Biosynthesis of Methylphosphomannosyl Residues in the Oligosaccharides of Dictyostelium discoideum Glycoproteins

EVIDENCE THAT THE METHYL GROUP IS DERIVED FROM METHIONINE*

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The phosphorylated oligosaccharides of Dictyostelium discoideum contain methylphosphomannosyl residues which are stable to mild-acid and base hydrolysis (Gabel, C. A., Costello, C. E., Reinhold, V. N., Kurtz, L., and Kornfeld, S. (1984) J. Biol. Chem. 259, 13762–13769). Here we present evidence that these methyl groups are derived from [methyl-3H]methionine, in vitro and [methyl-3H]S-adenosylmethionine in vitro. About 18% of the macromolecules secreted from vegetative cells labeled with [methyl-3H]methionine are released by digestion with preparations of endoglycosidase/peptide N-glycosidase F. The majority of the released molecules are sulfated, anionic high mannose-type oligosaccharides. Strong acid hydrolysis of the [3H]methyl-labeled molecules yields [3H]methanol with kinetics of release similar to those found for the generation of Man-6-P from chemically synthesized methylphosphomannose methylglycoside. Treatment of the [3H]methyl-labeled molecules with a phosphodiesterase from Aspergillus niger which is known to cleave this phosphodiester also releases [3H]methanol from a portion of the oligosaccharides. In vitro incorporation of [methyl-3H]S-adenosylmethionine into endogenous acceptors found in membrane preparations shows that the [3H]methyl group of the methylphosphomannose residues can be derived from this molecule.

The N-linked oligosaccharides of mammalian lysosomal enzymes contain residues of Man-6-P which are synthesized in a two-step process. First, GlcNAc-1-P is transferred from UDP-GlcNAc to selected Man residues to form a mild acid-labile phosphodiester (Man-6-P-GlcNAc), and second the GlcNAc residue is rapidly removed by a specific glycosidase to generate Man-6-P in monooester linkage. Both of these reactions occur in the Golgi and are essential for the transport of these newly synthesized enzymes to the lysosome (reviewed in Ref. 1).

The lysosomal enzymes of the slime mold Dictyostelium discoideum contain anionic N-linked oligosaccharides many of which are sulfated and also carry Man-6-P (2). These residues occur almost exclusively in an acid-stable phosphodiester different from the mammalian type (2). This phosphodiester has recently been identified as Man-6-P methyl ester (Man-6-P-OCH3) (3). The physiological function and the route of synthesis of this molecule are unknown, but it is likely that the phosphate and methyl groups are added after cotranslational glycosylation of the proteins (4). Therefore, these modifications probably occur during the transit of the proteins from the endoplasmic reticulum to the Golgi or to the lysosome (4). The sulfation of the oligosaccharides of α-mannosidase is known to occur in the Golgi (4).

Most of the known methyltransferase reactions use S-adenosylmethionine as a methyl group donor (reviewed in Ref. 5). Since methionine is converted to S-adenosylmethionine in a single step, [methyl-3H]Met is often used as a convenient precursor for the in vitro labeling of a variety of methylated molecules (5).

In this report we have examined the in vitro incorporation of [methyl-3H]Met into phosphorylated oligosaccharides derived from secreted glycoproteins and of [methyl-3H]AdoMet into endogenous acceptors. The results suggest that AdoMet can serve as a direct or indirect source of this methyl group.

EXPERIMENTAL PROCEDURES*

RESULTS

Labeling and Endo/PNGase F Digestion of Secreted Macromolecules—Cells of D. discoideum were grown in the presence of [methyl-3H]Met for 3 days and allowed to secrete lysosomal enzymes and other proteins into the culture medium (6). The secreted macromolecules were treated with Endo/PNGase F to release the susceptible N-linked oligosaccharides (7), and chromatographed on Sephadex G-50. As shown in Fig. 1, nearly 18% of the total [3H] label was released by this digestion. The released material occurred in two regions of the column. The major peak (R-1) coincided with the distribution of a similar enzymatic digestion [3H]Man-labeled secreted macromolecules (8) (not shown), and the second region (R-2) had an apparent size between a Man, GlcNAc oligosaccharide and a monosaccharide.

The total cell material from this labeling was similarly analyzed. Less than 3% of the total label was released in the R-1 region (data not shown). Thus, the secreted macromole-

* The abbreviations used are: AdoMet, S-adenosylmethionine; Endo/PNGase F, endoglycosidase preparation from Flavobacterium meningosepticum which contains endoglycosidase and peptide N-glycosidase activities; SDS, sodium dodecy1 sulfate; TCA, trichloroacetic acid; Man-6-P-OCH3, methylphosphomannosyl residue; HPLC, high performance liquid chromatography; Endo, endoglycosidase.

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2 Experimental Procedures are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2152, cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Experimental Procedures were digested with Endo/PNGase F and which required at least pool R-2 contained about 60% anionic molecules, most of totally stable to this treatment. The removal of sulfate esters molecules were considerably enriched in the Endo/PNGase F released radioactivity.

General Characterization of the R-1 and R-2 Fractions (Not Shown)—Greater than 98% of pool R-1 was anionic while pool R-2 contained about 60% anionic molecules, most of which required at least 200 mM NaCl for elution from QAE-Sephadex. About 38% of the R-2 molecules were cationic as shown by binding to Dowex 50.

About 56% of $^3H$ in R-1 was bound to ConA-Sepharose. This was increased to >90% by solvolysis, a procedure which is known to remove sulfate esters without breaking glycosidic linkages (2, 9). The $^3H$ label derived from [methyl-$^3H$]Met is totally stable to this treatment. The removal of sulfate esters also increased ConA-Sepharose binding of $^3H$-labeled oligosaccharides. R-2 did not interact with ConA-Sepharose either before or after solvolysis. We conclude that the R-1 molecules are high mannose-type, anionic oligosaccharides some of which are sulfated. Pool R-2 appears not to be typical of high mannose-type oligosaccharides and was not extensively characterized beyond this point.

Strong acid hydrolysis of the R-1 pool followed by HPLC analysis showed that none of the $^3H$ chromatographed with Man, fucose, or GlcNac. Therefore, the $^3H$ was not incorporated into the ring structure of the sugars themselves. Anion exchange HPLC resolves the R-1 oligosaccharides into molecules with 1-6 negative charges. No neutral oligosaccharides were found.

Kinetics of Strong Acid Hydrolysis and Identification of the Products—The Man-6-P-OCH$_3$ diester found in secreted glycoproteins is insensitive to phosphatase digestion and requires strong acid hydrolysis to generate a phosphatase-sensitive form (3).

If the [H]methyl label is incorporated into the Man-6-P-OCH$_3$, strong acid hydrolysis should produce Man-6-P and [H]methanol as products. Furthermore, it should be stable to base in contrast to more common methyl esters such as those found in carboxylic acids (3, 11). In fact, it is stable to 0.1 N NaOH for 4 h at 37 °C. Only 15% is degraded by 1 N NaOH at 80 °C for 10 h (data not shown).

The kinetics of acid-catalyzed hydrolysis of [H]Man-6-P-OCH$_3$ shows that 4 h of hydrolysis in 1 N HCl at 100°C is required to achieve >95% conversion of the diester to the monoester form, which is then fairly stable. The [H]methyl-labeled oligosaccharides were subjected to similar acid hydrolysis conditions and the loss of $^3H$ quantified by evaporation in the presence of a non-volatile internal standard ($^{14}$C) mannose. The results are shown in Fig. 2. About 90% of the label was hydrolyzed with a half-life of 95 min, and longer time points (not shown) of up to 24 h gave no further loss of $^3H$ label. This half-life was significantly longer than that of the chemically synthesized Man-6-P-OCH$_3$ ($t_{1/2}$ = 55 min). To determine whether this difference was due to the presence of a glycosidic linkage in sugars bearing the methyl group, the $\alpha$-methylglucoside of Man-6-OCH$_3$ was synthesized and the kinetics of its conversion to Man-6-P were measured (Fig. 2). The $T_{1/2}$ was calculated to be 66 min. Although the presence of the glycosidic linkage may influence the rate of hydrolysis slightly, it does not appear to totally account for the difference seen. Furthermore, when the oligosaccharides are desulfated by solvolysis prior to strong acid hydrolysis, the $t_{1/2}$ of [H] methyl-labeled oligosaccharides was still 95 min. It seems unlikely that the presence of the sulfate esters affects the rate of hydrolysis of the [H]methyl groups. The difference in the rate of hydrolysis may depend on structure of the oligosaccharide itself compared to the monosaccharide standard, Man-6-P-OCH$_3$. It is unlikely that the difference is due to an isotope effect ($^3H$ versus $^1H$) since this would only alter the rate by only ~3% (12). A similar acid treatment of the molecules from the R-2 region of the column indicated that only about 10% were labile and that the remainder was stable up to 9 h of hydrolysis.

To confirm that the $^3H$ label in the R-1 region was actually present in a methyl ester, the production of [H]methanol was confirmed via HPLC, as shown in Fig. 3. Note that the kinetics of the appearance of the product were the same as
FIG. 3. HPLC identification of [3H]MeOH from released oligosaccharides following acid hydrolysis. The R-1 released oligosaccharides (O) were hydrolyzed as described under "Experimental Procedures" and the resulting products were mixed with 600 cpm of [14C]MeOH (●) and injected on a C18 reversed phase HPLC column and eluted with 0.5 M NaH2PO4 buffer at pH 4.3 at a flow rate of 1 ml/min. Fractions were taken at 0.25-min intervals and counted immediately. The top panel shows the profile of unhydrolyzed sample and the middle and lower panels show the results of samples taken at 105 and 230 min of hydrolysis, respectively. See Fig. 2 for kinetics of appearance of [3H]MeOH. The arrow (↑) denotes the breakthrough volume.

FIG. 4. Digestion of oligosaccharides with one and two charges with A. niger phosphodiesterase. Oligosaccharides labeled with either [3H]Met or [3H]Man were treated by solvolysis to remove sulfate esters and then fractionated into molecules with one or two negative charges on QAE-Sephadex. The individual oligosaccharides were then digested with phosphodiesterase for various periods of time and then, in the case of the [3H]Man-labeled oligosaccharides, chromatographed on QAE-Sephadex or mixed with [3H]MeOH and evaporated for the [3H]Methyl-labeled molecules. The [3H]Man-6-P-OCH3 diester can be cleaved by a phosphodiesterase

The results of a time course of incorporation of [3H]AdoMet into crude membranes are shown in Fig. 5. Much of the label is incorporated into unknown molecules which loose the label on prolonged incubation. Mild base treatment which is sufficient to destroy carboxymethyl esters (11) shows that most of
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FIG. 5. Time course of incorporation of [methyl-3H]AdoMet into trichloroacetic acid-precipitable material. The crude membranes were incubated for various periods of time with [methyl-3H] AdoMet and treated as described under "Experimental Procedures," to determine the total (○), acid-stable (●), base-labile (▲), and base-stable (△) components.

The [3H]methyl is probably incorporated into such molecules. The label is also stably incorporated into molecules which are resistant to this base treatment. These products could include methylated amino acids and the Man-6-P-OCH₃ of the oligosaccharides. Strong acid hydrolysis (1 N HCl, 100 °C) sufficient to destroy Man-6-P-OCH₃, showed that a portion of the label was also stably incorporated into an acid-stable fraction. The incorporation was dependent on time and protein concentration and was totally abolished by 0.1% Triton X-100 and 1 mM non-labeled AdoMet (data not shown).

Subcellular Fractionation—The crude membranes were further fractionated using a step gradient of increasing sucrose concentrations, and the transferase activities measured in each fraction. N-Acetylglucosaminidase and α-glucosidase II served as markers of the lysosomal and rough endoplasmic reticulum fractions, respectively (4). The highest specific activity of transfer into ConA-Sepharose bindable material was associated with light density membranes which have been identified by others as Golgi (4) (Fig. 6).

Characterization of the in Vitro Products Released by Endo/PNGase F Digestion—About 5% of the total ³H could be released by Endo/PNGase F (Fig. 7). Most of this material was found in the R-2 region (see Fig. 1) which did not bind to ConA-Sepharose. The remainder (R-1) appeared to run in a position somewhat smaller than the oligosaccharides released by a similar digestion of labeled in vivo oligosaccharides. The products labeled in vitro from the R-1 region were both neutral and anionic and about 50% bound to ConA-Sepharose (not shown). Acid hydrolysis of the Endo/PNGase F material released volatile ³H (presumably methanol) with kinetics similar to those seen for the oligosaccharides synthesized in vivo (Fig. 8). Therefore, these molecules appeared to be the same as those labeled with [methyl-³H]Met in vivo. However, when the ConA bound oligosaccharides were analyzed by QAE-Sephadex chromatography, only non-sulfated species seemed to have been acceptors. This is shown by the fact that solvolysis treatment of the unfraccionated pool changed the QAE-Sephadex profile only slightly.

Based on these results we conclude that AdoMet can serve as a donor of the methyl group donor of the methyl phosphodiester in an in vitro assay. At this point, however, we do not know whether AdoMet is the immediate donor to a Man-6-P already located on the oligosaccharide at the time of methyl transfer or whether the methyl group is transferred together with phosphate.

DISCUSSION

The results of in vivo labeling presented here indicate that [methyl-³H]Met can donate a methyl group to the Man-6-P-OCH₃ containing oligosaccharides released by Endo/PNGase F digestion. These conclusions are based on the released by Endo/PNGase F digestion, binding to ConA-Sepharose, HPLC analysis of anionic species, kinetics of acid hydrolysis, base stability, release of ³Hmethanol, and sensitivity to A. niger phosphodiesterase (3). A very large proportion of the ³H incorporated into secreted macromolecules (90%) appears to be found in the methylphosphomannosyl residues. Although we do not know the absolute amounts synthesized during the labeling period, other data suggests that this phosphodiester may be plentiful on many secreted glycoproteins (8). The remaining 10% of the ³H was not found in the ring structure of the sugars themselves or in neutral oligosaccharides, but it is possible that some could be present as O-methyl ethers or as N-methyl substitutions of GlcN. Studies are in progress to answer these questions. These characterizations have been done only on the R-1 fractions (Fig. 1).

The nature of the R-2 fraction has not been investigated thoroughly, but its ability to be released by Endo/PNGase F suggests that it is derived from a GlcNAc-Asn linkage typical of N-linked oligosaccharides. The majority appears to be highly anionic and the insensitivity to strong acid hydrolysis suggests that it must be unrelated to the methylphosphomannosyl residues. A reasonable possibility is that the methyl group is present as an O-methyl ether or as an N-methyl-GlcN. Since the R-2 appears to be the major product of in vitro labeling using [³H]AdoMet, it is clearly an interesting and perhaps important molecule(s). In other labeling experiments using [2-³H]Man, [6-³H]GlcN, and ³⁵SO₄, small amounts of label are also found in the R-2 region. This would be consistent with a highly processed, charged, N-linked oligosaccharide. Previous results have shown that oligosaccharide processing α-mannosidases are not active in the vegetative state of Dictiostelium (16), although the lysosomal α-mannosidase is present at appreciable amounts and could degrade some oligosaccharides prior to secretion of the proteins.

³H. H. Freeze, unpublished observations.
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1.1 1.3 1.5
Sucrose Layer (M)

FIG. 6. Distribution of in vitro methylation products in cellular fractions. Crude membranes were fractionated on discontinuous sucrose gradients as described under "Experimental Procedures." Each fraction was then incubated with [methyl-3H]AdoMet for 1 h and the products were analyzed directly (D), after base treatment in 0.1 N NaOH at 37°C for 2 h (E and F), or acid treatment in 1 N HCl for 4 h at 100°C (G), or after digestion with Pronase and analysis on ConA-Sepharose (H). Each panel shows the percentage of the total counts having the indicated characteristic. N-Acetylglucosaminidase (B) and α-glucosidase (A) served as markers of the lysosomes and rough endoplasmic reticulum, respectively (4). The numbers on the bottom of the figure refer to the sucrose concentrations at the top and bottom of interphases of each fraction.

FIG. 7. Sephadex G-50 analysis [methyl-3H]AdoMet products released by Endo/PNGase F digestion. Crude membranes (650 µg) were incubated with 20 µCi of [methyl-3H]AdoMet at 0.65 µM for 4 h at 22°C and the products were precipitated with 9 volumes of cold acetone, dissolved in 1% sodium dodecyl sulfate and chromatographed on Sephadex G-50 to isolate the macromolecules. They were again precipitated with acetone, resolubilized, and treated with Endo/PNGase F and then chromatographed on the column to isolate the released oligosaccharides. The pools of R-1 and R-2 are indicated by the bars.

The in vitro incorporation of a portion of the 3H from [methyl-3H]AdoMet into a fraction which binds to ConA-Sepharose shows that AdoMet can serve as a donor for this reaction. We do not know if it is the immediate donor nor do we know if the synthesis of the methylphosphodiester occurs as a single or multi-step reaction. Of the several possible routes of synthesis, none would appear to be of the type seen for the mammalian lysosomal enzymes (1). Phosphorylation does not occur cotranslationally at the time of initial glycosylation (4). One possibility is that the phosphomethyl group is donated to the appropriate Man residues on an oligosaccharide in a single step. This would require the participation of a high energy donor such as a methylphosphodiester of ATP, although such a compound is not known to exist. In other experiments the inclusion of 1 mM ATP or [γ-32P]ATP did not stimulate incorporation of 3H into ConA bindable in vitro products or incorporation of 32P, respectively.

Alternatively, methylation of a Man-6-P residue already present on the oligosaccharide may occur. The initial phosphorylation might proceed through kinase-type reaction specific for an oligosaccharide. If an oligosaccharide kinase exists, the subsequent methyltransferase reaction must be extremely efficient since <2% of all of the oligosaccharides with Man-6-P are found in phosphomonoester linkage (8).

Yet another possibility is that the methylation of the phosphate displaces another group already in phosphodiester linkage. The use of methylation inhibitors would allow us to distinguish between these possibilities based on the presence of phosphate and its state of esterification; unfortunately, none of the eight inhibitors we tried were effective. No mutant strains of Dictyostelium lacking Man-6-P have been isolated, although many post-translational modification mutants have been screened in an enzyme-linked immunosorbent assay using the mammalian Man-6-P receptor and an antibody against it. Thus, the route of biosynthesis of this unusual phosphodiester remains unknown.

The cell fractionation studies suggest that oligosaccharide methylation occurs in the Golgi where the sulfation of α-mannosidase is known to occur (4). The inhibition of this reaction by 0.1% Triton X-100 suggests, but does not prove,
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Further work will be required to know if modifications of the oligosaccharides are causally related to the fate of the proteins bearing these unusual structures. A large number of mutant strains which are altered in the secretion, packaging, and developmental regulation of a variety of lysosomal enzymes will play a central role in answering this question (10, 21).

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EXPERIMENTAL PROCEDURES

Methods

Growth and Labeling of Cells. Strain AS-3 was used for all studies and it was grown in RM-5 medium as previously described (1). Cells in 25 ml of medium were labeled with [3H]methionine for 3 days and the cells and medium were harvested as described (8).

Release and Purification of Oligosaccharides. The secreted macromolecules were isolated by Sephadex G-50 chromatography in the presence of SDS and digested with Endo-Mannanase as described previously (6). The released oligosaccharides were collected and used as described.

September 191A-2X-3G and 14C-Methionine. [14C]methionine (1.5MBq-1) was a generous gift of Dr. Marc Kibrick and was radiolabeled by phosphorylation of [14C]methionine. Approximately 260,000 cpm of the [14C]methionine in 100 ml of methionine-containing medium was used for the experiment. The labeled [3H]methionine was taken at 1, 3, 5, 7, and 10 min after addition of the disaccharide to the amoebae. The culture was collected after 5 min with an overall yield of 1.4 MBq-1 (0.14 mCi). Repurification on Sephadex G-50 yielded an alkaline phosphatase-resistant fraction with a single radioactive charge and eluted from Sephadex G-200 with 1M NaCl. The disaccharide was stable and was converted to the phosphorylated species after the day as 1.0%.

The [14C]methionine was precipitated by addition of 1.0% of 1.0N hydrochloric acid and 0.5% of 1.0N sodium hydroxide and 0.5% hydrochloric acid (8). The sample was dissolved in 1% buffer and used as an internal marker to the incubated samples.

Preparation of Acid Phosphatase. The labeled oligosaccharides (1000 cpm) were mixed with approximately 200 cpm of [14C]methionine and hydrolyzed with 0.1 ml of 1.0N HCl at 100°C for various periods of time. The sample was then evaporated three times with 2 ml of 0.01 M HCl and then the sample was dissolved in 0.4 ml of water and counted. The residual [14C]methionine in the original sample was 0.4-0.5% of the input counts.

Base Hydrolysis. The sample was exposed to several different conditions previously described as described above. The conditions were: 0.16 M NaOH for 2 min at 3°C; 16 M NaOH for 19°C for up to 18 h.

Identification of [14C]methionine Following Acid Hydrolysis of the [3H]Releaser Oligosaccharides. The released oligosaccharides were hydrolyzed in 1M HCl as described above. At various times 2000 cpm of the samples were removed and placed on ice and then resuspended, mixed with 1000 cpm of 1.0M NaOH and injected into a 2500ml Versapak CIB reversed phase HPLC column (Alltech Associates) equilibrated in 0.10 HEPES pH 7.4 buffer, and fractions of 0.5 ml were taken at a flow rate of 1.0 cm/min at the same location buffer. HPLC buffer was added immediately to the samples and then counted.

Dissolution of Oligosaccharides in Phosphatase Digestion. Oligosaccharides labeled with [3H]methionine or [14C]methionine were dissolved in water and then digested with Aspergillus sp. Phosphatase. Oligosaccharides labeled with 2 or 3 negative charges were separated and individually treated with Aspergillus sp. Phosphatase (generously provided by Stuart Kibrick, Washington University School of Medicine, and with 1MBq-1 [3H]methionine, as previously described (3)). The low level of activity made it possible to carry out the incubations for a long period of time. At each of the time points, the individual fractions were chromatographed on Sephadex G-50 and the relative amount of radioactivity remaining in the respective fractions was measured.

Preparation of Cell-Free Extracts and Fractionation of Cell Components. Cells (50ng/ml) were grown to about 5.1 10^6/ml as described above and broken according to the method of Wierendorf et al. (4). In the second cell extract, a concentration of 3.5 10^6 cells/ml using a tight fitting Dounce homogenizer. The cell-free extract was obtained by using 100 10^6/g as 5 ml in an Eppendorf mixing buffer for 20 min through a 4.5 10^6/ml of 0.1M sucrose to 700 filter. The membrane was collected from the top of a 1.5 10^6/ml of a 2.1M sucrose and diluted to about 0.2M sucrose.

The beads were fractionated on a discontinuous sucrose gradient as described (4). The diethyl sucrose was labeled on gradient which consisted of 2.5-3.14 10^6/ml of 1.0M, 1.5M, 1.0M, and 0.2M sucrose to 700 filter. The sucrose gradients for the various assays, [3H]lysozyme (13M), and [3H]phosphatase, were used as markers of the lysosomes and the rough, respectively.

In Vivo Assay of Membrane Transferase. The methyl transferase activity of the homogenate, crude and homogenate membranes, and 1.0M sucrose were determined by diluting the preparations in 5.5 10^6/ml in protein and up to 5.5 10^6/ml were added to additional 0.2M sucrose buffer to the volume of 5.5 10^6/ml. Two micrograms of [14C]methylated specific activity (activity/10^6/ml) to 10^6/ml was added to the indicated samples for the indicated times. The samples were treated with 5.5 10^6/ml of 0.1M TCA and then removed and treated with 0.1M TCA and were washed with various amounts of [3H]methylated specific activity (activity/10^6/ml) to 10^6/ml was added to the indicated samples for the indicated times. The samples were then removed and washed with 0.1M TCA and then removed and treated with 0.1M TCA and were washed with 0.1M TCA.