The Large External Domain Is Sufficient for the Correct Sorting of Secreted or Chimeric Influenza Virus Hemagglutinins in Polarized Monkey Kidney Cells

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Abstract. MA104.11 rhesus kidney cells express several characteristics of polarized epithelial cells, including the formation of "domes" on impermeable substrates, the establishment of a transmonolayer electrical resistance when grown on collagen gels, the polarized maturation of influenza and vesicular stomatitis viruses, and the expression of the glycoproteins of those viruses at a single surface domain. The polarized expression of the influenza virus hemagglutinin (HA) is maintained in MA104.11 cells infected with SV40-derived vectors carrying a cDNA gene for either the wild-type influenza virus HA, a truncated HA gene encoding a secreted form of HA (HA^{sec}), or a chimeric gene encoding a hybrid protein with the external domain of the HA and the transmembrane and cytoplasmic domains of the vesicular stomatitis virus G protein (HAG). Thus, the recognition event separating glycoproteins, such as HA, destined for the apical surface from proteins, such as G, destined for the basolateral membranes involves features of the external domains of the proteins. The transmembrane and cytoplasmic domains of HA have no role in this process.

EUKARYOTIC cells direct a complex internal traffic of proteins moving from common sites of synthesis to separate final destinations. In polarized epithelial cells an aspect of this traffic is the transport of secreted and membrane-bound proteins to structurally distinct domains of the plasmalemma. These surface domains, delimited by the junctional complexes of the *zonula occludentes*, each contain unique sets of proteins required for their functional specialization (reviewed by Rodriguez-Boulan, 1983*a*; Simons and Fuller, 1985). Obviously, there are mechanisms for the recognition, sorting, and directional transport of these sets of proteins.

Polarized epithelial cells recognize not only their own proteins but also the glycoproteins of the enveloped viruses that infect them (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan and Pendergast, 1980; Herrler et al., 1981; Roth et al., 1983*a*; Rodriguez-Boulan, 1983*a*; Simons and Warren, 1984; Srinivas et al., 1986). During the early stages of infection of kidney epithelial cells with influenza viruses, the viral hemagglutinin (HA)[1] is expressed almost entirely at the apical cell surface where the virus buds (Rodriguez-Boulan and Pendergast, 1980; Fuller et al., 1983). Thus, the infected cell recognizes HA as belonging to the apical set of proteins, and this recognition has been shown to be independent of either glycosylation of HA (Green et al., 1981), or of the presence of other influenza virus proteins (Roth et al., 1983*b*). In the infected epithelial cell, the viral glycoproteins become the major content of the exocytic pathway and this has proven to be an enormous advantage for both morphological and biochemical characterizations of directional protein transport. For example, it has been established that HA is transported from the Golgi apparatus in small vesicles (Rodriguez-Boulan et al., 1984; Rindler et al., 1985) and that HA travels directly to the apical surface and does not pass through the basolateral domain (Misek et al., 1984; Matlin and Simons, 1984; Rindler et al., 1985). A protein that accumulates at the basolateral surfaces of epithelial cells, the G protein of vesicular stomatitis virus (VSV), also appears to be transported directly to its final location (Rindler et al., 1985; Pfeiffer et al., 1985; Salas et al., 1985) and is sorted in the absence of other VSV proteins (Stephens et al., 1986). HA and G proteins have been observed to colocalize in the cisternae of the Golgi apparatus in doubly infected epithelial cells (Rindler et al., 1984). Biochemical data indicate that G protein and the influenza neuraminidase, another apical glycoprotein (Jones et al., 1985) share the transport pathway at least as far as the terminal cisternae of the Golgi apparatus where sialic acid is added to G (Fuller et al., 1985). Thus, sorting of some surface glycoproteins occurs at, or between, the site of exit from the Golgi apparatus and the point of arrival at the cell surface.

The features of HA recognized by the cellular sorting apparatus could reside in the large external domain of HA exposed to the lumenal environment of the exocytic pathway,

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1. *Abbreviations used in this paper*: HA, hemagglutinin; pi., postinfection.

or could be present in the cytoplasmic domain and be available to interact directly with cellular components responsible for forming and/or targeting transport vesicles. To determine the domain of HA containing features important for sorting the protein to the apical cell surface, we have taken two approaches. (*a*) We have expressed a truncated HA gene lacking the sequences encoding the transmembrane and cytoplasmic domains and have studied the secretion of this product, HA^{sec}, in polarized cells. (*b*) We have constructed a chimeric HA glycoprotein gene in which sequences encoding the transmembrane and cytoplasmic domains of HA have been replaced by the analogous sequences from G protein (Roth et al., 1986). The chimeric protein encoded by this gene, HAG, was expressed from SV40 vectors in polarized cells and the surface distribution of the protein was determined by immunofluorescence and quantitative immunoelectron microscopy.

To date, the best vectors for introducing foreign glycoprotein genes into epithelial cells have been the simple enveloped viruses such as influenza, VSV, and Semliki Forest virus. However, the RNA genomes of these viruses are not amenable to genetic engineering. Thus, we have explored the use of recombinant virus vectors based on SV40, a simple DNA-containing virus that does not inhibit host cell macromolecular synthesis (Acheson, 1980). Such vectors have proven useful for the expression of cloned viral glycoprotein genes, as SV40 infection does not alter glycoprotein biosynthesis (Gething and Sambrook, 1981, 1982; Sveda and Lai, 1982; White et al., 1982; Hartman et al., 1982; Markoff et al., 1983; Roth et al., 1983*b*; Davis et al., 1983; Wills et al., 1984; Doyle et al., 1985; Jones et al., 1985; Zebedee et al., 1985; Roth et al., 1986; Gething et al., 1986). Since SV40 has a narrow host range limited to a few species of monkey cells, we have searched for a polarized epithelial cell line capable of supporting the replication of our expression vectors. We describe the advantages and limitations of such a cell line in our report.

Materials and Methods

Preparation of Virus Stocks

The A/Japan/305 strain of influenza virus was adapted to grow in MA104.11 cells by serial passage. VSV ts045, originally obtained from Dr. Sondra Schelessinger (Washington University, St. Louis, MO), was adapted to MA104 cells and grown as described elsewhere (Rodriguez-Boulan, 1983*b*). The construction of the vector SVEHAG is described in detail by Roth et al. (1986); the SVEHA and $SVEHA^{sec}$ vectors are those described by Gething and Sambrook (1981, 1982). Stocks of SV40 vectors and of the helper virus Dl1055 were prepared as previously described (Gething and Sambrook, 1981; Doyle et al., 1985).

Preparation of Filters Containing Collagen Gels

Millipore filter rings were prepared as described elsewhere (Misek et al., 1984) or by following a slightly modified protocol as described below, for the experiments with HA^{sec}. Nitrocellulose filters (SSWP 02500; Millipore Corp., Bedford, MA) with 3.0-μm diameter pores were boiled for 10 min to remove surfactants and mounted damp on plastic rings as follows. The open ends of 16-mm diameter polystyrene tubes (Sarstedt, Federal Republic of Germany) were dipped into chloroform and the softened plastic pressed to the nitrocellulose filter and allowed to harden; excess filter was excised and a 5-mm high plastic ring bonded to the filter was cut from the tube with a hot scalpel. The mounted filters were sterilized with 1.0% glutaraldehyde in H_2O for 30 min and then rinsed twice for 20 min in DME. A collagen gel was formed in the pores of the filter by soaking the filter in an ice-cold gelling solution (Elsdale and Bard, 1972) that contained ~1.0 mg/ml rat tail collagen. Excess gelling solution was removed and the gel allowed to form at 37°C. After gels had formed, the filters were stored in a 37°C incubator in DME containing 10% fetal calf serum. Before use, each batch of filters was tested for permeability to HA^{sec} that had been labeled with ^{125}I. Approximately 2.0% of the iodinated HA^{sec} was retained by the filters, a percentage equal to the proportion of the total medium present that was retained within the pores of the filter. Thus, adsorption of HA^{sec} to collagen-coated filters was minimal.

Measurements of Electrical Resistance of MA104.11 Cell Monolayers

Measurements of the electrical resistance of MA104.11 cell monolayers were made separately in the laboratories of E. Rodriguez-Boulan and M. Roth and were in good agreement. Electrical potential was measured across the monolayer with a high impedance voltmeter essentially as described by Cereijido et al. (1977). The resistance of filters containing collagen gels without cell monolayers was determined in each experiment and subtracted from that determined for filters with cells.

$SVEHA^{sec}$ Infection of MA104.11 Monolayers Grown on Filters

Filters with collagen gels were placed in 12-well culture dishes (Flow Laboratories, Inc., McClean, VA) and supported by 1-mm diameter glass beads fixed to the bottom of the well. MA104.11 cells that had been maintained several days past confluence on plastic culture dishes were trypsinized and seeded at a density sufficient to form a confluent monolayer, 1.3 × 10^5 cells/cm^2. Electrical resistance across the monolayer was measured daily. When resistance had risen to at least 200 ohms·cm^2, monolayers were infected with $SVEHA^{sec}$ virus, either from the top compartment, or through the filter. Virus was allowed to adsorb at 22°C in an atmosphere of 5% CO_2 for 4–6 h. The filters were immersed in DME containing 10% fetal calf serum and maintained at 37°C in an atmosphere of 5% CO_2.

Analysis of HA^{sec} Secretion

The medium of infected monolayers of MA104.11 cells on filters was changed to serum-free DME lacking methionine (Met−) for 30 min. To avoid creating a hydrostatic pressure beneath the monolayer that might lift the cells from the filter, medium was never removed from the top chamber when medium was present in the bottom chamber. The filters were moved to a new well and 250 μCi of [^{35}S]methionine in Met− medium at 37°C was added. After 30 min this labeling medium was removed and the filters were washed once in DME lacking serum and moved to a new well containing this medium. Two chase protocols were followed. (*a*) To determine the distribution of HA^{sec} in the apical or basal medium at different periods postinfection (pi.), either the chase was continued for 6 h in the same well, or after 3 h the medium was collected from the top and bottom filter chambers and the filter moved to a new well for another 3 h. After the chase, medium was collected from the top and bottom of the filter, and, if the filter had been shifted during the chase, medium from each side of the filter collected during the two chases was pooled. One-fourth of each sample was precipitated with 5 vol acetone containing 2% trichloroacetic acid. The remainder of each sample was immunoprecipitated with excess anti-HA and protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). (*b*) To investigate whether HA^{sec} accumulated in the filter after labeling at early periods pi., medium was collected from each side of the filter after chase intervals of 0, 2, and 6 h. For each sample, the filter was extracted first with 500 μl 50 mM Tris at pH 8.0 containing 1% NP-40 to release any HA^{sec} that remained within the cells. The filter was then extracted with 1% SDS in 50 mM Tris at pH 8.0. SDS extractions were performed at 37 or 75°C for 1 h, or at 98°C for 15 min (changing the temperature of the SDS extraction had no effect on the amount of HA^{sec} released from the filters), followed by vortexing for 2 min. The SDS extract was diluted to 500 μl with NET/GEL (150 mM NaCl, 50 mM Tris, pH 7.4, 10 mM EDTA, 0.25% gelatin, 0.05% NP-40) and left at 4°C overnight to allow complete diffusion of material from the pores of the filter. The filter was removed and the NP-40 and SDS extracts were combined and diluted to a 0.1% final concentration of SDS and immunoprecipitated. Immunoprecipitates were analyzed by PAGE (Laemmli, 1972). The gels were fluorographed (Bonner and Laskey, 1974) and exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C. For quantitation of HA^{sec} secretion, autoradiographs were scanned with a video densitometer (model 620; Bio-Rad Laboratories, Richmond, CA).

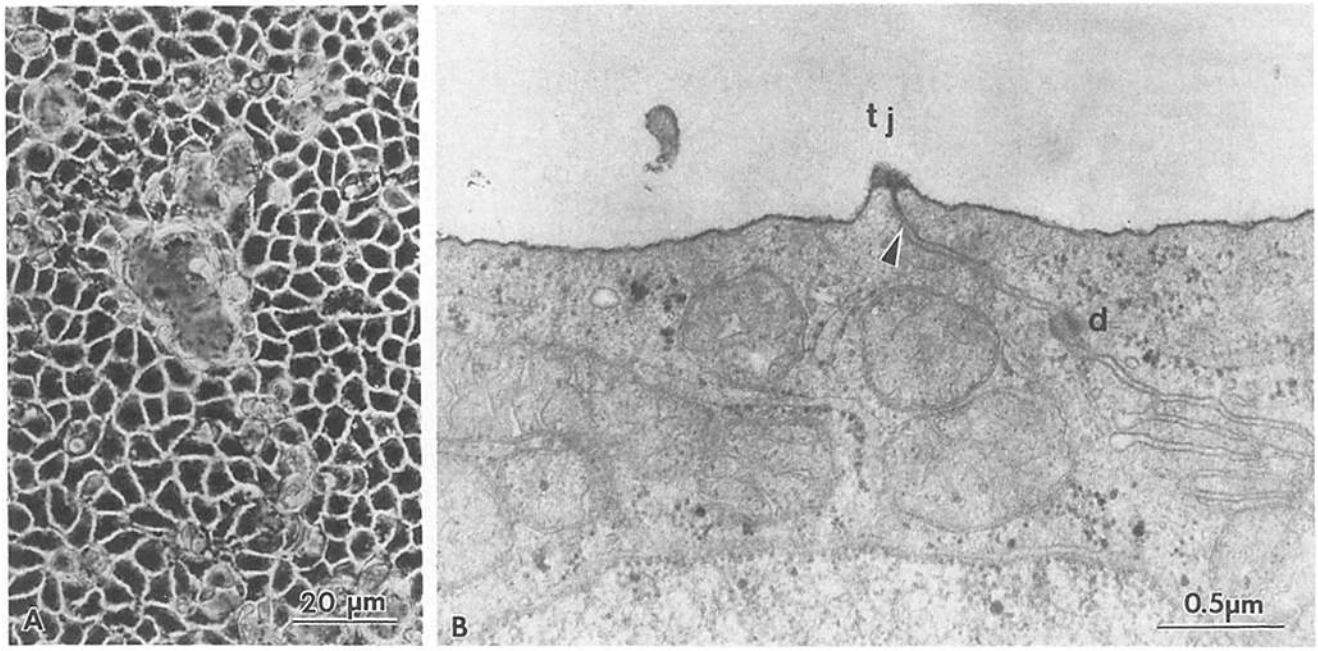


Figure 1. Domes and tight junctions in MA104.11 cells. When MA104.11 cells were grown on plastic substrata, domes started to develop 5 d after confluence (*A*). The presence of tight junctions was demonstrated by treating fixed confluent monolayers with ruthenium red (*B*). The dark reaction on the glycocalix in the apical surface stops at the level of the tight junction (*arrowhead*). Note the presence of a desmosome (*d*) and interdigitations in the lateral cell surface.

Only autoradiographs exposed within the linear response range of the film were used, and only samples for which the integrated optical density of the HA^{sec} band was 20× background were included in the quantitation.

Immunofluorescence

The procedures for immunofluorescence have been previously described (Rodriguez-Boulan, 1983*b*). Five-step immunofluorescence localization of HA on the apical and on the basolateral membranes was carried out on cells (grown on glass coverslips) fixed with 2% formaldehyde (freshly prepared from paraformaldehyde) and 0.05% glutaraldehyde in PBS. The fixed monolayers were incubated successively with: (*a*) a rabbit polyclonal antibody against the Japan HA (partially purified on a protein A-Sepharose column), (*b*) affinity-purified goat anti-rabbit IgG coupled to fluorescein (Cappel Laboratories, Cochraneville, PA), (*c*) 0.1% Triton X-100 in PBS to permeabilize the cells, (*d*) a monoclonal antibody specific for the Japan HA, and (*e*) affinity-purified goat anti-mouse IgG coupled to rhodamine (Cappel Laboratories). Semi-thin frozen sections were prepared essentially as described elsewhere (Salas et al., 1986) and stained for immunofluorescence as described above, but without the detergent treatment.

Electron Microscopy and Immunoelectron Microscopy

Samples that were not labeled with antibodies and protein A gold were fixed with 2% glutaraldehyde, postfixed with osmium tetroxide, dehydrated, and embedded in Epon. Thin sections were cut with a Sorvall MT-5000 ultramicrotome (Dupont Co., Wilmington, DE) and stained with lead citrate and uranyl acetate (Reynolds, 1963). For immunoelectron microscopy, colloidal gold particles of 8–12-nm diameter were prepared and coupled to protein A according to the procedure of Slot and Geuze (1985). Virus-infected monolayers on glass coverslips or on collagen-coated filters were fixed at appropriate times of infection with 2–3% formaldehyde (freshly prepared from paraformaldehyde) and 0.05% glutaraldehyde in PBS containing 0.1 mM Ca and 1 mM Mg. Filters were excised from the plastic rings with a scalpel and monolayers were scraped from the glass coverslip with a razor blade. After free aldehyde groups were quenched with 50 mM ammonium chloride in PBS, the filters were incubated at room temperature in the presence of HA-specific rabbit antibody (10–20 μg/ml) in PBS containing 0.1% BSA (PBS-BSA) for 2 h, washed while shaking with several changes of PBS-BSA, exposed for 2 h to colloidal gold–protein A (1:5 dilution), washed for 2 h in PBS-BSA and for 30 min in 0.1 M Na cacodylate (pH 7.4), and fixed (1 h to overnight) in 2% glutaraldehyde in Na cacodylate buffer containing 100 mM sucrose. Labeling efficiencies of basal antigens were identical when antibodies and colloidal gold were applied to the basal surface of filters in plastic ring assemblies, indicating that access to the basal surface was through the collagen-coated filters and not through breaks in the monolayers. After embedding in Epon, thin sections were mounted on Formvar-coated copper grids and photographed with a JEOL-100X electron microscope. The density of colloidal gold particles binding to the apical and to the basal surface was measured as a function of linear segments of the respective surface, measured with a Micro-Plan II image analyzer (Laboratory Computer Systems, Inc., Cambridge, MA). The background labeling of uninfected MA104.11 cells was observed to be very low, one to two gold particles per 10 μm of apical or basal membrane, and binding of protein A–gold to the collagen gel beneath cells was also minimal. In comparison, at early periods pi., average specific labeling of 10 μm segments of the apical surfaces of infected MA104.11 cells was 160, 120, and 50 for cells infected with the A/Japan influenza virus, SVEHA, and SVEHAG, respectively.

Results

MA104 Rhesus Kidney Cells, a Polarized Epithelial Cell Line that Supports SV40 Replication

SV40 replicates in kidney cells from African Green or Rhesus monkeys, but not in any of the well characterized epithelial cell lines such as Madin-Darby canine kidney (MDCK) or LLC-Pk1. Previously it was demonstrated by electron microscopy that epithelial cells within a mixed population of primary kidney cells derived from African Green monkey expressed HA in a polarized manner when infected with SV40 vectors carrying cDNA genes (Roth et al., 1983*b*). However, the presence of nonpolarized cell types in this preparation made quantitative experiments impossible. Thus, we have been searching for a continuous cell line that both

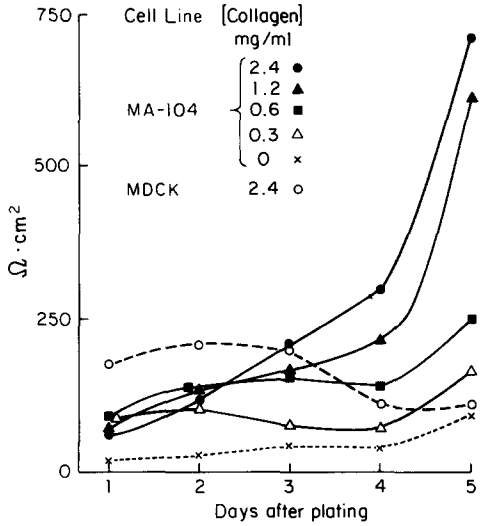


Figure 2. Development of transmonolayer electrical resistance by MA104.11 cells plated on collagen-coated nitrocellulose filters. MA104.11 cells were plated at confluence (150,000 cells/cm^2) on nitrocellulose filters coated with various concentrations of collagen in native gels. Transmonolayer electrical resistances were measured daily until the fifth day. Development of resistance was highly dependent on the collagen concentration for MA104.11, but not for MDCK cells.

retains the differentiated properties of transporting epithelia and is permissive for SV40 replication. One of the more easily distinguished characteristics of transporting epithelial cells in culture is the ability of these cells to transport water and salts across the basolateral membrane. The accumulation of fluid beneath the cells can become great enough to lift a section of monolayer free of its plastic substrate, resulting in the formation of a raised "blister" or "dome" of cells (Leighton et al., 1970; Rabito et al., 1978; Lever, 1979). MA104 rhesus kidney cells obtained from the laboratory of Paul Atkinson (Albert Einstein School of Medicine, Bronx, New York) were observed to form domes at low frequency. MA104 cells were subjected to two rounds of single-cell cloning and sublines were selected that formed domes when confluent (Fig. 1 *A*). One such line, MA104.11, was used for most of the studies in this report. The morphology of these cells was examined by electron microscopy. MA104.11 cells exhibited few microvilli and basal folds and abundant interdigitations, a morphology compatible with almost any nephron segment, with the exception of the proximal tubule. To directly demonstrate the junctional complexes of MA104.11 cells grown on an impermeable substrate, an electron-dense dye, ruthenium red, was added to cell cultures on plastic dishes that had been confluent for 4 d. Ruthenium red stained glycoconjugates present at the apical surface of MA104.11 cells up to the level of the tight junctions; no staining was detected on the lateral surface, indicating that the tight junctions were functional (Fig. 1 *B*).

MA104.11 cells were grown on nitrocellulose filters and tested for the presence of the transmonolayer electrical resistance characteristic of cells derived from renal tubules. The degree of electrical resistance developed by MA104.11 monolayers depended to some extent upon their substrate. MA104.11 cells would not form electrically tight monolayers on nitrocellulose filters with pore diameters of 0.8 μm or greater. However, when a native collagen gel was cast within the pores of the filter, MA104.11 cells formed confluent monolayers, even on filters with 8.0-μm diameter pores. Electrical resistance of these monolayers was low and slow to develop if the collagen concentration in the gel was <0.6 mg/ml (Fig. 2). However, at collagen concentrations of 1 mg/ml or higher, MA104.11 cells developed resistances of 700 ohms·cm^2 by 4 to 5 d after plating (Fig. 2).

To determine whether in MA104.11 cells, as in MDCK cells, viral budding and the expression of viral glycoproteins was polarized, monolayers confluent for 4–5 d were infected with either influenza virus or the ts045 mutant of VSV, which has a reversible temperature-sensitive defect in the viral glycoprotein (Lafay, 1974; Knipe et al., 1977). Infection with influenza was for 8 h; infection with ts045 was for 3 h at nonpermissive temperature (40°C) followed by 2 h at permissive temperature (32°C). Influenza virus budded with high preference from the apical surface while ts045 was selectively assembled from the basolateral plasmalemma (Fig. 3, *A* and *B*). Colloidal gold immunocytochemistry on infected monolayers grown on filters demonstrated that, as in MDCK cells, influenza HA was concentrated in the apical surface and VSV G protein in the basolateral surface (Fig. 3, *C* and *D*).

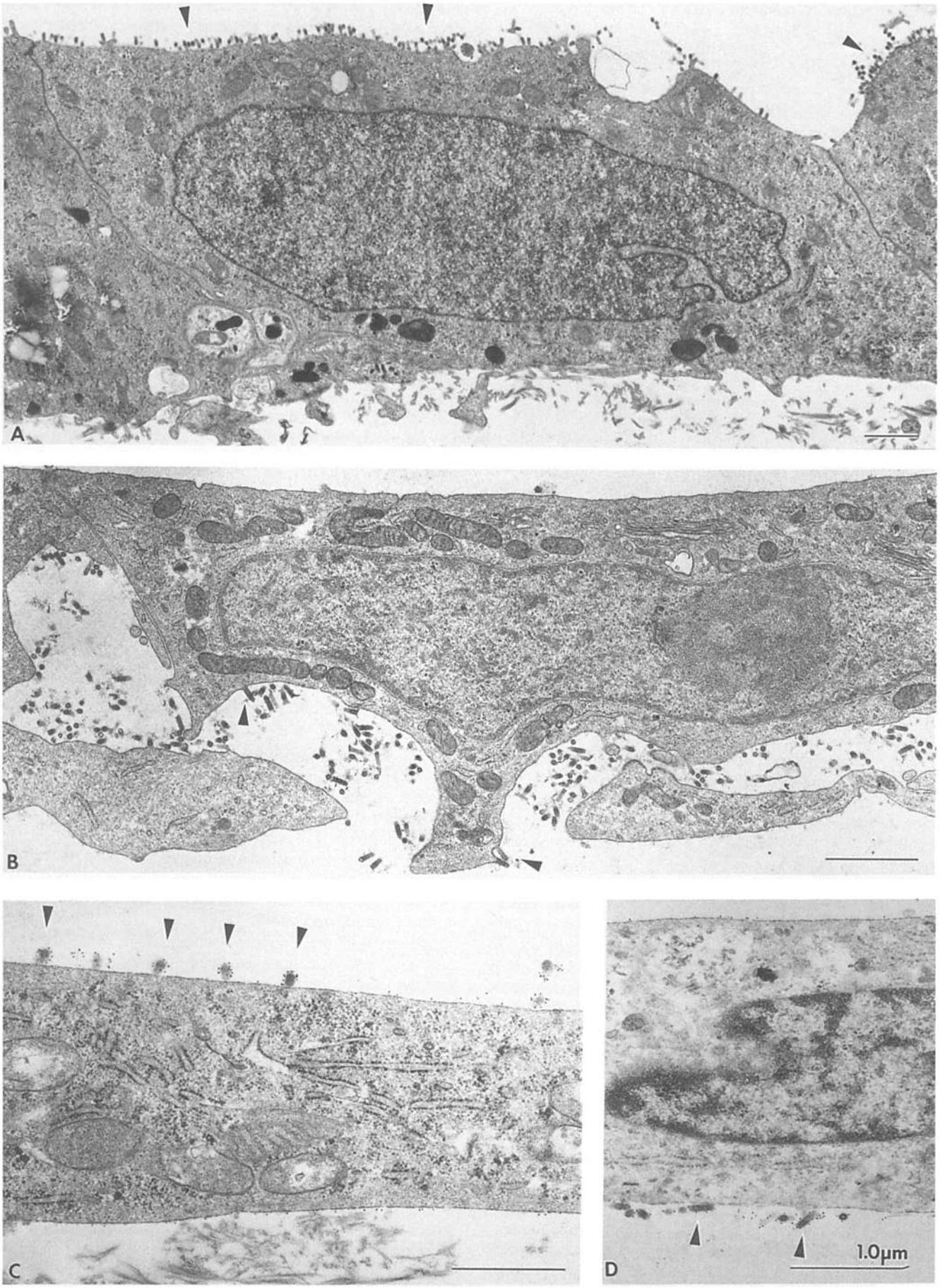
In summary, the surface polarity properties of MA104.11 cells resembled that of other well characterized epithelial cells in all of our assays.

Expression of HA in MA104.11 Cells Infected with SV40 Vectors Is Polarized

As expected of a rhesus kidney cell line, MA104.11 cells supported SV40 replication. Subconfluent, actively growing MA104.11 cells infected with a SV40 vector carrying a gene encoding the wild-type HA, SVEHA, produced 25% of the HA produced by CV-1 cells at the same multiplicity of infection (data not shown). Although confluent monolayers of cells are refractory to SV40 infection, 40 to 50% of MA104.11 cells that had been maintained for 3 to 5 d in confluent monolayers could be infected with SV40 vectors, as measured by immunofluorescence with an antibody specific for SV40 T antigen. Under these conditions HA was first detected at the cell surface 20 to 24 h pi., although at this period only 5% of the cells with bright nuclear fluorescence for T antigen contained surface fluorescence for HA (data not shown).

To determine that polarity of HA expression in MA104.11 cells was maintained after infection with SV40 vectors carrying the wild-type HA gene, MA104.11 monolayers that had

Figure 3. Polarity of virus budding and viral glycoprotein distribution in MA104.11 monolayers. Confluent MA104.11 monolayers grown on nitrocellulose filters coated with 2.4 mg/ml collagen were infected with influenza (*A* and *C*) or the temperature-sensitive mutant of VSV ts045 (*B* and *D*). Infection with influenza (MOI = 10) was for 7.5 h at 37°C; infection with ts045 was for 3 h at 40°C followed by 2 h at 32°C. *A* and *B* were fixed with 2% glutaraldehyde and processed for electron microscopy. *C* and *D* were prepared for immunoelectron microscopy as described in Materials and Methods. Note that influenza virions bud from the apical surface (*A* and *C*) and that VSV (ts045) virions are assembled from the basolateral one (*B* and *D*). Labeling with colloidal gold is observed predominantly on the apical surface for HA (*C*) and on the basal surface for VSV G protein (*D*). For both viruses, label tends to concentrate on virions.


A
B
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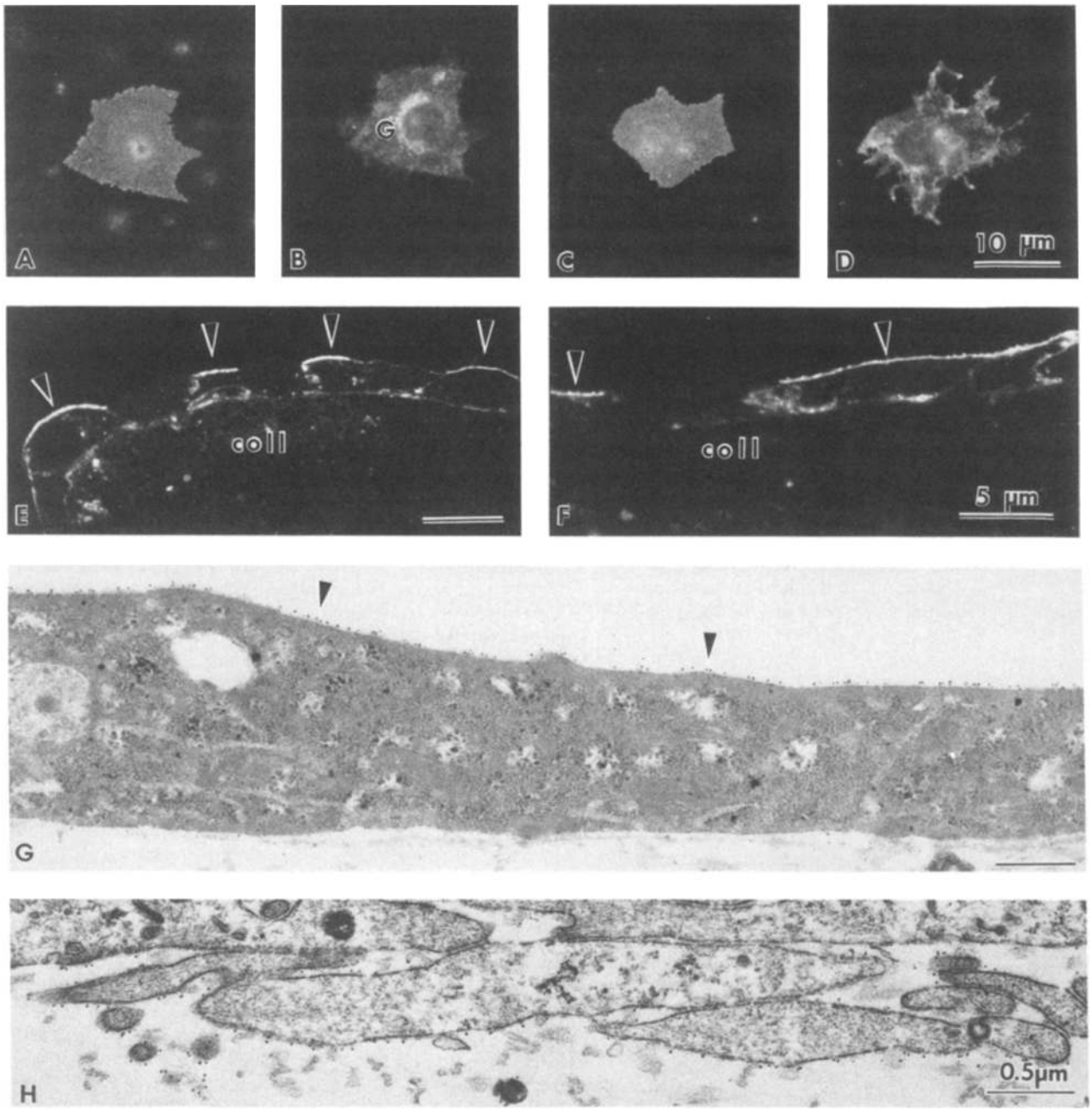

Figure 4. Polarized expression of influenza HA on the apical surface of MA104.11 cells. Confluent MA104.11 monolayers infected with SVEHA were maintained for 30 h (*A–G*) or for 38 h (*H*). (*A–D*) Fixed monolayers grown on glass coverslips were processed for double immunofluorescence by the five-step protocol. In this protocol, fluorescein stains only the apical surface (*A* and *C*) whereas rhodamine stains intracellular and basolateral HA (*B* and *D*, note the staining of the Golgi apparatus in *B*). For comparison, a cell in which HA is polarized (*A* and *B*) and a cell in which HA is not polarized (*C* and *D*) are shown. In the polarized cell (*A* and *B*), permeabilization allows staining of the Golgi apparatus (*B*) but no additional surface staining at lateral membranes, indicating that at the surface HA is limited to the apical domain. By contrast, the depolarized cell (*C* and *D*) has a large amount of HA in the basolateral surface. (*E–F*) Frozen sections (0.5-μm thickness) of MA104.11 monolayers grown on collagen gels (*coll*). Note the predominantly apical distribution of HA (*arrowheads*). Some cells, however, are starting to lose polarity of HA expression at foci in the basal and lateral membrane (*F*). (*G–H*) Protein A-gold immunocytochemistry of MA104.11 cells infected with SVEHA. In monolayers grown on collagen-coated filters and processed as described for colloidal gold immunocytochemistry (Materials and Methods) the majority of cells are labeled by anti-HA and protein A-gold predominantly at the apical surface (*G*). However, some cells that have begun to lose their polarity are labeled with protein A-gold at focal points in the basal surface (*H*). These areas probably correspond to the foci of basal fluorescence seen in other cells in *E* and *F*.

been confluent for several days were infected with the vector SVEHA and the cells were fixed for surface immunofluorescence 24–36 h later. HA at the cell surface was labeled with a rabbit anti-HA and a FITC-conjugated second antibody. The cells were then permeabilized by mild detergent treatment (Ash et al., 1977) and HA present within the cell or at basal and lateral membranes was labeled with a mouse monoclonal anti-HA and a RITC-conjugated second anti-

body (Fig. 4, *A–D*). Staining of the apical cell surface resulted in a planar, polygonal pattern with sharp edges where fluorescent cells contacted unstained neighboring cells (Fig. 4, *A* and *C*). In contrast, staining of HA at basolateral membranes resulted in a convoluted, three-dimensional shape (Fig. 4 *D*) that could not be contained within a single focal plane when viewed at high magnification. The fluorescence in the lateral membranes often extended for quite some distance beneath neighboring cells and was especially apparent when the neighboring cells contained no HA. A good indication that the detergent permeabilization had been effective was that the Golgi apparatus became brightly stained under these conditions (Fig. 4, *B* and *D*). The two patterns of fluorescence described above, the planar, polygonal staining of apical surfaces and the more extensive, convoluted staining of basolateral surfaces, were easily distinguished. Under the most synchronous conditions of infection that we were able to achieve, at 24 h pi., cells staining for HA at the apical surfaces and with little or no staining of HA in lateral membranes (as in Fig. 4, *A* and *B*) comprised 85% of the HA-positive cells. The percentage of unpolarized cells (i.e., those showing high amount of label in the basolateral membranes, as in Fig. 4, *C* and *D*) increased at later periods of infection. In less synchronous infections the percentage of HA-positive cells exhibiting polarity at any one time was lower, and the decrease in the percentage of polarized cells at later times was predictably less rapid.

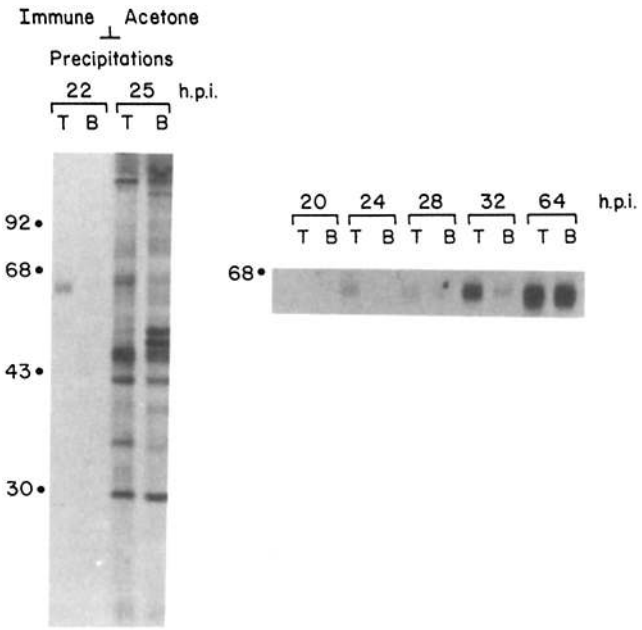

Figure 5. Polarized secretion of HA^{sec}. Polarized MA104.11 cell monolayers grown on filters containing collagen gels were infected with SVEHA^{sec}. At the times postinfection shown, the cells were labeled with [^{35}S]methionine and subjected to a pulse-chase protocol (Materials and Methods). At the end of the chase period, medium above (*T*) and below (*B*) the cells was collected and one-fourth was precipitated with acetone; the remainder was immunoprecipitated with anti-HA antibody. The panel on the left presents a comparison of the distribution of immunoprecipitated HA^{sec} with the distribution of endogenous MA104.11 polypeptides in an acetone precipitation of medium from SVEHA^{sec}-infected cells. The panel on the right presents a time course of HA^{sec} immunoprecipitated from medium above and below filters at the periods postinfection shown. Each time point is from a separate filter.

The polarized distribution of HA in MA104.11 cells infected with SVEHA is more easily seen when cells are presented in cross section (Fig. 4, *E* and *F*). MA104.11 monolayers grown on collagen gels were infected with SVEHA and fixed 30 h later. The monolayers with their collagen substrate were frozen and 0.5-μm sections were made. These were stained by indirect immunofluorescence with polyclonal anti-HA antibody. This technique allowed the antibody equal access to all cell membranes. In the monolayer sections shown in Fig. 4, the majority of HA-specific fluorescence is present at the apical cell surfaces. Some cells, however, show accumulation of HA at specific points of the basal membrane (Fig. 4 *F*).

The same results were observed by protein A–colloidal gold immunoelectron microscopy on monolayers grown on collagen-coated filters (Fig. 4 *G*). At late periods of infection, a larger percent of cells expressing HA on the apical cell surface also displayed considerable labeling of the basal surface (Fig. 4 *H*). To investigate whether the labeling of basal surfaces reflected the penetration of protein A–gold through the filter or whether the label gained access to basal surfaces through the opening of tight junctions during later periods of infection, anti-HA and protein A–gold was applied only to the basal side of filters supporting MA104.11 cells that had been infected with SVEHA^{sec} for 36 h. Under these conditions, only the basal cell surface was labeled (data not shown) and the amount of label at those surfaces was indistinguishable from that observed when antibody and protein A–gold was applied simultaneously to both apical and basal surfaces, as shown in Fig. 4 *H*. Thus, the collagen gel and filter presented no appreciable barrier to the passage of anti-HA or protein A–gold.

HA^{sec} Is Secreted from the Apical Surface during Early Periods of Infection of MA104.11 Cells with the SVEHA^{sec} Vector

Recognition events occurring during the sorting of HA to the apical surface of MA104.11 cells might take place within the lumen of organelles of the transport pathway, or might involve HA domains exposed to the cytoplasm. To address the question of which topological domains of HA are sufficient for sorting HA to the apical surface, we determined the pattern of secretion of a truncated HA from MA104.11 cells. This molecule, HA^{sec} (Gething and Sambrook, 1982), contains the entire HA ectodomain for which the three-dimensional structure has been solved (Wilson et al., 1981), and, in addition, contains 11 foreign amino acids. HA^{sec} is recognized by an antibody specific for the trimeric form of HA (data not shown) and is efficiently secreted from cells (Gething and Sambrook, 1982; Sambrook et al., 1985).

To collect polypeptides secreted from the two surface domains of MA104.11 cells, monolayers were grown on nitrocellulose filters with 3.0-μm diameter pores. Preliminary experiments established that the filters containing the collagen gel did not present a barrier to the passage of HA^{sec} and did not adsorb that protein. Medium containing HA^{sec} labeled with ^{125}I was added to either the top or bottom chamber and the labeled protein was allowed to diffuse across the filter. ^{125}I-HA^{sec} required 50 min to reach a con-

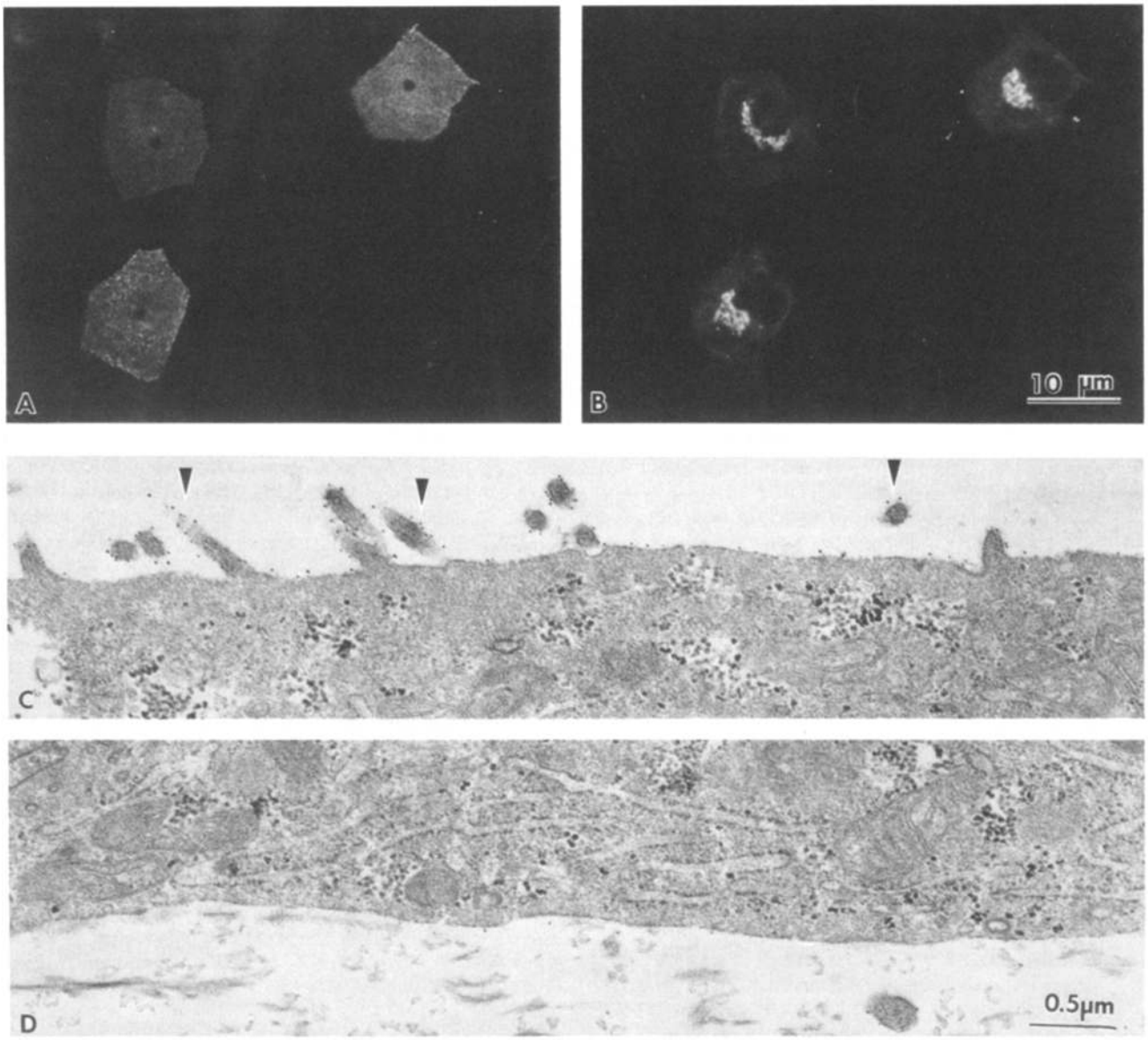

Figure 6. Polarized distribution of HAG in confluent monolayers of MA104.11 cells. Confluent monolayers of MA104.11 cells were infected with SVEHAG for 30 h and processed for five-step immunofluorescence (*A* and *B*) or for colloidal gold immunocytochemistry (*C* and *D*). Note (*A*) the staining of the apical surface and (*B*) the absence of basolateral labeling in Triton X-100–permeabilized cells (in spite of prominent labeling of the Golgi complex). *C* and *D* correspond to apical and basal regions of a single cell. Note the predominant labeling of the apical surface with colloidal gold (*arrowheads*).

stant transport rate across the filter. After a constant rate of ^{125}I-HA^{sec} transport was established, the amount of ^{125}I-HA^{sec} remaining with the filter was usually no more than 2.0% of the total present in the medium above and below the filter, an amount equal to the proportion of the total volume of applied medium that was included within the pores of the filter (data not shown).

MA104.11 cells were maintained for several days after reaching confluence and then removed from cell culture dishes with trypsin and EDTA and seeded into filter chambers at a density sufficient to form a monolayer overnight. Under these conditions, an electrical resistance could be detected across the monolayer after 24 h and this resistance rose to several hundred ohms·cm^2 after several days (see Fig. 2). On the third day, monolayers that had developed a good electrical resistance were inoculated with SVEHA^{sec} either from the apical side or through the filter to the basolateral membranes. Infection appeared equally efficient from both sides of the cell, indicating that virus particles could pass through the collagen gel within the pores of the filter. HA^{sec} could be detected in culture medium as early as 20–24 h pi. (Fig. 5). Beginning at this period, MA104.11 cells on filters were pulsed for 30 min with [^{35}S]methionine. After 30 min labeling, no HA^{sec} was detected in the culture medium (see Fig. 6) and thus no HA^{sec} was lost when the labeling medium was replaced with a chase medium containing excess nonradioactive methionine. The labeled cells were chased for a total of 6 h, a period sufficient for all HA^{sec} to be secreted from the cell and cross the filter (see Materials and Methods). Medium from the top and bot-

Table I. Secretion of HA^{sec} Is Polarized during Early Periods of Infection

Beginning of pulse	Percentage HA^{sec} in apical medium ± SD
h pi.	
22–25	94.7 ± 4.2
28	75.3 ± 5.5
30–35	66.0 ± 13.7
64–78	44.3 ± 2.0

Individual monolayers of MA104.11 cells grown on filters were infected with SVHA^{sec}, labeled with [^{35}S]methionine according to a pulse-chase protocol, and analyzed by autoradiography as described for Fig. 5. The autoradiographs were scanned by densitometry and results from a minimum of three monolayers were averaged for each period shown. The results were obtained from four separate infections.

tom filter chambers was collected, one-fourth of each sample was precipitated with acetone, and the rest was immunoprecipitated. The precipitates were analyzed by PAGE and autoradiography.

Fig. 5 shows the results for two filters from the same experiment labeled at the times shown. Most of the HA^{sec} labeled at 22 h pi. was precipitated from medium on the apical side of the monolayer; very little was detected in medium from the basal compartment of this filter. The total complement of polypeptides present in acetone precipitates of medium from the apical or basolateral compartments of a second MA104.11 monolayer infected with SVEHA^{sec} is shown in the second two lanes (Fig. 5). Two bands, at 65 and 35 kD, are prominent in medium from the apical side of the monolayer and are either absent or much more reduced in precipitates of medium from the basal side. Two bands at ~54 kD are present in medium from the bottom of the filter and are largely absent from medium from the top. Several higher molecular weight species are found preferentially in medium from the basal side of the cells, and several other polypeptides show a nonpolarized distribution. This asymmetric distribution of polypeptides secreted or shed from infected MA104.11 cells was also observed in medium from uninfected monolayers (not shown), and thus represents the pattern of endogenous MA104.11 proteins. During the period when HA^{sec} was secreted apically, endogenous MA104.11 proteins showed a polarized distribution, and, most importantly, passed through the filter. During later periods of infection this polarized distribution of endogenous MA104.11 proteins decreased, probably because of the loss of polarity in the infected proportion of the monolayer. This loss of polarity of endogenous proteins appeared to be a function of the virus infection rather than of the large amount of HA produced at later periods by the SVEHA^{sec} vector, since it also occurred during infection with wild-type SV40 (not shown).

A time course of HA^{sec} secretion from infected MA104.11 cells is shown in the second panel in Fig. 5. In this experiment individual filters were labeled at intervals beginning 20 h pi. by the pulse-chase protocol previously described, and HA^{sec} was immunoprecipitated from medium from each side of the monolayer. HA^{sec} is clearly secreted preferentially into medium on the apical side of the cells during the earliest periods of infection, with increasing amounts of HA^{sec} present in medium from the basal side of the monolayer at later times (Fig. 5, last lane). The distribution of HA^{sec} secreted during several such time course experiments was analyzed by densitometry of autoradiographs and the results are summarized in Table I. When first detected, HA^{sec} was transported vectorially to the apical surface with an efficiency that compares favorably with that observed for the membrane-bound HA (see Table II). During an infection of MA104.11 cells with SVEHA^{sec}, ~2×10^7 molecules of HA^{sec} were produced by each infected cell, of which 5%, or 10^6 molecules per cell were secreted during the period when 95% of HA^{sec} appeared in the apical medium. When the labeling period was delayed, the percentage of HA^{sec} secreted into the apical medium gradually decreased and the variation of results obtained with different monolayers increased, reflecting the asynchrony of infection of confluent monolayers of MA104.11 cells with SV40 vectors. At late periods during infection, HA^{sec} was secreted predominantly into the basal medium. The accumulation of large amounts of the protein in medium from the basal side of cells at these later periods directly demonstrates that HA^{sec} secreted from infected MA104.11 cells does cross the filter.

To be certain that the asymmetric distribution of HA^{sec} was not caused by a saturable adsorption of the protein to the filter, thereby preventing its release into the medium below the filter during early periods of infection, the cells and filters, after labeling and a 6-h chase, were extracted with detergent and the extract was immunoprecipitated. HA^{sec} remaining with the filter was observed only at later periods of infection and was never more than a small percent of the total produced, probably representing the proportion of the basal medium trapped within the filter pores (not shown). To establish that our extraction procedure resulted in a quantitative release of HA^{sec} from the cells and filters, we compared the amount of HA^{sec} that could be immunoprecipitated from the medium, cells, and filter at the end of a labeling period of 30 min, when HA^{sec} has not yet been transported through the Golgi apparatus, with the amount immunoprecipitated from medium, cells, and filters after a chase long enough for half of the HA^{sec} to be secreted into the apical medium. Immunoprecipitates from six monolayers labeled for 30 min and from six monolayers labeled 30 min and chased for 2 h were analyzed by PAGE, autoradiography, and densitometry. The mean optical density of total HA^{sec} immunoprecipitated at the end of the labeling period was 3.25 with a standard

Table II. Preferential Expression of Influenza HA on the Apical Surface of MA104.11 Cells Infected with Influenza Virus, or with SVEHA or SVEHAG Vectors

Virus/vector	Apical particle density / Basal particle density		Gold particles counted per cell	*n*
Monolayers grown on collagen-coated filters				
Influenza	7.82 ± 1.38	NS	334 ± 45	10
SVEHA	9.79 ± 1.92	NS	374 ± 69	11
SVHAG	8.03 ± 2.65	NS	113 ± 35	10
Monolayers scraped from plastic petri dishes				
SVEHA	10.73 ± 2.01	NS	260 ± 59	10
SVHAG	9.39 ± 2.38	NS	118 ± 42	14

Monolayers of MA104.11 cells confluent for 4 d, grown on collagen-coated filters or on plastic petri dishes were infected with influenza virus for 7.5 h or with SVEHA or SVEHAG for 30 h and fixed and processed for colloidal gold immunocytochemistry as indicated in Materials and Methods. Results are expressed as mean ± SEM. Significance of the difference between means was estimated with Student's *t* test. NS, no significant difference between the means. *n*, number of cells on which particles were counted.

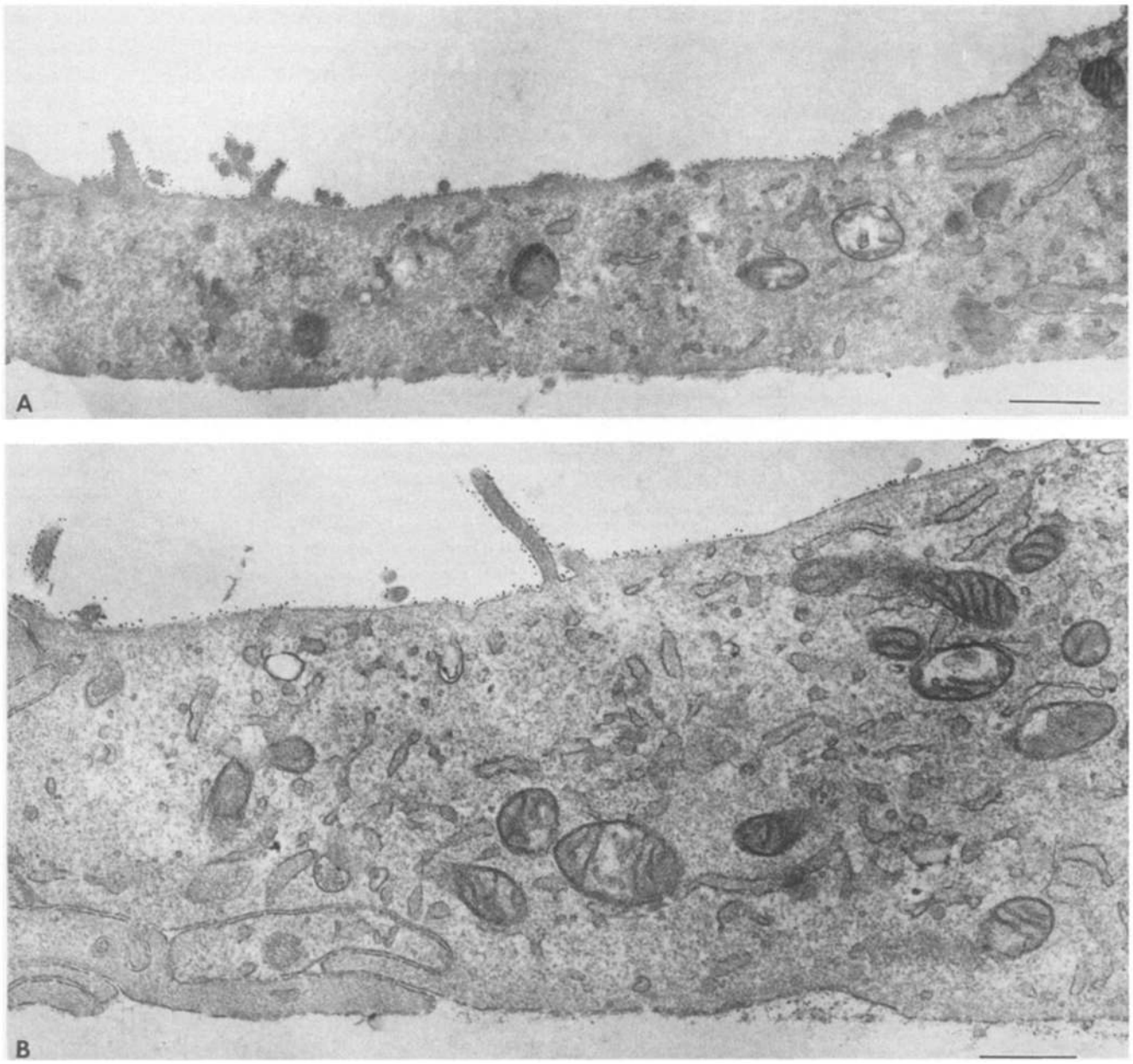

Figure 7. Polarized expression of HA and HAG in confluent monolayers of MA104.11 cells scraped from the substrate. Monolayers of MA104.11 cells confluent for 4 d were infected with SVEHA or with SVEHAG for 30 h, fixed with 2% formaldehyde (freshly prepared from paraformaldehyde) and scraped from the substrate. Immunospecific labeling is predominantly of the apical surface of these cells. Bar, 0.8 μm.

deviation of 0.46; on the same autoradiograph the mean optical density of total HA^{sec} immunoprecipitated after a 2-h chase was 3.20 with a standard deviation of 1.33. Thus, there was no significant difference between the amount of HA^{sec} recovered immediately after labeling or after a chase of several h. We therefore conclude that HA^{sec} is secreted in a polarized manner during the early period of infection of MA104.11 cells with $SVEHA^{sec}$.

A Chimera of HA and VSV G Protein Is Expressed at the Apical Surface of MA104.11 Cells

The sorting event by which the HA ectodomain is recognized by the traffic control apparatus of MA104.11 cells might involve the presence of a specific "address" on HA, or might occur due to the absence on HA of an address that targets other proteins to the basolateral membranes. It was of interest, therefore, to determine whether the presence of the transmembrane and cytoplasmic domains of a protein normally targeted to the basolateral membranes could redirect transport of the HA ectodomain in MA104.11 cells. Previously it had been shown that biologically active chimeric HAs could be expressed from genes in which sequences encoding the transmembrane and/or cytoplasmic domains of HA were replaced by those encoding the analogous domains of other viral glycoproteins (Doyle et al., 1985; Roth et al., 1986). One of these proteins, HAG, contains all of the HA

ectodomain for which the three dimensional structure was solved, 10 amino acids from the ectodomain of G protein, and the G transmembrane and cytoplasmic domains (Roth et al., 1986). Thus, this protein is a basolateral protein in the cytoplasm and within the lipid bilayer and is an apical protein in the extracytoplasmic compartment.

Confluent MA104.11 monolayers grown on glass coverslips or on millipore filters coated with a collagen gel were infected with the SVEHA vector carrying the wild-type HA gene or with SVEHAG carrying the gene for the HAG protein. At 25–35 h pi., the monolayers grown on coverslips were fixed with 2% formaldehyde and 0.05% glutaraldehyde and processed for five-step immunofluorescence (Materials and Methods). Monolayers grown on collagen-coated filters were fixed identically and processed for colloidal gold immunocytochemistry. Parallel gold immunocytochemical experiments were carried out on monolayers scraped after fixation from glass coverslips, as previously reported (Rodriguez-Boulan and Pendergast, 1980; Roth et al., 1983*b*). The results obtained are shown in Figs. 6 and 7. HAG was expressed preferentially on the apical surface of MA104.11 cells, as seen by both procedures.

A quantitative determination of the density of colloidal gold particles in the apical and in the basal surfaces of monolayers infected with both viruses or with influenza virus is shown in Table II. Wild-type influenza HA, produced either during normal infection with influenza virus or from cloned cDNA, was expressed at eight to ten times higher density on the apical than on the basal surface of MA104.11 cells. Similar results were observed for the expression of HAG. Thus, the amino acid sequences of G protein present in the HAG chimeric glycoprotein did not alter the polarized transport of the HA ectodomain to the apical cell surface.

Discussion

Characteristics of MA104.11 Cells Infected with Recombinant SV40 Vectors

We have characterized a clonal cell line, MA104.11, derived from MA104 rhesus monkey kidney cells. MA104.11 cells were permissive for SV40 replication and exhibited many characteristics of polarized epithelial cells, including formation of domes and electrically tight monolayers and the polarized maturation of enveloped viruses. When grown on a collagen gel supported by a nitrocellulose filter with large pores, MA104.11 cells either secreted or shed polypeptides into the medium in a polarized manner. The density of HA expression at the apical surface of MA104.11 cells in monolayers infected with influenza virus or with the SVEHA vector was about nine times the basal density level, as determined by colloidal gold immunocytochemistry. This ratio is somewhat lower than in MDCK cells, since in these cells the percent of basolateral HA is ~10% of the total and the basolateral surface area is four times the apical membrane area (Pfeiffer et al., 1985; Salas et al., 1986), which results in an apical/basal density ratio of approximately 40:1.

At present, foreign genes have been expressed in polarized cells through infection with enveloped viruses (Rodriguez-Boulan, 1983*a b*; Simons and Fuller, 1985) transfection with recombinant vectors (Kondor-Koch et al., 1985; Rissolo et al., 1985; Gottlieb et al., 1986; Roth, M. G., and E. Rodriguez-Boulan, unpublished results), and infection with vaccinia (Stephens et al., 1986), retrovirus (Mostov and Deitcher, 1986), or SV40 vectors. Infection with DNA-containing virus vectors has the advantages previously obtained with RNA-containing enveloped viruses. One can observe expression of a foreign gene in cells in polarized monolayers with no background of protein synthesized during periods when the cells are subconfluent. In addition, the relatively large amounts of protein produced and the high frequency of infected cells in the monolayer allow the application of techniques such as quantitative electron microscopy. A disadvantage of the virus infection is that the period during which transport is vectorial is transient, and the infection can be asynchronous. An advantage of the system of MA104.11 cells infected with recombinant SV40 vectors is that construction of the recombinant vectors and production of virus stocks is relatively easy. Thus, this system is particularly well-suited for screening chimeric or specifically mutated glycoprotein genes using the simple five-step immunofluorescence method. A limitation of the MA104.11-SV40 system, however, is that the infection is asynchronous. This limits the application of biochemical measures of polarity to those cells in the infected monolayer that first produce protein.

Intraluminal Recognition Required for Sorting HA

At early periods of infection of MA104.11 cells with the vector SVEHA^{sec} encoding a form of HA lacking its transmembrane and cytoplasmic domains, secretion of the HA^{sec} glycoprotein was into the apical medium. Such polarized secretion confirms earlier observations that HA is transported directly to the apical surface and must be sorted within the cell (Misek et al., 1984; Matlin and Simons, 1984; Rindler et al., 1985; Salas et al., 1985). In addition, our findings indicate that the transmembrane and cytoplasmic domains of HA are not required for correct sorting of HA. Similar observations have been reported for the cytoplasmic domain of the influenza neuraminidase (Jones et al., 1985).

Certain unidentified endogenous polypeptides were present in medium on both sides of MA104.11 monolayers in which 40% of the cells were infected with SVEHA^{sec}; others were observed only in the medium above or below the cells. It is likely that some, if not all, of these polypeptides are secretory proteins and that polarized MA104.11 cells are capable of secretion from both membrane domains, as has been recently demonstrated for MDCK cells (Kondor-Koch et al., 1985; Gottlieb et al., 1986). In addition, during the period when secretion of HA^{sec} was polarized, sorting of endogenous polypeptides to the basal surface continued in MA104.11 cells infected with SV40 vectors.

The secretion of certain proteins from a single membrane domain of a cell simultaneously secreting other proteins from both surfaces places several requirements on the cellular sorting mechanisms. There must be recognition events for sorting that occur within lumen of an organelle involved in intracellular transport and these events are likely to involve proteins that are directionally transported along both the apical and basolateral arms of the exocytic pathway. The existence of a class of proteins that is not sorted can be most easily explained if the lack of a sorting feature on those proteins allows transport to both membrane domains. In the sim-

plest case, sorted proteins would be excluded from one arm of the pathway and transported vectorially along the other, whereas unsorted proteins would lack the feature responsible for exclusion and gain entry nonspecifically to both. Sorted proteins could be excluded from the wrong pathway due to an intrinsic property (charge or state of aggregation), or might efficiently associate with other proteins (sorting receptors) that cause them to enter transport vesicles bound for a single surface domain. If one postulates that transport to one of the two surface domains occurs without a recognition event, then only those proteins that are specifically recognized can enter the directed pathway towards the other domain. This requires that proteins expressed at both surfaces would have to be inefficiently recognized and only partly sorted into the directed pathway.

HAG, a glycoprotein that has the HA ectodomain exposed to the lumen of the transport pathway and the membrane spanning and cytoplasmic sequences of G protein, is expressed preferentially at the apical surface of MA104.11 cells. We have observed that an antibody specific for the cytoplasmic domain of G protein recognizes the cytoplasmic domain of HAG; thus, HAG shares at least one cytoplasmic epitope with G protein. G protein, like HA, is a multimer and is thought to be a trimer (Kreis and Lodish, 1986). If the conformation of the cytoplasmic domain in HAG closely resembles the conformation of that domain in G itself, then there are two nonexclusive possibilities for the location of sorting addresses on HA and G protein: (*a*) the HA ectodomain could contain a dominant feature addressing HAG to the apical surface; (*b*) the feature of G protein recognized by the cellular sorting apparatus could be present in the G ectodomain and absent from the transmembrane and cytoplasmic domains. If recognition of membrane-bound proteins occurs in the lumen of an organelle of the exocytic pathway, it is possible that a common mechanism operates for both secreted and membrane proteins. This mechanism must be able to recognize proteins destined to be sorted and be able to direct them into separate transport vesicles. In other branches of the membrane traffic pathway, similar sorting events are mediated by adaptor or receptor proteins like the two mannose-6-phosphate receptors responsible for sorting lysosomal enzymes (Sahagian et al., 1981; Hoflack and Kornfeld, 1985), and the well described cell surface receptors of the endocytic pathway (Brown et al., 1983). Since HA and G protein appear to share transport compartments until the trans-Golgi cisternae or the trans-reticular network (Rindler et al., 1985; Fuller et al., 1985; Salas et al., 1985), it is tempting to suggest that a class of intracellular sorting receptors operates at this point in the pathway.

Location of a Sorting Address on HA

The features of glycoproteins recognized by the cell during sorting might be discrete, similar to the karyophilic signals identified for several proteins (Dingwall et al., 1982; Kalderon et al., 1984; Hall et al., 1984; Davey et al., 1985), or they might be some global property, such as a tendency to aggregate in a particular cellular environment, e.g., the low pH of the trans-Golgi cisternae (Anderson and Pathak, 1985; Schwartz et al., 1985). Presently there is little data available with which to distinguish these possibilities. In either case, recognition of proteins by intracellular sorting receptors would require interactions between some surface feature of the proteins and a receptor. Since it is well established that carbohydrate plays no role in the sorting of HA (Roth et al., 1979; Green et al., 1981; Meiss et al., 1982), our present results indicate that the sorting address on HA must involve amino acids on the surface of the ectodomain. A search for sequence homologies among a number of glycoproteins of the apical class for which the primary sequences are known (Pfieffer and Compans, 1984; Nakada et al., 1984; Blumberg et al., 1985*a*, *b*; Hiebert et al., 1985) reveals few clues to the nature or location of a sorting address. This suggests that such a feature, if it exists, is not likely to be a highly conserved continuous amino acid sequence like the nuclear transport signal of the large T proteins of polyoma viruses (Kalderon et al., 1984; Richardson et al., 1981). As an alternative to a continuous amino acid sequence, a sorting address might be composed of noncontinuous amino acids and be highly conformation-dependent, as has been suggested for the site on lysosomal enzymes recognized by the *N*-acetylglucosaminylphosphotransferase responsible for attaching the mannose-6-phosphate sorting signal (Kornfeld, 1986). Such a conformational address might even be present only transiently, in a particular intracellular environment.

The problem of locating a discrete surface feature that might be recognized as a sorting address is strictly analogous to mapping epitopes for monoclonal antibodies. It is important that mutations that disturb structure at a distance be distinguished from those that make a change in an informational feature itself. In the case of HA, the sorting address resides in the large ectodomain for which the structure is known (Wilson et al., 1981). Thus, HA would appear to be an ideal candidate for experiments designed to identify features recognized by the sorting mechanisms in epithelial cells.

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