Constitutive Apical Secretion of an 80-kD Sulfated Glycoprotein Complex in the Polarized Epithelial Madin–Darby Canine Kidney Cell Line

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Abstract. The biosynthesis, processing, and apical secretion of a group of polypeptides (Kondor-Koch, C., R. Bravo, S. D. Fuller, D. Cutler, and H. Garoff. 1985. *Cell.* 43:297–306) are studied in MDCK cells using a specific polyclonal antiserum. These polypeptides are synthesized as a precursor protein which has an apparent M_r of 65,000 in its high mannose form. This precursor is converted into a protein with an apparent M_r of 80,000 containing complex carbohydrates and sulfate. After intracellular cleavage of the 80-kD protein, the 35–45-kD subunits are secreted as an 80-kD glycoprotein complex (gp 80) linked together by disulfide bonds. Secretion of the protein complex occurs by a constitutive pathway at the apical surface of the epithelial monolayer. Since the immediate post-translational precursor, the 65-kD protein, is hydrophilic in nature as shown by its partitioning behavior in a phase-separated Triton X-114 solution, gp 80 is segregated into the apical exocytotic pathway as a soluble molecule. The proteolytic maturation of gp 80 is blocked in the presence of chloroquine and its secretion is retarded. The 80-kD precursor is released at the apical cell surface, demonstrating that proteolytic processing is not necessary for the apical secretion of this protein. If N-glycosylation is inhibited by tunicamycin treatment the protein is secreted in equal amounts at both cell surfaces, indicating a role of the carbohydrate moieties in the vectorial transport of this protein.

EPITHELIAL cells fulfill a dual function: they generate and maintain boundaries between the compartments of an organism and they condition the milieu on both sides of the epithelial layer by their capacity to vectorially secrete and transport selected substrates. The structural basis for this function is the differentiation of the plasma membrane into two distinct domains that differ from each other in protein and lipid composition (39) and the presence of tight junctions that separate the two plasma membrane domains and tie adjacent cells into a continuous sheet.

Vectorial secretion has been studied extensively in epithelia. In many epithelial cells polar secretion is accomplished by a specialized exocytotic pathway in which proteins destined for export are stored and concentrated in secretory vesicles and only released upon an extracellular stimulus (regulated exocytosis) (15, 30). Polarized secretion can, however, also occur constitutively as shown by the secretion of albumin, transferrin, and other proteins at the sinusoidal surface of liver parenchymal cells (17, 18).

We have used the polarized Madin–Darby canine kidney (MDCK) epithelial cell line as a model system to study vectorial secretion of proteins in transporting epithelial cells. This cell line displays, in culture, morphological and enzymatic properties characteristic of distal tubule cells (23). Furthermore, this cell line is well characterized with respect to its cell surface polarity, using either viral membrane proteins (34) or cellular plasma membrane proteins (33) as probes. Analysis of the asymmetric segregation of plasma membrane proteins at the cell surface and the vectorial discharge of secretory proteins at either side of the epithelial monolayer is facilitated by the ability of these cells to form tight monolayers of polarized cells on permeable supports such as nitrocellulose and polycarbonate filters (5, 9, 16, 25).

We have previously studied in these cells the secretion of an exogenous protein, lysozyme, synthesized from a recombinant gene in MDCK cells as well as the secretion of endogenous polypeptides (16). It has been shown that lysozyme is released in a nonpolarized fashion at both cell surfaces, whereas a group of endogenous 35–45-kD polypeptides is secreted at the apical cell surface. Since vectorial exocytosis in MDCK cells had so far been demonstrated only for membrane-spanning proteins it was important to test whether the apically secreted 35–45-kD polypeptides were transported to the cell surface as soluble molecules. Using specific antibodies, we have now identified the intracellular precursors of

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these peptides. We show here that the immediate posttranslational product is a soluble molecule, which is proteolytically processed to the 35–45-kD polypeptides after terminal glycosylation and sulfation. These polypeptides are secreted constitutively as an 80-kD protein complex at the apical cell surface. These results demonstrate that in MDCK cells soluble molecules can be segregated into a constitutive pathway leading to the apical cell surface. We further investigate the role of the proteolytic cleavage event and the N-linked carbohydrate groups in this process.

Materials and Methods

MDCK cells, strain II (19), baby hamster kidney (BHK)-21 cells (1), Semliki Forest virus, and anti-E2 antibodies were gifts of K. Simons and H. Garoff (European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany). Media and reagents for cell culture were obtained from Biochrom, Berlin, FRG and Gibco, Grand Island, NY; endoglycosidase H, tunicamycin, and chloroquine were from Sigma Chemical Co., St. Louis, MO; protein A-Sepharose was from Pharmacia Fine Chemicals, Piscataway, NJ. Polycarbonate Transwell filters were obtained from Costar, Cambridge, MA. [^{35}S]Methionine (>800 Ci/mmol; SJ.204) and [^{35}S]sulfate (25–40 Ci/mg; SJS.1) were purchased from Amersham Buchler, Braunschweig, FRG.

Culture of Cells on Plastic Dishes

MDCK cells were grown in culture medium containing MEM, supplemented with 10 mM Hepes (pH 7.3), 2 mM glutamine, 5% (vol/vol) FCS. BHK-21 cells were grown in culture medium containing G-MEM, supplemented with 10 mM Hepes (pH 7.3), 2 mM glutamine, 5% (vol/vol) FCS.

Culture of MDCK Cells on Filters

To allow separate access to the media on the two sides of an MDCK cell monolayer, cells were grown on a permeable support. Earlier (16) we have used 0.22-μm nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA; GSTF 02500) to study the secretion of lysozyme expressed from cDNA in MDCK cells. Control experiments had shown that the lysozyme molecule needed ~30 min to cross the filter and that it was not adsorbed to the filter material to any significant extent. Control experiments to evaluate the permeability of the nitrocellulose filters for gp 80, however, demonstrated that this protein was tightly adsorbed to the filter material; this resulted in an artificially low recovery from the basolateral medium.

We therefore chose for the study of the polarity of gp 80 secretion polycarbonate filters (Nucleopore Transwell TM 3412) because of their low affinity for this protein (see below).

MDCK cells were seeded at a density of 6×10^5 cells/cm^2 on 0.4-μm pore size polycarbonate filters and cultured in MEM supplemented with 10 mM Hepes, pH 7.3, 2 mM glutamine, 10% (vol/vol) FCS. Cells were fed with fresh medium every 24 h and were used for experiments 3 d after seeding at a density of 1.8×10^6 cells/cm^2.

To assess the permeability of the polycarbonate filters for gp 80, filters were preincubated in conditioned medium and mock pulse-chased. 1.5 ml of medium obtained from a [^{35}S]methionine-labeled MDCK cell monolayer containing either glycosylated or unglycosylated gp 80 were applied on top of the filter and 2.6 ml fresh medium to the other side and the filters were incubated at 37°C under gentle agitation. After various incubation times the media on both sides of the filter were collected, the filters extracted with cell lysis buffer, and the total samples were processed for immunoprecipitation. One individual filter was taken for each time point. Using this assay gp 80 was detected on the other side of the filter already after a 15-min incubation. After a 2-h incubation, 25% of the labeled gp 80 applied on top of the filter was recovered from the medium in the bottom chamber and 2–5% were recovered from the filter extracts. In contrast, when the same experiment was performed with filters covered with a confluent MDCK cell monolayer, no labeled gp 80 was recovered from either the basolateral medium or the cell lysates after a 2-h incubation.

Protein Purification and Generation of Specific Antibodies

Subconfluent monolayers of MDCK cells grown in 85-cm^2 culture flasks were rinsed twice in PBS and in serum-free medium (43) and were then incubated for 12 h at 37°C in 4.0 ml serum-free medium supplemented with antipain (1 μg/ml), benzamidine (1.75 μg/ml), and aprotinin (10 μg/ml). Every 10th flask was labeled with [^{35}S]methionine (100 μCi [^{35}S]methionine in 1.0 ml serum-free medium for 30 min) and then processed in an identical way. After the incubation, the media were collected and spun for 10 min at 1,000 *g*. The cleared media were concentrated 20-fold by ultrafiltration and dialyzed against 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. The proteins were separated by reverse-phase HPLC (material: wide pore C3; gradient: H_2O–0.1% fluoroacetic acid to H_2O–0.1% trifluoroacetic acid/75% acetonitrile). Fractions were lyophilized, taken up in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and aliquots were analyzed by SDS-PAGE, silver staining, and autoradiography. Fractions containing the secreted 35–45-kD peptides and no contaminating proteins were pooled. Approximately 100 μg purified proteins, as judged by the intensity of the Coomassie Blue–stained protein bands in the PAGE, were obtained from the medium of 120 culture flasks. They were used for the immunization of a rabbit following standard immunization protocols except that the first injection of the antigen was administered directly into the lymph nodes of the hind legs.

In Vivo Labeling of Proteins in MDCK Cell Cultures

MDCK cells were pulse labeled either as subconfluent monolayers grown in 35-mm plastic dishes (300 μCi [^{35}S]methionine/300 μl; 15 min at 37°C) or as 3-d-old cultures grown on 0.4-μm pore size polycarbonate filters (150 μCi [^{35}S]methionine/150 μl applied to the basolateral side of the inverted filter; 15 min at 37°C). Labeling was performed in MEM, supplemented with 2.2 g/liter $NaHCO_3$, 10 mM Hepes (pH 7.4), 0.2% BSA, and lacking methionine. After the pulse the cells were rinsed twice in the same medium containing excess methionine (150 mg/liter). Plastic-grown cells were chased for various periods of time in the same medium containing methionine (15 mg/liter). Cell lysates and media were collected and processed for immunoprecipitation as described before (16). Filter cultures were chased in serum-free medium (43) supplemented with 0.2% BSA (1.5 ml on the apical side, 2.6 ml on the basolateral side of the monolayer). After the various chase periods the media were collected, detergents and salts were added to the concentrations in the cell lysates, and the samples were processed for immunoprecipitation. The filters were removed from the chambers with a scalpel, placed in a 1.5-ml tube containing 0.5 ml of 0.5% SDS, 100 mM NaCl, 5 mM EDTA, 30 mM Tris-HCl, pH 8.1, and 5 μg/ml phenylmethylsulfonyl fluoride (PMSF) and boiled for 10 min (27). The liquid was removed and the filter reextracted with 0.5 ml 2.5% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl, pH 8.1, and 5 μg/ml PMSF using a short sonication. Both extraction media were pooled and processed for immunoprecipitation.

In vivo labeling in the presence of chloroquine or tunicamycin was performed with cells preincubated for 60 min at 37°C in culture medium containing chloroquine (200 μM) or tunicamycin (4 μg/ml were used to obtain a complete block of N-glycosylation in plastic-grown MDCK cells and a partial inhibition in filter-grown MDCK cells; to achieve a complete block of N-glycosylation in filter-grown MDCK cells 12 μg/ml had to be used). In these experiments, cells were pulsed for 15 min with 300 μCi [^{35}S]methionine. Same amounts of either drug were added to all media used in these experiments.

For labeling cells with [^{35}S]sulfate, plastic-grown monolayers were preincubated for 3 h at 37°C in MEM supplemented with 10 mM Hepes, pH 7.3, 2 mM glutamine, in which $MgSO_4$ had been replaced by $MgCl_2$. The cells were then labeled for 15 min at 37°C in the same medium containing carrier-free [^{35}S]sulfate (500 μCi/500 μl). After the pulse the cells were rinsed twice in medium containing excess $MgSO_4$ (2 g/liter) and were chased in the presence of normal amounts of $MgSO_4$ (0.2 g/liter).

Digestion of [^{35}S]Methionine-labeled Proteins with Endoglycosidase H

Subconfluent monolayers of MDCK cells were labeled with [^{35}S]methionine in the presence of chloroquine, chased for either 5 or 60 min, and cell lysates were collected. Proteins were immunoprecipitated and each sample was split into two portions; one was digested with endoglycosidase H and the other was mock digested as described by Zilberstein et al. (45) with the following modifications. The digestion was performed for 4 h with 16 mU of enzyme. Then another 16 mU of enzyme and 1/10 vol of 5 M NaSCN were added and the digestion was continued for 12 h.

Extraction with Triton X-114

A subconfluent monolayer of MDCK cells in a 3.5-cm dish was labeled with

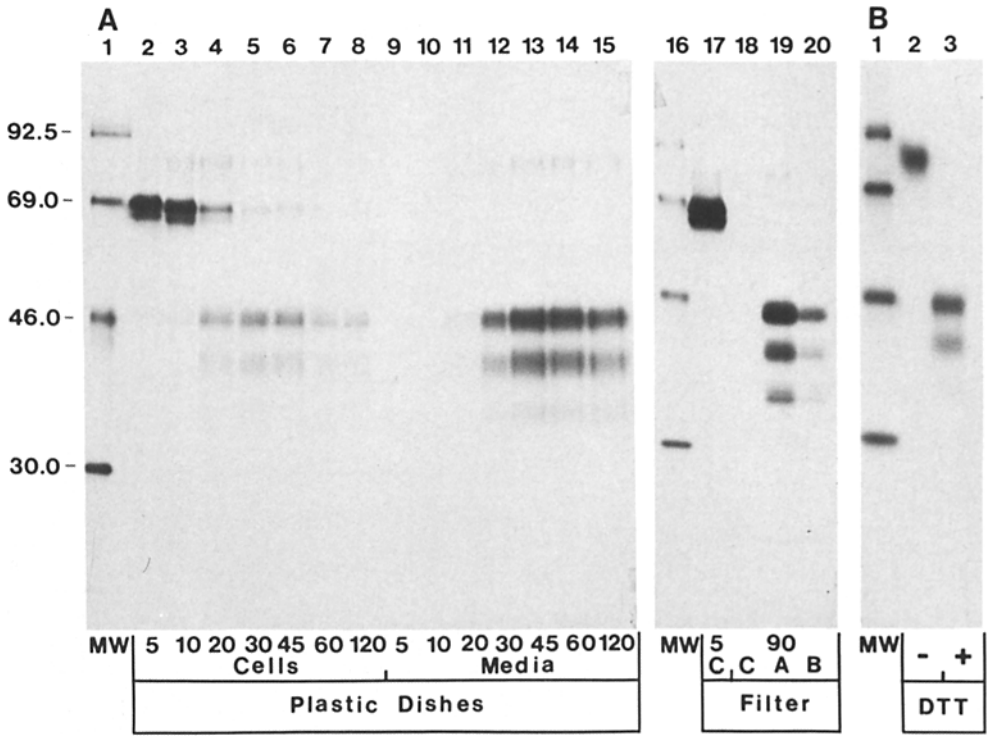

Figure 1. Pulse–chase analysis of the processing and secretion of gp 80 in MDCK cells. (*A*) Subconfluent plastic-grown monolayers (lanes *2–15*) and filter-grown monolayers (lanes *17–20*) of MDCK cells were pulse labeled with [^{35}S]methionine for 10 min and chased for the indicated periods up to 120 min. Lanes *2–8*, *17*, and *18* show the immunoprecipitated, reduced, and alkylated proteins in the cell lysates; lanes *9–15* in total; lane *19* in the apical medium; and lane *20* in the basolateral medium. Molecular mass standards are run in lanes *1* and *16*. (*B*) A subconfluent monolayer of MDCK cells was pulse labeled with [^{35}S]methionine, chased for 60 min, and the proteins in the medium were immunoprecipitated. Half of the sample was prepared for SDS-PAGE in the absence of DTT (lane *2*), and the other half in the presence of 1 mM DTT (lane *3*). Molecular mass standards are run in lane *1*.

[^{35}S]methionine (1 mCi/ml; 15 min). A subconfluent monolayer of BHK 21 cells in a 3.5-cm dish was infected with Semliki Forest virus (50 pfu/cell) as described by Saraste et al. (37). 4 h after infection cells were labeled with [^{35}S]methionine (200 μCi/ml; 30 min) and then chased for 30 min. Cells were rinsed twice in PBS and lysed at 0°C in PBS containing 0.5% Triton X-114, 4 μg/ml PMSF, and 10 mM iodoacetamide. Nuclei were spun down in an Eppendorf centrifuge at 0°C and the sample was divided into half. One half was directly used for immunoprecipitation; the other half was separated into a detergent and an aqueous phase according to the procedure of Bordier (4). Each phase was used separately for immunoprecipitation. Samples generated from MDCK cells were immunoprecipitated with anti-p35/p45 antibodies; samples generated from Semliki Forest virus-infected BHK 21 cells were immunoprecipitated with anti-E2 antibodies.

Other Methods

Immunoprecipitations were done as described before processing the entire cell lysate and media samples of one plastic-grown or one filter-grown MDCK cell culture (16). Equal amounts of serum were added to cell samples (1.0 ml) and media (1.0 ml) of plastic-grown MDCK and BHK cell cultures and to the apical (1.5 ml) and basolateral (2.6 ml) media of filter-grown MDCK cell cultures. Control experiments were performed to ensure that the efficiency of the immunoprecipitation was the same in all samples. PAGE was performed as described by Piccioni et al. (32) using 10% polyacrylamide slab gels. The procedure for fluorography has been described by Bonner and Laskey (3). The radioactivity in specific bands was quantitated by the method of Walter et al. (44).

Results

Biosynthesis, Processing, and Secretion of an 80-kD Glycoprotein Complex (gp80) in MDCK Cells

In a previous paper (16) we have described a group of acidic 35–45-kD polypeptides which are secreted from filter-grown monolayers of MDCK cells at the apical cell surface. From the analysis of in vivo–labeled proteins by two-dimensional PAGE it was clear that these polypeptides are synthesized as higher molecular mass precursor proteins. However, in the absence of specific antibodies we could only speculate on putative precursor molecules. Polyclonal antibodies have now been raised in rabbits against these polypeptides purified from serum-free MDCK cell medium by reverse-phase HPLC. The antiserum obtained is highly specific for the secreted 35–45-kD peptides and their precursor forms as judged by comparative immunoblot and fluorographic analyses of two-dimensional PAGE of in vivo–labeled MDCK cell lysates (data not shown) as well as by the analysis of immunoprecipitable proteins from MDCK cells and their media by one-dimensional PAGE (Fig. 1). Although the antibodies have been raised against the secreted peptides, they recognize all biosynthetic forms of the protein with apparently similar affinity as indicated by the quantitation of the pulse–chase experiments. We use this antiserum here to study biosynthesis, processing, and intracellular transport of the apically secreted polypeptides in MDCK cells.

Subconfluent monolayers of MDCK cells grown in culture dishes and filter-grown monolayers of MDCK cells were pulse labeled for 10 min with [^{35}S]methionine and chased for various time intervals at 37°C. Cells and media were collected, immunoprecipitated, and analyzed by SDS-PAGE and fluorography. As shown in Fig. 1 *A* the immediate post-translational form is a molecule with an apparent M_r of 65 kD. This precursor is converted into a protein with an apparent M_r of 80,000, which is only present in minor amounts in the cells since it is rapidly cleaved into the 35–45-kD polypeptides. The 80-kD protein can however be detected easily in the cell lysates of chloroquine-treated cells, since proteolytic maturation is inhibited under these conditions (see Fig.

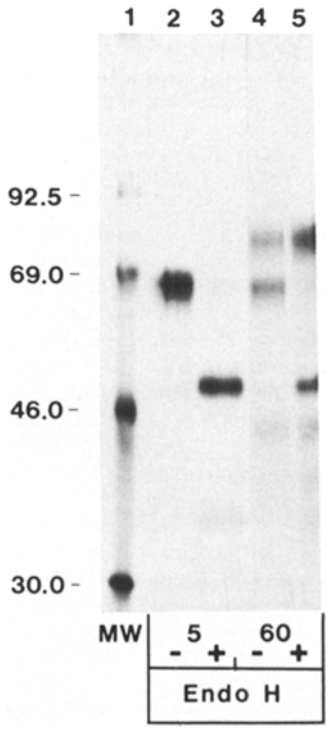

Figure 2. Analysis of the intracellular 65- and 80-kD precursor proteins with endoglycosidase H. Subconfluent plastic-grown monolayers of MDCK cells were pulse labeled with [^{35}S]methionine and chased in the presence of 200 μM chloroquine for 5 (lanes *2* and *3*) or 60 min (lanes *4* and *5*). The cells were lysed, the proteins immunoprecipitated, and the samples divided in half. One half was directly processed for SDS-PAGE (lanes *2* and *4*); the other half was digested with endoglycosidase H as described in Materials and Methods (lanes *3* and *5*).

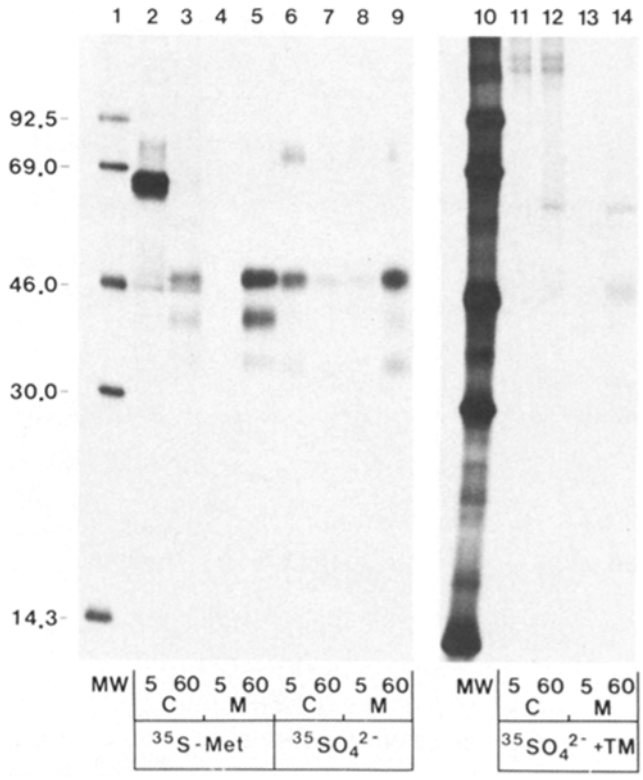

Figure 3. Analysis of immunoprecipitated [^{35}S]methionine- and [^{35}S]sulfate-labeled proteins. Subconfluent plastic-grown monolayers of MDCK cells were either pulse labeled with [^{35}S]methionine (lanes *2–5*) or with [^{35}S]sulfate in the absence (lanes *6–9*) or presence of tunicamycin (lanes *11–14*). After chase periods of 5 or 60 min, the proteins in the cell lysates (*C*) and media (*M*) were immunoprecipitated, reduced, and alkylated, and then processed for SDS-PAGE. The gel with the glycosylated proteins was exposed for 3 d, the gel with the samples from tunicamycin-treated cells for 30 d.

5). The 35–45-kD polypeptides are found in the cell lysates as early as 20 min after synthesis and are readily secreted. They can be detected in the medium of plastic-grown monolayers as early as 20 min after labeling. A faint band comigrating with the intracellular 80-kD precursor protein is also visible in the medium samples showing that the uncleaved 80-kD protein is also secreted, although in small amounts. Of the pulse-labeled protein, 50% is secreted at 30 min of chase; 60 min after synthesis only 10% is found in the cell lysates of plastic-grown cells. This does not change significantly at later times of chase.

If the cell lysate, the apical medium, and the basolateral medium of filter-grown monolayers at 90 min of chase are analyzed, 75% of the pulse-labeled gp 80 present in the 5-min cell sample is found in the apical medium, 20% is found in the basolateral medium, and 3% is recovered in the cell lysate (Fig. 1 *A*, lanes *17–20*; see also Fig. 7). This result shows that gp 80 secretion from filter-grown MDCK cells is polar. However, its sorting into the apical pathway of exocytosis does not occur with 100% efficiency, an observation that has also been made for the hemagglutinin of influenza virus, a marker protein of the apical plasma membrane domain in virus-infected MDCK cells (39).

The 35–45-kD polypeptides are secreted as an 80-kD protein complex, in which the polypeptide chains are linked together by disulfide bonds as shown by the analysis of the unreduced immunoprecipitated proteins by SDS-PAGE (Fig. 1 *B*). The unreduced secreted protein complex comigrates in SDS-PAGE with the intracellular, uncleaved 80-kD precursor protein.

Analysis of the carbohydrate structure of the two intracellular precursor proteins with endoglycosidase H demonstrates that the 65-kD form is sensitive to, whereas the 80-kD form is resistant to, digestion with endoglycosidase H (Fig. 2). Since endoglycosidase H cuts the Glc-NAC β-4 Glc-NAC bond in Asn-linked oligosaccharides with five or more mannose residues present in the endoplasmic reticulum but not the complex oligosaccharide units present in the Golgi stacks (12), the shift in apparent M_r is due to modifications of the protein during its migration from the endoplasmic reticulum to the cell surface, one of those being the maturation of its carbohydrate side chains.

Concomitant with the acquisition of endoglycosidase H resistance, the protein can be metabolically labeled with [^{35}S]sulfate. 5 min after labeling with sulfate, the 80-kD and the 35–45-kD polypeptides are found in the cell lysates (Fig. 3, lane *6*) and traces of these proteins can already be detected in the medium sample (Fig. 3, lane *8*). On gels loaded with [^{35}S]sulfate-labeled, immunoprecipitated proteins of tunicamycin-treated MDCK cells in which N-glycosylation is inhibited, we do not detect any protein bands comigrating with the unglycosylated forms even in prolonged exposures (Fig. 3, lanes *11–14*). gp 80 biosynthesis is not markedly decreased under these conditions as determined by the amount of [^{35}S]methionine incorporated during pulse labeling. Therefore the label is present predominantly in the N-linked oligosaccharides. This observation is in agreement with results obtained by Friedrich and Huttner (Friedrich, E., and W. B.

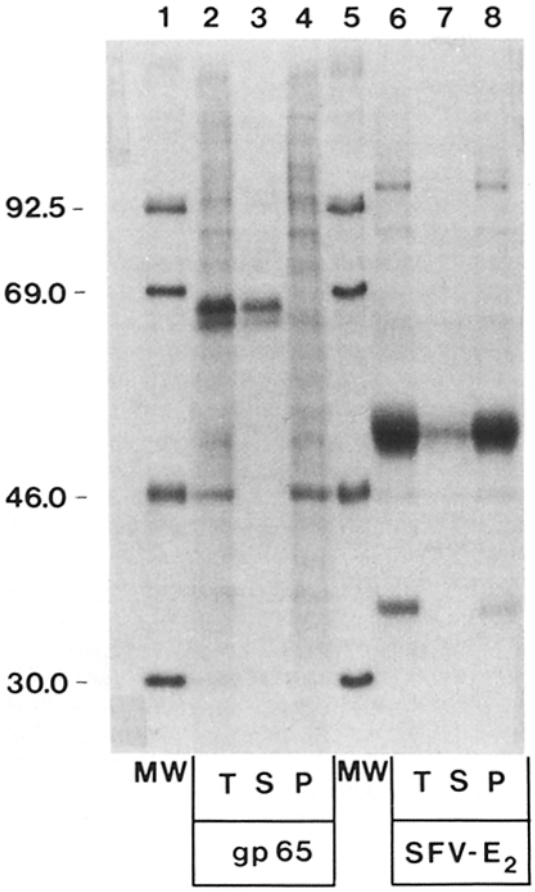

Figure 4. Analysis of the 65-kD precursor protein by Triton X-114 extraction. A subconfluent plastic-grown monolayer of MDCK cells was pulse labeled with [^{35}S]methionine, chased for 5 min, and processed for Triton X-114 extraction. Proteins were immunoprecipitated with antibody directed against the secreted 35–45-kD subunits (lanes *2–4*). A subconfluent monolayer of BHK-21 cells was infected with Semliki Forest virus, pulse labeled with [^{35}S]methionine 4 h after infection, chased for 30 min, and processed for Triton X-114 extraction (lanes *6–8*). Proteins were immunoprecipitated with anti-E2 antibodies. Lanes *2* and *6* show the unpartitioned samples (*T*); lanes *3* and *7* the aqueous phases (*S*); lane *4* and *8* the detergent phases (*P*). Molecular mass standards are run in lanes *1* and *5*.

Huttner, personal communication), who found that ~10% of the [^{35}S]sulfate incorporated into the 35–45-kD polypeptides was recovered as tyrosine sulfate whereas the bulk was alkali sensitive, suggesting linkage to carbohydrate.

To investigate whether the gp 80 complex is segregated into the apical exocytotic pathway as a membrane-bound or as a soluble protein, we tested if the protein exists in an amphiphilic form at any stage of its biogenesis. For this reason we performed a Triton X-114 extraction of the pulse-labeled 65-kD immediate posttranslational precursor protein. The partitioning of proteins between the aqueous and the detergent phase upon phase separation of dilute Triton X-114 solutions is taken as an operational distinction between amphiphilic and hydrophilic proteins (4). MDCK cells were pulse labeled and chased for 5 min. After solubilization of the cells in 0.5% Triton X-114 containing buffer at 4°C, half of the lysate was directly processed for immunoprecipitation. The other half was incubated at 30°C to separate into an aqueous and a detergent phase. Both samples were reextracted; the proteins were immunoprecipitated and analyzed by SDS-PAGE and fluorography. The partitioning of a viral membrane protein, the E2 protein of the Semliki Forest virus, was used as a control. The E2 protein is a component of the viral spike glycoprotein complex and spans the lipid bilayer once (10). BHK-21 cells infected with Semliki Forest virus were pulse labeled and chased as described in Materials and Methods. Cells were lysed in buffer containing 0.5% Triton X-114 and processed either directly or after phase separation for immunoprecipitation with an anti-E2 antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The 65-kD precursor protein was found exclusively in the aqueous phase, contaminating proteins as well as the E2 membrane protein were recovered in the detergent phase (Fig. 4). We conclude, that the 65-kD precursor behaves as a soluble protein in this analysis.

Effect of Chloroquine Treatment on the Processing and Secretion of gp 80 in MDCK Cells

Since treatment of cells with chloroquine has been shown to interfere with the proteolytic maturation of various glycoproteins and their intracellular transport (7, 26, 42), we wished to study its effect on gp 80 secretion. Specifically we wanted to determine whether the proteolytic processing of gp 80 is inhibited in the presence of chloroquine and whether gp 80 would be diverted from the apical into a basolateral or nonpolarized exocytotic pathway. Cells of plastic- and filter-grown monolayers were preincubated, pulse labeled, and chased in medium containing 200 μM chloroquine. Proteins in the cell lysates and media were immunoprecipitated and analyzed by SDS-PAGE and fluorography (Fig. 5).

In plastic-grown cells proteolytic processing of the 80-kD precursor protein is almost completely inhibited in the presence of chloroquine (Fig. 5, lanes *2–13*). gp 80 accumulates in its terminally glycosylated uncleaved precursor form in the cells. It can, however, be secreted at a low rate, as demonstrated by the protein bands in the media samples at 60 and 120 min of chase, which comigrate with the intracellular 80-kD protein. At 2 h of chase, 70–80% of the pulse-labeled, immunoprecipitable protein is present inside the cells, 20–30% is found in the medium. Significant intracellular degradation of the protein does not take place.

The analysis of immunoprecipitated proteins in the cell lysates and in the apical and basolateral media of filter-grown monolayers at 90 min of chase shows that the proteolytic processing of the 80-kD precursor is also inhibited in filter-grown cells, although to a lesser extent than in plastic-grown cells (Fig. 5, lanes *15–18*). Secretion of gp 80 from filter-grown cultures is delayed, not as dramatically, however, as in cultures grown on plastic support. At 90 min of chase, 15–20% of the pulse-labeled gp 80 present in the 5-min cell sample is recovered from the cell lysates, 80–85% from the apical medium, and 15% from the basolateral medium, a slightly more polar distribution than observed in untreated cells (Fig. 5, lanes *15–18*; see Fig. 7). These results demonstrate that chloroquine, although affecting processing and the kinetics of secretion, does not interfere with the polarity of gp 80 secretion. Proteolytic cleavage of the 80-kD precursor molecule is therefore not necessary for the apical secretion of the gp 80 complex in MDCK cells.

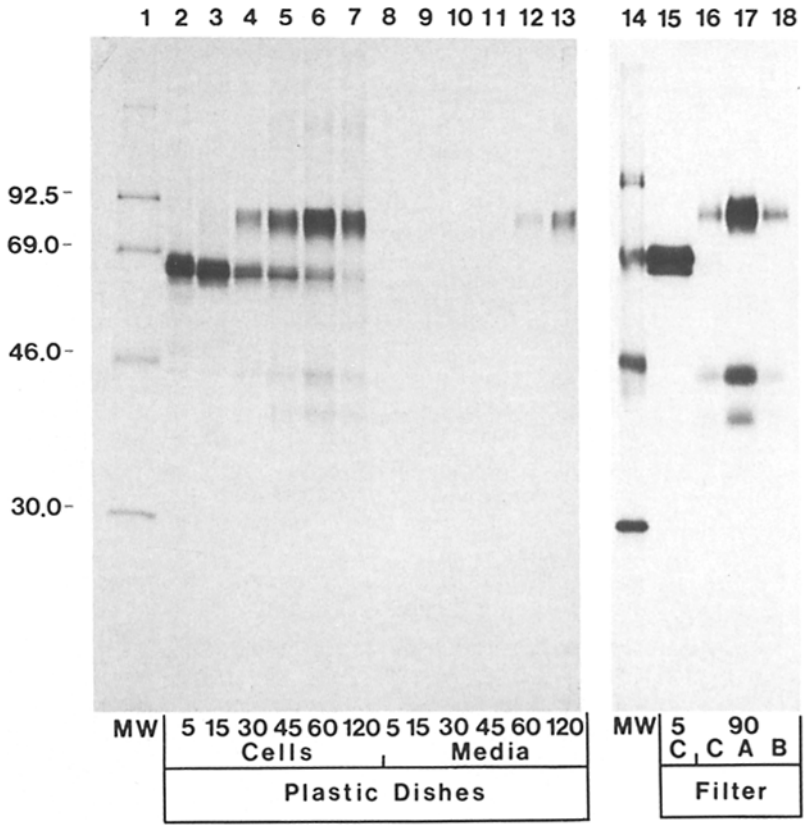

Figure 5. Pulse–chase analysis of the processing and secretion of gp 80 in the presence of chloroquine. Subconfluent plastic-grown monolayers (lanes *2–13*) and filter-grown monolayers (lanes *15–18*) of MDCK cells were pulse-labeled with [^{35}S]methionine and chased for the indicated periods in the presence of 200 μM chloroquine as described in Materials and Methods. Lanes *2–7*, *15*, and *16* show the immunoprecipitated, reduced, and alkylated proteins in the cell lysates; lanes *8–13* in total; lane *17* in the apical medium; and lane *18* in the basolateral medium. Molecular mass standards are run in lanes *1* and *14*.

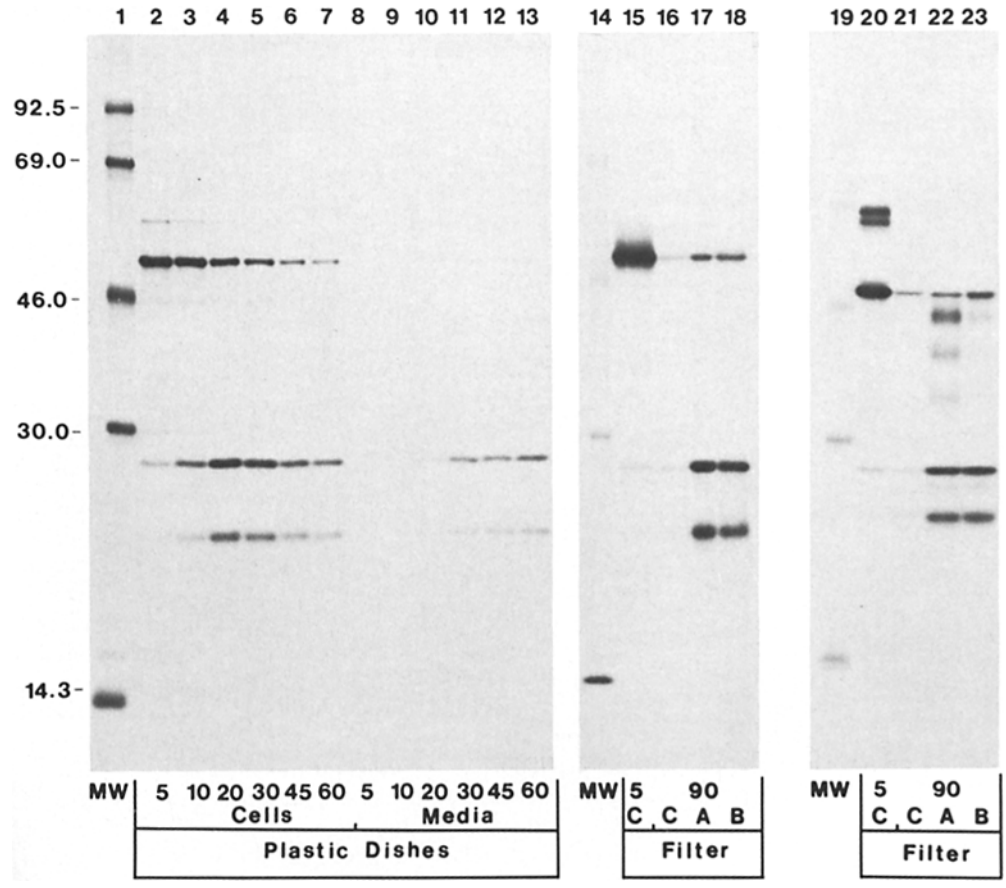

Figure 6. Pulse–chase analysis of the processing and secretion gp 80 in the presence of tunicamycin. Subconfluent plastic-grown monolayers (lanes *2–13*) and filter-grown monolayers (lanes *15–18* and *20–23*) of MDCK cells were pulse labeled with [^{35}S]methionine for 15 min and chased for the indicated periods in the presence of tunicamycin as described in Materials and Methods. The analysis of plastic-grown cells was done under conditions where N-glycosylation was completely inhibited; filter-grown cells were either used under conditions of complete (lanes *15–18*) or partial inhibition (lanes *20–23*). Lanes *2–7*, *15*, *16*, *20*, and *21* show the immunoprecipitated, reduced, and alkylated proteins in the cell lysates; lanes *8–13* in total; lanes *17* and *22* in the apical medium; and lanes *18* and *23* in the basolateral medium. Molecular mass standards are run in lanes *1*, *14*, and *19*.

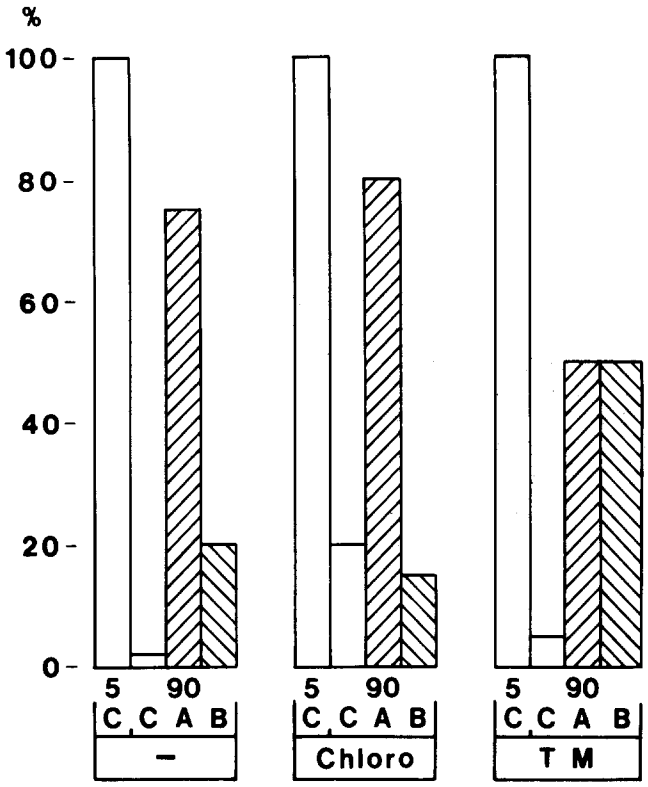

Figure 7. Quantitation of gp 80 present in the cell lysates, in apical medium, and in basolateral medium of untreated cells and cells treated with either chloroquine or tunicamycin. The radioactivity recovered in the gp 80 precursor present in the 5-min cell lysates was taken as 100%. The amount of radioactivity recovered in the individual samples at 90 min of chase was calculated as percentage of the amount present in the 5-min cell sample. Open bars (*C*) show the radioactivity recovered from the cell lysates; striped bars show the radioactivity recovered from the apical media (*A*) or basolateral media (*B*).

Effect of Tunicamycin Treatment on the Processing and Secretion of gp 80 in MDCK Cells

To examine the effect of N-linked carbohydrate units on the intracellular transport of gp 80 to the apical cell surface, MDCK cells were treated with tunicamycin. This drug inhibits the synthesis of the dolichol-linked oligosaccharide precursors and therefore prevents the transfer of oligosaccharide units to asparagine residues in the initial step of protein glycosylation (14). Incubation of MDCK cells grown in culture dishes in medium supplemented with tunicamycin (4 μg/ml) 1 h before and during the pulse and chase periods effectively blocks N-linked glycosylation as demonstrated by the increased mobilities of the precursor protein and the mature products in SDS-PAGE (Fig. 6). In the presence of tunicamycin a precursor protein with an apparent M_r of 50,000 is found at early times of chase. The concentration of the 50-kD protein in the cell lysates decreases as it is cleaved into peptides with apparent M_r of 27,000 and 23,000. The proteolytic processing of the precursor molecules takes place quickly after synthesis. We can detect the cleavage products already in the 5-min cell sample, i.e., 20 min after the onset of the labeling period. The unglycosylated peptides are rapidly secreted. They can be detected in the medium at 20 min of chase. At 60 min of chase, 30% of the pulse-labeled material is found in the cell lysates, and 25% is found in the medium. This does not change significantly with longer chases.

Nearly 50% of the gp 80 present in the 5-min cell sample can not be recovered at all after 60 min of chase. The low recovery of pulse-labeled gp 80 at late times of chase from plastic-grown MDCK cells could be due to a difference in the affinity of the antibody to the unglycosylated precursor and cleaved peptides, to an increased degradation of the unglycosylated cleaved peptides during sample preparation, or to an increase in the intracellular degradation of the unglycosylated protein. We can rule out the first two possibilities, since we do not observe this phenomenon in the analysis of unglycosylated proteins of filter-grown MDCK cells (Fig. 6, lanes *15–18*, and Fig. 7). We therefore think that increased intracellular degradation of the unglycosylated protein accounts for the low recovery of pulse-labeled material at late periods of chase and that this process is significant in plastic-grown but not in filter-grown cells.

If cell lysate, apical, and basolateral media of filter-grown MDCK cells preincubated, pulse labeled, and chased for 90 min in the presence of 12 μg/ml tunicamycin are analyzed, the unglycosylated protein was found in equal amounts in both media (Fig. 6, lanes *15–18*, and Fig. 7). When the same analysis was performed in the presence of 4 μg tunicamycin/ml only an incomplete block of N-glycosylation was achieved. Using this condition the glycosylated protein was recovered in a 75:20 ratio as in untreated cells, whereas the unglycosylated protein was recovered in a 50:50 ratio as observed during complete inhibition from the apical and basolateral medium of the same filter-grown cell monolayer (Fig. 6, lanes *20–23*). These results suggest that the N-linked carbohydrate groups play a role in the polar segregation of this secretory protein.

Discussion

The data presented in this paper demonstrate that soluble proteins can be sorted into a constitutive apical pathway of exocytosis in MDCK cells.

The protein studied is an 80-kD sulfated glycoprotein complex (gp 80) consisting of two polypeptide chains linked together by disulfide bridges. This protein may be identical to the one described by Gottlieb et al. (11). Their observation of a single 81-kD species in the cells and medium may result from the use of unreduced samples for SDS-PAGE or differences in processing between strains of MDCK cells.

Pulse–chase analysis shows that the kinetics of gp 80 secretion is typical for proteins released through a constitutive exocytotic pathway. Of the pulse-labeled protein, 50% is secreted within 30 min in MDCK cells. This lies within the range of half-times for secretion reported for albumin (23 min) in rat hepatoma cells (reference 41), and for albumin and antitrypsin (45 min), C3 complement (90 min), and antichymotrypsin (90 min) in a human hepatoma cell line (18). There is no evidence for intracellular storage of the 80-kD precursor protein or the mature protein complex.

Constitutive and polar exocytosis has been well characterized for plasma membrane proteins in MDCK cells. It has been shown that apical and basolateral membrane proteins are sorted inside the cell and not at the plasma membrane (5, 21, 24, 31) and that sorting occurs as a late step in the bio-

genesis of these proteins since they share the same pathway at least as far as the *trans*-Golgi compartment (8). The only example of constitutive and polar exocytosis of soluble proteins had been the secretion of proteins at the sinusoidal surface of the hepatocyte (15). It was therefore important to analyze whether gp 80 is segregated as a soluble or as a membrane-bound protein into the apical exocytotic pathway in MDCK cells. If segregation occurs after terminal glycosylation it could affect either the 80-kD precursor or the cleaved peptides. In the latter case, soluble proteins would be sorted, since the intracellular cleaved peptides are electrophoretically indistinguishable from the secreted forms. The same argument also applies to the 80-kD precursor. An 80-kD protein, which comigrates with the intracellular terminally glycosylated precursor protein can be detected in the medium of plastic-grown MDCK cell monolayers.

Direct evidence that gp 80 is segregated as a soluble protein was obtained by the analysis of the partitioning of the 65-kD immediate posttranslational precursor in a phase-separated Triton X-114 solution. Our data show that the 65-kD precursor protein is hydrophilic in nature since it does not partition into the detergent phase but is recovered exclusively from the aqueous phase. Hence gp 80 is segregated as a soluble protein into the apical pathway. This characterization shows that gp 80 belongs to the large group of secretory proteins, which are synthesized as soluble proforms and are proteolytically processed before release from the cells. However, in most cells vectorial release of these proteins is achieved via a regulated pathway. In this respect, gp 80 secretion in MDCK cells resembles that of albumin and C3 complement secretion in the hepatocyte (18).

When gp 80 secretion from filter-grown MDCK cells was analyzed after 90 min of chase, 75% of the newly synthesized protein was found in the apical medium, 20% was found in the basolateral medium, and 3% was recovered in the cell lysate. This distribution is similar to that observed for an apical membrane protein, the hemagglutinin of influenza virus (39) showing that sorting of this secretory protein occurs with an efficiency similar to that of an apical membrane protein.

It has been shown that intracellular transport processes are impaired by incubation of the cells with exogenous amines. These drugs inhibit endocytosis and receptor recycling and divert proteins from the regulated exocytotic and the lysosomal pathway into the constitutive exocytotic pathway (7, 26, 42). The mechanism by which they exert their effects is not clear. Based on the observation that vesicles of both the endocytotic and exocytotic pathways display an acidic pH (2, 6) and that exogenous amines raise the pH of these vesicles (22), the disturbance of a pathway by amines is taken as evidence that a low pH compartment is involved in that particular transport route. The increase in pH of acidic vesicles may also account for the inhibition of proteolytic cleavage in the maturation of various peptide hormones and neuropeptides by these drugs (6, 29, 36, 40). If proteolytic maturation of a protein were a prerequisite for the entry of the protein into a particular pathway the consequence of drug interference with the action of the processing protease would be a block in transport of the protein to its usual destination.

We tested this for the 80-kD protein in MDCK cells. Incubation of MDCK cells with 200 μM chloroquine inhibits the proteolytic processing of the 80-kD precursor molecule and decreases its rate of cell surface transport. The release of the gp 80 precursor is slow, but occurs at the apical cell surface. This result demonstrates that proteolytic cleavage of gp 80 is not necessary for the apical secretion of this protein and suggests the involvement of a low pH compartment in apical transport.

The role of carbohydrate moieties in the intracellular transport of plasma membrane and secretory proteins has been studied extensively. It has been demonstrated that the unglycosylated proteins show an increased intracellular degradation and a decreased efficiency of transport to the cell surface (13, 28, 38). Studies on the targeting of viral membrane proteins in either tunicamycin-treated or lectin-resistant MDCK cells demonstrated that N-glycosylation is not a prerequisite for the polar segregation of these proteins (13, 35). When analyzing gp 80 biosynthesis and secretion in the presence of tunicamycin we observed an increased intracellular degradation in plastic-grown cells but not in filter-grown cells. The efficiency of transport seems to be unaffected at least in filter-grown cells. We cannot determine the efficiency of transport in plastic-grown cells, since we cannot, in that analysis, distinguish whether the protein recovered at late times of chase in the cell lysates was located intracellularly or had been secreted basolaterally and was deposited between the cells and the plastic support.

Interestingly, the polarity of gp 80 transport is lost in the absence of N-glycosylation. gp 80 is released in equal amounts at both cell surfaces in tunicamycin-treated MDCK cells. This result demonstrates an important role for the N-linked carbohydrate chains and/or associated modifications in the apical secretion of this protein. This role may be either direct (as a sorting signal) or indirect (as a factor stabilizing a sorting competent conformation of the protein).

Together with our previous results (16) on the secretion of chicken lysozyme expressed in MDCK cells using an SV-40 vector, we have demonstrated the existence of two pathways of secretion in one cell type. Lysozyme is secreted at both cell surfaces with the same kinetics in equal amounts; the gp 80 complex is secreted at the apical cell surface. We do not know whether these proteins use the same vesicle populations. One possibility is that the apically directed lysozyme is transported in the same vesicles as the gp 80 complex and that the basolaterally directed lysozyme is routed in a different vesicle population. Another possibility is that apically and basolaterally directed lysozyme molecules migrate together in a vesicle population distinct from that transporting the gp 80 complex to the apical cell surface. Experiments involving comparative pulse–chase analysis of gp 80 and lysozyme secretion of filter-grown MDCK cell monolayers under the influence of drugs and low temperature (20) will help to elucidate this.

The presence of two secretory pathways in one cell requires specific sorting signals in the proteins of at least one pathway (15). Our results on gp 80 secretion in tunicamycin-treated cells argue that carbohydrates are involved in the signals needed for the apical secretion of this protein. We plan to pursue the identification of the pathway(s) requiring specific sorting signals and the characterization of the structures recognized by in vitro mutagenesis and expression of cDNA molecules coding for proteins secreted apically and/or basolaterally in MDCK cells.

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