Endocytosis via coated pits mediated by glycoprotein receptor in which the cytoplasmic tail is replaced by unrelated sequences

François Verrey*, Thierry Gilbert†, Thomas Mellow*, Gary Proulx*, and Kurt Drickamer*

*Department of Biochemistry and Molecular Biophysics
Columbia University
New York, New York 10032

†Department of Cell Biology and Anatomy
Cornell University Medical College
New York, New York 10021

Rat 6 fibroblast cell lines expressing wild-type chicken liver glycoprotein receptor (CHL) or chimeric receptors with alternate cytoplasmic tails were produced to study the role of the cytoplasmic tail in mediating receptor localization in coated pits and endocytosis of ligand. Cells expressing CHL or cells expressing a hybrid receptor that contains the cytoplasmic tail of the asialoglycoprotein receptor display high-efficiency endocytosis of *N*-acetylglucosamine-conjugated bovine serum albumin in experiments designed to measure an initial internalization step, as well as in studies of continuous uptake and degradation. Substitution of the cytoplasmic tail by the equivalent domain of rat Na,K-ATPase β subunit or by a stretch of *Xenopus laevis* globin β chain does not abolish endocytosis but decreases the endocytosis rate constant from 15%–16%/min to 2.4% and 6.5%/min, respectively. Electron microscopy was used to visualize the glycoprotein binding sites at the surface of Rat 6 cells transfected with the various receptors. The percentage of receptors found in coated areas ranged from 32% for CHL to 9% for the Na,K-ATPase hybrid, indicating that clustering in coated pits correlates with efficiency of endocytosis. We concluded that replacement of the CHL cytoplasmic

tail with unrelated sequences does not prevent, but decreases to varying extents, coated-pit localization and endocytosis efficiency. The construct with NH₂-terminal globin tail lacks a signal for high-efficiency localization in coated pits but nevertheless is directed to the pits by an alternative mechanism.

Introduction

Cell-surface receptors mediate rapid endocytosis of selected extracellular molecules. These receptors cluster in clathrin-coated pits, whereas proteins that remain stably at the cell surface are excluded from the coated portions of the plasma membrane. It appears that signals within the receptor polypeptides must direct them to the coated pits. Evidence for the identity of these signals has been obtained from studies of a number of receptors. Analysis of naturally occurring mutations at the low-density lipoprotein (LDL) receptor locus demonstrated that the cytoplasmic domain of this protein mediates collection in coated pits and rapid endocytosis (Brown and Goldstein, 1986). Site-directed mutagenesis and expression of an LDL receptor cDNA in hamster cells allowed Davis *et al.* (1987) to confirm the crucial role played by the cytoplasmic tail, and by a tyrosine residue within this domain, in conferring on the receptor the ability to perform rapid endocytosis. A specific role for the cytoplasmic tails of this and other endocytic receptors is also suggested by studies in which they have been shown to compete for binding to adaptor proteins of coated pits (Pearse, 1988). Interaction with adaptors is thought to mediate receptor collection in the pits.

Further evidence that a signal localized within the cytoplasmic portion of membrane proteins can mediate sorting was obtained by modifying the short cytoplasmic tail of influenza virus hemagglutinin. Introducing a tyrosine residue into the tail confers on hemagglutinin the ability to be internalized at a rate similar to that of endocytic receptors, whereas wild-type hemagglutinin is excluded from coated pits and is in-

Abbreviations used: BSA, bovine serum albumin; CHL, chicken hepatic lectin (chicken liver glycoprotein receptor); GBC, *Xenopus laevis* globin β chain–CHL chimeric receptor; LDL, low-density lipoprotein; Na,K-ATPase, Na⁺,K⁺-activated adenosine triphosphatase; NKC, Na,K-ATPase β subunit–CHL chimeric receptor; RC, RHL-1–CHL chimeric receptor; RHL-1, rat hepatic lectin-1 (rat asialoglycoprotein receptor).

ternalized very slowly (Lazarovits and Roth, 1988). The modified hemagglutinin tail was shown to compete for binding to adaptor proteins (Pearse, 1988), again suggesting that interaction with these proteins is responsible for the clustering in coated pits.

A morphological study of L-cells transfected with human transferrin receptor further underlined the role of interactions between cytoplasmic tails of endocytic receptors and cytoplasmic components of coated pits. This study demonstrated that hyperexpression of wild-type receptor increases the number of coated pits found at the cell surface, whereas hyperexpression of receptor with a cytoplasmic tail deletion does not (Iacopetta *et al.*, 1988).

The results just summarized provide evidence that a positive signal, consisting at least in part of a tyrosine residue, located within the cytoplasmic tail of plasma membrane proteins, can mediate the proteins' localization in coated pits. However, it should be noted that the transfection experiments with mutated LDL receptor—as well as more recent experiments on the 275-kDa mannose-6-phosphate receptor (Lobel *et al.*, 1989)—show that deleting part of the cytoplasmic tails of endocytic receptors or removing cytoplasmic tyrosine residues does not completely abolish their ability to internalize ligand. This suggests that the absence of a tyrosine in the cytoplasmic tail of plasma membrane proteins does not prevent a certain degree of coated-pit localization. In this case, other structural features of the membrane proteins might be involved in modulating the level of coated-pit localization.

Chicken liver receptor for partially deglycosylated proteins (CHL, chicken hepatic lectin) is an endocytic receptor composed of six identical polypeptides (Loeb and Drickamer, 1987). It shares with its mammalian homologue, the asialoglycoprotein receptor, a type II transmembrane orientation with a cytoplasmic N-terminus. Because the C-terminal 130 amino acid residues of each CHL polypeptide form the ligand-binding domain, only ~70 amino acids toward the N-terminus remain to provide membrane anchoring and intracellular routing signals. This structural simplicity makes CHL an ideal model for studying the role of different polypeptide domains in mediating receptor movement during endocytosis. It is also convenient that CHL expression is restricted to avian liver cells, so that wild-type fibroblast lines can be used as background-free recipients for transfection studies.

We show here that replacement of the cytoplasmic tail of CHL with unrelated sequences does not prevent chimeric receptors expressed in Rat 6 fibroblasts from entering the endocytic pathway through coated pits. However, these changes can modify the enrichment of receptors in coated pits and consequently their uptake rate. Both the rate of uptake of available surface receptors and the fraction of surface receptor available for endocytosis are dependent on the nature of the cytoplasmic tail.

Results

Generation of receptors with altered cytoplasmic domains

A cDNA for CHL has previously been expressed in rat fibroblasts and shown to direct the synthesis of a functional receptor (Mellow *et al.*, 1988). To probe the role of the cytoplasmic domain, this cDNA was initially modified to produce truncations of 18 or 21 amino acids at the intracellular N-terminus of the receptor. In vitro translation experiments in the presence of microsomes demonstrated that the mutated cDNAs direct the synthesis of membrane-anchored polypeptides having the expected size. However, screening of transfected cell clones by indirect immunofluorescence and immunoblotting showed that the truncated receptors are not efficiently expressed. This suggested that these mutants are poorly translated or unstable in cells, which precluded further functional investigations of these truncated receptors.

As an alternative approach to investigating the functional significance of the cytoplasmic tail, the 21 N-terminal amino acids of this domain were replaced with other sequences. Figure 1 compares the structure of wild-type CHL with the hybrid receptors that have been created. In the first construct (RC), the cytoplasmic domain of CHL was replaced by the equivalent domain of a related endocytic receptor, the major component of the rat asialoglycoprotein receptor (rat hepatic lectin-1 [RHL-1]; Holland *et al.*, 1984). This receptor is a mammalian homologue of CHL and shares the same transmembrane orientation (type II arrangement with N-terminus cytoplasmic). Although the extracellular domains of these receptors are homologous, there is no recognizable sequence similarity in their cytoplasmic segments. A second hybrid construct (NKC) was made with the cytoplasmic tail of rat Na⁺- and K⁺-activated adenosine triphosphatase (Na,K-ATPase) β subunit, the smaller subunit of the plasma

Figure 1. Organization of CHL and amino acid sequence of the cytoplasmic tail of wild-type and chimeric receptors. Amino acids are indicated in the single letter code and are numbered starting with the first amino acid of the mature wild-type protein. Amino acids from other proteins that were included in the chimeric receptor constructs are in **bold**. The hatched boxes represent transmembrane domains.

membrane sodium pump. This protein also has a type II transmembrane orientation but is not an endocytic receptor. For the third chimeric receptor, the cytoplasmic tail of CHL was replaced by the 11 N-terminal amino acids of the β subunit of *Xenopus* globin, a protein that is not a membrane protein.

High-affinity binding of GlcNAc-BSA by transfected cells

As an initial characterization of the function of the mutant receptors, surface expression of binding sites and binding affinities were measured by the use of radiolabeled *N*-acetylglucosamine-conjugated bovine serum albumin (^{125}I -GlcNAc-BSA) as ligand in the absence and

presence of excess unlabeled ligand; the difference between these values was taken as specific binding to the expressed receptor. No specific binding was detected on Rat 6 cells or on cell lines transfected with truncated receptor. Scatchard analysis of the binding experiments performed at 4°C showed a linear relationship between bound/free ligand and bound ligand for wild-type CHL and for the three chimeric receptors over a ligand concentration range of 0.1–60 nM. This allowed us to deduce the number of high-affinity binding sites and the dissociation constant (K_d) for each receptor (see Table 1). The only deviation from linearity was observed at the highest ligand concentration with receptor NKC. In this case, a slightly higher number of ligand molecules are bound at 60 nM

Table 1. Surface-binding sites for GlcNAc-BSA on Rat 6 cells expressing transfected CHL or chimeric receptors

Receptor	K_d (nM)	GlcNAc-BSA bound/cell (molecules $\times 10^3$)	Number of cell lines examined	Number of determinations
CHL	1.9 ± 0.7	7.1–25	2	4
RC	2.5 ± 0.9	6.9–65	4	5
NKC	2.4 ± 0.3	3.6–40	3	5
GBC	2.6 ± 2.3	6.8–14	3	4

Equilibrium binding at 4°C was performed in the absence and presence of a 20-fold excess of unlabeled ligand as described under Experimental procedures. The number of specific ligand-binding sites and the K_d were determined for each binding experiment by Scatchard analysis. The range for the number of surface-binding sites obtained for the different cell lines and the mean binding affinity $\pm$ standard deviation are indicated.

ligand concentration than expected for a single type of high-affinity binding site. The number of high-affinity binding sites found at the cell surface ranges from 3000 to 65 000/cell, depending on the cell line and on the transfected receptor. In spite of this wide variation, no correlation was found between number of binding sites and binding affinity.

In interpreting the binding data, it is important to bear in mind that CHL forms an oligomer with multiple carbohydrate-binding sites, each with moderate binding affinity. High-affinity binding observed with multivalent ligands can be explained by the simultaneous interaction of multiple GlcNAc residues in the ligand with oligomeric receptor. This complex situation makes different binding stoichiometries and affinities possible. The fact that wild-type and chimeric receptors each display a single type of high-affinity binding site, all with similar K_d s, suggests that all receptors can form oligomers with a similar number of carbohydrate-binding sites. The deviation from linearity observed in the Scatchard plot of receptor NKC, with an unexpectedly large number of binding sites at high ligand concentration, could be due to the presence of a receptor subpopulation presenting a lower number of carbohydrate-binding sites and consequently a lower affinity for the multivalent ligand. Because functional tests of endocytosis were performed with the use of ligand concentrations only a fewfold higher than the high-affinity K_d , the receptors corresponding to the high-affinity binding sites were measured in all cases.

Continuous uptake of ligand by transfected cells

Transfected cells were incubated with ^{125}I -GlcNAc-BSA (minus and plus excess unlabeled ligand) at 37°C for different times as a first approach to measuring their ability to internalize ligand by receptor-mediated endocytosis. At the end of the incubations, the amount of ligand bound at the cell surface, the amount retained in the cells, and the amount that was degraded and released into the medium were measured. An example of a time course for each receptor is shown in Figure 2. These experiments revealed two important facts:

1) Cell surface-bound ligand molecules rapidly reach a steady number. For all receptors, the number of binding sites observed after a 10-min incubation is $\geq 80\%$ of the value at later time points. The number of surface-binding sites remains stable over 5 h, indicating that the con-

tinuous uptake of ligand does not modify the size of the surface pool of receptors. The slight decrease in cell surface and intracellular radioactivity for receptor RC and CHL at 5 h can be accounted for fully by decreased ligand concentration in the incubation medium resulting from processing by the cells.

2) CHL and the three chimeric receptors confer on cells the ability to perform specific uptake of ligand. Intracellular radioactivity increases over the first hour of incubation and then remains stable. Degraded ligand reappears in the medium after a lag period of 10–30 min and subsequently increases nearly linearly over 5 h. However, the efficiency of the uptake is clearly lower for GBC and NKC than for wild-type receptor and RC. Rat 6 cells transfected with truncated receptor were used as a negative control for receptor-mediated endocytosis because they lack functional surface receptors for GlcNAc-BSA. For these cells, the amount of internalized and processed ligand is identical in the presence and absence of excess unlabeled ligand, indicating that no specific uptake takes place in the absence of binding sites at the surface.

Because a nearly steady state of ligand binding and uptake is rapidly reached for all functional receptors, uptake of GlcNAc-BSA can be considered, over the course of the experiment, as a continuous process that fits a first-order kinetic model, as shown previously for other endocytic receptors (Wiley and Cunningham, 1981; Schwartz *et al.*, 1982). This allowed us to calculate from the experimental data an endocytic rate constant k_e , which corresponds to the percentage of the cell surface-bound ligand molecules endocytosed per minute (see Experimental procedures). For all receptors, the values of k_e , calculated from the data of Figure 2, are highest during the first 10-min period, decrease over the next 20 min, and remain stable for the later time points. The higher initial uptake rate does not significantly influence the rate constant measured over long incubation periods.

To compare quantitatively the endocytic rate constant of wild-type and mutant receptors, we measured, in two or three cell lines for each receptor, the distribution of the ligand after a 2-h incubation at 37°C in the presence of 1 $\mu\text{g}/\text{ml}$ ^{125}I -GlcNAc-BSA (Table 2). The uptake rates calculated for both CHL and RC show that Rat 6 cells expressing these receptors internalize and degrade nearly 10 ligand molecules per hour per surface-bound ligand molecule (16% and 15%/min, respectively). This result is inter-

Figure 2. Continuous uptake of GlcNAc-BSA by Rat 6 cells transfected with CHL and chimeric receptor cDNAs. Cells were incubated with $1 \mu\text{g/ml}$ ^{125}I -GlcNAc-BSA for the indicated times. After washing the cells, the radioactivity of cell-surface-bound ($\square$), intracellular ($\circ$), and degraded ligand found in the medium (Δ) was determined. The scales for the radioactive ligand were chosen to obtain roughly similar levels of surface-bound ligand for all cell lines. Each point represents the mean of duplicate measurements. Nonspecific binding, measured in duplicates with 20-fold excess of unlabeled ligand, was subtracted from each value.

mediate between the numbers calculated for endocytosis of LDL by human LDL receptor expressed in hamster cells (7%–12%/min; Davis *et al.*, 1987) and for β -galactosidase endocytosis

by bovine mannose-6-phosphate receptor expressed in mouse L cells (40%/min; Lobel *et al.*, 1989), indicating that CHL performs endocytosis at a rate that is similar to other transfected

Table 2. Continuous uptake and degradation of GlcNAc-BSA by cell lines expressing transfected receptors

Receptor	Uptake rate constant k_e (%/min)	Number of cell lines examined	Number of determinations	Specificity ratio
CHL	16 ± 2	2	6	21 (9–41)
RC	15 ± 1	2	6	34 (14–67)
NKC	2.4 ± 0.3	3	5	7.9 (2.0–13)
GBC	6.1 ± 0.9	3	6	13 (2.2–23)

Continuous uptake experiments were performed over 2 h in the absence and presence of a 20-fold excess of unlabeled ligand. The specificity ratio represents specific uptake divided by uptake in the presence of excess unlabeled ligand. The mean is indicated for each receptor and the range is given in parentheses. Data for the specific uptake were used to calculate the endocytic rate constant as described under Experimental procedures. The means $\pm$ standard error from experiments performed on two to three cell lines per receptor are indicated.

endocytic receptors. Receptor GBC is also effective in mediating endocytosis of ligand, with an endocytosis rate constant only two to three times lower than that of CHL and RC. Receptor NKC is less efficient than receptor GBC by a factor of ~ 2 , but cells bearing this receptor still internalize 1.4 ligand molecules per hour per surface-bound molecule. As indicated by the specificity ratio in Table 2, even for this least efficient construct, receptor-mediated uptake was at least twofold greater than the unspecific uptake. Little variation of the endocytic rate constant was observed for one receptor expressed in different cell lines. Furthermore, there was no correlation of uptake rate constants with the number of cell-surface binding sites for any of the four receptors. Therefore, this parameter is suitable for the characterization of the endocytic function of a receptor.

Uptake of prebound ligand by transfected cells

Continuous uptake experiments measure a steady accumulation and degradation of ligand by cells expressing endocytic receptors. The derived ligand internalization rates (k_e) are not only dependent on receptor internalization kinetics but also on ligand binding, intracellular receptor-ligand dissociation, receptor recycling, and ligand routing to lysosomes or back to the cell surface. To focus on the internalization step, initial uptake experiments were performed using prebound ligand. Preliminary experiments showed that cells begin to internalize surface-bound ligand immediately on warming and that degraded ligand appears in the medium > 15 min after the temperature shift.

To analyze precisely the initial kinetics of uptake for the different receptors, short incubations at 37°C were performed on two or more cell lines per receptor. The average percentages of uptake at different time points are shown in Figure 3. Two features of this internalization time course had to be taken into account to fit theoretical curves to the experimental points. First, $\sim 10\%$ of the surface-bound ligand is not eluted from the cells by an acid wash when cells are not rewarmed after the binding step. We designate this fraction the "acid-resistant surface pool." Second, the rapid internalization process does not plateau at 100% but at 70%–80% of the total ligand initially bound at the cell surface. It is unlikely that the 20%–30% of ligand molecules that can be eluted by an acid wash after prolonged incubations are released

from inside the cells. Indeed, identical acid treatment in the continuous uptake experiments elutes a steady number of surface bound ligand, whether or not the cells have accumulated ligand. It is more likely that a fraction of surface-bound ligand is not participating in the rapid endocytosis. We designate this subpopulation the "residual surface pool" of ligand. The subpopulation that participates in the rapid uptake is referred to as the "rapid endocytosis pool."

By restricting the kinetic analysis of the early uptake to this last ligand pool, we obtained close fits to a first-order kinetic model. The parameters k_{re} (uptake rate constant for the rapid endocytosis pool) and $[LR]_{s,0}$ at t_0 (initial size of the rapid endocytosis pool) derived by this method are given in Table 3. The rate constant corresponds to the fraction of the rapid endocytosis pool that would be internalized in 1 min at the initial internalization rate. Knowing the size of the residual surface pool we can calculate an equivalent to k_e (the rate constant obtained from the continuous uptake situation), which gives the uptake rate relative to the total acid-sensitive surface pool (k'_e). This rate constant for prebound ligand (k'_e) of CHL-expressing cell lines (36%/min) is similar to the number obtained by Schwartz *et al.* (1982) for the endocytosis of asialo α_1 -acid glycoprotein by the asialoglycoprotein receptor of human hepatoma cell line HepG2 (46%/min). Furthermore, initial uptake curves of GlcNAc-BSA and transferrin by transfected Rat 6 cells are nearly superimposable for the first 3 min, before transferrin is recycled back to the cell surface (F. Verrey, unpublished data). This indicates that CHL in Rat 6 cells behaves similarly to an endogenous receptor with a type II transmembrane orientation.

Uptake experiments of prebound ligand are clearly not ideal because redistribution of surface proteins can take place at temperatures as cold as 0°C (Ziomek *et al.*, 1980) and because cooling followed by rewarming seems to have an effect on the number of coated pits (Anderson *et al.*, 1977a; Miettinen *et al.*, 1989). Nonetheless, the values for the rate constants obtained by this method are similar to the values obtained in continuous uptake experiments (see Tables 2 and 3) and confirm the hierarchy of uptake efficiency for the four receptors. This result also confirms the fact that the endocytosis rate constant for steady-state uptake of GlcNAc-BSA reflects mainly the internalization of ligand. The difference in initial uptake versus continuous uptake observed for CHL and re-

Figure 3. Uptake of prebound GlcNAc-BSA by Rat 6 cells transfected with CHL and chimeric receptor cDNAs. After binding ^{125}I -GlcNAc-BSA ($0.5 \mu\text{g/ml}$, minus and plus excess unlabeled ligand, 2 h at 4°C) to the cell surface and washing off unbound ligand, we allowed internalization to proceed at 37°C for the indicated times. The cells were then chilled, surface-bound and internalized ligand were measured, and values for specific binding and uptake were determined by subtracting competition controls. The internalized ligand is given as a fraction of the total ligand, and each point is the average of two to seven different experiments performed on two to three cell lines. The vertical bars represent standard errors. The horizontal lines separate three ligand pools: the acid-wash-resistant surface pool (AR), the rapid endocytosis pool (RE), and a residual surface pool (SR). The curves and the upper limit of RE have been derived from the experimental data by the use of a nonlinear least-squares fit program. First-order kinetics were assumed for the initial rapid uptake, which is restricted to a subpopulation of ligand (RE).

ceptor NKC might reflect the recycling of undegraded ligand to the cell surface: ligand molecules that are temporarily "hidden" inside the cells could appear internalized in the initial uptake assay but would not contribute significantly to the irreversible uptake and degrada-

tion measured in the continuous uptake assay. This phenomenon could also explain why the uptake rate constant appeared somewhat higher during the first 10 min than at later points of the continuous uptake experiments (see above).

Table 3. Uptake of prebound GlcNAc-BSA by cell lines expressing transfected receptors

Receptor	Rapid endocytosis pool (RE) (% of elutable ligand)	Internalization rate constant (relative to RE pool) k_{re} (%/min)	Endocytosis rate constant (relative to elutable ligand) k_e (%/min)
CHL	71	51	36
RC	67	22	15
NKC	34	11	3.7
GBC	61	9	5.5

The size of rapid endocytosis pool and the internalization rate constant k_{re} have been derived from the data presented in Figure 3 by using a nonlinear least-squares fit program. The rate constant k_e was calculated from k_{re} as described under Experimental procedures.

Localization of cell-surface GlcNAc-BSA binding sites

The hybrid receptors with nonreceptor cytoplasmic tail sequences internalize the ligand at an unexpectedly high rate; therefore, it was of interest to investigate whether internalization of GlcNAc-BSA by these chimeras is mediated by clathrin-coated pits. If this is the case, and if coated-pit localization is independent of the presence of ligand, then the fraction of surface-binding sites found over coated membrane should be proportional to the initial ligand uptake rate. To test this possibility, gold-labeled ligand was bound at 4°C to cells expressing the different receptors. After unbound ligand was removed by washing, cells were fixed and processed for electron microscopy. Nonspecific binding was measured by using a 100-fold excess of unlabeled ligand to compete with the gold conjugate and by testing a gold-labeled ligand with a carbohydrate moiety not recognized by the receptor (galactose-BSA).

Examples of micrographs from cells expressing the wild-type and the chimeric receptors are shown in Figure 4, and the quantitation of the gold particles is given in Table 4. Differences in total number of gold grains found in the tested cell lines are due to the different levels of receptor expression. The number of gold grains observed in the competition experiments was less variable and not significantly different from the number of gold particles observed when galactose-BSA-gold particles were used instead of GlcNAc-BSA-gold. Values for specific binding were obtained by subtracting the competition values from the total number of grains.

Rat 6 fibroblasts grown on cell culture dishes have narrow, footlike extensions on which ~21% of the nonspecifically bound gold grains are found. About 12% of the gold particles from test experiments are associated with these extensions, indicating that only a small percentage

of the total receptors are located on these processes. These extensions have been neglected in the measurement of the cell contour length.

The number of coated areas per millimeter cell contour is higher in Rat 6 cells (108–220 coated areas per millimeter cell surface length) than in human fibroblasts (30.4 ± 5.5 per millimeter; Anderson *et al.*, 1977a), giant HeLa cells (30–100 per millimeter; Bretscher and Thomson, 1983), mouse L-cells (11.5 per millimeter; Iacopetta *et al.*, 1988), or CHO cells (46–76 per millimeter; Miettinen *et al.*, 1989). Although there is some variability in the number of coated areas, it would be surprising if their number is significantly modulated by the presence of the transfected receptors or the ligand, because the level of transfected receptor expressed at the cell surface is very much lower than the level of transferrin receptor reported to induce an increase in coated-pit formation (Iacopetta *et al.*, 1988). To estimate the fraction of the cell surface occupied by coated pits, the size of coated areas was measured. The mean length of coated membrane segments is $0.31 \pm 0.12 \mu\text{m}$ ($n = 18$), with fully formed pits having, on average, ~25% longer coats than shallow pits. This result is comparable with the length measured in other cells (Anderson *et al.*, 1976, Bretscher *et al.*, 1980). Because 108–220 coated areas per millimeter cell contour were counted on the examined sections, it can be concluded that 3%–7% of the plasma membrane is coated. As expected, this value is somewhat more than reported for the other cells discussed above. This feature of Rat 6 cells increases the probability that a surface protein with a random distribution will be located in a coated pit. The high number of coated pits found in Rat 6 cells allows ready quantification of endocytosis events that would be close to background level in other cell types.

The same hierarchy as in the functional endocytosis experiments was found for the as-

sociation of the different receptors with coated pits, suggesting that internalization of CHL, as well as the chimeric receptors, takes place preferentially through coated pits. CHL and the chimeric receptor RC are most effectively concentrated, with 32% and 22%, respectively, of the specifically bound gold grains associated with coated pits. These numbers are similar to the results obtained for other endocytic receptors with the use of labeled ligand or anti-receptor antibodies: 50%–80% LDL receptor of human fibroblasts (Anderson *et al.*, 1976, 1977a,b), 58% of asialoglycoprotein receptor of rat hepatocytes (Wall and Hubbard, 1981), 25%–45% of transferrin receptor of K526 cells (Watts, 1985), and 12%–19% of human transferrin receptor expressed at very high level in mouse L-cells (Iacopetta *et al.*, 1988) are localized in coated areas. In cell lines expressing the receptors NKC and GBC, 9% and 14% of the labeling, respectively, is associated with coated areas. These numbers are slightly higher than would be expected if there were an absolute proportionality between the internalization rates and the extent of clustering in coated pits. Several factors could contribute to the proportionally high level of clustering observed with the receptors NKC and GBC. One possibility is that a fraction of the coated membrane areas observed by electron microscopy is not functionally active. Such inactive coated areas might be present throughout the experiment or they might appear during the cooling and fixation of the cells. Distribution of receptors with different tails between active and inactive coated areas might well differ. Another possibility is that a subpopulation of the ligand-receptor complexes does not dissociate on internalization and returns very rapidly to the cell surface, so that it would not appear as internalized, even in uptake experiments of prebound ligand.

Comparison of the results obtained in the absence of excess cold ligand with those obtained in the competition experiments, where <2% of the gold grains were found associated with coated areas, would suggest that all tested receptors, even NKC, are much enriched in these specialized membrane areas. However, because 3%–7% of the Rat 6 cell plasma membrane is clathrin coated, this result indicates that the number of gold grains found over coated areas in the competition experiment is less than proportional to the coated surface. We can therefore conclude from these experiments that not only CHL and the chimeric receptor RC, but also receptor GBC, are clearly concentrated in coated pits. Receptor NKC

might be slightly enriched but in any case is not excluded from coated areas.

Discussion

Mechanism for coated-pit localization of chimeric receptors

The fact that the hybrid receptor RC performs endocytosis almost as efficiently as CHL is perhaps not surprising, because its cytoplasmic tail is that of an endocytic receptor (RHL-1). It appears likely that these two receptors use the same high-efficiency pathway for endocytosis as the LDL receptor, the modified influenza virus hemagglutinin, or the mannose-6-phosphate receptor. Unlike these three proteins, CHL and receptor RC have NH₂-terminal cytoplasmic tails. Although both these type II membrane proteins share with the type I receptors the presence of a tyrosine residue within the cytoplasmic domain, there is at present no evidence whether the NH₂-terminal domains compete with the COOH-terminal peptides for binding to coated-pit adaptor molecules.

The chimeric receptors NKC and GBC, which have no tyrosine residues in their cytoplasmic domains, are less efficiently localized to coated pits than the two receptors with endocytic receptor tails. Nonetheless, a significant fraction of these chimeric receptors is still located in coated pits (9% and 14%, respectively) and this fraction is slightly higher than expected for random distribution at the cell surface (because 3%–7% of the cell surface of Rat 6 cells is coated). To explain this result, one can either postulate the presence of a positive signal for coated-pit localization within the cytoplasmic domain of GBC and NKC or, alternatively, raise the question whether a mechanism different from the interaction of a cytoplasmic signal with adaptor proteins could facilitate localization of these hybrid receptors in coated pits. Five fundamentally different mechanisms that could possibly produce the observed effect are discussed below. The results presented here are potentially consistent with any of these possibilities.

- 1) It could be envisaged that the Na,K-ATPase β subunit tail has some affinity for adaptor proteins in coated pits because there is evidence for endocytosis of Na,K-ATPase molecules (Pollack *et al.*, 1981; Jesaitis and Yguerabide, 1986; Fuch *et al.*, 1989). This possibility cannot be excluded, but appears less likely in view of the even more efficient coated-pit localization and internalization measured for

Figure 4. Localization of surface-binding sites for GlcNAc-BSA-gold particles in Rat 6 cells transfected with CHL and chimeric receptor cDNAs. After binding GlcNAc-BSA-gold particles (10 nm) for 2 h at 4°C and washing off unbound ligand, we processed cells for electron microscopy. In Rat 6 cells expressing CHL (A), 32% of specifically bound gold particles were

receptor GBC. The 11 N-terminal amino acids of globin β chain, which form the cytoplasmic tail of the receptor GBC, have been chosen as a neutral sequence with respect to endocytosis and contain no aromatic residue.

2) In addition to its cytoplasmic signal, CHL might have a second region within the membrane or in the extracellular domain that directs it to coated pits. This second signal could interact with another transmembrane protein that has a cytoplasmic signal for coated-pit localization. Interestingly, another chimeric receptor with Na,K-ATPase β subunit tail, transmembrane region, and neck attached to the CHL ligand-binding domain internalizes GlcNAc-BSA at a rate similar to that of NKC (Verrey, unpublished data). This result suggests that, if there is a "piggyback signal," it is unlikely to be localized in the transmembrane domain or neck of CHL.

3) Receptor clustering induced by multivalent ligand could direct the receptor to coated pits. This ligand-induced clustering hypothesis would imply that receptors are able to cluster in coated pits at 4°C. However, it has been difficult to document that oligomerization induced by multivalent ligand plays a role in coated pit-mediated internalization of plasma membrane proteins. Ligand-induced interaction of receptors seems to trigger rapid internalization of the epidermal growth factor receptor (Schlessinger, 1986); however, in this case, interaction between receptor molecules seems to be part of the regulatory mechanism, and crosslinking is not due to the multivalency of the ligand. Efficient internalization of the neutrophil Fc receptor type III (Fc γ RIII, CD16), is triggered by ligand-induced crosslinking of this receptor (which has a phosphatidylinositolglycan membrane-anchor) to the Fc γ RII receptor (which has a cytoplasmic peptide tail) (Selvaraj *et al.*, 1988). This type of mechanism is not likely for receptor NKC and GBC, because untransfected Rat 6 cells do not express receptors for GlcNAc-BSA to which ligand would crosslink them. Finally, antibody-mediated clustering and internalization of other transmembrane proteins, such as HLA or maltase, do not involve coated pits (Huet *et al.*, 1980; Kerjaschki *et al.*, 1984).

4) The receptor and the coated pits could be concentrated in domains of the cell surface,

thereby increasing the local concentration of receptors in the vicinity of coated pits. Such an effect could be obtained if recycling receptors are delivered to the plasma membrane only in the immediate vicinity of coated pits. Alternatively, receptors and pits could both be excluded from certain domains by diffusion barriers. The results obtained by Bretscher and Thomson (1983) on giant HeLa cells argue against this possibility, because they found a roughly continuous density of coated pits over the cell surface, although recycling receptors are returned to the leading edge of the cells. However, it is still possible that the receptors and the coated pits could be excluded from numerous microscopic surface domains not identifiable by conventional microscopic examination.

5) It is also possible that exclusion from coated pits might play a role in modulating the internalization rate of membrane proteins. This hypothesis implies, as does the previous one, that specific interaction with coated-pit components is not always a prerequisite for localization in coated areas of the plasma membrane. Space for other membrane proteins—which are not actively excluded—would be available in coated areas, beside the specific binding sites for endocytic receptors. However, this does not explain the relative enrichment of the chimeric receptors NKC and GBC in coated pits.

It is not clear which of these possibilities contributes to the efficiency of endocytosis and localization in coated pits displayed by the receptors GBC and NKC. However, the experimental results indicate that localization in coated pits is not exclusively a property of proteins with a tyrosine exposed on the cytoplasmic side of the membrane.

Significance of functionally distinct surface pools

When internalization of prebound ligand is measured, the presence of two populations of cell-surface receptors with distinct kinetic properties is revealed (rapid endocytosis pool and residual surface pool in Figure 3). These distinct pools cannot be distinguished by continuous uptake experiments or analysis of binding sites by electron microscopy after a 4°C in-

found associated with coated areas. In cell lines expressing chimeric receptors, this value was 22% for RC (B), 9% for NKC (C), and 14% for GBC (D) (see Table IV). The specificity of the binding was tested using a 100-fold excess of GlcNAc-BSA to compete with the gold conjugate. (E) An example of a competition experiment on a cell line expressing receptor CHL (same cell line as in A). Note the localization of gold particles on footlike extensions in this competition experiment. Gold particles and boundaries for coated regions are indicated by arrows and arrowheads, respectively. Bar = 0.25 μ m.

Table 4. Localization of surface-binding sites for GlcNAc-BSA-gold particles in Rat 6 cells expressing transfected receptors

Receptor	Coated areas/mm cell surface length	Gold particles over coated areas/mm cell surface length			Gold particles over uncoated membrane/mm cell surface length			Total gold particles/mm cell surface length			Percentage of specifically bound gold particles located over coated areas (%)
		Total	Comp.	Spec.	Total	Comp.	Spec.	Total	Comp.	Spec.	
CHL	181/108	43	1	42	172	84	88	215	85	130	32
RC	220/160	229	1	228	851	67	784	1080	68	1012	23
NKC	201/200	9	0	9	153	58	95	162	58	104	9
GBC	150/131	7	0	7	74	30	44	81	30	51	14

Binding of GlcNAc-BSA-gold particles at 4°C, fixation, electron microscopy, and quantification were performed as described under Experimental procedures. For each condition 18–42 cells, with a total surface length of 0.56–1.39 mm, were examined. The number of coated areas observed in absence and in presence of competing ligand is indicated. The number of gold particles is given for experiments performed in the absence (Total) and in the presence (Comp.) of unlabeled ligand. The difference represents specifically bound ligand (Spec.).

cubation. The warm-up experiments reveal that ~25% of CHL and of the chimeric receptors RC and GBC do not participate in the rapid, first-order internalization, but are removed more slowly from the cell surface. Because the receptors appear to be homogeneous by immunoblot analysis (data not shown), it is likely that interactions with other proteins or posttranslational modifications may be responsible. A first possibility is that the phosphorylation state of CHL determines its ability to undergo endocytosis. Indeed, it has been shown that 70% of CHL is in the phosphorylated state in chicken liver (Drickamer and Mamon, 1982). However, that the phosphorylation plays a role in determining the two receptor subpopulations seems unlikely, because the hybrid receptors RC and GBC, which show similar surface pools, have no phosphorylation sites in their cytoplasmic domains. A second possibility is that a subpopulation of the receptor-ligand complexes is unable to dissociate after internalization and returns rapidly to the cell surface. Consequently, a constant fraction of this subpopulation would always be at the cell surface. A third possibility is that a fraction of the surface receptors interacts directly or indirectly with cytoskeletal elements or other proteins that limit its mobility. A particular case of this third possibility is that carbohydrate-binding domains of receptors might bind to GlcNAc residues on the same cell, on other cells, or in the extracellular matrix, thereby leading to immobilization. Because each receptor oligomer contains multiple ligand-binding domains, this subpopulation of receptors would still be capable of binding exogenous ligand.

The residual surface subpopulation is much larger for receptor NKC (~60%) than for the three other receptors. This apparent effect may in part result from experimental factors peculiar to this receptor construct. Estimation of the size of the residual pool is less precise for NKC because of the slower initial uptake rate, which potentially renders recycling of ligand, timing of the measurements, and nonspecific effects because of cooling and rewarming proportionally more important. Alternatively, the large residual surface pool of receptor NKC could be the consequence of interactions of the Na,K-ATPase β subunit cytoplasmic tail with other structures (such as cytoskeletal components) that are restricted in their lateral mobility. These interactions could either be direct or through other proteins, such as Na,K-ATPase molecules (Jesaitis and Yguerabide, 1986; Nelson and Hamerton, 1989). In this case, the mobile subpopulation of NKC receptors would be integrated into coated pits as efficiently as receptor GBC (similar k_{re}). The relatively poor localization of receptor NKC to coated pits compared with receptor GBC, and the lower overall endocytosis efficiency of this receptor (k_e), would be a consequence of the larger pool of low-mobility receptor unique to this construct.

Implications of the RC construct for rat asialoglycoprotein receptor function

RHL-1, the major component of the rat asialoglycoprotein receptor, does not form receptor for the ligand asialo α_1 -acid glycoprotein in the absence of the second polypeptide RHL-2/3 (McPhaul and Berg, 1986; Braiterman *et al.*, 1989; Graeve *et al.*, 1990). However, the results

presented here demonstrate that replacement of the cytoplasmic domain of CHL with the cytoplasmic domain of RHL-1 produces a hybrid receptor that functions very similarly to wild-type CHL. The chimeric receptor is able to bind and internalize ligand and is efficiently localized in coated pits. These results show that the RHL-1 tail is able to make the necessary interactions to direct the receptor through the biosynthetic and endocytic pathways. It is interesting that CHL and the RC hybrid function almost identically, although the CHL and RHL-1 cytoplasmic tails have no detectable sequence similarity. In any case, the failure of cells transfected with RHL-1 to express a functional receptor for asialo α_1 -acid glycoprotein or galactose-BSA must be due to structural features of the rat receptor other than the cytoplasmic tail.

Experimental procedures

Material

Restriction enzymes and linkers were purchased from New England Biolabs (Beverly, MA). The neoglycoproteins galactose-BSA and GlcNAc-BSA were obtained from Pierce Chemical (Rockford, IL). GlcNAc- and galactose-BSA-gold colloidal particles (10 nm) were from E.Y. Labs (San Mateo, CA). Cell culture plates were purchased from Falcon, Becton Dickinson (Lincoln Park, NJ). Polybrene is a product of Aldrich Chemicals (Milwaukee, WI).

Recombinant DNA methodology

To generate cDNAs encoding truncated forms of CHL, a CHL cDNA (Mellow *et al.*, 1988) was cut with *AlwNI* and *HpaII* in the segment encoding the cytoplasmic tail. These fragments were ligated to a 5' fragment of the cDNA for the β chain of *Xenopus laevis* globin (Melton *et al.*, 1984) cut with *NcoI*, so that it provided an AUG codon within a favorable translation initiation context (Kozak, 1989).

Tail replacement chimeric receptors were obtained by ligating 5' restriction fragments of RHL-1 (Holland *et al.*, 1984), *X. laevis* globin, and rat Na,K-ATPase β subunit (Young *et al.*, 1987) cDNAs to a 3' fragment of CHL. This last fragment was generated by cutting with *AlwNI* and trimming with T4 DNA polymerase and encodes all but the 21 N-terminal cytoplasmic amino acids of the mature receptor. An RHL-1 cDNA fragment, encoding 36 N-terminal amino acids of the mature cytoplasmic tail, was prepared by cutting the cDNA with *MbolI* and trimming the overhang with T4 DNA polymerase. The missing nucleotide required to obtain ligation to the CHL fragment in frame was provided by adding a *PvuI* linker (New England Biolabs #1053) and cutting with *DpnI*. A rat Na,K-ATPase β subunit cDNA fragment encoding the 31 N-terminal amino acids of the mature cytoplasmic tail was obtained by cutting the DNA with *Sau3AI* and filling in with the Klenow fragment of *Escherichia coli* DNA polymerase I. A *Xenopus* globin cDNA fragment encoding the 11 N-terminal amino acids of the β chain was obtained by cutting with *BclI* and filling as above. The RHL-1 and globin cDNA fragments included 5' untranslated sequences and initiation codons, whereas the Na,K-ATPase construct used the initiation codon provided by the globin cDNA *NcoI* site (see above).

All constructs were made in vectors with an SP6 promoter (pSP64 or pGEM3) and were tested by *in vitro* transcription, followed by translation in a reticulocyte lysate system, immunoprecipitation, and polyacrylamide gel electrophoresis (Holland and Drickamer, 1986).

Expression of CHL and chimeric receptors in Rat 6 fibroblasts

The cDNA inserts encoding CHL, as well as the truncated and chimeric receptors, were transferred to pVcos, an expression vector derived from the Moloney murine leukemia virus (Maddon *et al.*, 1985). Rat 6 fibroblasts (Prasad *et al.*, 1976) were cotransfected with these constructs and pSV2neo, which contains a neomycin resistance gene (Southern and Berg, 1982), as described in Mellow *et al.* (1988).

Stable transfectants with a single copy of the expression construct were obtained by a second method, using pseudoviruses produced by ψ 2 cells. A G418 resistance gene under control of the Herpes virus thymidine kinase promoter (Episkopou *et al.*, 1984) was inserted into the *ClaI* site of the retroviral expression constructs, and ψ 2 cells (Mann *et al.*, 1983) were transfected as above. Fresh medium was added after 16–24 h, and 2 d later the medium containing pseudovirions was collected and filtered through 0.45- μ m filters. Polybrene was added to a final concentration of 8 μ g/ml and 1-ml aliquots were used to infect 50% confluent Rat 6 cells on 60-mm plates. Fresh medium was applied after a 2-h incubation at 37°C, and selection of infected cells was started 24 h later by adding medium containing 400 μ g/ml of G418. In some cases cells were split 1/20 48 h after infection. After 10–15 d, resistant colonies were cloned by the ring method.

G418-resistant cell lines produced by both techniques were tested for receptor expression by indirect immunofluorescence and immunoblotting (Mellow *et al.*, 1988). At least two cell lines from different transfection experiments were selected for each construct and used for functional tests.

Functional tests of CHL and chimeric receptors expressed in Rat 6 cells

The neoglycoprotein GlcNAc-BSA was radiolabeled with 125 I by the chloramine T method (Greenwood *et al.*, 1963) to a specific activity of 10^6 cpm/ μ g. Cells were plated on 24-well plates and grown 4–6 d to near or full confluency. Unless otherwise indicated, all incubations and washes were in carbonate-free Dulbecco's modified Eagle's medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 1% BSA at pH 7.6. Cell surface ligand was eluted from the cells by a 10-min wash at 4°C with 0.02 M acetic acid (pH 3.1), 0.5 M NaCl (Rothenberger *et al.*, 1987). Radioactivity remaining associated with cells was collected after solubilizing the cells for 30 min at room temperature in 1% sodium dodecyl sulfate. Medium was processed by the method of Goldstein and Brown (1974) to determine released acid-soluble degradation products. Samples were counted for 3 min on a Packard gamma counter. In all experiments, duplicates of each point were made with and without a 20-fold excess of nonradioactive ligand. The results for the competition (generally 5%–20% of the test) were subtracted from the test results to obtain values for specific binding sites. Numbers of cells in duplicate wells were determined by the use of a Neubauer hemacytometer.

Binding of GlcNAc-BSA. Cells were preincubated for 30 min at 4°C in incubation medium. The medium was removed by aspiration and replaced with incubation medium con-

taining 0.1, 0.5, 2.5, 12.5, and 60 nM radioactive ligand, in the presence and absence of a 20-fold excess of unlabeled ligand. After 2 h at 4°C, the medium was removed, cells were washed three times for 5–10 min with ligand-free incubation medium, and the total cell-associated radioactivity was counted. The results of the competition experiments were subtracted from the test results and the number of specific cell-surface binding sites and the dissociation constants were estimated for each individual experiment (Scatchard, 1949).

Continuous uptake of GlcNAc-BSA. Cells were preincubated at 37°C in incubation buffer for 30 min and incubated for different times with 1 µg/ml ¹²⁵I-GlcNAc-BSA (13.3 nM), in the presence and absence of a 20-fold excess of unlabeled ligand. At the end of the incubation, the medium was removed, and the cells were immediately chilled and washed three times with incubation buffer at 4°C. Cell-surface, intracellular, and secreted radioactivity were determined as above.

Uptake of prebound GlcNAc-BSA. Preincubation, binding of 0.5 µg/ml GlcNAc-BSA (minus and plus excess unlabeled ligand), and washes at 4°C were performed as in binding experiments. Internalization was initiated by adding prewarmed incubation buffer to the cells, which were then left at 37°C for different times. Rapid interruption of the uptake was obtained by aspiration of the warm medium and addition of prechilled acid wash buffer on ice. Cell-surface and intracellular radioactivity were measured as above; in certain experiments the medium from the 37°C incubation was also directly tested for radioactivity.

Calculation of endocytic rate constants

The following equation was used to calculate endocytic rate constants from the continuous uptake experiments

$$\frac{[LR]_i + [D]}{[LR]_s} = k_e T$$

The equation describes endocytosis as a first-order process in a situation with constant cell-surface ligand (Wiley and Cunningham, 1981). $[LR]_i$ represents all forms of intracellular ligand, $[D]$ the degraded ligand found in the medium, $[LR]_s$ the cell-surface-receptor-bound ligand, T the incubation time, and k_e the rate constant of endocytosis. This rate constant corresponds to the fraction of the ligand molecules bound at the cell surface (elutable with an acid wash) that is internalized per minute and allows comparison of receptor function independently of the number of cell-surface receptors and the time of incubation.

First-order kinetic curves were fitted to the results of initial uptake experiments to obtain values for the endocytic rate constant. A close fit to the experimental values was obtained once the rapid first-order kinetics were restricted to a rapid endocytosis surface pool, excluding an acid-resistant surface pool and a residual surface pool. The later pool of surface-bound ligand is internalized much more slowly and therefore only marginally affects determination of the rapid initial uptake rate constant. The first-order kinetic equation was adapted to our non-steady-state conditions

$$\frac{d[LR]_i}{dt} = k_{re}([LR]_{s_{re}} - [LR]_i)$$

which can be integrated to yield

$$[LR]_i = [LR]_{s_{re}}(1 - e^{-k_{re}t})$$

where $[LR]_i$ represents the intracellular ligand, $[LR]_{s_{re}}$ the initially cell-surface-bound ligand participating in the rapid

endocytosis, t the time at 37°C, and k_{re} the rate constant for endocytosis of the rapid endocytosis pool. This rate constant corresponds to the fraction of the rapid endocytosis pool that would be internalized at the initial internalization rate in 1 min. A modified version of the pharmacokinetic program MULTI (Yamaoka *et al.*, 1981) was used to fit theoretical curves to the experimental data and to derive values for the parameters $[LR]_{s_{re}}$ and k_{re} (see Figure 3 and Table III). Knowing the size of the residual surface pool ($[LR]_{s_r}$), we can calculate an equivalent to k_e

$$k'_e = k_{re} \frac{[LR]_{s_{re}}}{[LR]_{s_{re}} + [LR]_{s_r}}$$

The k'_e calculated by this method is the initial uptake rate constant for prebound ligand relative to the total acid-sensitive surface pool and can therefore be directly compared with the k_e obtained from continuous uptake experiments.

Electron microscopy

Cell culture, preincubation, binding of 4 µg/ml GlcNAc-BSA-gold (10 nm) for 2 h at 4°C, and three washes with incubation buffer were performed as in binding experiments. Nonspecific binding was assayed with 4 µg/ml galactose-BSA-gold and competition experiments were performed using a 20- or 100-fold excess of GlcNAc-BSA. After an additional wash with phosphate-buffered saline containing 0.2 mM CaCl₂ and 2 mM MgCl₂, cells were fixed at 4°C with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 30 min and rinsed with the same buffer. After postfixation in 1% OsO₄ in the same buffer for 2 h at 4°C, cells were rinsed quickly with distilled water, detached from the wells, and embedded in 1.5% agar. Samples were dehydrated in a series of graded ethanol solutions, infiltrated with propylene oxide, and embedded in Epon. Ultrathin sections of ~70 nm were cut and stained with uranyl acetate and lead citrate. The sections were examined with a JEM-100 CXII electron microscope (JEOL) at 80 kV.

In each experimental group, a minimum of 18 cells was analyzed. Micrographs of every cell were taken at a magnification of 3600–7200× and their contour length was measured by the use of a Sigma Scan program (Jandel). At a final magnification of 10⁵×, the total number of gold particles over the cell surface section, the number of gold particles over coated membrane areas, the number of gold particles on footlike processes, and the number of coated membrane areas were determined.

Acknowledgments

We thank Doug Melton for the globin cDNA, Jerry Lingrel for the Na,K-ATPase clone, and Bill Weis for helpful discussions. This work was supported by Grant CD-388 from the American Cancer Society to K.D., and by grants from the National Institutes of Health (GM-34107) and the American Cancer Society to E. Rodriguez-Boulton. F.V. is the recipient of a fellowship from the Swiss National Science Foundation. T.G. is the recipient of a fellowship from the Association pour la Recherche sur le Cancer (France). K.D. is recipient of a Faculty Research Award from the American Cancer Society.

Received: February 13, 1990.

Revised and accepted: April 12, 1990.

References

Anderson, R.G.W., Goldstein, J.L., and Brown, M.S. (1976). Localization of low density lipoprotein receptors on plasma

- membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc. Natl. Acad. Sci. USA* 73, 2434–2438.
- Anderson, R.G.W., Brown, M.S., and Goldstein, J.L. (1977a). Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell* 10, 351–364.
- Anderson, R.G.W., Goldstein, J.L., and Brown, M.S. (1977b). A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts. *Nature* 270, 695–699.
- Braiterman, L.T., Chance, S.C., Porter, W.R., Lee, Y.C., Townsend, R.R., and Hubbard, A.L. (1989). The major subunit of the rat asialoglycoprotein receptor can function alone as a receptor. *J. Biol. Chem.* 264, 1682–1688.
- Bretscher, M.S., Thomson, J.N., and Pearse, B.M.F. (1980). Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA* 77, 4156–4159.
- Bretscher, M.S., and Thomson, J.N. (1983). Distribution of ferritin receptors and coated pits on giant HeLa cells. *EMBO J.* 2, 599–603.
- Brown, M.S., and Goldstein, J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34–47.
- Davis, C.G., van Driel, I.R., Russell, D.W., Brown, M.S., and Goldstein, J.L. (1987). The low density lipoprotein receptor. Identification of amino acids in cytoplasmic domain required for rapid endocytosis. *J. Biol. Chem.* 262, 4075–4082.
- Drickamer, K., and Mamon, J.F. (1982). Phosphorylation of a membrane receptor for glycoproteins. *J. Biol. Chem.* 257, 15156–15161.
- Episkopou, V., Murphy, A.J.M., and Efstratiadis, A. (1984). Cell-specified expression of a selectable hybrid gene. *Proc. Natl. Acad. Sci. USA* 81, 4657–4661.
- Fuchs, R., Schmid, S., and Mellman, I. (1989). A possible role for Na⁺,K⁺-ATPase in regulating ATP-dependent endosome acidification. *Proc. Natl. Acad. Sci. USA* 86, 539–543.
- Goldstein, J.L., and Brown, M.S. (1974). Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.* 249, 5153–5162.
- Graeve, L., Patzak, A., Drickamer, K., and Rodriguez-Boulan, E. (1990). Polarized expression of functional rat liver asialoglycoprotein receptor in transfected Madin-Darby canine kidney cells. *J. Biol. Chem.* 265, 1216–1224.
- Greenwood, L.C., Hunter, W.M., and Glover, S.J. (1963). The preparation of ¹²⁵I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114–123.
- Holland, E.C., Leung, J.O., and Drickamer, K. (1984). Rat liver asialoglycoprotein receptor lacks a cleavable NH₂-terminal signal sequence. *Proc. Natl. Acad. Sci. USA* 81, 7338–7342.
- Holland, E.C., and Drickamer, K. (1986). Signal recognition particle mediates the insertion of a transmembrane protein which has a cytoplasmic NH₂ terminus. *J. Biol. Chem.* 261, 1286–1292.
- Huet, C., Ash, J.F., and Singer, S.J. (1980). The antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. *Cell* 21, 429–438.
- Iacopetta, B.J., Rothenberger, S., and Kühn, L.C. (1988). A role for the cytoplasmic domain in transferrin receptor sorting and coated pit formation during endocytosis. *Cell* 54, 485–489.
- Jesaitis, A.J., and Yguerabide, J. (1986). The lateral mobility of the (Na⁺,K⁺)-dependent ATPase in Madin-Darby canine kidney cells. *J. Cell Biol.* 102, 1256–1263.
- Kerjaschki, D., Noronha-Blob, L., Sacktor, B., and Fahrquhar, M.G. (1984). Microdomains of distinctive glycoprotein composition in the kidney proximal tubule brush border. *J. Cell Biol.* 98, 1505–1513.
- Kozak, M. (1989). The scanning model for translation: an update. *J. Cell. Biol.* 108, 229–241.
- Lazarovits, J., and Roth, M. (1988). A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. *Cell* 53, 743–752.
- Lobel, P., Fujimoto, K., Ye, R.D., Griffiths, G., and Kornfeld, S. (1989). Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. *Cell* 57, 787–796.
- Loeb, J.A., and Drickamer, K. (1987). The chicken receptor for endocytosis of glycoproteins contains a cluster of *N*-acetylglucosamine-binding sites. *J. Biol. Chem.* 262, 3022–3029.
- Maddon, P.J., Littman, D.R., Godfrey, M., Maddon, D.E., Chess, L., and Axel, R. (1985). The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell* 42, 93–104.
- Mann, R., Mulligan, R.C., and Baltimore, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33, 153–159.
- McPhaul, M., and Berg, P. (1986). Formation of functional asialoglycoprotein receptor after transfection with cDNAs encoding the receptor proteins. *Proc. Natl. Acad. Sci. USA* 83, 8863–8867.
- Mellow, T.E., Halberg, D., and Drickamer, K. (1988). Endocytosis of *N*-acetylglucosamine-containing glycoproteins by rat fibroblasts expressing a single species of chicken liver glycoprotein receptor. *J. Biol. Chem.* 263, 5468–5473.
- Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12, 7035–7056.
- Miettinen, H.M., Rose, J.K., and Mellman, I. (1989). Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. *Cell* 58, 317–327.
- Nelson, W.J., and Hammerton, R.W. (1989). A membrane-cytoskeletal complex containing Na⁺,K⁺-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* 108, 893–902.
- Pearse, B.M.F. (1988). Receptors compete for adaptors found in plasma membrane coated pits. *EMBO J.* 7, 3331–3336.
- Pollack, L.R., Tate, E.H., and Cook, J.S. (1981). Turnover and regulation of Na-K-ATPase in HeLa cells. *Am. J. Physiol.* 10, C173–C183.
- Prasad, I., Zouzas, D., and Basilico, C. (1976). State of the viral DNA in rat cells transformed by polyoma virus. *J. Virol.* 18, 436–444.
- Rothenberger, S., Iacopetta, B.J., and Kühn, L.C. (1987). Endocytosis of the transferrin receptor requires the cyto-

- plasmic domain but not its phosphorylation site. *Cell* 49, 423–431.
- Scatchard, G. (1949). The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51, 660.
- Schlessinger, J. (1986). Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell Biol.* 103, 2067–2072.
- Schwartz, A.L., Fridovich, S.E., and Lodish, H.F. (1982). Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line. *J. Biol. Chem.* 257, 4230–4237.
- Selvaraj, P., Rosse, W.F., Silber, R., and Springer, T.A. (1988). The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. *Nature* 333, 565–567.
- Southern, P.J., and Berg, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1, 327–341.
- Wall, D.A., and Hubbard, A.L. (1981). Galactose-specific recognition system of mammalian liver: receptor distribution on the hepatocyte cell surface. *J. Cell Biol.* 90, 687–696.
- Watts, C. (1985). Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. *J. Cell Biol.* 100, 633–637.
- Wiley, H.S., and Cunningham, D.D. (1981). A steady state model for analyzing the cellular binding, internalization and degradation of polypeptide ligands. *Cell* 25, 433–440.
- Yamaoka, K., Tanigawara, Y., Nakagawa, T., and Uno, T. (1981). A pharmacokinetic analysis program (multi) for microcomputer. *J. Pharmacobio-Dyn.* 4, 879–885.
- Young, R.M., Shull, G.E., and Lingrel, J.B. (1987). Multiple mRNAs from rat kidney and brain encode a single Na⁺,K⁺-ATPase β subunit protein. *J. Biol. Chem.* 262, 4905–4910.
- Ziomek, C.A., Schulman, S., and Edidin, M. (1980). Redistribution of membrane proteins in isolated mouse intestinal epithelial cells. *J. Cell. Biol.* 86, 849–857.