IDENTIFICATION OF AN OLIGOSACCHARIDE-SPECIFIC PHOSPHATE METHYLTRANSFERASE IN DICTYOSTELIUM DISCOIDEUM*

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The N-linked oligosaccharides on three lysosomal enzymes in Dictyostelium discoideum were found to contain mannose 6-phosphomethyl residues. We have identified and partially characterized a novel S-adenosylmethionine-dependent methyltransferase that is probably responsible for the synthesis of this unusual diester from Man-6-P. The enzyme selectively methylates the phosphate group of Man-6-P (Kₐ 4.3 mm). Glucose-6-P and fructose-1-P are relatively poor acceptors; however, the enzyme is inactive against a broad array of other phosphorylated compounds. Using model di-, tri-, and pentasaccharide acceptors that include portions of the three different branches of high mannose-type oligosaccharides, we found that the enzyme prefers terminal a₁-2 linked Man-6-P residues (Kₐ 0.15–1.25 mm) found on the known phosphorylated branches. The enzyme is membrane bound, has a normal pH optimum and cofractionates on sucrose gradients with GlcNAc-1-P transferase, which resembles its mammalian counterpart, and is, presumably, the first enzyme in the phosphorylation pathway. Based on the substrate specificity and colocalization with GlcNAc-1-P transferase, the phosphate methyltransferase is likely to be responsible for the generation of mannose 6-phosphomethyl diesters on Dictyostelium oligosaccharides.

Mannose 6-phosphate on the oligosaccharides of many mammalian glycoproteins is critical for their interaction with phosphomannosyl receptors (1). The best example of this is in the targeting of newly synthesized lysosomal enzymes (1, 2), but a diverse group of proteins including prolinier (3), thyroglobulin (4), transforming growth factor β-precursor (5), and the epidermal growth factor receptor (6), all contain mannose 6-phosphate as mono- or diesters. Man-6-P is added to oligosaccharides in a two-step pathway. First, GlcNAc-phosphotransferase uses UDP-GlcNAc to add GlcNAc-1-P to the 6-position of selected mannose residues (7). The transferase selects candidate acceptor proteins based on a common three-dimensional motif and the presence of terminal a₁→2 linked mannose residues on the oligosaccharide (8, 9). The acid-labile GlcNAc-P-Man diester is subsequently cleaved by a specific neutral pH α-N-acetylgalactosaminidase to yield the phosphomonoester (10). Proteins that present an available Man-6-P can then bind to either the cation-independent or cation-dependent phosphomannosyl receptors (1).

The vegetative cells of Dictyostelium discoideum also synthesize abundant amounts of phosphorylated N-linked oligosaccharides where Man-6-P occurs as an acid-stable phosphomethyl diester (Man-6-P-OC₃H₇) (11–13). Lysosomal enzymes that contain an acid-stable phosphodiester bind selectively to the mammalian cation-independent phosphomannosyl receptor (14, 15) but not to the cation-dependent receptor (16, 17). No comparable phosphomannosyl receptor has been found in Dictyostelium, and the role of Man-6-P in lysosomal enzyme targeting is unproven. The Dictyostelium and mammalian GlcNAc phosphotransferases closely resemble each other in that both require oligosaccharides with terminal a₁→2 Man residues and both add the first GlcNAc-1-P to the a₁→6 branch of the acceptor oligosaccharide. They differ from each other in that the Dictyostelium enzyme does not preferentially recognize mammalian lysosomal enzymes (9, 18).

We wanted to identify the remaining enzymes involved in the biosynthetic pathway as a prelude to functional deletion analysis. Our previous studies showed that the methyl group is derived from S-adenosylmethionine (AdoMet) (19), but there was no kinetic evidence for the presence of an acid-labile GlcNAc-P-Man intermediate, or for the mammalian "uncovering enzyme" that generates the phosphomonoester. Moreover, there is no biochemical precedent for a phosphate methyltransferase. Here we report the identification of a membrane-bound, phosphorylated oligosaccharide-specific, AdoMet-dependent phosphate methyltransferase that cofractionates with GlcNAc-1-P transferase.

EXPERIMENTAL PROCEDURES

Materials—Radiochemicals: [2-3H]mannose (20 Ci/mmol) and [methyl-3H]methionine were obtained from American Radiochemical Co., St. Louis, MO, and S-adenosyl [methyl-3H]methionine (82 Ci/ mmol) was obtained from Du Pont. [2-3H]Man-6-P-OCH₃ was synthesized by diazomethane treatment of [3H]Man-6-P as previously described (19). [3H]GlcNAc-1-P-F-Man₆Ome was prepared using Dictyostelium GlcNAc-phosphotransferase according to the procedure described by Lang et al. (18), except that the final UDP-GlcNAc concentration was reduced to 10 μM. N-Glycanase was purchased from Genzyme, Inc. Antibodies: peptide-specific mouse monoclonal antibodies against α-mannosidase, β-glucosidase, and acid phosphatase were obtained from Dr. James Cardelli, Louisiana State University (20, 21).

Cell Labeling and Immunoprecipitation of Lysosomal Enzymes—

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The abbreviations used are: Man-6-P-OCH₃, mannose-6-phosphomethyl diester; AdoMet, S-adenosylmethionine; Fuc, fucose; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.
Fig. 1. *N*-Glycanase digestion and analysis of methyl-3H]methionine-labeled lysosomal enzymes. Cells were labeled with methyl-3H]methionine in HL5 medium and α-mannosidase (panel A, 2800 cpm), β-glucosidase (panel B, 1850 cpm), and acid phosphatase (panel C, 3200 cpm) were precipitated from the medium or the cells (not shown). The immunoprecipitates were solubilized and incubated with or without 2 milliunits of N-glycanase. Following the digestion, both the control (open squares) and digests (closed circles) were analyzed by gel filtration chromatography on Sephadex G-50. The [3H]methionine-labeled oligosaccharides released from α-mannosidase immunoprecipitated from the cells (panel D) or the medium (panel E) were pooled, desalted, and analyzed by QAE-Sephadex chromatography. [3H]Mannose-labeled oligosaccharides from α-mannosidase (cells and medium) were similarly prepared and analyzed for comparison (panel F). The arrows on panel D refer to the beginning of step elutions with the indicated concentration of NaCl and represent oligosaccharides with 1, 2, 3, 4, 5, and >5 charges, respectively. Uncharged chains are eluted in the first three fractions.

Fig. 2. Crude subcellular fractionation of Man-6-P-dependent methyltransferase activity and QAE-Sephadex fractionation of 3H]methylation products. Crude sonicates of cells were prepared as described under "Experimental Procedures" and fractionated by centrifugation into 100,000 × g pellet and supernatant. Transferase assays of each fraction were conducted with various amounts of material from unfractionated lysates (panel A) or for the 100,000 × g (1 h) supernatant (panel B) or for the 100,000 × g (1 h) pellet (panel C) in the presence (○) or absence (□) of Man-6-P. The products synthesized in the crude sonicate were fractionated on QAE-Sephadex and eluted with 3 × 1.5-ml washes of 0, 35, or 125 mM NaCl in 2 mM Tris base. Authentic [3H]Man-6-P-OCH3 which carries one negative charge elutes exclusively in the first two 35 mM NaCl fractions. Results are shown as picomoles of Man-6-P-OCH3 formed during the assay.
g for 20 min on a Beckman TL-100 ultracentrifuge, resuspended at 25–40 mg/ml protein in 50 mM Tris-HCl, and immediately stored at 

-80°C.

**Phosphate Methyltransferase Assay—** Assays using Man-6-P were conducted in 100-μl volume in 1.5-ml microfuge tubes and contained 100,000 cpm of [methyl-3H]AdoMet at a final concentration of 5 mM Man-6-P, 50 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, and 100–400 μg of membrane protein. Samples were incubated for 1 h at room temperature (22°C) and then stopped by heating at 100°C for 2 min. The sample was diluted to 1 ml and spun at 10,000 × g on a Microfuge, and the supernatant was loaded onto a 0.5 × 1.5-cm column of Dowex-50 in a Pasteur pipette that was mounted on top of a similar column containing QAE-Sephadex equilibrated in 2 mM Tris base. The tandem column set was washed with 5 ml of water, and the Dowex-50 column was discarded at this point. The QAE-Sephadex column alone was washed with 4 ml of 35 mM NaCl in 2 mM Tris base, and the effluent was collected into a single 22-ml scintillation vial and counted with 15 ml of aqueous-compatible scintillation fluid. Background values of samples run in the absence of Man-6-P were subtracted and usually amounted to 10–15%.

**Oligosaccharide Phosphate Methyltransferase Assays—** The synthesis of the various phosphorylated substrates was previously described and their structures were documented by 1H NMR (22-26). The incubation conditions using these substrates were similar to those described above except that Man-6-P was omitted, and the final reaction volume was 20 μl using 30–50 μg of membrane protein. The reaction mixture was passed over the Dowex-50 column and then over a C-18 Sepak cartridge which was washed with 11 ml of water. The last 1-ml water wash was collected and counted to measure washing efficiency. The hydrophobic-labeled product was eluted 5 μl of MeOH and counted in a single scintillation vial and corrected for quenching by MeOH. The amount of radioactivity in the final water wash was subtracted from the MeOH wash. The C-18 cartridges were reused 10–15 times or until the water wash background values increased significantly. In all of the kinetic experiments, the same column was used for each individual oligosaccharide species tested. GlcNAc-Phosphotransferase Assay—Membranes were incubated with 100–300 μg of membranes and 100,000–200,000 cpm of UDP-[3H]GlcNAc as described previously (18).

**cAMP Phosphodiesterase and Alkaline Phosphatase Assays—** cAMP phosphodiesterase was assayed using 3'-5'-cyclic [3H]adenosine monophosphate, and alkaline phosphatase was assayed using 4-methylumbelliferyl phosphate as described previously (27).

**Characterization of Phosphate Methyltransferase Product—** The normal assay procedure was scaled up 4-fold in protein and run for 3 h to generate a large amount of product for characterization studies. The 35 mM NaCl fraction was passed over Dowex-50 (H-form) to remove sodium, and an aliquot was spotted on a 20-cm flexible thin plate next to chemically synthesized [3H]Man-6-P-OCH₃ and developed in 5:5:1:3 butanol/pyridine/water/acetic acid. Both lanes were cut into 0.5-cm sections and counted. Other sugar standards (5 μg each) on the plate were located by silver staining. A similar procedure was performed for a plate developed in ethyl acetate/acetic acid/water, 6:3:2. The product was also characterized by its susceptibility to acid hydrolysis in 1 N HCl at 100°C. Samples containing 1000 cpm of product were mixed with 100 cpm of 35S0₄ hydrolyzed, and the [3H]CH₃OH was removed in a shaker evaporator and the remaining radioactivity was normalized to 35S0₄ recovery. The conversion of [3H]Man-6-P-OCH₃ to [3H]Man-6-P was monitored by the increase in the percentage of [3H] that was converted from 1 to 2 negative charges. Under these conditions, <10% Man-6-P is dephosphorylated. Base hydrolysis was performed in 20 μl of 0.1 N NaOH at 37°C for 1–4 h.

**Subcellular Fractionation—** Cells were grown to mid-log phase, collected by centrifugation, washed once in cold 50 mM Tris-HCl pH 7.5, and resuspended in the same buffer at 1.5–2 × 10⁶ cells/ml and lysed by nitrogen cavitation at 1,000 psi. The unbroken cells were removed by 800 × g spin for 5 min, and lyase was spun at 10,000 × g for 20 min. This supernatant was removed and spun at 415,000 × g for 20 min to sediment a microsomal fraction (27). This pellet was resuspended in 2.0 ml of the same buffer using a 2-10 ml Strator and 15 strokes of a Dounce homogenizer and fractionated by two methods. In the first method, the resuspended pellet was layered on top of a step gradient consisting of 2.0 ml of sucrose at 0.88, 1.02, 1.17, 1.32, 1.45 M in 50 mM Tris-HCl, pH 7.5, and spun at 38,000 rpm for 2.5 h in an SW-50 rotor. This method was similar to a previously described method (27). In the second method, the resuspended microsomal pellet was placed on top of a linear sucrose gradient from 25 to 45% in sucrose and spun at...
**TABLE I**

| Effector | mM | % of control activity |
|----------|----|-----------------------|
| Cations  |    |                       |
| Ca²⁺     | 0.1| 48                    |
|          | 1.0| 100                   |
| Mg²⁺     | 0.1| 97                    |
|          | 1.0| 138                   |
| Mn²⁺     | 0.1| 107                   |
|          | 1.0| 125                   |
| Zn²⁺     | 0.1| 40                    |
|          | 1.0| 0                     |
| Co²⁺     | 0.1| 106                   |
|          | 1.0| 70                    |
| Anions   |    |                       |
| PO₄³⁻    | 1.0| 78                    |
|          | 20 | 38                    |
| SO₄²⁻    | 1.0| 34                    |
|          | 20 | 27                    |
| Ac⁻      | 1.0| 88                    |
|          | 20 | 62                    |
| Detergent|    |                       |
| None     |    | 53                    |
| Triton X-100 | 0.1 | 100            |
|          | 0.5| 113                   |
| Nonidet P-40 | 0.1 | 91             |
|          | 0.25| 100                 |
| Octyl-α-glucoside | 0.1 | 30             |
|          | 0.5| 0                     |
| Others   |    |                       |
| EDTA     | 1.0| 79                    |
|          | 5.0| 68                    |
| S-Adenosylhomocysteine | 0.1 | 36             |
|          | 0.5| 0                     |

**TABLE II**

Comparison of various acceptors for phosphate methyltransferase

| Acceptor* | Kₘ (mM) | Vₘₐₓ (pmol/h/mg) |
|-----------|---------|------------------|
| Man-6-P   | 4.3     | 400              |
| PM-α₁→2Man-OR | 0.15 | 1474         |
| PM-α₁→2Manα→6Man-OR | 0.47 | 1327         |
| PM-α₁→2Manα→2Man-OR | 1.25 | 1157         |
| PM-α₁→2Manα→3Man-OR | 16.7 | 2800         |
| PM-α₁→2Manα | 0.26 | 1502          |

* R = (CH₃)₂COOCH₃.

**FIG. 4.** Effects of time, protein concentration, pH, and temperature on methyltransferase activity. Panel A, samples containing 200 μg of protein were assayed for various periods of time at 22°C at pH 7.5. Panel B, samples were incubated for 1 h at 22°C with various amounts of membrane protein. Panel C, samples containing 200 μg of protein were incubated at 22°C for 1 h in 50 mM buffers at the pH indicated. (●, Tris; □, MES; Δ, HEPES). Panel D, samples containing 200 μg of protein were assayed for 1 h at different temperatures.

**FIG. 5.** Methylation of various phosphorylated compounds. Phosphate methyltransferase assays were conducted using indicated acceptor at 10 mM. All values were corrected for background with no added acceptor.

38,000 rpm for either 2.5 (24) or for 5.5 h. The fractions were analyzed for GlcNAc-phosphotransferase, Man-6-P phosphate methyltransferase both in the presence and the absence of Man-6-P. Assays for typical lysosomal enzymes including α-mannosidase and β-glucosidase (14) showed that >98% of the activity failed to cofractionate with the above enzymes. No neutral pH α₁→2 Man processing mannosidase could be detected in these fractions.
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RESULTS

Lyosomal Enzymes Contain Phosphomethylated Oligosaccharides—Previous studies showed that many anionic oligosaccharides in Dictyostelium contain Man-6-P-OCH₃ residues (13) and that individual lysosomal enzymes have an acid-stable diester (11). To determine whether the acid-stable diester in these enzymes is also a phosphomethyl group, vegetative cells were labeled with [methyl-³H]methionine and they were immunoprecipitated from both the cells and the growth medium using monoclonal antibodies against a-mannosidase, β-glucosidase, or acid phosphatase. These three proteins contain most of the [methyl-³H]methionine label in the polypeptide chain and a portion in the phosphorylated oligosaccharides. The proteins were digested with protease-free N-glycanase to release the N-linked oligosaccharides, and these were separated from the deglycosylated ³H-labeled peptides by gel filtration. Digestion specifically releases [³H]methyl-labeled material from each enzyme from the medium (panel E) were compared (Fig. 1, panel D) and from the cells (data not shown). The [³H]methyl-labeled N-linked oligosaccharides from α-mannosidase which was precipitated from either the cells (Fig. 1, panel D) or the medium (panel E) were compared to similarly released [³H]mannose-labeled chains (panel F) by QAE-Sephadex anion-exchange chromatography. The patterns are very similar except that no [³H]methyl label is found in neutral chains, as expected, since they do not contain Man-6-P (Fig. 1). The radioactivity was stable to mild-acid hydrolysis (0.01 N HCl, 100 °C, 30 min) and to base hydrolysis (0.1 N NaOH, 4 h at 37 °C), as previously shown for phosphomethylated oligosaccharides. We conclude that the mild acid-stable phosphodiester previously described in three lysosomal enzymes is probably also a methylphosphodiester.

Preliminary Experiments—To examine the biosynthesis of the phosphodiester, we assumed that GlcNAc-1-P phosphotransferase catalyzes the first step of the pathway to form GlcNAc-1-P-6-Man. GlcNAc is probably then cleaved by another enzyme to generate the phosphomonoester. AdoMet may activate or even be required for this cleavage, but finally, —CH₃ is transferred from AdoMet to Man-6-P to form Man-6-POCH₃. To search for an enzyme capable of converting the diester to the monoester, we synthesized [6-³H]GlcNAc-1-P-6-ManOMe from UDP-[³H]GlcNAc and α-methylmannoside using the Dictyostelium GlcNAc phosphotransferase. Cellular extracts were then assayed for the release of [³H]GlcNAc in the presence or absence of AdoMet. A small amount of substrate was converted to [³H]GlcNAc by a membrane-bound activity at neutral pH (data not shown), but AdoMet did not stimulate the activity. The enzyme is metal-ion independent and is completely inhibited by GlcNAc-1-P, but not by other hexose 1- or 6-phosphates. Further characterization of this enzyme is in progress, but its existence suggested that Man-6-P is directly phosphomethylated. To test this, [methyl-³H] AdoMet was added to total cell lysates in the presence or absence of Man-6-P. Label was transferred to material that contained only a single negative charge based on its elution from QAE-Sephadex with 35 mM NaCl (Fig. 2, panel D). To determine if the transferase activity was membrane-associated, assays were done on total cell lysate, supernatant, and resuspended pellet from a 100,000 x g centrifugation. The results clearly showed that the activity was highly enriched in the pellet fraction (Fig. 2). All subsequent characterizations were done with similar membrane preparations. The activity in the soluble fraction was not further characterized.

Product Characterization—The [³H]methylated product with one negative charge (from Fig. 2, panel D) was analyzed by thin layer chromatography as shown in Fig. 3. The single major peak coincided with the synthetic standard [³H]Man-6-P-OCH₃, and was well separated from other neutral and anionic sugars. Analysis in a second solvent system gave the same result (data not shown). The methylated product was stable to strong base hydrolysis (0.1 N NaOH, 4 h, 37 °C), but the methyl group was hydrolyzed with a t½ of 65 min in 1 N HCl at 100 °C, the same as authentic [³H]Man-6-P-OCH₃ (Fig. 3). Based on the Man-6-P dependence, charge, chromatographic behavior, base stability, and acid hydrolysis kinetics we conclude that the product formed from [methyl-³H] AdoMet in the presence of Man-6-P is Man-6-P-O-[³H]CH₃.

Optimization of Assay Conditions and General Characteristics of the Enzyme—As shown in Fig. 4, the assay is linear for at least 5 h (panel A) and between 0–600 µg of membrane protein (panel B). The pH optimum is 7.5 using either HEPES, Tris-HCl, or MES buffers (panel C), and the activity is highest at 22 °C (panel D), the normal growth temperature of Dictyostelium. The general characteristics of the methyltransferase activity are shown in Table I. It is not dependent on metal ions, but it is slightly activated by 1.0 mM Ca²⁺, Mn²⁺, or Mg²⁺. It is equally active in Triton X-100 or Nonidet P-40 but is totally inhibited by octyl-α-glucoside, Zn²⁺ ions, and 0.2 mM S-adenosylhomocysteine, the product of AdoMet-mediated methyltransferase reactions (26). Activity is partially inhibited by low concentrations of phosphate, sulfate, and acetate ions. The enzyme is totally stable for several months when stored at −70 °C in Tris-HCl buffer at 20–40 mg/ml, but loses >50% of the activity when stored at 4 °C overnight. Repeated freezing and thawing appeared to increase activity by 50%. Kₘ for Man-6-P is 4.3 mM and 5 µM for AdoMet. The Vₘₐₓ under optimal conditions is approximately 400 pmol/mg membrane protein (Table II).

Alternate Acceptors—To determine whether the methyltransferase is specific for phosphomethylation of Man-6-P, a number of other compounds containing phosphomonoesters were examined. The results presented in Fig. 5 show that most of them are not methylated to a significant extent when
Phosphate Methyltransferase Activity on Synthetic Oligosaccharides—Phosphorylation occurs only on selected Man residues in both Dictyostelium and mammalian cells. To determine whether methyltransferase preferentially recognizes specific phosphorylated residues in the context of a larger oligosaccharide, we tested model di-, tri-, and pentasaccharide acceptors that include portions of known phosphorylated chains. These substrates are linked to a hydrophobic arm [(CH₂)₅COOCH₃] for simple purification of the product on C-18 cartridges. To validate this method for our assay, we compared methyltransferase activity for a phosphorylated and non-phosphorylated mannobiose acceptor. The phosphorylated substrate was a much better acceptor for methyltransferase, and the product consisted almost entirely of material with one negative charge (85%), showing that the phosphate group was methylated (data not shown).

The results presented in Fig. 7 and in Table II show that present at 10 mM, but that a few other sugar phosphates including fructose-1-P and glucose-6-P could serve as weak acceptors; however, Man-6-P was clearly the best one (Fig. 6). Neither of these phosphorylated sugars (at 25 mM) competitively inhibits the methylation of the phosphorylated disaccharide described below (data not shown). We conclude that the methyltransferase has high specificity for Man-6-P.

Fig. 7. Comparison of phosphate methyltransferase activity using di-, tri-, and pentasaccharide acceptors. Membranes were incubated with increasing amounts of the indicated di-, tri-, or pentasaccharide acceptors or with Man-6-P. Selected portions of the data presented in panels A-C are expressed as double-reciprocal plots in panels D-F. In each case R = (CH₃)₂COOCH₃.

A

B

C

D

E

F

Acceptor (Mm)

1/S (mM)

pmole/hr/mg

pmole/hr/mg

1/pmol/hr/mg

1/pmol/hr/mg

1/pmol/hr/mg

1/pmol/hr/mg

1/S (mM)

1/S (mM)

1/S (mM)
phosphate methyltransferase has a marked preference for terminal Man-6-P residues that are linked α1→2 to an underlying Man residue. The $V_{\text{max}}$ is about 3-fold higher, and the $K_m$ is $>25$ times lower for Man-6-P. The linkage of the next sugar is also important. Those with α1→2 > α1→6 >> α1→3 Man whether this is part of a trisaccharide or pentasaccharide acceptor. The enzyme methylates a diphasphorylated pentasaccharide, but its activity is about the same as an acceptor containing P on the α1→3 branch, suggesting that phosphorylation on the branch inhibits methylation. As shown in Fig. 8, the substrate preference corresponds well, but not precisely (13, 34), with known locations of methylphosphate residues in *Dictyostelium*. Since Man processing is rare and uncharacterized in vegetative *Dictyostelium* cells (32), it is not known when the terminal Man on branch I is removed. This may be a significant issue because phosphates located in subterminal positions are poor acceptors (Fig. 7). Moreover, the unusual kinetic curves suggest that their interaction with the transferase is different than the others with Man-6-P in terminal positions, which show typical saturation kinetics. These results provide further evidence that this methyltransferase is involved in phosphorylated oligosaccharide synthesis.

**Subcellular Localization of the Phosphate Methyltransferase Activity**—We fractionated cells lysed by nitrogen cavitation into $10,000 \times g$ and $100,000 \times g$ pellets, and $100,000 \times g$ supernatant as described previously for the isolation of membranes that sulfate N-linked oligosaccharides on endogenous acceptors (27). The distribution and specific activities of phosphate methyltransferase and GlcNAc-1-P transferase were compared to alkaline phosphatase and cAMP phosphodiesterase as markers for the plasma membranes. Under these lysis conditions, the lysosomal enzymes are solubilized. As shown in Table III, >80% of both phosphotransferase and methyltransferase activities are found in the $100,000 \times g$ pellet, compared to the plasma membrane markers which are about equally divided between the $100,000 \times g$ and the $10,000 \times g$ pellet. The two transferases cofractionate in continuous (not shown) or discontinuous sucrose gradients (Fig. 9). The $100,000 \times g$ pellet was resuspended and applied to a gradient consisting of 0.85, 1.02, 1.17, 1.32, and 1.45 M sucrose, and individual fractions were assayed for GlcNAc phosphotransferase and phosphate methyltransferase. The activities cofractionate in the 1.17 and 1.02 M sucrose layers which is very similar to the distribution previously reported for N-linked oligosaccharide sulfation-competent membranes (27). Although *Dictyostelium* is thought to have a Golgi apparatus, no specific enzyme markers have been found.

**DISCUSSION**

Previous studies showed that *Dictyostelium* N-linked oligosaccharides released from a collection of secreted glycoproteins contain a novel Man-6-P-OCH$_3$ phosphomethylidiester (13). Cells metabolically labeled with [methyl-3H]methionine produced labeled oligosaccharides that were essentially identical to those labeled with [3H]Man (19). Here, we confirmed that the acid-stable phosphodiesterases previously described on the oligosaccharides of three frequently studied lysosomal enzymes is also Man-6-P-OCH$_3$ (11, 29). Very little of the Man-6-P occurs as either an acid-labile diester or a monoester in these molecules.

Protein, nucleic acid, and carbohydrate methylation are well known (30), but phosphomethylation has only been previously described in these oligosaccharides and in small nuclear RNA where it occurs as a 5' end cap on GTP (33). Identification of the *Dictyostelium* phosphate methyltransferase activity is the first enzyme of its kind and appears to be selective for Man-6-P. Fructose-1-P, which is a close structural analogue of Man-6-P (31), and glucose-6-P appear to be much weaker acceptors, but neither of these inhibits the methylation of the preferred oligosaccharide substrates even at 25 mM.

The strongest evidence supporting the role of the methyltransferase in phosphorylated oligosaccharide biosynthesis is its marked preference for methyl group transfer to specific

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**Fig. 8. Schematic drawing of an N-linked oligosaccharide and locations of known Man-6-P-OCH$_3$ residues and favored locations of phosphate methylation.** The boxed branches (I and III) and highlighted residues on this oligosaccharide indicate that these are favored locations for phosphorylation and methylation as determined by previous studies (13, 34) and the results presented here using model acceptors. Other residues may also be phosphorylated, but this has not been demonstrated.

**TABLE III**

| Fraction        | Protein | Methyltransferase | GlcNAc-1-P transferase | cAMP phosphodiesterase | Alkaline phosphatase |
|-----------------|---------|-------------------|------------------------|------------------------|----------------------|
|                  | mg      | %                 | pmol/mg/h$^a$          | %                      | pmol/mg/h$^a$        | %                    |
| $10,000 \times g$ pellets | 173     | 25                | 53                     | 15                     | 181                  | 19                   | 2,473                | 29                   | 36                   |
| $100,000 \times g$ pellets | 85      | 12                | 264                    | 85                     | 654                  | 81                   | 5,847                | 34                   | 33                   |
| $100,000 \times g$ supernate | 438     | 63                | 0                      | 0                      | 10,683               | 37                   | 28                   | 30                   |

$^a$ Specific activity.
Phosphate Methyltransferase in Dictyostelium

The function of phosphorylated oligosaccharides in lysosomal enzyme targeting in Dictyostelium is an unanswered question, primarily because there are no mutants strains that lack all Man-6-P. Many mutants show a partial loss of Man-6-P or a partial/complete loss of oligosaccharide sulfation, and still properly target their lysosomal enzymes, albeit more slowly. No phosphomannosyl receptor that binds phosphomethylated oligosaccharides has been identified (38), but similar studies have not yet been done with non-methylated intermediates. The similarities in substrate specificity of mammalian and Dictyostelium GlcNAc phosphotransferase are striking. Both recognize the same positions on the oligosaccharide acceptors and add the first and second GlcNAc-1-P residues to the same corresponding branches, respectively. They differ in that mammalian phosphotransferase recognizes the peptide portion of mammalian lysosomal enzymes, but the Dictyostelium enzyme does not. However, it is not known whether the Dictyostelium transferase recognizes the peptide portion of Dictyostelium lysosomal enzymes. As shown here, the methyltransferase has a strong preference for the known positions of phosphate groups. Based on these specificities and the fact that 98% of the Man-6-P occurs as Man-6-P-OCH$_3$ and not as GlcNAc-P-Man diester or Man-6-P monooester in lysosomal enzymes, it seems likely that phosphorylation/methylation are tightly coupled in vegetative cells. Typical N-linked oligosaccharide processing pathways usually produce variable amounts of the partially processed intermediates (40). It is very uncommon for 98% of the molecules to be converted into fully mature forms. The results found in Dictyostelium could imply that the reactions all occur without ever releasing the intermediates, such as a single multienzyme complex, or, alternatively, that the proper function of the phosphate group, whatever it is, requires methyl-diester covers. Now that the pathway is known, it will be possible to examine its regulation during development and to isolate mutants that are blocked at different steps in the pathway.

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**REFERENCES**

1. Dahms, N. M., Lobel, P., and Kornfeld, S. (1989) *J. Biol. Chem.* 264, 12115–12118
2. Kornfeld, S., and Mellman, I. (1989) *Annu. Rev. Cell Biol.* 5, 483–525
3. Lee, S.-J., and Nathans, D. (1988) *J. Biol. Chem.* 263, 3521–3527
4. Herzog, V., Neumuller, W., and Holzmann, B. (1987) *EMBO J.* 6, 555–560
5. Puchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., Kovacina, K. S., Roth, R. A., and Marquardt, H. (1988) *J. Biol. Chem.* 263, 14211–14215
6. Todderud, G., and Carpenter, G. (1988) *J. Biol. Chem.* 263, 17893–17896
7. Von Figura, K., and Hasilik, A. (1986) *Annu. Rev. Biochem.* 55, 167–193
8. Baranski, T. J., Faust, P. L., and Kornfeld, S. (1990) *Cell* 63, 281–291
9. Couso, R., Lang, L., Roberts, R. M., and Kornfeld, S. (1986) *J. Biol. Chem.* 261, 6526–6531
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10. Varki, A., and Kornfeld, S. (1981) J. Biol. Chem. 256, 9937–9943
11. Freeze, H. H., Yeh, R., Miller, A. L., and Kornfeld, S. (1983) J. Biol. Chem. 258, 14874–14879
12. Freeze, H. H., and Wolgast, D. (1986) J. Biol. Chem. 261, 127–134
13. Gabel, C. A., Costello, C. E., Reinhold, V. N., Kurz, L., and Kornfeld, S. (1984) J. Biol. Chem. 259, 13762–13769
14. Freeze, H. H., Yeh, R. Y., and Miller, A. L. (1983) J. Biol. Chem. 258, 8928–8933
15. Freeze, H. H. (1985) J. Biol. Chem. 260, 8857–8864
16. Hoflack, B., and Kornfeld, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4426–4432
17. Hoflack, B., Fugimoto, K., and Kornfeld, S. (1987) J. Biol. Chem. 262, 123–129
18. Lang, L., Couso, R., and Kornfeld, S. (1987) J. Biol. Chem. 261, 6320–6325
19. Freeze, H. H., and Wolgast, D. (1986) J. Biol. Chem. 261, 135–141
20. Ebelt, D. A., Bush, J., Dimond, R. L., and Cardelli, J. A. (1989) Arch. Biochem. Biophys. 273, 479–486
21. Bush, J. M., and Cardelli, J. A. (1989) J. Biol. Chem. 264, 7630–7636
22. Townsend, R. R., Hardy, M. R., Hindsgaul, O., and Lee, C. L. (1988) Anal. Biochem. 174, 459–470
23. Srivastava, O. P., and Hindsgaul, O. (1986) Carbohydr. Res. 155, 57–72
24. Srivastava, O. P., and Hindsgaul, O. (1987) Carbohydr. Res. 161, 196–210
25. Srivastava, O. P., and Hindsgaul, O. (1986) Can. J. Chem. 64, 2324–2330
26. Srivastava, O. P., and Hindsgaul, O. (1987) J. Org. Chem. 52, 2869–2875
27. Hohmann, H. P., Gerisch, G., Lee, R. W., and Huttner, W. B. (1985) J. Biol. Chem. 260, 13869–13878
28. Padh, H., Lavasa, M., and Steck, T. L. (1989) J. Cell Biol. 108, 865–874
29. Freeze, H. H., Koza-Taylor, P., Saunders, A., and Cardelli, J. A. (1989) J. Biol. Chem. 264, 19278–19286
30. Usdin, E., Borchardt, R. T., and Creveling, C. R. (1979) Transmethylation, pp. 1–631, Elsevier/North-Holland, Inc., New York
31. Kaplan, A., Achord, D. T., and Sly, W. S. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 2026–2030
32. Sharkey, D. J., and Kornfeld, S. (1991) J. Biol. Chem. 266, 18477–18484
33. Singh, R., and Reddy, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8280–8283
34. Couso, R., Van Halbeek, H., Reinhold, V., and Kornfeld, S. (1987) J. Biol. Chem. 262, 4521–4527
35. Madiyalakan, R., Jain, R. K., and Matta, K. L. (1987) Biochim. Biophys. Res. Commun. 142, 354–358
36. Oyama, M., Maeda, Y., and Takeuchi, I. (1984) Protoplasma 123, 152–159
37. Takemoto, K., Yamamoto, A., and Takeuchi, I. (1985) J. Cell Sci. 77, 93–108
38. Cardelli, J. A., and Dimond, R. L. (1988) in Protein Transfer and Organelle Biogenesis, pp. 363, Academic Press, New York
39. Judelson, H. S., and Dimond, R. L. (1988) Arch. Biochem. Biophys. 267, 151–157
40. Kornfeld, S., and Kornfeld, R. (1985) Annu Rev. Biochem. 54, 651–664