Polarized Endocytosis by Madin-Darby Canine Kidney Cells Transfected with Functional Chicken Liver Glycoprotein Receptor

Lutz Graeve,* Kurt Drickamer,† and Enrique Rodriguez-Boulan*

* Department of Cell Biology and Anatomy, Cornell University Medical College, New York 10021; and † Department of Biochemistry and Molecular Biophysics, Columbia University, New York 10032

Abstract. We have studied the expression of the chicken hepatic glycoprotein receptor (chicken hepatic lectin [CHL]) in Madin–Darby canine kidney (MDCK) cells, by transfection of its cDNA under the control of a retroviral promotor. Transfected cell lines stably express 87,000 surface receptors/cell with a $k_0 = 13$ nM. In confluent monolayers, $\sim 40\%$ of CHL is localized at the plasma membrane. 98% of the surface CHL is expressed at the basolateral surface where it performs polarized endocytosis and degradation of glycoproteins carrying terminal N-acetylglucosamine at an average rate of 50,000 ligand molecules/h. Studies of the half-life of metabolically labeled receptor and of the stability of biotinylated cell surface receptor after internalization indicate that transfected CHL performs several rounds of uptake and recycling before it gets degraded. The successful expression of a functional basolateral receptor in MDCK cells opens the way for the characterization of the mechanisms that control targeting and recycling of proteins to the basolateral membrane of epithelial cells.

Polarized epithelial cells act as a selective barrier in a variety of tissues, separating the external and the internal environment of the organism, yet allowing the directional transport of certain ions and macromolecules. This vectorial function is determined by the asymmetric localization of functional molecules into apical and basolateral plasma membrane domains, separated by continuous tight junctions (Rodriguez-Boulan and Nelson, 1989; Simons and Fuller, 1985). Current evidence indicates that newly synthesized plasma membrane proteins may use different transport pathways in different epithelia. In the renal kidney cell line Madin–Darby canine kidney (MDCK), apical and basolateral membrane proteins share a common route from the endoplasmic reticulum to the trans-Golgi network; here, they are sorted into different transport vesicles that fuse with the respective domain (for review, see Rodriguez-Boulan and Salas, 1989). In contrast, in the hepatocyte all plasma membrane proteins studied to date are first delivered to the basolateral domain, from where apical proteins are transcytosed to their final destination (Bartles et al., 1987; Bartles and Hubbard, 1988). A similar indirect pathway may operate in intestinal cells for at least some apical proteins (Hauri et al., 1979; Massey et al., 1987), although there is evidence for direct delivery in this system (Danielson and Cowell, 1985).

Plasma membrane proteins carry within their structure the information necessary for their polarized segregation and for additional rounds of sorting via endocytosis or transcytosis (Matlin, 1986; Mostov et al., 1986; Russell, 1987; Stephens and Compans, 1988; Iacopetta et al., 1988). The nature of these “sorting signals” is still unknown, but transfection experiments suggest that they, as well as the cellular decoding machinery, are highly conserved in different epithelial cells (Mostov and Deitcher, 1986; Wessels et al., 1989). The MDCK cell line is currently the best characterized model system to test this hypothesis. When grown on permeable support, MDCK cells display in vitro typical properties of natural epithelia (Cereijido et al., 1978; Louvard, 1980; Herzlinger and Ojakian, 1984; Rodriguez-Boulan, 1983, Balsarova-Stander et al., 1984; Vega-Salas et al., 1987). Expression of exogenous plasma membrane proteins in MDCK cells provides an excellent tool to study sorting events during biogenesis and recycling as well as structure/function relationships of receptors from other epithelial cell types, for which in vitro systems are not available yet (Mostov and Deitcher, 1986; Mostov et al., 1986, 1987).

The chicken liver glycoprotein receptor, also known as chicken hepatic lectin (CHL), mediates the uptake of glycoproteins carrying terminal N-acetylglucosamine residues (Ashwell and Harford, 1982). After internalization, the ligand is delivered to the lysosomal compartment for degradation, while the receptor recycles to the cell surface. The receptor is a hexamer formed of identical polypeptides of 207 amino acid residues, for which a type-2 transmembrane orientation has been established, with 23 aminoterminal residues being exposed to the cytoplasmic side and the carboxyterminal end lying in the ectodomain (Drickamer, 1981; Loeb and Drickamer, 1987). Homologous, but structurally

1. Abbreviations used in this paper: ASGP-R, asialoglycoprotein receptor; CHL, chicken hepatic lectin; GlcNAc-BSA, N-acetylgalactosamylated BSA; sulfo-NHS-biotin, sulfo-N-hydroxy-succinimido-biotin.
Materials and Methods

Materials

All cell culture media and supplements were purchased from Gibco Laboratories (Grand Island, NY). Electrophoresis chemicals were from Bio-Rad (Richmond, CA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The polyclonal rabbit antiserum against CHL has been described (Loeb and Drickamer, 1987). The polyvalent receptor was obtained by immunizing BALB/c mice with affinity-purified receptor. The most positive mouse was used for the preparation of mAb 4fl2 by standard procedures (Ausubel et al., 1987).

Cells

MDCK cells, strain II, in the 56th to 70th passage were grown in DME supplemented with 10% horse serum (HyClone Laboratories; Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml), with a 5% CO2 atmosphere at constant humidity. When grown on filters, 2–3 × 106 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 d with changes of medium every 2 d. To assess the integrity of the monolayer, [3H]thymidine (New England Nuclear, Boston, MA) was added to the medium in the apical compartment and the basal medium sampled and counted in a beta-counter (Beckman Instruments, Inc., Palo Alto, CA); chambers showing >1% of the applied counts in the basal medium after 1 h were discarded (Sargiacomo et al., 1989).

DNA Transfection and Isolation of Expressing Clones

The expression vector pCHL 223 has been recently described (Mellow et al., 1988). Plasmid pMV6 tk neo conferring resistance to the antibiotic G418 (Madden et al., 1985) was a gift from Dr. M. Chao (Cornell University Medical College, New York). 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 × 106, low passage) grown for 24 h in DME supplemented with 10% FCS were trypsinized and suspended in 1 ml of DME/10% FCS. The calcium-phosphate-DNA precipitate formed using 10-15 μg plasmid DNA was added to the cell suspension in a 10-cm tissue culture dish. After 30 min incubation at room temperature, DME containing 10% FCS and 100 μg/ml chloroquine was added and the cells were incubated at 37°C for 6 h. The cells were treated for 1 min at 37°C with a 15% (wt/vol) glycerol solution, washed, and incubated in DME/10% FCS for 2-3 d at 37°C until reaching confluency. The cells were split 1:8 into 14-cm dishes containing 20 ml DME supplemented with 10% horse serum and 500 μg/ml G418. The medium was changed every 3–4 d. After 16 d under selection, resistant colonies were trypsinized and cloned by limiting dilution into 96-well microtiter plates.

RIA and Indirect Immunofluorescence

Individual clones were screened for expression by indirect radioimmunoassay. Cells were plated on detachable 50-well plastic petri dishes (Lux, Miles Laboratories Inc., Naperville, IL), grown for 1–2 d and fixed in 2% (wt/vol) paraformaldehyde. After permeabilization with 0.075% (wt/vol) saponin, cells were incubated with a polyclonal rabbit antiserum directed against CHL. Cells were probed with 125Iprot A (0.5–2 × 106 cpm/ml; New England Nuclear) and counted in a gamma counter (Hewlett-Packard Co., Palo Alto, CA).

Procedures for indirect immunofluorescence of MDCK cells were as described (Rodriguez-Boulan, 1983). For intracellular staining, fixed cells were permeabilized with 0.075% saponin. Samples were photographed with an epifluorescence microscope (E. Leitz, Inc., Rockleigh, NJ) using 400 ASA film (Tri-X; Eastman Kodak Co., Rochester, NY). Exposure time was 30 s.

Immunoblotting

For immunoblotting, cells were lysed in 1 ml 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, for 30 min at 4°C. Nuclei were sedimented for 5 min and the supernatant was precipitated by addition of 9 vol of acetone for 30 min at −20°C. Protein samples were subjected to 10% or 12% SDS-PAGE according to Laemmli (1970) and transferred to nitrocellulose as described by Towbin et al. (1979). Nitrocellulose sheets were blocked with 3% (wt/vol) BSA, 2% (wt/vol) nonfat dry milk (Carnation), PBS containing 0.5% (vol/vol) Tween 20, and incubated with a rabbit antiserum against CHL (1:1,000) or mAb 4f12 and rabbit anti-mouse IgG followed by 125Iprot A (1 × 106 cpm) in the same buffer. Blots were exposed to film (XAR; Eastman Kodak Co.) at −80°C using an intensifying screen.

Biotin Assay for Polarity

A water-soluble biotin analogue, sulfo-N-hydroxy-succinimido-biotin (sulfo-NHS-biotin; Pierce Chemical Co., Rockford, IL), was employed to label selectively the apical or basolateral surfaces of filter grown monolayers. MDCK monolayers on filter chambers were washed 4 x with PBS (PBS containing 0.1 mM Ca2+ and 1 mM Mg2+) for 15 min at 4°C each. Sulfo-NHS-biotin (0.5 mg/ml in PBS; freshly diluted from a frozen stock of 200 mg/ml in DMSO) was added to either the apical or the basolateral compartment of the filter chamber. Compartments not 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 serum free medium (1×) and PBS/CM (3×). Filters were excised from the chamber with a scalpel and extracted with 1 ml 1% (vol/vol) Triton/CM, and incubated in a water bath at 80°C. Streptavidin was radiolabeled with Na125I (New England Nuclear) using chloramine T (Greenwood et al., 1963); typically a specific activity of ~5–10 μCi/μg was obtained. The specific activity of ~5–10 μCi/μg was obtained.

Biotin Assay for Endocytosis

A cleavable biotin analogue, sulfo-N-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce Chemical Co.) was used to label the basolateral surface of confluent monolayers as described above. Filters were washed twice with DME/0.2% BSA. Two filters were kept on ice, the others transferred to 37°C for various times. Incubation was stopped by...
transferring filters back to 4°C. After two washes in PBS/10% serum filters were incubated twice for 20 min in reducing solution: 310 mg glutathione (free acid) dissolved in 17 ml H2O; 1 ml of 1.5 M NaI, 0.12 ml of 50% NaOH and 2 ml of serum added just before use (Bretscher and Lutter, 1988). One filter was mock treated. After washing, free SH-groups were quenched in 5 mg/ml iodoacetamide in PBS/1% BSA for 15 min. Cell extracts were immunoprecipitated and analyzed as described above. 12% SDS-PAGE was performed under nonreducing conditions.

Antibody-binding Assay

Cells grown for 4 d on polycarbonate filters were fixed with 2% paraformaldehyde. Monolayers were permeabilized with 0.075% saponin, which was present in all subsequent steps. Permeabilized and nonpermeabilized monolayers were incubated with polyclonal mouse antiserum against CHL (diluted 1:100 in 5% rabbit serum/PBS) added to both sides of the filter for 2 h at room temperature. After extensive washing, 125I-labeled rabbit antimouse IgG (New England Nuclear; 1 μCi/filter, diluted in 5% rabbit serum/PBS) was added for 1 h to both sides of the filter. Filters were excised and counted.

Protease Assay

Clone IG12 cells were grown for 3–4 d on polycarbonate filters. Filters were washed in PBS/5 mM EGTA, excised and incubated in 5 mg/ml Pronase (Boehringer Mannheim GmbH; FRG) in PBS/5 mM EGTA for 2 h on ice. Control filters only received buffer. Digestion was stopped by addition of serum. Cells were pelleted and resuspended three times in PBS/10% serum and finally lysed in hot 2% SDS, 20 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, containing a protease inhibitor mix (2 mM PMSF, 2 μg/ml each of pepstatin, leupeptin, and antipain). Samples were boiled for 10 min and precipitated with acetone. After SDS-PAGE and transfer onto nitrocellulose, CHL was detected as described above. A monoclonal antibody directed against a 35-kD cytoskeletal protein was used to normalize for the protein amount loaded onto the gel.

Binding and Internalization Studies

N-acetyl-glucosylated BSA (GlcNAc-BSA; E-Y. Laboratories, Inc., San Mateo, CA) was radioactively labeled with Na[125I] (New England Nuclear) using the chloramine T procedure at a specific activity of 23-33 cpm/n mole. Binding medium consisted of 0.2% BSA in DME with 25 mM Heps replacing NaHCO3; the pH was adjusted to 7.2. Subconfluent MDCK cells grown for 2 d in 24-well plates were washed with MEM lacking Ca2+ and with binding medium at 4°C. Radiolabeled GlcNAc-BSA was added and cells were incubated for 60 min at 4°C. Nonspecific binding was determined at every ligand concentration by addition of 5 mM EGTA to the binding medium. After 5 washes with binding medium, cells were dissolved in 1 ml of 1 N NaOH and counted. One well was trypsinized to determine the cell number. For uptake experiments on subconfluent cells, cells were grown on 35-mm plates (1 × 106 cells/plate), washed as above and incubated with radiolabeled GlcNAc-BSA in binding medium at 37°C for 3 h. Following incubation, the cells were washed 5× in PBS containing 4 mM EDTA and processed as above. For binding studies on confluent monolayers, cells were grown for 6 d on polycarbonate filters (2.4 × 106 cells/Transwell). Preincubation was in binding medium for 1 h at 37°C. 2 μg/ml [125I]GlcNAc-BSA was added to either the apical or basolateral compartment. The opposite side received unlabeled ligand. Binding was for 2 h at 4°C after which filters were washed five times with binding medium. Filters were cut from the filter chamber with a scalpel and counted. For uptake experiments on confluent monolayers, cells were preincubated in binding medium at 37°C, after which radiolabeled ligand was added to either the apical or basolateral compartment for 1 h at 4°C. Filter chambers then were shifted to 37°C and incubated for various times. Apical and basolateral media were removed and tested for acid-soluble counts by the method of Goldstein and Brown (1974). Filters were washed as above, cut from the filter chambers, and counted.

Results

Expression of CHL in MDCK Cells

The chicken liver glycoprotein receptor (CHL) specific for glycoproteins containing terminal N-acetylgalactosamine residues has been recently expressed from a cDNA in rat fibroblasts, where it was able to bind and endocytose agalacto-orosomucoid (Mellow et al., 1988). Using the same expression vector, we transfected MDCK cells (via calcium-phosphate precipitation) along with pMV6 tk neo (Madden et al., 1985), a plasmid conferring resistance to the antibiotic G418. Transfected cells were grown for 16 d in selection medium, cloned by limiting dilution and screened for expression via an indirect radioimmunoassay of fixed, saponin-permeabilized cells using a polyclonal rabbit antisera directed against CHL followed by 125I-protein A. Four positive clones were expanded further and analyzed by immunoblot (Fig. 1). Three clones (IG12, 2A2, IBI) displayed a band with 25,000 M, the expected molecular mass for the chicken receptor (Mellow et al., 1988). Nontransfected cells (MDCK), uncloned CHL transfants (CHL), and clone 1H10 gave no signal. Most studies were performed with clone IG12, which expressed the largest amount of CHL. The receptor synthesized in MDCK cells was glycosylated; incubation of immunisolated CHL with peptide-N-glycopeptidase F resulted in a molecular mass shift of about 3 kD (data not shown). This is consistent with the fact that the chicken receptor is singly glycosylated on Asn67 (Drickamer, 1981).

To localize CHL in transfected MDCK cells, we first performed immunofluorescence studies on fixed, nonpermeabilized and permeabilized (0.075% saponin) cells (Fig. 2). For these studies a polyclonal mouse antiserum against CHL was used as the first antibody, because the rabbit antisera resulted in a strong unspecific staining on nontransfected

---

Figure 1. Expression of chicken hepatic lectin in transfected MDCK cells. Cell extracts of control cells (MDCK), transfected cells (CHL), and four isolated clones (IG12, 1H10, 2A2, IBI) were subjected to 10% SDS-PAGE. After transfer to nitrocellulose, the blot was incubated with polyclonal rabbit antisera directed against CHL followed by 125I-protein A. The fluorogram of the blot is shown.
MDCK cells, even after affinity purification of the antiserum. The mouse antiserum gave no background staining. On subconfluent cells expressing CHL, a relatively weak surface staining was observed (Fig. 2A). After permeabilization with saponin the perinuclear regions as well as the lateral plasma membranes were clearly stained (Fig. 2B). This indicates that a significant amount of CHL is localized intracellularly.

The amount of the receptor localized at the cell surface and intracellularly was quantitated by two different protocols, an antibody binding assay and a protease assay. For this, confluent monolayers were grown in polycarbonate filter chambers (Transwell) that allowed access to both cell surfaces. Confluent monolayers were fixed and surface and total amount of antigen were measured on nonpermeabilized and permeabilized cells using polyclonal mouse antiserum followed by radiolabeled rabbit anti-mouse IgG. Unspecific binding was determined on control cells not expressing CHL. 36% of the total amount of CHL measured in permeabilized cells was detected at the surface of nonpermeabilized monolayers (Table I). This indicates that ~2/3 of total CHL is localized intracellularly. A similar distribution was obtained by using a protease assay. Confluent monolayers were treated at 4°C with pronase for 2 h. Total CHL and the amount of receptor resistant to protease treatment were determined by SDS-PAGE, Western blot and incubation with mAb 4f12 or rabbit antiserum followed by [125I]protein A. An average of 61% of CHL was resistant to pronase treatment, consistent with the result of the antibody binding assay (Table I).

### Polarity of CHL in MDCK Cells

Although the surface localization of CHL in the chicken hepatocyte has not been analyzed, the rat hepatocyte homologue of CHL, the asialoglycoprotein receptor, is expressed exclusively at the sinusoidal; i.e., basolateral plasma membrane. To study the polarity of expression of CHL in the MDCK cell, a surface-labeling method and a ligand binding assay were employed.

The surface-labeling procedure uses a water soluble, membrane impermeant biotin analogue (sulfo-NHS-biotin), which when added either to the apical or basolateral side of confluent, filter grown monolayers labels only proteins exposed on that surface (Sargiacomo et al., 1989). Clone IGI2 cells were plated at high density in polycarbonate filter chambers and grown for at least 6 d. The integrity of the monolayer was assessed by measuring the [3H]inulin leak from the apical to the basolateral compartment, as previously described (Lisanti et al., 1988). Cells were labeled with sulfo-NHS-biotin from either the apical or basolateral side and cell extracts were prepared and immunoprecipitated with rabbit antiserum against CHL. After SDS-PAGE and transfer to nitrocellulose, the blot was probed with [125I]streptavidin under conditions minimizing nonspecific binding. The pattern obtained (Fig. 3) shows that the chicken receptor is only la-

### Table I. Distribution of CHL in Clone IGI2 Cells

| Localization | Antibody binding assay | Protease assay |
|--------------|------------------------|----------------|
| Surface      | 36%                    | 39%            |
| Intracellular| 64%                    | 61%            |

Antibody binding assay. Control and clone IGI2 cells were grown for 4 d on polycarbonate filters. Surface and total amount of antigen was determined by an antibody binding assay on fixed, nonpermeabilized and saponin permeabilized monolayers as described in Materials and Methods. All determinations were performed in duplicate. Standard errors of the mean were <3%. Specific values were obtained by subtracting binding to control cells. Values were expressed as a percentage of the total. The intracellular amount was obtained as the difference between surface and total amount. Values represent the mean of two independent experiments. Protease assay. Clone IGI2 cells were grown for 3-4 d on polycarbonate filters. Monolayers were treated with 5 mg/ml pronase at 4°C for 2 h as described in Materials and Methods. Cell extracts were analyzed by 12% SDS-PAGE and transferred to nitrocellulose. CHL was detected with mAb 4f12, followed by rabbit anti–mouse IgG and [125I]protein A or polyclonal rabbit antiserum against CHL followed by [125I]protein A. Bands were excised from the nitrocellulose and counted. Determinations were performed in duplicate or triplicate. Values were expressed as a percentage of the total amount (no pronase). Values given are the mean of three independent experiments, SEM <20%. The surface value was obtained as the difference between total and intracellular values.
beled when biotin is added from the basolateral side. The amount of surface receptor was quantitated with a ligand binding assay. N-acetyl-glucosamine-BSA (GlcNAc-BSA) was used as a ligand in these studies. In this neoglycoprotein, an average of 35 saccharide residues are coupled to each protein molecule, making this an efficient ligand for the chicken glycoprotein receptor. Initial binding studies were performed on subconfluent MDCK cells over a range of ligand concentrations at 4°C. Nonspecific binding was determined in the presence of EGTA to chelate Ca²⁺-ions. A typical binding curve is shown in Fig. 4. Scatchard analysis of the data reveals the presence of a single, homogeneous class of high affinity binding sites with an apparent Kₐ of 13 nM at ~87,000 sites/cell. These characteristics are similar to parameters observed in the chicken hepatocyte (Kₐ of 4 nM; 33,000 sites/cell) and in rat fibroblasts expressing CHL (Kₐ of 35 nM; 50,000 sites/cell) determined with agalacto-orosomucoid as ligand (Mellow et al., 1988). Binding studies on filter-grown monolayers were performed at a ligand concentration of 2 μg/ml. At this concentration about 40,000 ligand molecules were bound to each cell, 98% of them to the basolateral plasma membrane (Table II). This result clearly demonstrates that the chicken glycoprotein receptor is highly polarized in MDCK cells and able to bind ligand.

**Function of CHL in MDCK Cells**

To determine whether the receptor expressed in MDCK cells can also internalize and degrade bound ligand, a series of uptake and degradation studies were performed. Clone 1G12 and control MDCK were incubated with [¹²⁵I]GlcNAc-BSA added to either the apical or basolateral compartment of the filter chamber for 3 h at 37°C. Filters were washed under conditions that removed surface-bound ligand, and cell-associated radioactivity was determined. Cells expressing CHL did internalize GlcNAc-BSA in a polarized fashion; the amount of ligand internalized from the basolateral side was 15–30 times higher than the amount taken up from the apical side (data not shown). This is consistent with the basolateral localization of the receptor. Nontransfected MDCK cells showed no uptake. Competition studies revealed that the uptake was specific for proteins carrying terminal N-acetyl-glucosamine (Table III). These results clearly show that the uptake is mediated by the transfected receptor.

In the hepatocyte, glycoproteins internalized by the glycoprotein receptors are delivered to the lysosomes where they are degraded. Fig. 5 shows the result of an experiment in which the uptake and degradation of [¹²⁵I]GlcNAc-BSA by filter-grown clone 1G12 cells was followed over a time course of 6 h. In this case, the ligand was added only to the basolateral side. The cell-associated radioactivity reached a plateau after 1 h, increasing only marginally over the next 5 h. After a short lag period the amount of acid-soluble radioactivity released into the medium increased linearly over the time of the experiment; ~50,000 ligand molecules were degraded per cell in 1 h. Interestingly, the acid-soluble radioactivity was almost exclusively released to the basolateral compartment (Table IV), only 6% could be found apically after 6 h. This amount is comparable to the amount of [³H]inulin that leaks through the monolayer over such a time period. Only 0.1% of ligand added to the basolateral compartment was found on the apical side after 6 h of incubation (data not shown).

**Table I. Polarity of Binding of GlcNAc-BSA in Clone 1G12 Cells**

| Compartments | Molecules bound/cell | Percentage of total |
|--------------|----------------------|--------------------|
| Apical       | 728 ± 420            | 1.9                |
| Basolateral  | 37,864 ± 932         | 98.1               |

Control MDCK and clone 1G12 cells were incubated with 2 μg/ml [¹²⁵I]GlcNAc-BSA added to either the apical or basolateral side of the filter for 2 h at 4°C. The opposite side received unlabeled ligand. Filters were extensively washed in binding medium, excised, and counted in a gamma counter. All determinations were performed on duplicate filters. Nonspecific binding on control cells was subtracted (apical, 3,012 ± 420; basolateral, 7,816 ± 932). The data are representative of two independent experiments.

**Table II. Specificity of Uptake for GlcNAc-BSA in Clone 1G12 Cells**

| Competing ligand | Percentage of control uptake |
|------------------|----------------------------|
| None             | 100                        |
| 100 μg/ml GlcNAc-BSA | 7.2                       |
| 100 μg/ml Gal-BSA  | 94.5                       |

| Competing ligand | Percentage of control uptake |
|------------------|----------------------------|
| None             | 100                        |
| 100 μg/ml GlcNAc-BSA | 7.2                       |
| 100 μg/ml Gal-BSA  | 94.5                       |

Subconfluent MDCK cells (1 × 10⁶ cells/35-mm plate) were incubated for 3 h at 37°C in binding medium containing 2 μg/ml [¹²⁵I]GlcNAc-BSA in the absence or presence of competing and noncompeting ligand. Cells were washed with PBS containing 5 mM EGTA, dissolved in 1 M NaOH and counted in a gamma counter. All determinations were performed in duplicate. SEM <10%.

**Figure 3.** Polarity of chicken hepatic lectin in MDCK cells. Cells (clone 1G12) grown for 8 d on polycarbonate filter chambers were labeled from the apical (A) or basolateral (B) side with 500 μg/ml sulfo-NHS-biotin. Control filters received no biotin (−). Cell extracts were immunoprecipitated and analyzed by 10% SDS-PAGE. After transfer to nitrocellulose, the blot was probed with [¹²⁵I]streptavidin and autoradiographed with an intensifying screen at ~80°C.

**Figure 4.** Binding of [¹²⁵I]GlcNAc-BSA to MDCK cells expressing CHL. Subconfluent cells were incubated with ligand at various concentrations in 0.2% BSA/DME without or with 5 mM EGTA for 60 min at 4°C. Cells were washed five times with binding medium. Specific binding was taken as the difference between binding without and binding with EGTA. All points represent the mean of duplicate determinations.
The Journal of Cell Biology, Volume 109, 1989 2814

for 6 h at 37°C. Acid-soluble radioactivity in the apical or basolateral medium determined as described in Fig. 5. Nonspecific degradation was determined in one being the experiment shown in Fig. 5.

Radioactivity was set 100%. Values presented are the mean of two experiments, the presence of an excess of nonlabeled ligand. Total amount of acid-soluble radioactivity was determined as the difference between processing by cells expressing CHL and control MDCK cells. Each value represents one determination. The values are representative of three independent experiments.

shown). This transport was also observed in nontransfected MDCK cells and in the presence of an excess of unlabeled ligand, indicating that it was nonspecific.

From the values obtained above we can calculate a maximal cycle time for the CHL. Since confluent cells have ~40,000 surface binding sites and that comprises 36–39% of the total receptor number, ~100,000 receptor molecules are expressed per cell. Thus the maximal cycle time for the receptor is ~2 h. This comparatively long cycle time leaves the possibility open that CHL in MDCK cells is not recycling back to the cell surface but is transported to the lysosome along with the ligand where both get degraded. To exclude this possibility, the stability of metabolically labeled CHL was followed over 50 h under conditions of ligand processing. The initial amount of labeled CHL was degraded with a half-life of 16 h ± 3 h (SD) (data not shown). Thus, a mean lifetime of 23 h can be calculated for each receptor molecule. In a second approach, we directly demonstrated that internalized surface receptor is not degraded but stable over at least 4 h, supporting the notion that it is not transported to the lysosome along with the ligand. We labeled the surface receptor on the basolateral side with NHS-SS-biotin, a cleavable biotin analogue, which otherwise has comparable labeling characteristics as sulfo-NHS-biotin. The labeling of surface receptor only slightly impaired its efficiency in uptake and degradation (data not shown). The internalization of labeled receptor was monitored by removing extracellular label through cleavage of the disulphide linkage with glutathione as recently described (Bretscher and Lutter, 1988). The appearance of labeled receptor that is resistant to glutathione reduction was taken as a measure of its internalization. As shown in Fig. 6, all of the label on surface receptor is sensitive to reduction directly after labeling at 4°C (lane 2), but most of it becomes resistant after warming up the cells to 37°C (lanes 3–6). This indicates that most of the labeled surface receptor has been internalized. Nevertheless, the stability of the label over 4 h demonstrates that no receptor was degraded during this period. This is consistent with the notion that the receptor recycles back to the cell surface.

Discussion

We have expressed the chicken liver glycoprotein receptor CHL in the epithelial cell line MDCK. The receptor was almost exclusively localized on the basolateral plasma membrane where it bound and internalized ligand. The endocyted ligand was degraded at a rate of 1 ligand molecule/surface binding site/h. Thus, the transfected receptor seems to function efficiently in MDCK cells.

Several groups have recently reported the expression of functional glycoprotein receptors (from human, rat, and chicken origin) in fibroblasts and hepatoma cells (McPhaul and Berg, 1986; Mellow et al., 1988; Braiterman et al., 1989; Shia and Lodish, 1989). A very recent paper (Wessels et al., 1989) described the polarized expression of one of the two subunits of the human asialoglycoprotein receptor at the basolateral membrane of MDCK cells, but presented no evidence of functional activity. To our knowledge, the results with CHL reported here represent the first reconstitution of a functional and polarized basolateral receptor in an epithelial cell line.

CHL is structurally and functionally related to the human and rat asialoglycoprotein receptors (ASGP-Rs). CHL, however, is a simpler protein consisting of only one polypeptide species. Both ASGP-Rs comprise two forms, designated RHL-1 and RHL-2/3 in the rat, and H1 and H2 in human, making functional expression studies more complicated, since both subunits are apparently required for full func-

![Figure 5. Uptake and degradation of [125]GlcNAc-BSA by filter grown MDCK cells expressing CHL. Cells were preincubated with 2 μg/ml [125]GlcNAc-BSA added from the basolateral side for 60 min at 4°C. Uptake was initiated by transferring the filter chambers to 37°C. At the times indicated, cells were returned to 4°C and washed five times with PBS containing 4 mM EDTA. Filters were excised and counted. Basolateral medium was subjected to TCA precipitation. Acid soluble radioactivity was determined by the procedure of Goidstein and Brown (1974). Specific uptake and degradation was obtained as the difference between processing by cells expressing CHL and control MDCK cells. Each value represents one determination. The values are representative of three independent experiments.](image)

![Figure 6. Endocytosis of surface-labeled CHL. Confluent monolayers of clone G12 cells were labeled at 4°C from the basolateral side with NHS-SS-biotin. Filters were transferred to 37°C for different times or kept on ice. Filters were reduced at 0°C with sulfo-NHS-biotin. The labeling of surface receptor only slightly impaired its efficiency in uptake and degradation (data not shown). The internalization of labeled receptor was monitored by removing extracellular label through cleavage of the disulphide linkage with glutathione as recently described (Bretscher and Lutter, 1988). The appearance of labeled receptor that is resistant to glutathione reduction was taken as a measure of its internalization. As shown in Fig. 6, all of the label on surface receptor is sensitive to reduction directly after labeling at 4°C (lane 2), but most of it becomes resistant after warming up the cells to 37°C (lanes 3–6). This indicates that most of the labeled surface receptor has been internalized. Nevertheless, the stability of the label over 4 h demonstrates that no receptor was degraded during this period. This is consistent with the notion that the receptor recycles back to the cell surface.](image)

### Table IV. Polarity of Release of Processed Ligand by Filter Grown Clone 1G12 Cells

| Compartment   | Percentage of total |
|---------------|---------------------|
| Apical        | 6 ± 0.3             |
| Basolateral   | 94 ± 0.3            |

Cells were incubated with radiolabeled ligand added to the basolateral medium for 6 h at 37°C. Acid-soluble radioactivity in the apical or basolateral medium determined as described in Fig. 5. Nonspecific degradation was determined in the presence of an excess of nonlabeled ligand. Total amount of acid-soluble radioactivity was set 100%. Values presented are the mean of two experiments, one being the experiment shown in Fig. 5.
By analogy to homologous mammalian ASGP-Rs, a preferential sinusoidal localization is expected. In the rat hepatocyte, where the basolateral: apical membrane ratio is 7:1, 98% of the ASGP-R is localized on the basolateral plasma membrane (this represents a sevenfold concentration), as revealed by immunoelectron microscopy cytochemistry (Matsura et al., 1982; Hubbard et al., 1983). Our results indicate that, at steady state, 98% of CHL is localized on the basolateral surface of the MDCK type II cells used in this study, which display a basolateral/apical surface area ratio of 4:1 (von Bonsdorff et al., 1985; Vega-Salas et al., 1987). Thus, the receptor is 12 times more concentrated on the basolateral than on the apical domain in MDCK cells, as measured by the ligand binding assay used in this report. In addition, MDCK cells expressing CHL endocytosed and degraded ligand 15–30 fold more efficiently from the basal than from the apical side.

Subconfluent MDCK cells expressed 87,000 surface binding sites/cell for G1cNAc-BSA. In confluent cells, each cell bound 40,000 molecules of ligand at a ligand concentration of 30 nM (Table I). At this concentration, 70% of the binding sites are expected to be saturated (Fig. 4). This would result in a total surface receptor number of 57,000 for confluent cells. The difference between these two values might indicate that either subconfluent cells express more receptor at their surface, or that the measurement on filter grown monolayers underestimates the number of binding sites because of a reduced accessibility of ligand to the basal side. Nevertheless, one ligand molecule was degraded every hour/surface binding-site. This rate is about one fifth of the uptake rate described for Rat-1 fibroblasts expressing CHL (Mellow et al., 1988) and higher than the uptake rate reported for endogenous transferrin receptor in MDCK cells (each cell expresses 8,000–26,000 surface receptors [Kd = 2 nM] with an uptake of 6,000 transferrin molecules/hour at a ligand concentration of 5 nM [Fuller and Simons, 1986]). The relatively long half-life of CHL (16 h) and the fact that 40% of the total receptor is localized on the cell surface indicates that the continuous degradation of ligand can only be performed through recycling of the receptor. With a mean lifetime of 23 h and a maximal cycle time of 2 h every receptor has to perform at least 11 rounds of endocytosis and exocytosis before it gets degraded.

MDCK cells, like other epithelial cells, not only display an endocytotic pathway leading to the degradation of ligand in the lysosomes, but also are capable of performing transcytosis of receptor–ligand complexes from the basolateral to the apical compartment (Mostov and Deitcher, 1986; Maratos-Flier et al., 1987). The small amount of ligand transport from the basal to the apical side we observed was also displayed in the presence of an excess of nonlabeled ligand and by nontransfected MDCK cells. This suggests that this transport was either paracellular or by fluid phase transcytosis. Although we cannot completely exclude that a small fraction of ligand is transcytosed but remains bound to the receptor, our data demonstrate that sorting of ligand into the lysosomal degradation pathway is highly accurate.

Interestingly, the ligand degradation products were released preferentially to the basolateral side of filter-grown MDCK cells. There are two possible explanations for this result. One is that there exists a highly polarized export system from the lysosome to the basolateral side. In this regard, recycling back from a lysosomal or prelysosomal compartment to the cell surface has been demonstrated in macrophages and certain B lymphocytes, which partially degrade internalized antigen and then present it to effector cells (Unanue, 1984; Pernis, 1985). Alternatively, vectorial release of degraded product may reflect the basolateral localization of the neutral amino acid transport system described in LLC-PK1 and MDCK cells (Rabbit and Karish, 1982; Boerner et al., 1986).

To date, only one exogenous functional receptor had been studied in detail in the MDCK cell line; the polymeric immunoglobulin receptor (Mostov and Deitcher, 1986). After synthesis, this receptor travels to the basolateral domain. There it binds dimeric IgA, is then transcytosed to the apical side and, after proteolytic cleavage, is released as an IgA-secretory component complex. It was demonstrated that the deletion of the cytoplasmic domain of polymeric immunoglobulin receptor prevents endocytosis and leads to a direct transport to the apical domain (Mostov et al., 1986). A mutant receptor lacking both cytoplasmic and membrane-anchoring domains was secreted apically (Mostov et al., 1987). These observations indicate that the luminal domain of polymeric immunoglobulin receptor contains the information for targeting to the apical domain, whereas the cytoplasmic tail may direct the initial transport to the basolateral side. A similar approach to that used with polymeric immunoglobulin receptor may now be used to characterize the signals that direct the targeting, endocytosis, and recycling of CHL, a basolateral receptor.

We thank Andre Le Bivic for his advice and help in the preparation of mAb 4F12 and Doris Wall for critically reading the manuscript.

This work was supported by grants from the National Institute of Health (GM-34107, BRSF 507 RR05 396), American Cancer Society (BC-657), Grant in Aid from the New York Heart Association, and an Established Investigator Award from the American Heart Association to E. Rodriguez-Boulan, a grant from the American Cancer Society (CD-388) to K. Drick-amer, and a research stipend from the Deutsche Forschungsgemeinschaft to L. Graeve.

Received for publication 17 March 1989 and in revised form 21 August 1989.

References
Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. Annu. Rev. Biochem. 51:531–554.
Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current Protocols in Molecular Biology, Vol.

Graeve et al. Basolateral Endocytosis by Transfected CHL 2815
