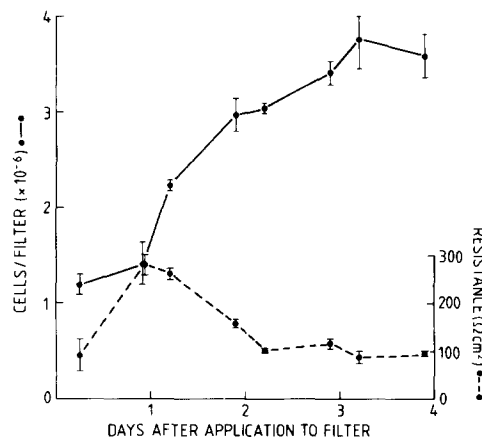FIGURE 1 Mini-Marbrook chamber for growing MDCK cells on a Millipore filter.

FIGURE 2 Growth of MDCK cells on Millipore filters. A suspension of MDCK cells (2.1 × 10^6 cells/chamber) was added to Millipore filters (0.45-μm pore size) mounted into mini-Marbrook chambers placed in a large Petri dish containing growth medium. At various times three filter cultures were removed and the electrical resistance measured. The filters were detached from the chambers, the monolayers fixed and stained with Hoechst dye, and the nuclei counted after photographing the mounted filters in a fluorescent microscope. The error bars represent standard deviations.

BSA, 0.35 g/l sodium bicarbonate instead of the usual 2.2 g/l, and 10 mM MOPS (3-[N-morpholino] propane sulfonic acid), 2 mM NaH$_2$PO$_4$, 10 mM TES (N-tris[hydroxymethyl] methyl 2-amino ethane sulfonic acid), and 15 mM HEPES adjusted to pH 7.4. ((−) methione MEM). The reduced bicarbonate and additional buffers were necessary to prevent pH fluctuations in the room air. BSA was added since transmonolayer resistance decays in the absence of protein (B. Gumbiner, personal communication). The cups were partially immersed in a 37°C water bath. Excess medium was aspirated until the inverted chambers settled to the bottoms of the cups. The basolateral part of the chamber (facing upwards) was aspirated free of medium and 250 μl (−)met MEM containing 50 μCi [^{35}S]met added. After a 5- or 10-min pulse label, all the medium and isotope in the cup was aspirated and the cup quickly filled with Earle's MEM containing 10 times the normal amount of unlabeled methionine (150 mg/l) as well as 0.2% BSA, the reduced bicarbonate concentration, and the buffer mix. Labeling was done from the basolateral surface (through the filter) because incorporation of methionine into protein was nearly 20-fold higher from the basolateral than from the apical domain. When chase incubations were at 20°C instead of 37°C, the cups containing the filter chambers were transferred to a 20°C water bath and filled with chase medium preincubated at 20°C. At the end of chase incubations, the filter chambers were washed by dipping in two changes of ice cold PBS and placed in individual cups on ice containing PBS.

Trypsin Assay: To detect cell surface hemagglutinin, filter cultures in individual plastic cups were immersed in ice cold PBS and oriented so that the side to be trypsinized was uppermost. Excess PBS was aspirated from the cups down to the level of the filter. Soybean trypsin inhibitor was added to the lower part of the chamber to block any trypsin that might leak through. The amount added was sufficient to give a final inhibitor concentration equal to the concentration of trypsin to be used in the assay (see Fig. 4). To allow the inhibitor to reach the apical surface of the monolayer when trypsinization was from the basolateral side, feet made from longitudinally cut plastic tubing were attached to the apical side of the chambers before inverting them in the cup. Feet were already present on the other side of the chamber. For proteolysis, trypsin solution (300 μl usually at 100 μg/ml in PBS) was added to the upper part of the chamber and left for 30 min at 0°C. Preliminary experiments using horseradish peroxidase as a probe suggested macromolecules could diffuse through the filter to the cell layer in as little as 1 min. To terminate proteolysis,

the trypsin solution was aspirated and the side of the culture treated with trypsin washed two times for 15 min with excess soybean trypsin inhibitor in PBS.

Antibodies: Specific antibodies to the N virus hemagglutinin were raised in rabbits by injection of protein micelles in the popliteal lymph node as described earlier (14). The serum was specific for hemagglutinin as judged by immunoblotting (2) of infected cell extracts separated on SDS PAGE and by immunoprecipitation of infected cells extracted first with SDS and then diluted with Triton X-100. When infected cells were extracted with a mixture of 0.1% SDS and 1.0% Triton X-100, the viral structural protein NP was co-precipitated either because some antibodies cross-reacted when NP was not denatured with SDS, or because NP associated with the hemagglutinin when cells were extracted under mild conditions. An IgG fraction was prepared by DEAE-cellulose chromatography (5) and stored in 50% (wt/vol) glycerol in PBS at −20°C.

Immunoprecipitation: Immunoprecipitation was performed as described earlier (16) except that filters were extracted on ice for 30 min directly in 0.1% SDS, 1.0% Triton X-100, 0.15 M NaCl, 20 mM Tris-Cl pH 8.6 containing the protease inhibitors aprotinin (10 μg/ml), iodoacetamide (1 mM), phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 μg/ml), antipain (1 μg/ml), benzamidine (17.5 μg/ml), and soybean trypsin inhibitor (10 μg/ml). Nuclei were removed by a 10 min spin in an Eppendorf (Hamburg, Federal Republic of Germany) microfuge at 4°C and antihemagglutinin IgG added to the supernatants.

Gel Electrophoresis: Gel electrophoresis on urea containing SDS polyacrylamide gels, sample preparation, and fluorography were as described before (16, 22).

Antibody Block of Virus Budding: Antihemagglutinin antiserum and preimmune serum were heated for 30 min at 56°C to inactivate complement and dialyzed overnight against PBS to remove the sodium azide preservative. Four confluent 35-mm dishes of MDCK cells were infected with ~20 pfu N virus/cell for 2.5 h at 37°C and 5% CO$_2$ in EMEM. Fresh medium (1.0 ml/dish) containing 1/10th the normal amount of methionine and 250 μCi [^{35}S]methione were added together with 20 μl preimmune serum or a 20 μl mixture of preimmune plus specific sera to give 1:200, 1:100, and 1:50 dilutions of the antihemagglutinin antiserum in the medium. Incubation was then continued for an additional 4.5 h. The media were collected and centrifuged for 5 min at 2,000 rpm and 5°C to remove any large debris. The cell monolayers were solubilized in a solution of 50 mM Tris-Cl pH 8.8, 2% SDS, and 5 mM EDTA. Aliquots of the media and the cells were counted on Whatman filter discs after trichloroacetic acid precipitation, and the protein concentration of the cell extract determined by the Lowry procedure. Second 0.5-ml aliquots of the medium were layered over a 175-μl cushion of 50% glycerol in 50 mM Tris-Cl pH 7.4, 100 mM NaCl in micro-SW 50 centrifuge tubes, and the virions concentrated by centrifugation at 150,000 g (40,000 rpm) in an SW50.1 rotor (Beckman Instruments, Palo Alto, CA) for 1 h at 4°C. The virus pellets were solubilized in SDS-gel sample buffer containing SDS, electrophoresed, and fluorographed.

Quantitation: Incorporation into the specific hemagglutinin polypeptides was measured by excising the precursor bands (HA + HA$_0$) and HA$_2$ from the dried gel using the autoradiograph as a template, solubilizing the bands in normal calf serum tissue solubilizer, and counting them as described (37). Percent cleavage of each sample was calculated as the quotient of twice the radioactivity in HA$_2$ and the total radioactivity in hemagglutinin (HA + HA$_0$ + 2HA$_2$) assuming that the HA$_2$ contains half the methionine present in the precursor molecule (38). A background value measured from a gel slice where no band was detected was subtracted from every determination. Control samples that were not trypsinized were usually not done since the amount of hemagglutinin cleaved in the absence of trypsin was small or nonexistent.

Electron Microscopy: MDCK monolayers on filters were fixed for electron microscopy in 0.5%–1.0% glutaraldehyde made in 0.1 M sodium cacodylate pH 7.4 for 20 min at room temperature and then transferred into 0.1 M sodium cacodylate at 4°C. Samples were postfixed with 1.0% OsO$_4$ in 0.1 M sodium cacodylate, for 1 h, stained en bloc in 2.0% aqueous uranyl acetate for 1 h at room temperature, dehydrated in a series of alcohols, and embedded in EPON 812. The blocks were sectioned and the sections stained with uranyl acetate and lead citrate (28) and examined in either a Philips 300 or a 400T electron microscope. For estimation of the relative surface areas of apical to basolateral membrane in randomly selected micrographs stereological analysis was performed as outlined by Weibel and Bolender (39). Details will be published elsewhere (von Bonsdorff, C.-H., S. Fuller, and K. Simons, manuscript in preparation).

Sources of Materials: Media and reagents for cell culture were purchased from Gibco Laboratories, (Grand Island, NY). [^{35}S]methionine had a specific activity of >37 TBq/mol and was from Amersham Corp. (Arlington Heights, IL). Worthington trypsin-TPCK (L-1-tosylamide 2-phenylethylchloromethylketone) and soybean inhibitor were obtained from Flow Laboratories,

Inc. (McLean, VA). Enhance was from New England Nuclear (Boston, MA), and NCS tissue solubilizer was from Amersham (Arlington Heights, IL). Protein A-Sepharose was a product of Pharmacia Inc. (Piscataway, NJ). Protease inhibitors were from Sigma Chemical Co. (St. Louis, MO). Hoechst dye no. 33258 was purchased from SERVA, (Heidelberg, Federal Republic of Germany). Eukitt was obtained from Vitromed (Basel).

RESULTS

Characterization of Cells Grown on Filters

MDCK cells were grown on 0.45-μm pore size Millipore filters mounted in mini-Marbrook chambers to permit independent access to the basolateral as well as apical plasma membrane domains. Following their application to the filter, the cells underwent about two rounds of division before reaching their maximum density after ~3 d (Fig. 2). The number of cells per unit area was about five times greater than the saturation density achieved when the cells are grown in plastic dishes, possibly because cells on filters were fed by medium from both the bottom and the top instead of only from the top as on plastic substrata. Cells in the more compact monolayer on filters were also more columnar than plastic grown cells (Fig. 3). Morphometric analysis indicated that the surface area of the apical plasma domain was 25.5 ± 0.05% of the total cell surface area.

Within a day after application of the cells to the filter, electrical resistance rose to a peak of nearly 300 Ω cm^2 (Fig. 2), and then declined to a stable value of ~100 Ω cm^2 after 2 to 3 d. Similar behavior was reported earlier for MDCK cells grown on collagen coated nylon discs (3, 7).

Optimization of the Trypsin Assay on Filter Cultures

RESISTANCE MEASUREMENTS: Previously published studies demonstrated that cell surface hemagglutinin could be detected by trypsinization at 0°C (16). Prior to use of the trypsin assay on filter cultures, experiments were performed to test the effects of infection and trypsinization on monolayer integrity as judged by the electrical resistance of the cultures. The concentration of trypsin used, 100 μg/ml, was the amount shown earlier to efficiently cleave apical hemagglutinin on plastic grown cells (16). As shown in Table I, neither trypsinization nor infection with influenza virus for 4 h caused a significant decline in resistance (13). Brief treatment of filter cultures with EGTA, which is known to open tight junctions (13) led on the other hand to nearly a 40% reduction in resistance (Table I). From these measurements it was concluded that the viral infection and the trypsin assay did not induce substantial paracellular leaks in the filter cultures.

Experiments on Pulse-labeled Filter Cultures

Use of the trypsin assay on filter cultures required that cleavage be equally efficient from both the apical and basolateral sides. Optimization from the basolateral side was particularly difficult because trypsin had to diffuse through the filter and along the lateral cell borders to be effective (see Fig. 4). If too little trypsin was added, it might bind to the filter or be inhibited by serum proteins previously adsorbed to the filter and thus never reach the bottoms of the cells. If too much trypsin was added large areas of the monolayer (with intercellular junctions intact) might be released from the filter, giving trypsin access to the apical domain. These problems were further complicated by the absence of a known trypsin
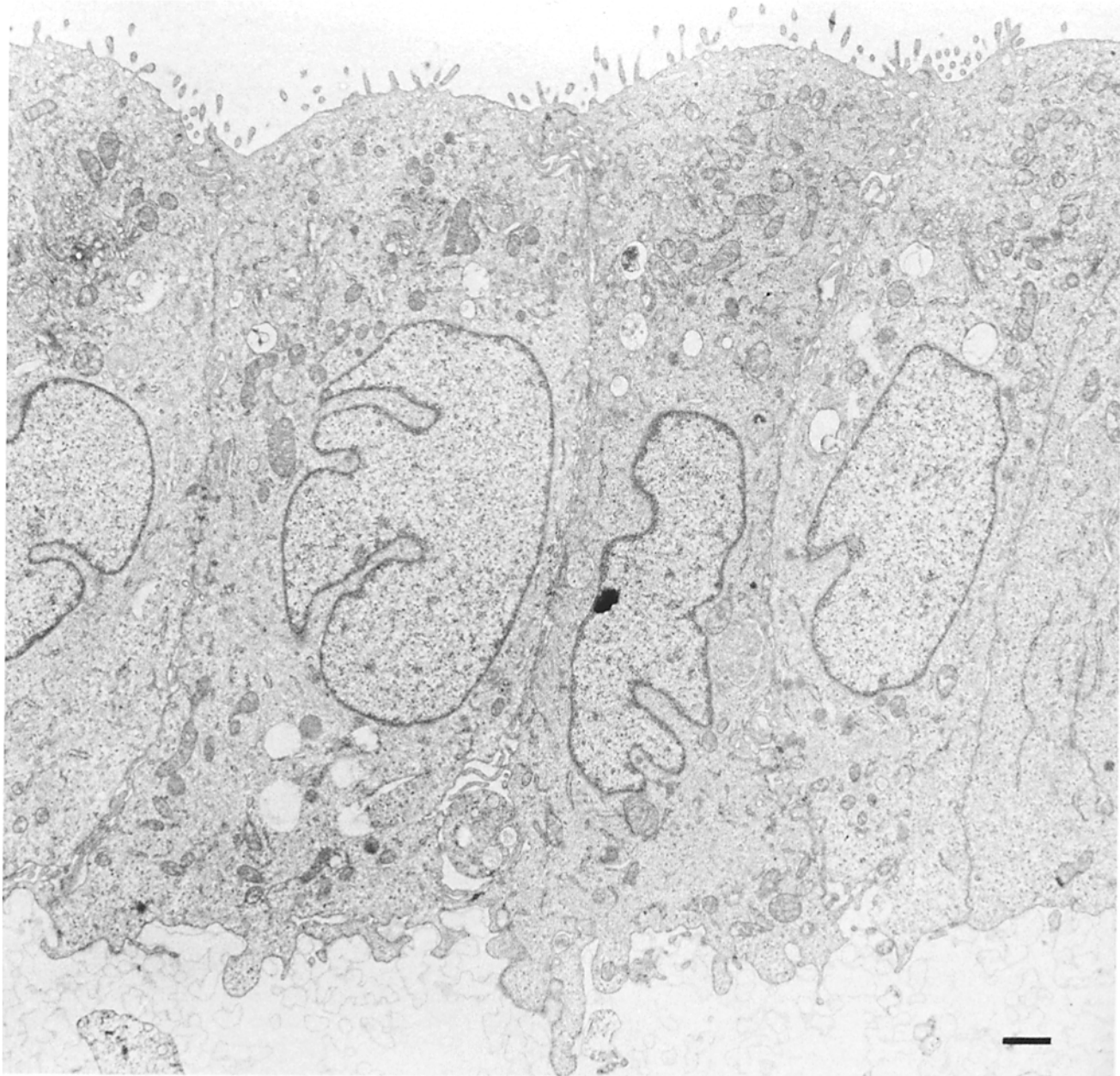
FIGURE 3 Morphology of MDCK cells grown on Millipore filters for 3 d. The apical surface is upwards and the filter is below the monolayer. Bar, 1 μm. × 7,150.

cleavable protein on the basolateral domain to use as a positive control.

To obviate these difficulties, the trypsinization conditions were established on filter cultures in which the hemagglutinin was pulse-labeled and chased for 90 min. By analogy with previous data from cells grown in plastic dishes (16), it was assumed that under these labeling conditions most hemagglutinin would be apical. Some cultures were then permeabilized by brief incubation with EGTA, and both impermeable (those not EGTA treated) and permeable cultures trypsinized apically or basolaterally at 0°C with enzyme concentrations between 10 and 500 μg/ml. In some cases the ability of soybean trypsin inhibitor to block cleavage of hemagglutinin by trypsin leaking across the monolayers was also tested (see Fig. 4).

It was reasoned that in impermeable cultures the optimum

trypsin concentration would cleave most labeled hemagglutinin when added to the apical side but almost no labeled hemagglutinin when added to the basolateral side. Such a result would indicate that trypsin at this concentration could efficiently cleave cell surface hemagglutinin, but was unable to cross impermeable monolayers. In permeable (EGTA treated) cultures, on the other hand, this optimum trypsin concentration would cleave nearly the same amount of hemagglutinin whether added from the apical or basolateral side. This would demonstrate that enough trypsin had been added basolaterally to penetrate the filter, diffuse along the lateral cell borders, and cross the permeable junctions. The labeled hemagglutinin on the apical side would act as a positive control and be cleaved by the basolateral trypsin as it leaked through the junctions to the apical domain.

The results of several such experiments are summarized in

TABLE I

Effects of Infection and Trypsinization on Resistance

Conditions	Trypsinization*	Permeabili-zation[‡]	Resistance (Ω cm^2)[§]
Mock-infected	—	—	104 ± 5.1
Mock-infected	apical	—	106 ± 5.2
Mock-infected	basolateral	—	101 ± 3.7
Mock-infected	—	+	68.0 ± 7.4
Infected[‖]	—	—	102 ± 4.7
Infected	apical	—	101 ± 6.0
Infected	basolatera	—	100 ± 4.8

* Trypsinization was for 30 min at 0°C with 100 μg/ml trypsin-TPCK in PBS with calcium and magnesium in cultures not permeabilized, and without calcium and magnesium in leaky cultures. An equal concentration of soybean trypsin inhibitor was on the opposite side.
[‡] Filter cultures were made leaky by treatment with 2 mM EGTA for 5 min at 37°C in PBS lacking calcium and magnesium.
[§] Resistance was measured as described in Materials and Methods with a current of 100 μA in PBS with calcium and magnesium, or without calcium and magnesium after EGTA treatment. Mean of three cultures shown $\pm$ SD.
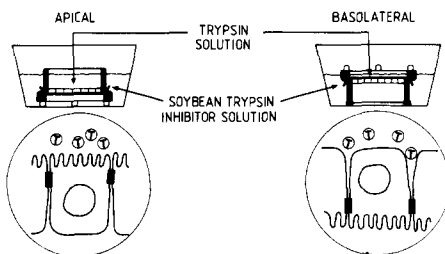[‖] Filter cultures were infected with influenza virus for 4 h at 37°C.

FIGURE 4   Trypsin assay of apical and basolateral domains in filter cultures. Mini-Marbrook chambers oriented such that the cell surface to be trypsin-treated is directed upwards. The circled drawings represent microscopic views of the monolayers and the distribution of trypsin (T) relative to the apical and basolateral domains.

Fig. 5. As shown previously, when the labeled hemagglutinin was entirely intracellular following a pulse-label but no chase incubation, exogenous trypsin did not cleave the precursor (Fig. 5, lanes 1–3). After a 90-min chase almost no hemagglutinin was cleaved in the absence of trypsin (Fig. 5, lane 4). In impermeable monolayers, addition of trypsin apically at 100 or 250 μg/ml at 0°C for 30 min with an equal concentration of soybean trypsin inhibitor on the opposite side resulted in a substantial amount of hemagglutinin cleavage apically (Fig. 5, lanes 5 and 11) but little cleavage from the basolateral side (Fig. 5, lanes 6 and 12). In filter cultures permeabilized with EGTA, 100 μg/ml of trypsin added to the apical domain cleaved nearly the same amount of hemagglutinin as in the impermeable cultures (Fig. 5, lanes 7 and 9). The same concentration of trypsin added from the basolateral side in permeable cultures cleaved substantially more hemagglutinin than in impermeable monolayers (Fig. 5, lane 8) even with soybean inhibitor on the opposite side. In the absence of soybean inhibitor, basolateral trypsin added to permeable cultures cleaved almost as much hemagglutinin as apical trypsin (Fig. 5, lane 10; compare with lane 9). Evidently, trypsin at 100 μg/ml added basolaterally in permeable cultures penetrated the filter and the monolayer and cleaved the apical hemagglutinin.

The conclusion from these data was that trypsin at a concentration of 100 μg/ml incubated with the filter cultures for 30 min at 0°C was sufficient to maximally cleave apical or basolateral hemagglutinin. In addition, these results dem-
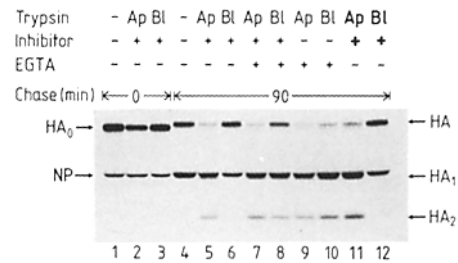

FIGURE 5   Controls of the trypsin assay on filter cultures. MDCK cells on 0.45-μm Millipore filters were infected with N virus for 4 h at 37°C and 5% CO_2. After washing, the individual cultures were pulse-labeled from the basolateral side (bottom, through the filters) with 50 μCi [^{35}S]methione for 5 min and, in some cases, chased for 90 min with medium containing excess unlabeled methionine in a 37°C water bath. The filter cultures were then washed in ice cold PBS-containing calcium and magnesium to stop intracellular transport. To make the tight junctions between cells leaky, some cultures were incubated 5 min at 37°C with 2 mM EGTA in PBS without divalent cations. To assay cell surface hemagglutinin, cultures were incubated 30 min on ice with a trypsin solution in PBS from either the apical (Ap) or basolateral (Bl) side, usually with an equal concentration of soybean trypsin inhibitor on the opposite side (see Fig. 4). Cultures that had been previously permeabilized by EGTA treatment were incubated with enzyme solutions made up in PBS lacking divalent cations. After blocking trypsin with soybean inhibitor, the monolayers were extracted with detergent, the hemagglutinin was isolated by immunoprecipitation, and the polypeptide pattern analyzed by SDS-gel electrophoresis and fluorography.

The various molecular forms of the hemagglutinin are indicated. HA_0 is the precursor with only core sugars; HA is the precursor with complex sugars; HA_1 and HA_2 are the products produced by trypsinization of cell surface hemagglutinin. HA_1 is poorly resolved from the viral internal protein NP, which contaminates the immunoprecipitates. Lanes 1–3, pulse-label but no chase; all hemagglutinin is inside the cell. Lanes 4–12, pulse-label and 90-min chase; hemagglutinin has reached the cell surface. Ap, apical trypsinization; Bl, basolateral trypsinization. Lanes 1 and 4, no trypsin, no soybean inhibitor. Lanes 2, 3, 5–8, 100 μg/ml trypsin, 100 μg/ml soybean inhibitor opposite. Lanes 9 and 10, 100 μg/ml trypsin, no soybean inhibitor opposite. Lanes 11 and 12, 250 μg/ml trypsin, 250 μg/ml soybean inhibitor opposite (bold-face lettering). Notice the increased cleavage of hemagglutinin in lanes 8 and 10 where the leaky junctions have allowed trypsin to penetrate from the basolateral to apical domain.

onstrated that soybean trypsin inhibitor at a concentration equal to that of trypsin on the opposite side of the filter could reduce artifactual cleavage due to leakage of trypsin across the monolayer. These conditions were adopted for the remaining experiments described in this paper.

Appearance of Pulse-labeled Hemagglutinin on the Apical and Basolateral Domains

EXPERIMENTS AT 37°C: To examine the site on the plasma membrane where newly synthesized hemagglutinin first appears, infected filter cultures were pulse-labeled for 5 min and chased for various times at 37°C. At each time point two samples were taken. One was trypsinized apically and the other basolaterally. The cells were then solubilized in detergent solution, hemagglutinin immunoprecipitated, and the samples analyzed by SDS gel electrophoresis and fluorography.

As shown in Fig. 6a, hemagglutinin was first apparent on the plasma membrane in samples trypsinized apically. Cell
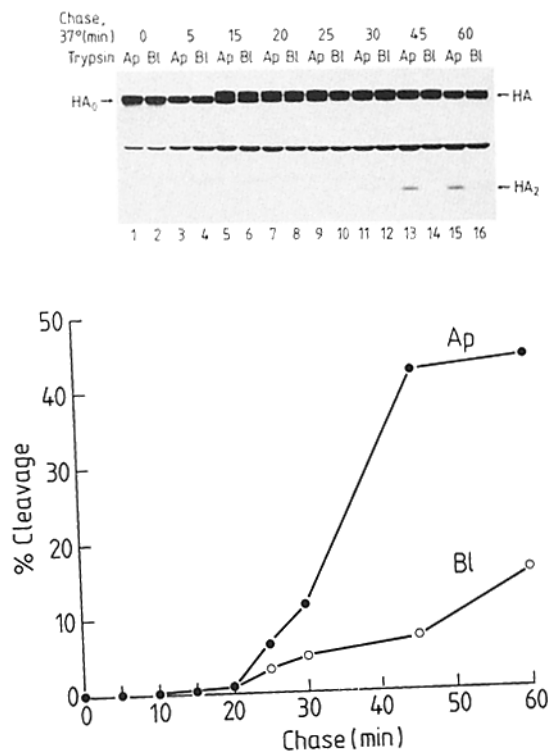
FIGURE 6 Transport of hemagglutinin to the cell surface at 37°C. MDCK cells on filters were infected with N virus, pulse-labeled for 5 min with [^{35}S]methione, and chased for up to 60 min at 37°C (see Fig. 5). Duplicate cultures were sampled at each time point and treated with trypsin apically or basolaterally for 30 min at 0°C with trypsin inhibitor opposite. Hemagglutinin polypeptides were analyzed by fluorography after immunoprecipitation and SDS-gel electrophoresis. a, fluorograph; b, percent cleavage of hemagglutinin calculated by cutting out the precursor HA_0 and HA and the cleavage product HA_2 and counting the radioactivity in each band (see Materials and Methods). Notice that apical (●) exceeds basolateral (○) cleavage at every time point.
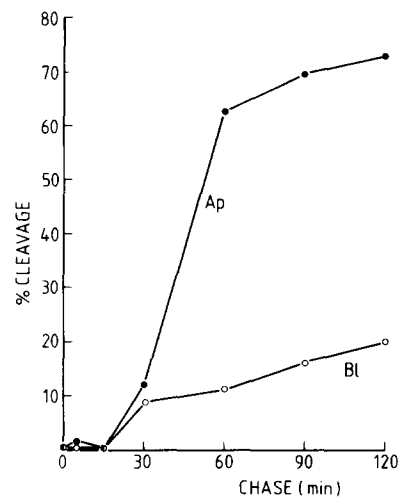

FIGURE 7 Transport of hemagglutinin at 37°C, longer chase incubations. The experiment described in the legend to Fig. 6 was repeated with longer chase incubations.

surface hemagglutinin could be detected as early as after a 25-min chase and approached a constant value after 45 to 60 min. These kinetics were identical to those determined earlier for cells grown on plastic (16). In samples trypsinized basolaterally, there was also progressive appearance of hemagglutinin but in much smaller amounts than in the apical samples. At every time point the amount of apical hemagglutinin exceeded that on the basolateral domain (Fig. 6b).

To examine the distribution of hemagglutinin on the cell surface as steady state conditions were approached, pulse-labeled filter cultures were chased for extended periods up to 2 h and then trypsinized and analyzed. The results are shown in Fig. 7. Again, at all time points apical exceeded basolateral hemagglutinin.

Within the time resolution of this analysis, it seemed that the bulk of the newly made hemagglutinin initially reached the plasma membrane at the apical domain. A significant fraction was also detected simultaneously on the basolateral domain.

EXPERIMENTS AFTER SYNCHRONIZATION AT 20°C: Since a fraction of the cell surface hemagglutinin was detected on the basolateral domain, it was not possible from the previous experiments to rule out a model in which most hemagglutinin transiently appears on the basolateral domain and then is rapidly transferred to the apical domain. One

prediction of this model is that newly made hemagglutinin would be first concentrated on the basolateral domain and only later on the apical domain. To help eliminate this possibility, additional experiments were performed in which the ability to detect small amounts of hemagglutinin on the cell surface was increased. Cells were pulse-labeled for 10 min instead of 5 and chased for 2 h at 20°C. Under these conditions the hemagglutinin is transported to the Golgi complex and acquires complex sugars, but does not reach the plasma membrane (16). When the temperature is raised to 37°C, the hemagglutinin rapidly goes to the cell surface. This approach yields increased sensitivity by doubling the labeling time and then concentrating the labeled hemagglutinin at a distal intracellular site.

As illustrated in Fig. 8, the results from this approach were similar to those obtained earlier. Hemagglutinin was detected on the cell surface after 5 min incubation at 37°C (Fig. 8b). Basolateral hemagglutinin was also seen. At all time points apical exceeded basolateral hemagglutinin.

Experiments were also performed in which trypsin was present at the basolateral surface during the entire chase period. If a significant amount of the hemagglutinin ultimately found on the apical domain traversed the basolateral domain during the chase, then it would be expected to be cleaved by the trypsin continuously present. The previous experiment was repeated but additionally, in a third sample from each time point, trypsin (100 μg/ml) was added to the basolateral medium during the last 10-min incubation at 20°C to allow it to diffuse through the filter, and then left there during the subsequent incubation at 37°C. As shown in Fig. 9, the amount of cleavage in apically treated samples exceeded at all times the amount of cleavage in either the basolaterally or continuously trypsinized filter cultures. The kinetics of hemagglutinin appearance were different in this experiment because the samples were slowly warmed from 20 to 37°C. The slight increase in cleavage in the continuously incubated sample relative to the basolateral sample was probably from the effects of trypsinization at 37°C on the integrity of the monolayer. Still this amount of basolateral cleavage was not sufficient to account for all the hemagglutinin which ultimately can be detected apically.

Chase,20°(min)   0 ◄——————————— 120 ——————————►
Chase,37°(min)   0   0   5   10   15   25   30
Trypsin Ap Bl Ap Bl Ap Bl Ap Bl Ap Bl Ap Bl Ap Bl
HA —   — HA
                                                     — HA₂
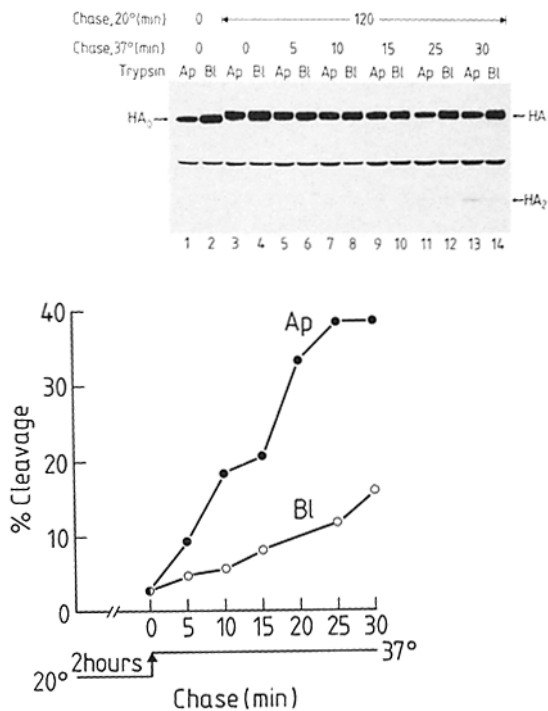   1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 8   Appearance of hemagglutinin on the cell surface after synchronization of the pulse-label at 20°C. Infected MDCK cells in filter cultures were pulse-labeled at 37°C for 10 min with 50 µCi [³⁵S]methione and chased for 2 h at 20°C by addition of precooled chase medium and incubation in a 20°C water bath. The temperature was then raised once again to 37°C by changing the medium and transferring the cultures to the 37°C water bath. At each time point duplicate samples were taken and trypsinized apically or basolaterally with soybean inhibitor opposite. Hemagglutinin polypeptides were analyzed by fluorography after immunoprecipitation and SDS gel electrophoresis. a, fluorograph; b, quantitation from the gel. Ap, apically trypsinized; Bl, basolaterally trypsinized.

Effects of Specific Antibodies on Apical Hemagglutinin Appearance

All the previous experiments assume that hemagglutinin on the basolateral domain is equally sensitive to trypsin as apical hemagglutinin. Although it was unlikely that trypsin would fail to cleave hemagglutinin on the basolateral domain, another approach using antibodies to perturb possible transfer of hemagglutinin from the basolateral to apical domain was attempted.

A rabbit antiserum specific for the hemagglutinin (see Materials and Methods) was first titered against infected MDCK cells grown in plastic dishes to determine a concentration that would block virus budding from the cells. All dilutions tested, 1:50, 1:100, and 1:200, completely inhibited appearance of virions in the medium (as judged by isolation of radioactive virions) (data not shown). Although indirect, this assay at least indicated that sufficient antibody was present at these dilutions to bind to the cell surface and exert an effect on virus production.

Filter cultures were then infected, pulse-labeled, and incubated at 20°C for 2 h. Specific antibodies were added to the basolateral side of one set of filters at a final dilution of 1:100 during the last 30 min at 20°C to give them time to diffuse through the filter to the cell monolayer before labeled hemagglutinin reached the cell surface. Preimmune antiserum

Chase,37°(min) ——0 ——•—— 5 ——•—— 10 ——•—— 15 ——•——20——•
Trypsin Ap Bl Co Ap Bl Co Ap Bl Co Ap Bl Co Ap Bl Co
HA —   — HA
                                                          — HA₂
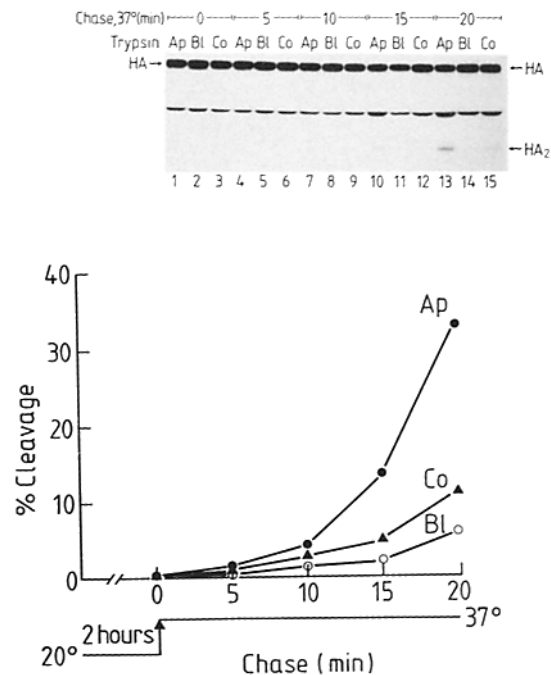   1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 9   Cleavage of hemagglutinin by trypsin continuously present on the basolateral side. Infected MDCK cells on filters were pulse-labeled and chased for 2 h at 20°C as described in the legend to Fig. 8, and then slowly warmed to 37°C by transferring the culture from the 20°C water bath to one at 37°C. Trypsin (100 µg/ml) was added to the basolateral side of one set of cultures with an equal concentration of trypsin inhibitor on the opposite side 10 min prior to the shift from 20 to 37°C to allow the trypsin to penetrate the filter. At each time point, one culture trypsinized continuously in this way and two other cultures were sampled. The continuously trypsinized culture was treated with trypsin inhibitor to block further trypsinization. The other two were treated apically or basolaterally with 100 µg trypsin/ml at 0°C for 30 min with trypsin inhibitor opposite. Hemagglutinin polypeptides were analyzed by immunoprecipitation, SDS gel electrophoresis, and fluorography. a, fluorograph; b, quantitation from radioactivity on the gel. Ap, apically trypsinized (at 0°C); Bl, basolaterally trypsinized (at 0°C). Co, continuously trypsinized (at 37°C). Note that the apical cleavage is always greater than either the continuous or basolateral samples. The different shape of the curve in b relative to that shown in previous figures is due to the slow warming from 20 to 37°C.

at the same dilution was added to a second (control) set of filters. The cultures were then warmed to 37°C, samples taken at various times, and both sets of filters trypsinized apically at 0°C. As shown in Fig. 10, the antibodies had no effect on apical hemagglutinin appearance.

DISCUSSION

The data presented here indicate that the apical domain is the site on the plasma membrane where most of the influenza virus hemagglutinin appears initially following its synthesis. A model in which the hemagglutinin has to pass the basolateral domain prior to reaching the apical domain seems unlikely from these results. If this model were true, then trypsin continuously present on the basolateral side during a chase of pulse-labeled hemagglutinin should cleave most of the hemagglutinin on its way to the apical cell surface. This was not the case (Fig. 9). In addition, specific antibodies present basolaterally did not inhibit the appearance of hemagglutinin apically (Fig. 10). For a basolateral component to go unde-
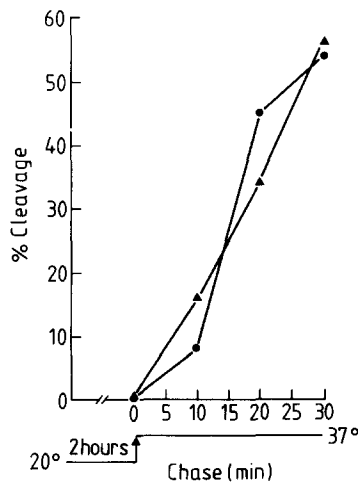
FIGURE 10. Effects of basolateral antihemagglutinin antiserum on apical hemagglutinin appearance. Infected MDCK cells on filters were pulse-labeled and chased for 2 h at 20°C as described in the legend to Fig. 8, and then slowly warmed to 37°C by transferring them from a 20 to 37°C water bath. Prior to raising the temperature, heat inactivated antihemagglutinin antiserum was added to the basolateral side of one set of cultures to a final dilution of 1:100. Preimmune serum was added to the parallel (control) cultures at the same concentration. At each time point, one culture from each set was sampled. To assay hemagglutinin on the apical domain, both sets were then trypsinized apically with 100 µg trypsin/ml for 30 min at 0°C, and the polypeptides analyzed by immunoprecipitation, SDS-gel electrophoresis, and fluorography. Only the percent cleavage calculated from counting bands off the gel is shown here. ▲ specific antiserum; ●, control (preimmune) serum.

tected in these assays, the resident time would have to be exceedingly short. Also unlikely is a model in which the hemagglutinin first appears uniformly distributed over the plasma membrane, and is then concentrated apically by sorting during membrane recycling (15, 21). At all time points during the pulse-chase experiments, it was observed that the amount of apical exceeded the amount of basolateral hemagglutinin. If insertion of the hemagglutinin had occurred in proportion to surface area of the plasma membrane domains, then the surface area of the apical domain would have to exceed that of the basolateral domain by more than threefold to produce the observed data. In fact, estimates of the relative surface areas from electron micrographs of cells grown on filters suggest the opposite: the basolateral surface area is about threefold greater than the apical.

These conclusions differ from those reported earlier by Quaroni et al. (23, 24) and Hauri et al. (9). In their experiments the routing of total fucosylated proteins and the specific apical enzyme sucrase-isomaltase to the cell surface in rat enterocytes was examined by in vivo pulse-labeling and cell fractionation. They concluded that a large proportion of the apical components first appeared basolaterally and were then moved apically. However, it is difficult from their experimental approach to rule out cross-contamination between cytoplasmic vesicles carrying plasma membrane proteins to the apical domain and vesicles derived from the basolateral surface.

MDCK cells and other epithelial cell lines have previously been grown on Millipore filters to study ion and solute transport (3, 8, 19). The first use of Millipore filters mounted in mini-Marbrook chambers was by Richardson and Simmons (29) to demonstrate in MDCK cells the polarity of surface components that could be iodinated by lactoperoxidase. With this system, both sides of the cell are equally and independently accessible. Labeling with radioactive methionine from the basolateral side, which is only possible on filters or other permeable supports, is at least an order of magnitude more sensitive than labeling from the apical side because the amino

acid uptake system is concentrated on the basolateral domain (Balcarova-Ständer et al., submitted; 25, 26). In addition, cells grown on filters are morphologically better organized and grow to a higher density than those grown on plastic substrata. They have a more columnar shape and asymmetric arrangement of organelles (Fig. 3).

The filter culture system used in this study is not simple to manipulate. Millipore filters are opaque so that confluence must either be assessed indirectly by measurement of electrical resistance, or the monolayer fixed and observed by fluorescent staining or after making the filter transparent. For judging the leakiness of the monolayer, electrical resistance is not completely satisfactory since a filter that passes ions may still be impermeable to macromolecular reagents such as trypsin. Conversely, a filter with a local imperfection permitting an enzyme to cross may still have a significant electrical resistance. Assays for monolayer tightness must, therefore, be carefully tailored to the experiments they are designed to control. Lastly, Millipore filters mounted in mini-Marbrook chambers are very susceptible to damage from handling during the experiment. Leaks are easily induced, for example, by hydrostatic pressure from the basolateral side which may force the cells away from the filter.

The experiments described here suggest that some hemagglutinin is also present on the basolateral domain. The magnitude of this component cannot be accurately determined using the trypsin assay because small trypsin leaks from the basolateral side would lead to an overestimate. Basolateral hemagglutinin has been independently detected by immunofluorescence of filter grown cells (6) and by immunoperoxidase staining of plastic grown cells (Bainton, D., and K. Matlin, unpublished observations), and estimated to be at least 6% of the total cell surface hemagglutinin by radiometric techniques (Pesonen, M., and K. Simons, unpublished observations).

There is also evidence that other apical proteins have a small basolateral component in both MDCK and LLC-PK₁ cells (Balcarova-Ständer et al., submitted; 25, 26). Whether this is true also for epithelia in vivo is not yet clear. A number of studies have previously detected normal apical proteins on the basolateral domain (4, 9, 18, 23, 24,). These data are derived from cell fractionation and may therefore be explainable by cross-contamination. In a recent careful look at the distribution of leucine aminopeptidase on isolated hepatocyte plasma membranes by immunogold labeling, 85% of the specific label was localized on the (apical) bile front (35). Labeling on the other domains was not above background levels, but the authors were unable to conclude that aminopeptidase was completely absent from these parts of the plasma membrane. More work is clearly needed to assess the significance, if any, of misplaced apical proteins.

The results reported here suggest that the newly synthesized hemagglutinin and probably other apical proteins are sorted prior to reaching the plasma membrane. Complementary data on basolateral proteins are required to strengthen this conclusion. Recent experiments on secretion from MDCK cells are consistent with this idea (Kondor-Koch et al., submitted). When MDCK cells were stably transformed with a chicken lysozyme cDNA (a nonglycosylated secretory protein), the enzyme was released equally on both sides of the cells. This indicates that two exocytic pathways exist, one directly to the apical and one directly to the basolateral domain. Future work will be focused on identifying the cellular organelle from which the two pathways diverge.

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