Polarized Expression of Functional Rat Liver Asialoglycoprotein Receptor in Transfected Madin-Darby Canine Kidney Cells*

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The rat liver asialoglycoprotein receptor or rat hepatic lectin (RHL) consists of two polypeptide species, a major one designated RHL-1 and a minor one designated RHL-2/3, which exists in two differentially glycosylated forms. We have studied the biosynthesis, targeting, and function of the different forms after transfection of their cDNAs into the polarized Madin-Darby canine kidney cell line. In cells expressing only RHL-1, newly synthesized protein undergoes rapid intracellular degradation and is not detected at the cell surface. In contrast, RHL-2/3 when transfected alone is much more stable and is expressed at the basolateral surface of filter-grown cells. When both forms are expressed together, newly synthesized RHL-1 escapes rapid degradation and is detected at the basolateral surface. In double transfectants a functional receptor is formed that specifically endocytoses and degrades ligand at the basolateral side.

The hepatic asialoglycoprotein receptor (ASGP-R) binds and internalizes desialylated plasma glycoproteins that carry terminal galactose or N-acetyl-galactosamine residues. The ligands are endocytosed and delivered to the lysosomal compartment for degradation while the receptor recycles to the cell surface (Ashwell and Harford, 1982; Geuze et al., 1986). 96% of the surface receptor is localized on the basolateral domain of the rat hepatocyte (Matsuura et al., 1982; Hubbard et al., 1983). Cloning and sequencing of the rat and human ASGP-Rs have demonstrated a similar structure. The rat ASGP-R contains two polypeptide species, designated RHL-1 and RHL-2/3; RHL-2/3 exists in two forms which are differentially glycosylated. Both proteins are "type-2" membrane proteins, anchored to the membrane by a stretch of 25 hydrophobic amino acids near the amino terminus. The amino acid sequences of RHL-1 and RHL-2/3 are 50% identical; the major difference is an 18-amino acid insertion in the cytoplasmic domain of RHL-2/3 (Holland et al., 1984; Halberg et al., 1987). The human ASGP-R is composed of two forms designated H1 and H2, which are 70% and 67% identical in amino acid sequence to RHL-1 and RHL-2/3.

Despite extensive work, the precise nature of the functional rat ASGP-R is still elusive. Cross-linking experiments have initially suggested that RHL-1 and RHL-2/3 are not physically linked (Holland et al., 1987, Halberg et al., 1987). On the other hand, immunoprecipitation studies indicated that the ASGP-R complex at the cell surface contains noncovalently linked RHL-1 and RHL-2/3 (Sawyer et al., 1988). Similarly, cDNA transfection experiments in a nonpolarized hepatoma cell line have suggested that both subunits are essential for expression of a functional receptor that is capable of internalizing asialoangliosomucoid (McPhaul and Berg, 1986). Nevertheless, cells expressing RHL-1 alone cannot perform uptake, binding, and degradation of galactosylated poly-L-lysine, a synthetic high affinity ligand (Braiterman et al., 1989). Studies with the analogous human ASGP-R have indicated that H1 and H2 form a heterooligomer and that expression of both forms is essential for a functional receptor (Bischoff et al. 1987; Shia and Lodish, 1989). Furthermore, H2 expressed in NIH 3T3 fibroblasts was rapidly degraded intracellularly unless coexpressed with H1, which indicates that both subunits interact early in the biosynthetic pathway (Shia and Lodish, 1989). Recently, the H1 subunit was expressed in MDCK cells. It was localized at the basolateral domain, but a function of this subunit was not described (Wessels et al., 1989).

In this study we have analyzed the biosynthesis, polarity, and function of the two forms of the rat liver ASGP-R expressed after single and double transfection of their cDNAs into the polarized MDCK cell line (Rodriguez-Boulan, 1983a; Simons and Fuller, 1985; Rodriguez-Boulan and Salas, 1989). We show that in cells transfected only with RHL-1, the protein is rapidly degraded intracellularly, while RHL-2/3 is targeted to the basolateral plasma membrane. In cells expressing both subunits, RHL-1 is significantly more stable, and expressed together with RHL-2/3 at the basolateral domain. Only in double transfectants was a functional receptor capable of internalizing and degrading ligand in a polarized fashion detected.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials—**MDCK cells, strain II, in the 56th to 70th passage, were grown in Dulbecco's minimum essential medium (DME) (GIBCO) supplemented with 10% horse serum (HyClone, Logan, UT), penicillin (100 units/ml), and streptomycin (100 μg/ml),...
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with an air-5% CO₂ atmosphere at constant humidity. When grown on filters, 3 x 10⁵ cells were seeded on (24.5-mm diameter, 0.4-μm pore size) polycarbonate membranes (Transwell chambers, Costar, Cambridge, MA) and cultured for at least 6 days with changes of medium every other day. The “tightness” of the monolayer was monitored as described (Lisanti et al., 1986; Sargiacomo et al., 1989).

All chemicals were by Sigma unless indicated otherwise. Polyclonal rabbit antisera directed against RHL-1 and RHL-2/3 have been described elsewhere (Halberg et al., 1987).

**DNA Transfections and Isolation of Expressing Clones—**Expression constructs containing full length copies of the cDNAs for RHL-1 and RHL-2/3 (Holland et al., 1984; Halberg et al., 1987) under the control of the promoter in the left long terminal repeat of Moloney murine leukemia virus (Mo-MuLV) were analogous to those previously used to transfect chicken liver glycoprotein receptor in rat fibroblasts (Mellow et al., 1988). Transfection of MDCK cells was performed by a modification of the calcium-phosphate precipitation procedure described by Graham and van der Eb (1973). The ratio of selectable to nonselectable DNA was 1:10. MDCK cells (2 x 10⁵ cells, low passage) grown for 24 h in DME supplemented with 10% fetal bovine serum (GIBCO) were trypsinized and suspended in 1 ml of medium. The calcium-phosphate-DNA precipitate (0.5 ml) formed using 10–15 μg of plasmid DNA was added to the cell suspension in a 10-cm tissue culture dish. After 30 min of incubation at room temperature, medium containing chloroquine (100 μg/ml) was added, and the cells were incubated at 37°C for 15 min. Filters were treated for 1 min at 37°C with a 15% (v/v) glycerol solution, washed, and incubated until reaching confluence. The cells were split 1:8 into 14-cm dishes containing 20 ml of DME supplemented with 10% horse serum and 500 μg/ml G418 (GIBCO). After 10–14 days under selection, resistant colonies were trypsinized and cloned by limiting dilution into 96-well microtitre plates. Individual clones were screened for expression by radioimmunooassay and indirect immunofluorescence. Only clones with more than 90% of cells expressing RHL-1 and/or RHL-2/3 were propagated further.

**Immunofluorescence and Radioimmunooassay—**Procedures for indirect immunofluorescence of transfected MDCK cells were as described (Rodriguez-Boulan, 1983). For intracellular staining, fixed cells were permeabilized with 0.075% (v/v) saponin. Samples were photographed with a Leitz Orthofluor fluorescence microscope (E. Leitz, Rockleigh, NJ) using 400 ASA Kodak Tri-X film (Eastman Kodak Co.). Exposure time was 30 s.

For screening of clones by radioimmunooassay, cells were plated on detachable 90-mm plastic Petri dishes (Lux, Miles Laboratories, Elkhart, IN). grown for 1–2 days, fixed, permeabilized, and reacted with the first antibody as for indirect immunofluorescence. Cells were probed with ¹²⁵I-protein A (0.5–2 x 10⁶ cpm/well, Du Pont-New England Nuclear) and counted in a gamma counter (Hewlett-Packard).

**Metabolic Labeling of MDCK Cells, Immunoprecipitation, and Immunoblotting—**For labeling MDCK cells grown on plastic dishes (35 mm), 150 μCi/300 μl [³⁵S]methionine/cysteine (Trans³⁵S-label, ICN, Irvine, CA) was added to subconfluent monolayers in methionine and cysteine-free DME supplemented with 0.2% (w/v) bovine serum albumin (BSA), and 10 μM Hepes, pH 7.3. In pulse-chase experiments, labeling periods were 15 min, after which DME containing 10 times normal concentration of methionine and cysteine was added for 5 min, which was then replaced by DME, 0.2% BSA, 10 mM Hepes, pH 7.3. MDCK cells grown on Transwell polycarbonate filters were labeled with 150 μCi/150 μl of Trans³⁵S-label added to the basal side of inverted filters in a humid chamber (Balcarova-Staender et al., 1988). Filters were treated as described from the apical side for 30 min in 1% Nonidet-P-40, 0.5% sodium deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4 (lysis-buffer). Nuclei were removed by centrifugation for 5 min in an Eppendorf centrifuge. The supernatant was then removed by aspiration, and the apical side of the filter was washed three times with PBS/CM (PBS containing 0.5% Tween 20). After lysis, the monolayers grown in polycarbonate filter-chambers for 6 days were labeled for 20 h with Trans³⁵S-label (150 μCi/ml) in DME containing 10% of the normal concentration of methionine and cysteine. Cells were washed at 4°C with phosphate-buffered saline, 0.1 mM CaCl₂, 1 mM MgCl₂ (PBS/CM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride 100 μg/ml in PBS/CM was added either to the apical or basolateral side. Trypsin inhibitor (100 μg/ml in PBS/CM) was added to the opposite side. Incubation was on ice for 1 h. Control cells received only PBS/CM. Monolayers were washed twice for 5 min with 100 μg/ml trypsin-inhibitor added to both sides of the filter and several times with PBS. Extraction was in lysis buffer containing 100 μg/ml trypsin inhibitor.

**Biotin Assay for Polarity—**Sulfo-N hydroxy succinimidobiotin (sulfo-NHS-biotin; Pierce) was employed to label selectively the apical or basolateral surface of filter grown monolayers (Sargiacomo et al., 1989). MDCK monolayers on filter chambers were washed four times with PBS/CM for 15 min at 4°C each. Sulfo-NHS-biotin (0.5 mg/ml in PBS/CM; freshly diluted from a frozen stock of 200 mg/ml in dimethyl sulfoxide) was added to either the apical or basolateral compartment of the filter. Portion of cells receiving sulfo-NHS-biotin were filled with PBS/CM. Labeling was for 20 min at 4°C and repeated twice. After the final labeling, filter chambers were washed with DME (one time) and PBS/CM (three times). Filters were permeabilized with 0.1% Nonidet-P-40, 0.5% sodium deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4, and one wash in H₂O, and the samples were eluted by boiling the pellets in SDS-PAGE sample buffer. Eluted protein was separated by SDS-PAGE and transferred to nitrocellulose. Blots were blocked for 2 h with 3% BSA, 2% non-fat dry milk in PBS/TGG (PBS containing 0.5% Tween 20, 10% (v/v) glycerol, 1 mM glucose) at room temperature. ¹²⁵I-Streptavidin (1 x 10⁶ cpm/ml in PBS/TGG containing 0.3% BSA) was allowed to bind for 1 h at 4°C. The blots were washed with PBS/CM, and after several washes, blots were washed with PBS/CM and air-dried at -80°C. Streptavidin (Bethesda Research Laboratory) was radiolabeled with Na₂¹²⁵I (New England Nuclear) using chloramine T (Greenwood et al., 1963); typically a specific activity of approximately 5−10 μCi/μg was obtained.

**Degradation Studies—**Galactosylated BSA (Gal-BSA; E-Y Laboratories; San Mateo, CA) was radioactively labeled with Na₂¹²⁵I (New England Nuclear) using chloramine T procedure at a specific activity of 2−3 μCi/μg. Binding medium consisted of 0.2% BSA in DME with 25 mM Hepes replacing NaHCO₃; the pH was...
adjusted to 7.2. Filter grown cells (2.4 x 10^6 cells/Transwell) were preincubated for 1 h in hving medium at 37 °C, after which labeled ligand was added to either the apical or basolateral compartment. After incubation, apical and basolateral media were removed and tested for acid-soluble radioactivity by the method of Goldstein and Brown (1974). Filters were washed five times with PBS containing 4 mM EDTA in the cold, cut from the filter chambers, and counted.

**RESULTS**

**Expression of RHL-1 and RHL-2/3 in Singly Transfected MDCK Cells**

Constructs containing full length cDNAs for RHL-1 and RHL-2/3 under the control of a retroviral promoter were transfected into MDCK cells along with pMV6 tk neo (Maddon et al., 1985), a plasmid conferring resistance to the antibiotic G418. Transfected cells were grown in the presence of 500 µg/ml G418 for 10–14 days. G418-resistant colonies were screened for expression via indirect immunofluorescence on fixed, detergent-permeabilized cells using a polyclonal anti-serum directed against the respective receptor form and rhodamine conjugated goat anti-rabbit serum or via radioimmunoassay using ^125I-protein A. Of the clones resistant to G418, 10–30% expressed RHL-1 or RHL-2/3. Several clones, in which all cells express the protein, were obtained for each receptor form. Expression was stable over at least 10 passages.

**Biochemical Characterization of the Established Lines—**

RHL-1 and RHL-2/3 forms synthesized by transfected MDCK cells were analyzed and compared with rat liver forms by SDS-PAGE of membrane protein fractions extracted and concentrated by Triton X-114 condensation, followed by immunoblotting using polyclonal rabbit antisera and ^125I-protein A (Fig. 1). MDCK cells expressing RHL-1 showed a major band with an apparent Mr of 41,000 (Fig. 1A, lane 1) comigrating with rat liver RHL-1 (Fig. 1A, lane R), and a minor band of 38,000. The clone expressing RHL-2/3 displayed two bands, a relatively sharp one at 42 kDa and a broad one around 70 kDa (Fig. 1B, lane 2/3). RHL-2 and RHL-3 of rat liver migrate at 49 and 60 kDa in the gel system used.

In order to determine whether the observed differences in molecular weight between rat liver and MDCK forms of RHL are due to differences in glycosylation, we studied the effect of digestion of the RHL forms by endo H and peptide-N-glycosidase F. Endo H cleaves only oligosaccharides of the high mannose type, while peptide-N-glycosidase F cleaves all N-linked oligosaccharides (Tarentino and Maley, 1974; Tarentino et al., 1985). MDCK cells expressing RHL-1 and RHL-2/3 were metabolically labeled with TranS-S-label for 3 h, cell lysates were immunoprecipitated, and the immunoprecipitates digested with endoglycosidases. Non-digested and digested proteins were analyzed by SDS-PAGE and fluorography (Fig. 2A). In parallel, rat liver microsomes were solubilized with detergent and digested with endo H or peptide-N-glycosidase F. After SDS-PAGE, proteins were transferred to...
nitrocellulose and probed with sera against RHL-1 and RHL-2/3 followed by 125I-protein A (Fig. 2B). Peptide-N-glycosidase F digestion of RHL-1 and RHL-2/3 from either MDCK or rat liver resulted in forms of 30 kDa (RHL-1) and 35 kDa (RHL-2/3), the mass expected for the unglycosylated polypeptides (Holland et al., 1984; Halberg et al., 1987). Thus, the differences in molecular weight between MDCK and rat liver forms appear to be due to differential glycosylation. Endo H digestion of RHL-1 in MDCK cells revealed that the 38-kDa form, which was the predominant form under these labeling conditions, was endo H sensitive, while the 41-kDa form gave rise to two bands of 40 and 36 kDa (Fig. 2A). This indicates that the 41-kDa form has partially acquired complex oligosaccharides. This is more clearly shown in Fig. 2C, where a pulse-chase protocol was employed. Cells were labeled with Tran35S-label for 15 min and chased for 5 min and 2 h. At 5 min chase RHL-1 (38 kDa) was endo H-sensitive. After 2 h chase, it shifted to 41 kDa and acquired partially endo H-resistant oligosaccharides. Rat liver RHL-1 was largely endo H-resistant. It showed a small shift upon endo H digestion, compatible with the presence of perhaps one high mannose oligosaccharide (Fig. 2B).

In rat liver both RHL-2 and RHL-3 were endo H resistant, i.e. carried complex oligosaccharides (Fig. 2B). In MDCK cells only the 70-kDa form (RHL-3) was endo H resistant, while the 42-kDa form was endo H-sensitive (Fig. 2A).Therefore and for reasons that will become apparent below, the 42-kDa form will be referred to as RHL-2' throughout the paper. Together, these results indicate that while the polypeptide backbones of the ASGP-R forms synthesized in MDCK cells and rat hepatocytes are identical, the processing of the oligosaccharides differs considerably.

Localization of Transfected ASGP-R—In order to localize RHL-1 and RHL-2/3 in MDCK cells, we carried out indirect immunofluorescence on non-permeabilized and saponin-permeabilized cells (Fig. 3). Intracellularly, RHL-1 and RHL-2/3 appeared concentrated in the perinuclear region (Fig. 3, B and D). At the cell surface, only RHL-2/3 was detected. This could be observed in both permeabilized and non-permeabilized cells (Fig. 3, C and D). Surface staining was not detected in cells expressing RHL-1 (Fig. 3A). There are two possible interpretations for this result: that RHL-1 transport to the cell surface is blocked intracellularly, or that RHL-1 is delivered to the plasma membrane but is immediately shed into the medium.

Bio synthesis of RHL-1 and RHL-2/3—The finding that only RHL-2/3 was expressed on the cell surface of transfected MDCK cells while RHL-1 was not, led us to investigate the biosynthesis and processing of both forms in MDCK cells using pulse-chase experiments. Subconfluent monolayers were labeled for 15 min with Tran35S-label and chased in medium containing non-radioactive methionine and cysteine for various times. After immunoprecipitation with either RHL-1 and RHL-2/3 antiserum, samples were analyzed by SDS-PAGE and fluorography (Fig. 4, A and B). The pulse-chase experiments revealed that newly synthesized RHL-1 (38 kDa) was processed to the 41-kDa form, which disappeared rapidly, with a half-life of less than 2 h (Fig. 4A). Cells expressing RHL-2/3 synthesized a precursor of 42 kDa (RHL-2'), which eventually was processed to the 70-kDa form (RHL-3). Coincident with the shift in molecular weight, RHL-2/3 acquired endo H-resistant oligosaccharides (Fig. 2, and data not shown). This shows that in MDCK cells RHL-2' is the kinetic precursor of RHL-3. This situation is different from the one in the rat hepatocyte, where differential glycosylation results in two endo H-resistant forms which are both expressed at the cell surface (Halberg et al., 1987; Sawyer et al., 1988).

In MDCK cells, RHL-2/3 is considerably more stable than RHL-1, with almost no loss within 6 h and a substantial portion of RHL-3 detectable even after 18 h chase. In order to exclude the possibility that RHL-1 was proteolytically cleaved at the cell surface and shed into the medium, a pulse-chase experiment was performed with MDCK cells grown on polycarbonate filters. This allowed us to analyze the apical and basolateral media for the presence of soluble forms of RHL-1. The kinetics of degradation of RHL-1 on confluent, filter-grown cells were similar to the ones observed in plastic grown cells. No soluble forms of RHL-1 were detected in the apical or basolateral medium (data not shown). This suggests that RHL-1 is not transported to the cell surface but degraded intracellularly. Degradation was unimpaired when cells were

Fig. 3. Immunofluorescence localization of RHL-1 and RHL-2/3 in nonpermeabilized and permeabilized cells. Cells were grown for 2 days on glass coverslips and fixed in 2% paraformaldehyde. Cells were permeabilized with 0.075% saponin, which was present in all subsequent steps. Staining was via indirect immunofluorescence using an affinity purified serum against RHL-1 and a normal serum against RHL-2/3, followed by rhodamine-conjugated goat anti-rabbit serum. Surface (A) and intracellular (B) staining of cells expressing RHL-1, surface (C), and intracellular (D) staining of cells expressing RHL-2/3. No surface staining was obtained in RHL-1 expressing cells (A). Control cells gave no staining. Bar, 10 μm.

Fig. 4. Biosynthesis and processing of RHL-1 and RHL-2/3 in MDCK cells. Subconfluent MDCK cells (1-2 x 10⁶ cells) were labeled for 15 min with Tran35S-label (150 μCi/dish) and chased for the times indicated. Cells were extracted with lysis buffer and the post-nuclear supernatant was immunoprecipitated with either anti-RHL-1 (A) or anti RHL-2/3 (B) serum. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Molecular mass markers from top to bottom: 205, 103, 67, 42, 28 kDa.
treated with chloroquine, a drug known to inhibit lysosomal function (data not shown). Further work is needed to elucidate the mechanism by which RHL-1 is degraded intracellularly.

**Polarity of Surface Expression of RHL-2/3**—Indirect immunofluorescence on semithin frozen sections and two biochemical assays were employed to analyze the polarized surface expression of RHL-2/3. Cells were grown for 3–4 days on collagen-coated coverslips, fixed, and processed for semithin frozen sectioning. Immunostaining of 0.5-μm sections showed a clear basolateral localization of the antigen (Fig. 5A). Control cells gave no such staining (data not shown).

It has been shown that cell surface hemagglutinin in influenza virus-infected MDCK cells can be sensitively assayed by trypsinization of filter-grown cells from either the apical or basolateral side of the filter under conditions which do not open tight junctions (Matlin and Simons, 1984). We employed this protease polarity assay to determine the surface localization of RHL-2/3. Cells were plated at high density in polycarbonate filter chambers, grown at confluence for 5–6 days and labeled for 20 h with Tran-35S-label. Trypsin (100 μg/ml) was added to either the apical or basolateral side, and filters were incubated for 1 h at 4 °C. Digestion was stopped with trypsin inhibitor, cell extracts were immunoprecipitated and analyzed by SDS-PAGE. The fluorogram (Fig. 5B) shows that 70–80% of RHL-3 was digested when trypsin was added to the basolateral side, which is consistent with a preferential basolateral localization. RHL-2' was not digested, indicating that it is localized only intracellularly.

A method for labeling cell surface proteins was employed to confirm this result (Lisanti et al., 1988; Sargiacomo et al., 1989). Cells on polycarbonate filters were labeled with sulfo-NHS-biotin, a water-soluble biotin-analogue, either from the apical or basolateral side. Cell extracts were immunoprecipitated, analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with 125I-streptavidin. The pattern obtained showed a strong labeling of RHL-3 when sulfo-NHS-biotin was added to the basolateral side, compared with a weak labeling on the apical side (Fig. 5C, lanes 1 and 2). Quantitation by counting of the bands excised from the nitrocellulose revealed that more than 90% of cell surface RHL-3 was localized on the basolateral domain. RHL-2' could not be detected at the cell surface. The band at 49 kDa (Fig. 5C, hc) also labeled in non-transfected MDCK cells represents the heavy chain of immunoglobulins which binds iodinated streptavidin without prior biotinylation (see Fig. 6D). The increased labeling of this band from the basolateral side suggests that heavy chain from the medium may have been preferentially adsorbed to the basolateral side of the cells/filter and thus been additionally biotinylated.

**Coexpression of RHL-1 and RHL-2/3 in Doubly Transfected MDCK Cells**

The observation that in single transfectants RHL-1 was not expressed at the cell surface of MDCK cells but was rapidly degraded intracellularly, suggests that coexpression of RHL-2/3 might be essential for RHL-1 to be transported to the cell surface. This possibility was investigated by transfection of MDCK cells with both cDNAs. Several clones that expressed both subunits were isolated by criteria of immunofluorescence and immunoblot analysis. The immunoblot revealed the synthesis in double transfectants of all RHL-forms that were expressed in single transfectants, namely RHL-1, RHL-2', and RHL-3 (Fig. 6A, lanes 1 and 3). Additionally a band migrating at approximately 47 kDa was detected (arrow in Fig. 6A, lane 3). This form may resemble rat liver RHL-2 that migrates at 49 kDa.

Pulse-chase experiments with single and double transfectants revealed two clear differences between them: (i) RHL-1 in double transfectants was significantly more stable than in single transfectants (Fig. 6B, left panel). The half-life as determined by densitometry of the fluorogram was more than 6 h compared with two in single transfectants (data not shown). (ii) RHL-2' was chased into two different forms, RHL-3 and the 47-kDa form that might correspond to rat liver RHL-2 (Fig. 6B, right panel). Stability of RHL-2/3 was similar in single and double transfectants (data not shown). These results suggest that coexpression of both forms "rescues" RHL-1 at least partially from rapid intracellular degradation, and furthermore that it alters the processing of the precursor RHL-2' to give rise to two final forms as in the hepatocyte. In order to show that the additional form in double transfectants is actually part of a functional receptor complex, receptors from singly and doubly transfected cells were affinity purified by binding to galactose-Sepharose at 25
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A Functional Receptor Is Expressed in Doubly Transfected MDCK Cells—Recent studies in non-polarized hepatoma cells have indicated that expression of both RHL subunits is essential for the formation of a functional ASGP-R (McPhaul and Berg, 1986). Nevertheless, cells expressing only RHL-1 were able to perform endocytosis of galactosylated poly-L-lysine, but not of asialoorosomucoid, the most widely used ligand for functional studies (Braiterman et al., 1989). Receptor from singly and doubly transfected cells was able to bind to galactose-Sepharose (Fig. 6C).

In order to determine if a functional receptor capable of endocytosis was expressed in singly and doubly transfected MDCK cells, uptake and degradation studies were performed on various clones. Galactosylated bovine serum albumin (Gal-BSA) was used as a ligand in most experiments; in this neoglycoprotein an average of 40 saccharide residues are covalently linked to each protein molecule. Gal-BSA was degraded with efficiency similar to asialoorosomucoid by positive clones (data not shown). Cells were grown for 6 days on polycarbonate filters and incubated for 6 h with 125I-labeled ligand (2 μg/ml). The basolateral medium was analyzed for acid-soluble radioactivity as a measure of ligand degradation (Goldstein and Brown, 1974). MDCK cells release degraded ligand at a low rate while the two clones expressing both subunits (1A10, 2C7) showed degradation rates severalfold higher (up to 10-fold higher, in some experiments) (Table I). The time course of ligand uptake and degradation was followed over a period of 6 h in MDCK and clone 1A10 cells (Fig. 7). The uptake in 1A10 cells was most significant during the initial hour after which it only
degradation was linear over the 6-h time course (Fig. 7B). 

Determination of ligand observed in double transfectants was moderately affected by an excess of non-labeled competing (Gal-BSA) or GlcNAc-BSA. In contrast, in 1A10 cells, Gal-BSA almost restored control levels, while GlcNAc-BSA had no effect (Table II). Furthermore, when the degradation assay was performed in the presence of antisera against RHL-1 and RHL-3, the antibodies strongly interfered with the degradation by 1A10 cells, while non-immune serum did not (Table II). None of these sera had any effect on the nonspecific degradation by MDCK cells. These experiments strongly suggest that the higher levels of degradation observed in double transfectants are mediated by the transfected receptor.

In order to determine if the uptake of ligand is polarized in clone 1A10 cells, as would be expected from the polarized surface expression of RHL-1 and RHL-3 (Fig. 6D), we added ligand either to the apical or basolateral side of filter-grown MDCK and clone 1A10 cells. After 1 h of preincubation in binding medium, 125I-Gal-BSA (2 μg/ml) was added to the basolateral compartment of control MDCK and clone 1A10 cells, (i) either with no competitor or with 200 μg/ml unlabeled Gal-BSA or GlcNAc-BSA and (ii) either with no addition or with either polyclonal rabbit antiserum directed against RHL-1 and RHL-2/3 (1:200 dilution each) or nonimmune serum (1:50 dilution). Incubation was for 6 h. Processing of samples was as described in Table I. All determinations were performed on duplicate filters, standard errors of the mean were less than 10%. Each experiment was performed twice. Values are expressed as % of total degradation by clone 1A10 cells (i, 100% = 7603 ligand molecules/cell/h; ii, 100% = 3576 ligand molecules/cell/h).

### Table II

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### Table III

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\* Total (apical and basolateral) values were combined.

\* Ratio of total degradation when ligand was apically added versus when ligand was added basolaterally.

DISCUSSION

We have expressed cloned cDNAs for the two forms of the rat liver asialoglycoprotein receptor in the polarized dog kidney MDCK cell line, which normally does not express this receptor, in order to dissect the roles played by the two forms in polarized targeting and function in an epithelial environment. In rat liver, ASGP-R is predominantly localized at the...
problems, we transfected the cDNAs for RHL-1 and RHL-2/3 separately into MDCK cells and studied their biosynthesis, processing, and targeting to the plasma membrane in permanently expressing clonal cell lines.

RHL-1 was not detected at the surface of MDCK cells (Fig. 3), but it underwent rapid intracellular degradation shortly after its synthesis (Fig. 4). This unexpected behavior was reproduced in two independently derived clones (data not shown) which makes it unlikely that a general cellular transport defect has been selected RHL-2/3, in contrast, was transported efficiently to the cell surface (Fig. 3).

The considerable difference in molecular weight of RHL-2/3 expressed in rat liver and in MDCK cells can be explained by differential N-linked glycosylation, as indicated by endoglycosidase digests (Fig. 2). In MDCK cells, RHL-2' was the kinetic precursor of RHL-3 and the molecular mass shift from 42 to 70 kDa was concomitant with the acquisition of endo H-resistant oligosaccharides (Fig. 4 and data not shown). Only RHL-3 was expressed at the cell surface of MDCK cells.

In the rat hepatocyte both forms are endo H resistant and are expressed on the cell surface, suggesting that they derive from a common precursor by differential glycosylation (Halberg et al., 1989; Sawyer et al., 1988).

Semithin frozen sections and two different biochemical procedures indicated a preferential basolateral localization of RHL-3 in MDCK cells (>90% of the surface receptor, according to the biotin labeling procedure), in agreement with the localization in the hepatocyte (Fig. 5). Thus, at least with RHL-2/3 the targeting information can be decoded by the MDCK cell sorting machinery. Recently, it was demonstrated that one form of the human ASGP-R (H1) is also targeted to the basolateral plasma membrane of MDCK cells (Wessels et al., 1989). Furthermore, we have recently shown that the related chicken glycoprotein receptor, which is a hexamer consisting of only one type of polypeptide, is expressed at the basolateral domain of MDCK cells, where it is employed in endocytosis of ligand. This indicates that the mechanisms that mediate basolateral protein sorting in hepatocytes and kidney cells and between different species are similar. Nevertheless, although RHL-3 was expressed at the basolateral surface of MDCK cells, it alone was not capable of performing endocytosis of ligand (Table 1).

To reconstitute a functional receptor in MDCK cells both, RHL-1 and RHL-2/3, had to be expressed together. The coexpression of RHL-2/3 had a significant effect on the stability of RHL-1. RHL-1 in double transfectants displayed a significantly longer half life than in single transfectants (6 as opposed to 2 h; Fig. 6). Furthermore, RHL-1 was expressed together with RHL-3 at the cell surface in double transfectants. This indicates that RHL-1 and RHL-2/3 might interact early in the biosynthetic pathway and that for RHL-1 this interaction might be crucial to prevent its rapid degradation. A similar behavior of receptors, consisting of two or more subunits has been recently described for the human ASGP-R and the T cell receptor. In the case of the T cell receptor, a seven subunit protein, only completely formed heptamers were correctly targeted to the cell surface, while all partial complexes were rapidly destroyed intracellularly (Minami et al., 1987; Sussman et al., 1988). In transfected fibroblasts expressing only the α-chain or α-β-complexes of the T cell receptor this degradation even took place in the endoplasmic reticulum (Lippincott-Schwartz et al., 1988).

When the two forms of the human ASGP-R were expressed in fibroblasts, the H2 form was rapidly degraded intracellularly unless it was coexpressed with H1 (Shia and Lodish, 1989). This result is comparable with our observation with one striking difference. In the human ASGP-R/fibroblast model system H1 (homologous to RHL-1) is the "rescuing" protein, while in the rat ASGP-R/MDCK system RHL-2/3 (homologous to H2) fulfills this role. At the moment we can only speculate on whether this difference is due to the different cell lines used or reflects different molecular organizations of the human and rat receptors. Cross-linking studies with the purified rat liver ASGP-R have provided evidence that RHL-1 and RHL-2/3 form homooligomers, probably hexamers; no mixed oligomers were detected using this approach (Halberg et al., 1987). On the other hand, immunoprecipitation studies with the rat ASGP-R revealed that at least at the plasma membrane a heterotypic complex is formed (Sawyer et al., 1988). Cross-linking in human Hep G2 cells did reveal heterodimers and trimers that contained both forms, H1 and H2 (Bischoff et al., 1988). This might indicate differences in molecular structure between the human and the rat ASGP-R. Nevertheless, one group has reported that in hepatoma cells RHL-1 alone can reach the surface and can be employed in binding, uptake, and degradation of a high specificity ligand, indicating that there also might be cell-specific differences (Braiterman et al., 1989). Clearly, more work is needed to understand these observations.

The other interesting observation in doubly transfected cell lines was the appearance of an additional form of RHL-2/3 which like RHL-3 seems to be derived from the precursor form RHL-2'. Its molecular weight and its ability to affinity purify with the receptor suggests that it might be a form similar to RHL-2 in rat ASGP-R, although it does not acquire complete endo H-resistance (data not shown).

Initial attempts to detect a functional ASGP-R in MDCK cells by binding studies using either asialoorosomucoid or galactosylated bovine serum albumin (Gal-BSA) as ligands failed. This was probably due to the small number of surface binding sites expressed on subconfluent cells and a high nonspecific binding of ligand to the MDCK cells. Nevertheless, we were able to demonstrate specific uptake and degradation of ligand by the double transfectants, but not by cells expressing only one form (Table I), thus confirming the results obtained in nonpolarized cells (McPhaul and Berg, 1986; Shia and Lodish, 1989). This degradation was specific for galactose containing glycoprotein and was inhibited by antibodies to the receptor. Furthermore, it showed a striking polarity because it was only observed when the ligand was added basolaterally. However, the rate of degradation was comparatively low. Only 2,500–10,000 molecules/cell/h were processed with Gal-BSA as ligand. Similar rates were observed with asialoorosomucoid as ligand (data not shown). This degradation rate is only one-tenth of the rate we observed in MDCK cells which exhibits the same polarity as in the

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Functional Asialoglycoprotein Receptor Expression in MDCK

hepatocyte and mediates endocytosis at the basolateral surface.

REFERENCES

Polarized expression of functional rat liver asialoglycoprotein receptor in transfected Madin-Darby canine kidney cells.
L Graeve, A Patzak, K Drickamer and E Rodriguez-Boulan


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