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REVIEW ARTICLE

Receptor-mediated endocytosis

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Introduction

In 1934, Lewis described the process of pinocytosis – an invagination of the plasma membrane enclosing a small droplet of fluid. Pinocytosis appeared to be a constitutive process, whereas phagocytosis described some years earlier by Metchnikoff was regulated. Only within the last two decades has it been recognized that (i) during pinocytosis cells internalize vast amounts of membrane, indeed, more membrane than they synthesize [which led to the concept of membrane recycling (Steinman *et al.*, 1983; refer to Schneider *et al.*, 1979*a*, *b*)] and (ii) that receptors mediate selective uptake of both macromolecules and particles. While the term endocytosis has been used to describe these processes, the term receptor-mediated endocytosis is usually reserved for specific uptake of soluble molecules. Broadly speaking, receptor-mediated endocytosis involves the movement of bound ligands from the cell surface to the interior. Whereas some ligands (e.g. ligands specific for the galactose, mannose, mannose 6-phosphate and LDL receptors) are targeted to lysosomes often subserving the nutritional needs of the cell, others (e.g. transferrin and IgG) are recycled to the plasma membrane or targeted to specific plasma membrane domains. Ligands may also be modified on entering the cell [e.g. proteolysis in macrophage endosomes (Diment & Stahl, 1984) or lysosomes, resialylation by passage through the Golgi or removal of iron in the case of transferrin (Klausner *et al.*, 1983; Dautry-Varsat *et al.*, 1983; Harding & Stahl, 1983)]. Intracellular transport and processing vary markedly between different receptor–ligand systems and different cell types, but these systems may be placed in four general categories (Fig. 1, Table 1).

1. Receptors that recycle but target their ligand to lysosomes. This category includes the LDL (Goldstein *et al.*, 1979), asialoglycoprotein (Gal/GalNAc) (Schwartz *et al.*, 1982), mannose 6-phosphate (Fischer *et al.*, 1980*a*, *b*), and mannose receptor systems (Stahl *et al.*, 1980).

2. Receptors that recycle but do not target their ligand to lysosomes. The ligand remains attached to the receptor during its transit through the cell. In polarized cells this may result in transport of ligands between the apical and basolateral surfaces (transcytosis or diacytosis). Examples include transferrin and IgG (in suckling rat ileum) (Abrahamson & Rodewald, 1981).

3. Receptors that do not recycle and target their ligand to lysosomes, including the EGF receptor (Carpenter & Cohen, 1976), the insulin receptor (Kasuga *et al.*, 1981, Carpentier *et al.*, 1978, 1979) (insulin receptors may recycle in some cells) and the Fc receptor (in macrophages) (Mellman & Plutner, 1984) (Fc receptors may recycle under different conditions in some cells).

4. Receptors that do not recycle and do not transport ligands to lysosomes. Transport of IgA represents a unique mechanism, transcytosis of IgA results in cleavage and loss of the IgA receptor (Kuhn & Kraehenbuhl, 1979; Mostov & Blobel, 1982).

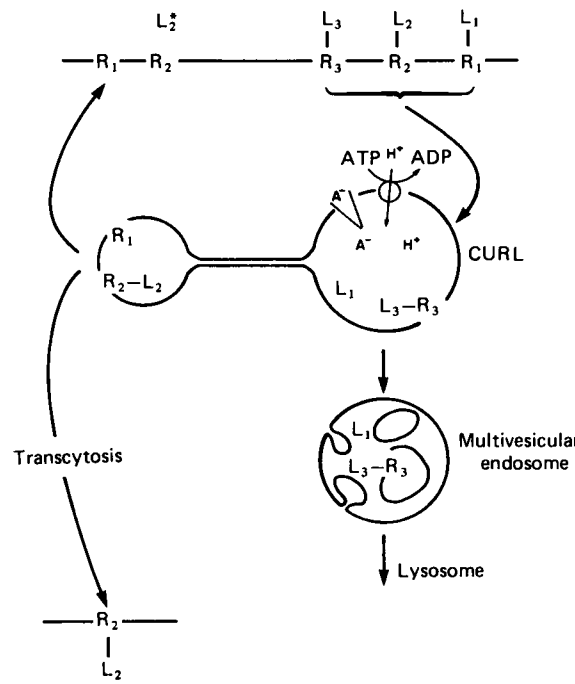

Fig. 1. Model showing the pathways followed by receptors and ligands during receptor-mediated endocytosis

Receptor–ligand complexes enter the cell via coated pits and coated vesicles. Soon after entering the cell clathrin coats are shed from the coated vesicles and ligand molecules are localized in large electron-luscent vesicles (endosomes or CURL). Nucleotide-dependent acidification coupled to anion transport lowers the internal pH of the endosome. Ligands which dissociate from their receptors at low pH (L_1) accumulate in the vesicle lumen, other ligands (L_2, L_3) stay bound to their receptors. Receptors that recycle (R_1, R_2) are concentrated in arm-like extensions of the endosome and return to the cell surface. Those that do not (R_3) remain with the endosome which vesiculates prior to fusion with secondary lysosomes. Any contained receptors and ligands are digested.

Abbreviations used: LDL, low-density lipoprotein; EGF, epidermal growth factor; CURL, compartment for the uncoupling of receptor–ligand complexes; DIDS, di-isothiocyanostilbenesulphonic acid.

Table 1. Types of transport and processing of receptor-ligand systems

Type	Cell type	Amount of receptors at cell surface amine sensitive
1. Receptor recycled; ligand to lysosomes		
Mannose receptor	Macrophage	+
Galactose receptor	Hepatocyte	+
LDL receptor	Fibroblast	±
Mannose 6-phosphate receptor	Fibroblast	+
α_2-Macroglobulin receptor	Fibroblasts	+
2. Receptor recycled; ligand recycled		
IgG (neonatal gut)	Rat ileum	−
Transferrin	Reticulocyte	−
	Fibroblasts	−
(Mannose)*	Macrophages	−
(Galactose)*	Hepatocytes	−
(LDL)*	Fibroblasts	−
IgG monomer	Macrophages	−
3. Receptor demise; ligand to lysosomes		
EGF	Fibroblasts	
Insulin	Adipocytes	
	Lymphocytes	
Human choriogonadotropin	Follicular cells	
IgG multimer	Macrophages	
4. Receptor demise; ligand not to lysosomes		
IgA	Hepatocyte	−

* Short cycle (Townsend *et al.*, 1984; Simmons & Schwartz, 1984; Greenspan & St. Clair, 1984; Tietze *et al.*, 1982).

There are two new 'organelles' which play important roles in mediating the process of endocytosis. First, coated vesicles, identified in 1964 (Roth & Porter, 1964) appear to concentrate and internalize certain plasma membrane proteins while excluding others. Secondly, there is a class of intracellular pre-lysosomal structures which have been shown to be responsible for sorting receptors from ligands. These structures have been referred to as endosomes or more recently CURL (compartment for the uncoupling of receptor–ligand complexes) (Geuze *et al.*, 1983*a*) and receptosomes (Pastan & Willingham, 1981).

Receptor-mediated endocytosis involves a number of membrane fusion steps. Coated vesicles deliver receptor–ligand complexes to the endosome; here tubules and vesicles rich in receptors form as a consequence of receptor–ligand sorting. These vesicles bud away from the endosome and fuse with the plasma membrane or, alternatively, fuse with secondary lysosomes or elements of the Golgi. Very little is known about the mechanisms that control the formation and transport of these vesicles. Mechanistically, since different receptors target to different destinations, one might imagine the cytoplasmic domain of a receptor binding intermittently to contractile proteins. At another level of organization it is important to consider the role of transmembrane electrochemical gradients in driving receptor movement in a selective way. There is a large body of information indicating that weak bases block receptor movement in cells, putatively by neutralizing acid intracellular compartments. Ionophores produce similar but not identical effects (Stein *et al.*, 1984). These and other data suggest that transmembrane gradients are important determinants of receptor recycling.

A major determinant of ligand sorting in cells is the striking pH dependence of ligand binding to receptors. Endosomal membranes contain proton-pumps that acidify the endosomal contents. Within acidic endosomes, many ligands dissociate from their receptors and then make their way separately to secondary lysosomes. Other ligands may remain bound to their receptor at low pH and remain with the receptor during its transit through the cell. The pH dependency of ligand binding also serves to mediate transcellular transport of protein ligands across cells. IgG binds avidly to receptors in the gut at low pH and is released at neutral pH on the basolateral side of the cells.

Many physiologically important macromolecules enter cells by receptor-mediated endocytosis; unfortunately, a detailed account of each receptor–ligand system is beyond the scope of this Review. Instead the text concentrates on the main pathways of receptor-mediated endocytosis. The involvement of the clathrin coated pit, coated vesicle and endosome is reviewed in detail. The Review also deals with possible mechanisms whereby the endosome may achieve its sorting function.

Receptor distribution and ligand transport

Receptor distribution. Receptors involved in receptor-mediated endocytosis have been found both at the cell surface and associated with intracellular membranes. Cell surface binding sites can be determined by measuring the number of radiolabelled ligand molecules that can bind to a cell at 4 °C, a temperature that effectively arrests endocytosis. It is worth noting that many ligands are multivalent and ligand binding studies may underestimate the number of receptors. Fortunately many of the receptors under discussion have been purified to

homogeneity and radiolabelled receptor-specific antibodies can be used to provide an independent estimation of receptor numbers.

Many cells have intracellular pools of ligand-specific binding sites. Pricer & Ashwell (1976) demonstrated the binding of asialo-orosomucoid to Golgi and lysosomal membranes isolated from rat liver; these observations were confirmed by studies on solubilized hepatocytes. These cells contain a total of 860000 receptors of which only 80000 reside in the plasma membrane (Steer & Ashwell, 1980). Similar results have been provided by Weigel & Oka (1983) using hepatocytes permeabilized with digitonin. Human fibroblasts contain an intracellular pool of binding sites specific for mannose 6-phosphate; this pool contains 80% of the total receptors found in the cell (Fischer *et al.*, 1980*a*, *b*). Macrophages contain an internal pool of mannose glycoprotein binding sites and mannose phosphate specific sites that can be revealed by incubating cells with saponin (Wileman *et al.*, 1984; Shepherd *et al.*, 1984). Receptor distribution within cells has also been studied by electron microscopy. Antibody bound to the asialoglycoprotein receptor can be visualized if frozen sections are incubated with colloidal gold absorbed to Protein A. Geuze *et al.* (1982) show localization of receptors along the sinusoidal membrane of hepatocytes and also in association with internal membranes. A quantitative analysis of gold bead distribution shows that 35% of the receptors are confined to the plasma membrane; the rest are found near Golgi, smooth endoplasmic reticulum and endosomal membranes (Geuze *et al.*, 1983*b*).

The pathway taken by the ligand. Radiolabelled ligands bound to the cell surface at 4 °C are rapidly internalized when cells are warmed to 37 °C. A detailed study of the internalization of ^{125}I-asialo-orosomucoid has been made by Schwartz *et al.* (1982). They calculate an internalization half-life of 2.2 min for the uptake of asialo-orosomucoid on board the galactose receptor of Hep G-2 cells. Similar rates of internalization have been shown for the clearance of surface-bound LDL by fibroblasts (Basu *et al.*, 1981) or uptake of mannose glycoproteins by macrophages (Stahl *et al.*, 1980). An alternative way to study the transport of ligands during endocytosis is to fractionate cell homogenates on Percoll gradients (Merion & Sly, 1983; Harford *et al.*, 1983*a*; Wileman *et al.*, 1984). During the first 5 min of uptake radiolabelled ligands are concentrated in buoyant membrane fractions rich in the plasma membrane marker enzyme alkaline phosphodiesterase. A two-step Percoll gradient protocol (Merion & Sly, 1983) has shown the transfer of ligand molecules to a membrane fraction with a density intermediate between that of plasma membrane and the lysosome. After 20–30 min incubation ligands following pathway 1 or 3 (Fig. 1) are transferred from these vesicles to more dense vesicles containing lysosomal enzymes. At this point trichloroacetic acid-soluble radioactivity is detected in the cell culture medium.

Ligand molecules absorbed to colloidal gold (Maxfield *et al.*, 1978; Handley *et al.*, 1981; Geuze *et al.*, 1983*b*; Pastan & Willingham, 1981), ferritin or horseradish peroxidase (Wall *et al.*, 1980) have been used in conjunction with electron microscopy to study the morphology of structures that contain ligand molecules on their way to lysosomes. At 4 °C ligands bind to specialized areas of the plasma membrane known as coated pits (Anderson *et al.*, 1977*a*; Maxfield *et al.*, 1978). These are invaginations in the plasma membrane which appear 'bristle-coated' by electron microscopy (Fig. 2*a*). Coated pits are thought to localize selectively certain membrane proteins in the pits and mediate their internalization into coated vesicles (Fig. 2*b*). Shortly after entering the cell, coated vesicles lose their coats and ligand molecules are seen in a complicated arrangement of large smooth-surfaced vesicles and tubular structures referred to as endosomes (Figs. 2*d* and 2*e*). This endosomal localization of ligand may be analogous to the appearance of radiolabelled ligand in vesicles of intermediate density on Percoll gradients. Within 30 min ligands that are degraded (Fig. 1, pathways 1 and 3) are seen in electron-dense structures that stain positively for acid phosphatase, showing transfer of the ligand to lysosomes (Fig. 2*f*).

Coated pits and coated vesicles

The coated pit plays an important role in the selective movement of receptors and, correspondingly, in the uptake of ligands (Goldstein *et al.*, 1979). Morphological evidence has shown that LDL (Anderson *et al.*, 1977*a*; Handley *et al.*, 1981), lysosomal enzymes (Willingham *et al.*, 1981), asialoglycoproteins (Wall *et al.*, 1980; Wall & Hubbard, 1981; Zeitlin & Hubbard, 1982), EGF (Carpentier *et al.*, 1982), α_2-macroglobulin (Willingham *et al.*, 1979) and transferrin (Harding *et al.*, 1983) are all internalized via coated pits and vesicles. Biochemically, isolated coated vesicles have been shown to contain receptors for mannose 6-phosphate, transferrin, and LDL (Pearse, 1982; Campbell *et al.*, 1983). Morphological evidence suggests that a number of ligands can enter the cell via the same coated pit (Maxfield *et al.*, 1978; Carpentier *et al.*, 1982). Coated pits not only concentrate receptor molecules but selectively exclude other plasma membrane proteins (Bretscher *et al.*, 1980; Bretscher, 1983). For example, it has been calculated that the θ antigen of 3T3 fibroblasts is excluded at a level of 99% from the coated regions of the fibroblast plasma membrane (Bretscher *et al.*, 1980).

Formation of clathrin-coated domains on the plasma membrane and the subsequent dissociation of clathrin from coated vesicles prior to fusion with endosomes probably plays a regulating role in receptor-mediated endocytosis. The mechanisms involved in clathrin coat assembly and dissassembly have received much attention and most studies of these processes have used isolated coated vesicles. These can be prepared from tissue homogenates by repeated centrifugation of membrane fractions in sucrose or ^2H_2O gradients (Pearse, 1975, 1976).

Structure of coated vesicles. Electron microscopic examination of purified coated vesicles shows that the vesicle membrane is surrounded by a polygonal protein lattice with a morphology similar to that seen coating vesicles within intact cells (Heuser *et al.* 1980; Kanaseki & Kadota, 1969) (Fig. 2*b*). The major polypeptide of coated vesicles, clathrin, has a molecular mass of 180000 Da (Pearse, 1976); other proteins present are two polypeptides of 36000 and 33000 Da known as the clathrin light chains and a polypeptide of 110000 Da. Mild biochemical manipulations [0.5 M-Tris (pH 7.0)/2 M-urea] (Keen *et al.*, 1979) can cause the coat to dissociate from the membrane vesicle. Under these conditions the

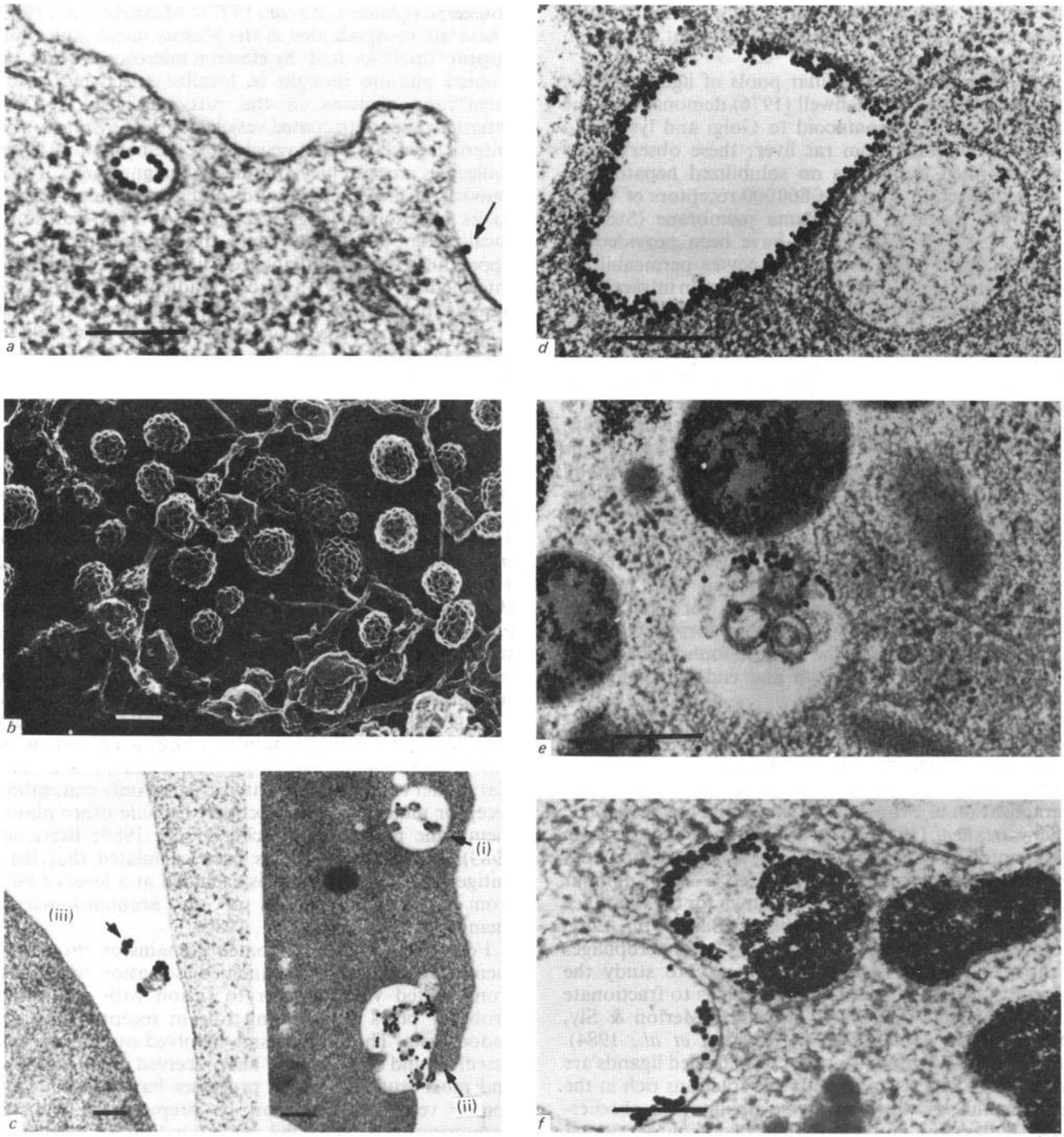

Fig. 2. Morphology of structures involved in receptor-mediated endocytosis

(*a*) Shows mannose–bovine serum albumin-coated colloidal gold bound to coated areas of a macrophage plasma membrane (arrow), and also concentrated in a small coated vesicle near the cell surface (bar = 200 nm). (*b*) A view of the inside of the plasma membrane from a lymphocyte showing the budding of clathrin-coated structures (bar = 100 nm). (*c*) Exocytosis of colloidal gold-transferrin particles by rat reticulocytes. Transferrin-coated gold particles are seen bound to the surface of inclusion vesicles within multivesicular endosomes (i). Endosomes fuse with the plasma membrane (ii) and inclusion vesicles, with their bound ligand, are shed from the cell (iii) (bar = 200 nm). (*d*)–(*f*) Endosomes and multivesicular bodies. Bone-marrow derived macrophages were allowed to ingest mannose–bovine serum albumin adsorbed to small colloidal gold particles (7–8 nm diameter) and then chased for 5 h at 37 °C in normal medium. This procedure heavily labelled secondary lysosomes. The cells were then incubated at 37 °C with large (15 nm) mannose-albumin–gold particles. (*d*) and (*e*) After 10 min incubation, 15 nm gold particles are localized in large electron-luscent vesicles with arm-like extensions (*d*) and inclusion vesicles (*e*) (bar = 200 nm). (*f*) After 20 min incubation 15 nm gold particles are seen in lysosomes previously labelled with small (7–8 nm) gold particles (bar = 200 nm).

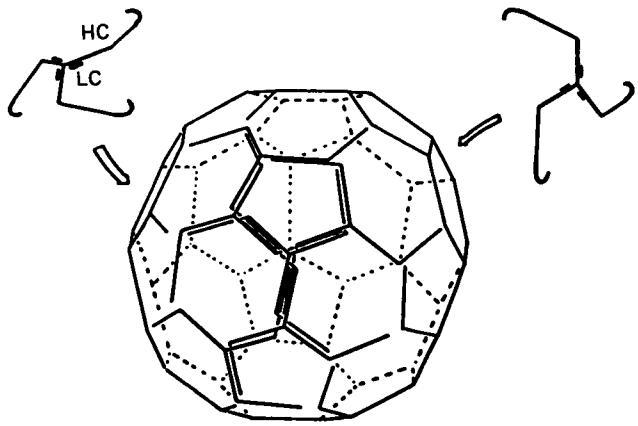

Fig. 3. Diagrams of clathrin trimers (triskelions) and their packing arrangement in a cage (HC, heavy chain; LC, light chain)

Reproduced from Harrison & Kirchhausen (1983).

dissociated clathrin exists as a trimer containing three 180000 Da polypeptides in association with three clathrin light chains (Kirchhausen & Harrison, 1981; Kirchhausen *et al.*, 1983). These trimers can be visualized by electron microscopy and have a characteristic triskelion morphology (Unanue *et al.*, 1981; Ungewickell & Branton, 1982) (Fig. 3). The 110000 Da protein remains with the now stripped membrane vesicle.

Assembly of coated vesicles. Triskelions incubated at pH 6.0 will rapidly polymerize to form polygonal clathrin cages. This reassembly phenomenon has proved to be useful in the determination of the domains of the triskelion that are responsible for cage construction. Digestion of clathrin cages with trypsin does not affect cage morphology, but selectively converts the 180 kDa clathrin peptide to a polypeptide of 110 kDa (Schmid *et al.*, 1982). If the proteolysed cages are dissociated and the resulting triskelions are examined by electron microscopy they are found to have truncated legs; the distal third of each leg having been removed by the proteinase. The observation that these modified triskelions do not lose their ability to reassemble into cages has suggested that the central domain of the triskelion is involved in cage construction. Digestion of cages with elastase, on the other hand, selectively proteolyses the clathrin light chains. Elastase-digested cages also remain intact, but after cage dissociation the triskelions are unable to reassociate into cages; instead they form a heterogeneous polygonal lattice (Schmid *et al.*, 1982; Kirchhausen & Harrison, 1981; Lisanti *et al.*, 1981). Monoclonal antibodies raised against the clathrin light chains have been used in conjunction with electron microscopy to determine where the light chains bind to the triskelion (Kirchhausen *et al.*, 1983). The results from these experiments suggest that the clathrin light chains bind near the center of the triskelion. The observation that removal of clathrin light chains prevents cage formation and affects the handedness of triskelions suggests that the light chains pull the distal portion of the triskelion legs into the correct position during cage assembly.

Membrane vesicles stripped of their clathrin coats can be used to study the factors affecting the coating of membranes by clathrin. Triskelions added to stripped vesicles bind with high affinity to the membrane and rapidly form polygonal clathrin coats (Hanspal *et al.*, 1984). Triskelions do not bind to inside-out red blood cells (erythrocytes do not have coated pits) or unstripped coated vesicles. Interestingly, truncated triskelions formed by limited proteolysis of cages by trypsin also bind to stripped vesicles suggesting that, as with cage assembly, it is the triskelion hub that is important for lattice construction. Polyacrylamide-gel electrophoresis of stripped vesicles shows the presence of two major polypeptides, one of 55000 Da thought to be tubulin (Wiedenmann & Mimms, 1983), the other of 110000 Da. Limited proteolysis of stripped vesicles by elastase selectively digests the 110000 Da protein and the resulting vesicles are unable to bind clathrin triskelions (Unanue *et al.*, 1981). It is thought that the 110000 Da membrane protein may be the point of attachment for triskelions and may promote clathrin lattice formation.

Disassembly of coated vesicles. Coated vesicles containing receptor–ligand complexes lose their clathrin coats shortly after budding away from the plasma membrane. Clathrin coats on membranes would be expected to be stable because, *in vitro*, triskelions spontaneously coat stripped vesicles or condense to form clathrin cages. Vesicle uncoating may be considered, therefore, to be energetically unfavorable and would require an enzyme and an energy source. Patzer *et al.* (1982) have studied vesicle uncoating by mixing coated vesicles with crude cytosolic extract in the presence or absence of ATP. They show that both ATP and cytosol are required to uncoat vesicles. An assay for clathrin uncoating was developed by Schlossman *et al.* (1984). Purified clathrin was radiolabelled and allowed to assemble into empty cages. Uncoating activity was detected by following the release of soluble radioactive clathrin from intact cages; the latter could be sedimented by high-speed centrifugation. They used this assay to follow the purification of cytosolic factors involved in vesicle uncoating. Passage of bovine brain cytosol over DEAE-cellulose, hydroxyapatite (to remove actin) and ATP–agarose was sufficient to purify a single polypeptide of 70000 Da which had ATP-dependent uncoating activity. This enzyme is referred to as 'uncoating ATPase'. It appears that the enzyme binds to intact clathrin coats or cages and then hydrolyses ATP. Triskelions are released from the cage in a stoichiometric complex with the uncoating ATPase (Braell *et al.*, 1984). These triskelion–enzyme complexes are unable to reassociate into cages. Electron microscopy of the complexes shows a bulge at the hub of the triskelion, suggesting that this may be the enzyme binding site. Uncoating ATPase is relatively abundant in cytoplasm (0.1% of the total protein) and is present in the same molar concentration as clathrin (Goud *et al.*, 1985). Cytoplasmic clathrin may exist as a complex with the uncoating enzyme; the mechanisms which disrupt, these complexes, allowing new coat assembly, remain to be elucidated. It is interesting that, although the concentration of clathrin in different cell types is constant at approx. 0.1% of the total cytoplasmic protein, the percentage of cytoplasmic clathrin that is assembled into cages varies (Goud *et al.*, 1985). The fraction of assembled clathrin is highest in endocytically and exocytically active cells. It is possible

that a cell can recruit clathrin from an unassembled pool to form coated pits and vesicles when stimulated to secrete or endocytose proteins.

Endosomes

Endosome terminology and definitions. The discovery of a new organelle responsible for transportation of receptors and ligands has provided problems of terminology and definition. Receptosome has been used to distinguish the organelle from vesicles formed during fluid-phase pinocytosis and phagocytosis (Pastan & Willingham, 1981, 1983). In some aspects this term is inappropriate because fluid phase markers enter vesicles that contain receptors and it is not yet clear that all vesicles formed throughout the endocytic pathway contain receptors. For example, some receptor-free vesicles may form to fuse with lysosomes. Helenius *et al.* (1983) use the term endosome to describe vesicles formed during endocytosis. There is now a growing catalogue of endosomes with names assigned on the basis of morphology, e.g. multivesicular or tubulovesicular endosomes (Harding *et al.*, 1984), endosomes I, II and III (Helenius *et al.*, 1983) or, alternatively, named according to the position of the organelle within the cell, e.g. peripheral or juxtanuclear endosomes (Hopkins, 1983, 1985). Geuze *et al.* (1983*a*) use the term CURL to describe endosomal vesicles with distinctive curled tubular extensions. CURL is probably analogous to endosomes II and III. In most cell types endosomes show variable cellular distribution and heterogeneous morphology and the above definitions have become blurred and, as a consequence, are difficult to apply rigidly.

Endosome isolation and characterization. Endosomes, probably as a result of their complex composition and morphology, have proved very difficult to isolate. The labelling of endosomes with radioactive ligands has allowed identification for fractionation experiments. A period of ligand uptake is chosen that allows the endosomal vesicles to be labelled but does not allow the ligand to reach the lysosomes. The cells or tissue are homogenized and analysed by standard cell fractionation techniques (Hadjiivanova *et al.*, 1984; Saermark *et al.*, 1985). At present there is no enzyme recognized as a marker for endosomal membranes. Organelle purity is assessed on the basis of separation of vesicles containing radioactivity from enzyme activities associated with Golgi, secondary lysosomes or plasma membrane. Purity can also be assessed using electron microscopy if endosomes contain an electron-dense ligand. Dickson *et al.* (1983) report a 37-fold purification of endosomes obtained from human KB cells labelled with ^{125}I-EGF. Secondary lysosomes were removed from cell homogenates using Percoll gradients; vesicles were then passed through Sephacryl S-1000 to separate endosomes from galactosyltransferase activity. Subsequent centrifugation on sucrose gradients gave vesicles containing only trace amounts of plasma membrane and lysosomes.

An alternative means of purifying endosomes has been to fill the endosomal compartment with a compound that can change the density of the organelle. A successful change in endosome equilibrium density has been reported by Courtoy *et al.* (1984). They perfused rat liver with horseradish peroxidase bound to galactosylated bovine serum albumin. The conjugate entered the endosomal compartment via the hepatocyte galactose receptor (Quintart *et al.*, 1984). The homogenized liver was fractionated by using sucrose gradients and a crude vesicle fraction containing high peroxidase activity was taken and incubated with 3,3-diaminobenzidine and H_2O_2. The 3,3-diaminobenzidine polymerized in vesicles containing horseradish peroxidase and caused a major shift in equilibrium density. These denser vesicles could then be separated from non-endosomal vesicles by centrifugation on a second sucrose gradient. This resulted in a 250-fold purification of endosomes with respect to homogenate.

The partially purified endosome preparations described have been analysed for their lipid and protein content. Endosomes, like the plasma membrane, contain a relatively high cholesterol/phospholipid ratio (Dickson *et al.*, 1983). Polypeptide analysis by polyacrylamide-gel electrophoresis has revealed a protein pattern different from purified plasma membrane or lysosomes with major bands at 120, 92, 68 and 55 kDa. Immunoprecipitation studies showed the presence of receptors for mannose-6 phosphate and transferrin.

Endosome function. Having outlined some of the terminology of receptor-mediated endocytosis it should be possible to follow the pathway of the ligand through the endosome system and assign particular functions to these different structures. During the first 2 min ligands leave coated vesicles and enter small peripheral vesicles and tubules (peripheral endosomes, endosome I) and are closely associated with the limiting membrane of the vesicles, suggesting that they are bound to their respective receptors. During the next 8–10 min intralumenal acidification and receptor–ligand dissociation takes place (Harford *et al.*, 1983*a*; Wileman *et al.*, 1985). The morphology of endosomes changes greatly during this period and this undoubtedly has functional significance. Endosomes become larger and gain arm-like extensions. The structures formed during this period are referred to as CURL. The observation that the tubules are enriched for receptors and the vesicle lumen contains dissociated ligand makes this an attractive acronym for a subset of endosomes carrying out the initial steps in receptor–ligand sorting. The lumen of CURL appears to contain little soluble protein and has a volume which is very much larger than the precursor tubules and vesicles. It is thought that endosomes swell rapidly during this phase, possibly as a result of the active pumping of ions into the vesicle lumen during acidification. At the same time the swollen endosome lumen acts as a volume sink to trap dissociated ligand molecules for eventual transfer to lysosomes. Ligands which remain bound to their receptor at acidic pH would be expected to concentrate in the tubules of the endosome and recycle with the receptor (category 2, Fig. 1). At present it is not possible to determine the mechanism whereby receptors concentrate in the tubules of CURL. They may, for example, be actively clustered into specialized domains in a manner analogous to coated pit formation. There is, as yet, no evidence for the presence of clathrin at the tips of the tubules. Alternatively, receptor–ligand complexes may be present initially in the tubules of CURL and it is the ligand that moves by diffusion into the swelling endosome during acidification. Rome (1985) argues that segregation is a passive phenomenon; since 90% of the membrane of CURL is found in the tubules, receptors allowed to move

randomly through the membrane would be found 90% enriched in the tubules.

During the later stages of endocytosis (6–15 min) endosome morphology changes again and ligand molecules appear in multivesicular bodies, some of which have tubular extensions (multivesicular or tubulovesicular endosomes). In polarized cells these vesicles are found in the juxtanuclear area. The inclusion vesicles may form by extrusion of the endosome membrane into the lumen of CURL. It is thought that receptors and possibly ion pumps and channels are lost from CURL at this point. The multivesicular endosome, with its contents destined for degradation (Haigler *et al.*, 1979), fuses with lysosomes, while receptors and other proteins to be spared degradation return to the plasma membrane.

Endosome biogenesis. It is not yet clear how the ligand is transported between endosomes that have very different morphologies. The question arises as to whether endosomes are stable organelles, perhaps akin to the Golgi apparatus, or whether the endosome is a transient collection of vesicles and tubules which, after accomplishing its sorting function, is transferred *en bloc* to the lysosomes.

Two models have been proposed by Helenius *et al.* (1983). In the first endosomes are considered to be stable organelles which would require small vesicles to pass ligand between them and the cell surface and lysosomes. Vesicles derived from coated vesicles, perhaps peripheral endosomes, would deliver receptor–ligand complexes to CURL. After receptor–ligand dissociation vesicles would take clustered receptors back to the cell surface. A second group of vesicles would carry small droplets of fluid containing dissociated ligand to lysosomes. In the second model, endosomes and their contents move together through the cell. Peripheral endosomes form as a result of fusion between incoming vesicles and grow in size. Arm-like extensions localize clustered receptors, while the endosome body envaginates to form the multivesicular endosome which ultimately fuses with lysosomes.

There are arguments for and against both models. A vesicle shuttle would be the most convenient means of transporting clustered receptors to and from the endosome. On the other hand, small vesicles would be an extravagent means of transporting the endosomal contents to lysosomes since they have a high surface-to-volume ratio. The available data favour a model where the endosome matures to deliver itself and its contents to lysosomes. Functional proteins, which would necessarily be spared from degradation, e.g. receptors, ion channels, proton pumps, are clustered and packaged into small vesicles for transport back to the cell surface or to an alternative intracellular destination.

Kinetics of ligand uptake

Receptor recycling. In general the uptake of ligands classified as Type 1 (Table 1) by receptor-mediated endocytosis proceeds linearly over extended periods even in the presence of protein synthesis inhibitors. The number of ligand molecules internalized by cells far exceeds the total binding capacity of the surface and intracellular pools. These observations have suggested that receptors recycle between the plasma membrane and the cell interior to allow the continuous uptake of ligand. It is possible to estimate the recycling times for some receptors. Alveolar macrophages internalize approx. 1.8×10^6 molecules of mannose–bovine serum albumin per cell in 1 h at 37 °C. Macrophages possess only approx. 400000 mannose receptors, so to account for this rate of uptake each receptor must be reused every 12 min (Wileman *et al.*, 1984). Similar calculations suggest that the asialoglycoprotein receptor returns to the cell surface within 7.5 min of its internalization in Hep G2 cells and recycles 250 times in its lifetime (Schwartz & Rup, 1983).

Is there any additional evidence that receptors can move from the cell surface to the intracellular pool and back out again? It has not yet been possible to demonstrate that an individual receptor molecule can complete the whole pathway, but there is evidence that receptors leave the plasma membrane on binding their ligand and that these receptors are rapidly replaced by receptors moving from the cell interior. For example, internalization of ^{125}I-asialo-orosomucoid by Hep G-2 cells is accompanied by a rapid depletion of cell surface asialoglycoprotein receptors, followed by a rapid recovery of cell surface binding activity within 4 min (Ciechanover *et al.*, 1983). Interestingly, this transient loss of surface receptors is ligand-specific, since Hep G-2 cells do not lose cell surface transferrin or insulin receptors during endocytosis of asialoglycoproteins. Direct evidence of receptor internalization has been provided at the electron microscope level using receptor-specific antibodies complexed to colloidal gold. Geuze *et al.* (1982) demonstrate the transfer of hepatocyte asialoglycoprotein receptors from the plasma membrane to small vesicles during the uptake of asialo-orosomucoid and Hopkins (1983) shows the internalization of transferrin receptors during the uptake of transferrin by epidermoid carcinoma cells.

The rapid recovery of cell surface binding activity that follows receptor–ligand internalization suggests that receptors quickly return to the cell surface or are replaced by insertion of internal receptors into the plasma membrane. The movement of internal receptors to the cell surface has been demonstrated by Stahl *et al.* (1980). Alveolar macrophages lose their surface mannose glycoprotein receptors when exposed to trypsin at 4 °C. Mannose binding activity remains low if the cells are maintained at 4 °C. If the cells are briefly warmed to 37 °C there is a rapid recovery of cell surface binding activity. The interpretation is that warming the cells allows internal receptors to move to the plasma membrane. The movement of internal transferrin receptors to the plasma membrane has been demonstrated at the electron microscopy level using colloidal gold–transferrin probes (Harding *et al.*, 1983) and antibodies to the receptor itself (Hopkins, 1983). It appears that, in epidermoid carcinoma cells, internal transferrin receptors are introduced into specific domains of the plasma membrane (Hopkins, 1983, 1985).

Since many ligands are transferred to lysosomes and digested while their receptors recycle, a mechanism must exist whereby receptors and ligands are separated so that receptors may be spared lysosomal digestion and return to the cell surface functionally intact. Insight into a possible mechanism for receptor–ligand dissociation and segregation has come from the following observations. (i) Agents that neutralize acid intracellular compartments block endocytosis. (ii) The affinity of a number of ligands for their receptors is pH-dependent and low at acid pH. Acidification of an intracellular compartment containing receptor–ligand complexes would enhance receptor–

ligand dissociation and allow differential receptor–ligand sorting. Support for this proposal has been provided initially by studies using amines that dissipate intracellular pH gradients, and later by direct demonstration of an endosomal proton pump (Tycko & Maxfield, 1982).

Amines and proton ionophores inhibit receptor-mediated endocytosis. Weak bases such as chloroquine, NH_4Cl and methylamine diffuse into cells across membranes in their uncharged form and accumulate in acidic intracellular compartments where they become protonated and raise intravesicular pH (reviewed by Dean *et al.*, 1984). Proton ionophores such as monensin also dissipate intracellular pH gradients; they allow protons to equilibrate across membranes by exchange with cations, for example monensin exchanges protons preferentially with Na^+ while nigericin exchanges protons for K^+. As a result the biological effects of proton ionophores are more complex than those of amines, since ion gradients across membranes may alter during neutralization of acidic compartments. Incubation of cells with amines or chloroquine inhibits endocytosis of mannose glycoproteins (Tietze *et al.*, 1980) asialoglycoproteins (Tolleshaug & Berg, 1979; Schwartz *et al.*, 1984), lysosomal enzymes (Gonzalez-Noriega *et al.*, 1980), α_2-macroglobulin (Van Leuven *et al.*, 1980), while monensin inhibits endocytosis of LDL (Basu *et al.*, 1981), asialoglycoproteins (Berg *et al.*, 1983; Harford *et al.*, 1983*a*) and mannose glycoproteins (Wileman *et al.*, 1984). In all these studies reduced ligand endocytosis is attributed to a loss of receptors from the cell surface. On warming cells to 37 °C surface-bound ligand is rapidly internalized (Basu *et al.*, 1981; Harford *et al.*, 1983*a*; Berg *et al.*, 1983; Schwartz *et al.*, 1984; Wileman *et al.*, 1984). Surface LDL receptors accumulate within perinuclear vacuoles as a consequence of monensin treatment (Basu *et al.*, 1981) and asialoglycoprotein receptors redistribute from the plasma membrane to the cell interior of Hep G-2 cells in response to chloroquine (Schwartz *et al.*, 1984).

A number of studies have shown that amines and proton ionophores exert their effect by disrupting endosome function. Percoll gradient fractionation of amine or monensin-treated cells shows ligands trapped in light membrane fractions (Harford *et al.*, 1983*a*; Wileman *et al.*, 1984) with a density similar to that of endosomes (Merion & Sly, 1983). Harford *et al.* (1983*b*) followed intracellular dissociation of asialo-orosomucoid from the galactose receptor and showed that more than half the ligand dissociated from the receptor within 20 min of entering the cell. Since the ligand had insufficient time to reach lysosomes they concluded that receptor–ligand dissociation was a pre-lysosomal event. This dissociation was blocked by monensin. Direct evidence for endosomal acidification has been provided by Tycko & Maxfield (1982) using fluorescein-labelled ligands. The pH-dependent excitation profile of fluorescein-labelled probes can be used to determine the pH of intracellular compartments (Ohkuma & Poole, 1981). Fluorescein-labelled α_2-macroglobulin and asialoglycoprotein enter an acidic (pH 5.5) environment shortly after entering the cell but before being delivered to lysosomes, indicating that endosomes can acidify prior to fusion with lysosomes. The acidic nature of the endosome has also been demonstrated at the electron microscopy level. Amines accumulate in acid vesicles and antibodies raised against amines can be used as immunohistochemical tools to identify acidic compartments (Anderson *et al.*, 1984; Anderson & Pathak, 1985; Schwartz *et al.*, 1985). These studies demonstrate antibody staining of endosomes, lysosomes and elements of the trans Golgi.

It is still not clear why the bulk of internalized receptor–ligand complexes are unable to return to the cell surface during amine treatment. (There is a small pool of receptor–ligand complexes which can return to the surface but, as will be described later, they follow a pathway that is not affected by amines.) Evidence that most receptor–ligand complexes are unable to recycle in the presence of amines is provided by Tietze *et al.* (1980, 1982). Macrophages treated with amines accumulate a pool of mannose-glycoproteins which is inaccessible to a dissociating medium (EDTA + α-methyl mannoside). If receptor–ligand complexes were recycling the ligand would leave the receptor at the cell surface under dissociating conditions, producing a recovery of ligand-binding activity. Only 20% of pre-internalized ligands could be released from the cells under external dissociating conditions, which suggests that the bulk of the receptor–ligand complexes are trapped inside. Another interesting observation is that some receptors recycle constitutively in the absence of added ligand (Basu *et al.*, 1981; Tietze *et al.*, 1982; Schwartz *et al.*, 1984; Wileman *et al.*, 1984; Watts, 1985). These receptors are trapped within the cell when intravesicular acidification is prevented. Since these receptors cannot be trapped as a result of being immobilized with their ligand, the results imply that acidification may also be required for the retrieval of unoccupied receptors in preparation for transport back to the cell surface.

Receptors that follow pathway 1 (Fig. 1) are trapped within cells irrespective of the agent dissipating endosomal pH gradients. In the case of transferrin (pathway 2, Fig. 1) the situation is slightly different. The receptor recycles normally in cells treated with amines (Ciechanover *et al.*, 1983; Klausner *et al.*, 1983; Harding & Stahl, 1983) or in cells with a genetic defect in endosomal acidification (Klausner *et al.*, 1984). Surprisingly, cells treated with monensin are unable to recycle transferrin receptors, and receptors and ligand accumulate in swollen multivesicular bodies close to the Golgi (Stein *et al.*, 1984). Monensin exchanges protons for Na^+, and it may be that an increased lumenal [Na^+], or a consequent change in membrane potential, prevents exit of transferrin receptors from the para Golgi compartment. Baeniziger & Fiete (1982) have shown previously that removal of Na^+ ions from the cytoplasm of hepatocytes prevents delivery of endosomal ligand to lysosomes. An updated model for the transferrin cycle is slowly emerging. It appears that there may be fast (8 min cycle) and slow (2 h cycle) recycling pathways. The slow pathway, seen during prolonged incubation with transferrin, involves passage of the receptor through elements of the trans Golgi (Snider & Rogers, 1985; Hopkins, 1983). There is evidence that this compartment may be less acidic (pH 6.5) than earlier endocytic vesicles (Yamishiro *et al.*, 1984), suggesting that ion gradients rather than pH gradients may have a predominant effect on receptor–ligand sorting in this compartment. The question arises as to whether this slow component is truly a receptor-mediated pathway or whether it is a cycle followed by all membrane proteins (Snider & Rogers, 1985; Widnell *et al.*, 1982).

Coated vesicles and endosomes contain ATP-dependent proton pumps and anion channels

A number of laboratories have investigated the acidification mechanisms of coated vesicles and endosomes. Experiments designed to measure the lumenal pH of coated vesicles rely on the trapping of a weak base by protons pumped into the coated vesicle; thus the accumulation of [^{14}C]methylamine (Forgac *et al.*, 1983) or the accumulation and subsequent quenching of Acridine Orange fluorescence within coated vesicles (Stone *et al.*, 1983, 1984; Van Dyke *et al.*, 1984) can be used as a measure of intralumenal acidification. It is important that these experiments are conducted using highly purified coated vesicles and control experiments have to show that weak bases are not trapped by contaminating lysosomes, Golgi vesicles or mitochondria. In contrast to coated vesicle preparations, it has not been possible to prepare endosomes devoid of Golgi or lysosomal vesicles and alternative methods of measuring intravesicular pH have been devised. It is possible to introduce selectively a fluorescent probe into the endosomal compartment by allowing cells to internalize a fluorescent ligand for a short period of time, thereby avoiding transfer of the ligand to lysosomes. Endocytic vesicles can then be separated from cytoplasm and secondary lysosomes by homogenization and subsequent centrifugation on Percoll gradients (Galloway *et al.*, 1983; Merion & Sly, 1983; Merion *et al.*, 1983). An alternative approach has been to introduce fluorescent α_2-macroglobulin into cells and then to permeabilize selectively the plasma membrane with low concentrations of digitonin (Yamashiro *et al.*, 1983). This allows the cytoplasm surrounding the endosome compartment to be washed away; any existing pH gradient is dissipated by a brief treatment with monensin before the cells are equilibrated with a physiological buffer. The above experiments have shown that the acidification of coated vesicles and endosomes shares many features in common.

The lumen of coated vesicles (Forgac *et al.*, 1983; Stone *et al.*, 1982; Van Dyke *et al.*, 1984) and endosomes (Galloway *et al.*, 1983; Merion *et al.*, 1983; Yamashiro *et al.*, 1983) will acidify (ΔpH 0.6 units) in the presence of millimolar concentrations of ATP. This proton gradient is dissipated by proton ionophores, suggesting that these organelles actively lower their lumenal pH by means of an ATP-dependent proton pump. An alternative explanation is that acidification is indirect and occurs as a result of a Na^+ or K^+ gradient created by a Na^+,K^+-ATPase followed by Na^+/H^+ exchange. This mechanism is thought to be unlikely, since coated vesicles and endosomes acidify equally well in the absence of either Na^+ or K^+ and proton translocation is not affected by sodium vanadate, an inhibitor of the Na^+,K^+-ATPase of the plasma membrane (Forgac *et al.*, 1983; Van Dyke *et al.*, 1984; Galloway *et al.*, 1983; Yamashiro *et al.*, 1983). Moreover, Yamashiro *et al.* (1983) show that endosomal acidification is unaffected by amiloride, a drug that inhibits Na^+/H^+ exchange. The coated vesicle and endosomal ATPases are also distinct from the F_1–F_0 ATPase of mitochondria, since vesicle acidification is unaffected by efrapeptin, oligomycin or NaN_3 (Stone *et al.*, 1983; Forgac *et al.*, 1983; Van Dyke *et al.*, 1984; Galloway *et al.*, 1983).

It has yet to be determined whether or not the import of protons into the endosomes creates a membrane potential. The electrogenic properties of the lysosomal proton pump have been studied in some detail but some confusion still remains. Schneider (1983) suggests that proton pumping is coupled to the co-transport of phosphate ions and that the pump is therefore electroneutral. In contrast, Harikumar & Reeves (1983), using a potential-sensitive fluorescent dye, demonstrate the generation of a membrane potential during lysosomal acidification. This potential is dissipated in the presence of permeant anions (Cl^-). The absence of purified endosomes precludes the direct measurement of endosomal membrane potential; nevertheless, there is evidence suggesting that endosomal and coated vesicle acidification requires counter-ion movement. For example, acidification of endosomes and coated vesicles is inhibited in media where Cl^- has been replaced by poorly permeable anions such as gluconate or sulphate (Xie *et al.*, 1983; Van Dyke *et al.*, 1984; Galloway *et al.*, 1983); moreover, anion channel inhibitors such as DIDS can inhibit endosomal acidification (Yamashiro *et al.*, 1983) and duramycin can block proton translocation in coated vesicles (Stone *et al.*, 1984). The role of an anion channel in coated vesicle acidification has been studied in some detail by Stone *et al.* (1984). They show that coated vesicles accumulate ^{36}Cl when incubated with ATP and that this accumulation can be blocked by concentrations of duramycin that block acidification.

It is possible that endosomal acidification creates a membrane potential that slows down the rate of proton transport. This potential can be short circuited by Cl^- conductance, and in the absence of permeant anions acidification is inhibited. The ability to generate a membrane potential by regulating a Cl^- channel during acidification may have functional significance. The membrane potential may provide the driving force for receptor movement and may, in conjunction with pH changes, participate in fusion processes. A proton pump with similar properties has been demonstrated in vesicles isolated from the Golgi, an organelle which also has a transport and sorting functions (Glickman *et al.* 1983).

ATP-dependent acidification of endosomes is thought to accelerate receptor–ligand dissociation and subsequent receptor-ligand sorting within the cell. ATP-dependent dissociation of receptor-ligand complexes has been shown to occur within macrophage endosomes (Wileman *et al.*, 1985); dissociation is inhibited by proton ionophores, further suggesting that it is intravesicular acidification that drives dissociation. ATP-dependent receptor–ligand dissociation was blocked by the agents above that block the acidification of endosomes and coated vesicles, and dissociation was insensitive to oligomycin, NaN_3 and sodium vanadate. As expected, the data strongly suggest that the ATPase responsible for receptor–ligand dissociation is the same as that which powers acidification. ATP-dependent receptor–ligand dissociation was also blocked by anion channel inhibitors DIDS and duramycin and occurred poorly if Cl^- was replaced by gluconate. It would appear that receptor–ligand dissociation within endosomes, like coated vesicle acidification, requires the participation of an anion channel.

Alternative pathways

Ligand recycling. The text above draws on many examples from studies of ligands which dissociate from their receptors at low pH and are delivered to lysosomes

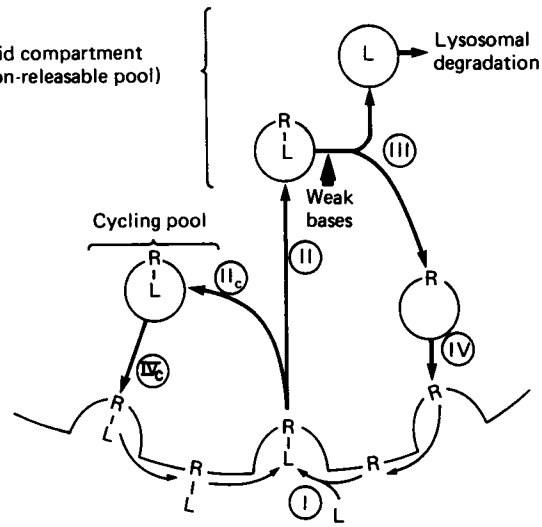

Fig. 4. Receptor–ligand recycling in the short cycle

Receptors bind ligand at the cell surface (I); most of the ligand travels to an endosome which is able to acidify (II); the receptor and ligand dissociate and the ligand is degraded (III) while the receptor returns to the cell surface (IV). Other receptor-ligand complexes enter an endosome which does not acidify (II_c), receptor and ligand do not dissociate and return to the cell surface together (IV_c).

(Pathway 1, Fig. 1). Other ligands remain attached to their receptors in an acid environment, avoid lysosomes, and recycle to the cell surface (Pathway 2, Fig. 1). A good example is transferrin. Diferric transferrin is internalized and converted into apotransferrin, since iron dissociates from transferrin under the influence of acid pH. Apotransferrin retains a high affinity for the transferrin receptor within the endosome and recycles back to the cell surface. At pH 7.4 only diferric transferrin retains a high affinity for the receptor, apotransferrin dissociates to be replaced by diferric transferrin and the cycle continues (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983; Harding & Stahl, 1983; Morgan, 1981).

The short cycle (diacytosis, retroendocytosis, receptor–ligand Recycling). Some ligands which would normally be expected to be delivered to lysosomes return to the cell surface intact on board their receptor (Fig. 4). Tietze *et al.* (1982) first demonstrated this by incubating cells containing previously internalized ligands with EDTA or competitive ligands, the former acting to bind Ca^{2+} which is required for ligand binding and the latter to displace receptor-bound ligand as it returns to the cell surface. While a small amount of ligand was shed to the extracellular compartment upon further incubation in the absence of dissociating media, a substantial portion was externalized in its presence. These findings suggested that a portion of internalized receptor–ligand complexes must normally return to the surface intact – and only in the presence of conditions which enhance receptor–ligand dissociation does one actually observe net transfer to the extracellular compartment. Similar observations have been made with the galactose receptor of hepatocytes (Simmons & Schwartz, 1984) and the LDL receptor of fibroblasts (Greenspan & St. Clair, 1984; Aulinskas *et al.*, 1985). Workers using the galactose receptor for their studies have referred to this as diacytosis, whereas similar phenomena with LDL receptors has been called retroendocytosis. Tietze *et al.* (1982) referred to this phenomena as receptor–ligand cycling as opposed to receptor recycling. Incubation of cells with ligand in the presence of amines increases the number of receptor–ligand complexes within cells but does not increase the flux of receptor–ligand complexes back to the cell surface. This suggests that there are two functionally distinct pools of receptors (Fig. 4). There are those which would normally deliver their ligand to lysosomes; these are sensitive to the effects of amines and need to pass through an acidic compartment to return to the cell surface. The other receptor pool cycles independently of intracellular acidification and returns the ligand to the cell surface. A population of endosomes defective in acidification has been described recently (Wileman *et al.*, 1985); approx. 20% of macrophage endosomes that contain receptor–ligand complexes cannot acidify on addition of ATP. The observation that the latter receptor–ligand complexes do not dissociate suggests that they enter endosomes that possibly lack proton pumps. Alternatively, some newly formed endosomes may randomly fuse back to the plasma membrane before acidification can occur.

In several papers over the past few years Regoeczi *et al.* (1982*a*, *b*) have nicely demonstrated that asialotransferrin is taken up by hepatocytes *in vivo* and that some of the ligand is re-expressed at the cell surface. Here the mechanism appears to be slightly different to that described above. It is now known that transferrin binds to the transferrin receptor both at neutral pH and at acid pH – so what was initially thought to be the recycling of galactose-receptor–ligand complexes may well be the intracellular transfer, under acid conditions, of ligands from one receptor (galactose) to another (transferrin). Nevertheless, these studies have been influential in pointing out that the transferrin-receptor–ligand complexes may move through the trans Golgi compartment following internalization, since the recycled transferrin molecules are resialylated. Such a model has been supported by Snider & Rogers (1985) who show that the transferrin receptor, deprived of its sialic acid residues at the cell surface, is resialylated during endocytosis and recycling. The nature of the ligand may also influence the fate of receptors to which they bind. For example, it has been shown that polyvalent ligands target Fc receptors to lysosomes, whereas monovalent ligands direct receptor–ligand complexes back to the cell surface (Mellman *et al.*, 1984). It should be mentioned that the Fc receptor with polyvalent ligand bound is thought not to recycle.

Receptor degradation and receptor shedding. It is still not clear how receptors and other membrane-bound proteins are delivered to lysosomes (category 3, Fig. 1). Receptors which are degraded in lysosomes (e.g. receptors for EGF and insulin) must be segregated from domains of the endosome that recycle allowing them to target with the lumenal contents of the endosome destined for degradation in lysosomes (Haigler *et al.*, 1979). It is possible that these proteins are extruded from limiting membrane into the membrane of the inclusion vesicles observed in the lumen of multivesicular endosomes (Harding *et al.*, 1985). These inclusion vesicles would then be sorted with other lumenal contents and target to lysosomes. The transferrin receptor is usually recycled following internalization, but in reticulocytes some transferrin receptors are not

Table 2. Molecular biology of receptors entering the cell via receptor-mediated endocytosis

Ligand	Species	Complete primary amino acid sequence deduced from:	Distribution of amino acid residues				Intracellular pathway (see Table 1)	Reference
			Cytoplasmic domain	Cytoplasmic terminal	Membrane spanning domain	Extracellular domain		
EGF	Human	cDNA	542	*C*	23	621 (8% cysteine)	Type 3	Ullrich *et al.* (1984)
LDL	Human	cDNA	50	*C*	22	767 (15% cysteine)	Type 1	Yamomoto *et al.* (1984)
Transferrin	Human	cDNA; gene	61	*N*	28	632	Type 2	McClelland *et al.* (1984)
Asialoglycoprotein	Rat	Peptide sequencing	—	*N*	30	—	Type 1	Drickamer *et al.* (1984)
	Human	cDNA	—	*N*	—	—		Spiess *et al.* (1985)
IgA/IgM transcytosis	Rabbit	cDNA	103	*C*	23	629	Type 4	Mostov *et al.* (1984)
Insulin	Human	cDNA	403 (β-subunit)	*C*	22	930 (16% cysteine)	Type 3	Ebina *et al.* (1985)
						735 from α-subunit 195 from β-subunit		Ullrich *et al.* (1985)

recycled but are lost from the cell during differentiation into erythrocytes. This receptor loss apparently also occurs via extrusion of receptors into the membrane of inclusion vesicles (Pan & Johnstone, 1983; Harding *et al.*, 1984; Pan *et al.*, 1985). These cells have a paucity of lysosomes, however, and multivesicular endosomes do not seem to fuse with lysosomes. Their lumenal contents are uniquely lost from the cell by exocytosis of multivesicular endosomes rather than lysosomal degradation. Exocytosis of colloidal gold transferrin bound to the transferrin receptor of reticulocytes is shown in Fig. 2(*c*).

Future perspectives

Research into the molecular biology of receptor-mediated endocytosis is moving forward at a fast pace. During the last 2 years the complete amino acid sequences of six receptors have been deduced (Table 2) and others are soon to follow. The sequence data show that the majority of each receptor's amino acids are incorporated into the extracellular, or ligand-binding, domain. In some cases this domain contains high concentrations of cysteine residues and this may be of functional significance. For example, cysteine crosslinking may stabilize the ligand-binding domain as it passes through the acidic environment of the endosome (Sudhof *et al.*, 1985) or alternatively, intermolecular crosslinks may form as a consequence of ligand binding allowing receptor clustering and capping at the cell surface (Ebina *et al.*, 1985). A membrane-spanning region of approx. 20–30 amino acid residues is attached to the extracellular domain anchoring the receptor to the plasma membrane. The large cytoplasmic tails of the EGF and insulin receptors house the tyrosine-specific protein kinases of these receptors (Cohen *et al.*, 1980; Ullrich *et al.*, 1984; Kasuga *et al.*, 1982; Roth & Cassell, 1983) and these regions share sequence homology with one another. Other sequence homologies have provided some striking discoveries about the receptors themselves. For example, the EGF receptor shares sequence homologies with viral transforming proteins (Downward *et al.*, 1984), and the primary amino acid sequence of the IgA/IgM transcytosis receptor shows that the receptor itself has multiple immunoglobulin-like domains (Mostov *et al.*, 1984). Although the EGF precursor and LDL receptor show some domain-specific homologies (Yamomoto *et al.*, 1984; Sudhof *et al.*, 1985), sequence homologies between receptors is sparse. This is not altogether surprising since the receptors, where sequence data are available, bind different ligands and follow different endocytic pathways through the cell. One feature they share in common is the ability to bind to clathrin coated pits. Unfortunately sequence homologies defining a clathrin binding domain have not been found.

The molecular biology of the receptors will provide part of the story. Cell lines defective at different stages of endocytosis have also provided useful tools. For example J.D. cells (Anderson *et al.*, 1977*b*) have LDL receptors that are unable to cluster in coated pits. An analysis of the amino acid sequence of the cytoplasmic tail of these defective receptors may help to define clathrin-binding domains (Lehrman *et al.*, 1985). Cell lines defective in endosomal acidification have been produced (Merion *et al.*, 1983; Robbins *et al.*, 1984; Marnell *et al.*, 1984). The observation that these defects only affect endosomal acidification and not acidification of lysosomes has suggested that the ATPases responsible for proton translocation may be different for these two organelles. The transmembrane signals, for example, ion fluxes or phosphorylation events, that are triggered by the binding of the ligand will be the focus of much research. The functional components of the endosome, its array of ion channels and proton pumps need to be isolated and hopefully reconstituted *in vitro* to study ion fluxes during acidification. Important questions remaining to be answered focus on the control of the many membrane fusion events that occur during receptor-mediated endocytosis. Firstly, how do receptors and ligand become concentrated in domains of the endosomes that are labelled for different destinations? How do membrane tubules or vesicle shuttles know when to fuse with their target organelles? The isolation of organelle-specific fusogens from the cytoplasm and the study of ion fluxes before and during fusion offer exciting areas of research.

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