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

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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.

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
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 (Mr = 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 NH2-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 (Mr = 30–40,000) and unglycosylated species (Mr = 19,000). The observation that MAL2 glycosylation was sensitive to treatment with endoglycosidase 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,
An essential role for MAL2 in transcytosis | de Marco et al. 39

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
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 early 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 canalculus (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 a way that transcytosing molecules accumulate in apical recycling endosomes and further transport to the canalicular surface is reduced (Apodaca et al., 1994; van Ijzenendoorn 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 3. Double label immunofluorescence analysis of MAL2 and transcytosing 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.
An essential role for MAL2 in transcytosis

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 allowed 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...
perinuclear endosomes but is essential for exit of plgR-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%
The 19-mer phosphorothioate oligonucleotide MAL2/AS (5′-GCCGCGCATGCTGGCGGTG-3′) (Isogen Bioscience BV) was designed to anneal to endogenous MAL2 mRNA. The phosphorothioate oligonucleotide MAL2/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 × 10⁶ cells suspended 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 (data not published).

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 plgR was provided by Dr. P. Brandtzaeg (University of Oslo, Oslo, Norway). Transfection of HepG2 cells with plasmid DNA was performed by electroporation using an Electo Cell Manipulator 600 (BTX). Stable transfectants were selected by treatment with 0.2 μM 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 transfected 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 32P-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 μM 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 assay

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 plgR were treated twice with a 100-fold excess of asialofetuin for 30 min at 4°C to block IgA internalization after 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 plgR, 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.

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