A Specific Sorting Signal Is Not Required for the Polarized Secretion of Newly Synthesized Proteins from Cultured Intestinal Epithelial Cells

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Abstract. Caco-2 cells, derived from human colon, have the morphological, functional, and biochemical properties of small intestinal epithelial cells. After infection with enveloped viruses, influenza virions assembled at the apical plasma membrane while vesicular stomatitis virus (VSV) particles appeared exclusively at the basolateral membrane, similar to the pattern observed in virus-infected Madin–Darby canine kidney (MDCK). When grown in Millicell filter chamber devices and labeled with [^{35}S]methionine, Caco-2 monolayers released all of their radiolabeled secretory products preferentially into the basal chamber. Among the proteins identified were apolipoproteins AI and E, transferrin, and α-fetoprotein. No proteins were observed to be secreted preferentially from the apical cell surface. The lysosomal enzyme β-hexosaminidase was also secreted primarily from the basolateral surface of the cells in the presence or absence of lysosomotropic drugs or tunicamycin, which inhibit the targetting of lysosomal enzymes to lysosomes. Neither of these drug treatments significantly affected the polarized secretion of other nonlysosomal proteins. In addition, growth hormone (GH), which is released in a nonpolar fashion from MDCK cells, was secreted exclusively from the basolateral membrane after transfection of Caco-2 cells with GH cDNA in a pSV2-based expression vector. Similar results were obtained in transient expression experiments and after selection of permanently transformed Caco-2 cells expressing GH. Since both β-hexosaminidase and GH would be expected to lack sorting signals for polarized exocytosis in epithelial cells, these results indicate that in intestinal cells, proteins transported via the basolateral secretory pathway need not have specific sorting signals.

EPITHELIAL cells, in addition to their roles in the formation of barriers and in the transport of nutrients, are responsible for the synthesis and secretion of many important proteins. These products, after synthesis in the rough endoplasmic reticulum and transport through the Golgi apparatus, are secreted by the polarized cells from the surfaces appropriate for their sites of action (cf. reference 43). For example, digestive enzymes synthesized by the pancreas are exported from the apical plasma membrane of acinar cells for transport via the pancreatic ducts to the duodenum (23). Other proteins, such as albumin, are synthesized by hepatocytes and released from the sinusoidal surface (47), the equivalent of the basolateral membrane of other polarized epithelial cells (10, 22). In principle, the sorting process that addresses plasma membrane proteins to the apical and basolateral surfaces could also function in the polarized delivery of secretory products. However, the mechanisms that serve to distinguish these products and direct them to the appropriate plasma membrane domain for discharge have yet to be elucidated.

The secretory products of epithelial cells, depending on their origin, may be transported via regulated or constitutive routes (24). Some cell types, like those of the acinar cells of the pancreas, package their products in secretory granules for discharge only upon an appropriate stimulus. In other epithelia, such as hepatocytes, the secretory products including serum proteins are released in a continuous manner. While the requirement for a protein to be packaged in a storage granule of an epithelial cell is sufficient to insure its polarized exocytosis along with the rest of the granule contents, upon the fusion of the granule with the appropriate cell membrane, it is not clear what processes may govern the vectorial transport of constitutively secreted proteins. These proteins are believed to be carried to the cell surface in the same vesicles that serve to transport plasma membrane proteins (46).

The secretion of proteins of the constitutive pathway by polarized epithelial cells has been examined both in vivo and in vitro. In perfused rat liver, Sztul et al. (47) investigated the entry of albumin into the bile (i.e., through the apical = canilicular surface) and concluded that the kinetics of its appearance in the cannulated bile duct were too slow to represent direct secretion from the hepatocytes. Its presence in the bile is likely to be due to paracellular transport as well as nonspecific transcytosis across the hepatocyte from the blood (25). These data imply that the secretion of albumin occurs in a polarized fashion directly to the sinusoidal membrane

from the Golgi apparatus of the cell. By contrast, Madin–Darby canine kidney (MDCK) cells were more variable in their ability to secrete proteins in a polarized fashion. While an endogenously synthesized protein was found to be secreted only from the apical membrane, exogenous proteins, such as chick oviduct lysozyme and rat growth hormone, introduced into MDCK by transfection of appropriate cDNAs in expression vectors, were released in a nonpolarized fashion from both membrane surfaces (17, 26). Most significantly, the liver form of $alpha_{2u}$-globulin, a product of hepatocytes found in the plasma and thus presumably exported selectively from the sinusoidal surface (42), was also secreted by MDCK cells from both surfaces equally (17). The sorting signal that this protein should possess for polarized deliver in hepatocytes was not recognized by the kidney-derived epithelial cells, despite the fact that the transfected MDCK released an endogenous secretory protein specifically into the medium of the apical chamber.

These results suggest that there are tissue-specific sorting recognition systems and raise the question of what mechanisms might operate to direct secretion in the epithelial cells of such organs as the liver, which is the source of most of the proteins and lipoproteins of the serum, and the intestine, which is also known to assemble lipoproteins and to secrete them into the intercellular spaces. To investigate these issues, we have used another epithelial cell line, Caco-2, derived from human colon carcinoma but exhibiting the morphological and biochemical properties of small intestinal absorptive cells (11, 36). These cells form microvilli and tight junctions, and they synthesize and secrete a number of apolipoproteins, as is characteristic of the small intestine (48). By studying the polarized exocytosis of these products in detail and investigating the secretion of other proteins, such as β-hexosaminidase and growth hormone, we provide evidence that secretory proteins need not possess specific sorting signals for transport along the basolateral secretory pathway in intestinal cells.

Materials and Methods

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DME (4.5 g/liter glucose; KC Biological, Inc., Lenexa, KS) containing 20% FBS (Gibco, Grand Island, NY), 15 mM Hepes, 200 U/ml penicillin and streptomycin (Irvine Scientific, Santa Ana, CA), polymyxin B (200 U/ml; Sigma Chemical Co., St. Louis, MO), and 5 µg/ml fungizone (E. J. Squibb, Princeton, NJ) in a 95% air/5% CO_2 incubator. For plating in Millicell chambers (Millipore Corp., Bedford, MA), confluent monolayers in 75 cm² flasks (all plasticware was from Corning Glass Works, Corning, NY) were detached with 0.5% trypsin 0.2% EDTA (Gibco) and the cell suspension plated at 10^6 cells per 3 ml of medium in the inside of 30-mm chambers that had been presoaked for 30 min in medium and placed in 6-well dishes. After 16 h, the medium was removed, the filters with attached cells were washed three times in DME, and 1.5 ml fresh culture medium was added to the inside and outside. The medium was changed every 2–3 d. Under these conditions, the cells remain as a monolayer (as viewed in the electron microscope) and the electrical resistance measured across the monolayer reached 250–500 ohm·cm² after 4–6 d (48). No differences were observed in either the polarized secretion of newly synthesized proteins or in the polarized assembly of enveloped viruses if the cells were plated on filters at lower densities (4–8 × 10^4/cm²) and used 14–25 d later (not shown).

Viral Infections and Electron Microscopy

Caco-2 cells plated at 2 × 10^5 cells per 35-mm dish and grown 2 d past

confluency (8 d) were infected as described for MDCK cells (38). Briefly, monolayers were rinsed twice with DME and infected with influenza/WSN or vesicular stomatitis virus (VSV; Indiana stain) in DME containing 15 mM Hepes at a multiplicity of infection of 15–20 (determined on MDCK) in 0.4 ml of the same medium. After a 1-h infection period, the medium was replaced (2 ml) and the incubation continued for an additional 5 h in the case of VSV or 6.5 h for influenza. Monolayers were then rinsed two times in ice-cold 0.1 M cacodylate and fixed for 1 h in the same buffer with 2% glutaraldehyde (Polysciences, Inc., Warrington, PA) at 4°C. The cells were then scraped from the dishes and pelleted in a microcentrifuge (Brinkman Instruments Co., Westbury, NY) and the fixation continued overnight. The samples were processed for electron microscopy by standard procedures; the sections were produced on an ultramicrotome (model No. MT-2B; Sorvall Instruments Div., Newtown, CT) and viewed and photographed in a Phillips 300 electron microscope operated at 80 mV. Quantitation of the virions on the two surfaces was made on photographs of 15 infected cells for VSV (1,039 total) and 29 cells for influenza (681 total), chosen at random.

Electrical Resistance Measurements

These were made essentially as described (7, 38, 48) using Ag/AgCl electrodes mounted on a ringstand in such a way as to allow one pair of recording and current electrodes to enter the medium inside the Millicell chamber and one pair outside the insert in the culture dish. The current required to effect a 3.3-mV potential difference across the monolayer was recorded on a microampmeter, and the resistance calculated according to Ohm's law. Readings on the filters alone were <25 ohm·cm².

Transfection of Caco-2 Cells

The insertion of the cDNA for growth hormone (GH)[1] (originally provided by Dr. John Baxter, University of California, San Francisco, CA) into pSV2 (34) and its expression under the control of the SV40 early promoter has been described (17). Transfection was carried out as detailed previously (18) using a modification of the calcium phosphate precipitation procedure (8). For permanent transformants, pSV2-neo (1 µg/dish; 32, 34) was included. Briefly, Caco-2 cells at three-fourths confluency in 75-cm² flasks were treated for 3 min at 4°C with trypsin-EDTA and then incubated with 75 µg/dish of CsCl gradient-purified plasmid in the precipitation medium for a total of 8 h. After a 15% glycerol shock, cultures were allowed to recover for 22 h. The cells were then trypsinized and plated either into Millicell chambers, as described above, for transient expression experiments or diluted and plated onto 100-mm dishes for selection of permanent transformants. After 48 h, these dishes had their medium replaced with medium that consisted of a 1:1 mixture of fresh and conditioned medium from Caco-2 cells that contained G418 (Gibco) at 400 µg/ml final concentration. The slow-growing, neomycin-resistant colonies proved difficult to subclone; therefore, after 3 wk in culture, the remaining colonies were trypsinized and replated together in T25 flasks. These mixed cultures were then continuously grown, expanded, and plated onto Millicell chambers in standard culture medium supplemented with G418 (200 µg/ml). Before labeling, monolayers were incubated for 18 h with sodium butyrate (10 mM) in the medium to enhance expression of the gene (16, 17).

Radiolabeling of Cells and Immune Precipitation

Millicell chambers with confluent monolayers of Caco-2 cells were rinsed three times in 1.5 ml per side of methionine-free MEM (Irvine Scientific) containing 20 mM Hepes and left in the third rinse for 30 min. The medium was then replaced with met-free MEM containing 100–250 uCi/ml [^{35}S]-methionine (500 Ci/mmol; New England Nuclear, Boston, MA) added to the basal chamber and the incubation continued for 4–6 h. The inhibitors NH₄Cl (Fisher Scientific Co., Pittsburgh, PA) and chloroquine (Sigma Chemical Co.) were added during the 30-min preincubation as well as during the labeling period itself. Tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) was present for 1.5 h before the preincubation period and thereafter. The medium was collected separately from each side, FBS added (final concentration = 2%) along with EDTA (7.5 mM), and cell debris removed by centrifugation for 2 min in a microcentrifuge (Fisher Scientific Co.). The medium was then transferred to another tube and heated to 100°C for 2 min and recentrifuged. For TCA precipitation, 1 ml of 10% TCA was added to 50–100 µl of sample with 40 mM unlabeled methionine. The precipitates were sedimented by centrifugation and washed three times in
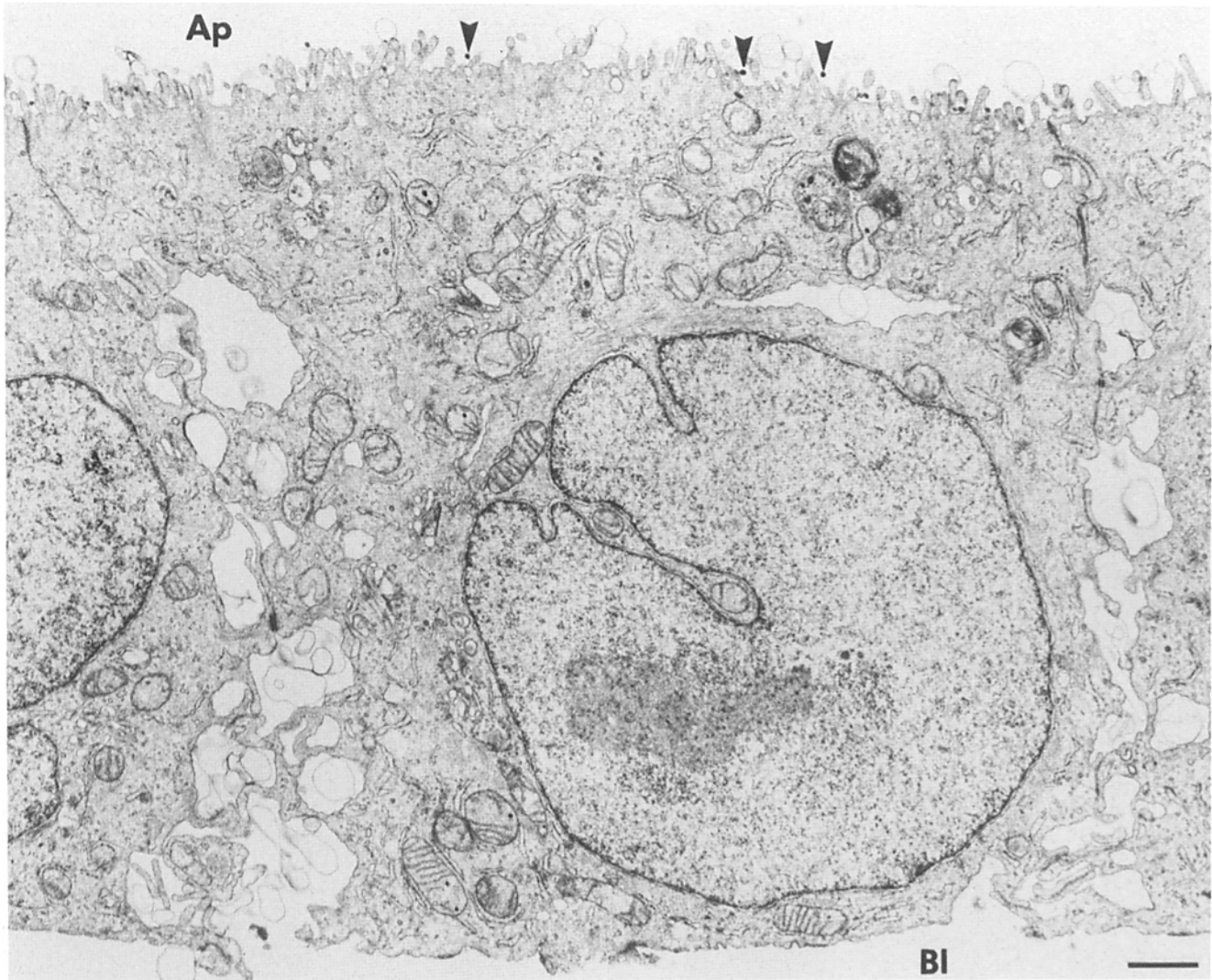
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1. *Abbreviation used in this paper*: GH, growth hormone.

Figure 1. Assembly of influenza virions on the apical surface of infected Caco-2 cells. Monolayers on 35-mm dishes were infected (moi = 15) with influenza and taken for electron microscopy 7.5 h post infection. In this cell typical of the monolayer, virions (*arrowheads*) are seen associated with the apical (*Ap*) plasma membrane while the basolateral (*Bl*) surface was free of such particles. Bar, 1 µm.

5% TCA. Immune precipitations were carried out by adding 2–10 µl of antiserum to 0.5–1.4-ml samples of the remaining medium. The antisera were provided by Dr. H. Samuels, NYU Medical Center, New York (baboon anti–rat growth hormone), Dr. Elizabeth Neufeld, UCLA Medical Center, Los Angeles, CA (rabbit anti–human β-hexosaminidase A), and Drs. R. Gregg and B. Brewer of the National Institutes of Health, Bethesda, MD (goat anti–human apo E). Rabbit anti–human apo AI was purchased from Calbiochem-Behring Corp. Rabbit antiserum against human α-fetoprotein and transferrin was from Dako Scientific (sold by Accurate Chemical & Scientific Corp., Westbury, NY). After an overnight incubation at 4°C, 10 µl of preswollen protein A-Sepharose (Sigma Chemical Co.) was added and 2 h later the samples were washed five times in 190 mM NaCl, 50 mM Tris, 6 mM EDTA, 2.5% Triton X-100, 0.2% SDS, pH 8.5, by centrifugation and resuspension. Radioactive molecular mass standards were purchased from Bethesda Research Laboratories (Bethesda, MD). Samples were analyzed by NaDodSO₄-PAGE using standard procedures (28). Fluorography was performed using EN³HANCE (New England Nuclear) according to the manufacturer's instructions and visualized using Kodak X-Omat film (Eastman Kodak Co., Rochester, NY). Scanning densitometry was performed on appropriate exposures using a densitometer (Hoefer Scientific Instruments, San Francisco, CA) with a chart recorder (Perkin-Elmer Corp., Norwalk, CT). Relevant peaks were cut from the chart paper and quantitated by weighing. To normalize for experiment-to-experiment variability, the 90-kD protein (of unknown identity) was also scanned and the percentage of

basolateral secretion is included in the figure legends. It was chosen because it is a sharp, well-separated band in TCA precipitates that is generally unaffected by the drug treatments employed.

Results

Polarity of Viral Budding in Caco-2 Cells

Caco-2 cells have been shown to synthesize and transport several microvillar hydrolases to their apical plasma membranes, as assessed by immunofluorescence microscopy (21). They would appear, therefore, to have the mechanisms for the sorting of apical and basolateral proteins that are characteristic of epithelia in vivo and of other epithelial cell lines, such as MDCK, derived from canine kidney. To test this notion directly, monolayers of Caco-2 cells were infected with the enveloped viruses, influenza and VSV, which in MDCK and other cells of epithelial origin, assemble their virions on the apical or basolateral surfaces, respectively (40). As depicted in the electron micrographs of Figs. 1 and 2, Caco-2
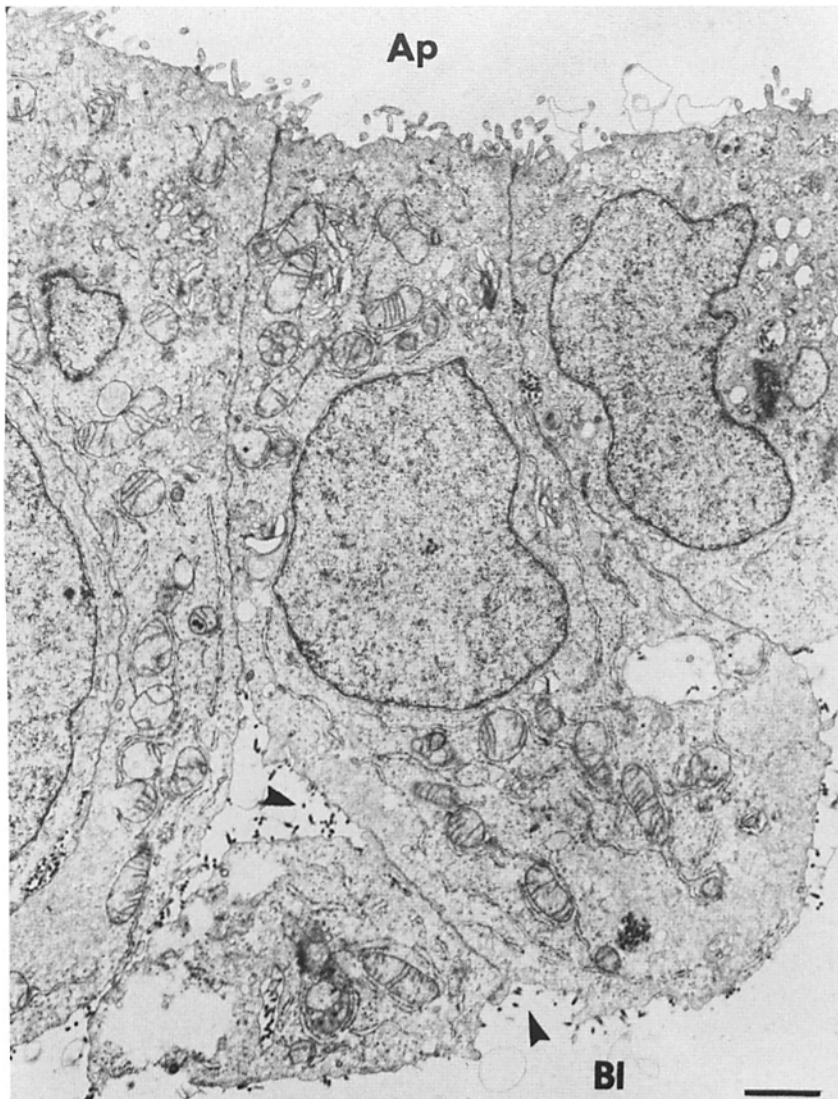
Figure 2. Assembly of VSV virions on the basolateral surface of infected Caco-2 cells. Confluent monolayers on 35-mm dishes were infected with VSV (moi = 20) and taken for electron microscopy 6 h post infection. The bullet-shaped virions (*arrowheads*) were almost exclusively found proximal to the lateral and basal (*Bl*) aspects of the plasma membrane whereas the apical (*Ap*) cell surface was free of particles. Bar, 0.88 μm.

cells manifested the same domain specificity for viral budding previously observed in MDCK cells, and this was observed soon after the cells reached confluence. Influenza virions (∼95%) were associated with the apical pole of the cells (Fig. 1), and VSV particles (Fig. 2) appeared almost exclusively at basolateral surfaces (∼99%).

Polarized Secretion of Newly Synthesized Proteins by Caco-2

Caco-2 cells synthesize and secrete a variety of products into the medium as observed after metabolic labeling of the cells and analysis by NaDodSO₄-PAGE and autoradiography (Fig. 3). Some of these products have been identified as apolipoproteins (48). When Caco-2 cells were cultured on Millipore filters mounted in plastic chambers that allow access to medium on both sides of the monolayer, the prominent [^{35}S]methionine-labeled products obtained after TCA precipitation were observed almost exclusively in the basal chamber, representing those secreted from the basolateral plasma membrane (Fig. 3). Specific proteins, known to be bonafide secretory proteins and not derived from any plasma membrane precursor by shedding or proteolytic cleavage,

were also identified by immune precipitation. For example, apolipoproteins AI and E were secreted exclusively (>92%) out of the basolateral side of the cell (Fig. 3 *A*), as were α-fetoprotein (80%) and transferrin (>93%) (Fig. 3 *B*). No radiolabeled products were detected exclusively or even preferentially in the medium of the apical chamber.

Polarized Secretion of a Lysosomal Enzyme

The well-developed basolateral secretory pathway of Caco-2 cells was defined above using proteins normally found in the serum. It is conceivable that such proteins, as well as the unidentified prominent radiolabeled products, all possess sorting signals specifying polarized secretion. We therefore studied the secretion of a protein that would not ordinarily be considered a serum protein, the enzyme β-hexosaminidase, which, like other lysosomal enzymes (cf. reference 27), is thought to be targetted to lysosomes in the cell by a mannose-6-phosphate–dependent mechanism (19, 20). In fibroblasts, some of the enzyme escapes transport to the lysosome and enters the secretory pathway (37). As is evident in the autoradiographs depicted in Fig. 4, Caco-2 cells also synthesize and secrete β-hexosaminidase, which was detected
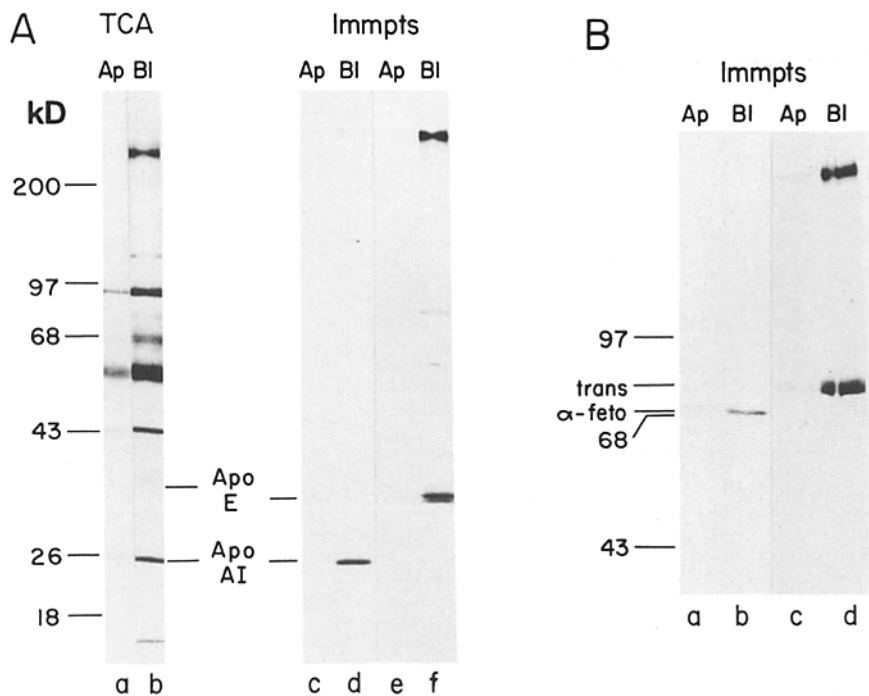
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Figure 3. Polarized secretion of newly synthesized proteins from Caco-2 cells. Cells grown for 7 d in Millicell chambers and having electrical resistance of >300 ohm·cm² were continuously labeled with [³⁵S]methionine for 4 h, as described in Materials and Methods, and subjected to NaDodSO₄-PAGE and autoradiography. (*A*) Medium was collected from the apical (*Ap*; lanes *a, c,* and *e*) and basolateral (*Bl*; lanes *b, d,* and *f*) chambers. Lanes *a* and *b* are TCA precipitates of an aliquot (100 µL) of collected medium, while other samples (1.4 ml) were immune precipitated (*Immpts*) with antibodies to apolipoprotein AI (*Apo AI*; *c* and *d*) and E (*Apo E*; *e* and *f*). Samples were run on a 7-13% gradient polyacrylamide gel. (*B*) In this experiment, the apical (*a* and *c*) and basal (*b* and *d*) medium were subjected to immune precipitation with anti-α-fetoprotein (*α-feto*; *a* and *b*) or antitransferrin (*trans*; *c* and *d*) antibodies and separated by electrophoresis on an 8% polyacrilamide gel. The positions of molecular mass standards are indicated in the margin for each gel. In each case, the secretory products of the cells were predominantly found in the basal compartment resulting from secretion from the basolateral surface: 92% basolateral for apo AI and E, 80% for α-fetoprotein, 93% for transferrin, and 91% for the 90 kD protein (lanes *a* and *b*).

primarily in the basolateral chamber after radiolabeling of monolayers cultured on filters and immune precipitation with specific antibody. The enzyme was observed to migrate on the polyacrylamide gels in a characteristic triplet with a prominent band representing the beta subunit 63 kD (19, 37). The amount of β-hexosaminidase secretion was increased two- to threefold by the lysosomotropic amines NH₄Cl and chloroquine, which raise lysosomal pH and cause lysosomal hydrolases to be shunted from their normal pathway to the secretory route for export from the cell (15, 19, 41). The inclusion of these drugs in the labeling medium did not alter the polarized secretion of β-hexosaminidase, which was virtually only detectable in the basolateral chamber (Fig. 4). Neither NH₄Cl (up to 30 mM) nor chloroquine (up to 300 µM, not shown) had any effect on the basolateral appearance of any of the major TCA precipitable products (Fig. 4 *B*). Furthermore, these lysosomotropic drugs did not increase the release or influence the polarized secretion from Caco-2 cells of apolipoproteins AI or E, α-fetoprotein, and transferrin, as determined after immune precipitation (not shown). These data are consistent with the results of Matlin (30) for influenza HA and Caplan et al. (4) for the Na⁺,K⁺ ATPase demonstrating that the sorting of apical and basolateral membrane proteins in MDCK cells is not a pH-dependent process. However, the recent report by Caplan et al. (5), indicating that laminin secretion by MDCK cells incubated with NH₄Cl is nonpolarized, implies that for some secretory proteins destined for the basolateral surface, the pH of the Golgi apparatus is important. With regard to the influence of the lysosomotropic agents to the process of basolateral secretion, the kidney-derived MDCK cells and the intestinally derived Caco-2 cells appear to differ, an indication

that the mechanisms for sorting basolaterally directed secretory products may also be very different in the two epithelial cell types (see Discussion).

The polarity of secretion of β-hexosaminidase was similarly unaffected by preincubation of the cells with tunicamycin (Fig. 5). Tunicamycin is an inhibitor of N-linked glycosylation, and, therefore, it prevents the man-6-PO₄ oligosaccharide modification of lysosomal enzymes (41, 49). Lysosomal enzymes synthesized in the presence of the drug are inefficiently transported to the lysosome and are secreted from the cell. The 57-kD form of the beta subunit of β-hexosaminidase produced by Caco-2 in the presence of tunicamycin is the same size as the protein secreted from fibroblasts and subsequently treated with endoglycosidase H to remove N-linked oligosaccharide side chains (37). This form of hexosaminidase should not, therefore, be capable of interacting with the man-6-PO₄ receptors, and the basolaterally directed export of the hexosaminidase cannot be attributed to interactions with these receptors, which are present both in intracellular organelles and on the plasma membrane of many cell types, including hepatocytes (14).

Polarized Secretion of GH by Transfected Cells

The secretion of β-hexosaminidase in the basolateral direction suggests that polarized secretion by Caco-2 cells requires no specific signal, since it is presumed that a lysosomal enzyme would lack such an addressing signal. To provide a more direct test of this hypothesis, the cDNA encoding for GH was introduced into Caco-2 by transfection. GH is one of a variety of secretory products introduced into MDCK cells by recombinant DNA techniques (17, 26). Like chick oviduct lysozyme, prochymosin (rennin, a product of
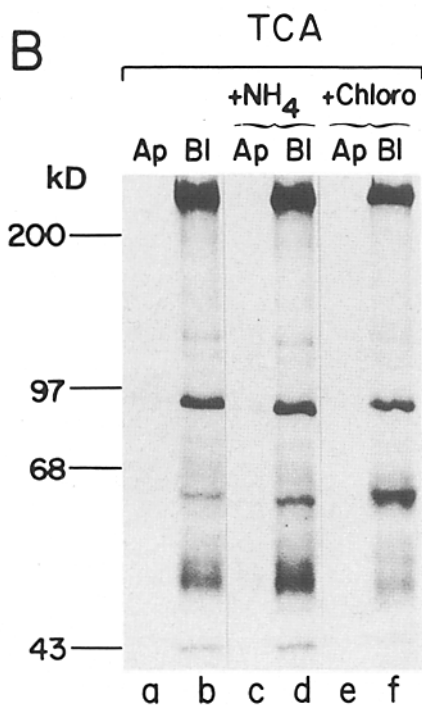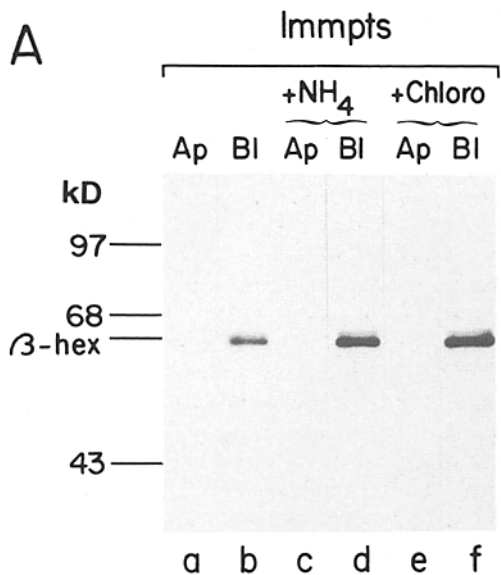
Figure 4. Polarized secretion of β-hexosaminidase from Caco-2 cells in the presence or absence of lysosomotropic amines. Monolayers on Millicell chambers 6 d after plating were labeled as described in Fig. 3. The apical (*Ap*; *a*, *c*, and *e*) and basolateral (*Bl*; *b*, *d*, and *f*) media were either immune precipitated with anti-β-hexosaminidase (*A*; 1.4-ml sample) or TCA precipitated (*B*; 50 μL) and subjected to NaDodSO₄-PAGE on an 8% gel. Cells were incubated in the absence of inhibitors (*a* and *b*), in the presence of 30 mM NH₄Cl (*c* and *d*), or in the presence of 100 μM chloroquine (*chloro*; *e* and *f*) during a 30-min preincubation as well as during the labeling period. In all cases, β-hexosaminidase was observed to be primarily in the basal chamber (*A*): 85% in the untreated sample, 94% for NH₄Cl, and 96% for chloroquine. The inhibitors had no effect on the overall pattern of secretion: 96% of the 90-kD protein was detected in the basal medium in the absence of drugs, 93% in NH₄Cl-treated samples, and 94% after incubation with chloroquine (*B*).
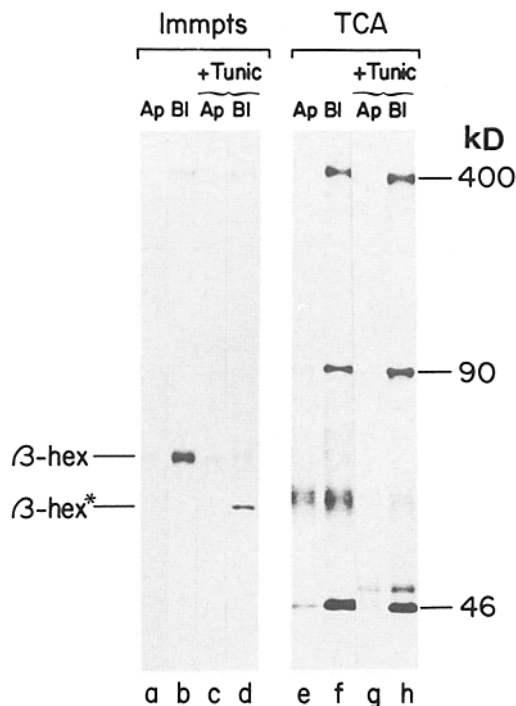
Figure 5. Polarized secretion of β-hexosaminidase in the presence of tunicamycin. Caco-2 cells grown in Millicell chambers for 7 d were preincubated for a total of 2 h in the presence (*c* and *d*) or absence (*a* and *b*) of 2 μg/ml of tunicamycin (*tunic*) and then labeled for 6 h as outlined in Materials and Methods. Medium from the apical (*Ap*; *a*, *c*, *e*, and *g*) or basal (*Bl*; *b*, *d*, *f*, and *h*) chambers was collected and subjected to immune precipitation with anti-β-hexosaminidase (1.4 ml; *a–d*) or to TCA precipitation (100 μl; *e–h*). Samples were subsequently separated by NaDodSO₄-PAGE on gradient (7–13%) gels. Although tunicamycin caused a shift in the apparent molecular mass of β-hexosaminidase (*β-hex*) from 63 to 57 kD (*β-hex**), the protein continued to be found primarily in the basolateral chamber; 83% in the untreated sample and 94% for the 57-kD form synthesized in the presence of the inhibitor. Similarly, 87% of the 90-kD protein (lanes *e–h*) was observed in the basal compartment in the untreated control, and 86% in the tunicamycin-treated sample. Indicated on the right margin are the positions of migration of the 90-kD protein as well as two other major endogenous secretory products.

bovine gastric glands), and alpha₂ᵤ-globulin, it was secreted in a nonpolarized fashion from MDCK cells and was observed in both the apical and basal medium compartments. But unlike these other proteins, GH is not a product of polarized epithelial cells in vivo but is instead synthesized and packaged into secretory vesicles by pituicytes. For these reasons, GH was deemed to lack a specific epithelial plasma membrane domain–sorting signal.

Caco-2 cells were transfected with the cDNA-encoding GH in transient expression experiments. In addition, neomycin-resistant transformants were isolated after cotransfection with pSV2-neo that stably expressed GH. When the cells were plated in Millicell chambers, the radiolabeled GH that was produced was immune precipitated only from the medium of the basal chamber (Fig. 6). The polarized secretion was observed in both the transiently expressing and permanently transformed samples.
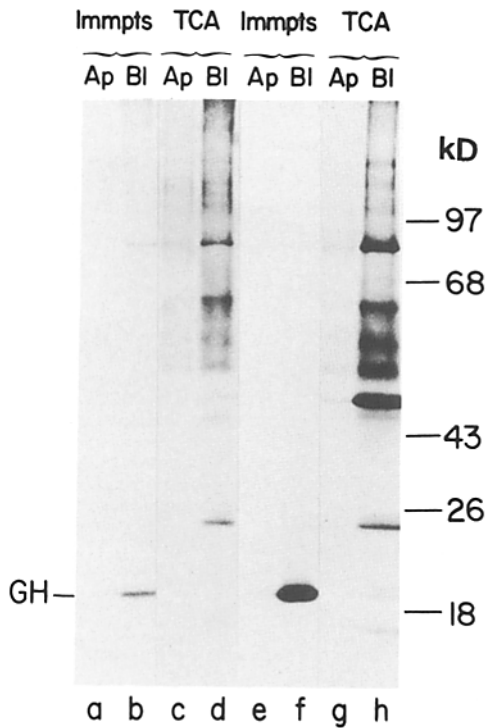
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Immpts TCA Immpts TCA
Ap Bl Ap Bl Ap Bl Ap Bl

kD
—97
—68
—43
—26
GH—
—18

a b c d e f g h

Figure 6. Secretion of GH from transfected Caco-2 cells. Cells in 75-cm² flasks were transfected with pSV2-GH as described in Materials and Methods. In transient expression experiments (*a–d*), cells were plated into Millicell chambers the day after transfection and labeled 4 d later for 5 h with [³⁵S]methionine. For permanent transformants (*e–h*), the neomycin-resistant derivatives were labeled 6 d after plating in the chambers. In all cases, sodium butyrate (10 mM) was added to the culture medium 16 h before labeling. Shown in the figure are immune precipitates (*a*, *b*, *e*, and *f*) using anti–GH antibodies and TCA precipitates (50 μL; *c*, *d*, *g*, and *h*). The transiently expressing cells incorporated radioactive label poorly but nonetheless, the secreted GH was found (93%) in the basal chamber (*b*) with very little in the apical compartment (*a*). Similarly, the GH produced by permanently transformed Caco-2 was observed in the basal compartment (compare *f* with *e*) almost exclusively (94%). The transfection protocol itself did not affect the continued basal secretion of endogenously synthesized Caco-2 proteins (compare *d* with *c*, and *h* with *g*): 91% of the 90 kD protein, for example, was found in the basolateral chamber in each case.

Discussion

Caco-2 cells were demonstrated to secrete a variety of newly synthesized products preferentially from their basolateral surfaces. These included transferrin, α-fetoprotein, and apolipoproteins AI and E, as well as a number of unidentified proteins. No proteins were secreted specifically from the apical surface. This property of the cultured intestinal epithelial cells is likely to mirror that of enterocytes of the small intestine in vivo, which synthesize such products as lipoproteins and would be expected to be able to segregate these proteins and to transport them out of the basolateral membrane and into the lymph (6). Indeed, the expression of α-fetoprotein in Caco-2 indicates that these transformed cells are typical of an embryonic form of the intestine, resembling in part embryonic hepatocytes (44), which share a common origin from the primitive gut tube (45). It is reasonable to suggest

that Caco-2 cells are a model not only for secretion by intestinal absorptive cells but also for secretion of albumin and other serum components into the perisinusoidal space by hepatocytes.

It is not known whether all of the aforementioned secretory products of Caco-2 cells retain sorting signals specifying basolateral transport. The observation that β-hexosaminidase and GH also are exported via the basolateral pathway provides strong evidence, however, that a specific sorting signal is unnecessary. β-hexosaminidase is ordinarily targetted to lysosomes, although some of the newly synthesized enzyme escapes this pathway and is instead secreted from the cell (19). The secretion of β-hexosaminidase has not been directly examined in other polarized epithelial cell lines, but the lysosomal enzymes cathepsin D and β-glucuronidase are not sorted in MDCK cells and are secreted from both the apical and basolateral surfaces (5; Compton, T., R. Andy, and D. D. Sabatini, unpublished observations), as is also the case for GH (17). MDCK cells do, however, support the vectorial secretion of an endogenous protein of 81 kD (17) from the apical surface and laminin from the basolateral domain (5). These results demonstrate that in the two cell types, secretory proteins that lack sorting signals are handled differently. Caco-2 cells, in contrast to the kidney-derived MDCK, export them solely along the basolateral secretory pathway. Because there is so little information available regarding the molecular nature of sorting information, it cannot be formally excluded that lysosomal enzymes and pituitary hormones do have putative basolateral addressing sequences recognized by a sorting system present in Caco-2 but absent in MDCK cells. It is unlikely, however, that a highly specific mechanism would have evolved to recognize proteins of such widely divergent origin.

The preference for basolateral secretion in Caco-2 cells may reflect the pattern of apical-basolateral traffic in the cell. That is perhaps 90% of the membrane protein transport in intestinal cells goes to the basolateral surface; the secretory proteins, free in the lumen of the Golgi apparatus, enter vesicles bound for the cell surface in a stochastic manner that reflects the volume of traffic to both membranes. However, the synthesis of microvillar membrane constituents represents a significant fraction of total membrane synthesis in enterocytes in vivo. In EM autoradiographic studies using ³H-fucose to label newly synthesized glycoproteins and glycolipids, the overall representation of silver grains over the apical cell surface of small intestinal absorptive cells was 50% or more of the total over the plasma membrane, an amount inconsistent with a basolateral traffic flow model (2, 3).

Of course, any discussion of the overall cellular membrane traffic depends on whether the sorting of apical and basolateral proteins in intestinal epithelial cells occurs intracellularly. In theory, all plasma membrane proteins of epithelial cells, even those destined for the apical domain, might first be transported to the basolateral domain and only subsequently be redistributed. It is even conceivable that vesicles carrying these proteins initially fuse at random over the entire plasma membrane and that the ratio of apical to basolateral delivery reflects the available surface area. In both cases, the secretory proteins would initially follow the same route as membrane proteins. These mechanisms have been ruled out in the case of MDCK cells. A number of laborato-

ries have demonstrated in these kidney-derived cells that the sorting of protein destined for the apical and basolateral membranes occurs intracellularly, and is a late Golgi or post-Golgi event (4, 13, 31, 32, 35, 38, 39). In the intestine, the site of sorting has not yet been established. Investigations using immunolocalization of apical proteins and subcellular fractionation during pulse–chase labeling have also supported the idea of intracellular sorting (9, 12). However, Massey et al. and Moktari et al. (29, 33) have evidence based on immune precipitation of membrane fractions that contradicts this view and instead suggests that apical proteins are first targetted to the lateral plasma membrane. In liver, similar studies using subcellular fractionation have also provided evidence for a transient basolateral plasma membrane intermediate in the intracellular transport pathway of proteins destined for the bile canilicular membrane (1). The Caco-2 cell line should provide an in vitro system similar to MDCK to test more directly whether there are differences in the mechanisms for sorting of membrane proteins between epithelial cell types.

The existence of a basolaterally directed pathway for secretory proteins lacking sorting signals raises the issue of whether the same might not hold true for membrane proteins. Such a hypothesis is attractive since the residents of the basolateral plasma membranes of epithelial cells are very common proteins found in nonpolarized cells as well, while characteristic apical membrane proteins tend to be present only on epithelial cells. In this way, apical membrane proteins would contain specific signals for transport while basolateral proteins would represent those that lack these sorting signals. An unambiguous test of this hypothesis would require that a membrane protein lacking a specific sorting signal be introduced into the polarized epithelial cell lines. If the model is correct, such a membrane protein would be expected to appear solely on the basolateral plasmalemma.

References

1. Bartles, J. R., H. M. Ferracci, B. Stieger, and A. L. Hubbard. 1987. Biogenesis of the rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. J. Cell Biol. 105:1241–1251.
2. Bennett, G., E. Carlet, G. Wild, and S. Parsons. 1984. Influence of colchicine and vinblastine on the intracellular migration of secretory and membrane glycoproteins. III. Inhibition of intracellular migration of membrane glycoproteins in rat intestinal columnar cells and hepatocytes as visualized by light and electron-microscope radioautography after ³H-fucose injection. Am. J. Anat. 170:545–566.
3. Blok, J., L. A. Ginsel, A. A. Mulder-Stapel, J. U. M. Onderwater, and W. Th. Daems. 1981. The effect of colchicine on the intracellular transport of ³H-fucose-labelled glycoproteins in the absorptive cells of cultured human small-intestinal tissue. Exp. Cell Res. 215:1–12.
4. Caplan, M. J., H. C. Anderson, G. E. Palade, and J. D. Jamieson. 1986. Intracellular sorting and polarized cell surface delivery of (Na⁺,K⁺) ATPase, an endogenous component of MDCK cell basolateral plasma membranes. Cell. 46:623–631.
5. Caplan, M. J., J. L. Stow, A. P. Newman, J. A. Madri, H. C. Anderson,
M. G. Farquhar, G. E. Palade, and J. D. Jamieson. 1987. Dependence on pH of polarized sorting of secreted proteins. Nature (Lond.). 329:632–635.
6. Cardell, Jr., R. R., S. Badenhausen, and K. R. Porter. 1967. Intestinal triglyceride absorption in the rat. J. Cell Biol. 34:123–155.
7. Cereijido, M., E. S. Robbins, W. J. Dolan, C. A. Rotunno, and D. D. Sabatini. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. J. Cell Biol. 77:853–880.
8. Chu, G., and P. A. Sharp. 1981. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation. Gene (Amst.). 13:197–202.
9. Danielsen, E. M., and G. M. Cowell. 1985. Biosynthesis of intestinal microvillar proteins: evidence for an intracellular sorting taking place in, or shortly after, exit from the Golgi complex. Eur. J. Biochem. 152:493–499.
10. Evans, W. H. 1980. A biochemical dissection of the functional polarity of the plasma membrane of the hepatocyte. Biochim. Biophys. Acta. 604:27–64.
11. Fogh, J., J. M. Fogh, and T. Orfeo. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. 59:221–226.
12. Fransen, J. A. M., L. Ginsel, H.-P. Hauri, E. Sterchi, and J. Blok. 1985. Immuno-electronmicroscopical localization of a microvillus membrane disaccharidase in the human small-intestinal epithelium with monoclonal antibodies. Eur. J. Cell Biol. 38:6–15.
13. Fuller, S. D., R. Bravo, and K. Simons. 1985. An enzymatic assay reveals that proteins destined for the apical and basolateral domains of an epithelial cell line share the late Golgi compartments. EMBO (Eur. Mol. Biol. Organ.) J. 4:297–307.
14. Geuze, H. J., J. W. Slot, G. J. A. M. Strous, A. Hasilik, and K. von Figura. 1984. Ultrastructural localization of the mannose-6-phosphate receptors in rat liver. J. Cell Biol. 98:2047–2054.
15. Gonzalez-Noreiga, A., J. H. Brugg, V. Talkad, and W. S. Sly. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol. 85:839–852.
16. Gorman, C. M., B. H., Howard, and R. Reeves. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. 11:7631–7648.
17. Gottlieb, T. A., G. Beaudry, L. Rizzolo, A. Colman, M. Rindler, M. Adesnik, and D. D. Sabatini. 1986. Secretion of endogenous and exogenous proteins from polarized MDCK cell monolayers. Proc. Natl. Acad. Sci. USA. 83:2100–2104.
18. Gottlieb, T. A., A. Gonzalez, L. Rizzolo, M. J. Rindler, M. Adesnik, and D. D. Sabatini. 1986. Sorting and endocytosis of viral glycoproteins in transfected polarized epithelial cells. J. Cell Biol. 102:1242–1245.
19. Hasilik, A., and E. F. Neufeld. 1980. Biosynthesis of lysosomal enzymes in fibroblasts—synthesis as precursors of higher molecular weight. J. Biol. Chem. 255:4937–4945.
20. Hasilik, A., and E. F. Neufeld. 1980. Biosynthesis of lysosomal enzymes in fibroblasts—phosphorylation of mannose residues. J. Biol. Chem. 255:4946–4950.
21. Hauri, H.-P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxer. 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. J. Cell Biol. 101:838–851.
22. Hubbard, A. L., D. A. Wall, and A. Ma. 1983. Isolation of hepatocyte plasma membranes. I. Presence of three major domains. J. Cell Biol. 96:217–229.
23. Jamieson, J. D., and G. E. Palade. 1971. Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. J. Cell Biol. 50:135–158.
24. Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. Science (Wash. DC). 230:25–32.
25. Kloppel, T. M., W. R. Brown, and J. Reichen. 1986. Mechanisms of secretion of proteins into bile: studies in the perfused rat liver. Hepatology (Baltimore). 6:587–594.
26. Kondor-Koch, C., R. Bravo, S. D. Fuller, D. Cutler, and H. Garoff. 1985. Exocytotic pathways exist to both the apical and basolateral cell surfaces of the polarized epithelial cell MDCK. Cell. 43:297–306.
27. Kornfeld, S. 1986. Trafficking of lysosomal enzymes in normal and disease states. J. Clin. Invest. 77:1–6.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.
29. Massey, D., H. Feracci, J.-P. Gorvel, A. Rigal, J. M. Soulie, and S. Maroux. 1987. Evidence for the transit of aminopeptidase N through the basolateral membrane before it reaches the brush border of enterocytes. J. Membr. Biol. 96:19–25.
30. Matlin, K. S. 1986. Ammonium chloride slows transport of the influenza virus hemagglutinin but does not cause mis-sorting in a polarized epithelial cell line. J. Biol. Chem. 261:15172–15178.
31. Matlin, K. S., and K. Simons. 1984. Sorting of an apical plasma membrane glycoprotein occurs before it reaches the cell surface in cultured epithelial cells. J. Cell Biol. 99:2131–2139.
32. Misek, D. E., E. Bard, and E. Rodriguez-Boulan. 1984. Biogenesis of epithelial cell polarity: intracellular sorting and vectorial exocytosis of an

apical plasma membrane glycoprotein. *Cell.* 39:537-546.

33. Moktari, S., H. Feracci, J.-P. Gorvel, Z. Mishal, A. Rigal, and S. Maroux. 1986. Subcellular fractionation and subcellular localization of aminopeptidase N in rabbit enterocytes. *J. Membr. Biol.* 89:53-63.

34. Mulligan, R. C., and P. Berg. 1980. Expression of a bacterial gene in mammalian cells. *Science (Wash. DC).* 209:1422-1427.

35. Pfeiffer, S., S. D. Fuller, and K. Simons. 1985. Intracellular sorting and basolateral appearance of the G protein of vesicular stomatitis virus in MDCK cells. *J. Cell Biol.* 101:470-476.

36. Pinto, M., S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. 1983. Enterocyte-like differentiation and polarization of the human carcinoma cell line Caco-2 in culture. *Biol. Cell.* 47:323-330.

37. Proia, R. L., and E. F. Neufeld. 1982. Synthesis of β-hexosaminidase in cell-free translation and in intact fibroblasts: an insoluble precursor chain in a rare form of Tay-Sachs disease. *Proc. Natl. Acad. Sci. USA.* 79:6360-6364.

38. Rindler, M. J., I. E. Ivanov, H. Plesken, E. J. Rodriguez-Boulan, and D. D. Sabatini. 1984. Viral glycoproteins destined for the apical or basolateral plasma membrane domains traverse the same Golgi apparatus during their intracellular transport in Madin-Darby canine kidney cells. *J. Cell Biol.* 98:1304-1319.

39. Rindler, M. J., I. E. Ivanov, H. Plesken, and D. D. Sabatini. 1985. Polarized delivery of viral glycoproteins to the apical and basolateral plasma membranes of Madin-Darby canine kidney cells infected with temperature-sensitive viruses. *J. Cell Biol.* 100:136-151.

40. Rodriguez Boulan, E. J., and D. D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model for the study of epithelial polarity. *Proc. Natl. Acad. Sci. USA.* 75:5071-5075.

41. Rosenfeld, M. G., G. Kreibich, D. Popov, K. Kato, and D. D. Sabatini. 1982. Biosynthesis of lysosomal hydrolases: their synthesis in bound polysomes and the role of co- and post-translational processing in determining their subcellular distribution. *J. Cell Biol.* 93:135-143.

42. Roy, A. K., and O. W. Neuhaus. 1966. Proof of the hepatic synthesis of a sex-dependent protein in the rat. *Biochim. Biophys. Acta.* 127:82-87.

43. Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for incorporation of proteins in membranes and organelles. *J. Cell Biol.* 91:1-22.

44. Sell, S., M. A. Longley, and J. Boulter. 1985. α-fetoprotein and albumin gene expression in brain and other tissues of fetal and adult rats. *Brain Res.* 22:49-53.

45. Severn, C. B. 1971. A morphological study of the development of the human liver. I. Development of the hepatic diverticulum. *Am. J. Anat.* 131:133-146.

46. Strous, G. J., R. Willemsen, P. van Kerkhof, J. W. Slot, H. J. Geuze, and H. F. Lodish. 1983. Vesicular stomatitis virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. *J. Cell Biol.* 97:1815-1822.

47. Sztul, E. S., K. S. Howell, and G. E. Palade. 1983. Intracellular and transcellular transport of secretory component and albumin in rat hepatocytes. *J. Cell Biol.* 97:1582-1591.

48. Traber, M. G., H. J. Kayden, and M. J. Rindler. 1987. Polarized secretion of newly synthesized lipoproteins by the Caco-2 human intestinal cell line. *J. Lipid Res.* 28:1350-1363.

49. Von Figura, K., M. Rey, R. Prinz, B. Voss, and K. Ullrich. 1979. Effect of tunicamycin on transport of lysosomal enzymes in cultured skin fibroblasts. *Eur. J. Biochem.* 101:103-109.