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Cell Surface Carbohydrates and Cell Development

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Chapter 2

**ROLE OF OLIGOSACCHARIDES IN THE INTRACELLULAR
AND INTERCELLULAR TRAFFICKING OF MAMMALIAN
GLYCOPROTEINS**

Ajit Varki

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I. SCOPE OF THE CHAPTER

Almost all cellular glycoproteins are confined to membrane-limited compartments, with their oligosaccharide side chains facing away from the cytosol. Extracellular glycoproteins that enter the cell by endocytosis from the plasma membrane also maintain this topology. This chapter will review the role of sugar chains in the trafficking of glycoproteins, both in the biosynthetic pathway and in the endocytic process. Traffic via the following routes will be considered: from Golgi apparatus to lysosomes, from cell surface to endosomal compartments, and from endoplasmic reticulum (ER) to Golgi apparatus. In almost all the systems considered in this chapter, a *soluble* glycoprotein is picked up by a *membrane-bound* lectin

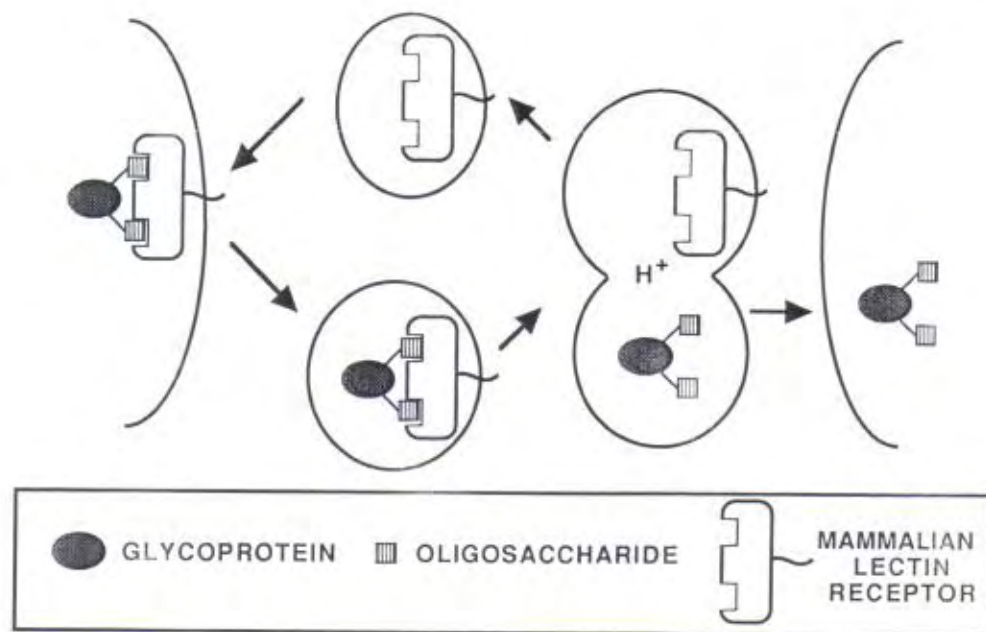


FIGURE 1. Features common to the receptor pathways discussed in this chapter. A soluble glycoprotein is recognized by a multivalent membrane-bound lectin receptor that binds to multiple sugar residues on the protein. The receptor-ligand complex is invaginated into a vesicle which delivers the complex to another compartment by targeted fusion. The receptor and ligand separate under the influence of acidic pH, the free receptor recycles, and the ligand is delivered to its final destination.

receptor via recognition of the oligosaccharide chains of the glycoprotein. The receptor-ligand complex is then invaginated into a vesicle which delivers the ligand to another subcellular compartment by targeted fusion (see Figure 1). In every case, both receptor and ligand are multivalent, and the receptor is capable of recycling. Many detailed reviews concerning these carbohydrate-specific receptors have been published over the past few years.¹⁻¹² It is not the intention of this chapter to reduplicate these excellent and detailed discussions; rather, an attempt is made to provide an overview of all these systems with emphasis upon the historical background and the development of concepts. Particular attention is given to the best defined of these pathways, in which mannose 6-phosphate (M6P) residues mediate trafficking of lysosomal enzymes to lysosomes. The other receptors involved in carbohydrate-mediated recognition and subcellular trafficking are then considered together, in less detail. A short discussion follows on the possible role of glucose residues on *N*-linked oligosaccharides in modulating trafficking of newly synthesized glycoproteins from the ER. Finally, an attempt is made to compare and contrast the discovery of the different pathways, and the lessons that they hold for the future.

II. THE INTRACELLULAR ITINERARY OF GLYCOPROTEINS

Until recently, glycosylation was felt to be restricted to proteins in the ER-Golgi pathway,^{13,14} and its subsequent ramifications (see Figure 2 for a current summary of these pathways). While clear exceptions to this rule have been found,¹⁵ this chapter will focus only upon the role of oligosaccharides in trafficking along these routes. Newly synthesized *soluble* glycoproteins spend a variable period of time in the ER,^{16,17} and then traverse the stacks of the Golgi apparatus¹⁴ by vesicle-mediated transport.¹⁸ From the Golgi, they can be delivered to the lysosomes or be secreted into the surrounding milieu via constitutive or

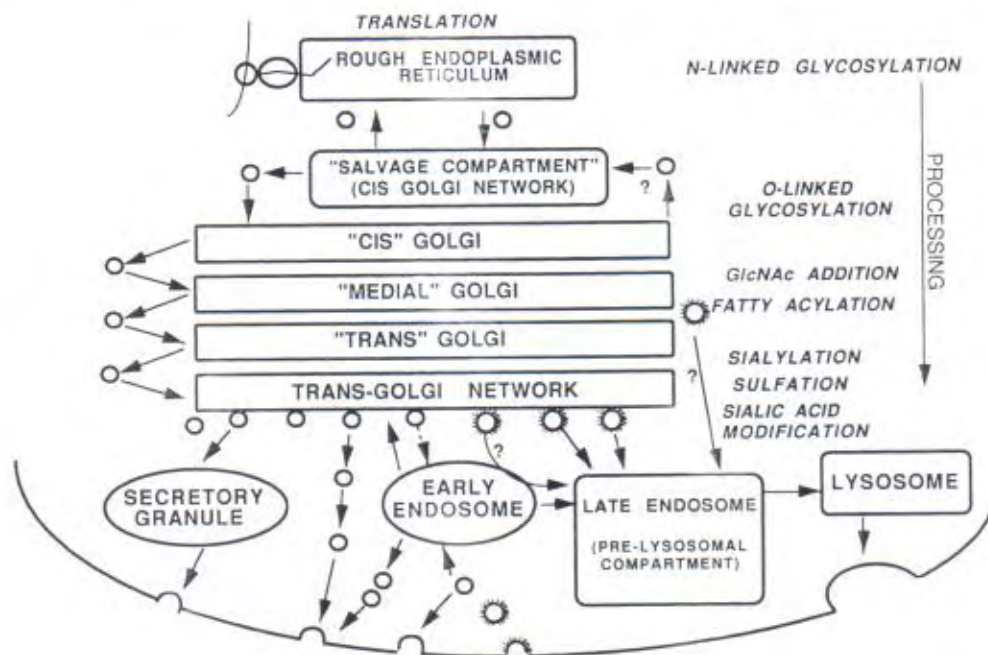


FIGURE 2. Common subcellular pathways followed by glycoproteins. Newly synthesized glycoproteins originating from the rough endoplasmic reticulum (ER) pass by vesicular traffic through the stacks of the Golgi apparatus, and are then sorted to various destinations, including the lysosomes, the plasma membrane, the secretory granules, and the constitutive exocytic pathway. Along this route, a variety of modifications are carried out, some of which are indicated in the figure (in relation to the proposed locations where they are believed to occur). Glycoproteins entering the cell via the endocytic pathway can intersect with those following the biosynthetic route in various situations, as depicted in the figure. Question marks indicate pathways that have been less frequently reported (see text for discussion). The fuzzy border indicates vesicles believed to be clathrin-coated.

regulated secretory pathways. Exogenous glycoproteins can also enter the cell by receptor-mediated endocytosis,^{19,20} ultimately arriving in the lysosomes or returning to the surface, sometimes via the *trans*-Golgi apparatus (see Figure 2). Throughout these journeys, the oligosaccharides on glycoproteins are known to undergo substantial modifications. The details of these changes are best characterized for the *N*-linked oligosaccharides,²¹ and are frequently used as convenient "signposts" to *trace* the itineraries of glycoproteins. However, in only a few instances have these changes been shown to *direct* the subcellular traffic of these proteins.

III. ROLE OF PHOSPHORYLATED OLIGOSACCHARIDES IN THE DELIVERY OF GLYCOPROTEINS TO THE LYSOSOMAL COMPARTMENT

A. HISTORICAL BACKGROUND

The lysosomal enzymes are a family of soluble acid hydrolases that traverse the same ER-Golgi pathway as secretory proteins, but are eventually sorted into the lysosomal compartment. The role of M6P residues in directing this process is the best-established example of oligosaccharide-mediated trafficking. To the skeptic, it is the *only* case in which oligosaccharide-mediated targeting has been conclusively shown to be vital for the survival of an organism. Thus, it is worthwhile to review the historical background leading to the discovery of this pathway. During the 1960s, clinical and pathological analyses of various human genetic diseases termed "storage disorders" indicated a common feature: that they

were caused by failure of the degradation of normal cellular components which accumulated in the lysosomes.^{22,23} One group of storage disorders, termed the "mucopolysaccharoidoses", was characterized by accumulation of sulfated "mucopolysaccharides" (now called glycosaminoglycans), which could be labeled by [³⁵S]sulfate. Neufeld and co-workers demonstrated that cultured fibroblasts from patients with these disorders reproduced the "storage" phenotype *in vitro*.²⁴ This abnormal accumulation of labeled mucopolysaccharides could be corrected by co-cultivation with normal fibroblasts. The process was shown to be due to soluble "corrective factors" secreted into the media of the normal cells. Furthermore, fibroblasts from one clinicopathological type of "mucopolysaccharoidosis" could cross-correct the cells from another type of disorder; for example, cells or secretions from "Hurler" syndrome fibroblasts could correct cells from Hunter syndrome fibroblasts.²⁵ As facile assays for lysosomal hydrolases became available, it was found that each of these clinicopathological entities was due to the lack of a specific enzyme, and that the "corrective factors" were the lysosomal enzymes themselves.^{26,27} Further investigation showed that these enzymes existed in two forms: a "high-uptake" form that was responsible for the cross-correction and a "low-uptake" form that was not capable of correction.^{23,28}

B. THE MYSTERY OF I-CELL DISEASE

Leroy and others had reported an unusual human genetic disorder characterized by prominent granules in cultured fibroblasts. Because of these prominent "inclusion bodies", the disorder was termed "I-cell disease".²⁹ These cells were characterized by lack of not one, but almost all of the lysosomal enzymes. This was not due to a failure of synthesis of these molecules, but rather to failure of their delivery to the lysosome. Thus, most of the lysosomal enzymes made by these cells were directly secreted into the media where they could be easily detected.³⁰ Hickman and Neufeld made the seminal observation that the secreted enzymes of I-cell disease fibroblasts were not taken up by other cells.³¹ Thus, I-cells were not capable of cross-correcting other cell types. On the other hand, I-cells could take up the enzymes secreted (in smaller quantities) by normal cells. They proposed that I-cell disease was caused by failure to add a specific recognition marker common to all lysosomal enzymes which could be responsible for their normal trafficking to the lysosomes. Since uptake could be destroyed by periodate treatment of the enzymes, they predicted that this common marker contained carbohydrate.³² Direct binding studies on fibroblasts indicated the presence of saturable, high-affinity receptors that could account for the uptake phenomenon.^{33,34}

C. DISCOVERY OF THE MANNOSE 6-PHOSPHATE PATHWAY FOR THE TRAFFICKING OF LYSOSOMAL ENZYMES

The next major breakthrough was the observation by Kaplan, Achord, and Sly that the uptake of "high-uptake" lysosomal enzymes could be blocked by the sugar mannose 6-phosphate (M6P) and its stereoisomer fructose-1-phosphate.³⁵ While millimolar concentrations of these sugars were required for complete blockade, similar concentrations of a variety of other monosaccharides and their phosphate esters had no comparable effect. A phosphorylated mannooligosaccharide from the yeast *Hansenula holstii*, that contained α -linked M6P, was even more potent in its ability to block uptake.³⁶ Since α -linked mannose (Man) residues were already known to occur on high mannose-type *N*-linked oligosaccharides of many glycoproteins, it was predicted that such residues might be phosphorylated on lysosomal enzymes. This prediction was corroborated by the effects of alkaline phosphatase, which abolished "high-uptake" activity,³⁵ and tunicamycin, which caused increased secretion of lysosomal enzymes from cells.³⁷ Other evidence soon appeared: Jourdan's group showed that bovine testicular glycoproteins that inhibited uptake contained M6P;³⁸ Natowicz et al. showed that M6P was present in "high-uptake" forms of lysosomal enzymes and that uptake

was proportional to the M6P content of fractions of the enzymes;³⁹ Hasilik and Neufeld demonstrated the phosphorylation of oligosaccharides of lysosomal enzymes in intact cells by metabolic labeling with ³²Pi;⁴⁰ and von Figura and Klein confirmed that M6P was present on endo- β -*N*-acetylglucosaminidase H (endoH)-sensitive oligosaccharides from a lysosomal enzyme.⁴¹

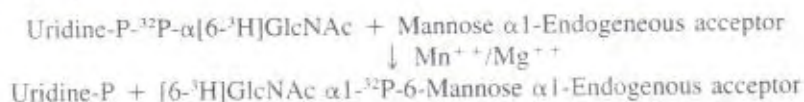
D. STRUCTURAL STUDIES OF PHOSPHORYLATED *N*-LINKED OLIGOSACCHARIDES

The next important step was the structural elucidation of the phosphorylated oligosaccharides of lysosomal enzymes. Using methods they had previously established for metabolic labeling of *N*-linked oligosaccharides with [2-³H]Man, Tabas and Kornfeld⁴² isolated the endoH-sensitive *N*-linked oligosaccharides of newly synthesized β -glucuronidase, and fractionated them by negative charge. While several molecules contained phosphate esters, the majority were found to be "blocked" by outer *N*-acetylglucosamine (GlcNAc) residues. Removal of these outer GlcNAc residues by mild acid was required for exposure of the phosphomonoesters on the underlying Man residues. Since endoH can release only high mannose-type oligosaccharides,⁴³ it was concluded that these molecules bore Man-P-GlcNAc sequences on such oligosaccharides. The same kind of phosphodiester linkage was reported by Hasilik and von Figura on other lysosomal enzymes.⁴⁴

The amount of labeled phosphorylated sugar chains obtained by immunoprecipitation of a single lysosomal enzyme was insufficient for further structural analysis. However, release of labeled oligosaccharides by endoH from whole-cell material permitted the detailed structural characterization of these novel molecules. Using this source, Varki and Kornfeld described a diverse family of phosphorylated high mannose-type oligosaccharides.^{45,46} While individual molecules bore only one or two phosphate residues, five possible locations were identified (see Figure 3), with the individual residues existing as either phosphomonoesters or phosphodiesters. Treatment with an α -*N*-acetylglucosaminidase confirmed the identity of the "blocking" monosaccharide in the phosphodiester groups, and its α -anomeric linkage. The sugar chains bearing the phosphodiesters showed less processing (removal of Man residues),²¹ indicating that they were the biosynthetic precursors of those with the phosphomonoesters. Similar structural studies were reported by Natowicz, Baenziger, and Sly on human spleen β -glucuronidase;⁴⁷ some differences were noted in the position of the phosphate esters.

E. ENZYMIC BASIS FOR THE GENERATION OF THE PHOSPHOMANNOSYL RECOGNITION SIGNAL

Most cellular phosphorylation events are mediated by ATP-dependent kinases. However, attempts to demonstrate such an activity against lysosomal enzyme acceptors were a failure. The correct enzymatic mechanisms were largely worked out by the groups of Kornfeld and von Figura. The novel GlcNAc α 1-P-6 Man α 1-X sequence allowed prediction of a phosphorylation mechanism utilizing UDP-GlcNAc as a donor. Using [β -³²P]UDP-GlcNAc, Reitman and Kornfeld⁴⁸ demonstrated the following reaction in rat liver, utilizing endogenous acceptor glycoproteins:



Exogenously added lysosomal enzymes could also be used as acceptors in this reaction. The ³²P-labeled glycopeptides from the reaction bound to Concanavalin A Sepharose, indicating that the label was probably on high mannose-type oligosaccharides. A similar approach was taken by Hasilik, Waheed, and von Figura to demonstrate this GlcNAc-phosphotransferase activity in human fibroblasts.^{49,50} However, since "high-uptake" activity

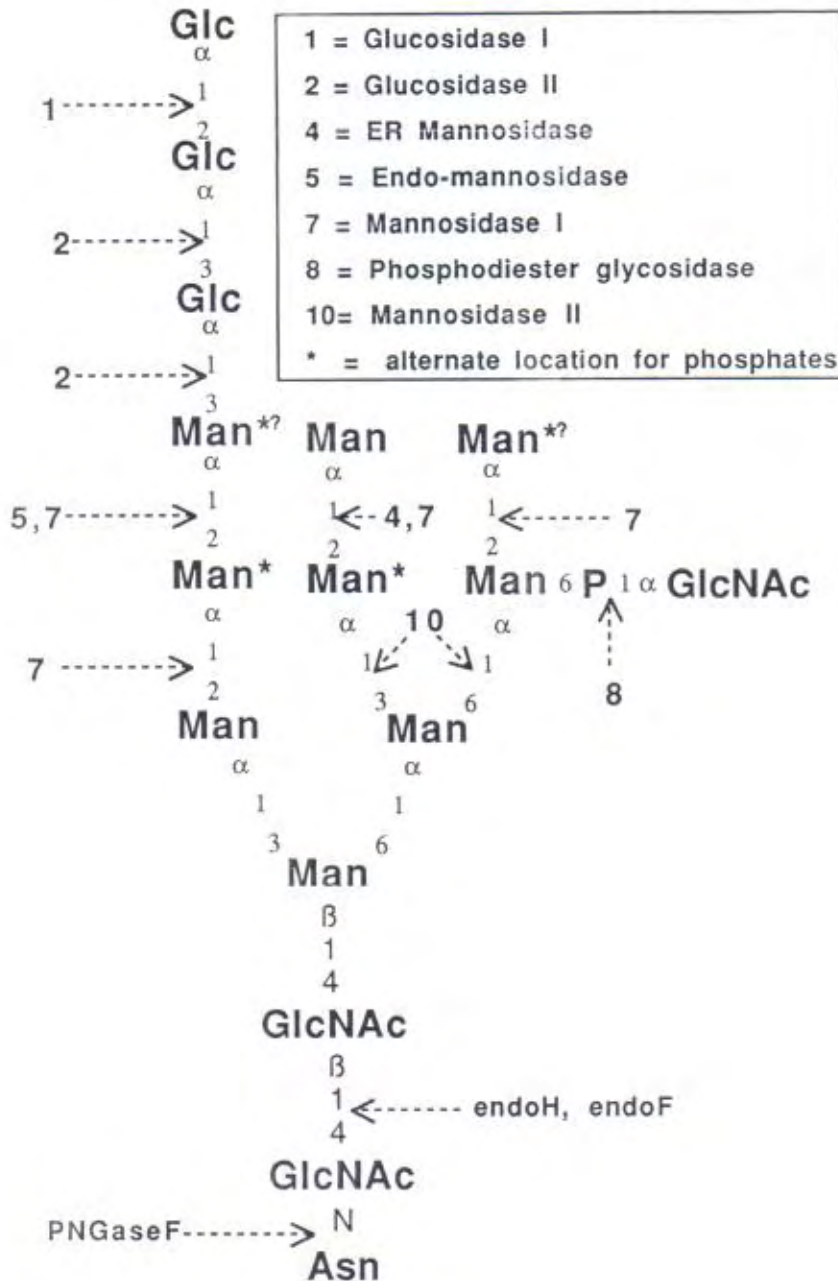


FIGURE 3. Composite structure of the oligosaccharide initially transferred to glycoproteins in *N*-asparaginyl linkage, along with sites of phosphorylation and actions of various enzymes. The linkages of the various residues of this composite oligosaccharide are indicated, along with a single phosphodiester-linked GlcNAc residue which is selectively expressed on lysosomal enzymes. Alternate positions of the phosphodiesters are indicated, as are the sites of action of various mammalian and bacterial enzymes.

of lysosomal enzymes was destroyed by alkaline phosphatase, this phosphatase-resistant phosphodiester could not be responsible for enzyme trafficking. Furthermore, structural studies had predicted a precursor-product relationship of these molecules with phospho-monoester-bearing oligosaccharides.⁴⁵ This predicted the existence of an enzyme capable of

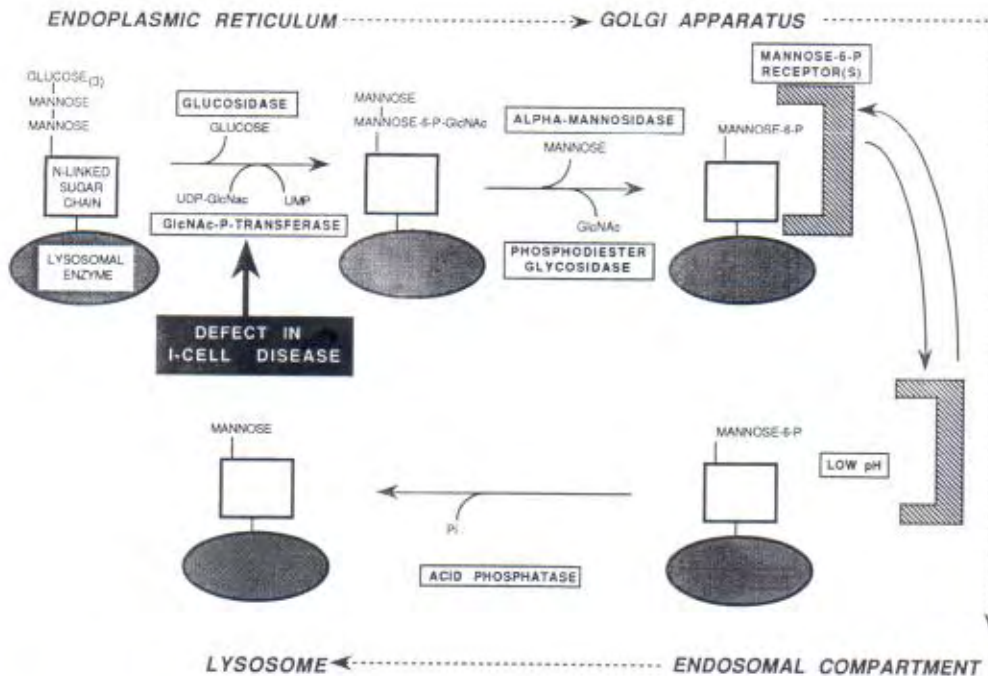


FIGURE 4. Outline of the mannose 6-phosphate pathway for targeting of newly synthesized lysosomal enzymes to lysosomes. The processing of the *N*-linked oligosaccharides of lysosomal enzymes to generate the phosphomannosyl recognition marker and the trafficking of the phosphorylated enzymes are outlined in a simplified and stylized fashion (see text and Figure 5 for more details). The site of the enzymatic defect in I-cell disease (and in pseudo-Hurler polydystrophy) is indicated.

removing the outer GlcNAc residue. Such an enzyme (initially called α -*N*-acetylglucosaminyl phosphodiesterase) was described by Varki and Kornfeld.⁵¹ In keeping with its putative role, it was enriched in the Golgi apparatus and had a neutral pH optimum. Several groups then partially purified and characterized this enzyme and showed that it was capable of acting on phosphodiester-linked GlcNAc at different positions on the *N*-linked oligosaccharide and on a variety of model compounds containing similar sequences.⁵²⁻⁵⁴ Subsequent studies of the enzymatic mechanism by ¹⁸O incorporation showed evidence for a glycosidase (with specific recognition of the phosphodiester) rather than a phosphodiesterase. The enzyme was thus renamed α -*N*-acetylglucosaminyl-1-phosphodiester glycosidase.⁵⁵ Subcellular fractionation studies by several groups confirmed that the two enzymes are primarily located in the Golgi apparatus.^{50-53,56-58} However, more recent evidence suggests that a portion of the phosphotransferase activity might be in a post-ER, pre-Golgi compartment.^{16,59}

Thus, the "phosphomannosyl recognition marker" of lysosomal enzymes is generated by the sequential action of these two enzymes on the high mannose-type oligosaccharides of lysosomal enzymes, along with the action of the processing α 1,2 mannosidase in the Golgi apparatus (see Figures 4 and 5).⁶⁰ Pulse-chase studies confirmed the order of events^{59,61} and indicated that more than one oligosaccharide on a given lysosomal enzyme could be phosphorylated. In keeping with the structural studies,⁴⁵ when dual phosphorylation of an oligosaccharide occurred, the first diester was always attached to the α 1-6-linked side of the molecule (see Figures 3 and 5).⁵⁹ Eventually the phosphomonoesters were lost, presumably upon exposure to a phosphatase in an acidified compartment^{61,62} (see Figure 4). However, loss of phosphate residues appears to be cell type specific: in some cells, the phosphorylated form of the enzymes persists and exogenous factors can affect the extent and rate of dephosphorylation.^{63,64}

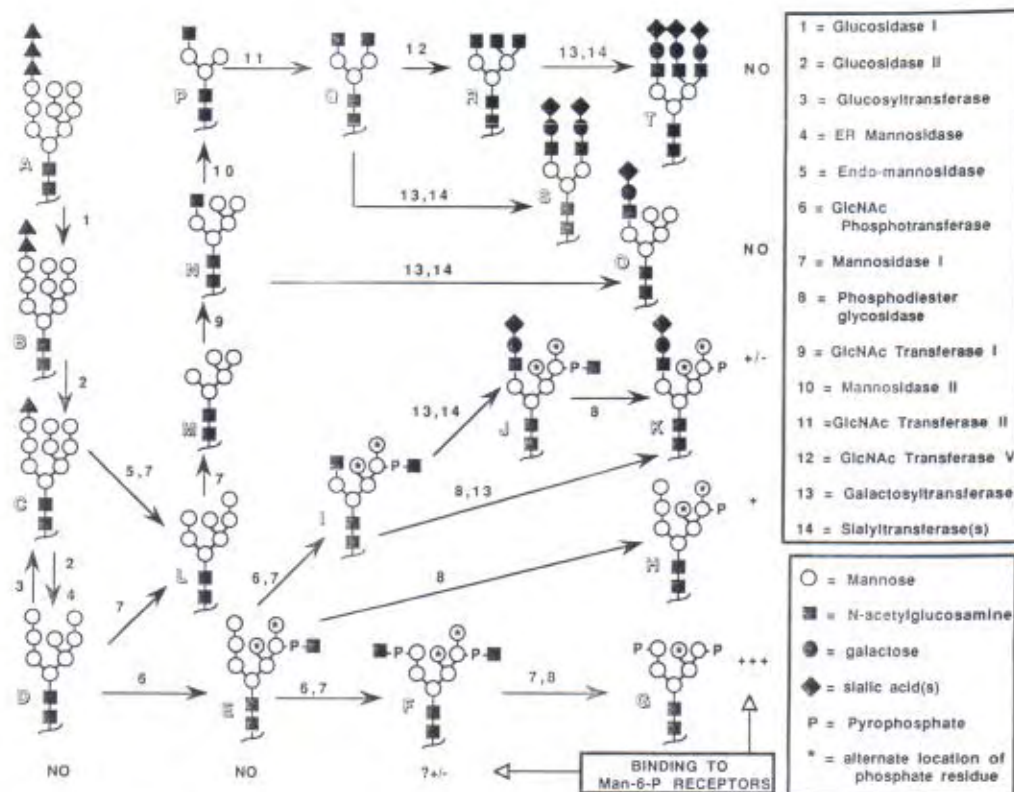


FIGURE 5. Some pathways for processing of *N*-linked oligosaccharides: generation of the phosphomannosyl recognition marker and interactions with the M6P receptors. The dolichol-linked oligosaccharide with three glucose residues, nine Man residues, and two *N*-acetylglucosamine residues is transferred *en-bloc* to an *N*-asparaginyl linkage on a newly synthesized glycoprotein (see structure A at the left upper corner, and Figure 3 for linkages). The glucose residues are removed by the sequential action of glucosidase I and II (structures B, C, and D). The last glucose residue can also be replaced by a specific glycoprotein glucosyltransferase, causing a deglycosylation:reglycosylation cycle (structures C and D) which terminates in favor of glucose removal. Additional reactions that can occur in the ER include the removal of one α 1,2-linked Man residue (see structure D), and the addition of a single GlcNAc phosphodiester to one of three Man residues on the side of the molecule attached α 1,6 to the β -linked Man (structure E).

In the Golgi apparatus, the singly phosphorylated molecules can have a second GlcNAc phosphodiester added to the side of the oligosaccharide that originally bore the glucose residues (structure F). Removal of the outer GlcNAc residues by the phosphodiester glycosidase and further processing of Man residues can result in generation of molecules with one or two phosphomonoester residues and 5 to 7 Man residues (structures G and H). The phosphate esters are mostly found on the Man residues that were initially subterminal (i.e., below an α 1,2-linked Man residue). However, they have also been found on some outer residues, particularly on the α 1,6-linked side of the molecule (see figure for alternate locations of the phosphate esters). The extent of Man removal by the Golgi α -mannosidase is restricted by the position of the phosphate esters. Thus, structures G and H represent only two of several possible structures bearing one or two phosphomonoesters. Also shown in the figure are some of the pathways followed by oligosaccharides that do not become phosphorylated. In the Golgi, further processing occurs with removal of the several α -linked Man residues, and addition of GlcNAc, Gal, and SA residues to generate typical complex-type and hybrid-type oligosaccharides (structures L through T). In some cell types hybrid phosphorylated molecules are also found which have complex-type sialylated lactosamine units on the α 1,3-linked side, and M6P residues on the α 1,6-linked side, as phosphomonoesters or phosphodiesters (structures I, J, and K).

Binding studies of several of these oligosaccharides (released by endoH) with purified M6P receptors have demonstrated the relative affinities indicated in the figure. The best ligand is the molecule at the lower right corner (structure G) that carries two terminal phosphomonoester groups on an oligosaccharide with six to seven Man residues. The next best ligand in the molecule has one phosphomonoester (structure H). Diester-containing molecules and hybrid-type molecules have little or no binding affinity for the receptors.

The GlcNAc phosphotransferase prefers one or more α 1,2-linked Man residues on the substrate oligosaccharide.^{65,66} However, phosphorylation tends to occur predominantly on inner Man residues. Removal of the outer Man residues by the Golgi α -mannosidase then follows, concomitant with the "uncovering" of the phosphomonoester. Thus, the final product recognized by the M6P receptors (see below, and Figures 4 and 5) usually contains *terminal* M6P residues on smaller high mannose-type oligosaccharides (5-7 Man residues). Mannose 6-phosphate is the 2-epimer of glucose 6-phosphate, which is not well recognized by the M6P receptors. Therefore, a M6P residue in an oligosaccharide should not be recognizable if it is substituted at the 2-position by an outer Man residue. Thus, removal of such an outer Man residue is probably necessary to create a recognizable phosphomannosyl recognition marker (see Figures 4 and 5). Removal of the glucose residues is also required before the second phosphodiester can be added to the α 1,3-linked arm of the molecule.⁶⁷ More recently, Lazzarino and Gabel have shown that phosphorylation is a two-step reaction in which addition of the second phosphodiester residue also requires the removal of certain Man residues.^{59,60}

F. THE ENZYMATIC BASIS FOR I-CELL DISEASE (MLII) AND PSEUDO-HURLER POLYDYSTROPHY (MLIII)

The availability of enzyme assays allowed analysis of fibroblasts from patients with I-cell disease (also called Mucopolipidosis II, or MLII) and a milder variant with a similar phenotype called pseudo-Hurler polydystrophy (also called Mucopolipidosis III, or MLIII). In all such cases examined to date, the defect has been in the GlcNAc phosphotransferase,^{49,68-70} the first enzyme in the pathway (see Figure 4). The enzyme deficiency is less severe in MLIII, explaining the milder disease in these patients. Family studies demonstrated the heterozygous state for these disorders: obligate heterozygotes showed a partial enzyme deficiency and slightly elevated levels of serum lysosomal enzymes, but no clinical phenotype.^{71,72} Studies of metabolically labeled fibroblasts corroborated the failure of phosphorylation in these diseases.^{68,73} The assay for GlcNAc phosphotransferase has also been used for prenatal diagnosis of I-cell disease.⁷⁴ Thus, it appears almost certain that GlcNAc phosphotransferase deficiency is the primary genetic disorder in these diseases. However, ultimate proof will require the cloning and analysis of the gene(s) encoding this enzyme activity in normal and disease states. Unfortunately, purification of this enzyme has proven to be difficult.

Until recently, no genetic defect had been discovered in the second enzyme in the pathway, the phosphodiester glycosidase. However, a normal Lebanese individual with elevated levels of lysosomal enzymes has recently been shown to have a partial deficiency of the phosphodiester glycosidase.⁷⁵ Fibroblasts from individuals in this family also showed elevated secretion of lysosomal enzymes which were of the "low-uptake" type, and treatment of these enzymes with purified phosphodiester glycosidase restored uptake. This presumably represents a heterozygous state for phosphodiester glycosidase deficiency; a homozygous state has yet to be discovered.

It was observed early on that lysosomal enzymes from I-cell disease fibroblasts and tissues carried more complex-type *N*-linked oligosaccharides, and were more heavily sialylated.⁷⁶⁻⁷⁸ The simplest explanation is that phosphate residues block the normal action of the processing Golgi mannosidases required for generation of the complex chains. Thus, failure of phosphorylation in I-cell disease would allow removal of the Man residues, resulting in further processing to sialylated antennae.²¹ However, it is also possible that a change in the itinerary of the lysosomal enzymes in I-cell disease causes them to spend more time in regions of the Golgi apparatus where late processing reactions take place (see discussion below concerning the itinerary of the M6P receptors).

G. LESSONS LEARNED FROM VARIANTS OF MLII AND MLIII

While α -linked Man residues were required for GlcNAc phosphotransferase action, the simplest related compound α -methylmannoside was found to be a poorer substrate than a high mannose-type oligosaccharide, which in turn was poorer than a native lysosomal enzyme.^{69,79} Other glycoproteins bearing high mannose-type oligosaccharides were also not particularly good substrates. These data suggested that the GlcNAc phosphotransferase specifically recognized lysosomal enzymes. Direct assays comparing different glycoprotein acceptors proved that this was indeed the case.⁷⁹ Since saturating concentrations of the α -methyl-mannoside acceptor could be inexpensively achieved, it was routinely used in screening assays of the phosphotransferase. In most cases of MLII and MLIII, assays with monosaccharide or intact lysosomal enzyme acceptors gave congruent results. However, fibroblasts from one pair of siblings with MLIII gave completely discordant results, showing *normal* activity with the α -methylmannoside acceptor, and *markedly decreased* activity with lysosomal enzyme acceptors.⁶⁹ The phosphotransferase from these patients appears to be present in normal catalytic amounts, but fails to recognize lysosomal enzymes as special acceptors for phosphorylation. This provided genetic evidence for a specific and distinct mechanism for recognition of lysosomal enzymes by the phosphotransferase. The molecular basis of this variant enzyme remains to be determined, but the defect is presumably in a recognition site (or subunit) rather than in the catalytic site (or subunit).

As might be expected for sporadic inherited disorders, this group of diseases is genetically very heterogeneous. Several complementation groups among MLII and MLIII patients have been identified by Miller, Shows, and others, using cell fusion and heterokaryon analysis.^{72,80-82} One group coincides with the variant phosphotransferase described above and another is a heat-labile form of the enzyme. Differences in the apparent size of the enzyme complex in various cases of MLII and MLIII have also been reported.⁸³ A related observation is the correction of phosphotransferase activity and lysosomal sorting in some MLII and MLIII cell lines when they are grown in 88 mM sucrose.⁸⁴ The possibility of stabilization of enzyme activity by sucrose has been suggested.⁸⁵ Final explanations for the behavior of these mutant enzymes will have to await the molecular cloning of the normal phosphotransferase gene(s).

Another novel variant of I-cell disease was reported by Thomas et al.⁸⁶ A male child with the phenotype of moderately severe I-cell disease was shown to be composed of a mosaic of cells that were either homozygous or heterozygous for deficiency of the GlcNAc phosphotransferase. The developmental basis for this unusual mosaicism remains unexplained. This rare experiment of nature does carry one important lesson: that *complete* "cross-correction" of lysosomal enzymes between all normal and deficient cells cannot occur, even when *both* cell types are growing *together* in the same multicellular organism. However, as demonstrated recently for α -glucosidase targeting to heart and skeletal muscle,⁸⁷ it may be possible to correct specific deficiencies in specific tissues using a phosphorylated lysosomal enzyme.

H. STRUCTURAL BASIS FOR SELECTIVE RECOGNITION OF LYSOSOMAL ENZYMES BY THE GLCNAc PHOSPHOTRANSFERASE

Such studies indicated that phosphorylation of lysosomal enzymes must involve specific recognition of the polypeptide. Since the high mannose-type oligosaccharides of lysosomal hydrolases are not different from those of other glycoproteins entering the ER-Golgi pathway, this recognition is crucial for the selective trafficking of the enzymes. The cloning and sequencing of several lysosomal enzyme genes⁸⁸ failed to reveal homologies in their primary sequence that could explain recognition by the GlcNAc phosphotransferase. Furthermore, denatured lysosomal enzymes did not act as specific acceptors, indicating that features of secondary or tertiary structure are crucial for recognition.^{79,89} In this regard, recent elegant experiments of Kornfeld, Baranski, and Faust have involved the systematic "swapping" of

sequences between homologous enzymes such as cathepsin D (which is recognized by the GlcNAc phosphotransferase) and pepsinogen (which is not normally phosphorylated). The data indicate that specific lysine residues in a certain tertiary domain of Cathepsin D and the proximity of an adjacent peptide loop can explain the recognition by the phosphotransferase.^{90,91} Addition of these features to pepsinogen was sufficient to convert this secretory protein into a phosphorylated molecule that was directed to the lysosomes. Further analysis of the secondary and tertiary structure of other lysosomal enzymes will be required to confirm that these structural motifs comprise a universal recognition marker for phosphorylation.

I. IDENTIFICATION AND CHARACTERIZATION OF RECEPTORS FOR THE PHOSPHOMANNOSYL RECOGNITION MARKER

In order for the phosphomannosyl recognition marker to dictate trafficking of an enzyme to the lysosome, it must be recognized by specific receptors. The first candidate M6P receptor was isolated from bovine liver by Sahagian and Jourdain,⁹² using affinity chromatography on a yeast phosphomannan. Shortly thereafter, the same receptor was isolated from other sources by several other investigators, using a variety of affinity methods.^{46,93,94} This molecule had an apparent molecular weight by SDS-PAGE of greater than 210 kDa, and bound M6P in the absence of cations. Hoflack and Kornfeld observed that certain cells deficient in this receptor^{95,96} still showed M6P-inhibitable binding of lysosomal enzymes to membranes.⁹⁷ This led to the discovery of a second M6P receptor of molecular weight 46 kDa which required cations for optimal binding.⁹⁸

A complete cataloguing of endoH-sensitive phosphorylated oligosaccharides from a macrophage cell line⁴⁶ revealed a bewildering array of structures, including some with sialylated lactosamine units on the nonphosphorylated antennae of hybrid molecules (see Figure 5 for some examples). The availability of such labeled molecules permitted a detailed analysis of their interaction with purified immobilized M6P receptors.^{46,94,99} As predicted from the earlier studies, the receptors bound with highest affinity to oligosaccharides with two phosphomonoesters (structure G, Figure 5), and poorly to molecules bearing GlcNAc-P-Man phosphodiester (structures E and F). Binding to molecules carrying one phosphomonoester was intermediate (structure H). Interestingly, monophosphorylated molecules carrying the ester on the α 1,3-linked side of the oligosaccharide (not shown in Figure 5) bound better than those with the ester on the α 1,6-linked side (see Figure 3 for linkages). However, the former were obtained from the Thy E⁻ glycosylation mutant,⁶⁷ and do not occur in the normal cells.

In vitro removal of the "blocking" GlcNAc residues from molecules carrying two phosphodiester (structures E and F, Figure 5) caused improved binding to the receptors, but not to the extent expected. When such artificially generated molecules with phosphomonoesters were also treated with α -mannosidase, binding became similar to native molecules with phosphomonoesters.⁴⁶ This confirmed the prediction from the structural studies (see above, Section E) that mannose processing is important for generating high-affinity binding. Finally, with hybrid molecules bearing phosphomonoesters and sialic acids, the presence of sialic acid on the other antenna caused decreased binding. These findings with isolated oligosaccharides were confirmed and extended by several investigators who also studied uptake by cells.^{94,100-102} By and large, the uptake properties of various oligosaccharides mirrored their behavior with the isolated M6P receptors.

More recently, the oligomeric state and stoichiometry of binding of the two purified receptors have been extensively investigated by the groups of Kornfeld and von Figura.^{1,103-107} It appears that the small cation-dependent receptor (CD-MPR) exists mainly as a dimer, with each monomer component binding one residue of M6P. However, monomeric and tetrameric forms were also found, and the equilibrium between the three forms was dependent upon temperature, pH, and the presence of M6P.^{107,108} The large cation-independent receptor

(CI-MPR) appears to be primarily in a monomeric state (although the presence of oligomers has not been ruled out). Somewhat surprisingly, this much larger monomer binds only two residues of M6P, implying that only two of its 15 repeating units (see below) are involved. These results can explain earlier observations in which oligosaccharides with two phosphomonoesters had affinities not much worse than that of native lysosomal enzymes, whereas molecules with single phosphomonoesters bound less well. Since intact lysosomal enzymes can have more than one oligosaccharide bearing M6P,⁶¹ it is possible that two appropriately spaced monophosphorylated oligosaccharides could together provide a high-affinity ligand for the receptors. This possibility has not been directly tested because of the difficulty in obtaining model glycoproteins with each possible combination.

J. WHAT IS THE DEFINITION OF THE PHOSPHOMANNOSYL RECOGNITION MARKER?

In the final analysis, the "phosphomannosyl recognition marker" is a term encompassing a large family of M6P-bearing oligosaccharides, each with varying degrees of affinity for the receptors (see Figure 5). The number, nature, and physical distribution of such oligosaccharides on each protein molecule could further alter their relative affinities for the two receptors. More recent work by Sahagian¹⁰⁹⁻¹¹¹ and Gabel¹¹² on Cathepsin L indicates that the extent to which the "recognition marker" is functionally recognized *in vivo* can also depend upon its level of synthesis, the rate of its secretion, the number of phosphorylated oligosaccharides, and the effect of the polypeptide in masking and altering the affinity of oligosaccharides. Thus, any given cohort of newly synthesized lysosomal enzymes can be expected to represent a *spectrum* of affinities, in a continuum from the lowest affinity to the highest affinity. When this complexity is superimposed upon factors such as the number and availability of the receptors, the differences in the properties of the two receptors, and the density of overall traffic through the Golgi apparatus, it is clear that there is great flexibility in this trafficking mechanism. This flexibility is manifested in the varying extent to which different cell types target different M6P-containing proteins to their lysosomes.

K. MOLECULAR CLONING OF THE M6P RECEPTORS

Nucleotide sequences encoding both of the M6P receptors have now been cloned and extensively characterized from several species (see References 1, 2, and 113 through 117 for detailed discussions, and Table 1 for comparison of the two receptors). Derived amino acid sequences show that both molecules are type-I membrane glycoproteins, with amino-terminal hydrophobic signal sequences, large extracytoplasmic domains, single transmembrane hydrophobic regions, and relatively small carboxyl terminal intracytoplasmic domains. The extracytoplasmic domains of both receptors have potential N-linked glycosylation sites (some of which are known to be used), and the intracytoplasmic domains have sequences that are potential substrates for protein kinases. The two receptors are clearly homologous to one another. The large receptor has 15 contiguous repetitive units of approximately 145 amino acids each, that have partial identity to one another. The small receptor has a single such domain with homology to the repeating domains of the large receptor. Mutagenesis studies have served to identify specific residues of these receptors involved in M6P binding¹¹⁸ and subcellular trafficking (see below).

L. RELATIVE ROLE OF THE TWO M6P RECEPTORS IN LYSOSOMAL ENZYME TARGETING

Cells naturally lacking the CI-MPR, or that are depleted of this receptor by specific antibodies, secrete a substantial portion of their lysosomal enzymes.¹¹⁹⁻¹²¹ Expression of the cDNA encoding this receptor corrected the secretion defect in such cells^{122,123} On the other hand, when antibodies against the CD-MPR are added to such cells, the remaining enzymes

TABLE 1
Comparison of the Two Mammalian Mannose 6-Phosphate Receptors

| Feature | Cation-independent (large) receptor | Cation-dependent (small) receptor |
|---|--|--|
| Type of protein/reported subunit molecular weight (by SDS-PAGE) | Type I membrane glycoprotein >210 kDa | Type I membrane glycoprotein 46 kDa |
| Subunit molecular weight based on cDNA cloning and derived sequence | 275 kDa | 28 kDa |
| Optimal pH for binding | 6.0—7.0 | 6.0—6.5 |
| Cation dependence for binding | No | Mn ⁺⁺ > Mg ⁺⁺ = Ca ⁺⁺ (? not required for tetramer) |
| Domain structure (based on cDNA cloning and derived sequence) | 15 homologous repeating units of ~145 amino acids | Single 155-amino acid unit homologous to repeating units of large receptor |
| Native oligomeric state | Monomer (?oligomer) | Dimer or tetramer |
| Stoichiometry of binding to M6P | Two per monomer | One per monomer |
| Kd for oligosaccharide with 2 M6P residues | 2 × 10 ⁻⁹ M | 2 × 10 ⁻⁷ M |
| Binding of other ligands | | |
| Methylphosphomannose residues | Yes | No |
| Insulin-like growth factor II | Yes (no in chicken/xenopus) | No |
| Subcellular distribution | Endosomal compartments (major) Cell surface (minor) Golgi apparatus (variable) | Endosomal compartments (major) Cell surface (minor) Golgi apparatus (variable) |
| Role in biosynthetic pathway | Yes | Yes |
| Role in endocytic pathway | Yes | No (?except at high density) |

Note: The data are summarized from work on human, bovine, murine and chicken receptors by several investigators (see text for literature references on specific subjects).

are secreted, suggesting that the latter receptor can also mediate targeting.¹²⁴ However, overexpression of the CD-MPR in cells lacking the CI-MPR resulted in only a partial correction of the secretion defect.¹²⁵ These results suggest that while the CI-MPR is the major determinant of trafficking in the biosynthetic pathway, the CD-MPR also contributes a significant component. However, when the CD-MPR was overexpressed in cells containing the CI-MPR, *increased secretion* of lysosomal enzymes resulted.¹²⁶

The minor route of traffic of lysosomal enzymes is from the cell surface, via endosomes to the lysosomes (see below for details). Even though a portion of the CD-MPRs are known to recycle to the cell surface,¹²⁷ it appears that only the CI-MPR is responsible for traffic of enzymes along this route in cultured cells.¹²⁴ However, when the CD-MPR is strongly overexpressed in cells, it does appear to be capable of mediating uptake from the surface.¹²⁵ These differences may relate to the very narrow pH optimum of binding of the CD-MPR and/or its oligomeric state, which in turn may depend upon many other factors (see above).

At the present time, it is not possible to reconcile all of these findings into a clear-cut summary. It is likely that the CI-MPR is the major determinant of targeting to the lysosome, while the CD-MPR appears to *modulate* the pathway in the direction of either retention *or* secretion, perhaps dependent upon a variety of factors such as its oligomeric state, level of expression, subcompartmental pH values, temperature, divalent cation availability, the amount of the CI-MPR present, and differences in the affinities of the two receptors for multivalent ligands (see Table 1). Analysis of the situation is further complicated by the fact that the CI-MPR also serves as a receptor for insulin-like growth factor II, and that its subcellular distribution can be altered by a variety of apparently unrelated factors (see below). In the final analysis, different combinations of amounts and locations of the two receptors, together

with the spectrum of affinities of the "phosphomannosyl recognition marker" on various molecules (see above), could be responsible for the highly variable physiology of lysosomal enzyme trafficking in different cell types.

M. INTRACELLULAR DISTRIBUTION AND ITINERARIES OF THE M6P RECEPTORS AND THEIR LIGANDS

Several investigators have studied the biosynthesis, intracellular distribution, and trafficking of the two M6P receptors.^{105,128-135} Each receptor is synthesized as an N-linked glycoprotein which traverses the ER-Golgi pathway to the Golgi apparatus, the plasma membrane, and the endosomal compartments (see below). In the cell types studied to date, the majority of the receptors are found in an internal location (see below). However, several investigators^{120,127,136,137} have presented direct and indirect evidence that the cell surface and internal pools of receptors must be constantly mixing with one another.

These itineraries of the M6P receptors fit reasonably well with the proposed itineraries of the lysosomal enzymes themselves. Most lysosomal enzyme molecules bear some complex-type chains, including sialic acids,^{76-78,138,139} which indicate that they have passed through the late part of the Golgi apparatus. Secondly, in some cell types, most of the M6P receptors are found in the late part of the Golgi apparatus, and in adjacent endosomal compartments.^{135,140-142} Based upon such lines of evidence, some investigators have concluded that all newly synthesized lysosomal enzyme molecules traverse the entire Golgi pathway and exit from the *trans*-Golgi network to a "prelysosomal compartment" (analogous to "late endosomes").^{140,143} However, others have suggested that in some cell types, the lysosomal enzymes can exit the Golgi apparatus earlier.^{144,145} The relative importance of these two routes in different cell types has not been clearly defined. Furthermore, recent data about the behavior of the CD-MPR (see above) suggest that at least some of the newly synthesized enzymes might first enter an earlier endosomal compartment.¹²⁶ Another finding that does not fit the current scheme is that lysosomal enzymes secreted in the presence of ammonium chloride have a decreased rate of "uncovering" of their phosphodiester groups.¹⁴⁶ Regardless of the specific details, it appears likely that the bulk of the newly synthesized lysosomal enzymes are diverted from the Golgi apparatus to the endosomal compartments by specific binding to one or the other of the MPRs. Here they part company with the receptors, which recycle to the cell surface or the Golgi apparatus.

There are now other examples of proteins that follow such a pathway, from the Golgi to the endosomes. In the case of the invariant chain associated with the MHC class II complex, a similar controversy exists as to whether the protein is delivered from the Golgi to the late endosome or to the early endosome.¹⁴⁷⁻¹⁴⁹ One way to reconcile all these apparently disparate models is to suggest that they are all partially correct (see Figures 2 and 6). It is possible that in the exocytic pathway, the diversion of the M6P receptors (or the invariant chain) to the endosomal compartments is a stochastic process governed by multiple factors, that can be protein specific and cell-type specific. Thus, the probability of diversion from the default pathway of secretion to the endosomal compartments might increase progressively across the Golgi stack, reaching a maximum at the *trans*-Golgi network. Thereafter, the probability of diversion might progressively fall as the protein nears the cell surface. However, some molecules that escape diversion at the Golgi level could still exit into early endosomal compartments that are closer to the cell surface. This compartment could send the molecules back inward to the late endosomal compartment, or send them on to the cell surface. The same logic could be applied to the endocytic pathways (see below). Thus, the probability of diversion from incoming receptors back to the surface or to the exocytic pathways might be stochastic and specific for a given protein in a given cell type, progressively decreasing as the molecules pass further into the cell. Such a model could also account for events such as early "diacytosis" of the asialoglycoprotein receptor (see below), and

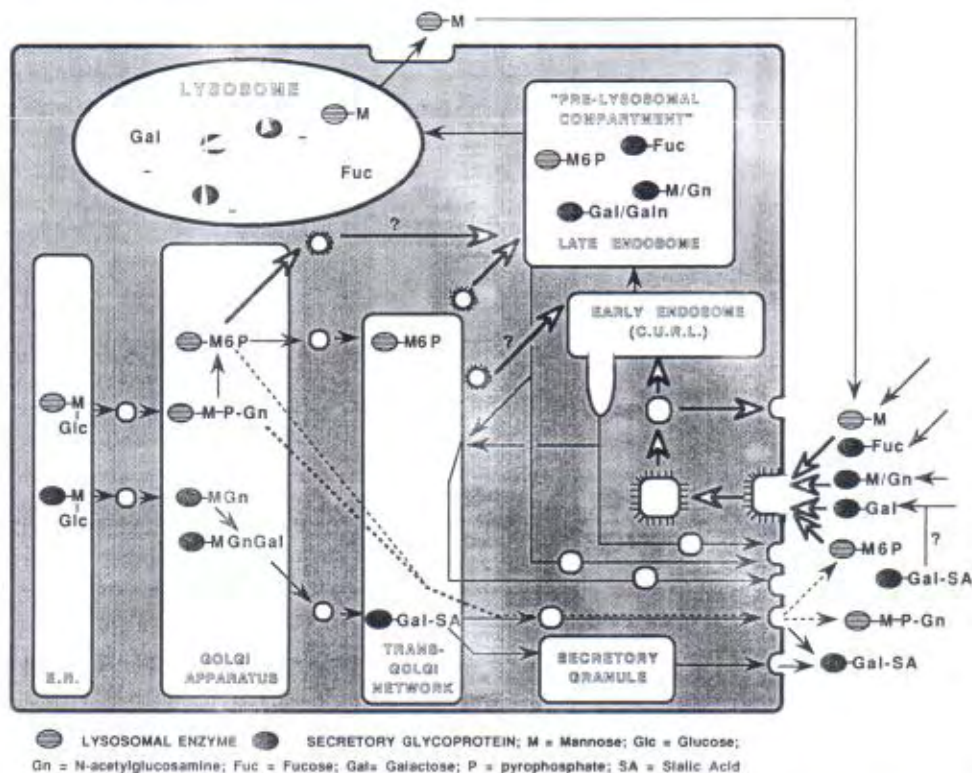


FIGURE 6. Overview: role of carbohydrates in intracellular and intercellular trafficking of glycoproteins. This figure presents a composite simplified view of pathways occurring in and between a variety of different cell types. The glycoproteins are depicted with only the crucial terminal sugars that mediate recognition events. The open arrows represent the only steps at which carbohydrate recognition events are conclusively known to direct subcellular traffic. Upon encountering lower pH in the endosomal compartments, the ligands are dissociated from their receptors. The dotted lines indicate minor pathways in most cell types. Question marks indicate pathways that have been less frequently reported (see text for discussion). The fuzzy border indicates vesicles believed to be clathrin-coated.

the return to the cell surface of the M6PR via the *trans*-Golgi complex (see Figures 2 and 6).

In many cells, a minority of the newly synthesized lysosomal enzymes completely escape sorting into the lysosome, and are directly secreted into the medium carrying M6P residues. Since a small but significant portion of the CI-MPRs are found at the cell surface, the secreted enzymes are subject to recapture by the same cell or presumably by adjacent cells bearing similar receptors. In this case, the itinerary of the bound molecules appears to follow the classic coated pit/coated vesicle/endosomal pathway,^{19,20,150,151} eventually arriving in the lysosome (see Figures 2 and 6). The relative importance of this "secretion-recapture" pathway (originally proposed many years ago by Neufeld) might vary, depending upon the situation. For example, some activated macrophages and neutrophils secrete a substantial portion of their newly synthesized enzymes into the medium. It is possible that in inflammatory situations, it is useful for such secreted enzymes to be returned to lysosomes from the extracellular fluid via the M6P pathway or perhaps the Man/Fuc receptor pathway (see Section IV.I.).

N. EVIDENCE FOR A DISTINCT RECEPTOR-ENRICHED INTERNAL COMPARTMENT

There is general agreement that M6P receptors are not found in mature lysosomes, but

that they drop off their lysosomal enzyme cargo at an endosomal compartment. Such a staging area was further defined by Griffiths et al. and termed the "prelysosomal compartment".¹⁴⁰ This receptor-rich compartment was shown to be the site of intersection of newly synthesized lysosomal enzymes with incoming ligands endocytosed from the cell surface, and its acidic internal pH was felt to be responsible for the unloading of the enzymes from the M6P receptors.^{140,141} It now appears that this compartment may be synonymous with the "late endosomal" compartment identified by other investigators studying the pathways of endocytosis.^{20,152} More recent evidence suggests that it may also be the site where autophagic and pinocytotic vesicles first encounter lysosomal enzymes.¹⁵³

Having unloaded their cargo in the endosomal compartment, the M6P receptors shuttle back to the Golgi apparatus for a new round of enzyme pick-up. The contents of this compartment would then either fuse with, or become classic "primary lysosomes" exemplified by the presence of the lysosomal membrane glycoproteins and lysosomal enzymes, and the *absence* of the M6P receptors.² In an attempt to isolate this compartment, Messner et al. demonstrated that a subcellular fraction greatly enriched in both MPRs could be selectively purified using an antibody specific for the cytoplasmic tail of the CI-MPR.¹⁵⁴ The physical isolation of this compartment now paves the way for a structural and functional understanding of its role in trafficking. This approach may also allow distinction of any subtle differences in the distribution of the 46-kDa receptor.¹³⁵

O. MOLECULAR DETERMINANTS OF TRAFFICKING OF THE M6P RECEPTORS

As described above, the M6P receptors have a subcellular distribution that is unique from all other known cellular receptors. While endocytosis of these receptors from the cell surface and delivery from the *trans*-Golgi network involves clathrin-coated vesicles,^{131,150,155} the recent experiments of Draper et al. suggest that the recycling of the receptors back from the endosomal compartment to the *trans*-Golgi does not involve clathrin.¹⁵⁶

What is it that determines the distinctive subcellular localization of these receptors and their itineraries? With the cloning of the receptors, the molecular dissection of this question became possible. The expression of variant forms of the receptors using selected deletions and site-directed mutagenesis has been carried out, and the functions of these mutant molecules analyzed. These interesting studies^{1,2,123,133,157} deserve a detailed and separate review, but can only be briefly summarized here. With regard to the CI-MPR, the evidence suggests that the major determinants of endocytic trafficking for the CI-MPR are encoded in the sequence Tyr-Lys-Tyr-Ser-Lys-Val (amino acids 2318-2324) of the cytoplasmic tail of the receptor.¹⁵⁷ The tyrosine residues could be substituted with phenylalanine with no loss of activity, indicating the requirement for any aromatic amino acid at these positions. Interestingly, the crucial elements of this signal (an aromatic side chain separated from a bulky hydrophobic chain by two amino acids) could also be identified in the cytoplasmic tails of a number of other membrane receptors known to undergo internalization.¹⁵⁷ In the case of the CD-MPR, two distinct internalization signals were found in the cytoplasmic domain;¹³³ one is similar to that found in the CI-MPR, while another involving two phenylalanine residues is similar to the internalization signals of some other endocytosing receptors. Both motifs are characterized by having hydrophobic residues in the first and last positions of the critical sequences.

How exactly do the cytoplasmic domains direct the itineraries of the receptors? Pearse and colleagues have shown that the cytoplasmic tail of the CI-MPR can bind specifically to two "adaptor" proteins, which in turn are known to interact with clathrin.¹⁵⁸ Interestingly, the two adaptors show selective distribution to the plasma membrane and the Golgi complex respectively, the two sites where trafficking decisions of the receptor must be determined. Thus, it is reasonable to predict that their binding to the receptor "tail" might mediate its

selective concentration in the appropriate clathrin-coated pit. In support of this notion, mutation of two of the tyrosine residues in the cytoplasmic tail of the receptor eliminated interaction with one of the adaptors.¹⁵⁸ Others have found evidence for serine phosphorylation of the cytoplasmic tail of the CI-MPR at residues 2421 and 2492 by a casein-kinase II-like enzyme.¹⁵⁹ Furthermore, changes in the distribution of the CI-MPR induced by phorbol esters are associated with changes in serine phosphorylation,¹⁶⁰ and the binding of IGF-II to this receptor induces coupling with the guanine nucleotide-binding protein G_{i-2} ¹⁶¹ via a specific cytoplasmic segment.¹⁶² These data suggest that protein kinases and G-proteins could also be involved in modulating the traffic of this molecule.

Some other experiments suggest that the extracellular domain of the receptors can also play a role in determining their itinerary. Thus, "swapping" the external domain of the CI-MPR for that of the EGF receptor resulted in an itinerary more like that of the latter.¹⁶³ It is possible that while the cytoplasmic tail determines endocytosis and targeting to the endosomal compartments, the extracellular domain might determine further traffic from the endosomes to the Golgi apparatus.

P. THE IGF-II RECEPTOR IS IDENTICAL TO THE CATION-INDEPENDENT M6P RECEPTOR

Insulin-like Growth Factor II (IGF-II) was known to bind to the receptor for IGF-I, and also to an independent specific receptor of reported size 220 to 250 kDa. Molecular cloning of the IGF-II specific receptor revealed the surprising fact that it is identical to the CI-MPR.¹⁶⁴⁻¹⁶⁷ A large number of studies have since been reported concerning the potential interactions between these seemingly disparate ligands: only a brief synopsis can be presented here. While it is clear that the two ligands bind to distinct sites on the receptor, there have been conflicting reports regarding interactions between the two systems.^{121,167-175} In various studies, the two ligands were found to have either synergistic or antagonistic actions, sometimes depending upon whether intact lysosomal enzyme or free M6P was used. It has also been suggested that the redistribution of the receptor upon insulin stimulation could explain some of the known metabolic effects of this hormone on protein degradation by altering the trafficking of lysosomal enzymes.^{176,177}

It has been demonstrated that the CI-MPRs of the chicken and *Xenopus* do not bind IGF-II, although their cells respond to IGF-II.¹⁷⁸⁻¹⁸⁰ This finding makes it less likely that the overlap in binding specificity is of vital importance to animal cells in general. Furthermore, it has been recently noted that the mouse CI-MPR is maternally imprinted (i.e., expressed only from the maternal chromosome), and is close to or identical to the *Tme* locus, the only known maternal effect mutation in mice.¹⁸¹ The defect is nuclear encoded, and abnormal embryos that inherit a deletion of the *Tme* locus from their mother die at day 15 of gestation. However, unlike mice that are defective in the IGF-II gene, which are viable but small in size, the *Tme* embryos are edematous but normal in size. These data suggest that it is the M6P-binding component of this receptor that is vital for survival.

Q. CORROBORATIVE DATA FROM MUTANT AND VARIANT CELL LINES

Much of the work described above has been carried out in normal fibroblasts or in established hematopoietic cell lines. Useful information has also been obtained by analysis of a variety of other cell types. Some naturally occurring cell lines are partly or completely deficient in the CI-MPR,¹¹⁹ while others secrete a large fraction of their enzymes in spite of having the receptor.⁹ On the other hand, Sertoli cells of the testis synthesize very little of the CD-MPR.¹⁸² Thus far, cells deficient in both the MPRs have not been found. Recent work has shown that in polarized epithelial cells, the distribution of these receptors is even more specialized.¹⁸³ The subcellular itinerary of the M6P receptors can also depend upon the function of a given cell type. For example, in the case of the osteoclast (a specialized

form of macrophage involved in bone resorption), lysosomal enzymes are bound to the CI-MPR but are carried to the apical surface of the cell where they are unloaded into an acidic space outside the cell.¹³⁴

Useful data has also been derived from a variety of mutant cell lines (reviewed in Reference 9). Lymphoma cells with mutations at specific steps in N-linked glycosylation were studied for the trafficking of their lysosomal enzymes via these pathways.⁶⁷ In general, the results have corroborated nicely with the conclusions from wild-type cells; thus, removal of the glucose residues is required for the addition of the second phosphate residue, and some mutants with truncated oligosaccharides are still capable of targeting enzymes even though they cannot make diphosphorylated sugar chains. A series of novel CHO (Chinese Hamster Ovary) mutants with abnormalities in lysosomal enzyme trafficking were isolated and characterized by Robbins, Krag, and others.¹⁸⁴⁻¹⁸⁷ However, these mutants do not have specific abnormalities in the M6P pathway, but rather have pleiotropic defects in endosomal acidification, endocytosis, or glycosylation.

R. LYSOSOMAL ENZYMES FROM *D. DISCOIDEUM* ARE TAKEN UP BY HUMAN FIBROBLASTS

The slime mold *D. discoideum* is known to secrete a large fraction of its lysosomal enzymes into the medium, particularly under conditions of glucose starvation. Freeze and others demonstrated that these secreted enzymes showed the "high-uptake" phenotype with human fibroblasts in culture, and that this uptake could be blocked by M6P.^{188,189} In keeping with this finding, the CI-MPR could be purified using these enzymes as affinity ligands.⁴⁶ However, it was subsequently shown that the *D. discoideum* oligosaccharides had a novel methylphosphomannose sequence that was being recognized for uptake^{190,191} and that the CD-MPR was unable to recognize this structure.⁹⁹ This also explained why the "high-uptake" activity of these enzymes could not be destroyed by alkaline phosphatase. Somewhat surprisingly, these eucaryotic organisms do not appear to utilize the same pathway for targeting of their lysosomal enzymes.¹⁹² No receptor for phosphorylated Man residues has been found in these organisms, despite the presence of a GlcNAc phosphotransferase that recognizes α 1,2-linked Man residues, and the generation of the identical reaction product.¹⁹³ Notably, this phosphotransferase does not show the specific recognition of lysosomal hydrolases seen with the mammalian enzyme.

S. ALTERNATE PATHWAYS FOR THE TARGETING OF LYSOSOMAL ENZYMES

It is clear that the phosphomannosyl recognition marker plays a crucial role in the trafficking of newly synthesized lysosomal enzymes to lysosomes in cell types such as the fibroblast. However, several lines of evidence suggest that alternate mechanisms must exist in some other cell types. Even in the most severe cases of I-cell disease, some cells and tissues (e.g., liver and circulating granulocytes) have essentially normal levels of intracellular enzymes.^{71,77,78,194} Likewise, B-lymphoblast lines established from patients with I-cell disease do not show the complete phenotype of enzyme deficiency seen in fibroblasts.^{195,196} It is also of note that lysosomal enzymes are successfully targeted in lower eucaryotes such as *Saccharomyces*, *Trypanosoma*, and *Dictyostelium*, without the aid of an identifiable M6P receptor pathway.^{192,197,198} One interpretation is that the M6P pathway for trafficking of lysosomal enzymes is a specialized form of targeting superimposed upon some other basic mechanisms that remain undefined.¹⁹⁴

Two lysosomal enzymes, acid phosphatase and β -glucocerebrosidase, are not affected, even in I-cell disease fibroblasts, implying that they do not require the phosphomannosyl recognition marker pathway for trafficking to lysosomes in any cell type. In the case of acid phosphatase, Von Figura and others have shown that this enzyme begins life as a membrane-

bound protein, and follows novel pathways in getting to the lysosome.¹⁹⁹ Once in the lysosome, it is proteolytically cleaved to generate the mature soluble form that remains there. β -Glucocerebrosidase is also membrane associated, does not show phosphorylation of its oligosaccharides,²⁰⁰ and is targeted to lysosomes independent of this pathway. Likewise, integral membrane proteins of the lysosome such as the lamp/lgp proteins do not require the phosphomannosyl recognition marker pathway for trafficking to lysosomes.²

T. OTHER POTENTIAL ROLES FOR MANNOSE 6-PHOSPHATE-BEARING OLIGOSACCHARIDES

Mannose 6-phosphate esters were first found on the oligosaccharides of classical lysosomal enzymes and were thought to be specific for these proteins. However, they have since been reported on a variety of other proteins, some of which are nonlysosomal. The first such variant noted was the major excreted protein (MEP) of transformed fibroblasts²⁰¹ which is now known to be the acid protease, cathepsin L. The studies of Sahagian¹⁰⁹⁻¹¹¹ and Gabel¹¹² suggest that steric hindrance by the peptide backbone and/or the presence of only one oligosaccharide may cause a failure of recognition of the mature marker by the M6P receptors. However, no obvious difference was found in the oligosaccharide of cathepsin L between the normal and the transformed state, suggesting that growth-induced changes in the lysosomal transport system are also important in explaining the secretion.¹¹¹ Another acid hydrolase with M6P that takes the secretory route is uteroferrin,²⁰² a purple, iron-containing tartarate-resistant acid phosphatase found by Roberts et al. in uterine secretions of pseudo-pregnant sheep. In this case, the data suggest that failure of removal of the blocking GlcNAc residues is the cause for secretion.^{63,89} More recently, these investigators have shown M6P in diester linkage on uteroferrin-associated basic proteins²⁰³ and on uterine "milk" proteins.²⁰⁴ Again, the significance of these observations to the physiology of these proteins is unknown. Another acid hydrolase, cathepsin B, is known to have a dual cellular distribution, with a membrane-associated form in early endosomes and a soluble form in the lysosomes.²⁰⁵ The role of M6P residues in determining this dual localization has not been investigated.

M6P has also been found on the TGF- β precursor,²⁰⁶ but is lost in the mature form of this growth factor. Since acid treatment is traditionally used to "activate" the precursor *in vitro*, it has been suggested that M6P may serve to target it to an acidic compartment for activation in the intact cell. In fact, a recent study demonstrates that the cellular activation of TGF- β seen in co-cultures of bovine aortic and smooth muscle cells is inhibited by M6P and by antibodies against the CI-MPR.²⁰⁷ Other nonlysosomal proteins that carry M6P are proliferin,²⁰⁸ thyroglobulin,^{209,210} the EGF receptor,²¹¹ and a fungal peroxidase.²¹² More recently, the varicella virus glycoprotein has been shown to contain M6P on its complex-type sugar chains.²¹³ It has been suggested that this form of M6P originates from an entirely distinct pathway. Thus, the significance of finding M6P on nonlysosomal proteins is unclear in most cases. One should not necessarily assume that the phosphomannose residues on all of these proteins are involved in intracellular trafficking. Just as phosphorylation of serine residues has diverse biological roles, it is possible that M6P might be utilized for more than one purpose in a complex multicellular organism. Further investigation of each of these situations is needed, with an open mind to all possibilities.

A final group of unexplained observations involve the effects of M6P, its stereoisomer fructose 1-phosphate, and in some cases the phosphorylated yeast mannans on various biological phenomena. These include the inhibition of natural killer cell-mediated cytotoxicity,²¹⁴ the abrogation of lymphocyte binding to the high endothelial venule,²¹⁵ the stimulation of neuronal growth,¹⁷² and the inhibition of central nervous system inflammation in allergic encephalomyelitis.²¹⁶ The implication is that M6P-containing oligosaccharides are somehow involved in mediating these complex cellular interactions. However, direct proof

for this is still lacking in all of the cited cases, and alternate explanations for the phenomena are possible.

IV. ROLE OF MAMMALIAN OLIGOSACCHARIDES IN THE INTERCELLULAR TRAFFICKING OF GLYCOPROTEINS

The discovery of the M6P pathway resulted from studies of uptake of lysosomal enzymes into intact cells. These observations were actually presaged by the description of other carbohydrate-specific uptake phenomena. In each case, mammalian lectins were eventually identified that targeted their ligands from the cell surface via the coated pit/coated vesicle/endosome pathway^{19,20} to eventual degradation in the lysosome. This group of mammalian lectins are considered together in the following sections, in considerably less detail than that accorded to the M6P receptors.

A. DISCOVERY OF THE HEPATIC ASIALOGLYCOPROTEIN RECEPTOR

The first of these lectins to be discovered was the asialoglycoprotein receptor of hepatocytes. In studying the biology of the serum protein ceruloplasmin, Ashwell, Morell, and others sought ways to introduce a long-lived isotope into the protein. They introduced ³H-label into glycoproteins by reduction with tritiated borohydride, following either galactose oxidase treatment²¹⁷ or mild periodate cleavage of the side chains of the sialic acids.²¹⁸ In the former case, it was necessary to remove the outer sialic acid residues to permit the action of galactose oxidase. Surprisingly, the serum half-life of intravenously injected ceruloplasmin was dramatically shortened by such removal of sialic acids.²¹⁹ This was caused by recognition of the exposed galactose (Gal) or *N*-acetylgalactosamine (GalNAc) residues by a liver-specific process.^{220,221} Thus was born the concept that specific carbohydrate sequences could be recognized by tissue-specific receptors which could mediate uptake of the protein carrying these sugar chains. Affinity chromatography on immobilized asialoglycoproteins then permitted purification of the "asialoglycoprotein receptor" responsible for this phenomenon^{222,223} and its eventual molecular cloning (see Table 2, and below).

Not surprisingly, asialoglycoproteins were present only in very small amounts in normal mammalian serum. In contrast, an inhibitor assay showed that avian species had high serum levels of asialoglycoproteins.²²⁴ Unlike mammalian liver, the avian liver was found to have a lectin specific for terminal *N*-acetylglucosamine (GlcNAc) rather than Gal.^{224,225} Notably, GlcNAc is the sugar found immediately beneath β -Gal residues in complex type *N*-linked oligosaccharides (see Figure 5). Thus, removal of both sialic acids and β -linked Gal residues is required for recognition by the avian hepatocyte receptor. This receptor has also been purified^{225,226} and molecularly cloned (see Table 2, and below).

B. DISCOVERY OF THE MANNOSE RECEPTOR OF MACROPHAGES

This sugar-specific receptor was also discovered somewhat serendipitously. Based on the early recognition that lysosomal hydrolases could be taken up by cells, the hope was born that lysosomal storage disorders could be treated by administration of purified enzymes. As a first step towards such a goal, Stahl and others administered lysosomal enzymes and other glycoproteins intravenously into rats. They discovered that uptake was occurring in a highly specific fashion, into the macrophages of the reticuloendothelial system, particularly the spleen and the liver.²²⁷⁻²³⁰ However, this uptake process was not unique to lysosomal enzymes, and was found for a variety of other glycoproteins with high mannose-type oligosaccharides.²³¹⁻²³² The receptor responsible for this process was then demonstrated in isolated macrophages and shown to bind synthetic glycoproteins with terminal Man and fucose (Fuc) residues.²³³⁻²³⁵ This Man/Fuc receptor has since been purified to homogeneity from several sources²³⁶⁻²³⁹ and molecularly cloned (see Table 2, and below).

TABLE 2
Membrane-Bound Animal Lectins That May be Involved in Subcellular Trafficking of Glycoproteins

| Lectin | Protein structure | Predominant cell type expressed in | Terminal monosaccharides recognized | Functional pathways* |
|----------------------------------|----------------------------------|---|-------------------------------------|----------------------|
| Cation-independent M6PR | 275-kDa monomer(?) | Many cell types | Mannose 6-phosphate | A & B |
| Cation-dependent M6PR | 46-kDa homodimer | Most cell types | Mannose 6-phosphate | A |
| Asialoglycoprotein receptor | Heterodimer of 48 kDa and 42 kDa | Mammalian hepatocyte | GalNAc>Gal>>Glc | B |
| Chicken hepatic lectin | Hexamer with 25-kDa subunit | Avian hepatocyte | GlcNAc>>Man,Glc | B |
| Gal/GalNAc receptor | Homooligomer of 42 kDa | Peritoneal macrophages | Galactose = GalNAc ?sialic acid | B/?C |
| Man/Fuc receptor | 180-kDa monomer | Macrophages, liver endothelial cells? | Mannose>Fucose >>GlcNAc | B/C |
| Gal/Fuc receptor | 77—88-kDa monomer | Kupffer cells | Galactose or fucose | ?B/?D |
| Sialic acid receptor | 175—185-kDa (monomer?) | Macrophages from bone marrow/lymph node | Sialic acids | B/?D |
| Thyroid GlcNAc-specific receptor | 45-kDa homotrimer | Thyroid membranes | GlcNAc>>Gal | B? |

* A: Golgi apparatus to endosome (pre-lysosomal compartment); B: coated pit to coated vesicle to uncoated vesicle to early endosome (CURL); C: phagocytosis of particles; and D: adhesion to other cells or substratum.

C. IDENTIFICATION, PURIFICATION, AND CHARACTERIZATION OF OTHER CARBOHYDRATE-SPECIFIC RECEPTORS

Since these early observations, similar "uptake" or binding phenomena have uncovered several other carbohydrate-specific membrane-bound receptors. Listed in Table 2 are those receptors for which some molecular details have since emerged. Affinity chromatography with the cognate carbohydrate ligands has permitted complete purification of almost all of these molecules. A Kupffer cell receptor specific for Fuc (and Gal) residues was discovered, purified, and cloned by Hill and co-workers.²⁴⁰⁻²⁴⁴ More recently, a macrophage Gal/GalNAc specific lectin that is homologous, but not identical to the hepatocyte asialoglycoprotein receptor, has been described, purified, characterized, and cloned by the groups of Kawasaki and Schauer.²⁴⁵⁻²⁴⁷ This receptor is present only on certain types of macrophages such as those from the peritoneal cavity. Another receptor expressed on macrophages in the bone marrow appears to recognize sialic acid as its ligand,^{248,249} while one described in thyroid membranes recognizes GlcNAc residues.²⁵⁰ Unlike the M6P receptors which are widely distributed among many cell types, all the others listed in Table 2 show a relative or absolute cell-type specificity in expression. This suggests that they may have tissue-specific functions.

Biosynthetic studies^{251,252} have shown that all of these receptors are glycoproteins synthesized in the ER as membrane-bound polypeptides, and pass through the Golgi apparatus, eventually reaching the plasma membrane and/or endocytic compartments. In those cases studied, each receptor molecule was found to undergo hundreds of rounds of endocytosis and recycling before final degradation (see below). The binding properties of the purified receptors have been studied in great detail by several groups, using both natural ligands and synthetic neoglycoproteins. In most cases, the type, number, and spacing of the cognate sugars appear to be important in binding affinity.^{223,240,242,253-257} In some cases, the binding properties of the receptors changed somewhat in the purified state compared with predictions

made from intact cells. It also appears likely that multiple receptors can participate in the recognition and uptake of glycoproteins by intact cells. For example, neoglycoproteins terminating in the sequence Gal β 1-4(Fuc α 1-3)GlcNAc β - are recognized by both the asialoglycoprotein receptor and the Man/Fuc receptors of liver cells,^{240,258} and asialotransferrin is recognized by both the transferrin and asialoglycoprotein receptors.²⁵⁹

D. MOLECULAR CLONING OF THE CARBOHYDRATE-SPECIFIC RECEPTORS UNCOVERS A COMMON STRUCTURAL MOTIF

Several of these receptors have been cloned and expressed^{243,260-265} in different cell types. With the availability of sequence information on these proteins, some of the confusion about their relationships to one another has been resolved (see Table 2). Drickamer was the first to recognize a specific sequence motif shared by all of these polypeptides (the M6P receptors are the only exception) which he has termed the "carbohydrate recognition domain" (CRD) (reviewed in Reference 6). Because all of these receptors required calcium for their action, he named them "C-type" lectins (in contrast to the "S-type" lectins such as the soluble β -galactoside binding lectins that require thiols for their activity). Since then, "C-type" lectin-like domains have been recognized in a variety of other proteins, including those that dictate traffic of whole cells (e.g., the lymphocyte homing receptors, see Chapter 3).

Unlike the M6P receptors, all of these lectins share common features of type II membrane proteins, having a small aminoterminal cytoplasmic domain, a single hydrophobic transmembrane anchoring region, and a large carboxylterminal extracellular domain containing the carbohydrate-binding region. However, they vary widely in molecular size, subunit composition, and sugar binding specificity (see Table 2 and below). With the exception of the asialoglycoprotein receptor, these receptors appear to be multimers derived from single gene products. The asialoglycoprotein receptor appears to arise from two unique polypeptide chains which share a 70 to 80% homology, which are dependent upon one another for optimal surface expression and ligand binding, and exist functionally as heterooligomers.^{263,266-271} Another common feature of these receptors appears to be the presence of multiple carbohydrate-binding regions within each extracytoplasmic domain.^{265,272,273} Since such multiplicity is compounded by oligomerization, it is not surprising that binding affinities are markedly affected by the number and spacing of the cognate sugars on the ligand.

Taken together, the results of molecular cloning indicate that these proteins share a common ancestry, but have diverged considerably during evolution.²⁴⁴ Not surprisingly, the degree of homology is better when the receptors bind similar ligands; for example, the Gal/GalNAc lectin of peritoneal macrophages has a 50 to 75% homology with the two subunits of the asialoglycoprotein receptor of hepatocytes.²⁴⁷ Once again, the M6P receptors appear to be the exception to the rule, having little homology to the other carbohydrate-specific membrane receptors.

E. A COMMON ENDOCYTIC PATHWAY IS FOLLOWED BY ALL THE RECEPTORS

While most of these receptors show cell type-specific expression, the general pathways they follow once they have bound ligands appear to be common.^{150,235,274-279} Ligand-receptor complexes cluster in clathrin-coated pits, which become coated vesicles, in a manner similar to that of all receptor-mediated endocytosis.^{3,19,20,131,155,280} In the cases studied, the internalization reaction appears to be a pseudo-first-order process, with a constant fraction of the ligand being internalized per unit time.³ Following internalization, the clathrin-coated vesicles undergo rapid uncoating and then fuse together with the early endosomal compartment where the ligands separate from the receptors under the influence of acidic pH (see below). This compartment has been termed by some as the "Compartment of Uncoupling of Receptor and Ligand" or CURL.²⁷⁷ Morphologic evidence indicates that free receptor

molecules tend to cluster together in tubular extensions, distinct from the main vesicular compartment that contains the released ligand. The great majority of the receptor molecules then recycle back to the cell surface to undergo another round of endocytosis. The ligands are next delivered to a late endosomal compartment where they probably meet with newly synthesized lysosomal enzymes arriving from the Golgi apparatus,¹⁴⁰ eventually reaching the lysosome for degradation (see Figure 6).

Depending upon the receptor, the ligand, and the cell type, a portion of the endocytosed ligand-receptor complex can also return to the cell surface prior to encountering the acid pH of the early endosome. This phenomenon, termed "diacytosis" or "retrocytosis", appears to be a physiological process that can be modulated by a variety of factors.²⁸¹⁻²⁸³ The significance of this apparently futile cycle is unclear at this time. A systematic search for this retrocytosis pathway has not been made for several of the other carbohydrate-specific endocytosis receptors (including the M6P receptors).

Some receptor molecules that have released their ligands appear to pass through the *trans*-Golgi compartment before returning to the cell surface (reviewed in Reference 284). This is suggested by experiments in which receptors become resialylated during endocytosis.^{127,137,183,285} While only a small fraction of the receptors follow this path with each round of endocytosis, it eventually involves most of the molecules. In the case of the asialoglycoprotein receptor, a small portion of the diacytosed ligand also becomes resialylated.²⁸⁶ Again, the significance of this pathway to the normal functioning of the receptors is unclear. Evidence from others suggests that this is a special feature of recycling receptors not shared by all cell surface sialoglycoproteins.²⁸⁷

Detailed studies of the number, turnover, recycling, and distribution of some of these receptors have been carried out by many investigators using a variety of tools and conditions (see References 264, 278, 279, and 288 through 298 for some examples). The reader is referred to these papers and to recent excellent reviews on the subject.^{3-5,8,10,12,20} The entire process of internalization occurs within a few minutes, and final delivery of the ligand to the lysosome is often complete within 20 to 30 min. There are relative differences between the receptors in both the quantitative and kinetic aspects of recycling. Early intermediates in internalization and fusion with endosomal compartments have also been isolated and reconstituted *in vitro* using a variety of tools.^{296,299,301} Recent work has shown that the timing of uncoupling of receptor and ligand can be different for various receptors endocytosed at the same time.^{151,302} As with the M6P receptors, studies of genetically engineered receptors indicate that while endocytosis involves their cytoplasmic tails, alternative or parallel mechanisms do exist.³⁰³ More recently, morphological evidence has suggested that the endosomal compartments may have complex tubular interconnections³⁰⁴ and that multivesicular bodies may traverse this system, playing a crucial role in determining the fate of various receptors and their ligands. Thus, the functional and two-dimensional data previously observed may represent a three-dimensional reality that is much more complex than originally imagined. Also, as discussed earlier, the pathways followed by these receptors might be stochastically determined by multiple factors, rather than rigidly defined.

F. THE IMPORTANCE OF INTRA-ORGANELLE pH AND THE EFFECT OF WEAK BASES

A common characteristic of all the carbohydrate-specific receptors described in this review (including the M6P receptors) is that they discharge their ligands at acidic pH. Thus, the first encounter with low pH dissociates the receptor from the ligand, permitting the former to recycle, while the latter is irreversibly consigned for delivery to the lysosomes (see Figure 1). For receptors following the coated pit/coated vesicle pathway from the cell surface (see Figures 2 and 6), this critical drop in pH is first encountered in the early endosome.^{151,305,306} In the case of newly synthesized lysosomal enzymes arriving from the

Golgi apparatus, the critical pH is presumably first encountered upon arrival in the late endosomal or prelysosomal compartment.^{20,305} In both cases, the majority of the freed-up receptor molecules then return to their original location where the pH is once again near neutral. This provides a simple mechanism for the reutilization of some of the receptors. Although a systematic analysis of this issue has not been done, it appears that the receptors most sensitive to a fall in pH tend to drop off their ligands the earliest in the endocytic pathway.^{151,302} This is in keeping with the progressive fall in pH en route from the early endosome to the late endosome to the lysosome.³⁰⁵ Perhaps not surprisingly, the M6P receptors appear to be the last to part company with their ligands,¹⁵¹ presumably in the late endosome. Unlike the other receptors, this also allows them to enter a pool from which they can return to the *trans*-Golgi region.

The pH dependence of ligand binding also provides a logical explanation for the effects of "lysosomotropic amines" such as chloroquine and ammonium chloride on receptor traffic (see References 298 and 306 through 311 for examples). These bases become protonated and trapped in naturally acidic compartments raising the intraluminal pH not only in the lysosomes, but also in the endosomal compartments where the receptor-ligand uncoupling normally occurs. Similar effects are seen with the proton ionophore, monensin.^{312,313} Thus, it appears that receptor-ligand complexes remain uncoupled and are therefore unavailable for further rounds of recycling. This effect is held in common with many other types of receptor mediated endocytosis,^{19,305} and has been used to dissect events in the endocytic pathway.

G. ARE THESE CARBOHYDRATE-SPECIFIC RECEPTORS INVOLVED IN THE BIOSYNTHETIC PATHWAY?

As described earlier, the *major* proportion of the M6P receptors directs newly synthesized enzymes from the Golgi apparatus to the late endosome, while a *minor* proportion is found on the cell surface and follow an endocytic itinerary similar to that of other carbohydrate-specific receptors. It is therefore natural to question whether the other receptors have a role in directing glycoprotein traffic in the biosynthetic pathway. Although some supportive experiments have been reported, there is as yet no convincing evidence that this is the case. In keeping with this view, the distribution of these lectins is quite different from that of the M6P receptors, being found mostly in the plasma membrane and the early part of the endocytic pathways. However, a significant fraction of the asialoglycoprotein receptors in normal liver and in hepatoma cells has been noted in the *trans*-Golgi network, in a pool which is cycloheximide resistant and "silent"; that is, not participating in the repeated rounds of endocytosis from the surface.^{10,302} It is possible that newly synthesized glycoproteins that fail to get sialylated in the *trans*-Golgi might be diverted for lysosomal destruction by this pool of receptors. In this manner, the asialoglycoprotein receptors could serve a "proof-reading" function for newly synthesized molecules that are incompletely glycosylated. Similar possibilities could apply to the other carbohydrate-specific receptors. Of course, it is also possible that such silent internal pools of receptor are actually used for rapid up-regulation on the cell surface under various conditions.

H. SOME SPECIAL CONSIDERATIONS REGARDING THE ASIALOGLYCOPROTEIN RECEPTOR

An early observation was that upon treatment of intact hepatocytes or the purified asialoglycoprotein receptor with neuraminidase, the binding activity disappeared.³¹⁴ The explanation appears to be that the oligosaccharides of the receptor itself are susceptible to the action of the neuraminidase. Thus, the receptor tends to bind to itself or to other adjacent membrane proteins, making it unavailable for binding soluble ligands. More recent experiments have suggested that this artificial phenomenon could be the analogue of a natural process by which receptor function is modulated by its own state of sialylation.³¹⁵

Based upon its location, specificity and itinerary, it is natural to speculate that the asialoglycoprotein receptor has a role in determining the serum half-life of glycoproteins and circulating hormones. The detection of increased levels of asialoglycoproteins in some liver diseases supports this notion.³¹⁶ However, direct proof of this role in the normal organism is still lacking. One can envisage that de-sialylation of a circulating plasma glycoprotein is a stochastic process related to the number of times it has had the opportunity to encounter a neuraminidase. The removal of a few sialic acid residues by such an enzyme would be sufficient to commit the glycoprotein for removal by the liver. The difficulty has been in identifying the putative neuraminidase(s) involved in such a process, and in showing that their action is indeed the determinant of the serum half-life of glycoproteins. A variation of this hypothesis has been suggested by the experiments of Tavassoli and others.^{317,318} In this work, liver endothelial cells were shown to be capable of taking up fully sialylated glycoproteins, desialylating them, and passing them on by transcytosis for uptake by the hepatocyte asialoglycoprotein receptor. In this scenario, it is the liver endothelium that contains the relevant neuraminidase, and is therefore the primary determinant of the serum half-life of glycoproteins. This hypothesis deserves further investigation.

I. SOME SPECIAL CONSIDERATIONS REGARDING THE MANNANOSE/FUCOSE RECEPTOR

This receptor appears to function primarily in the endocytic pathway of the macrophage, presumably targeting proteins for degradation. However, there is also evidence that it may serve to target larger particles; for example, microorganisms or particles for phagocytosis and destruction or even intercellular transfer.^{4,273,319} This could be particularly important for cells that reside on mucosal surfaces (e.g., alveolar and peritoneal macrophages). It has also been postulated that the circulating soluble Man/GlcNAc lectin (a distinct protein) might cooperate with the macrophage receptor to eliminate certain circulating microorganisms. In keeping with these hypotheses, the monocyte precursors of macrophages do not express the Man/Fuc receptor until they are activated.⁴

In a manner analogous to the asialoglycoprotein receptor, an abnormal increase in endogenous ligands (e.g., by inhibitors of *N*-linked oligosaccharide processing) also causes a functional loss of this receptor.³²⁰ Lee and colleagues have reported that neoglycoproteins carrying multiple Man residues actually stimulate secretion of presynthesized lysosomal enzymes.³²¹ This phenomenon appears to have the same sugar specificity as the Man/Fuc receptors and occurs regardless of whether the neoglycoprotein is degradable (Man-BSA) or nondegradable (Man-poly-D-lysine). The significance of this observation to the normal biology of this receptor remains somewhat uncertain.

J. OTHER POTENTIAL BIOLOGICAL ROLES FOR CELL SURFACE LECTINS

Primarily because of their ease of use, the model compounds used for study of these lectins have been soluble glycoproteins. As mentioned above, there is some evidence that the mannose receptor of macrophages could play a role in recognizing mannan-bearing pathogens and aid in their phagocytosis.⁴ It is possible that some of the other receptors on macrophages (e.g., the Gal/GalNAc receptor of peritoneal macrophages and the Gal/Fuc receptor of Kupffer cells) play a similar role against as yet unidentified pathogens. Another potential role for these receptors could be in the interaction of cells with their substratum or with other adjacent cells. Experiments with immobilized carbohydrates have shown that such interactions can be demonstrated *in vitro*.³²²⁻³²⁴ These sugar-specific interactions are presumably occurring via cell surface lectins similar or identical to those described in this review. It remains to be demonstrated if there are natural counterparts to these phenomena *in vivo*. Good candidates may be the Gal/Fuc receptor of Kupffer cells, which failed to mediate endocytosis when cloned and expressed in heterologous cells, and the sialic acid-

binding hemagglutinin of bone marrow macrophages, which was found to be concentrated at contact points with other bone marrow cells.³²⁵

K. A NOTE ABOUT SOLUBLE MAMMALIAN LECTINS

All of the carbohydrate binding receptors discussed in this review are membrane-anchored proteins with hydrophobic transmembrane regions that require detergent for solubilization. A variety of soluble mammalian lectins have also been described, both of the "C-type" and of the "S-type", that recognize glycoproteins with terminal sugars such as sialic acid, galactose, or mannose (reviewed in References 6 and 326). There is at present no evidence that any of these lectins dictate trafficking of glycoproteins within or between cells. This is perhaps not surprising, given their water-soluble nature and their subcellular location (either cytosolic or extracellular). However, it is possible that following binding to extracellular ligands, some of these receptor-ligand complexes may be taken up by cells (e.g., the circulating Man/GlcNAc lectin).³²⁷

Soluble forms of several of the membrane-bound receptors have been generated *in vitro* by selective proteolysis, permitting isolation of carbohydrate recognition domains that retain their natural binding properties.^{6,328} Naturally occurring soluble forms of some of these receptors have also been reported, presumably resulting from similar proteolytic events *in vivo*.^{329,330} The biological significance of such soluble molecules is uncertain. More recently, genetic engineering has permitted the production of truncated soluble forms of these receptors which also retain their binding properties.^{6,117,153,257}

V. IS THERE A ROLE FOR OLIGOSACCHARIDES IN THE DELIVERY OF GLYCOPROTEINS FROM THE ER TO THE GOLGI APPARATUS?

A. HISTORICAL BACKGROUND

The discovery that *N*-linked glycosylation occurred co-translationally in the ER (reviewed in Reference 331) and that the oligosaccharide underwent significant early modification ("processing") in the ER (reviewed in Reference 21) led to the suggestion that transit of newly synthesized glycoproteins to the Golgi apparatus might be controlled by these sugar chains. Such speculation was fueled by early observations with the antibiotic tunicamycin,³³² which prevents *N*-linked glycosylation by interfering with the formation of the lipid-linked oligosaccharide precursor (reviewed in Reference 333). Several proteins whose *N*-linked glycosylation was prevented by tunicamycin failed to be expressed^{334,335} or to be secreted.³³⁶⁻³³⁸ However, several exceptions to this rule soon appeared,³³⁹⁻³⁴³ ruling out an absolute requirement for *N*-linked glycosylation in this segment of the itinerary of glycoproteins. In retrospect, it appears that these effects of tunicamycin are caused by failure of proper folding of proteins. Thus, proteins that happened to misfold because of the lack of co-translational *N*-linked glycosylation become insoluble and/or susceptible to the ER protease systems.^{16,344}

B. DOES GLUCOSE REMOVAL FROM *N*-LINKED OLIGOSACCHARIDES MODULATE PROTEIN EXIT FROM THE ER?

The transit time of glycoproteins from the ER to their final destination can be highly variable.^{16,17,245} The primary determinant of this variability appears to be the time spent by the protein in the ER, the "transitional ER", and in a "salvage compartment" prior to exit to the Golgi apparatus (reviewed in References 16 and 17). The structural basis for this variability is not clear, but probably includes multiple factors such as the time required for completion of folding, disulfide bond formation, oligomerization, and oligosaccharide processing. An event peculiar to the ER is the removal of three glucose residues that are part of the *N*-linked oligosaccharide initially transferred *en-bloc* to glycoproteins. The removal

of these glucose residues is catalyzed by two ER-specific enzymes, glucosidase I and II (see Figures 3 and 5). With the rare exception of some trypanosomatid protozoa,³⁴⁶ these glucose residues and their subsequent ordered removal are highly conserved throughout the eucaryotes. On the other hand, they are the only components of the transferred oligosaccharide that have never been found on mature glycoproteins under normal circumstances.

The significance of this highly conserved addition and removal of glucose residues has remained obscure. Recent evidence from Parodi and others indicates that after transfer to the protein, these glucose residues may actually be in a dynamic equilibrium between removal by glucosidase II and replacement by a specific ER glucosyltransferase.³⁴⁷⁻³⁴⁹ This finding can explain the puzzling observation that removal of the *innermost* glucose residue is greatly delayed in pulse-chase studies, in spite of the fact that it is mediated by the same enzyme (glucosidase II) that acts on the *middle* residue.^{350,351} This apparent delay in the removal of the last glucose residue can now be explained by a constant cycle of deglycosylation and reglycosylation. Since the final product leaving the ER is always devoid of glucose residues, this cycle must end in favor of the glucosidase rather than the glucosyltransferase. In keeping with this conclusion, the glucosyltransferase works much better on denatured glycoproteins than on native folded molecules.³⁴⁹ Thus, it could be hypothesized that prior to the final folding of a glycoprotein in the ER, it is at constant risk of being reglycosylated. However, once it has reached its native folded state, the transferase can no longer act, and the glucosidase would complete the removal of the final glucose residue.

Thus, the final removal of glucose residues could possibly be a rate-limiting factor in exit of proteins from the ER. In keeping with this hypothesis, the oligosaccharides of temperature-sensitive mutants of viral glycoproteins are monoglucosylated when retained in the ER at the nonpermissive temperature.³⁵¹ Likewise, specific inhibitors of the processing glucosidases have been shown to alter the exit time of proteins from the ER.³⁵² On the other hand, in cells treated with such agents, some proteins do eventually leave the ER, carrying the unremoved glucose residues. Likewise, cell lines with decreased glucosidase II activity can have mature proteins carrying glucose residues.^{198,353,354} Thus, while removal of glucose residues could be important in modulating exit of proteins from the ER, it cannot be an absolute requirement. An intermediate possibility is that permanent removal of *some* glucose residues from a protein is sufficient to permit exit from the ER. In this regard, it is noteworthy that all mutants in glucosidase II reported to date are "leaky" (having some complex type oligosaccharides), suggesting that complete lack of glucose removal might be lethal. However, this "leakiness" does not necessarily represent the action of residual glucosidase II in the mutants. Spiro and colleagues have recently noted a novel endomannosidase in the Golgi apparatus that can remove a glucosyl-mannose disaccharide from monoglucosylated oligosaccharides (see Figure 5).³⁵⁵ The action of this enzyme on glucosylated molecules that exit the ER has been directly demonstrated in intact cells.^{356,357} The existence of this "fail-safe" mechanism for removal of the glucose residues further implies that it normally fulfills some important role.

The potential role of glucose removal in modulating glycoprotein trafficking along the ER-Golgi pathway remains a matter for further study. If the last glucose residue indeed serves as a specific signal, one would predict the existence of a receptor for this structure, or a receptor for the product of glucose removal (a terminal α 1,2-linked Man on the α 1,3-linked arm of the oligosaccharide; see Figure 5, structure D). A search for such receptors by affinity chromatography and further analysis of glycoprotein traffic in the glucosidase II mutants is worthwhile.

VI. SUMMARY: ROLE OF CARBOHYDRATE-SPECIFIC RECEPTORS IN THE INTRACELLULAR AND INTERCELLULAR TRAFFICKING OF GLYCOPROTEINS

Shown in Figure 6 is a composite view of intracellular trafficking pathways that occur in a variety of different cell types. In this review, the possible and probable roles that the oligosaccharide units of glycoproteins play in directing or modulating these pathways have been discussed. It is very clear that newly synthesized lysosomal enzymes with the mature phosphomannosyl recognition marker are diverted by M6P receptors from the stream of newly synthesized glycoproteins, to endosomal compartments and thence to the mature lysosome. Although this diversion appears to occur primarily from the *trans*-Golgi network in many cell types, there is some evidence that it can also occur earlier in the Golgi apparatus, and it is possible that some diversion can occur later (see Figures 2 and 6). A minority of the M6P receptors are also present on the cell surface, where they can mediate uptake of lysosomal enzymes carrying the mature phosphomannosyl recognition marker. In the latter case, the enzymes in question can originate either from the same cell or from another cell (see Figure 6). A minority of the proteins that become phosphorylated fail to have the outer blocking GlcNAc residues removed. Such molecules cannot bind the M6P receptors, are secreted directly from the cell, and are presumably fated to remain in an extracellular location. Certain proteins (e.g., uteroferrin) are more prone to follow this pathway than others in the same cell. Depending on the cell type, the expression level of the two M6P receptors, and other factors, a portion of the enzymes can also be secreted carrying the mature phosphomonoester recognition marker.

With regard to the other carbohydrate-specific receptors (specific for Gal, Man, GlcNAc, and Fuc), there is as yet no evidence that they can divert newly synthesized molecules from their outward-bound flow. Rather, it appears that these receptors serve to remove molecules with the appropriate terminal sugars from the surrounding milieu. Such molecules can obviously originate not only from the same cell, but also from other cells, both nearby and distant. In most cases, the origin of the natural ligands for these receptors is uncertain. However, in one specific case, the interaction of lysosomal enzymes with the macrophage mannose receptor, some reasonable speculation is possible. Several cell types are known to discharge their lysosomal contents upon specific stimulation; for example, activation of macrophages in inflammatory lesions. These enzymes may no longer carry their phosphomannosyl recognition marker because it has been destroyed by acid phosphatase in the lysosome. However, they do generally carry high mannose-type oligosaccharides that allow them to bind to the Man/Fuc receptor of macrophages. Thus, lysosomal enzymes released in a variety of circumstances could be salvaged from the extracellular space and sent back to the lysosomes of macrophages (see Figure 6). The relative importance of such a pathway in the homeostasis of the whole organism has yet to be defined.

It is also possible that some of these receptors play a more important role in cell-cell interaction than in subcellular trafficking. However, the importance of such interactions has yet to be directly demonstrated in the intact organism. Finally, the suggestion that removal of glucose residues on *N*-linked oligosaccharides might modulate exit of glycoproteins from the ER is based on several lines of circumstantial evidence, and remains to be fully investigated.

VII. FUTURE DIRECTIONS

While a great deal of information has accumulated regarding the carbohydrate-specific pathways discussed in this review, many fundamental questions remain unanswered. Some

of these questions are posed below as possible directions for future investigation. What are the specific biological roles of the tissue-specific and carbohydrate-specific receptors for Gal, GalNAc, Man, GlcNAc, and Fuc? What are their natural ligands for the endocytic pathway? Do any of them function to traffic ligands in the biosynthetic pathway? Do any of them have a bona fide role in cell-cell adhesion? What is the basis for their tissue-specific expression? Are there naturally occurring mutants in pathways other than the M6P targeting system? If so, what are their phenotypes?

Likewise, questions remain about the M6P pathway. Why are there two M6P receptors? What determines their relative importance in the trafficking of lysosomal enzymes? What is the significance of the cation dependence and narrow pH range of the 46-kDa M6P receptor? What are the precise subcellular itineraries of each of these receptors? What is the molecular structure of the GlcNAc phosphotransferase? Is the recognition motif for phosphorylation of lysosomal enzymes recently discovered in cathepsin D universal to other lysosomal enzymes? How does the GlcNAc phosphotransferase phosphorylate multiple oligosaccharides on the same protein? Why is M6P present on nonlysosomal proteins such as thyroglobulin, proliferin, and the TGF- β precursor? Are there roles for M6P distinct from the intracellular trafficking of proteins? What is the evolutionary distribution of the M6P recognition pathway? Is it present in plants?

These and many other questions pose interesting challenges for the future. It is worth pointing out that, while several of the mammalian lectin receptors were discovered before the M6P receptors, we know much less about their specific biological significance. What is the reason for this disparity? A likely reason can be found in comparing the history of their discovery, as described in this review. The identification of the M6P receptors was a culmination of a series of discoveries in which analysis of a naturally occurring mutant (I-cell disease) led to analysis of function and identification of structures, which finally led to the discovery of the receptors. In contrast, all of the other mammalian lectins were discovered primarily by virtue of their binding to specific carbohydrate sequences. Thus, in these cases, the path of discovery has been from phenomenon to receptor to structure. In the absence of specific mutants deficient in these pathways, it has therefore been difficult to assign specific biological roles for them in the intact organism. It may be necessary to generate artificial mutants in complex multicellular systems (e.g., transgenic mice) to uncover the biological roles of these highly specific receptors in the normal functioning of the mammalian organism.

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