The MAL proteolipid is an integral membrane protein identified as a component of the raft machinery for apical sorting of membrane proteins in Madin-Darby canine kidney (MDCK) cells. Previous studies have implicated lipid rafts in the transport of exogenous thyroglobulin (Tg), the predominant secretory protein of thyroid epithelial cells, to the apical surface in MDCK cells. We have examined the secretion of recombinant Tg and gp80/clusterin, a major endogenous secretory protein not detected in Triton X-100 insoluble rafts, for the investigation of the involvement of MAL in the constitutive apical secretory pathway of MDCK cells. We show that MAL depletion impairs apical secretion of Tg and causes its accumulation in the Golgi. Cholesterol sequestration, which blocks apical secretion of Tg, did not alter the levels of MAL in rafts but created a block proximal to Tg entrance into rafts. Apical secretion of gp80/clusterin was also inhibited by elimination of endogenous MAL. Our results suggest a role for MAL in the transport of both endogenously and exogenously expressed apical secretory proteins in MDCK cells.

Segregation of newly synthesized proteins into distinct vesicular carriers destined for the apical or basolateral subdomains is fundamental for the establishment and maintenance of polarity in epithelial cells. Basolateral targeting of transmembrane proteins is mediated by sorting signals in their cytoplasmic tail that might or might not be related to dileucine- or tyrosine-based sorting signals (1, 2). In contrast, apical sorting of the influenza virus hemagglutinin (HA)â and proteins attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor appears to be mediated in polarized thyroid cells (9, 10). However, glycation does not constitute a universal apical sorting signal, as unglycosylated transmembrane proteins are also delivered to the apical surface (11), and even normally glycosylated membrane proteins are able to reach the apical surface in the absence of glycosylation (12–14).

MAL is a nonglycosylated integral membrane protein of 17 kDa containing multiple hydrophobic segments (15). Endogenous MAL is localized at steady state, predominantly in the apical zone of polarized epithelial cells (16), and resides in lipid rafts in epithelial MDCK and Fischer rat thyroid (FRT) cells (16, 17). In MDCK cells in which endogenous MAL was depleted, a role has been demonstrated for MAL as an element of the apical sorting apparatus necessary for transport of apical membrane proteins (18–20). This emphasizes that MAL is the first integral membrane component of the machinery for apical transport of membrane proteins to be identified. Specialized secretory proteins are cotranslationally delivered to the luminal throughout the entire exocytic pathway. The lack of domains that could provide targeting signals directly via incorporation into rafts or recognition by cytosolic machinery in secretory proteins indicates that luminal sorting features are responsible for their polarized transport. N-glycans were shown to act as an apical sorting signal for a number of secretory proteins (21), leading to the suggestion that specialized lectins were involved as sorting machinery (22). However, as there are examples of apical sorting of secretory proteins in the absence of glycation (23, 24), it is clear that this mechanism cannot be applied to all secretory proteins. It is more likely that specialized machinery recognizes conformational features in the secretory proteins that control segregation into distinct vesicular carriers destined for the apical or basolateral surface (25).

Specialized epithelial cells may export newly synthesized secretory proteins either using a constitutive pathway similar to that present in other epithelia or through a regulated pathway involving storage in secretory granules (26). Thyroglobulin (Tg), the thyroid hormone precursor product, is apically secreted by thyroid epithelial cells. Although Tg appears to use the regulated pathway in these cells, Tg is also predominantly released from the apical surface when expressed in polarized MDCK cells, which lack such a pathway (27). This indicates that luminal sorting features present in Tg can be decoded also in MDCK cells. Although Tg lacks a direct means of membrane...
attachment, a small percentage of intracellular Tg molecules was found to be associated with the Triton X-100-insoluble raft fraction in MDCK cells, suggesting that one or more membrane components bind Tg to the rafts during biosynthetic transport (27). Delivery of Tg to the apical surface was dependent on normal cholesterol levels supporting a role for the rafts in Tg transport (27). In contrast to Tg, clusterin/gp80, the major apically secreted endogenous protein of MDCK cells, is exclusively detected in the Triton X-100-soluble fraction during biosynthetic transport in MDCK cells (28). Despite that fact, normal apical sorting of gp80 requires intact cholesterol levels (6). In this study we show that MAL depletion impaired apical secretion of Tg and caused its retention in the Golgi. Apical secretion of gp80 was also impaired, indicating a role for MAL in apical transport of both endogenously and exogenously expressed apical secretory proteins in MDCK cells.

EXPERIMENTAL PROCEDURES

Materials—The DNA construct expressing rat Tg has been described previously (29). Rabbit polyclonal antibodies to Tg were obtained from Dr. P. R. Larsen (Brigham and Women’s Hospital, Boston, MA). The mouse monoclonal antibody (mAb) 9E10 against the c-Myc epitope EQKLVISEED was obtained from the American Type Culture Collection. The rat mAb 2E5 specific to dog MAL has been described previously (18). The anti-calanxin mAb was purchased from Transduction Laboratories (Lexington, KY). Peroxidase-conjugated secondary anti-IgG antibodies were supplied by Pierce. Fluorescein- and Texas Red-conjugated antibodies were from Southern Biotech (Birmingham, AL). Antibodies to the 58-K Golgi marker, Triton X-100, methyl-β-cyclodextrin (CD), and cycloheximide were purchased from Sigma.

Cell Culture Conditions, Cell Treatments, and Transfections—The MDCK II cells stably expressing recombinant Tg have been described previously (27). Cells were grown on Petri dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37 °C in an atmosphere of 5% CO2. Depletion of cellular cholesterol was carried out by treating the cells with 10 mM CD for 30 min 4 h before analysis (30). The construct expressing a human (h) MAL protein lacking the four amino acids contiguous with the initial methionine residue and bearing the 9E10 c-Myc epitope at the NH2 terminus (hMAL-9E10); the control (AM) and antisense (AS) phosphorothioate oligonucleotides were supplied by Pierce. Fluorescein- and Texas Red-conjugated antibodies were from Southern Biotech (Birmingham, AL). Antibodies to the 58-K Golgi marker, Triton X-100, methyl-β-cyclodextrin (CD), and cycloheximide were purchased from Sigma.

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Detergent Extraction Procedures—Lipid rafts were isolated by standard procedures (5). Cells grown to confluency in 100-mm dishes were rinsed with phosphate-buffered saline and lysed for 20 min in 1 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 at 4 °C. The lystate was scraped from the dishes with a cell lifter, the dishes were rinsed with 1 ml of the same buffer at 4 °C, and the lystate was homogenized by passing the sample through a 22-gauge needle. The extract was finally brought to 40% sucrose in a final volume of 4 ml and sequentially overlaid with 6 ml of 30% sucrose and 2 ml of 5% sucrose. Gradients were centrifuged for 18 h at 39,000 rpm at 4 °C in a Beckman SW41 rotor. The opalescent band at the 5–30% sucrose interface, containing rafts, was collected as the Triton X-100 insoluble fraction, whereas the 40% sucrose layer containing the load was harvested as the Triton X-100 soluble fraction.

Analysis of Polarized Secretion—MDCK cells were seeded at confluency in 100-mm dishes and incubated with 10 mM glycine for 5 min to quench the aldehyde groups. The cells were then permeabilized or not with 0.2% Triton X-100, rinsed, and incubated for 1 h with goat anti-mouse or anti-rat IgG antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (ECL, Amersham Biosciences, Inc.).

For immunoprecipitation of MAL, antibodies were pre-bound overnight at 4 °C to protein G-Sepharose in 10 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, 1% Triton X-100. Cell extracts were incubated for 4 h at 4 °C with a control antibody bound to protein G-Sepharose, and the supernatant was immunoprecipitated by incubation for 4 h at 4 °C with the appropriate antibodies bound to protein G-Sepharose. After collection, the immunoprecipitates were washed six times with 1 ml of 10 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, and 1% Triton X-100 and analyzed by SDS-PAGE under reducing conditions. To detect 35S labeling, dried gels were finally exposed to Fuji film imaging plates (Fuji Photo Film Co., Tokyo, Japan). For analysis of endoglycosidase H resistance, immunoprecipitates were incubated overnight at 37 °C in the presence or absence of 0.05 units/ml endoglycosidase H (Roche Molecular Biochemicals) before analysis by SDS-PAGE. Quantitative analyses were done using the Image program (Research Services Branch, National Institutes of Health).

Immunofluorescence Analysis—MDCK cells grown on coverslips were fixed in 4% paraformaldehyde for 15 min, rinsed, and treated with 10 mM glycine for 5 min to quench the aldehyde groups. The cells were then permeabilized or not with 0.2% Triton X-100, rinsed, and incubated with 3% bovine serum albumin in phosphate-buffered saline for 15 min. Cells were then incubated for 1 h with the indicated primary antibody. After several washings, blots were incubated for 1 h with goat anti-mouse or anti-rat IgG antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (ECL, Amersham Biosciences, Inc.).

RESULTS

We described previously that apical secretion of Tg requires intact lipid rafts (27). As MAL has been demonstrated to be a component of the machinery for the raft-dependent apical transport of membrane proteins in MDCK cells (18–20), we thought that MAL might also mediate the apical transport of secretory proteins. To address this point, we first compared the intracellular distribution of Tg and MAL in MDCK cells (Fig. 1). Although much of the intracellular Tg distributed in the endoplasmic reticulum as revealed by colocalization with cal-
A

**Fig. 2.** MAL depletion in MDCK cells reduces apical secretion of recombinant Tg. A, normal MDCK cells or MDCK/hMAL-ΔN cells expressing recombinant Tg were transfected with control (AM) or antisense (AS) oligonucleotide, plated onto 24-mm-diameter tissue culture inserts, and incubated at 37 °C for 48 h. Cells were labeled metabolically with a mixture of [35S]methionine/cysteine for 30 min and incubated in the absence of the radioactive precursors. After 8 h, the apical (a) and basolateral (b) culture media were collected, and the cells were lysed (Lys). The incorporation of [35S] label into protein in the cell lysate was determined in each case. The values obtained were used for correcting the volumes of the culture media and the cell lysate. The endogenous (H9004) or the exogenous (hMAL-ΔN) MAL levels in these cells were examined by immunoblot analysis with mAb 2E5 or 9E10, respectively. The results of one representative experiment of five performed are shown. B, quantitative analysis of the effect of MAL depletion on the polarized release of Tg. The intensity of the Tg signal in the immunoprecipitates from the apical (black bars) and basolateral (gray bars) culture media and the cell lysate (white bars) from experiments in which MAL was depleted to different extents were quantified; the values obtained were expressed in arbitrary units. C, the percentage of Tg secreted on the apical side in cells transfected with the antisense oligonucleotide relative to that in cells transfected with the control oligonucleotide is represented. The results of some representative experiments are shown. The effects observed in other experiments in which MAL was depleted to different extents (not included in the figure) were consistent with those shown.

nexin, an endogenous membrane protein of the endoplasmic reticulum, a small fraction of Tg colocalized with MAL in the Golgi region. This distribution of Tg is consistent with our previous biochemical analysis showing that there are two pools of intracellular Tg in MDCK cells, one major pool that is sensitive to endoglycosidase H digestion and is excluded from lipid rafts and a minor pool that is both endoglycosidase H-resistant and associated with lipid rafts (27).

To approach directly the possible role of MAL in Tg transport, we used specific antisense oligonucleotides to deplete the levels of endogenous MAL as described previously (18, 20). MDCK cells stably expressing Tg were transfected with either control AM or antisense oligonucleotides. 48 h later, cells were metabolically labeled with [35S]methionine/cysteine and incubated at 37 °C in the absence of radioactive precursors. After 8 h, medium was recovered from both the apical and basolateral compartments, and the cell monolayer was lysed. After immunoprecipitation with anti-Tg antibodies, Tg was visualized by autoradiography of the immunoprecipitates. The extent of MAL depletion obtained was quantified by densitometric scanning of immunoblots of the cell lysates probed with anti-MAL mAb 2E5. A representative experiment in which MAL levels dropped to ∼6% of those in control cells is shown in Fig. 2A. Whereas, under the conditions of chase used, ∼95% of the labeled Tg was recovered from the apical compartment in cell transfected with the control oligonucleotide, depletion of MAL reduced the levels of apical secretion of Tg to one-third and caused a concomitant increase in cell-associated Tg. To confirm that the observed effects were due to MAL depletion, we used MDCK/hMAL-ΔN cells, which express a modified MAL protein in which levels are insensitive to treatment with antisense oligonucleotides (20). Fig. 2A shows that the exogenous expression of hMAL-ΔN allowed normal apical secretion of Tg and prevented its accumulation within the cell, despite the drop in the endogenous MAL content. A quantitative analysis of the amount of Tg in the apical and the basolateral compartments and that present in the cell lysate is shown in Fig. 2B. It is of particular note that in MAL-depleted cells the total Tg recovered was less than that in control cells, probably because of progressive degradation of the retained protein. The compilation of the different experiments presented in Fig. 2C shows a clear correlation between MAL depletion and progressive reduction of Tg secretion into the apical medium.

To gain further insight into the effect of MAL depletion on Tg transport, we compared the intracellular distribution of Tg in cells with normal and depleted levels of MAL (Fig. 3). As newly synthesized Tg requires ∼8 h to be fully secreted (27), intracellular Tg can be detected at steady state by immunofluorescence analysis in control MDCK cells. To trace the Tg that accumulates in the cells specifically because of MAL depletion and to distinguish it from newly synthesized Tg, we treated the cells with cycloheximide for 8 h to inhibit protein synthesis and to allow the pre-existing Tg to be secreted. Fig. 3 shows that this treatment indeed allowed the secretion of most of the pre-existing Tg, as revealed by the reduced Tg staining found in the control cell cultures treated with cycloheximide (Fig. 3,
on coverslips, and incubated at 37 °C.

Top panels show the effects of one representative experiment of three performed are shown. Bar, 5 μm.

**Fig. 3.** Tg is retained intracellularly in MDCK cells with reduced levels of MAL. MDCK cells expressing exogenous Tg were transfected with control (AM) or antisense (AS) oligonucleotide and were incubated at 37 °C for 48 h. Cells were then left untreated or were treated with 100 ng/ml cycloheximide (CHX) for 8 h and subjected to double label immunofluorescence analysis with anti-Tg and anti-58K antibodies. The results of one representative experiment of three performed are shown.

**Fig. 4.** Biochemical analysis of intracellular Tg in MDCK cells with reduced levels of MAL. MDCK cells expressing exogenous Tg were transfected with control (AM) or antisense (AS) oligonucleotide and were incubated at 37 °C for 48 h. Cells were labeled metabolically for 30 min and incubated in the absence of the radioactive precursors. After 8 h, the culture supernatant (Sup) containing the radiolabeled secreted Tg was harvested, and the cells were lysed (Lys). After immunoprecipitation with anti-Tg antibodies, the immunoprecipitates from the cell lysate were resuspended in denaturing buffer, divided and placed in two different tubes, and incubated in the presence (+) or absence (−) of endoglycosidase H (endo H). The asterisk indicates the position of a specific protein band on the blotting that could be detected in the immunoprecipitates. Note that whereas secreted Tg was endoglycosidase H-resistant (R) regardless of the levels of MAL, intracellular Tg was completely sensitive (S) in cells treated with the control oligonucleotide (AM) or partially resistant to endoglycosidase H digestion in cells with the levels of MAL depleted by treatment with the antisense oligonucleotide (AS).

Middle panels compared with the cells incubated in the absence of the protein synthesis inhibitor (top panels). Consistent with the biochemical results presented in Fig. 2, depletion of MAL with antisense (AS) oligonucleotides produced a detectable intracellular accumulation of Tg (Fig. 3, bottom panels). It is of particular note that the retained Tg colocalized significantly with the 58-K Golgi marker in MAL-depleted cells, suggesting that elimination of MAL produces partial accumulation of Tg in the Golgi. To confirm this observation by biochemical means, we compared the resistance to endoglycosidase H digestion of the secreted and the intracellular Tg from cells with normal or depleted levels of MAL (Fig. 4). Secreted Tg was endoglycosidase H-resistant, corresponding to the mature protein, regardless of the levels of MAL. However, whereas intracellular Tg was nearly 100% endoglycosidase H-sensitive in control cells, in cells with depleted levels of MAL ~50% of the protein acquired endoglycosidase H resistance, consistent with the observed partial accumulation of the protein in the Golgi (Fig. 3).

We had previously described that cholesterol sequestration with CD inhibits secretion of Tg in MDCK cells (27). To establish a connection between the effect of cholesterol sequestration and that of MAL depletion, we analyzed the effect of CD on the presence of Tg and MAL in lipid rafts. Fig. 5 shows that the reduction of apical secretion of Tg caused by treatment with CD was not due to reduced partitioning of MAL into lipid rafts but...
rather to a diminished incorporation of newly synthesized Tg in those specialized microdomains. This might be interpreted as meaning that CD treatment blocks access of newly synthesized Tg to the rafts containing MAL.

To analyze the effect of MAL depletion on the secretion of gp80, the major endogenous secretory protein of MDCK cells, cells were labeled metabolically with [35S]methionine/cysteine and incubated at 37 °C in the absence of radioactive precursors. After 8 h, medium was recovered from both the apical and basolateral compartments, equivalent aliquots from the apical and basolateral samples were fractionated by SDS-PAGE, and gp80 was visualized by autoradiography. A representative experiment in which MAL levels dropped to ~7% of those in control cells is shown in Fig. 6A. Under the conditions used, most of the secreted gp80 was recovered from the apical compartment in cells transfected with the control oligonucleotide, consistent with the reported apical targeting of gp80 in normal MDCK cells. Consistent with the pattern of Tg secretion presented in Fig. 2, apical secretion of gp80 was greatly reduced in MAL-depleted cells. The exogenous expression of hMALΔN allowed normal apical secretion of gp80 despite the drop in the endogenous MAL content. A quantitative analysis of the amount of gp80 secreted into the apical and basolateral compartments is shown in Fig. 6B.

**DISCUSSION**

Segregation of secretory proteins into distinct containers destined for either the apical or basolateral surface should be mediated through recognition of sorting determinants by membrane receptors specific for either the apical or basolateral pathways in the lumen of the exocytic compartments. The receptor-secretory protein complex is assumed to gain access to the appropriate vesicular carrier using the same type of sorting signal as that employed by membrane cargo. Secretory granules of pancreatic acinar cells contain rafts, and apical secretion of zymogens by the regulated secretory pathway requires normal levels of cholesterol (31). Carboxypeptidase E is a raft-associated protein that has been proposed to function as a sorting receptor for some prohormones in the regulated secretory pathway (32). Definitive identification of secretory protein receptors in the constitutive secretory pathway remains elusive, although overexpression experiments of two different apical secretory proteins indicate that apical sorting of this type of protein is a saturable and signal-mediated process probably involving competition for sorting receptors (33). Our previous study of MDCK and FRT cells indicates that the secretory Tg protein becomes integrated into lipid rafts during biosynthetic transport as would be expected if its putative receptor uses the same raft-mediated mechanism of apical transport as the cargo HA molecule (27). The MAL protein is the first integral membrane member to be identified of the sorting machinery necessary for apical transport of HA in MDCK cells (18–20). In this study, we have used Tg as a model of raft-associated secretory protein to analyze the role of MAL in the transport of this type of protein in MDCK cells, which lack the regulated secretory pathway. We have observed that apical secretion of Tg is impaired in MDCK cells with reduced levels of MAL. At least a fraction of newly synthesized Tg accumulates in the Golgi under those conditions, as revealed by immunofluorescence analysis and acquisition of resistance to endoglycosidase H digestion, suggesting that egress of Tg from the Golgi is affected by MAL depletion. The observed accumulation of Tg in the Golgi is consistent with the proposed role of MAL in formation of the transport vesicles destined for the apical surface (34, 35).

Cholesterol sequestration with CD inhibits secretion of Tg in MDCK cells, implicating lipid rafts in Tg transport (27). The same treatment did not affect the residence of MAL in rafts, probably because of its strong association with those specialized membranes as revealed by its resistance to solubilization even at a high concentration of detergent (36). In contrast, incorporation of newly synthesized Tg into rafts was greatly diminished by CD treatment, suggesting a weak association of the protein with the rafts. This, in turn, suggests diminished recruitment of protein or lipid components that serve as Tg receptors (37).

FRT cells were thought to be defective in raft-mediated apical transport as, unlike the case in MDCK cells, the majority of GPI-anchored proteins do not become integrated into rafts during transport and are targeted to the basolateral membrane (38, 39). The fact that gp80 is apically secreted in FRT cells and the observation that gp80 is not detected in the raft fraction during biosynthetic transport to the apical surface in MDCK cells led to the suggestion that apical sorting of gp80 takes place by a raft-independent transport pathway (28). Recent evidence indicates that FRT cells assemble functional rafts for apical transport of HA and certain GPI-anchored proteins (20, 40). Moreover, similar to HA targeting, cholesterol depletion inhibits apical secretion of gp80 in MDCK cells (6). Thus, although gp80 is not detected in the Triton X-100-insoluble raft fraction (28), this protein appears to require intact rafts for transport. We have found that, in addition to raft-associated Tg, efficient delivery of gp80 to the apical medium is also dependent on MAL levels consistent with the reported intracellular retention of gp80 in MDCK cells in which MAL levels were reduced (19).

The putative sorting receptors for Tg and gp80 appear to behave as membrane components associated with and not associated with Triton X-100 insoluble lipid rafts, respectively. It has been reported previously that apical transport of membrane proteins associated (HA and GPI-anchored proteins) or not associated (gp114, p75 neurotrophin receptor, and dipeptidylpeptidase IV) with lipid rafts, as well as the bulk of membrane proteins, are all sensitive to reduction of MAL levels (18–20). The fact that MAL depletion affects apical transport of
raft- and non-raft-associated proteins might be explained by two alternative models (Fig. 7). The first model (Fig. 7A) proposes that only one pathway exists for most apical proteins with raft- and non-raft-associated proteins together in the same vesicle and directly involving MAL. Our current model postulates that the sorting sequences present in the carboxyl terminus of MAL might direct the formation of the transport vesicles by polymerization of vesicular coats (35). This action could produce a transient and weak stabilization in rafts of proteins such as gp80 that are not normally found in the raft fractions at steady state. This transient stabilization is not detectable by the standard insolubility assay using extraction with Triton X-100 (28), although it might be detected using the zwitterionic detergent CHAPS (6). The second model (Fig. 7B) proposes the existence of two different apical pathways, one with raft- and non-raft-associated proteins and directly involving MAL and a second type of vesicle with proteins excluded from these rafts but either directly or indirectly requiring a functional MAL-mediated pathway. Whichever model is correct, the evidence in the present study shows that, regardless of their solubility in Triton X-100, MAL is required in MDCK cells for apical transport and directly involving MAL is required in MDCK cells for apical transport of endogenous and exogenous secretory proteins.

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