Published Online: 7 October, 2002 | Supp Info: http://doi.org/10.1083/jcb.200206033
Downloaded from jcb.rupress.org on September 22, 2019

JCB Report

MAL2, a novel raft protein of the MAL family, is an essential component of the machinery for transcytosis in hepatoma HepG2 cells

María C. de Marco,[1] Fernando Martín-Belmonte,[1] Leonor Kremer,[2] Juan P. Albar,[2] Isabel Correas,[1] Jean P. Vaerman,[3] Mónica Marazuela,[4] Jennifer A. Byrne,[5] and Miguel A. Alonso[1]

[1]Centro de Biología Molecular "Severo Ochoa" and [2]Departamento de Immunología and Oncología, Centro Nacional de Biotecnología, Universidad Autónoma de Madrid and Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049 Madrid, Spain
[3]Christian de Duve Institute of Cellular Pathology, Université Catholique de Louvain, B-1200 Brussels, Belgium
[4]Departamento de Endocrinología, Hospital de la Princesa, 28006 Madrid, Spain
[5]Oncology Research Unit and the University of Sidney Department of Pediatrics and Child's Health, The Children's Hospital, Westmead, NSW, 2145, Australia

Transcytosis is used alone (e.g., hepatoma HepG2 cells) or in combination with a direct pathway from the Golgi (e.g., epithelial MDCK cells) as an indirect route for targeting proteins to the apical surface. The raft-associated MAL protein is an essential element of the machinery for the direct route in MDCK cells. Herein, we present the functional characterization of MAL2, a member of the MAL protein family, in polarized HepG2 cells. MAL2 resided selectively in rafts and is predominantly distributed in a compartment localized beneath the subapical F-actin cytoskeleton. MAL2 greatly colocalized in subapical endosome structures with transcytosing molecules en route to the apical surface. Depletion of endogenous MAL2 drastically blocked transcytotic transport of exogenous polymeric immunoglobulin receptor and endogenous glycosylphosphatidylinositol-anchored protein CD59 to the apical membrane. MAL2 depletion did not affect the internalization of these molecules but produced their accumulation in perinuclear endosome elements that were accessible to transferrin. Normal transcytosis persisted in cells that expressed exogenous MAL2 designed to resist the depletion treatment. MAL2 is therefore essential for transcytosis in HepG2 cells.

Introduction

Transcellular transport of external material involves a specialized pathway known as transcytosis, consisting of endocytosis of cargo on one side of the epithelial barrier, its vectorial traffic in vesicular carriers across the cell, and subsequent delivery to the other side. In addition to translocating external material, the transcytotic pathway is used as an indirect route for targeting membrane proteins to the apical surface via the basolateral membrane. Despite considerable advances in the elucidation of transcytosis steps (Mostov et al., 2000), little is known about the integral protein machinery implicated in the postendocytic events and in the organization of specialized subcellular compartments involved.

Address correspondence to Miguel A. Alonso, Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. Tel.: 34-91-397-8037. Fax: 34-91-397-8087. E-mail: maalonso@cbm.uam.es

Key words: hepatocytes; transcytosis; lipid rafts; MAL family; polarized transport

Simultaneous to the indirect transcytotic route, polarized epithelial MDCK cells transport newly synthesized proteins to the apical surface directly from the Golgi. A direct transport pathway appears to be mediated by integration of cargo protein into specialized glycolipid and cholesterol-enriched membrane microdomains or rafts that subsequently originate vesicular carriers destined for the apical surface (Simons and Wandinger-Ness, 1990). MAL is a nonglycosylated integral membrane protein of 17 kD containing four hydrophobic segments (Alonso and Weissman, 1987) that exclusively resides in rafts (Puertollano et al., 1999). Using MDCK cells whose endogenous MAL was depleted, an essential role has been demonstrated for MAL as an element of the machinery necessary for transport of apical proteins through the direct route (Cheong et al., 1999; Puertollano et al., 1999; Martín-Belmonte et al., 2000, 2001).

MAL is the founder member of a family of proteins (the MAL protein family) with structural and biochemical similarities (Pérez et al., 1997). MAL2 is a novel member of the

© The Rockefeller University Press, 0021-9525/2002/10/37/8 $5.00

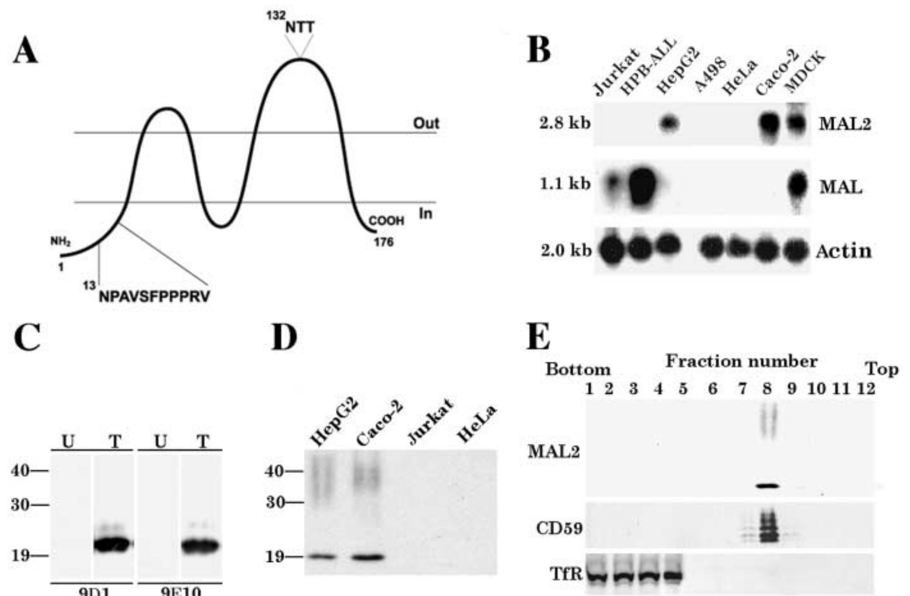

Figure 1. **Characterization of human MAL2 with a newly generated monoclonal antibody.** (A) Schematic model of the predicted structure of MAL2 with indication of the position of the consensus *N*-glycosylation site present in the molecule and the NH_2-terminal peptide selected for the preparation of antibodies. (B) Expression of the *MAL2* gene in different cell lines. Total RNA from the indicated cell lines was hybridized to DNA probes specific to MAL2, MAL, or β-actin. (C) Characterization of a novel mAb to MAL2. To assay the specificity of mAb 9D1, protein extracts from untransfected (U) or from transfected (T) COS-7 cells transiently expressing MAL2 tagged with the c-Myc 9E10 epitope were subjected to immunoblot analysis with either mAb 9D1 or the antitag mAb 9E10. Since COS-7 cells are negative for *MAL2* gene expression (unpublished data), no reaction was observed with endogenous proteins of COS-7 cells. (D) mAb 9D1 detects endogenous MAL2. Membrane fractions from the indicated cell lines were subjected to immunoblot analysis with anti-MAL2 mAb 9D1. (E) Identification of endogenous MAL2 in lipid raft fractions in HepG2 cells. Cells were extracted with 1% Triton X-100 at 4°C and subjected to centrifugation to equilibrium in sucrose density gradients. Aliquots from each fraction were analyzed by immunoblotting with anti-MAL2 mAb 9D1, and antibodies to CD59, used as a raft marker, and to TfR, a transmembrane protein excluded from rafts. Fractions 1–4 represent the 40% sucrose layer and contain the bulk of cellular membranes and cytosolic proteins, whereas fractions 5–12 represent the 5–30% sucrose layer and contain the rafts.

MAL family identified recently (Wilson et al., 2001). In our initial experiments, we detected *MAL2* gene expression in hepatoma HepG2 cells and in the epithelial MDCK and Caco-2 cell lines, all of which use the transcytotic pathway to a greater (HepG2) or lesser extent (MDCK and Caco-2) to target membrane proteins to the apical surface. Since we considered the hypothesis that MAL2 may act as machinery for transcytosis plausible, we undertook a study of MAL2 function in hepatoma HepG2 cells, which are a paradigm for the study of the transcytotic pathway in a cellular context deprived of other apical routes of transport for single transmembrane and glycosylphosphatidylinositol (GPI)*-anchored proteins (Bastaki et al., 2002).

Results and discussion

The MAL2 protein resides in lipid rafts in hepatoma HepG2 cells

Human MAL2 is a 176-residue (M_r = 19,000) protein with four putative transmembrane domains (Fig. 1 A) and ~36% identity with MAL at the amino acid level (Wilson et al., 2001). To identify a suitable human model cell system for studying MAL2, we performed Northern blot analyses using a panel of human cell lines. Canine MDCK cells were analyzed in parallel. Fig. 1 B shows that MAL2 mRNA species were expressed in hepatoma HepG2 cells and intestinal Caco-2 cells, consistent with the reported expression of MAL2 mRNA in the corresponding tissues (Wilson et al., 2001). MAL2 transcripts were undetectable in all other human cell lines analyzed. Canine MDCK cells were the only cell line positive for both MAL and MAL2 mRNA expression.

The NH_2-terminal peptide indicated in Fig. 1 A was chosen to generate a mAb to human MAL2. The 9D1 hybridoma clone was identified as producing antibodies to MAL2 by the selective detection of MAL2 in COS-7 cells transiently expressing c-Myc–tagged MAL2 (MAL2-Myc) (see Fig. 4 A) but not in untransfected cells (Fig. 1 C). Consistent with the observed expression of the *MAL2* gene (Fig. 1 B), mAb 9D1 recognized endogenous MAL2 in HepG2 and Caco-2 cells but not in Jurkat or HeLa cells (Fig. 1 D). No cross-reactivity was observed with the canine protein in MDCK cells (unpublished data). Contrary to observations of exogenous MAL2 in COS-7 cells, the endogenous MAL2 protein in HepG2 and Caco-2 cells migrated as a mixture of glycosylated (M_r = 30–40,000) and unglycosylated species (M_r = 19,000). The observation that MAL2 glycosylation was sensitive to treatment with endoglycosydase H (unpublished data) supports the use of the unique consensus site of *N*-glycosylation present in the MAL2 molecule (Fig. 1 A). Fig. 1 E shows that endogenous MAL2 was selectively detected in the raft fraction of HepG2 cells. As controls, we observed that raft fractions contained the GPI-anchored protein CD59 and excluded the transferrin (Tf) receptor (TfR), which were taken as representatives of markers associated and nonassociated with lipid rafts, respectively.

Subcellular distribution of MAL2 in polarized HepG2 cells

Hepatocytes are specialized polarized cells whose plasma membrane is subdivided into bile canalicular and sinusoidal domains, equivalent to the apical and basolateral surfaces,

*Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; pIgR, polymeric immunoglobulin receptor; Tf, transferrin; TfR, Tf receptor.

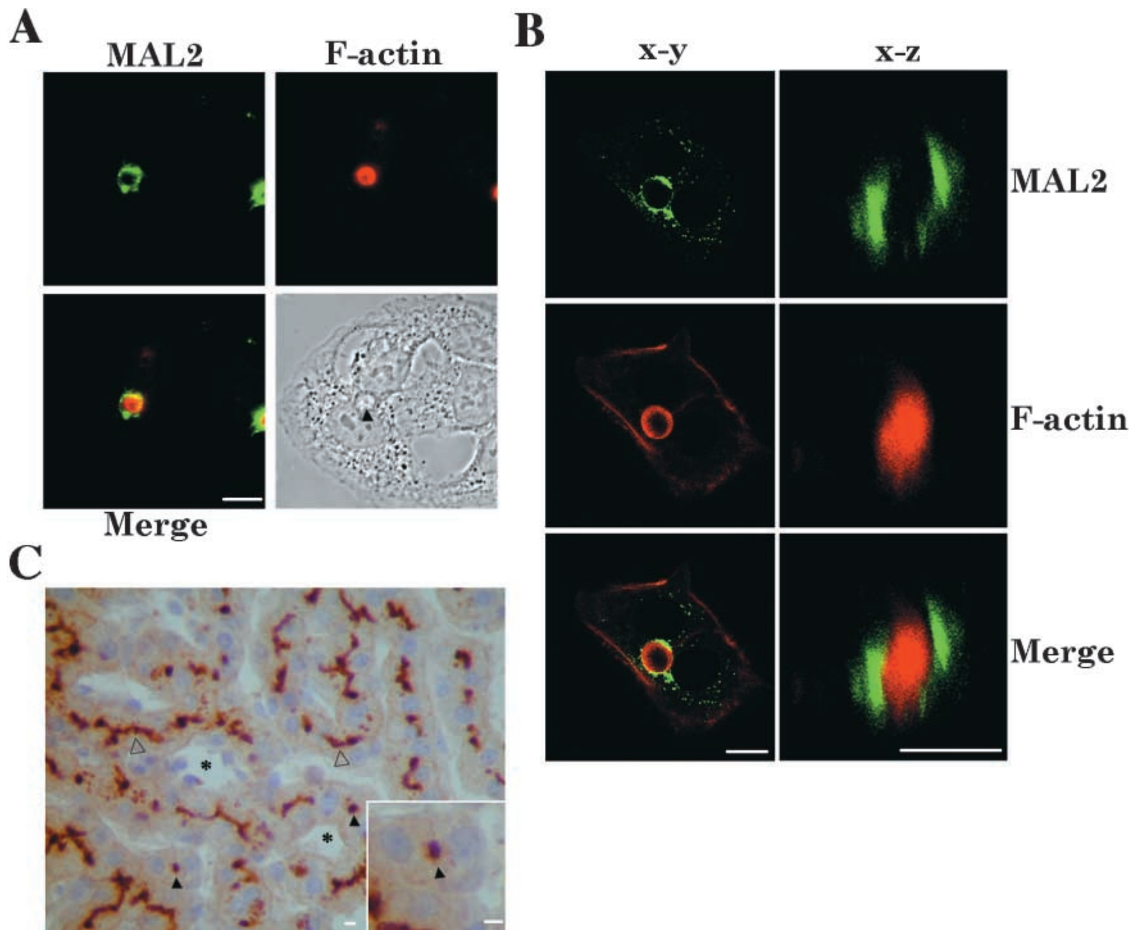

Figure 2. **Distribution of MAL2 in polarized HepG2 cells and human liver.** (A) HepG2 cells were double stained for MAL2 and F-actin and analyzed using a conventional fluorescence microscope. A phase–contrast image from the same field was taken to visualize the bile canaliculus (arrowhead). (B) HepG2 cells were double stained for MAL2 and F-actin and analyzed by confocal microscopy. Optical horizontal x,y and vertical x,z sections corresponding to planes in the middle of the cell or in the center of the bile canaliculus, respectively, are shown. (C) Liver tissue sections were subjected to immunohistochemical analysis with anti-MAL2 mAb 9D1 and counterstained with hematoxylin to visualize nuclei. Reactivity was found exclusively in the bile canaliculi, which appear as dots or lines depending on whether they were sectioned transversely (filled arrowheads) or longitudinally (open arrowheads) and absent from the sinusoidal membrane (asterisks). The inset shows at higher magnification a transversely sectioned bile canaliculus stained for MAL2. Bars, 5 μm.

respectively, of polarized epithelia. The canalicular surfaces of apposing hepatocytes delimit the bile canaliculi, which are small channels involved in bile secretion, whereas the sinusoidal surfaces face specialized capillary-like vessels, referred to as sinusoids, and establish contacts between adjacent cells. The species specificity of mAb 9D1 to the human MAL2 protein, the ability of HepG2 cells to polarize in vitro delimiting closed spaces reminiscent of bona fide bile canaliculi (Sormunen et al., 1993), and the fact that polarized trafficking in this cell line is simpler than in other cell lines (Bastaki et al., 2002) led us to choose HepG2 cells for further study.

The canalicular membrane is highly enriched with actin filaments, and so F-actin staining with fluorescent phalloidin can be used to visualize this membrane subdomain (Zegers et al., 1998). 48 h after plating, the percentage of HepG2 cells forming bile canaliculi was estimated to be ~30% as assayed by F-actin staining. We have confined our analysis exclusively to this polarized cell population. Fig. 2 A shows that MAL2 was mostly detected in the canalicular membrane region as revealed by double-label immunofluorescence analysis of MAL2 and F-actin and parallel imaging under a phase–contrast microscope. In the nonpolarized cell population, MAL2 was distributed in the perinuclear region (unpublished data). Confocal microscopic analysis (Fig. 2 B) in a horizontal x,y plane shows that MAL2 mostly distributed in polarized cells beneath the actin belt that surrounds the bile canaliculus. In addition, the presence of low levels of MAL2 in perinuclear vesicular structures was observed in some of the confocal planes. The vertical x,z view shows that, although actin is also detected inside the bile canalicular space, consistent with the presence of actin in microvilli protruding in the bile canaliculi, MAL2 was only found beneath the canalicular actin cytoskeleton. Consistent with the distribution observed in polarized HepG2 cells, MAL2 was exclusively detected at the bile canalicular membrane region by immunohistochemical analysis of human liver tissue sections (Fig. 2 C).

We next compared the distribution of MAL2 with those of two types of transcytosing molecules known to pass through apically localized recycling endosome structures en route to the canalicular surface (Schell et al., 1992; Apodaca

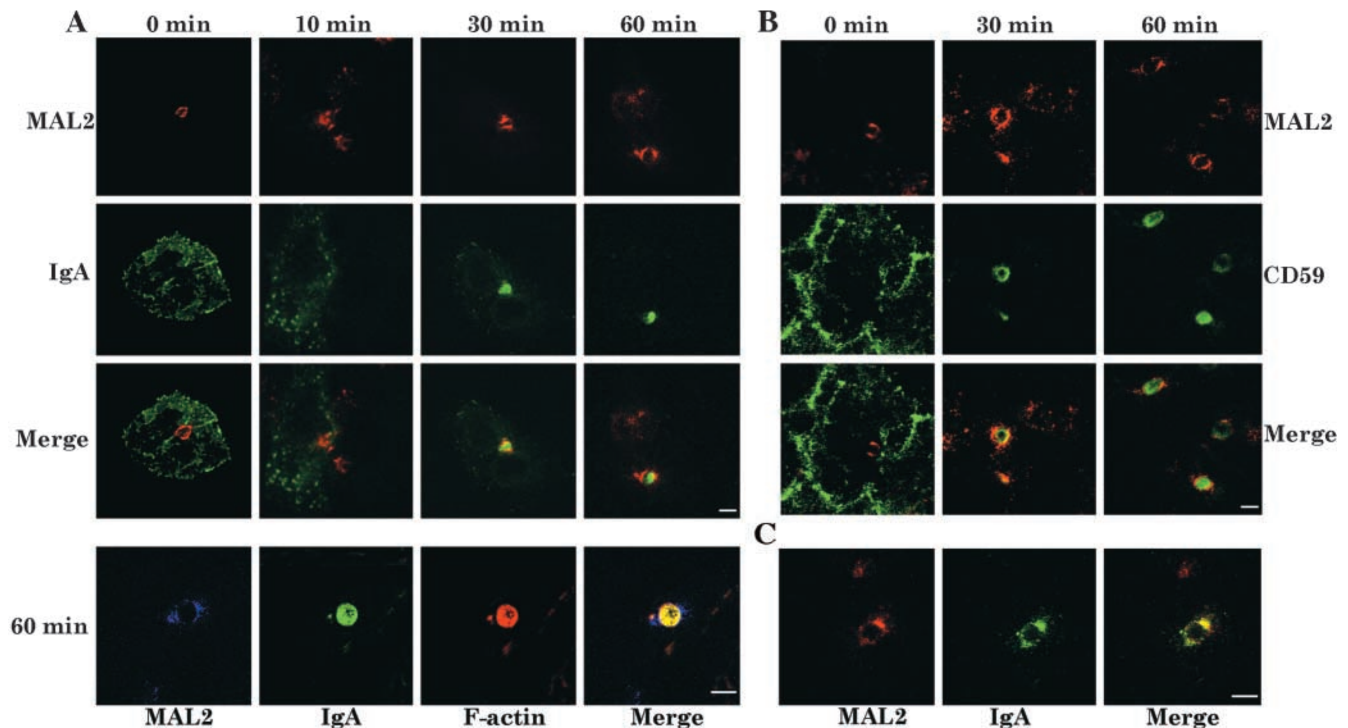

Figure 3. **Double label immunofluorescence analysis of MAL2 and trancytosing molecules.** (A) HepG2 cells stably expressing pIgR were incubated with IgA for 30 min at 4°C, washed, and placed at 37°C for the indicated times. Cells were then subjected to double label immunofluorescence analysis as indicated to detect MAL2 and IgA or were triply labeled to also detect F-actin. (B) HepG2 cells were incubated for 30 min at 4°C with anti-CD59 mAb, washed, and incubated at 37°C for the indicated times. Cells were then subjected to double label immunofluorescence analysis to detect MAL2 and CD59. (C) HepG2 cells stably expressing pIgR were incubated with IgA for 30 min at 4°C, washed, and incubated at 37°C for 60 min in the presence of 33 μM nocodazole. Cells were then washed, incubated for 120 min at 18°C in the absence of nocodazole, and subjected to double label immunofluorescence analysis to detect MAL2 and IgA. In all cases, the images presented are the composite projection of all the frames obtained by confocal microscopic analysis of the cells. Bars, 5 μm.

et al., 1994). First, we followed the transport of polymeric IgA bound to the polymeric immunoglobulin receptor (pIgR), as a prototypical transcytotic system (Mostov and Deitcher, 1986), using HepG2 cells that stably expressed the human pIgR molecule. The second system analyzed consisted of the basolateral-to-apical transcytosis of the endogenous GPI-anchored molecule CD59. IgA and anti-CD59 antibodies were bound to their respective target molecule on the surface of HepG2 cells at 4°C. The two ligands were internalized and concentrated in peripheral basolateral endosomes upon incubation at 37°C, and after 30 min, they had mostly accumulated in apical endosomes and colocalized with endogenous MAL2. After 60 min, both molecules were detected in the bile canaliculus (Fig. 3, A and B). Nocodazole treatment impedes transport of pIgR from peripheral basolateral early endosomes to downstream compartments of the transcytotic pathway but does not affect IgA internalization in MDCK cells (Hunzinker et al., 1990; Apodaca et al., 1994). Transcytotic transport to the canalicular membrane is sensitive to incubation of cells at 18°C in such a way that transcytosing molecules accumulate in apical recycling endosomes and further transport to the canalicular surface is reduced (Apodaca et al., 1994; van IJzendoorn and Hoekstra, 1998). To look for better conditions of colocalization of MAL2 and transcytosing IgA, HepG2 cells with surface-bound IgA were incubated for 60 min at 37°C in the presence of nocodazole to accumulate IgA in peripheral basolateral early endosomes and then for 120 min at 18°C in the absence of the drug to allow synchronized trafficking of the complex to apical recycling endosomes, while simultaneously preventing its exit therefrom. Fig. 3 C shows a nearly complete colocalization of MAL2 with IgA under these conditions.

Depletion of endogenous MAL2 in HepG2 cells using an antisense oligonucleotide-based strategy

To address directly the possible role of MAL2 in transcytotic transport, we designed a 19-mer phosphorothioate oligonucleotide complementary to the sequence surrounding the AUG translation initiation site of human MAL2 mRNA (Fig. 4 A) for use as an antisense MAL2 oligonucleotide (MAL2/AS). As a control oligonucleotide, we used the antisense oligonucleotide MAL/AS used previously for depleting endogenous MAL from MDCK (Puertollano et al., 1999). Although the control oligonucleotide or other oligonucleotides with the same composition as MAL2/AS but different sequence did not affect MAL2 levels, transfection of oligonucleotide MAL2/AS greatly diminished the amount of endogenous MAL2 in HepG2 cells (Fig. 4 B). The MAL2 levels obtained in cells transfected with oligonucleotide MAL2/AS were routinely 2–10% of those found in control cells. The levels of CD59 and the TfR chosen as representatives of proteins included in or excluded from lipid rafts, respectively, were not affected by this treatment.

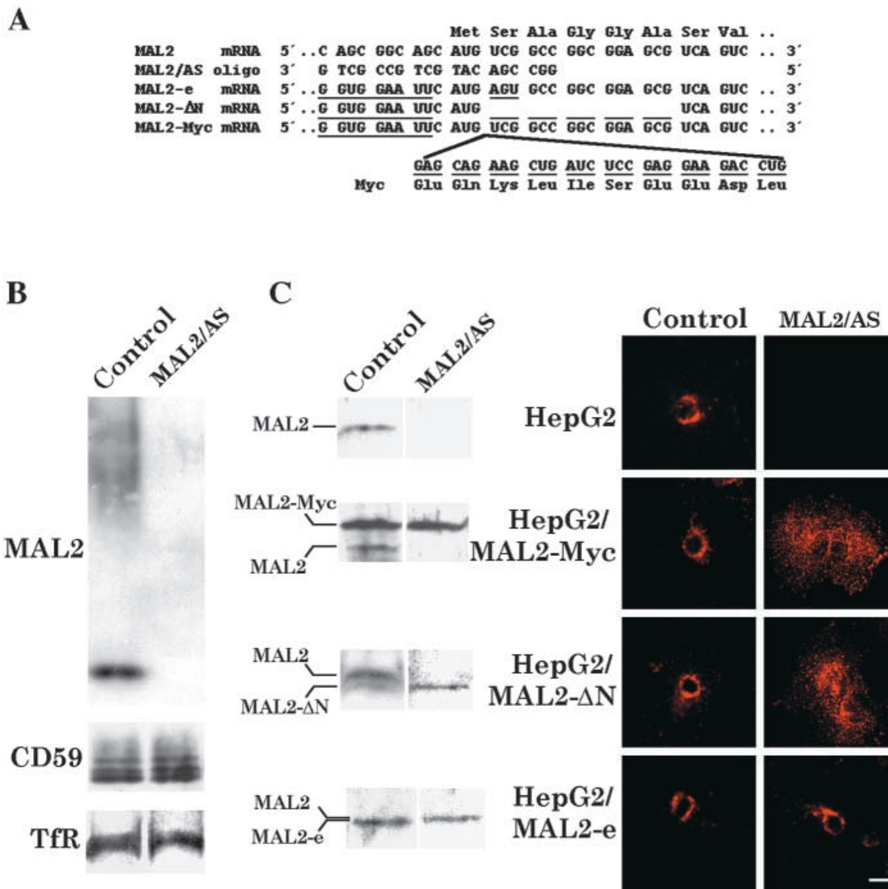

Figure 4. **Depletion of endogenous MAL2 by transfection with an antisense phosphorothioate oligonucleotide.** (A) The sequence of the antisense oligonucleotide used in MAL2 depletion experiments (MAL2/AS) and its alignment with wild-type MAL2 mRNA and the recombinant MAL2 mRNA species expressed in HepG2 cells are shown. Note that the changes introduced in the recombinant MAL2 transcripts prevent pairing with oligonucleotide MAL2/AS. The singly underlined residues correspond to sequences in the vector located immediately upstream of the inserted cDNA sequence. The doubly underlined residues in the coding sequence indicate nucleotides replaced by an equivalent triplet (MAL2-e), deleted (MAL2-ΔN) or added (MAL2-Myc). (B) HepG2 cells were transfected with control or MAL2/AS oligonucleotide and incubated at 37°C. After 72 h, cell extracts were subjected to immunoblot analysis with anti-MAL2 mAb 9D1 and with anti-CD59 and TfR antibodies. (C) Normal HepG2 cells or cells stably expressing the indicated exogenous products were transfected with control or MAL2/AS oligonucleotide and incubated at 37°C. After 72 h, cells were processed for immunoblot and immunofluorescence analyses with anti-MAL2 mAb, which recognizes both endogenous and exogenous MAL2 species. For simplicity, only the part of the blots corresponding to the unglycosylated MAL2 species is shown. Note that due to the deletion (MAL2-ΔN) or insertion (MAL2-Myc) of sequences the modified MAL2 proteins migrates faster or slower, respectively, than endogenous MAL2. Several independent cell clones (>3) from each type of cell transfectant were assayed and gave the same results. The immunofluorescence images correspond to the composite projection of all the frames obtained by confocal microscopic analysis of the cells. Bar, 5 μm.

As a control for the specificity of the effect of oligonucleotide MAL2/AS, we designed different DNA constructs producing MAL2 transcripts with an altered 5′ untranslated region and modifications in the first nucleotides of the coding region (silent substitutions, deletions, or insertions) that prevent pairing with the antisense MAL2/AS oligonucleotide. The constructs encoded an intact MAL2 protein (MAL2-e) or MAL2 proteins modified by either a five–amino acid deletion (MAL2-ΔN) or the addition of the Myc epitope (MAL2-Myc) in the sequence contiguous with the initial methionine residue. The modifications introduced did affect neither the glycosylation nor the incorporation of the molecule into rafts (unpublished data). HepG2 cells that stably expressed each of the MAL2 proteins were used to assay the effect of the antisense MAL2/AS oligonucleotide on the levels of exogenous and endogenous MAL2. We found MAL2 expression in the presence of antisense oligonucleotide MAL2/AS in all the transfectants as assayed by immunoblot and immunofluorescence analyses (Fig. 4 C). Although the staining and distribution of MAL2 was unaltered by transfection with the control oligonucleotide, the antisense oligonucleotide MAL2/AS rendered MAL2 levels almost undetectable in normal HepG2 cells. In the transfectants treated with control oligonucleotides, the exogenous protein presented a distribution identical to that of the endogenous protein. However, under conditions of endogenous MAL2 depletion only the transfectants expressing the intact protein (HepG2/MAL2-e cells) presented the typical subcanalicular distribution of endogenous MAL2.

MAL2 depletion blocks transcytosis of pIgR and CD59 in HepG2 cells

Fig. 5 A shows that internalization of IgA in HepG2 cells was not affected in cells treated with the antisense MAL2/AS oligonucleotide despite the reduction of MAL2 levels, since IgA accumulated in small peripheral basolateral endosome structures in the continuous presence of nocodazole, regardless of the levels of MAL2. However, whereas pIgR-IgA moved to apical endosomes and the bile canaliculi after removal of nocodazole and incubation at 37°C in the cells transfected with the control oligonucleotide, the complex mostly shifted to large endosomes with perinuclear location and accumulated in this compartment in cells with depleted levels of MAL2. The accumulated IgA colocalized partially with Tf in the same structures under conditions that allow trafficking of Tf from basolateral early endosomes to downstream compartments, including perinuclear endosomes (Fig. 5 B). Therefore, MAL2 is not required for transport of pIgR-IgA from the sinusoidal membrane to peripheral and

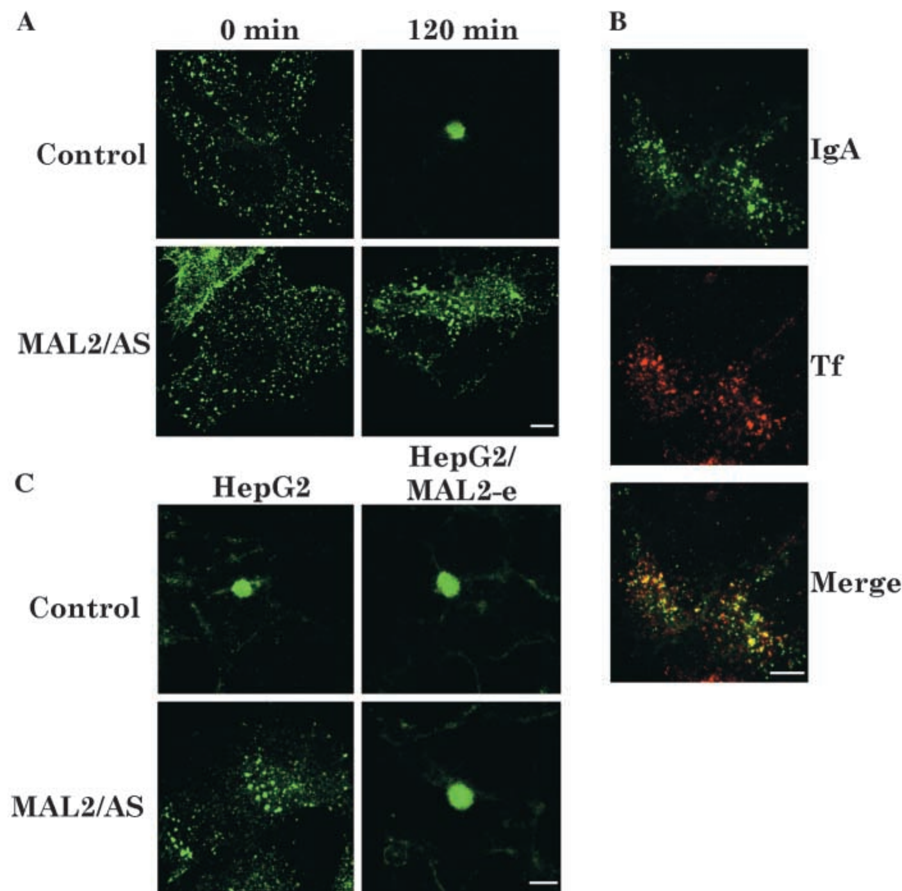

Figure 5. **MAL2 depletion blocks transcytosis of both pIgR-IgA and CD59 in HepG2 cells.** (A) HepG2 cells stably expressing pIgR were transfected with either control or MAL2/AS oligonucleotide, plated, and incubated at 37°C for 72 h. Cells were incubated in the presence of IgA for 60 min at 4°C and then were treated with 33 μM nocodazole for 60 min at 37°C. After removal of nocodazole (0 min), cells were incubated at 37°C for 120 min in the absence of the drug. Cells were stained with anti-IgA antibodies to determine the distribution of IgA. In parallel, mAb 9D1 was used to visualize the effect of the antisense oligonucleotide on MAL2 levels (unpublished data). One representative experiment out of ten performed is shown. (B) HepG2 cells in which IgA was accumulated in perinuclear endosomes by depletion of MAL2 were incubated with Tf for 10 min at 37°C, washed, and incubated for 10 min at 37°C to allow trafficking of internalized Tf to downstream compartments. Cells were then subjected to double label immunofluorescence analysis to detect IgA and Tf. (C) Normal HepG2 cells or HepG2 cells stably expressing MAL2-e were transfected with either control or MAL2/AS oligonucleotide, plated, and incubated at 37°C for 72 h. After binding of anti-CD59 mAb at 4°C, cells were incubated at 37°C for 60 min and fixed. After permeabilization, cells were stained with secondary antibodies to visualize the antibody bound CD59 complexes. One representative experiment out of six performed is shown. In all cases, the images presented are the composite projection of all the frames obtained by confocal microscopic analysis of the cells. Bars, 5 μm.

perinuclear endosomes but is essential for exit of pIgR-IgA from perinuclear endosomes to transit to downstream apical endosome compartments.

To confirm that MAL2 has a more general role in transcytosis, we examined the effect of MAL2 depletion in transcytosis of CD59. In addition, to demonstrate that the effects on transcytosis were due to MAL2 depletion and not to spurious effects of the antisense MAL2/AS oligonucleotide, we took advantage of the selectivity of MAL2/AS in blocking expression of endogenous but not exogenous MAL2 in HepG2 cells stably expressing recombinant MAL2 proteins. Fig. 5 C shows that CD59 transcytosis was also blocked by MAL2 depletion and that the exogenous expression of intact MAL2 resistant to the depletion treatment allowed normal transcytotic transport of CD59 despite transfection with the antisense MAL2/AS oligonucleotide. Consistent with their dispersal in cells whose endogenous MAL2 was depleted (Fig. 4 C), the MAL2 proteins with a modified NH_2 terminus were not able to substitute endogenous MAL2 in CD59 transcytosis (unpublished data).

MAL and MAL2 as elements of the integral membrane protein machinery for raft-mediated trafficking

Although the use of direct routes of transport to the apical membrane appears to be restricted, the indirect transcytotic route constitutes a common pathway for most polarized cells. The fact that MAL and MAL2 are expressed and access rafts independently of each other suggests that the two proteins perform distinct tasks in the cell. In epithelial MDCK cells, MAL depletion causes accumulation at the Golgi region of cargo normally delivered to the apical membrane through the direct route (Cheong et al., 1999; Martín-Belmonte et al., 2001). MAL2 depletion in HepG2 cells blocks apical transport of transcytosing molecules at perinuclear endosomes. Therefore, both MAL and MAL2 are members of the machinery of polarized transport: MAL plays an essential role in the direct apical transport pathway, whereas our results presented here show that MAL2 is required for the indirect transcytotic route.

Materials and methods

Materials

The hybridoma producing mAb 9E10 to the c-Myc epitope was purchased from the American Type Culture Collection. The rabbit polyclonal antibodies to human Tf and pIgR were from Dako, and those to the human TfR were from Zymed Laboratories. Mouse mAb to CD59 (MEM-43/5, IgG2a) was provided by Dr. V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). Fluorescent phalloidin was obtained from Molecular Probes. Nocodazole and human Tf were purchased from Sigma-Aldrich.

Cell culture conditions, DNA constructs, and transfections

Human hepatoma HepG2 cells (Sormunen et al., 1993) were grown on Petri dishes in DME supplemented with 10% FBS (GIBCO-BRL), penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in an atmosphere of 5%

CO_2/95% air. The 19-mer phosphorothioate oligonucleotide MAL2/AS (5′-GGCCGACATGCTGCCGCTG-3′) (Isogen Bioscience BV) was designed to anneal to endogenous MAL2 mRNA. The phosphorothioate oligonucleotide MAL/AS used in control experiments has been described previously (Puertollano et al., 1999). Oligonucleotides (50 μM) were introduced into HepG2 cells by electroporation of 2×10^6 cells resuspended in 200 μl of culture medium buffered with 10 mM Hepes, pH 7.0, and supplemented with 10% FBS and with 20 μg of salmon sperm DNA as a carrier. The cell suspension was placed in a 4-mm gap cuvette, and the transfection was performed with the equipment set up at 200 V, 480 ohms, and 960 μF. Under these conditions, the actual pulse length, as defined by the equipment manufacturer, ranged from 40 to 50 ms. After electroporation, 1 ml of culture medium with 10% FBS was added, and the cells were left at room temperature for 10 min. Finally, cells were plated out at subconfluent levels. Parallel experiments to measure the uptake of oligonucleotides using phosphorothioate oligonucleotides labeled at the 5′ end with Texas red indicated that the transfection efficiencies varied between 95–99% of cells as assayed by immunofluorescence analysis (unpublished data).

To express modified MAL2 mRNA species, the cDNA containing the coding sequence of MAL2 (Wilson et al., 2001) was amplified by PCR using 5′ oligonucleotide primers containing sequences designed with the appropriate modifications and an oligonucleotide primer specific for its 3′ end. The amplification products were cloned into the pCR3.1 DNA eukaryotic expression vector (Invitrogen). The cDNA construct expressing human pIgR was provided by Dr. P. Brandtzaeg (University of Oslo, Oslo, Norway). Transfection of HepG2 cells with plasmid DNA was performed by electroporation using an Electro Cell Manipulator 600 (BTX). Stable transfectants were selected by treatment with 0.5 mg/ml G-418 sulfate (Sigma-Aldrich) for at least 4 wk after transfection. Cell clones were screened by immunofluorescence analysis and/or reverse transcription coupled to the PCR using oligonucleotide primers corresponding to sequences in the vector flanking the cloning sites used. The clones that proved to be positive were maintained in drug-free medium.

Preparation of monoclonal antibodies to MAL2

The peptide corresponding to amino acids 13–28 of the MAL2 molecule was coupled to keyhole limpet hemocyanin. Spleen cells from mice immunized with the peptide were fused to myeloma cells and plated onto microtiter plates. The hybridoma clone 9D1, which produced antibodies that recognized MAL2 in membrane extracts from COS-7 cells transiently expressing tagged MAL2, was selected.

Northern blot analysis

20 μg total RNA from the indicated cell lines were denatured in 50% formamide and 2.2 M formaldehyde at 65°C, electrophoresed, and transferred to nylon membranes. RNA samples were hybridized under standard conditions to the indicated ^{32}P-labeled cDNA probes. Final blot washing conditions were 0.2 × SSC/0.1% SDS (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at 65°C.

Detergent extraction procedures and immunoblot analysis

HepG2 cells grown to confluency in 100-mm dishes were rinsed with PBS and lysed for 20 min in 1 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 at 4°C. Lipid rafts were prepared essentially as described by Brown and Rose (1992). Fractions of 1 ml were harvested from the bottom of the tube, and aliquots were subjected to immunoblot analysis. Samples were then subjected to SDS-PAGE in 15% acrylamide gels under reducing conditions and transferred to Immobilon-P membranes (Millipore). After sequential incubation with the primary and secondary antibodies, blots were developed using an ECL Western blotting kit (Amersham Biosciences).

Immunofluorescence and immunohistochemical analyses

HepG2 cells were fixed in 4% paraformaldehyde for 15 min, rinsed, and treated with 10 mM glycine for 5 min. The cells were then permeabilized with 0.2% Triton X-100, rinsed, and incubated with 3% BSA in PBS for 15 min. Cells were incubated for 1 h with the indicated primary antibodies, rinsed several times, and incubated for 1 h with the appropriate fluorescent secondary antibodies (Southern Biotech). Controls to assess labeling specificity included incubations with control primary antibodies or omission of the primary antibodies. As indicated in each case, images were obtained using either a Bio-Rad Laboratories Radiance 2000 Confocal Laser microscope or a conventional fluorescence microscope (ZEISS).

For immunohistochemical analysis, normal human liver tissue was fixed for several hours in 10% neutral buffered formalin and subjected to tissue processing and paraffin embedding. 5-μm tissue sections were blocked with a control antibody, and then sections were sequentially incubated with anti-MAL2 9D1 mAb and peroxidase-conjugated anti–mouse IgG antibodies. Sections were developed with 0.5 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide and counterstained with hematoxylin.

Transcytosis assays

The polymeric human IgA stock employed consisted of a mixture of 70% dimeric IgA and 30% of heavier IgA polymers, mostly trimers and tetramers. IgA transcytosis was analyzed essentially as described by van IJzendoorn and Hoekstra (1998). Briefly, HepG2 cells stably expressing exogenous pIgR were treated twice with a 100-fold excess of asialofetuin for 30 min at 4°C to block IgA internalization through asialoglycoprotein receptors, and then incubated with 50 μg/ml IgA at 4°C for 30 min, washed extensively, and incubated at 37°C. Under these conditions HepG2 cells expressing pIgR, but not normal HepG2 cells, were able to internalize IgA. After cell fixation and permeabilization, IgA was detected using anti–human IgA antibodies coupled to fluorescein. To analyze the transcytotic transport of CD59, HepG2 cells were incubated with anti-CD59 mAb for 30 min at 4°C, washed extensively, and incubated at 37°C. Cells were fixed and permeabilized, and the antibody bound to CD59 was detected using a fluorescent anti–mouse IgG2a antibody.

We thank S. Gómez, C. Sánchez, and F. Roncal for technical help.

M.C. de Marco is the recipient of a predoctoral fellowship from the Ministerio de Ciencia y Tecnología. This work was supported by grants from the Comunidad de Madrid (08.3/0025/2000; 08.5/0066/2001.1), Ministerio de Ciencia y Tecnología (PM99-0092), Fondo de Investigación Sanitaria (01/0085-01/02), and Fundación Eugenio Rodríguez Pascual. We also acknowledge an institutional grant from the Fundación Ramón Areces to Centro de Biología Molecular "Severo Ochoa."

Submitted: 6 June 2002
Revised: 3 September 2002
Accepted: 5 September 2002

References

Alonso, M.A., and S.M. Weissman. 1987. cDNA cloning and sequence of MAL, a hydrophobic protein associated with human T-cell differentiation. *Proc. Natl. Acad. Sci. USA.* 84:1997–2001.

Apodaca, G., L.A. Katz, and K.E. Mostov. 1994. Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. *J. Cell Biol.* 125:67–86.

Bastaki, M., L.T. Braiterman, D.C. Johns, Y.-H. Chen, and A.L. Hubbard. 2002. Absence of direct delivery for single transmembrane apical proteins or their "secretory" forms in polarized hepatic cells. *Mol. Biol. Cell.* 13:225–237.

Brown, D.A., and J.K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* 68:533–544.

Cheong, K.H., D. Zacchetti, E.E. Schneeberger, and K. Simons. 1999. VIP17/MAL, a lipid raft-associated protein, is involved in apical transport in MDCK cells. *Proc. Natl. Acad. Sci. USA.* 96:6241–6248.

Hunzinker, W., P. Mâle, and I. Mellman. 1990. Differential microtubule requirements for transcytosis in MDCK cells. *EMBO J.* 9:3515–3525.

Martín-Belmonte, F., R. Puertollano, J. Millán, and M.A. Alonso. 2000. The MAL proteolipid is necessary for the overall apical delivery of membrane proteins in the polarized epithelial Madin-Darby canine kidney and Fischer Rat Thyroid cell lines. *Mol. Biol. Cell.* 11:2033–2045.

Martín-Belmonte, F., P. Arvan, and M.A. Alonso. 2001. MAL mediates apical transport of secretory proteins in polarized epithelial Madin-Darby canine kidney cells. *J. Biol. Chem.* 276:49337–49342.

Mostov, K.E., and D.L. Deitcher. 1986. Polymeric immunoglobulin receptor expressed in MDCK cells trancytoses IgA. *Cell.* 46:613–621.

Mostov, K.E., M. Verges, and Y. Altschuler. 2000. Membrane traffic in polarized epithelial cells. *Curr. Opin. Cell Biol.* 12:483–490.

Pérez, P., R. Puertollano, and M.A. Alonso. 1997. Structural and biochemical similarities reveal a family of proteins related to the MAL proteolipid, a component of detergent-insoluble membrane microdomains. *Biochem. Biophys. Res. Commun.* 232:618–621.

Puertollano, R., F. Martín-Belmonte, J. Millán, M.C. de Marco, J.P. Albar, L. Kremer, and M.A. Alonso. 1999. The MAL proteolipid is necessary for normal apical transport and accurate sorting of the influenza virus hemagglutinin in

Madin-Darby canine kidney cells. *J. Cell Biol.* 145:141–145.

Schell, M.J., M. Maurice, B. Stieger, and A.L. Hubbard. 1992. 5′ nucleotidase is sorted to the apical domain of hepatocytes via an indirect route. *J. Cell Biol.* 119:1173–1182.

Simons, K., and A. Wandinger-Ness. 1990. Polarized sorting in epithelia. *Cell.* 62: 207–210.

Sormunen, R., S. Eskelinen, and V. Letho. 1993. Bile canaliculus formation in cultured HepG2 cells. *Lab. Invest.* 68:652–662.

van IJzendoorn, S.C.D., and D. Hoekstra. 1998. (Glyco)sphingolipids are sorted in sub-apical compartments in HepG2 cells: a role for non-Golgi-related intracellular sites in the polarized distribution of (glyco)sphingolipids. *J. Cell Biol.* 142:683–696.

Wilson, S.H., A.M. Bailey, C.R. Nourse, M.G. Mattei, and J.A. Byrne. 2001. Identification of MAL2, a novel member of the MAL proteolipid family, through interactions with TPD52-like proteins in the yeast two-hybrid system. *Genomics.* 76:81–88.

Zegers, M.M.P., K.J.M. Zaal, S.C.D. van IJzendoorn, K. Klappe, and D. Hoekstra. 1998. Actin and microtubules are involved in different membrane traffic pathways that transport sphingolipids to the apical surface of polarized HepG2 cells. *Mol. Biol. Cell.* 9:1939–1949.