Steps in the phosphorylation of the high mannose oligosaccharides of lysosomal enzymes

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Abstract The phosphomannosyl recognition marker of acid hydrolases, which mediates their translocation to lysosomes, has been shown to be synthesized in two steps. First, *N*-acetylglucosamine 1-phosphate is transferred to an acceptor mannose by UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase, resulting in a phosphate group in diester linkage between the outer *N*-acetylglucosamine and the inner mannose. Next, an α-*N*-acetylglucosaminyl phosphodiesterase removes the *N*-acetylglucosamine, leaving the phosphate in monoester linkage with the underlying mannose residue. This exposed phosphomannosyl residue serves as the essential component of a recognition marker which leads to binding to high-affinity receptors and subsequent translocation to lysosomes. We propose that the first enzyme in this scheme, *N*-acetylglucosaminylphosphotransferase, catalyses the initial, determining step by which newly synthesized acid hydrolases are distinguished from other newly synthesized glycoproteins and thus are eventually targeted to lysosomes. The absence of this enzyme activity, as in inclusion-cell (I-cell) disease and pseudo-Hurler polydystrophy, precludes the receptor-mediated targeting of newly synthesized acid hydrolases to lysosomes. As a consequence, the enzymes are secreted into the extracellular milieu.

It is now well established that adsorptive pinocytosis of lysosomal enzymes is mediated by phosphomannosyl residues on these enzymes (Kaplan et al 1977, Sando & Neufeld 1977, Kaplan et al 1978, Ullrich et al 1978, Natowicz et al 1979, Distler et al 1979, von Figura & Klein 1979, Bach et al 1979, Fischer et al 1980a, b, Hasilik & Neufeld 1980a, b, Gonzalez-Noriega et al 1980). These residues are the essential components of a recognition marker that is necessary for binding to high-affinity receptors on the cell surface and for

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subsequent translocation to lysosomes (Hickman et al 1974). Initially adsorptive pinocytosis was believed to be the major route by which newly synthesized acid hydrolases were delivered to lysosomes (Neufeld et al 1977). Recently Sly and co-workers have demonstrated that most of the receptor is located intracellularly rather than on the cell surface (Fischer et al 1980a). From this finding and other evidence, these investigators have proposed that the primary function of this receptor system is to mediate the intracellular transport of acid hydrolases from the Golgi to lysosomes (Sly & Stahl 1978, Sly et al 1981). According to this model adsorptive pinocytosis serves as an alternative route for enzyme delivery to lysosomes.

Our work has been concerned with two basic aspects of this pathway. First, we have investigated the biochemical steps in the phosphorylation of the mannose residues of lysosomal enzymes and, second, we have attempted to determine the basis by which the cell selectively phosphorylates the mannose residues of lysosomal enzymes. Our initial goal was to elucidate the structure of the phosphorylated oligosaccharide units present on lysosomal enzymes because these structures might provide clues about the biosynthetic steps in phosphorylation. We labelled cells in tissue culture with [2-^{3}H]mannose and then we isolated the lysosomal enzyme β-D-glucuronidase (EC 3.2.1.31) by immunoprecipitation. The [2-^{3}H]mannose-labelled oligosaccharide units of the enzyme were then purified and structurally analysed. This approach offered several advantages: it enabled us to determine the structure of the units by using extremely small amounts of material; and it allowed us to define the kinetics of phosphorylation in the intact cell. Previous workers had established that the initial glycosylation of asparagine-linked oligosaccharides occurs by the *en bloc* transfer of a high mannose-type oligosaccharide to the nascent polypeptide chain (Kiely et al 1976, Parodi & Leloir 1979). With the [2-^{3}H]mannose label one could therefore follow the fate of the oligosaccharide units of newly synthesized lysosomal enzymes as they proceeded from their site of synthesis in the rough endoplasmic reticulum to their final destination in the lysosomes.

When we analysed the phosphorylated oligosaccharide units of newly synthesized β-D-glucuronidase, we found that most of the phosphate groups were present in diester linkage between the sixth OH group of mannose residues in the underlying oligosaccharide and the first carbon of outer, α-linked *N*-acetylglucosamine residues (Tabas & Kornfeld 1980). A similar finding was also made by Hasilik et al 1980. Our studies of the structure of these unusual oligosaccharides (Varki & Kornfeld 1980b) showed that the phosphates are linked to five different mannose residues on the oligosaccharide, and that individual molecules may contain one or two phosphates, thus generating many isomers. A composite picture is shown in Fig. 1, where the asterisks identify the mannose residues that can be phosphorylated in the

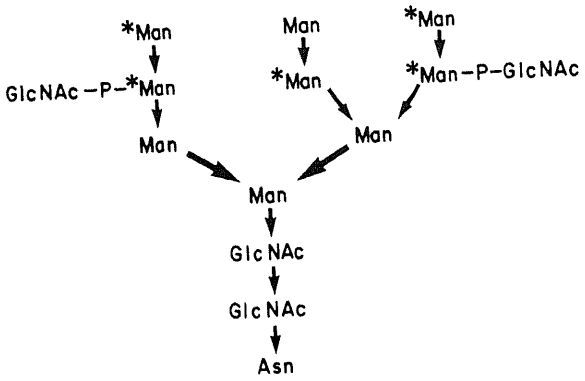

FIG. 1. Structure of a phosphorylated high mannose oligosaccharide that contains two phosphodiester units. The asterisks identify the mannose residues that are phosphorylated in various isomers (after Varki & Kornfeld 1980b).

various isomers. The most common sites for phosphorylation of oligosaccharides that have two diesters are also shown.

The finding of phosphodiester-linked moieties on the oligosaccharides of newly synthesized β-D-glucuronidase suggested the existence of a novel biosynthetic pathway, which is outlined in Fig. 2. In this scheme the

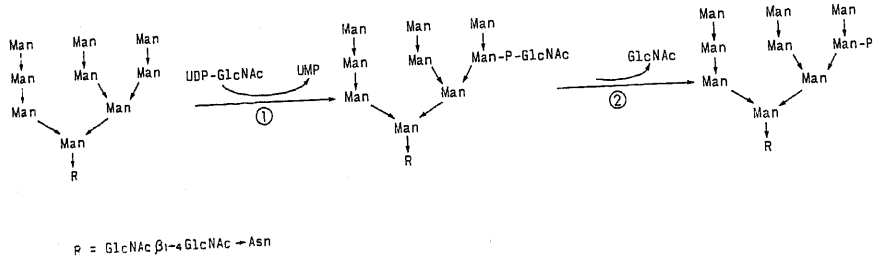

FIG. 2. Proposed mechanism for the phosphorylation of the high mannose units of acid hydrolases. The enzymes involved are (1) UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosaminyl-1-phosphotransferase and (2) α-*N*-acetylglucosaminyl phosphodiesterase (R = *N*-acetylglucosamine $\beta 1 \rightarrow 4$ *N*-acetylglucosamine $\rightarrow$ asparginine).

phosphomannosyl residues are synthesized in two steps. First, *N*-acetylglucosamine l-phosphate is transferred to an acceptor mannose to generate a phosphate group in diester linkage between the outer 'blocking' *N*-acetylglucosamine and the inner mannose. Next, the *N*-acetylglucosamine is removed, leaving the phosphate in monoester linkage to the underlying mannose residue. This mechanism is compatible with the finding that alkaline phosphatase-susceptible phosphomonesters are essential for high-affinity binding to the receptors (Kaplan et al 1977).

In order to prove that the diesters are actually converted to monoesters *in vivo* (i.e. by 'uncovering'), we examined the kinetics of the phosphorylation pathway in the murine macrophage line P388D$_1$ (Goldberg & Kornfeld 1981). Cells were incubated with [2-^{3}H]mannose for 15–20 min and then chased in unlabelled media for various times up to 5 h. β-D-Glucuronidase was immunoprecipitated and its oligosaccharide units were examined (for extent of phosphorylation and uncovering) by chromatography of the oligosaccharides on quaternary aminoethyl-Sephadex using an elution system that separates neutral oligosaccharides from oligosaccharides with one or two phosphomonoesters or phosphodiesters. The results are shown in Fig. 3. At the end of the 20-min labelling period most of the β-D-glucuronidase oligosaccharides were neutral; the only detectable phosphorylated species was a small amount of oligosaccharide with one covered phosphate. During the first 40 min of the chase, the amount of oligosaccharide with one covered phosphate greatly increased, and material having two covered phosphates became detectable, as did material with uncovered phosphates. The total extent of phosphorylation increased from 5% at the end of the pulse period to 20% after 40 min of chase and to 25% after 80 min of chase. As the chase proceeded, most of the phosphodiesters were converted to phosphomonoesters, as predicted by the scheme shown in Fig. 2. Since the total amount of labelled mannose in newly synthesized β-D-glucuronidase increased only slightly during the first 40 min of the chase period, the increase in the amount of phosphorylated species during this time must have resulted from phosphorylation of neutral high-mannose-type oligosaccharides transferred to the enzyme during the 20 min pulse. This finding demonstrates that phosphorylation is a post-translational event which takes place on protein-bound oligosaccharide and not on lipid-linked oligosaccharide precursors.

In our most recent experiments we have attempted to define the state of the oligosaccharides on the lysosomal enzymes when these molecules first bind to the high-affinity intracellular receptors. To approach this question we did a pulse-chase experiment similar to the one described above, but in this case we isolated a total cell-membrane fraction at each time point. This fraction, which contained the high-affinity receptor with its bound ligand, was treated with mannose 6-phosphate to release the bound lysosomal enzymes. The oligosaccharide units of the released glycoproteins were then analysed and compared to the phosphorylated oligosaccharides that remained in the soluble fraction of the cell. The results were striking. There was a tremendous enrichment in oligosaccharides with one and two phosphomonoesters in the material released from the receptor with mannose 6-phosphate. In contrast, most of the enzyme in the soluble fraction contained oligosaccharides with phosphodiester units. These data indicate that conversion of phosphodiesters to phosphomonoesters precedes binding to the intracellular receptors.

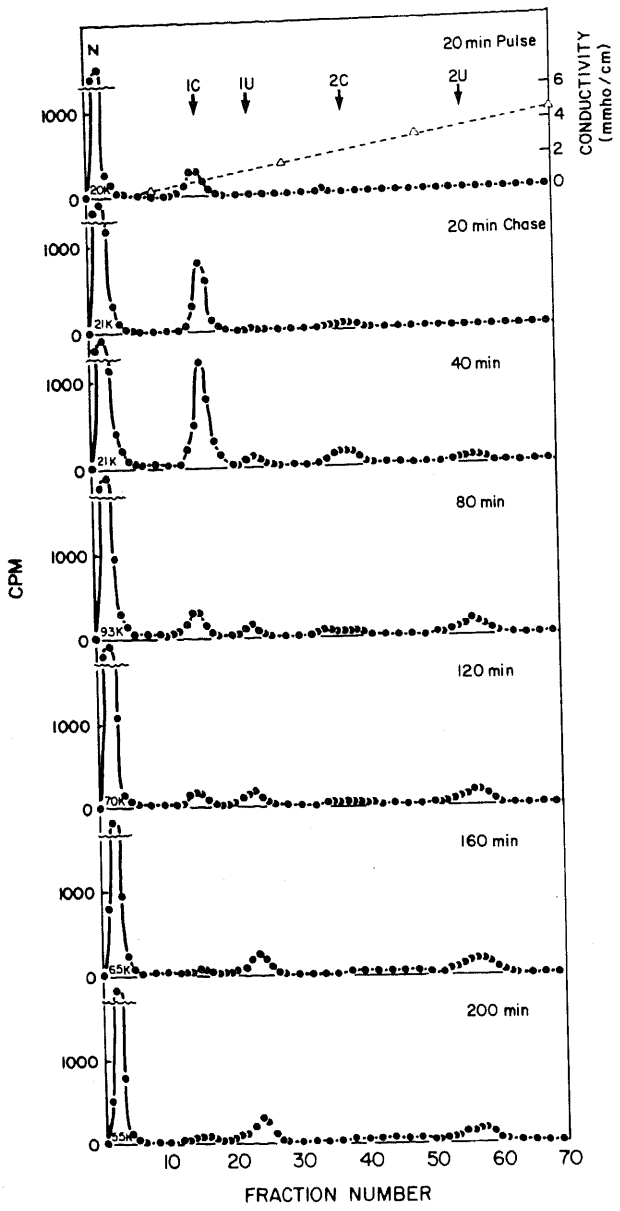

FIG. 3. Kinetics of phosphorylation of oligosaccharides in β-D-glucuronidase. Mouse P388D$_1$ cells were incubated with [2-^{3}H]mannose for 20 min and then chased in unlabelled media. At the indicated times β-D-glucuronidase was immunoprecipitated and its oligosaccharide units were isolated and fractionated on quaternary aminoethyl-Sephadex. The column was eluted with a

UDP-*N*-Acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase

As shown in Fig. 2, the proposed biosynthetic pathway requires two enzymes: an *N*-acetylglucosaminylphosphotransferase and an α-*N*-acetylglucosaminyl phosphodiesterase. Assays for each of these enzymes have been developed and the enzyme activities have been detected in a number of tissues. UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase activity has been detected in homogenates of Chinese hamster ovary cells, human diploid fibroblasts and rat liver (Reitman & Kornfeld 1981a, Hasilik et al 1981). Using [β-^{32}P]UDP-[^{3}H]*N*-acetylglucosamine as donor, we showed that the enzyme transfers *N*-acetylglucosamine 1-phosphate to the sixth OH group of mannose residues in high mannose-type oligosaccharides of lysosomal enzymes. The enzyme is not inhibited by tunicamycin or stimulated by dolichol phosphate, indicating that the reaction does not proceed via a dolichol-pyrophosphoryl-*N*-acetylglucosamine intermediate. Fibroblasts from patients with inclusion-cell (I-cell) disease (mucolipidosis II) and pseudo-Hurler polydystrophy (mucolipidosis III) are severely deficient in this enzyme activity (Hasilik et al 1981, Reitman et al 1981, Varki et al 1981). These autosomal recessive storage diseases are characterized by a general failure to target acid hydrolases to lysosomes in spite of normal rates of acid hydrolase synthesis (Hickman & Neufeld 1972) and by the failure to incorporate [^{32}P]phosphate into newly synthesized acid hydrolases (Hasilik & Neufeld 1980b, Bach et al 1979). The absence of the *N*-acetylglucosaminylphosphotransferase explains the lack of phosphorylation of the newly synthesized acid hydrolases. This failure to generate the phosphomannosyl recognition signal precludes the receptor-mediated targeting of the newly synthesized acid hydrolases to lysosomes, and consequently the enzymes are secreted into the extracellular milieu.

One of the most intriguing questions about this system concerns the basis for the specificity of the selective phosphorylation of the oligosaccharide units of lysosomal enzymes. If we assume, as the data indicate, that lysosomal enzymes and non-lysosomal glycoproteins are each glycosylated with the identical oligosaccharide precursor, then we must explain why only the lysosomal enzymes are phosphorylated. The simplest explanation is that all

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linear gradient of pyridinium acetate, pH 5.3, from 2 mM to 500 mM. The neutral oligosaccharide peaks are cut off at the top; total counts per min (cpm) are given under each peak. The abbreviations are: N, neutral oligosaccharide standard; 1C, oligosaccharide standard containing one covered phosphate; 1U, standard containing one uncovered phosphate; 2C and 2U, standards containing two covered and two uncovered phosphates, respectively (from Goldberg & Kornfeld 1981).

lysosomal enzymes share a common protein recognition site or marker and that this marker causes one of two possible effects. First, it could lead to the segregation of newly synthesized lysosomal enzymes into a specialized subcellular compartment which contains the *N*-acetylglucosaminylphosphotransferase. In this case the transferase would not have to recognize lysosomal enzymes specifically but, rather, it would phosphorylate the oligosaccharide units of any glycoprotein in the compartment. The other possibility is that the *N*-acetylglucosaminylphosphotransferase is able to distinguish between lysosomal and non-lysosomal enzymes on the basis of this common protein

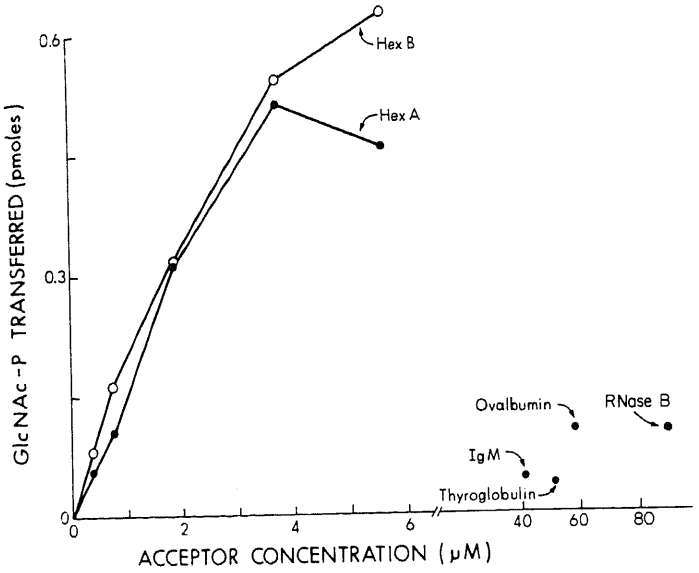

FIG. 4. Activity of *N*-acetylglucosaminylphosphotransferase towards various glycoproteins containing high mannose-type oligosaccharides. The two lysosomal enzyme acceptors were human placental β-hexosaminidase A and B (after Reitman & Kornfeld 1981b).

recognition marker. To evaluate this, we partially purified the *N*-acetylglucosaminylphosphotransferase from rat liver and determined its ability to phosphorylate the oligosaccharide units of a series of lysosomal and non-lysosomal glycoproteins (Reitman & Kornfeld 1981b). Fig. 4 shows the results of a typical experiment using glycoprotein acceptors that have in common the presence of high mannose-type oligosaccharide units. It is evident that the lysosomal enzymes β-hexosaminidase A and B are the best acceptors, being active at very low protein concentrations. In contrast, none of the four non-lysosomal glycoproteins is a good acceptor. The data in Table 1 show some of the kinetic parameters of the enzyme towards various

acceptors. The apparent K_m values for the three lysosomal enzymes are in the low micromolar range whereas the K_m for ribonuclease B is approximately 100-fold greater. The acceptor activity of the other non-lysosomal glycoproteins was so low that it was not possible to determine their apparent K_m and V_{max} values. Table 1 also presents the kinetic parameters for two carbohydrate acceptors, α-methylmannoside and Man_{5-8}*N*-acetylglucosamine oligosaccharide. These molecules have very high apparent K_m values, being 10^3–10^4-fold greater than those of lysosomal enzymes. Their V_{max} values are 10–100-fold greater than those of the glycoprotein acceptors. The relative catalytic efficiency (V_{max} divided by apparent K_m) of the transferase towards the various acceptors is also shown in Table 1. The three lysosomal enzymes are phosphorylated at least 100-fold more efficiently than either ribonuclease B, Man_{5-8}*N*-acetylglucosamine oligosaccharide or α-methylmannoside. This preference of the *N*-acetylglucosaminylphosphotransferase for lysosomal enzymes in these *in vitro* assays demonstrates the remarkable specificity of a glycosyltransferase for the class of glycoproteins on which it acts *in vivo*.

TABLE 1 Kinetic parameters of *N*-acetylglucosaminylphosphotransferase activity towards various acceptors

	Apparent K_m mM	V_{max}[a]	*Relative acceptor activity*[b]
Human placental β-hexosaminidase A	0.020	251	163
Human placental β-hexosaminidase B	0.006	270	610
Porcine hepatic α-N-acetylglucosaminidase	0.009	181	258
Bovine pancreatic ribonuclease B	0.9	116	1.6
Man_{5-8}*N*-acetylglucosamine oligosaccharide	32	3103	1.2
α-Methylmannoside	113	8940	1.0

[a]pmol *N*-acetylglucosamine 1-phosphate transferred $h^{-1}mg^{-1}$ enzyme protein. [b]The relative acceptor activity is defined as the V_{max} divided by the apparent K_m, normalized to α-methylmannoside (after Reitman & Kornfeld 1981b).

To probe further the substrate specificity of the *N*-acetylglucosaminylphosphotransferase, we denatured the lysosomal enzymes by heat and then used them as acceptors. In all instances the denatured enzyme lost its ability to serve as an acceptor of *N*-acetylglucosamine 1-phosphate even though the protein remained in solution. Although the loss or modification of some heat-labile moiety cannot be ruled out, we believe these results strongly suggest that the conformation of the acceptor protein is recognized by

N-acetylglucosaminylphosphotransferase. In other words, the *N*-acetylglucosaminylphosphotransferase appears to be specific for a particular protein conformation that is unique to lysosomal enzymes. The nature of this conformation requirement is obscure at present. Since both ribonuclease B (with an exposed oligosaccharide unit [Baynes & Wold 1976, Tarentino et al 1974]) and free high mannose-type oligosaccharides are poor acceptors, it is unlikely that accessibility of the carbohydrate chain to the transferase is the sole conformational requirement for recognition.

The specificity of the *N*-acetylglucosaminylphosphotransferase towards lysosomal enzymes makes it unnecessary to postulate a mechanism for the specific segregation of newly synthesized acid hydrolases which would precede exposure to the *N*-acetylglucosaminylphosphotransferase. From these data we propose that the *N*-acetylglucosaminylphosphotransferase is the initial and determining enzyme for the pathway that eventually results in the segregation of acid hydrolases into lysosomes.

α-*N*-Acetylglucosaminylphosphodiesterase

An α-*N*-acetylglucosaminylphosphodiesterase capable of removing the 'covering' *N*-acetylglucosamine residues has been purified from rat liver (Varki & Kornfeld 1981) and human placenta (Waheed et al 1981a). The enzyme is distinct from a previously described lysosomal α-*N*-acetylglucosaminidase, which can also remove covering *N*-acetylglucosamine residues (Varki & Kornfeld 1980a, Waheed et al 1981b), and it is active against *N*-acetylglucosamine residues that are α-linked to an underlying phosphate, although it is inactive against *p*-nitrophenyl α-*N*-acetylglucosaminide. Furthermore, the activity is inhibited by PO_4 ions. Recently we have obtained evidence that the enzymic reaction may proceed by a glycosidase mechanism rather than by a true phosphodiesterase mechanism. We are pursuing this further to determine if a change in the nomenclature of the enzyme is appropriate.

Subcellular localization of *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminylphosphodiesterase

Both the *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminylphosphodiesterase have been localized to Golgi-enriched smooth membranes (Waheed et al 1981b, Varki & Kornfeld 1980a). We have succeeded in separating both activities from the trans Golgi marker galactosyltransferase by subjecting mouse lymphoma and $P388D_1$ macrophage membranes to

centrifugation on a continuous sucrose density gradient. The precise location of these two enzyme activities is not known.

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DISCUSSION

Sabatini: Are the substrates that you use enzymes that are recovered from secretory fluids and not from lysosomes?

Kornfeld: They are from various sources: some are from tissues; some are from secretions.

Sabatini: Would the enzyme that has reached the lysosomes, lost the phosphate and, in most cases, also lost a peptide piece, be a substrate?

Kornfeld: Yes. The enzymes that we use as acceptors have lost their pro-piece, so this piece evidently does not contain the information necessary for oligosaccharide phosphorylation. It is possible, however, that if the pro-piece were still present, the enzymes might be even better substrates.

Sabatini: Although the enzymes that have lost the peptide can be phosphorylated because they have a recognition segment for phosphorylation, is it possible that they cannot be taken to the lysosomes because they have lost the piece for address to the lysosomes?

Kornfeld: We have not phosphorylated a molecule *in vitro* and shown that it is taken up by cells and targeted to lysosomes. However, all the studies of Dr Sly and others in this field, on acid hydrolase uptake and targeting, utilize enzymes that have lost their pro-piece. Yet these enzymes go into lysosomes. Furthermore, in I-cell disease, the only known defect is the lack of phosphorylation, and in fibroblasts, at least, targeting of newly synthesized acid hydrolases to lysosomes does not occur.

Sabatini: But in the liver cells is the requirement for phosphomannose an open question?

Kornfeld: Yes.

Sly: We have some data on isolated oligosaccharides which agree with your findings on the binding to the receptor (K. Creek & W. Sly, unpublished observations). The diphosphorylated oligosaccharide itself is subject to pinocytosis and is delivered to lysosomes. In fact, a single Endo-H-released oligosaccharide with two phosphates as monoesters is endocytosed very efficiently by fibroblasts and is delivered to lysosomes. The signal for targeting is thus present in a single oligosaccharide.

Sabatini: So the signal is not only necessary for uptake but is also sufficient for delivery to lysosomes?

Sly: I don't know if we can say that. Although the phosphorylated oligosaccharide by itself is delivered to lysosomes this doesn't rule out the possibility that a portion of the polypeptide could also contribute to binding.

Sabatini: The observation shows only that the mature enzyme could reach the liver lysosome by pinocytosis; it doesn't show that it would reach the liver lysosomes by the intracellular route, which may require the peptide.

Dean: Have you looked at a variety of other hydrolytic enzymes as possible substrates? And do you have evidence for any late processing which is specific to secretion in the $P388D_1$ cells?

Kornfeld: We haven't studied any non-lysosomal hydrolases except ribonuclease B. We are finding some unusual oligosaccharides with hybrid-type

structures on the secreted lysosomal enzymes. I don't think that there are any unique steps; it is just a matter of the phosphate changing the normal sequence of processing by the usual processing enzymes.

Jourdian: What is the nature of the hybrid molecules?

Kornfeld: The phosphorylated hybrid oligosaccharides have features of both high mannose and complex-type molecules. The phosphate is present on the high mannose portion and presumably prevents mannose processing. On the other side of the molecule we find the sequence: sialic acid to galactose to *N*-acetylglucosamine, which is typical of a complex-type molecule.

Jourdian: Are the hybrid oligosaccharides susceptible to the action of endohexosaminidase H?

Kornfeld: Some are.

Jourdian: We find that the greatest affinity or avidity of binding of β-galactosidase fractions to the phosphomannosyl receptor is for those that contain hybrid-type oligosaccharides.

Baker: In general terms, to what extent is protein synthesis, followed by post-translational modification, relevant to making membranes recycle? To what extent are these processes generating any part of the 'motor' for recycling? Or is membrane recycling driven totally independently? Is it correct to say that there is a membrane 'conveyor belt' operating independently of the nature and quantity of molecules loaded onto it?

Kornfeld: I don't think that glycosylation is at all concerned with membrane recycling. In certain circumstances one can block glycosylation with tunicamycin and show that normal cell growth occurs or, with certain viruses, that normal virus assembly takes place. I would consider that the glycosylation reactions serve to put recognition tags on proteins for targeting and for influencing their physical properties, but these reactions do not directly affect membrane recycling.

Baker: Is protein synthesis driving the system at all?

Palade: The present evidence indicates that a conveyor belt operates continuously, and probably at a constant rate, between the endoplasmic reticulum and the Golgi complex (Jamieson & Palade 1967) and between the Golgi complex and the lysosomes (Friend & Farquhar 1967); you also mentioned this in your paper, Dr Kornfeld. The rate of movement from the Golgi complex to the cell surface and from the surface to lysosome, however, can be regulated in many cells. To the extent so far tested, these activities do not depend on sustained protein synthesis (Jamieson & Palade 1968).

Kornfeld: There is plenty of evidence that receptors migrate from the surface internally and back out again in the presence of inhibitors of protein synthesis (see also p 109-119).

Palade: The conveyor belts operate continuously and some of them can be regulated over very large ranges, from a high level, as in secretagogue-

stimulated exocytosis, to a basic level, as for miniature potentials recorded at resting neuromuscular junctions (Katz 1969).

Bretscher: If β-glucuronidase has three high mannose oligosaccharide chains, and if any one of them can get phosphorylated, does that imply that the protein has three recognition sites for the enzyme that puts on the phospho-*N*-acetylglucosamine?

Kornfeld: Perhaps it does. All three of the oligosaccharide units of β-glucuronidase may be phosphorylated. It could be that there is a recognition marker for each glycosylation site.

Bretscher: Perhaps this would make it a very restrictive sequence.

Rothman: Have you analysed the products of the enzyme incubations to find out which oligosaccharides are present, and the rates at which they are glycosylated?

Kornfeld: We have not tried that, but hope to do so.

Goldstein: Assuming that you found a mutation that destroys the activity of the phosphodiesterase or uncovering enzyme, what cellular fate would you predict for the uncovered lysosomal enzymes? Would they be secreted extracellularly?

Kornfeld: Oligosaccharides with the uncovered phosphates clearly bind with much higher affinity to the receptor than do species with the covered phosphates, but that doesn't mean that molecules with covered phosphates have no affinity for the receptor. Most of the lysosomal enzymes are tetramers: they have an average of 12 oligosaccharides per molecule. This means that there could be a very large number of diesters on one molecule which might produce a high enough affinity to allow some of the enzyme molecules to bind to the receptor. This could explain why the lack of the uncovering enzyme might not be associated with a clinically apparent disease. In assays of about 50 fibroblast cell lines from patients with I-cell disease and pseudo-Hurler polydystrophy, we have not found any line that is deficient in uncovering activity. Every line we have tested has had a defect in the phosphorylating enzyme.

Sly: We have done some uptake experiments on oligosaccharides with covered phosphate to see if they are taken up (K. Creek & W. Sly, unpublished observations). The results agree with what you say. Although they are taken up, the uptake is very low, with about 30-fold less efficiency than the uptake for those with two uncovered phosphates. However, if one had a multivalent ligand, containing several groups that bind with low affinity, it might still be segregated. This is certainly true for low density lipoprotein (LDL) substituted with many pentamannosyl monophosphate groups. LDL with one such group has low affinity, but LDL with 40 such groups binds 1000-fold better, and is taken up very efficiently.

Goldstein: Dr Sly, you proposed several years ago that the function of

lysosomal acid phosphatase was to remove the mannose 6-phosphate from lysosomal enzymes, thereby trapping the enzymes within lysosomes. Do you still believe that?

Sly: Yes. I believe that lysosomal acid phosphatase cleaves the phosphate from the recognition marker, and this forms a trap for delivered enzyme in the lysosome.

Sabatini: In I-cell disease do the non-phosphorylated enzymes become terminally glycosylated?

Kornfeld: Yes.

Sabatini: In that case, terminal glycosylation is probably normally prevented by the phosphate. Do you agree?

Kornfeld: I think that is true.

Sabatini: This then further supports the idea that the phosphate must be added at a site that precedes the one where terminal glycosylation takes place.

Kornfeld: Yes it does.

Cohn: What are the rates of synthesis of some of these enzymes in I-cell disease compared with normal cells?

Kornfeld: The studies of Vladutiu & Rattazzi (1979) would indicate that the rate of lysosomal enzyme synthesis in I-cell disease is normal.

Cohn: Has this been done carefully with specific antibodies against the enzyme?

Kornfeld: No; the studies have not been done using antibodies. These investigators followed the increase in total enzyme activity of growing fibroblasts and found that I-cell fibroblasts appear to make the same amount of enzyme as normal fibroblasts but secrete most of it into the medium.

Geisow: Do you know whether tunicamycin causes secretion of newly synthesized lysosomal enzymes? Since tunicamycin blocks addition of carbohydrate, the enzymes might be released.

Kornfeld: We haven't done those experiments with lysosomal enzymes but one would predict that tunicamycin should cause secretion.

Sabatini: We have recently done that and found that tunicamycin causes massive secretion of completely unprocessed lysosomal polypeptides (Rosenfeld et al 1982).

Geisow: It has been found in many systems, although not exclusively, that blocking glycosylation by tunicamycin or 2-deoxyglucose causes proteolysis to occur in non-lysosomal secretory proteins. Could this be because failure of a recognition step causes the newly synthesized acid hydrolases to enter a secretory pathway, rather than to be picked up and taken to the lysosome?

Kornfeld: Possibly, although the pH in the secretory vesicles would not be optimal for lysosomal proteases. The susceptibility of non-glycosylated glycoproteins to proteolysis appears to be mainly a function of the particular

protein involved. The results obtained with tunicamycin are extremely variable depending on the system studied. Even within the same system the results may be variable. For instance, in one strain of vesicular stomatitis virus (VSV), tunicamycin totally blocks the migration of the G protein to the surface because the protein aggregates, whereas with another variant of VSV the G protein is expressed normally on the cell surface in the presence of tunicamycin (Gibson et al 1979). It appears that each individual glycoprotein has its own requirement for glycosylation in terms of its ability to maintain its normal physical properties.

Geisow: In some cases, however, the oligosaccharide chain is added to a large molecule, so one would imagine that there was minimal perturbation of conformation of that molecule.

Sly: What is your tentative assignment of the processing steps and the binding to receptors? Do you believe that the phosphotransferase is a late endoplasmic reticulum (ER) enzyme?

Kornfeld: Our studies of enzyme localization utilize membranes separated on the basis of density, and we cannot extrapolate from these experiments to the morphological compartment without other evidence. The *N*-acetylglucosaminylphosphotransferase could be in the transitional zone of the ER or in the cis portion of the Golgi. I don't think it is in the trans Golgi.

Sly: Where do you think the receptors are occupied?

Kornfeld: We don't know for sure, but our kinetic data are consistent with the molecules going onto the receptor when they reach the trans Golgi.

Hubbard: How does the receptor distribute in that gradient?

Kornfeld: We are just starting to do those experiments.

Sabatini: I would interpret the findings of Dr Sly to mean that the receptor is mainly in microsomes derived from the ER. If that were true then, teleologically, I would expect the phosphate to be uncovered in the ER.

Sly: We have some fractionation data which suggested that the bulk of the receptor was in the ER, or at least sedimented with the ER, and that that receptor was occupied (Fischer et al 1980). If uncovering of the phosphate doesn't occur until the Golgi, and uncovering is required to bind to receptors, I believe that our interpretation of those experiments may be wrong, and perhaps lysosomes contaminated the fraction that we called ER. I hope that Dr Kornfeld's fractionation work will provide an answer to this question, but it is not clear yet.

Kornfeld: Phosphate uncovering doesn't occur until 30–40 min after the protein has been made, so if the receptors are in the ER the protein has to be sitting in the ER all this time. In addition it appears that mannose trimming also occurs before the molecules go onto the receptor. Since our data indicate that the 'uncovering' enzyme and the processing α-mannosidase are localized

in the Golgi elements, it seems unlikely that the newly synthesized lysosomal enzymes go onto the receptor in the ER.

Sabatini: Have you looked at the effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on these processes?

Kornfeld: We are just starting to do that.

Jourdian: Binding of oligosaccharides, or glycoproteins containing mannose 6-phosphate blocked with *N*-acetylglucosamine, to receptors is extremely poor. If one chemically synthesizes D-mannopyranose-6-(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate) one finds that it is a very poor inhibitor of binding. These results suggest that removal of the *N*-acetylglucosamine residue is required for binding.

Rothman: Dr Kornfeld said that he isolated 'hybrid' molecules from secretions and it has just been suggested that hybrids are better substrates for binding. Are such hybrid oligosaccharides found in lysosomes; and are they found in larger amounts in secretions than other phosphorylated forms that lack terminal sugars?

Kornfeld: We have looked carefully for these species only in secretions. We are now starting to look in lysosomes, but that is more difficult because as soon as the molecules reach the lysosome the phosphate is removed. One therefore cannot accumulate these species in lysosomes and then purify them.

Sly: Then why is the hybrid binding better?

Jourdian: It seems to bind better than the isozymic forms that carry mannose-rich chains which contain exposed mannose 6-phosphate residues.

Sly: Is it better than the binding of oligosaccharides that contain two uncovered phosphates?

Jourdian: I can't answer that.

Kornfeld: What are you comparing it with?

Jourdian: We have eluted highly purified enzyme from diethylaminoethyl cellulose (DEAE) with increasing concentrations of sodium chloride, and we have arbitrarily divided the eluted enzyme into fractions that exhibit low, intermediate, high and very high uptake when added exogenously to cultures of skin fibroblasts (Sahagian et al 1981). We compared the carbohydrate content of the low and intermediate fractions to that of the very high-uptake fractions and found that the oligosaccharide portion of the very high-uptake fractions released with endohexosaminidase H contained increased concentrations of sialic acid and galactose (J. Distler et al, unpublished work).

Kornfeld: But you are not determining which oligosaccharide on the molecules is responsible for the high-uptake behaviour. Those same molecules might also have species with two uncovered phosphates.

Jourdian: That is possible.

Cohn: Does the secreted molecule often have phosphate associated with it?

Kornfeld: Yes.

Cohn: Do you think, then, that the secreted molecule never 'sees' the lysosome and that it goes by a separate intracellular pathway?

Kornfeld: That probably varies with the cell line being studied. We have recently made an observation that seems to complicate the issue considerably. We have found that the mouse P388D_1 cells do not have detectable mannose 6-phosphate binding protein, and while they secrete half the enzyme that they make, they retain the other half, presumably in lysosomes, although we haven't isolated lysosomes from these cells. In other cell types that *do* have detectable receptor, very little enzyme is secreted. In addition, in I-cell disease several cell types seem to have normal levels of lysosomal enzymes even though the phosphorylating enzyme is absent. So there may be alternative ways of getting acid hydrolases into lysosomes, including pathways that are independent of the phosphomannosyl recognition marker. We are starting to screen a variety of cell types to determine which of them have mannose 6-phosphate receptor and how that correlates with secretion.

Sabatini: Randy Schekman has worked with yeast cells, which contain a vacuole that may be equivalent to a lysosome, because it contains acid hydrolases which I believe bear phosphate groups linked to mannoses. He has characterized many interesting mutants that are unable to secrete proteins at the cell surface (Novick et al 1981, Esmon et al 1981). He has found that secretory mutants are still capable of segregating carboxypeptidase Y to the vacuole. This suggests that passage of this hydrolase to the cell surface is not required for it to reach lysosomes. In addition, I understand that there are mutant yeasts which are unable to add the phosphate and yet the enzyme still reaches the vacuoles.

Kornfeld: Do you know if those vacuoles contain only lysosomal enzymes?

Sabatini: I don't know, and one could question whether they can be regarded as equivalent to lysosomes.

Palade: How long is the interval between the time of enzyme synthesis and the time of arrival of the same enzyme in the lysosomes?

Kornfeld: We have not directly traced the enzymes into lysosomes. The best marker we have of arrival in lysosomes is the loss of phosphate from the oligosaccharides. If one accepts that the loss occurs in the lysosomes, then it takes approximately one hour for newly synthesized enzyme to reach the lysosomes. This is consistent with the studies of Skudlarek & Swank (1981) who showed that the loss of the pro-piece from newly synthesized enzyme begins at about one hour.

Sabatini: But that evidence applies only to β-glucuronidase (Rosenfeld et al 1982). For other enzymes it takes hours and hours to completely remove the pro-pieces.

Palade: The evidence for rapid transit along the secretory pathway is obtained from certain cell types that produce secretory proteins and discharge

them continuously into the extracellular medium. Such proteins begin to appear within the medium after considerably shorter times (15–20 min). Therefore, lysosomal enzymes must be contained and delayed in some compartment (perhaps the Golgi) which is different from the compartments involved in the secretory traffic. When exactly is the G protein acylated?

Kornfeld: I don't know for this cell line.

Rothman: In Chinese hamster ovary cells fatty acid is added almost precisely 10 min after synthesis. Our studies (reported in my paper, p 120-137) indicate that at least two glucose residues are removed before the fatty acid is added, but that the fatty acid is added before any mannoses are removed.

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