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The Phosphomannosyl Recognition System for Intracellular and Intercellular Transport of Lysosomal Enzymes

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Speculation that specific receptors might play a role in pinocytosis and in transport of acid hydrolases to lysosomes originated from observations made by Neufeld and co-workers in the late 1960s. Initially, they observed that normal fibroblasts secreted "corrective factors" (later identified as acid hydrolases) that, when added to fibroblasts from patients with mucopolysaccharidoses, could be taken up by these mutant fibroblasts with resultant correction of their storage abnormalities [1–4]. The selectivity and saturability of the uptake process suggested an adsorptive pinocytosis mechanism [3] and, in turn, implied the presence of a recognition marker on the enzyme and pinocytosis receptors on the cell surface. The inappropriate extracellular excess and intracellular deficiency of multiple hydrolytic activities in I-cell disease fibroblasts led Hickman and Neufeld [5] to propose that a whole family of lysosomal enzymes share a common recognition marker necessary for their uptake by cell surface receptors. This early work focused attention exclusively on the role of the recognition marker on acid hydrolases and its receptor in the recapture of secreted acid hydrolases. However, more recent evidence suggests that both the recognition marker and its receptor play a significant role in the intracellular transport of newly synthesized acid hydrolases to lysosomes.

EVIDENCE FOR PHOSPHORYLMANNOSE IN THE "COMMON RECOGNITION MARKER" FOR UPTAKE OF LYSOSOMAL ENZYMES

Early studies by several laboratories produced evidence for the internalization of acid hydrolases by cultured fibroblasts [1, 6–10]. Kinetic studies suggested that the endocytosis of acid hydrolases fulfills two requirements for a receptor-mediated, active transport process [6, 11–19]. First, selectivity was evident from

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the fact that only certain forms of acid hydrolases (called "high-uptake" forms) were taken up at rates that greatly exceeded the rate of nonspecific fluid- or bulk-phase endocytosis [4, 6, 17, 20–22]. Experiments with β-glucuronidase showed the high-uptake forms to be relatively more acidic than the poorly pinocytosed low-uptake forms of the enzyme [20, 21]. Second, saturability of the uptake process has been demonstrated with several high-uptake enzymes [6, 11–19].

Studies by Kaplan et al [11] with human platelet β-glucuronidase provided the first evidence that "high-uptake"acid hydrolases are phosphoglycoproteins, and that phosphohexose is an essential component of the recognition marker on the enzyme for the fibroblast pinocytosis receptor. Alkaline phosphatase treatment of high-uptake β-glucuronidase destroyed its uptake activity and converted the enzyme to less acidic forms without diminishing its catalytic activity [11]. The suggestion that the phosphate on the high-uptake enzyme is linked to mannose was based on the finding that mannose-6-phosphate (Man-6-P) and phosphomannose-containing yeast mannans were potent competitive inhibitors of β-glucuronidase pinocytosis.

Phosphohexosyl recognition is not limited to pinocytosis of β-glucuronidase from platelets [12]. In fact, pinocytosis of β-glucuronidase from all human sources examined was inhibited by Man-6-P and diminished by prior treatment of the enzyme with alkaline phosphatase [12]. Similar findings with β-galactosidase and β-hexosaminidase from platelets indicated that this type of phosphohexosyl recognition component is present on other platelet hydrolases as well [12]. A still further case for generality of phosphohexosyl recognition of acid hydrolases by fibroblast receptors was provided by studies on β-hexosaminidase from fibroblast secretions. These studies excluded the possibility that the phosphohexosyl recognition component is limited to blood platelet enzymes which, as contaminants, might contribute to the small amounts of high-uptake enzyme in other organs. The high-uptake β-hexosaminidase from fibroblast secretions had the same properties as high-uptake β-hexosaminidase from platelets and as high-uptake β-glucuronidase from all sources tested [12]. Although there were no precedents for 6-phosphomannose in mammalian glycoproteins, these observations providing indirect evidence for a Man-6-P-containing recognition component in pinocytosis of lysosomal glycosidases were soon corroborated and extended to include several other acid hydrolases from various sources [13–15, 23]. These results supported the generality of a phosphomannosyl-type recognition marker on human lysosomal enzymes and suggested that phosphomannose is an essential part of the common, carbohydrate-containing [19, 24] recognition marker for uptake that had been originally proposed by Hickman and Neufeld [5] and thought to be missing, defective, or masked in I-cell disease [5].

DIRECT EVIDENCE FOR MANNOSE-6-PHOSPHATE ON HIGH-UPTAKE LYSOSOMAL ENZYMES

The body of indirect evidence suggesting that lysosomal acid hydrolases contain phosphomannosyl groups important for their interaction with and uptake by specific pinocytosis receptors was subsequently extended by several laboratories that provided direct evidence for Man-6-P in the recognition marker of the enzymes. Von Figura and Klein [25] demonstrated release of acidic oligosaccha-

rides (which were susceptible to degradation by alkaline phosphatase, α-mannosidase, and β-N-acetylglucosaminidase) following endoglycosidase H treatment of high uptake α-N-acetylglucosaminidase. This endoglycosidase H treatment converted the enzyme to a form no longer susceptible to adsorptive pinocytosis, and the authors concluded that the recognition marker was present on high-mannose oligosaccharide chains bearing phosphorylated mannose and/or N-acetylglucosamine residues at the nonreducing termini. Using an enzymatic assay for Man-6-P, Natowicz et al [26] showed 1) that acid hydrolysis of high-uptake human spleen β-glucuronidase releases Man-6-P, 2) that all of the Man-6-P on the enzyme is present on endoglycosidase H-sensitive oligosaccharides, and 3) that the Man-6-P content of the enzyme varies directly with its susceptibility to pinocytosis by fibroblasts. Sahagian et al [27] also reported Man-6-P on β-galactosidase from bovine testes, and Distler et al [15] demonstrated Man-6-P in glycopeptides from bovine testicular glycoproteins that had been originally purified as inhibitors of enzyme pinocytosis. It has also recently been demonstrated that phosphate is incorporated into acid hydrolase precursors [28–30].

ARE OTHER STRUCTURAL FEATURES INVOLVED IN ENZYME RECOGNITION?

Further insight into the nature of the recognition, binding, and pinocytosis of acid hydrolases by fibroblast receptors came from studies using yeast phosphomannans as model ligands [31, 32]. Studies by Kaplan et al [11] demonstrated competitive inhibition of acid hydrolase pinocytosis by certain phosphomannans from Saccharomyces cerevisiae. Since phosphomonoester forms demonstrated superior inhibitory properties compared to phosphodiester or dephospho forms of these mannans [31], it appears that phosphate, in the form of exposed mannosyl 6-phosphate residues, is required for optimal inhibition of enzyme uptake. If this 6-phosphomannose group is blocked, then interaction with the receptor and inhibitory potency are dramatically reduced. Comparison of the effects of mild acid hydrolysis on the inhibitory potency of native phosphomannan from S cerevisiae and Hansenula holstii also suggested that the degree of inhibitory potency may be related to the size of the blocking group. Thus, the native phosphomannan from S cerevisiae mutant X2180-mnnl, which contains only a single mannose blocking the phosphate group [33], is 4-fold less potent as an inhibitor than the phosphomonoester [31]. By contrast, the inhibitory potency of a phosphomonoester fragment of Hansenula holstii phosphomannan is 1,000-fold more than the native molecule [31], where the blocking group is at least a tetrasaccharide [34, 35].

Although Man-6-P is structurally analogous to the group on lysosomal acid hydrolases that interacts with fibroblast receptors, it may not be the only structural feature of the recognition site contributing to the high affinity of the enzyme for its receptor. The apparent Km for β-glucuronidase pinocytosis was estimated by Kaplan et al [11] to be $\sim 6 \times 10^{-9}$ M, whereas the apparent Ki for Man-6-P is 4 orders of magnitude greater [11]. A similar difference between the Ki of Man-6-P and the Km for α-L-iduronidase was shown by Sando and Neufeld [13]. These findings indicate that either there are structural features of the recognition marker in addition to phosphomannose, or that binding of lysosomal enzymes involves more than one Man-6-P recognition marker.

One possibly important structural feature of the recognition marker was suggested by Distler et al [15], who reported that Man $\alpha 1 \rightarrow 2$ Man was manyfold more potent as an inhibitor of enzyme pinocytosis than mannose, and that the disaccharide P-6-Man $1 \rightarrow 2$ Man was present in partial hydrolysates of glycopeptides that had been isolated as inhibitors of enzyme pinocytosis. By contrast, Kaplan et al [31] demonstrated that Man-6-P in $\alpha 1 \rightarrow 3$ linkage to mannose at the nonreducing terminus of pentamannosyl monophosphate was not significantly more inhibitory than Man-6-P as a monosaccharide. In the structure for phosphorylated oligosaccharides reported by Varki and Kornfeld [36], three of the five potential sites of phosphorylation were on mannose residues linked $1 \rightarrow 2$ to mannose. Characterized phosphorylated oligosaccharides are not yet available in sufficient amounts to permit a direct correlation of structure with high-affinity binding.

Studies on yeast phosphomannans [31, 32] suggested that high-affinity binding to phosphomannosyl receptors might involve an interaction of more than one Man-6-P on high-uptake enzyme with one or more cell surface receptors. Mild acid hydrolysis of native H. holstii phosphomannan yields two principal products [34, 35], a monovalent pentamannosylmonosphosphate, $(Man)_5$-P, and a larger molecular weight polyphosphomonoester (PPME) fragment, which are quite different in their potency as pinocytosis inhibitors [30]. The large PPME, which can be regarded as multivalent with many exposed 6-phosphomannose groups, is 100-fold more potent as an enzyme pinocytosis inhibitor per mole of phosphate (100,000-fold more potent per molecule) than is $(Man)_5$-P [32]. Moreover, the rate and kinetics of $(Man)_5$-P uptake are no greater than can be explained solely by nonspecific, fluid-phase endocytosis. This suggests that although $(Man)_5$-P is an inhibitor of adsorptive pinocytosis of acid hydrolases, it is very poorly taken up by the process it inhibits. By contrast, PPME does appear to be taken up by adsorptive pinocytosis: Its uptake is saturable and, at low concentrations, at least 30-fold greater than can be explained by the rate of fluid-phase endocytosis alone [32].

The simplest interpretation for the differences in properties of $(Man)_5$-P and PPME is that the PPME is a multivalent ligand that can interact with multiple receptors on the surface of fibroblasts. Such a multivalent ligand could be taken up more rapidly for two reasons. First, multivalency could confer higher binding affinity leading to a larger fraction of occupied receptors at low ligand concentrations. Second, multivalent ligands might actually stimulate adsorptive pinocytosis by cross-linking more than one receptor. Several examples for multivalency in the recognition and uptake of ligands by cell surface receptors have been demonstrated [37, 38], and Murray and Neville [39] have recently shown that a multivalent "neoglycoprotein" can be constructed that is taken up very efficiently by phosphomannosyl enzyme receptors. They chemically modified low density lipoprotein (LDL) by covalently attaching pentamannosyl phosphate groups to the lysine amino groups of LDL (40–50 molecules per LDL). As had been the case with the PPME, the synthetic multivalent LDL ligand had an affinity (Kd 2×10^{-9} M) 4–5 orders of magnitude greater than that of pentamannosyl monophosphate. Like the PPME, the Man-6-P-LDL inhibited enzyme pinocytosis, was efficiently taken up by fibroblasts, and its uptake was inhibited by Man-6-P. Karson et al [40] also constructed a multivalent Man-6-P-substituted albumin that was taken up by the same receptor system.

Similarities in the uptake of PPME and β-glucuronidase suggested that both ligands are pinocytosed by the same receptors [32]. The uptake of both is inhibited by Man-6-P and diminished by prior treatment with alkaline phosphatase. Moreover, just as PPME competitively inhibits pinocytosis of purified high-uptake human β-glucuronidase by fibroblasts, so also is the pinocytosis of the PPME fragment competitively inhibited by the high-uptake enzyme. This raises the question of whether acid hydrolases are similar multivalent ligands that induce their own pinocytosis by binding to multiple cell surface receptors. For instance, β-glucuronidase is a glycoprotein and appears to be a tetramer of identical 75,000-mol wt subunits [41]. Multivalent interactions with receptors could result from several possibilities: 1) interaction of single 6-phosphomannose groups on different protein subunits; 2) more than one oligosaccharide chain, each of which could bear a 6-phosphomannose moiety; or 3) multiple 6-phosphomannose moieties could be present on a single oligosaccharide chain. In this regard, Natowicz et al [26] have demonstrated up to 4.4 mol of 6-phosphomannose per mole high-uptake enzyme, and Varki and Kornfeld [36] have shown that phosphorylation can occur on at least five separate mannose residues on the high-mannose-type oligosaccharides of acid hydrolases and that individual oligosaccharides can have one, two, and perhaps even three phosphate residues.

It is therefore clear that acid hydrolases could indeed be multivalent ligands, and their uptake could depend on interactions with multiple pinocytosis receptors. However, an alternate possibility is that high-uptake acid hydrolases are monovalent ligands that bind to receptors with higher affinity than 6-phosphomannose owing to other structural features of the enzyme or recognition marker. Thus, the pinocytosis of the multivalent PPME phosphomannan fragment could be interpreted as a result of its binding to and cross-linking several phosphomannosyl enzyme receptors. However, the observation that the inhibitory potency of high-uptake enzyme for PPME pinocytosis is dramatically reduced by prior treatment with endoglycosidase H to release phosphomannosyl-containing oligosaccharides from the enzyme [32] is compatible with the notion that high-uptake enzyme can act as multivalent ligand.

LYSOSOMOTROPIC AMINES IMPAIR RECEPTOR REUTILIZATION

Further insight into the properties of acid hydrolase pinocytosis has come from studies on the effect of lysosomotropic amines on fibroblasts, results of which led to several predictions concerning the role of the recognition marker and its receptor in enzyme transport. Several laboratories have found that chloroquine inhibits pinocytosis of exogenous enzyme [42–45]. Gonzalez-Noriega et al [44] recently reported experiments suggesting that this inhibition is due to inhibition of reutilization of cell-surface receptors following internalization of enzyme-receptor complexes. Lysosomotropic amines not only inhibited pinocytosis of exogenous enzyme, but also caused normal fibroblasts to secrete large amounts of acid hydrolases—ie, approximately to the same level of secretion observed in I-cell disease fibroblasts. The lysosomotropic amines appeared to cause normal fibroblast lines [44, 46] and any non-I-cell disease fibroblast lines tested [44] to divert newly synthesized enzymes to the extracellular medium. By contrast, these agents did not enhance the already high levels of secretion seen in I-cell disease

fibroblasts or those from patients with mucolipidosis III, who are thought to have a similar defect. Although amine treatment of normal cells mimicked the I-cell phenotype, the enzyme secreted by these cells was not a recognition-defective, I-cell-like enzyme. In fact, it was greatly enriched for high-uptake enzyme forms [44]. These observations suggested that amines block the normal segregation of newly synthesized enzymes even though the enzymes have normal recognition markers, and suggested a mechanism by which the intracellular traffic pathway for delivery of enzymes to lysosomes was disrupted.

The amines are known to diffuse through membranes and become protonated within lysosomes [47]. As the lysosomal membrane is relatively impermeable to the protonated amines, which become entrapped or exit slowly, the intralysosomal pH is increased from 4.5 to above 6.0 [48]. The rise in pH is thought to inhibit the release of receptor-bound enzyme, so that with time all receptors are saturated [44]. On the basis of these observations, Gonzalez-Noriega et al [44] suggested that the delivery system for lysosomal enzymes may depend on the pH-dependent release of enzyme from receptor in lysosomes to permit free receptors to be reutilized. By raising the intralysosomal pH, amines could block receptor reutilization by interfering with receptor–ligand dissociation. In this view, once all of the receptors are saturated with ligands that cannot dissociate, the amines have effectively produced the equivalent of a receptor-negative phenotype, with all subsequently synthesized enzyme failing to be transferred to lysosomes, and instead, being secreted.

Tietze et al [49] and Schlesinger et al [50] have described similar results on the effect of amines in inhibiting binding, endocytosis, and dissociation of receptor–ligand complexes in receptor-mediated transport of mannose glycoconjugates by macrophages. They further proposed that, following internalization, receptor–ligand complexes enter one of two functionally distinct compartments [49–51]. From one of these, receptor–ligand complexes appear to cycle back to the cell surface where dissociation of intact ligand may occur. From the other, ligand remains intracellularly and appears to be transferred to acid intracellular compartments where it is degraded.

An alternate possibility for the action of amines was suggested by experiments of Helenius et al [52], showing that certain enveloped virus–vesicle fusion processes, which are important to viral multiplication and normally take place in lysosomes, are pH-dependent and blocked by amines. This raises the possibility that amines might disrupt traffic of lysosomal enzymes by interfering with some pH-dependent vesicle–vesicle fusion process on which enzyme delivery and receptor reutilization depend. In any case, it is clear that amines divert most newly synthesized enzymes to the extracellular medium, strengthening the view that most enzyme segregation normally occurs via an intracellular, receptor-mediated process.

PROPERTIES OF PHOSPHOMANNOSYL ENZYME RECEPTORS ON CELL SURFACES AND MEMBRANES

We have [44, 53–55] proposed a model in which most newly synthesized lysosomal enzymes rely on the phosphomannosyl recognition marker to be segregated from other products of the endoplasmic reticulum. This model and the results on the effects of lysosomotropic amines discussed above, led to several pre-

dictions on the distribution of phosphomannosyl enzyme receptors in mammalian cells. These predictions included: 1) the presence of a large pool of internal receptors; 2) that most of these receptors must be located in the lumen of prelysosomal fractions; 3) that receptors present in prelysosomal fractions must be occupied by endogenous acid hydrolase, whereas receptors present in lysosomes should be free; and 4) that endogenous enzyme, bound to prelysosomal endomembranes and displaceable by Man-6-P, must contain the phosphomannosyl recognition marker and be more susceptible to phosphomannosyl-mediated pinocytosis than the large pool of free acid hydrolases located mainly in the lysosomal fraction.

Cell surface phosphomannose receptors were originally inferred by the selectivity, saturability, and specific Man-6-P inhibitability of acid hydrolase pinocytosis. Later, Rome et al [56, 57] directly demonstrated binding of α-L-iduronidase to fibroblasts that had been detached by trypsin and allowed to recover partially in suspension culture. They estimated 14,000 enzyme-binding sites per cell. Gonzalez-Noriega et al [44] studied direct binding of human spleen β-glucuronidase to attached fibroblasts and estimated 36,800 specific (Man-6-P-inhibitable) binding sites per cell. Both studies showed that the properties of enzyme-binding sites on the cell surface shared the specificity and kinetic properties previously described for the receptors that mediate adsorptive enzyme pinocytosis. Similar results have recently been described by Kaplan and Pannell [58] for fibroblast cell surface binding of α-mannosidase from secretions of Dictyostelium discoideum.

The receptor site for acid hydrolases in membranes prepared from human fibroblasts [59] shares many properties with the receptor site demonstrated [11–14, 44, 56, 57] in intact cells. β-glucuronidase from human spleen specifically binds to the fibroblast membrane receptors [59]. The binding is saturable with increasing enzyme concentration and is competitively inhibited by Man-6-P and the phosphomonoester phosphomannan fragment from H holstii, which inhibits enzyme pinocytosis by fibroblasts. Binding is specific for high-uptake forms of lysosomal enzymes, ie, prior treatment of the acid hydrolase with alkaline phosphatase and/or endoglycosidase H (which destroy or remove the phosphomannosyl recognition marker) greatly reduces enzyme binding. Divalent cation is not required for enzyme binding to the membrane receptors. Dissociation of bound enzyme from membranes receptors is very slow at neutral pH ($t_{1/2} \sim 11$ h), but is greatly accelerated in a concentration-dependent manner by the addition of mannose-6-phosphate, or by lowering the pH to levels comparable to intralysosomal pH. Finally, the amount of specific β-glucuronidase binding to fibroblast membrane receptors is linearly dependent on the amount of added membrane protein, and can be destroyed by pretreatment of the membranes with trypsin. Therefore, the enzyme binding properties of the membranes from disrupted fibroblasts [59] are similar in every respect tested to the properties of the enzyme binding sites implicated in adsorptive pinocytosis of lysosomal enzymes.

EVIDENCE THAT RECEPTORS MUST BE RECYCLED

Comparison of the maximum number of enzyme binding sites at the cell surface and the maximum velocity of adsorptive enzyme pinocytosis provided important information in understanding the role of the recognition marker and its receptor in enzyme transport. Gonzalez-Noriega et al [44] showed that the number

of enzyme molecules pinocytosed rapidly exceeds the maximum number of binding sites measured on the cell surface. Calculations based on the amount of enzyme maximally bound to the cell surface and the maximum rate of internalization established that the cell surface receptors must be replaced or reutilized approximately every 5 minutes. Similar results have been presented by Rome et al [56]. Moreover, a constant rate of enzyme uptake was obtained even when cells were incubated in the presence of cycloheximide for up to 3 hours [44]. Thus, synthesis of new binding sites is apparently not required for enzyme internalization during this time. This suggests that there is either a large internal pool of enzyme-binding sites containing at least 36 times the number present on the cell surface and/or that some of the cell surface sites are reutilized. Binding studies on both cell surfaces and total cell membranes of fibroblasts pretreated with or without trypsin to destroy binding sites at the cell surface indicate the presence of an internal pool of binding sites 4 times greater than that found at the cell surface [59]. The relationship of the large pool of enzyme-binding sites on intracellular membranes to those on the cell surface is not currently known. However, even if all of the internal receptors were in equilibrium with the cell-surface receptors and able to replace the cell-surface receptors internalized during enzyme pinocytosis, the total number of receptors (intracellular and cell surface) is insufficient to explain the kinetics of enzyme pinocytosis in the presence of cycloheximide without invoking reutilization of receptors following enzyme pinocytosis [44, 56, 59].

SUBCELLULAR AND ORGAN DISTRIBUTION OF PHOSPHOMANNOSYL ENZYME RECEPTORS

The finding that a majority (~ 80%) of phosphomannosyl enzyme receptors in fibroblasts are present on intracellular membranes is consistent with the hypothesis that these receptors play a role in regulating the intracellular transport of newly synthesized acid hydrolases, segregating these enzymes from other products of the endoplasmic reticulum, and directing them to lysosomes. Additional insight into the distribution and role of phosphomannosyl enzyme receptors was gained from studies on enzyme receptors in membranes from rat organs and in subcellular fractions of rat liver [60]. The binding of human fibroblast secretion β-hexosaminidase B to membranes prepared from rat liver was found to have properties identical to those described above for cell surface receptors of intact human fibroblasts and receptors on membranes from disrupted human fibroblasts [60]. Of the total phosphomannosyl enzyme receptors in rat liver, approximately 90% were found to be present in endoplasmic reticulum, Golgi apparatus, and lysosomes (80%, 7%, and 5% respectively), and only about 10% of the activity was found in the plasma membranes [60]. Binding activity in nuclei (0.2%) and mitochondria (1.3%) was negligible. The receptors in these fractions also displayed two different kinds of latency. The first was classical latency where binding sites are enclosed within vesicles and subsequently exposed by permeabilizing the vesicles with detergent. Binding of enzyme to rat liver fractions before and after detergent treatment thus showed that the phosphomannosyl enzyme receptor appeared to be on the inside surface of vesicles derived from the endoplasmic reticulum, lysosomes, and Golgi apparatus, and on the outer surface of vesicles and sheets derived from the plasma membrane. A second type of

latency was suggested by binding data on detergent-treated fractions that had been washed first with mannose-6-phosphate, a treatment that had been shown previously [44, 56, 59] to displace receptor-bound enzymes from membranes of human fibroblasts. A greater than 6-fold increase in binding of added enzyme was seen in liver total homogenates and endoplasmic reticulum fractions after the Man-6-P wash. The Golgi apparatus demonstrated about a doubling in specific binding after the same treatment, whereas there was only a slight increase in the binding to plasma membrane and lysosomes. These results suggested that more than 80% of the phosphomannosyl enzyme binding sites of the endoplasmic reticulum and about half of the binding sites in the Golgi apparatus were occupied by endogenous, Man-6-P displaceable ligand, whereas only 10% of the binding sites in the plasma membrane and lysosomes were so occupied. Thus, in these cell fractions, the intracellular receptors appeared to be occupied by endogenous enzymes and presented an occupancy gradient that ran downhill from endoplasmic reticulum to lysosomes.

Use of β-hexosaminidase derived from the subcellular rat liver fractions as a marker for endogenous rat acid hydrolases provided insight into the localization of endogenous enzyme in relation to vesicle membranes [60]. Greater than 80% of the endogenous enzyme in each subcellular fraction was sedimentable. Prior treatment with Man-6-P greatly reduced the amount of sedimentable enzyme in the plasma membrane (from 86% to 20%), but enzyme in the other fractions was largely unaffected, suggesting that most of the endogenous enzyme in plasma membrane was bound to the outer surface of vesicles whereas enzyme in the other fractions was enclosed in vesicles and not accessible to Man-6-P. Detergent treatment without added Man-6-P greatly reduced the percentage of sedimentable enzyme in the total homogenate and lysosomes (which comprise 80% of the enzyme in the total homogenate). About half of the enzyme in Golgi apparatus was also rendered nonsedimentable. A similar relationship of lysosomal acid hydrolases to endomembranes has previously been reported by Tsuji et al [61] following hypotonic shock of rat liver fractions, and by Goldstone et al [62, 63] following freezing and thawing of rat kidney fractions. The enzyme released by detergent alone is apparently vesicle-enclosed but not receptor-bound endogenous rat liver acid hydrolase. Finally, the combination of a detergent treatment to open vesicles with a Man-6-P treatment to free receptor-bound enzyme rendered most of the enzyme nonsedimentable in every subcellular fraction tested. Together these results are interpreted to mean that most of the endogenous acid hydrolases in endoplasmic reticulum, and about half of the enzyme in the Golgi apparatus, were specifically bound to receptors on the inner surface of vesicles.

The above results are compatible with the model that lysosomal enzymes are transported by membrane receptors in the endoplasmic reticulum, through the Golgi apparatus to lysosomes, where they are released [53, 59, 60]. Previous studies [21] suggested that the recognition marker is removed from acid hydrolases following their delivery to lysosomes. Thus, it was predicted that a total homogenate of rat liver might contain two qualitatively different types of endogenous enzyme: 1) a large fraction of enzyme in the lysosomes, not receptor-bound and releasable by treatment with detergent only; and 2) a small fraction of the total enzyme, representing newly synthesized enzyme enroute to lysosomes, receptor-bound, and displaceable by detergent in the presence of Man-6-P. Since this

latter enzyme is apparently bound to phosphomannosyl enzyme receptors, it was predicted that it should contain the phosphomannosyl-recognition marker for uptake and be susceptible to pinocytosis by fibroblasts [60]. After preparation of these two enzyme fractions, it was observed that the Man-6-P-inhibitable uptake of the "bound" enzyme was 45 times the rate of pinocytosis of the "soluble" enzyme, again consistent with predictions based on the view that intracellular receptors transport phosphomannosyl enzymes [60].

If the Man-6-P recognition marker and its receptor provide a general mechanism for delivery of acid hydrolases by lysosomes, one might expect that the pathway should not be restricted to one cell or tissue type and that phosphomannosyl enzyme binding activity is present in membranes prepared from homogenates of any cell or tissue type that uses this pathway for enzyme delivery. Indeed, phosphomannosyl enzyme binding activity with comparable affinities and with the properties of the phosphomannosyl enzyme receptor on human fibroblasts can be demonstrated in each of nine different rat and human organs tested, including testes, brain, kidney, spleen, liver, lung, and muscle [60]. These studies suggest that the phosphomannosyl enzyme receptor is widely distributed in rat and human tissues as predicted above. Recent observations by Freeze et al [64] that Dictyostelium discoideum secretes acid hydrolases that are recognized by human fibroblast phosphomannosyl enzyme receptors suggest that similar acid hydrolase transport pathways may be present as well in other eukaryotes besides mammals.

RECEPTOR SOLUBILIZATION AND PURIFICATION

Solubilization and purification of phosphomannosyl enzyme receptors should greatly aid the analysis of the way in which they are able to carry bound ligand to lysosomes. Sahagian et al [65, 66] have recently radiolabeled phosphomannosyl receptors from bovine liver. Membranes prepared by differential centrifugation from a total homogenate of bovine liver were solubilized with Triton X-100, incubated with high-uptake bovine testicular β-galactosidase, and precipitated with anti-β-galactosidase antibody [65, 66]. Material precipitated by antibody was then treated with Man-6-P, and the protein released by this treatment was radioactively labeled with ^{125}I. This labeled material was then used as a marker for large-scale preparations of bovine liver phosphomannosyl enzyme receptors by phosphomannan-Sepharose affinity chromatography. SDS-gels of the bovine receptor suggested a molecular weight of 215,000 [65, 66].

The phosphomannosyl enzyme receptor is an integral membrane protein; it is not displaced by changes in ionic strength (Fischer and Sly, unpublished), and requires detergent to be solubilized [65–67], (Fischer and Sly, unpublished). When soluble detergent–protein complexes are diluted below the detergent's critical micellar concentration, the detergent dissociates readily from the protein and dissolves in the aqueous phase, thus causing the proteins to aggregate [68–69]. This aggregation can be controlled in the presence of phosphatidylcholine vesicles so that the detergent-solubilized receptors retain their ability to bind acid hydrolases (Fischer and Sly, unpublished). Thus, we have developed an assay in which membranes are used as a source of receptor, the receptor is solubilized with detergent (Zwittergent 3-12), and the detergent is removed from the protein by dilution in

the presence of phosphatidylcholine liposomes. The aggregated protein, reconstituted into liposomes, is then sedimented after precipitation with 37.5% (final concentration) acetone. The precipitate can then be resuspended, incubated with acid hydrolase, and the bound enzyme separated from the free enzyme by centrifugation.

Using this approach, phosphomannosyl enzyme receptors have been successfully solubilized from human spleen membranes (Fischer and Sly, unpublished). Greater than 90% of the binding activity originally present in the membranes and solubilized by detergent can be recovered adsorbed to the phosphatidylcholine liposomes. Binding of β-hexosaminidase to the solubilized receptors is saturable, inhibitable by Man-6-P (but not Man-1-P or Glc-6-P), and shares the kinetics and specificity of membrane receptors. The binding assay is linear with receptor added and depends upon the presence of receptor in the liposomes; liposomes without added receptor do not bind the enzyme. A similar assay has recently been described by Steiner and Rome [67] for binding of α-L-iduronidase to receptors solubilized from Swarm rat chondrosarcoma cells and adsorbed onto liposomes composed of crude lecithin and stearylamine. The Triton X-100 solubilized receptors from these cells were purified to apparent homogeneity on a phosphomannan-Sepharose affinity resin [67] and comigrated on SDS-slab gels with the bovine liver receptor [65, 66].

BIOSYNTHESIS AND PROCESSING OF PHOSPHOMANNOSYL RECOGNITION MARKERS ON LYSOSOMAL ENZYMES

Recent studies on the biosynthesis of lysosomal acid hydrolases have greatly advanced understanding of the structure and processing of the phosphomannosyl recognition marker. Tabas and Kornfeld [70] demonstrated that ^{3}H-mannose-labeled oligosaccharides of β-glucuronidase in mouse lymphoma cells consist of "high-mannose-type" units in which mannose residues located at or near the nonreducing termini are linked by phosphodiester bonds to another moiety, α-N-acetylglucosamine. Varki and Kornfeld [36] then showed that phosphorylation can occur on at least five separate mannose residues and that individual molecules can contain up to three phosphate residues. Subsequent studies have suggested that acid hydrolases are phosphorylated by the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to 6-position hydroxyls on mannose residues of high mannose-type oligosaccharides on the enzymes [71, 72]. The UDP-N-acetylglucosamine: Glycoprotein N-acetylglucosamine-1-phosphotransferase responsible for this transfer has been shown to be deficient in fibroblasts from patients affected with I-cell disease (mucolipidosis II) and pseudo-Hurler polydystrophy (mucolipidosis III) [71–74]. The phosphate residues blocked by N-acetylglucosamine are initially resistant to phosphatase [36, 70, 75]. However, a second enzymatic activity in the biosynthesis and processing of the acid hydrolase phosphomannosyl recognition marker has been identified and partially purified. This enzyme hydrolyzes the phosphodiester bond, removing the blocking N-acetylglucosamine from the phosphate, and renders the phosphate sensitive to phosphatase [75–77].

What relation do the steps involved in biosynthesis of the phosphomannosyl

recognition marker, including the blocked phosphate residues and the phosphodiesterase activity that removes the blocking N-acetylglucosamine groups, have to the transport of newly synthesized acid hydrolases to lysosomes? The first step in the biosynthesis of the recognition marker is the cotranslational transfer of $(Glc)_3(Man)_9(GlcNac)_2$ from lipid-linked intermediates to nascent acid hydrolases entering the cisternal space of the endoplasmic reticulum [78]. This step is rapidly followed by removal of the terminal glucose trimer [78]. It is not clear, however, at what point phosphorylation of the high-mannose-type units on the acid hydrolases occurs. The results from our laboratory discussed above indicated the presence of phosphomannosyl enzyme receptors in the endoplasmic reticulum of rat liver and suggested that these receptors are occupied by endogenous acid hydrolases, which can be displaced by Man-6-P. This would imply that newly synthesized acid hydrolases may be phosphorylated shortly after synthesis in the endoplasmic reticulum or in a specialized compartment that sediments with the endoplasmic reticulum.

Other studies have shown by morphometric and marker enzyme analysis that the Golgi apparatus typically comprises only about 1% or less of the particulate protein in a total homogenate of rat liver [79, 80], and that enzyme activities with an exclusive or primary Golgi apparatus localization (eg, galactosyltransferase) should have specific activities in this subcellular fraction enriched 80–100-fold or more relative to the total homogenate [79, 80]. Subcellular fractionation studies of rat liver demonstrated that the α-N-acetylglucosaminyl phosphodiesterase described above is somewhat enriched in the Golgi apparatus [75, 76]. Varki and Kornfeld [75] have reported an enrichment of 20-fold for this enzyme in Golgi apparatus, whereas Waheed et al [76] reported enrichments of 19-fold in the Golgi apparatus (which was enriched 80-fold in galactosyltransferase), 1.7-fold in smooth microsomes, and 0.9-fold in the rough microsomes. Although the specific activity might appear low in the smooth and rough microsomes, those fractions actually were found to contain a significant amount of the total α-N-acetylglucosaminyl phosphodiesterase activity [76, 77]. Thus, the distribution of this enzyme appears similar, though not identical, to Golgi apparatus galactosyltransferase, but between a third and half of the total enzyme present in the homogenate is apparently in pre-Golgi apparatus membranes [76, 77]. Similarly, although the activity of the glycoprotein N-acetylglucosaminyl phosphotransferase was found to be enriched in Golgi apparatus membranes, this phosphorylating activity has been demonstrated to be present in significant levels in smooth and rough endoplasmic reticulum membranes as well [76].

These results suggest that phosphorylation and processing of the phosphomannosyl recognition marker on newly synthesized acid hydrolases may occur as early as in the endoplasmic reticulum, or as late as in the Golgi apparatus. As discussed above, endoplasmic reticulum fractions prepared from rat liver by the procedure of Morré [79, 80] contain phosphomannosyl enzyme receptors apparently occupied by endogenous rat acid hydrolases [60], suggesting that at least a fraction of newly synthesized enzyme obtains the phosphomannosyl recognition marker in this fraction shortly after synthesis. Endogenous ligand released by Man-6-P treatment of endoplasmic reticulum fractions prepared by the method of Sandberg et al [81] is a potent inhibitor of fibroblast secretion β-hexosaminidase binding to human spleen membranes (Grubb and Sly, unpublished), again sugges-

ting the possible presence of phosphorylated ligand as early as the endoplasmic reticulum.

The presence of the phosphomannosyl recognition marker and its receptor not only clearly influences the fate and compartmentalization of acid hydrolases, but may also influence the processing of the oligosaccharide chains on lysosomal enzymes. Many secretory glycoproteins contain "complex-type" [78] oligosaccharide chains. These chains are the end result of a series of processing steps on oligosaccharide chains which were initially high-mannose-type chains when transferred cotranslationally from lipid-linked intermediates to nascent glycoproteins entering the cisternal space of the endoplasmic reticulum [78]. These high-mannose-type oligosaccharide chains are first trimmed to smaller mannose-containing core oligosaccharide, and then built back up into complex-type chains by the action of several glycosyl transferases in the Golgi apparatus region, which add N-acetylglucosamine, galactose, and sialic acid to the oligosaccharide chains [78]. The Man-6-P recognition marker has been shown to be present on high-mannose-type oligosaccharide chains [30, 70]; ie, the Man-6-P bearing oligosaccharides have not been processed to complex-type oligosaccharides. However, I-cell enzymes, which lack the recognition marker, have been shown to possess oligosaccharide chains with features that suggest further processing to complex-type. Initially, excess sialic acid (the terminal sugar in complex-type oligosaccharide chains) was reported in I-cell secretion enzymes [82]. More recently, data on carbohydrate composition of I-cell enzymes revealed sugars found predominantly in complex-type oligosaccharide chains [83–85]. We have found that I-cell secretion enzyme is quantitatively retained on ricinus-communus-Sepharose columns, suggesting that the oligosaccharide chains contain galactose (a monosaccharide also found in chains that have been processed further) [54]. In contrast, only 10% of β-hexosaminidase secreted by normal fibroblasts (up to 30% of the enzyme secreted in the presence of amines) was specifically adsorbed to this galactose-recognizing lectin [54]. When the ricin adsorbed enzyme secreted by normal fibroblasts was eluted from ricin-Sepharose columns and tested for uptake, Man-6-P-inhibitable pinocytosis was observed. Treatment of the eluted enzyme with endoglycosidase H destroyed the suceptibility to pinocytosis of the enzyme, without reducing its ability to be rebound to ricin-Sepharose columns [86].

The above observations suggest that hexosaminidase secreted by normal cells can contain high-mannose-type, Man-6-P-bearing oligosaccharides (that are endoglycosidase H-sensitive), and galactose-containing oligosaccharides (that have been further processed to complex-type). This could be explained, if the presence of the Man-6-P moieties on normal lysosomal enzymes either directly, or by binding to the phosphomannosyl receptor, prevents further processing of the phosphomannosyl oligosaccharides into complex-type chains [54]. In this regard, it is known that an α-1,2-mannosidase is necessary for pruning the high-mannose-type oligosaccharide chains down to the core before building them back up into complex chains [78]. Goldberg and Kornfeld [87] have reported that the phosphorylated species of β-glucuronidase in mouse macrophages contain nine mannoses, but no glucose residues. These results suggest that phosphorylation of oligosaccharides on newly synthesized enzymes occurs after the action of glucosidases I and II after synthesis in the endoplasmic reticulum [78, 87], but before the action of the early Golgi apparatus α-1,2-mannosidase [87]. From these

observations, one can infer that phosphorylation of acid hydrolase oligosaccharides may occur prior to transport to the Golgi apparatus, or alternatively, in a specialized smooth membrane compartment such as GERL [88].

Reitman and Kornfeld [72] have demonstrated that thyroglobulin glycopeptides also act as acceptors in in vitro assays of the glycoprotein N-acetylglucosaminyl phosphotransferase, which synthesizes the phosphomannosyl recognition marker. Thus, at least in vitro, the transferase does not appear to have absolute specificity for the oligosaccharide units of acid hydrolases. Assuming that this enzyme is reponsible for the phosphorylation of the oligosaccharide units of acid hydrolases, there must be some mechanism that allows the enzyme to distinguish acid hydrolases from other glycoproteins that appear to have identical oligosaccharide units. Hasilik and Neufeld [88–91] have reported that newly synthesized acid hydrolases contain polypeptide chains larger than the subunits of purified tissue enzymes and that these precursors are trimmed to their eventual size over a period of hours or days [88–91]. One possibility is that the peptide fragments removed during proteolytic processing of lysosomal enzymes may serve as a second type of recognition marker, either for the transferase itself, or for directing the newly synthesized enzymes to a specialized compartment containing the transferase.

It is unclear at this time whether acid hydrolases containing phosphomannosyl residues blocked by α-N-acetylglucosamine can bind to the phosphomannosyl enzyme receptor. Endogenous β-hexosaminidase displaced by Man-6-P from a combined endoplasmic reticulum, lysosome, mitochondria fraction of rat liver prepared by the method of Leelevathi et al [92] has been found to demonstrate Man-6-P-inhibitable binding to phosphomannosyl enzyme receptors in membranes prepared from human spleen (Fischer and Sly, unpublished). However, when this same enzyme was assayed for susceptibility to pinocytosis by human fibroblasts, it had the properties of a low uptake enzyme (Fischer and Sly, unpublished). Pretreatment of this poorly pinocytosed enzyme with partially purified α-1-N-acetylglucosaminyl phosphodiesterase [75] enhanced its uptake 4–5-fold (Fischer and Sly, unpublished) and further suggested that the untreated enzyme, which apparently binds but is not taken up, contains blocked phosphate residues. Hasilik et al [71] have also reported that fibroblast secretion β-hexosaminidase, which had been pretreated with phosphatase to remove any unblocked phosphate residues, has the properties of a low-uptake enzyme unless it is also pretreated with phosphodiesterase to remove blocking N-acetylglucosamines. These results suggest that removal of the blocking GlcNac residues to expose phosphomonoesters of mannose is required to induce pinocytosis by phosphomannosyl enzyme receptors. It is thus possible that blocked phosphomannosyl groups can bind to receptors, but that removal of the phosphate is necessary to generate a high-affinity or high-energy interaction wih receptors leading to vesicularization and uptake. An analogous situation may be necessary to stimulate intracellular budding off of vesicles containing receptor-bound enzyme from the Golgi apparatus or GERL for transport to lysosomes. Previous results [31, 32] demonstrating that phosphodiester forms of phosphomannans can interact with phosphomannosyl enzyme receptors, but that this interaction is much stronger when groups blocking the phosphomannose moieties are removed, lends support to this view.

THE INTRACELLULAR PATHWAY FOR RECEPTOR-MEDIATED SEGREGATION AND TRANSPORT OF LYSOSOMAL ENZYMES: SUMMARY

Taken as a whole, the data to date support the hypothesis that the receptor phosphomannosyl recognition marker system that serves in the pinocytosis of exogenously added acid hydrolases also plays a role in delivery of endogenous enzymes to lysosomes by a direct intracellular route [44, 53–55, 59, 60]. As discussed earlier, the suggestion that free, secreted enzyme is a transport intermediate [22] seems to hold true for only a small fraction of the total enzyme pool [93]. This is demonstrated by 1) the lack of a precursor–product relationship between extracellular and intracellular enzyme in pulse chase experiments [89, 93], and 2) failure to deplete significantly intralysosomal enzyme levels by agents known to interfere with uptake, including Man-6-P [8, 53, 89] or immobilized antibodies [94]. It thus appears more likely that most newly synthesized acid hydrolases bind to phosphomannosyl enzyme receptors on endomembranes and are transported to lysosomes by vesicles which bud off from the endoplasmic reticulum or GERL [44, 53–55, 59, 60]. Alternatively, a fraction of the enzyme may travel, tightly bound to receptors, onto the plasma membrane and back into the cell by endocytosis [94]. There are certain similarities in both of these proposed pathways, including the binding of newly synthesized acid hydrolases to intracellular phosphomannosyl enzyme receptors, with segregation from other products of the endoplasmic reticulum. The major difference is that in one case vesicles containing receptor-bound enzyme go directly to lysosomes, whereas in the other, vesicles go first to the plasma membrane. One might infer that the fraction of enzyme that reaches the cell surface is not large because growth of cells in 10 mM Man-6-P, which displaces over 90% of bound enzyme from the cell surface in less than 10 minutes at 37°C [44], does not detectably deplete cells of intracellular enzyme [86]. However, if Man-6-P is significantly less effective in displacing the receptor bound biosynthetic transport intermediate than in displacing added high-uptake enzyme, or if the time that the receptor-bound intermediate spends on the cell surface en route to lysosomes is too brief to permit displacement by Man-6-P, the conclusions drawn from failure to deplete cells of enzyme by growth in the presence of competitive inhibitors could be incorrect. Resolution of the importance of a plasma membrane intermediate will require pulse-chase, autoradiographic, election microscopic, and subcellular fractionation and endomembrane flow kinetic studies.

In summary, the results presented here, in concert with results obtained in other laboratories, suggest a general model for the transport of newly synthesized acid hydrolases from the endoplasmic reticulum to lysosomes. Although the data to date are limited in their resolution of the precise localization of certain intracellular events by the resolution of the analytical techniques employed, and several important questions are still outstanding (such as how the cell decides which glycoproteins will receive the phosphomannosyl recognition marker), the natural life history of lysosomal enzymes (Fig. 1) and the general model for the role of phosphomannosyl-enzyme receptors in enzyme pinocytosis and in transport of acid hydrolases to lysosomes in mammalian cells may be summarized as follows: 1) translation of acid hydrolase mRNA on membrane-bound ribosomes in the

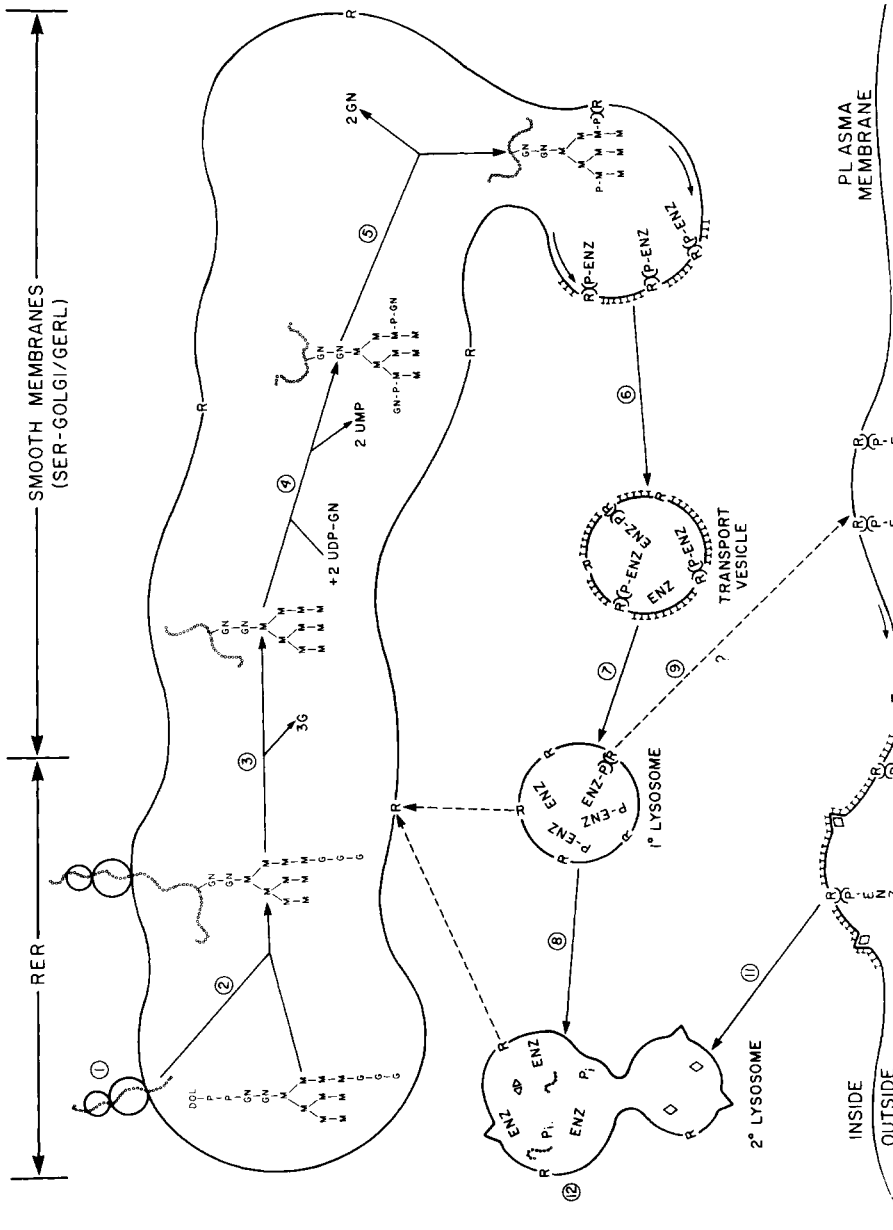

Fig. 1. Processing and transport of lysosomal acid hydrolases. For details, see text. (M, mannose; G, glucose; GN, N-acetylglucosamine; P, phosphate; ENZ, enzyme; R, phosphomannosyl receptor; DOL, dolichol).

rough endoplasmic reticulum; 2) cotranslational transfer of $(Glc)_3(Man)_9(GlcNac)_2$ from lipid-linked intermediates to asparagine residues of nascent acid hydrolase chains; 3) removal of glucose; 4) transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to the 6-position hydroxyl of from one to three mannose residues on the high-mannose type oligosaccharide chains; 5) release of blocking N-acetylglucosamine residues by phosphodiesterase to expose monoester phosphorylmannose groups creating a "high-uptake" enzyme form; 6) enzymes bound to intracellular phosphomannosyl-receptors collect into vesicles which bud off of the Golgi apparatus or GERL; 7) as the pH falls below pH 6 in primary lysosomes, enzymes dissociate from receptors, and free receptors can be reutilized; 8) once in lysosomes, acid phosphatase releases phosphate inactivating the recognition marker, and acid protease trims off excess polypeptide; 9) alternatively, some receptor bound enzyme may travel to the cell surface; 10) unbound enzyme or enzyme lacking the recognition marker (as in I-cell disease) is secreted; 11) some cell types may pinocytose secreted high-uptake acid hydrolases; 12) the secondary acid hydrolases participate in the degradation activities of the lysosome and die after prolonged exposure to their proteolytic neighbors.

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