*Original Article*

# Modifications of lysosomal enzymes in *Dictyostelium discoideum*

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## Abstract

This paper has two purposes. The first is to review the past studies on the structure, biosynthesis, and immunological properties of a class of glycoproteins, the lysosomal enzymes, in *Dictyostelium discoideum.* The second purpose is to present new data on the analysis of mutant strains altered in the biosynthesis of the lipid-linked precursor of N-linked oligosaccharides, and on the characterization of new carbohydrate antigenic determinants found on multiple proteins in *Dictyostelium.* We will also show how a combination of genetic, biochemical and immunochemical approaches have been used to unravel a portion of the glycosylation pathway in *Dictyostelium.*

The long-term goal of these studies is to use *Dictyostelium discoideum* as a model system to understand the functions of a variety of glycoconjugates in a multicellular organism. The existence of a large number of mutant strains which are altered in a variety of cellular functions, development and the posttranslational modification of multiple proteins, offers a great opportunity to explore this area.

## Introduction

Our understanding of the structure and the biosynthesis of complex glycans in mammalian cells has increased greatly during the last decade (1–3). Many general principles of biosynthesis and a large number of related structures have been described (1, 3). Moreover, as analytical methods have improved, new structures, unusual linkages and entirely new sugars are being reported frequently (4–7). The expression of many of these glycan structures is often cell, tissue, or developmental stage specific (8–13). In fact, it is generally assumed and often reported that the complex carbohydrates have great potential to encode a wealth of biological specificity at all levels of complexity and development. Yet, the current approaches have had limited success in demonstrating that these structures carry highly specific information. There are very few concrete examples which show that a specific arrangement of sugars performs a specific biological function which is of positive benefit to the organism. The most notable examples of demonstrated specificity are: the targeting of newly synthesized mammalian lysosomal enzymes to the lysosomes by the interaction of phosphorylated N-linked oligosaccharides with the phosphomannosyl receptor (14, 15); and, the roles of oligosaccharins in plant development and defense (16, 17). It would be surprising if these diverse examples of carbohydrate specificity were unique among biological systems. If others do exist, what approaches can be used to demonstrate them and to evaluate their physiological importance?

One of the most powerful approaches used to understand the biosynthesis of complex molecules and their mechanisms of regulation is the analysis of mutants which are altered in various proteins, processes, or functions. This type of approach has been invaluable in dissecting the pathway of mam-

malian N-linked oligosaccharide biosynthesis using lectin resistant tissue culture cell lines (18–21). Many of these lines lack individual sugar residues or even entire classes of oligosaccharides, and yet such mutations are not lethal, at least under laboratory conditions. This observation suggests that many of the crucial functions of glycoconjugates may occur at the multicellular level. Unfortunately, cloned mammalian tissue culture cell lines cannot develop into multicellular organisms. Therefore, it may be necessary to analyze mutants of multicellular organisms which are altered in the synthesis of the glycoconjugates. Detecting and analyzing such mutations might be difficult since they could be lethal. On the other hand, an organism, which has an optional multicellular stage might have significant advantages as a model system.

The eukaryotic amoeba *Dictyostelium discoideum* can serve as an appropriate model system for this study because it has two major advantages over many other systems currently under investigation. The first advantage is that a population of genetically homogeneous cells can be induced to undergo a non-obligatory, synchronous multicellular development (22, 23). The developing organism exhibits many properties typical of multicellular organisms: at least three types of cell-cell adhesion systems (24), finely regulated tissue proportioning, positional information, along with temporally and spatially regulated expression of at least 200 proteins (25). During development the majority of amoeba differentiate into cellulose encased spores that crown a tapering stalk composed of evacuolated cells. Each of the cell types is morphologically, functionally, and biochemically distinct with each type expressing a series of unique gene products not found in the other (25). The occurrence of vegetative and developmental states permits the analysis of the glycoconjugates at either the single- or multicellular stages.

The second advantage of *Dictyostelium* as a model system is that it is a haploid organism. This makes it relatively easy to produce and screen for mutant strains which are altered in any number of physiological and developmental processes, including the biosynthesis and function of the glycocconjugates. Many mutant strains have already been isolated in a number of individual enzymes, cellular proteins and in physiological processes. In work from this laboratory several of them have been found to be defective in the biosynthesis and processing of N-linked oligosaccharides, as will be discussed below.

Most of the available information about the glycoconjugates of *Dictyostelium* is confined to glycoproteins. Limited studies have also been done on glycolipids (26–29) and on the complex glycans of the surface sheath and the structural components of the stalk and spore cells (30–36). The current discussion will focus on glycoproteins with N-linked oligosaccharides, but it should be emphasized that the composition, structure, and the function of the extracellular matrices is nearly completely unknown. The future study of these complex glycans may be extremely rewarding.

## General features of protein glycosylation in Dictyostelium

Essentially all studies of the oligosaccharides of *Dictyostelium* glycoproteins are concerned with the N-linked oligosaccharides, although there is some evidence for the presence of the O-linked variety (6, 37, 38). In *Dictyostelium*, the N-linked oligosaccharides are composed of residues of GlcNAc, Man, and Fuc (39). Phosphoryl dolichol-like derivatives of Man and Glc have been described and are thought to act as intermediates in the biosynthesis of the N-linked oligosaccharides (26–28). Protein glycosylation occurs cotranslationally by *enbloc* transfer of a $Glc_3Man_9GlcNAc_2$ oligosaccharide from a dolichol-like lipid precursor similar to that described for many eukaryotic systems (1, 2). However, it should be pointed out that the specific structure of the lipid-linked precursor has not been proven in *Dictyostelium*. The oligosaccharide is processed following its transfer to Asn residues on the proteins by the sequential removal of the three Glc residues by a set of two neutral pH, membrane associated glucosidases found in the rough endoplasmic reticulum (40). Glucosidase I appears to cleave the terminal $\alpha$-1,2 linked residue and $\alpha$-Glucosidase II removes the 2 $\alpha$-1,3-linked residues of Glc (40). Further processing of the oligosaccharides by removal of Man residues is minimal in vegetative cells. There is no evidence for the presence of either sialic acids or the multi-aentennary complex-type chains found in higher organisms.

Some of the *Dictyostelium* oligosaccharides do undergo extensive modification by the addition of phosphate, sulfate, methylesters (41–44) and peripheral GlcNAc or Fuc (45) residues to selected sugar residues.

During development the pattern of glycosylation changes dramatically (39, 45, 46). The N-linked oligosaccharides are still derived from the lipid-linked precursor, but many of them are highly processed by the removal of a large portion of the Man residues (39). Phosphorylation and sulfation decrease (46–48). These changes in the glycosylation of many proteins may be important in the appearance and disappearance of various carbohydrate antigens which are associated with different stages of development (48–50).

Thus, *Dictyostelium* appears to synthesize N-linked oligosaccharides which are derived from structures typical of those made by many eukaryotic cells, but many of the types of processing and modifications are different than those typical of well characterized mammalian cells.

Two glycoprotein systems have been intensively studied biochemically, genetically and immunochemically. These are: 1) the lysosomal enzymes and, 2) the plasma membrane glycoproteins which may mediate cell adhesion during development. The remainder of this discussion will focus on the lysosomal enzymes and their carbohydrate modifications.

## Structure and biosynthesis of lysosomal enzymes

### Historical perspectives

At least eight lysosomal enzymes are developmentally regulated in *Dictyostelium,* and they are often used as markers of different developmental stages (22). Their expression requires new protein synthesis and prior RNA synthesis. A variety of mutant strains altered in the temporal or morphological features of development also show altered accumulation of the various enzymes (22). In an attempt to understand the functional roles of these regulated proteins, strains which carried mutations in the structural gene of several different enzymes were analyzed to determine the effects on either vegetative growth or development. The surprising result was that the great majority of the mutations in several different enzymes showed only minor effects on the kinetics or morphology of development (22, 23). Thus, it would appear that the highly regulated enzymes do not play crucial roles in successful development under laboratory conditions.

The lysosomal enzymes of *Dictyostelium* are glycoproteins which bind to Concanavalin A (43, 51). They have much received attention recently since a variety of studies suggested that the oligosaccharides might be involved in developmental regulation and cell-type specific expression of the various enzymes, and on the expression of unusual antigenic determinants shared by multiple lysosomal enzymes. Furthermore, the discovery of Man-6-P in the lysosomal enzymes of *Dictyostelium* hinted that the intracellular targeting of the newly synthesized enzymes might also depend on carbohydrate modifications similar to those in mammalian cells (41).

Since a large number of mutant strains showed altered accumulation of one or more of the lysosomal enzymes it appeared that common post-translational steps might be affected in these mutants.

### Biosynthesis and targeting of lysosomal enzymes

### General features of mammalian cells

The biosynthesis of lysosomal enzymes in mammalian tissue culture cell lines has been intensively studied during the last decade. The general approach involves pulse labeling cells with an appropriate precursor, usually [$^{35}$S]methionine, followed by a chase in the absence of label. The enzyme is then precipitated by a specific antibody and analyzed by SDS-PAGE combined with flourography (52, 53). The general conclusions which have emerged from these studies are that lysosomal enzymes contain signal sequences and are synthesized on membrane bound polysomes. The proteins are cotranslationally glycosylated with N-linked oligosaccharides, discharged into the endoplasmic reticulum where the signal sequence is cleaved and the enzymes are routed from the RER through the Golgi to the lysosome. The lysosomal enzymes are synthesized as larger precursors and then proteolytically cleaved into mature-sized subunits either just before or after arrival at the lysosome.

The N-linked oligosaccharides are also processed during the transit of the lysosomal enzymes through the Golgi. Following their arrival in the Golgi for further processing, the path of the lysosomal enzymes diverges from that of other glycoproteins. They are targeted to the lysosome by a set of reactions specific to lysosomal enzymes. The presence of a sequence of amino acids common to lysosomal enzymes allows them to interact with a binding site on another protein, UDP-GlcNAc:lysosomal enzyme, GlcNAc-1-P transferase (54). The enzyme catalyzes the transfer of GlcNAc-1-P from UDP-GlcNAc to the 6-position of selected Man residues on the oligosaccharide. This forms an acid-labile phosphodiester, GlcNAc-P-Man. The GlcNAc residue is rapidly cleaved by a specific $\alpha$-N-acetylglucosaminidase to yield a phosphomonoester of Man-6-P (14, 54). The oligosaccharide on the lysosomal enzyme binds to a receptor in the Golgi and the enzyme is transported to the lysosome.

This mechanism of lysosomal enzyme targeting occurs in several types of mammalian cells, but it is clear that there is at least one additional targeting mechanism. Some cell types do not phosphorylate the lysosomal enzymes and yet they still localize them correctly. The nature of this other mechanism is not known (55, 56). In one case, glucoscerebrosidase, the oligosaccharide is not phosphorylated and the enzyme remains firmly bound to the membrane during its transit to the lysosome. Even after arrival in the lysosome the protein remains membrane associated, in contrast to most lysosomal enzymes which are soluble (57).

*Dictyostelium*

The biosynthesis and targeting one lysosomal enzyme, $\alpha$-mannosidase, has been studied in detail in *Dictyostelium*. The protein is first synthesized as a 120KDa precursor, co-translationally N-glycosylated at 9−10 sites, and eventually proteolytically cleaved into 58KDa and 60KDa mature subunits (58−60). The mature enzyme is found as a tetramer of about 235KDa. It is not clear in which cellular compartment the proteolytic cleavage occurs, but some evidence has suggested that it may occur prior to its arrival in the lysosome (60). The majority of the oligosaccharides are highly modified by the addition of sulfate, phosphate and phosphomethylesters (41, 44, 61), and probably

peripheral GlcNAc residues. Another enzyme $\beta$-Glucosidase has been less extensively studied. It is synthesized as a precursor of 105KDa and is cleaved into a mature form of 100KDa (62). It is also modified by the addition of sulfate and phosphate (43).

The discovery of Man-6-P in the N-linked oligosaccharides of these enzymes suggested that they might be routed to the lysosome by a phosphomannosyl receptor system. *Dictyostelium* contains a GlcNAc-1-P transferase which will carry out the transfer of this group from UDP-GlcNAc to an artificial exogenous acceptor $\alpha$-methyl-mannoside, much as the mammalian transferase will do (63). However, the *Dictyostelium* transferase does not possess the binding site specifically required for the interaction with lysosomal enzymes, and cannot phosphorylate most mammalian lysosomal enzymes (64). It is not known if the transferase recognizes *Dictyostelium* lysosomal enzymes. In addition, *Dictyostelium* does not contain the $\alpha$-N-acetylglucosaminidase needed to cleave the GlcNAc-P-Man phosphodiester (64). Several groups have unsuccessfully tried to identify a phosphomannosyl receptor in *Dictyostelium* (66). Thus, all of the available enzymological studies at the present time do not support the existence of the typical Man-6-P targeting system known for mammalian cells. So, how are the Man-6-P residues made and what function do they serve?

Part of the answer may lie in the fact that the Man-6-P residues of *Dictyostelium* are not found as phosphomonoesters but instead exist in an unusual acid-stable phosphodiester, methylphosphomannose, Man-6-P-OCH$_3$ (44). The mammalian GlCNac-P-Man diester is extremely acid-labile, (0.02 M HCl, 100°, 20 min) but strong acid hydrolysis (1 N HCl, 100°, 4 hrs) is needed to completely hydrolyze Man-6-P-OCH$_3$ to Man-6-P and MeOH (65). The pathway of biosynthesis is not known, but the -CH$_3$ group is derived from S-adenosylmethionine and the (phospho?)methylation appears to occur in the Golgi (65). Several mechanisms are theoretically possible: 1) a two-step reaction using an oligosaccharide specific kinase, followed by the methylation of the phosphomonoester formed in the first step; 2) a two-step reaction involving a short-lived intermediate diester such as GlcNAc-P-Man followed by the S-adenosylmethionine dependent displacement of the first diester formed or; 3) a single step transfer

of P-OCH$_3$ from a high energy donor. There is no evidence at present which eliminates any of these possibilities.

Recent studies by Meirendorf *et al.*, show that the precursor of $\alpha$-mannosidase is firmly bound to the membrane until it is cleaved into the mature subunits (58). Also, Wood and Kaplan have presented evidence recently that the proteolytic cleavage may occur in or near the Golgi and not in the lysosome (60). The membrane associated precursor cannot be removed from the membrane by low pH or elution with high salt, chaotrophic salts, Man-6-P or dextran sulfate. The failure of Man-6-P to solubilize the precursor suggests that it is not bound to a phosphomannosyl receptor while similar treatments solubilize lysosomal enzymes bound to the phosphomannosyl receptor in mammalian cells (67, 68). These results obviously do not offer support in favor of the involvement of the Man-6-P in the targeting of the lysosomal enzymes in *Dictyostelium*. Again it must be sized that there is evidence for the presence of Man-6-P-OCH$_3$, not Man-6-P in the lysosomal enzymes of this organism. The important question of the mechanism of lysosomal enzyme targeting is still open.

The lysosomal enzymes are secreted during vegetative growth in axenic medium and during development (69). There are two independent secretion pathways. One involves the simultaneous release of many acid hydrolases by what appears to be the fusion of intact lysosomes with the plasma membrane. The other mechanism involves the constituitive secretion of the precursors (of $\alpha$-mannosidase and $\beta$-glucosidase) without first passing through the lysosomal compartment (58, 60). It is not known whether this second type of secretion has any functional significance or whether it simply reflects the inefficiency of the targeting system. A large number of mutant strains have been isolated which are altered in the secretion process (70). Many of the mutants undersecrete the enzymes while some oversecrete the unprocessed precursors (71).

In summary, the general features of the synthesis *Dictyostelium* lysosomal enzymes are much like those of mammalian cells; however, the phosphorylation of the oligosaccharides and the mechanism of targetting of these enzymes to the lysosome appear to be different. At the present time, the in-

volvement of the N-linked oligosaccharides in targeting has not been demonstrated.

*Analysis of sulfated oligosaccharides*

The lysosomal enzymes are also modified by the addition of sulfate esters to the oligosaccharides. The presence of sulfate presents some problems for the structural analysis of the oligosaccharides because sulfate blocks the release of the majority of them by Endo H digestion. In fact, only about 25% of the oligosaccharides derived from a pool of three purified lysosomal enzymes can be released from the glycopeptides, but all can be released after the sulfate is specifically removed by solvolysis (43). More recently, another endoglycosidase prepraration, Endo/PNGaseF, has been used to release the majority of the fully sulfated oligosaccharides from the intact proteins (Fig. 1). This has permitted an analysis of the types of sulfate esters and their location on the sugar ring (6).

The identification and quantitation of sulfated sugars presents some formidable problems. First, harsh chemical treatments such as methylation analysis or hydrazinolysis which are routinely used for the structural analysis of oligosaccharides lead to the loss of certain types of sulfate esters (71a, 72). Second, there are no known sulfatases which cleave sulfate esters from intact oligosaccharides. Finally, the presence of the sulfate esters can block oligosaccharide digestion by exo-glycosidases even when sulfate is found on a neighboring sugar residue (73). The identification of sulfated sugars has been achieved by partial acid hydrolysis followed by chromatography (74). Since the stability of the sulfate ester bond is usually less than the majority of the glycosidic linkages, most of the sulfate is lost during acid hydrolysis. This makes accurate quantitation of the sulfated sugar impossible.

Two approaches were successfully used to characterize and ultimately identify the sulfated sugar residues in the *Dictyostelium* oligosaccharides. The first approach was to measure the kinetics of acid catalyzed hydrolysis and the second was to assess the sensitivity of the sulfate esters to base hydrolysis. The kinetics of acid hydrolysis of sulfate esters depend on their orientation on the ring structure (75). Sulfates linked to primary hydroxyl groups are relatively stable ($t_{1/2} = 90-120$ min in 0.25 N HCl at 100°C), while those in axial orienta-
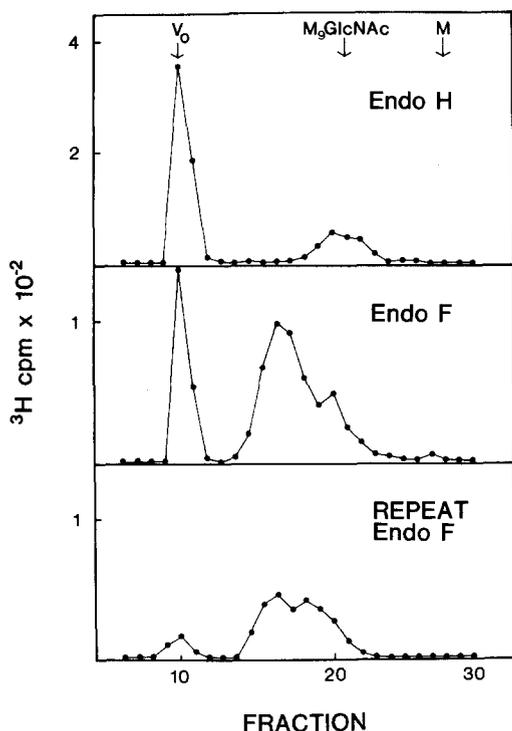
*Fig. 1.* Sephadex G-50 analysis of endoglycosidase digestions of [³H] Man labeled glycosidases from *D. discoideum.* A mixture of three purified glycosidases was denatured and digested with Endo H or withEndo/PNGaseF (16). The digest was then chromatographed on Sephadex G-50, and samples counted for radioactivity. The material remaining in the $V_o$ after digestion was precipitated with acetone, retreated with Endo/PNGaseF and analyzed again on Sephadex G-50. The Endo H resistant material was also treated with Endo H again, but no further release occurred. The $V_o$ was determined with bovine serum albumin, and the $V_{100}$ with Man and Man$_9$GlcNAc showed the elution position of the oligosaccharide.

tion are less stable ($t_{1/2} = 60 - 84$ min), and finally those in equatorial linkage are labile ($t_{1/2} = 6 - 25$ min). N-sulfate esters are extremely acid labile, being completely destroyed within an hour in 0.04 N HCl at 100 °C (76). The half-lives of all the sulfate esters are relatively indifferent to the presence of other substitutions on the ring.

Some sulfate esters are also sensitive to base hydrolysis, but only in two cases: when the sulfate is located in the 6-position with an unsubstituted 3-OH position (or vice versa); and when there is an unsubstituted -OH group trans to the sulfate ester (77, 78). This means that base hydrolysis is dependent on the presence of other substitutions on the

sugar residue. In the first case, the release of the sulfate group is accompanied by the formation of a 3,6 anhydro sugar (Fig. 2). The ring strain labilizes the glycosidic linkage to mild-acid hydrolysis and will result in preferential cleavage of the oligosaccharide at that point (79). Complete acid hydrolysis of the base treated oligosaccharides leads to the formation of the free 3,6 anhydrosugar. In the second case, as shown in Fig. 3, base causes epoxide formation, and subsequent acid hydrolysis accompanied by Walden inversion results in the formation of equal amounts of the original desulfated parent sugar and an appropriate epimer of the parent sugar. For instance, when either Man-3-or -4-SO$_4$ is treated with base and then hydrolyzed, idose is formed. The appearance of this sugar is diagnostic of the present of one or both of these sulfate esters. Precise identification of the sulfated sugar is possible if the expoxide derivative is treated with sodium methoxide as shown in Fig. 3.

These approaches were used to study [³⁵S]-labeled oligosaccharides prepared from secreted glycoconjugates by digestion with Endo/PNGaseF preparations. The great majority of the sulfate was released by acid hydrolysis with kinetics expected for esters located on primary hydroxyl groups. Furthermore, most of this material was also sensitive to base hydrolysis, suggesting that it occurred on a residue that was unsubstituted at the 3-OH position. The possibilities were only Man-6-SO$_4$ and GlcNAc-6-SO$_4$. Man-6-SO$_4$ was identified as [³H] 3,6 anhydromannitol and quantified by HPLC analysis of the biosynthetically [³H]Man labeled oligosaccharides in a variety of fractions. This is the first report of Man-6-SO$_4$ in a biological system (6). By comparison with other structural and compositional data, the results showed that this residue could account for most of the sulfate found in the released oligosaccharides. Subsequently, this residue was also found to account for all of the sulfate in the major oligosacharide released from purified α-mannosidase or a mixture of three purified lysosomal enzymes (61).

The function of the sulfate residues is not known. It may be important for protection of the protein against proteolysis. It is clear that the sulfated sugars play a crucial role in defining an antigenic determinant found in multiple proteins in *Dictyostelium* (see below).
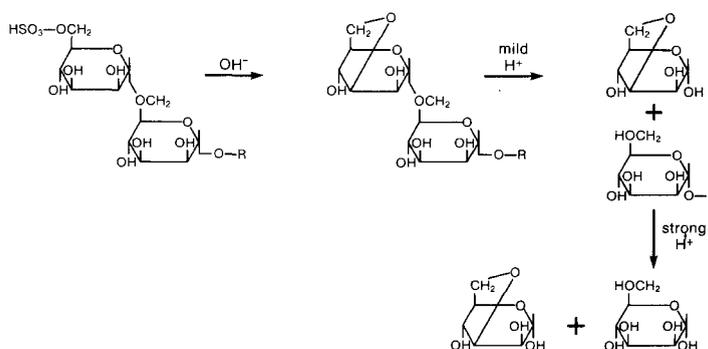
*Fig. 2.* Formation of 3,6 anhydromannose from Man-6-SO$_4$. The reactions involved in the formation of 3,6 anhydromannose from Man-6-SO$_4$ by treatment with base are shown. Mild acid hydrolysis of the base-treated material preferentially cleaves the oligosaccharide at the glycosidic linkage involving the anhydrosugar. Strong acid hydrolysis cleaves all the glycosidic linkages, but does not destroy the 3,6 anhydro sugar.

## Other modifications of lysosomal enzymes

Since a large portion of the lysosomal enzymes are secreted during vegetative growth, the secretions have been used as a convenient source of lysosomal enzymes. Analysis of the [$^3$H]GlcN labeled oligosaccharides shows the presence of GlcNAc residues beyond the chitobiose core (Couso and Kornfeld, manuscript in preparation; Freeze, unpublished observations) (Fig. 4). Based on nuclear
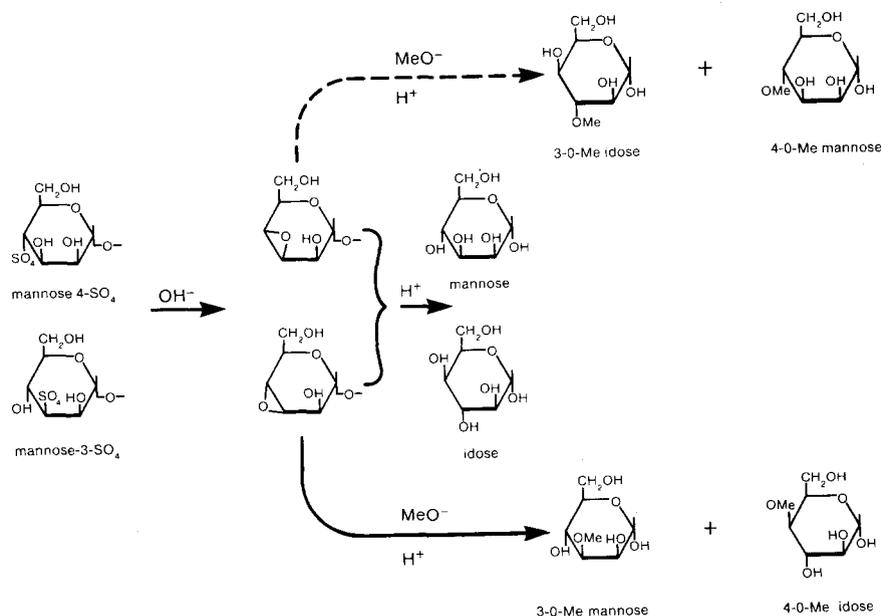


*Fig. 3.* Identification of sulfated sugars following epoxide formation. Sulfate esters are sensitive to base when they are adjacent to a trans hydroxyl group. In this example, Man-3-SO$_4$ and Man-4-SO$_4$ both have the appropriate structures required for the sulfate to be base-sensitive. Both generate 3,4 epoxide rings within the sugar ring. Strong acid hydrolysis of either one leads to the formation of equal amounts of Man and Idose (Id). Thus, the appearance of Id is diagnostic of the presence of one or both of the sulfated sugars. Positive identification of which sugar sulfate is present can be accomplished by sodium methoxide treatment of the base-treated material followed by acid hydrolysis. Each of the original sulfated sugars will produce a characteristic set of products, as shown.

magnetic resonance studies by Kornfeld and Couso, the residues appear to occupy the bisecting position on the 3,6 di-substituted, branched Man residues. As shown in Fig. 4 the GlcNAc residue could be at either positions A or B. The results of Couso and Kornfeld show that the majority of the substitution is at position 'B'. This is the first report of a bisecting GlcNAc at this position. All previous studies on oligosaccharides from mammalian cells have found this type of substitution at only position A (80).

At least two lysosomal enzymes are known to contain residues of GlcNAc-P-Serine (81), which are thought to be derived from the transfer of GlcNAc-1-P from UDP-GlcNAc to the hydroxyl group of serine residues in the polypeptide chain. The linkage is labile to mild-acid hydrolysis yielding GlcNAc and phosphoserine. Base treatment gives GlcNAc-1-P and serine. It is not known if this substituent is permanent once it is added to the protein. Like the other modifications, the function is unknown.

Some of the neutral N-linked oligosaccharides from total cellular secretions also appear to contain fucose. However, carbohydrate compositions of purified lysosomal enzymes do not show the presence of appreciable amounts of fucose (82).

## Immunochemical studies of the lysosomal enzymes

### Discovery of shared determinants

By the mid 1970s β-glucosidase, β-N-acetylglucosaminidase, and α-mannosidase had all been purified to homogeneity and mutants in the structural gene had been isolated for each of the proteins (22, 23). Antisera were prepared against each of several purified native or denatured enzymes, or their individual subunits excised from polyacrylamide gels. Surprisingly, each antiserum precipitated at least seven other lysosomal enzyme activities (83, 84). While none of the proteins shared any common subunits, the antibodies might recognize a shared sequence on the proteins perhaps involved in lysosomal enzyme targeting. This, however, proved unlikely since the antigen recognition was sensitive to periodate oxidation, but not to proteolytic digestion. Careful examination of the antibody preparations by preabsorption with any

of several different antigens showed that each of the antigens could remove all of the antibodies which recognized the group of enzymes. Furthermore, the antibody titration curves of all of the enzymes were identical. The conclusion was that the proteins shared a common determinant(s) and that it was likely to be a highly immunogenic carbohydrate.
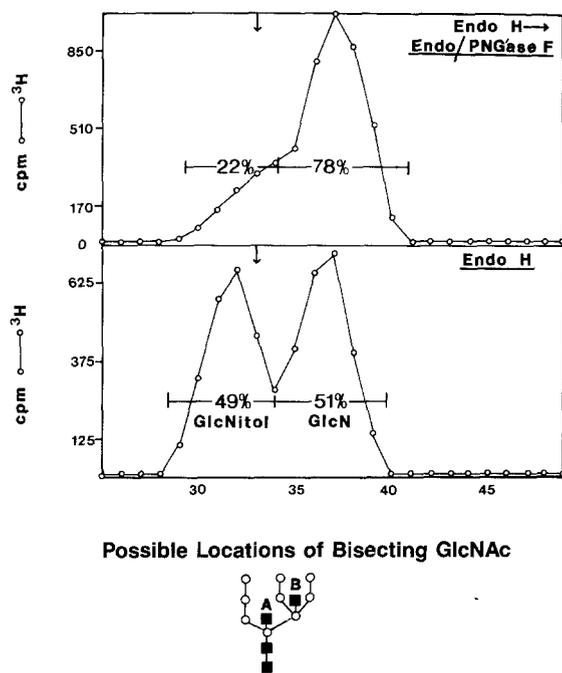


Possible Locations of Bisecting GlcNAc

Fig. 4. Analysis of acid hydrolysates of [³H]GlcN labeled oligosaccharides released by endoglycosidase digestions. (A) Oligosaccharides from secreted glycoproteins were metabolically labeled with [³H]GlcN and released by digestion with Endo H. The released oligosaccharides were separated from those which were not cleaved. The intact resistant proteins were then treated with Endo/PNGaseF and the released oligossaccharides were isolated (Endo H – – – > Endo/PNGaseF). Each of the pools of the intact released oligosaccharides was then reduced to generate a single N-acetylglucosaminitol residue per oligosaccharide. This was followed by total acid hydrolysis to yield glucosaminitol (from the terminal GlcN) or glucosamine from any non-reduced, non-terminal residue. The hydrolysates were then chromatographed on paper and the radioactivity determined in 1 cm sections of each strip. The presence of radioactivity in the reduced and unreduced forms of both the Endo H and the Endo H – – – > Endo/PNGaseF digestions shows the presence of a large amount of GlcNAc residues beyond the chitobiosyl core. Possible locations of a bisecting GlcNAc residue. GlcNAc residues in position 'A' are known to occur in mammalian glycoproteins, while position 'B' is the location of the GlcNAc residue on the *Dictyostelium* oligosaccharides. (Symbols are as shown in Fig. 9).

*The shared determinant: common antigen 1 (CA1)*

To characterize this shared determinant more thoroughly, monoclonal antibodies were prepared using partially purified lysosomal enzyme preparations as immunogens (85). Two-dimensional PAGE analysis of the precipitated proteins showed that many of the proteins secreted during growth contained the shared determinant, while relatively few intracellular proteins did. Another property of the secreted immunogenic proteins was that they were extremely negatively charged some having isoelectric points estimated to be less than 3.5. The proteins could be labeled effectively with [$^{35}$S] $SO_4$, and some with $^{32}PO_4$ (85). Previous work had shown that the lysosomal enzymes contained both of these substituents on the oligosaccharides (41, 43). It seemed likely that the oligosaccharide might be part of the antigenic site.

To test this hypothesis glycopeptides were prepared from β-glucosidase by Pronase digestion and used as competitors of the immunoprecipitation of another enzyme containing the shared determinant (86). The crude glycopeptides and those with two to six charges were very effective competitors, but neutral oligosaccharides or those with only a single charge did not compete. When the sulfate esters were removed from the glycopeptides or the oligosaccharides by gentle solvolysis, none were effective competitors. Oligosaccharides with up to four sulfate esters were no better competitors than those with two. Therefore, it appeared that two residues of sulfate were required to define the shared determinant. To determine if the critical sulfate esters were in mild acid sensitive linkages the oligosaccharides were treated with mild acid under conditions that destroy at least 75% of all equatorial sulfate esters, but only about 10% of primary linked esters. The treated oligosaccharides showed only about a 10% decrease in potency in blocking CA1. The results suggest that all of the sulfate esters needed to define the shared determinant CA1 are in primary linkageas; Man-6-$SO_4$ is a likely candidate (48).

*Common antigens CA2 and CA3*

Other shared determinants also exist, and several of these have been characterized using an approach similar to that described for CA1 (48). Fifty-seven of the 60 hybridoma lines that produced monoclonal antibodies against multiple lysosomal enzymes were specific for CA1. The three others recognized distinct carbohydrate epitopes. Based on competitive inhibition studies using various sulfated and non-sulfated monosaccharides and polysaccharides from various sources or oligosaccharide/glycopeptides from *Dictyostelium,* one of the antibodies recognizes a sulfated or unsulfated GlcNAc residue(s). This is called common antigen 2 or CA2. The sulfate is not an absolute requirement as it is in the case of CA1, but sulfated oligosaccharides are better competitors that non-sulfated ones. Neutral and non-sulfated anionic oligosaccharides from the lysosomal enzymes or glycopeptides from ovalbumin or ovomucoid are good competitors, too. It seems unlikely that a GlcNAc residue in the core region would be highly antigenic. Since it is clear that the oligosaccharides of secreted proteins have bisecting GlcNAc in a position different than that seen in mammalian cells, it is possible that this GlcNAc residue (see Fig. 4B, GlcNAc residue 'B') might be critical for this determinant. An interesting feature of this antibody is that it recognizes only denatured (boiled) protein. Native β-glucosidase or whole cell proteins are not recognized (48).

Two other antibodies have a specificity different from either CA1 or CA2. This epitope is called CA3 and occurs on the carbohydrate chains of lysosomal enzymes and other proteins in *Dictyostelium.* These antibodies like those that recognize CA2 react only with denatured (boiled) protein. At present we have very little information on the nature of the determinant. A widely diverse group of monosaccharides, oligosaccharides and glycopeptides all weakly inhibit antigen-antibody interaction. It is clear that the antibody recognizes a substituent on an N-linked oligosaccharide, since Endo/PNGaseF treated β-glucosidase does not compete as well as the glycosylated form. Furthermore, each of the different antibodies that recognize CA1, CA2, and CA3 compete with each other for binding to the lysosomal enzymes, while an antibody specific for the protein portion does not compete with and is not competed by any of these antibodies (48).

Using a series of sequential precipitations with each of the classes of antibodies in all of the different orders, it is evident that cellular and secreted
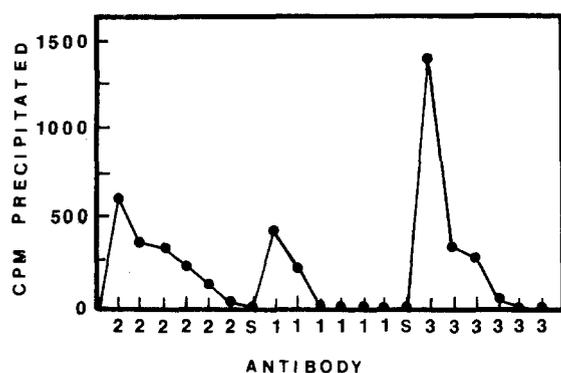
*Fig. 5.* Example of serial precipitation of proteins using common antigen antibodies. [$^{35}$S]Methionine labeled proteins from wild-type strain AX3 were sequentially precipitated by monoclonal antibodies directed against epitopes CA2, followed by CA1 and finally by CA3. Each antibody was used six times followed by Pansobrin precipitation to remove any residual antibody. Similar precipitations using these antibodies, but done in different orders allows the determination of the relative amount of protein which contains any combination of the antigens.

proteins contain various combinations of CA1, CA2, and CA3 (48). Some contain only one or another of the determinants while others contain all three. A typical example of the sequential precipitation is shown in Fig. 5. When the antibodies are used in all of the possible orders to precipitate proteins, the relative amounts of protein containing each of the epitopes can be estimated. Precipitation by a combination of all the antibody completely removes all Concanavalin A binding proteins from the medium. On the other hand, nearly half of the Con A binding intracellular proteins are not precipitated by the three sets of antibodies. Thus it is clear that the antigens are not evenly distributed among all proteins.

*Other shared determinants*

Gustafson *et al.*, have suggested that the GlcNAc-P-Serine is the epitope recognized by polyclonal antisera against lysosomal enzymes (87). Clearly, there are multiple antibodies which recognize different shared determinants present on the lysosomal enzymes. At this point, no monoclonal antibodies have been isolated which specifically recognize GlcNAc-P-Serine, but it is quite possible that a minor set of antibodies in rabbit antiserum may recognize such a determinant. Recently Couso

and Kornfeld have described an enzyme activity which will transfer GlcNAc from UDP-GlcNAc to endogenous acceptors (manuscript in preparaton). This activity is distinct from the one which uses $\alpha$-methyl mannoside as an acceptor.

Bozzarro *et al.*, have isolated monoclonal antibodies which recognize multiple proteins via a shared determinant (88), but they have not tested these against lysosomal enzymes. The binding of one of these preparations to its antigen appears to be preferentially inhibited by GlcNAc compared to other monosaccharide inhibitiors. This antibody may be similar to those that recognize CA2.

## Mutants altered in the modification of N-linked oligosaccharides

*Dictyostelium* is especially well suited for the generation and analysis of mutant strains. This approach has now been applied to the analysis of the biosynthesis of glycoproteins, the secretion and targeting of lysosomal enzymes and the expression of the shared determinants. In this section we will discuss some of these results.

The first post-translational modification mutant isolated in *Dictyostelium* was in the modA locus (89). It was initially discovered by screening mutagenized cells for a deficiency in vegetative lysosomal $\alpha$-mannosidase activity. Several other glycosidases also showed lower specific activities, and most importantly, each was less anionic than the wild-type enzyme. The mutation was mapped to linkage group I, which was different from the locations of any of the known structural genes for the effected enzymes. Subsequently, glycopeptides in many glycoproteins of the mutant strain were found to be deficient in Man-6-P and sulfate esters (42). Since almost 30% of the glycopeptides were altered by the mutation, it was unlikely that only lysosomal enzymes were affected. The primary defect was later shown to be the result of a deficiency in the N-linked oligosaccharide processing $\alpha$-1,3 glucosidase, Glucosidase II (40). As a result, the glucosylated branch of the oligosaccharide chain cannot be further modified by the addition of either phosphate or sulfate residues. The other branches of the oligosaccharides appear to be normal. The mutation causes a decreased stability of the various lysosomal enzymes, but the rate of

processing of either α-mannosidase or β-glucosidase through the rough endoplasmic reticulum is normal (90). In contrast, the inhibition of oligosaccharide processing Glucosidase I by 1-deoxynojirimycin slows the processing of several mammalian glycoproteins (91, 92).

Lysosomal enzymes purified from the modA mutant have been helpful in assessing the structural requierments needed for the binding of the slime mold lysosomal enzymes to the mammalian phosphomannosyl receptor (82). Since phosphorylation cannot occur on the unprocessed branch, only a single Man-6-P-OCH$_3$ residue can be added to the oligosaccharide. When these enzymes are used as ligands for binding to mammalian phosphomannosyl receptor in the membranes of fibroblasts, they are taken up about 10% as fast as the normal enzyme which has predominently two residues of Man-6-P-OCH$_3$. These results show that two residues of Man-6-P-OCH$_3$ are needed for effective uptake of the ligands.

Five mutant strains (HL240−244) have also been isolated with lack CA1 (85). Although, the strains grow somewhat more slowly than the wild-type, the loss of the shared determinant is not crucial for survival. Similarly, four of the strains will undergo development, but they are slow in competing development and usually only about 20% of the aggregates make final fruiting bodies. Strain HL-240 does not develop beyond aggregation. It is clear

that the shared determinant is not crucial for development, and equally clear that development is not normal.

One of these strains, HL-244, appears totally deficient in the sulfation of proteins as determined by SDS-PAGE. The other four strains are 30−50% reduced in the sulfation of TCA precipitable material. To define the shared determinant more precisely, a partial structural analysis of the oligosaccharides of the mutant strains was needed.

Metabolically labeled oligosaccharides were analyzed from strains HL-241 and HL-243 following Endo/PNGaseF digestion of secreted proteins. The oligosaccharide patterns of each of the two strains are very similar to each other in many respects. The ratio of SO$_4$ to Man is lower compared to that in the wild-type. Both contain anionic and neutral oligosaccharides, but the number of charges per oligosaccharide is much less in the mutants than in the wild-type, suggesting that there are fewer sulfate and/or phosphate residues per oligosaccharide. Both mutants contain sulfate esters as indicated by the change in QAE-Sephadex patterns following solvolysis to remove the sulfate esters (Fig. 6). Moreover, both mutant strains still contain Man-6-SO$_4$. Clearly, the mere presence of SO$_4$ does not define CA1, which is missing in these strains. To further characterize the oligosaccharides, the size of the neutral species was analyzed by amine-adsorption HPLC (Fig. 7). The results
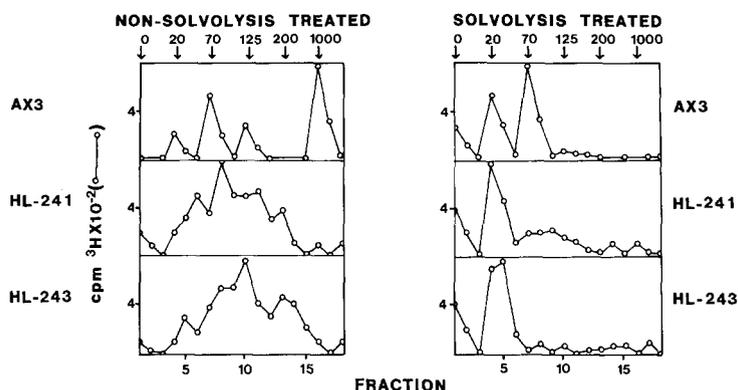


Fig. 6. QAE-Sephadex analysis of anionic oligosaccharides of strains AX3, HL-241 and HL-243 before and after solvolysis. Anionic oligosaccharides were released from secreted proteins labeled with [³H]Man by digestion with Endo/PNGaseF. The oligosaccharides were analyzed by QAE-Sephadex chromatography before and after solvolysis to remove sulfate esters. Oligosaccharides of wild-type cells. which have 0, 1, 2, 3, 4 and 5−6 negative charges are eluted from QAE-Sephadex by 0, 20, 70, 125, 200 and 1 000 mM NaCl respectively. Following solvolysis, the negative charge is due to the presence of methylphosphomannosyl residues on the oligosccharides.
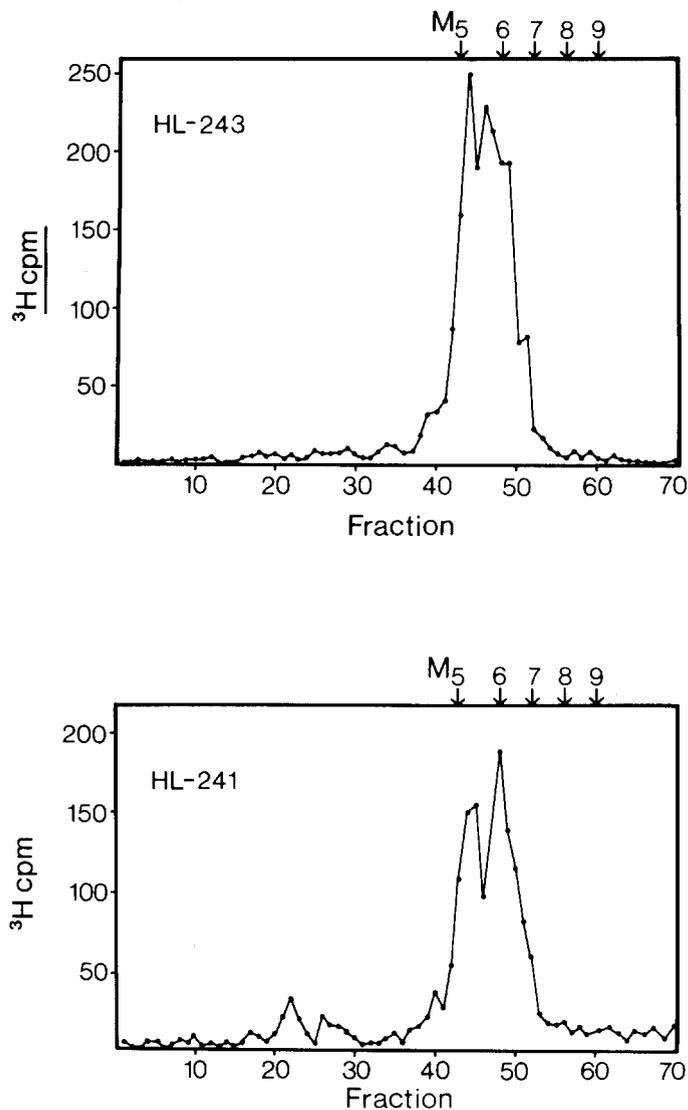
Fig. 7. HPLC analysis of neutral oligosaccharides from strains HL-241 and HL-243. The neutral oligosaccharides released from each of the two mutant strains was applied to an AX-5 column and eluted with a gradient of 35−65% water in acetonitrile. The numbers at the top refer to standards with a single GlcNAc-itol residue and the indicated number of Man (M) residues. Neutral oligosaccharides from wild-type cells are nearly all Man9−10 sized.

showed that both mutant strains produced smaller species of size $Man_{5-6}GlcNAc$, compared to the wild-type of $Man_{9-10}GlcNAc$ (6).

One explanation for the smaller size of the neutral oligosaccharide from the mutants is that they are degradation artifacts produced after the proteins were secreted into the medium. Normally the phosphate and sulfate residues might 'protect' the oligosaccharide from degradation in the medium,

and the failure to add the anionic groups could render them sensitive to random degradation. To eliminate this possibility, we decided to isolate the lipid-linked oligosaccharide to determine its size and characterize the structure. The precursor was isolated by standard methods (93), and the oligosaccharide was hydrolyzed from the lipid by mild-acid, leaving both of the core GlcNAc residues on the oligosaccharide. When the size of the oligosaccha-
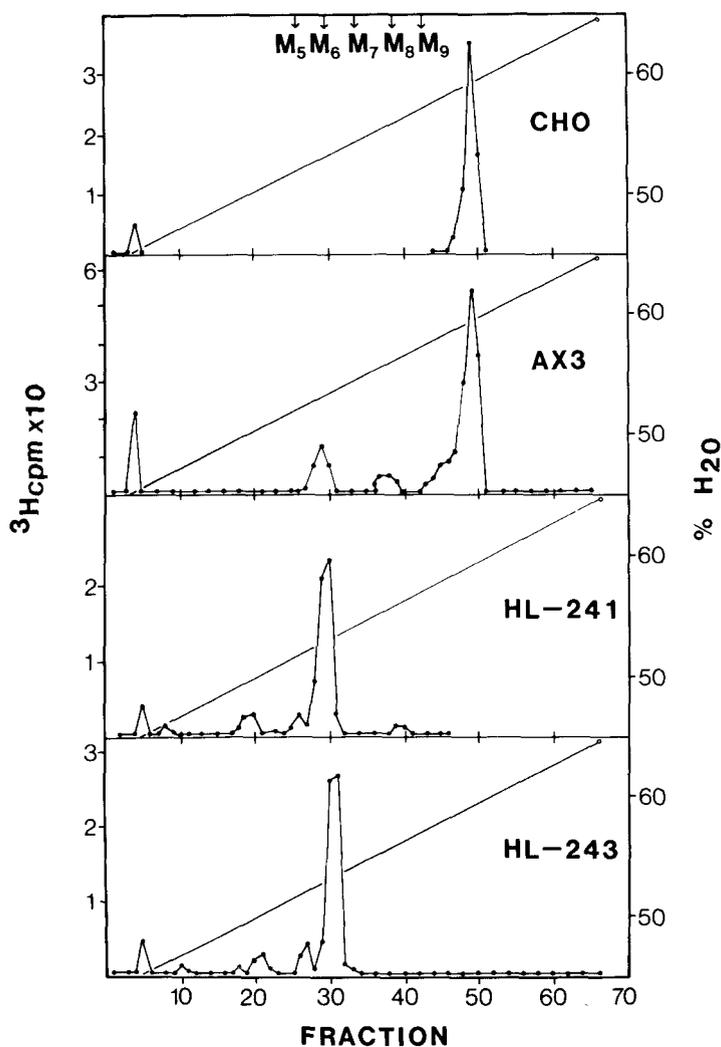
Fig. 8. HPLC analysis of lipid-linked oligosaccharides. The lipid-linked oligosaccharides were purified from Chinese hamster ovary cells (CHO) and from strains AX3, HL-241 and HL-242 (85). The oligosaccharides were released by mild-acid hydrolysis, chromatographed on Concanavalin A Sepharose, eluted, and reduced. They were then analyzed on an AX-5 column together with standards described in Fig. 7. Note that the standards were released with Endo-H and have only a single GlcNAc residue, while the samples have two GlcNAc residues since they were released from the lipid by hydrolysis.

ride was measured by HPLC (94) it was found to be considerably smaller than that isolated from the wild-type cells. By comparison with standards the size was estimated to be equivalent to a $Man_5GlcNAc_2$ (Fig. 8). Thus, it was clear that a truncated precursor of the oligosaccharides was synthesized in the mutant strains; and this undoubtedly was insufficient to accomodate the number or orientation of the Man-6-$SO_4$ residues required to define the shared determinant, CA1.

Preliminary experiments suggest that the complete $\alpha$-mannosidase digestion yields only Man and a trisaccharide-sized structure which is probably Man-$\beta$-GlcNAc-$\beta$-GlcNAc. This would suggest that the oligosaccharide is not glucosylated. By analogy with the biosynthetic order of addition of the Man residues to the mammalian lipid-linked oligosaccharide (95), the structure of the oligosaccharide could be:

```
      Man
       |
α1,2   |
       |
      Man
       |
α1,2   |
       |
      Man                    Man
         \                  /
α1,3      \                /   α1,6
           \              /
            \Man         /
             |   β1,4
            GlcNAc
             |   β1,4
            GlcNAc
```

This structure has not been proven. Further work is needed to confirm it and to determine the nature of the primary defect. However, it this structure is correct, then it would suggest that the shared determinant of CA1 requires critical Man-6-SO$_4$ residues located on the missing branches; the mere presence of Man-6-SO$_4$ is not sufficient to define the antigen. Furthermore, proteins from cells carrying the modA mutation still have CA1 determinant. This is significant because this mutation affects the significant because this mutation affects the processing of the glucosylated branch and is known to prevent its phosphorylation. It is likely that it also prevents normal sulfation of this branch. Taken together these results suggest that the sulfation of the glucosylated branch is not critical for CA1 while those on the branches missing in HL-241 and HL-243 are necessary. The other shared determinants are still present in these strains, and this must mean that those Man residues are not required features of the other determinants. So, we can now use these mutants to define the minimal structures required to define the other shared determinants.

The creation and analysis of mutant strains defective in the production of carbohydrate antigens is a novel approach to defining glycoconjugate structure. Since this can be correlated with the effects on particular proteins, processes or on development, it offers a possible insight into the func-

tion of the particular structure. In the case of the shared determinant CA1, it permitted the selection of mutants in the synthesis of the lipid-linked oligosaccharide. In the case of strain HL-244 it may show a mutant defective in the ability to transfer sulfate to normal sized oligosaccharides.

Using the monoclonal antibodies against CA2 and CA3 we can now screen for mutant strains which lack these determinants. Since the strains which lack CA1 still have CA2 and CA3, it is likely that mutant strains which lack CA2 or CA3 will have different set of primary defects than those that eliminate CA1.

## Developmental changes in modification of N-linked oligosaccharides

Although the function of the shared determinants of lysosomal enzymes is not known they all appear to be developmentally regulated, and they are not equally distributed between stalk and spore cells in the mature fruits. In the case of CA-1, its synthesis ceases after about 10 h of development and overall sulfation decreases. The phosphorylation of the oligosaccharides on the lysosomal enzymes is also reduced as shown by their lack of uptake into human fibroblasts via the phosphomannosyl receptor (47). It is interesting that a membrane bound, neutral pH α-mannosidase is induced during this part of development (96). This enzyme is thought to be involved in the trimming of the mannose residues to produce the smaller oligosaccharides seen during later development. A second membrane bound, neutral pH α-mannosidase also appears somewhat later (97). This second enzyme has different substrate specificity and inhibitor sensitivities than the first one. The appearance of these two mannosidases correlate well with the appearance of lysosomal enzymes which are considerably less charged than those found in vegetative cells. It is reasonable that the increased processing of the oligosaccharides results in the less charged enzymes, but this is not proven. The isolation of strains which lack either or both of these α-mannosidases may be able to shed some light on their function and on the importance of trimmed oligosaccharide structures during development.

The lysosomal enzymes synthesized later in de-

velopment appear to differ in their modifications in pre-stalk and pre-spore cells based on differences in their electrophoretic mobility when isolated from the two cell types (46). This may indicate alternate processing of the proteins or the oligosaccharides in the different cell types. If the oligosaccharides are processed differently in the different cell types, the analysis of mutant strains altered in the modifications of the antigens, the lipid-linked oligosaccharides or the processing α-mannosidases may be invaluable in determining the importance of the precise oligosaccharide structure to proper modifications of these enzymes.

Recently Free and his associates have shown that during development there is an unequal distribution of several lysosomal enzymes into two classes of vesicles which have different densities (98). One has the density expected for lysosomes and the other co-fractionates with an organelle called the pre-spore vesicle which is found only in cells normally destinated to become spore cells (23). These results suggest that developed cells have two populations
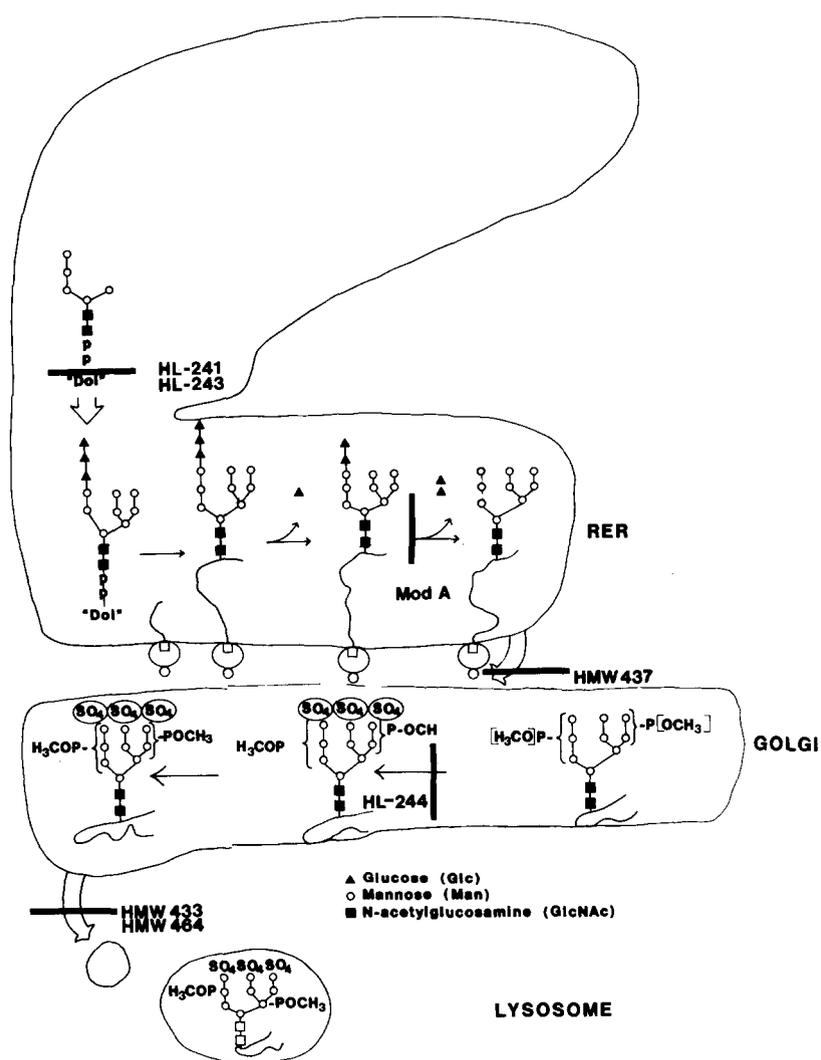


Fig. 9. Schematic diagram of the processing of glycoproteins in *Dictyostelium discoideum*. The processing of the oligosaccharides as described in the text is shown. The location of the sulfate esters on any particular residue is not intended. Refer to the text for details on the mutants which block processing of oligosaccharides or of the lysosomal enzyme α-mannosidase.

of physically separable vesicles while only a single class is found in vegetative cells. It is possible that alternative targeting routes are being followed, but this is an open question at the moment. Judelson and Dimond (48) have recently shown that both α-mannosidase and β-glucosidase synthesized after about 12 h of development lack the ability to bind to the phosphomannosyl receptor, and presumably have oligosaccharides which are partially processed. Moreover, these enzymes are *not* proteo-lytically cleaved or targeted to the lysosome; instead they are secreted to the outside of the cell. Although this is not proof of the importance of the oligosaccharide in the targeting of the lysosomal enzymes, it is consistent with the involvement of the oligosaccharide.

Up to this point we have discussed only carbohydrate antigens which disappear during development, but new carbohydrate antigens also appear on multiple proteins only during development. The exploration of general nature of these other determinants has been very important in understanding the nature of cell-cell interaction and cellular cohesiveness during development (49, 50).

### Summary of lysosomal enzyme processing

Figure 9 shows a schematic summary of the steps involved in the biosynthesis and processing of lysosomal enzymes and other glycoproteins. We have also shown the location of the lesions found in several mutant strains. Mutant strains HMW-437, -433 and -464 all carry defects in the structural gene for α-mannosidase 1 (71). The α-mannosidase of strain HMW-437 is arrested in the RER, while those of the other two strains fail to leave the Golgi, but all are glycosylated in accord with their sub-cellular location (71).

At present there are over 40 mutant strains which are altered in the post-translational modification of single or multiple lysosomal enzymes. Ultimately, our goal is to identify the primary defects in many of these strains. In some, the defect is already known to be in the structural gene for the lysosomal α-mannisidase (α-Man1) as mentioned above. These mutants will be useful for the exploration of the effects of amino acid sequence on proteolysis and oligosaccharide modification. As for the other mutations that effect multiple lysosomal

enzymes, they are often abberant in the packaging or secretion of these proteins from the cells. In these complex interactions, cause and effect will not be easily discernible. The continued systematic analysis of these mutants should give us a wealth of information on the structure, biosynthesis and, hopefully, the biological roles of the N-linked oligosaccharides in the growth and development of *Dictyostelium*. This in turn may provide clues for understanding the function of glycoconjugates in other multicellular systems.

### Abbreviations

Man, mannose; Glc, glucose; GlcNAc, N-acetylglucosamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RER, rough endoplasmic reticulum; Man-6-P, Mannose-6-phosphate; Man-6-P-OCH$_3$, methylphospho-mannose; Endo/PNGaseF, a preparation containing endoglycosidase F and peptide N-glycosidase F isolated from *F. meningiosepticum;* Endo H, endoglycosidase H from *S. plicatus;* Man-6-SO$_4$, mannose-6-sulfate.

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