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Partial Purification and Characterization of β-Mannosyltransferase from Suspension-Cultured Soybean Cells†

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ABSTRACT: The β-mannosyltransferase that catalyzes the synthesis of Man-β-GlcNAc-GlcNAc-PP-dolichol from GDP-mannose and dolichyl-PP-GlcNAc-GlcNAc was solubilized from microsomes of suspension-cultured soybean cells by treatment with 1.5% Triton X-100 and was purified about 700-fold by chromatography on DEAE-cellulose, hydroxylapatite, and a GDP affinity column. The purified enzyme was reasonably stable in the presence of 20% glycerol and 0.5 mM dithiothreitol. The enzyme required either detergent (Triton X-100 or NP-40) or phospholipid for maximum activity, but the effects of these two were not additive. Thus, either phosphatidylcholine or Triton X-100 could give maximum stimulation. In terms of phospholipid stimulation, both the head group and the acyl chain appeared to be important since phosphatidylcholines with 18-carbon unsaturated fatty acids were most effective. The purified enzyme had a sharp pH optimum of 6.9–7.0 and required a divalent cation. Mg^{2+} was the best metal ion with optimum activity occurring at 6 mM, but Mn^{2+} was reasonably effective while Ca^{2+} was slightly stimulatory. The K_m for GDP-mannose was calculated to be 1.7×10^{-6} M and that for dolichyl-PP-GlcNAc-GlcNAc about 9×10^{-6} M. The enzyme was inhibited by a number of guanosine nucleotides such as GDP-glucose, GDP, GMP, and GTP, but various uridine and adenosine nucleotides were without effect. The purified enzyme was apparently free of α-1,3-mannosyltransferase (and perhaps other mannosyltransferases) and dolichyl-P-mannose synthase since the only product seen from dolichyl-PP-GlcNAc-GlcNAc and GDP-mannose was Man-β-GlcNAc-GlcNAc-PP-dolichol. No activity was observed when dolichyl-P-mannose replaced the GDP-mannose.

It is now well established that lipid-linked saccharides play an intermediate role in the biosynthesis of the oligosaccharide chains of the asparagine-linked glycoproteins of eucaryotic cells (Struck & Lennarz, 1980; Elbein, 1979). Thus, a large number of studies have been done with membrane preparations from various cells and tissues in order to follow the incorporation of GlcNAc, mannose, and glucose into the various

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lipid-linked oligosaccharides (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). Such experiments have resulted in an understanding of the sequence of reactions that lead to the formation of the final lipid-linked oligosaccharide $Glc_3Man_9(GlcNAc)_2$-diphosphodolichol. However, in spite of our knowledge about the sequence of reactions, we know relatively little about the individual enzymes that catalyze these reactions. Such information is of considerable importance in order to understand how this pathway is regulated in the cell and also to understand how these membrane-bound transferases interact with other membrane components.

In animal cells, the GlcNAc-1-P transferase that catalyzes the first step in the lipid-linked saccharide pathway, i.e., the formation of dolichyl-PP-GlcNAc, has been solubilized and partially purified from several different sources (Heifetz et al., 1979; Keller et al., 1979; Plouhar & Bretthauer, 1983; Villemez & Carlo, 1980). Unfortunately, this enzyme has thus far proven to be too unstable for extensive purification, and therefore, studies on possible regulation are difficult. Nevertheless, experiments with a microsomal enzyme preparation from chick retina showed that the formation of dolichyl-PP-GlcNAc was stimulated 7–10-fold by the addition of dolichyl-P-mannose to incubation mixtures (Kean, 1982). Similar results were obtained with the partially purified GlcNAc-1-P transferase from pig aorta (Kaushal & Elbein, 1985). This enzyme is also stimulated by phospholipids (Plouhar & Bretthauer, 1983), and this could also represent a mechanism of regulation.

Besides the GlcNAc-1-P transferase, the mannosyltransferase that forms dolichyl-P-mannose (dolichyl-P-mannose synthase) has been purified extensively from yeast (Haselbeck & Tanner, 1982) and from rat liver microsomes (Jensen & Schutzbach, 1985). In addition α-1,2-mannosyltransferase (Schutzbach et al., 1980) and α-1,3-mannosyltransferase (Jensen & Schutzbach, 1981) were purified extensively from liver. Recently, we purified the β-mannosyltransferase from aorta and examined the properties of this enzyme. Thus far, there is no evidence that any of the mannosyltransferases are involved in regulation although their inhibition by guanosine nucleotides might exert some control on the lipid-linked saccharide pathway (Grant & Lennarz, 1983). In this paper, we describe the 700-fold purification of the β-mannosyltransferase from soybean cells grown in culture. The purified enzyme is fairly stable under appropriate conditions and therefore should be amenable to a variety of studies. Various properties of this enzyme are described including its stimulation by phospholipids and its inhibition by a variety of guanosine nucleotides.

Experimental Procedures

Materials. GDP-[U-^{14}C]mannose (282 mCi/mmol) and UDP-[6-^{3}H]GlcNAc (20 Ci/mmol) were purchased from New England Nuclear Co., while GDP-[U-^{14}C]glucose (263 mCi/mmol) was from International Chemical and Nuclear Co. Bio-Beads SM-2, Bio-Gel P-4 (200–400 mesh), and hydroxylapatite were from Bio-Rad Laboratories, and DE-52 was obtained from Whatman Chemical Separation, Ltd. Periodate-oxidized GDP, adipic acid, hydrazine hydrate, sugar nucleotides, nucleotides, phospholipids, Triton X-100, NP-40, sodium deoxycholate, octyl β-glucoside, cyanogen bromide activated Sepharose, and dolichyl phosphate were purchased from Sigma Chemical Co., and dodecyl β-maltoside was from Calbiochem. Synthetic phosphatidylcholines were from Avanti Polar Lipids, Inc. β-Mannosidase was purified from *Aspergillus fumigatus* as previously described (Elbein et al., 1977). All other chemicals were from reliable chemical sources and were of the best grade available.

Preparation of Plant Membrane Fraction. Soybean (Glycine max., L) cells were grown in 500-mL Erlenmeyer flasks, each containing 100 mL of B-5 medium (Chu & Lark, 1976) for 7 days at 28 °C. The composition of this medium was as described in the reference. The cells were harvested by filtration and washed with 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, containing 8% sucrose, 0.5% poly(vinylpyrrolidone), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, and 0.5 mM dithiothreitol. The cells were then suspended in the same buffer (0.5 g of cells/mL), placed in an ice bucket, and disrupted by sonic oscillation for 3–5 min. The lysate was centrifuged at 3000*g* to remove large particles, and the supernatant liquid was centrifuged at 105000*g* for 1 h to obtain the membrane fraction.

Solubilization of the β-Mannosyltransferase. The microsomal fraction obtained as described above was suspended in 20 mM Tris buffer, pH 7.0, containing 20% glycerol and 0.5 mM dithiothreitol and treated with Triton X-100, at a final concentration of 1.5%. The suspension was kept in ice for 15 min with occassional mixing in a Teflon homogenizer. The suspension was then centrifuged for 60 min at 105000*g*, and the supernatant liquid was gently removed and used as the solubilized enzyme for purification studies as described under Results. All preparations were done at 0 °C, unless otherwise stated.

Preparation of Dolichyldiphospho-GlcNAc-GlcNAc and Man-β-GlcNAc-GlcNAc-diphosphodolichol. The dolichyldiphospho-GlcNAc-GlcNAc that was used as the substrate for the β-mannosyltransferase was prepared according to a previously described procedure (Kaushal & Elbein, 1986). The Man-β-GlcNAc-GlcNAc-diphosphodolichol was prepared by incubating the partially purified β-mannosyltransferase with dolichyldiphospho-GlcNAc-GlcNAc and GDP-mannose, in the assay system described below. The product, containing Man-β-$(GlcNAc)_2$-PP-dolichol, was isolated by solvent extraction in $CHCl_3$–CH_3OH (1:1) and saponified in $CHCl_3$–CH_3OH–H_2O (10:10:3) containing 0.1 N NaOH at 37 °C for 15 min to destroy contaminating phospholipids. At the end of this time, the mixture was adjusted to a $CHCl_3$–CH_3OH–H_2O mixture of 1:1:1 by the addition of water, and the lower layer and interface were removed, concentrated to dryness, and redissolved in $CHCl_3$–CH_3OH–H_2O (10:10:3). To this fraction, a small amount of radioactive (20 000 cpm) dolichyl-PP-$(GlcNAc)_2$, prepared as previously described (Kaushal & Elbein, 1986b), was added as a marker. The mixture (in 10:10:3) was applied to a DEAE-cellulose (in acetate) column that had been previously equilibrated with $CHCl_3$–CH_3OH–H_2O (10:10:3). The column was washed with this solvent, and the lipids were eluted with a 0–0.2 M gradient of ammonium formate in the same solvent. The Man-β-$(GlcNAc)_2$-PP-dolichol emerged at about 0.12 M ammonium formate. This lipid was further purified by thin-layer chromatography on silica gel plates in $CHCl_3$–CH_3OH–NH_4OH–H_2O (65:35:4:4).

Assay of β-Mannosyltransferase. The dolichyl-PP-$(GlcNAc)_2$ (1.4 μg) was added to assay tubes in $CHCl_3$–CH_3OH (1:1), and the solvent was removed under a stream of air. Then, 100 μL of Triton X-100 (0.2%) was added, and the lipids were dispersed by sonication. Then, 8 mM $MgCl_2$, 20 mM Tris buffer, pH 7.0, and 0.1 mL of enzyme solution were added, and the final volume was adjusted to 0.4 mL with water. The reaction was initiated by the addition of 0.025 μCi of GDP-[^{14}C]mannose. The tubes were incubated for 30 min. The reactions were stopped by the addition of 1 mL of $CHCl_3$,

1 mL of CH_3OH, and 0.6 mL of water, and the mixtures were shaken vigorously. The phases were separated by centrifugation, and the lower phase was removed and saved. The upper layer and interface were reextracted with another 1 mL of $CHCl_3$. After thorough mixing, the phases were separated by centrifugation, and the lower phase was combined with the lower layer from the first extraction. This $CHCl_3$ layer contained the Man-β-$(GlcNAc)_2$-PP-dolichol, and an aliquot of this fraction was taken for the determination of radioactivity.

When the effects of phospholipids were measured, the phospholipids were added to the assay tubes in an organic solvent (usually hexane) along with the dolichyl-PP-$(GlcNAc)_2$. The solvent was removed under a stream of air (or nitrogen), and 0.1 mL of water (rather than detergent) was added. The other reaction components were added, but not the enzyme, and the mixtures were sonicated to disperse the lipids. The dimyristoyl, dipalmitoyl, and distearoyl derivatives of phosphatidylcholine were dispersed in an ultrasonic bath that was maintained above the phase transition temperatures of the lipids, i.e., 35, 45, and 60 °C, respectively. After the lipids had been dispersed, the enzyme and GDP-mannose were added to initiate the reactions.

Assay of α-Mannosyltransferases. The dolichyl-PP-$(GlcNAc)_2$-β-Man was used as the acceptor of mannose from GDP-mannose for the estimation of α-mannosyltransferases in the various fractions from the DEAE column. The other reaction components and incubations were as described for the β-mannosyltransferase assay. The reaction was initiated by the addition of 0.1 μCi of GDP-[^{14}C]mannose, and the formation of products was measured as described for β-mannosyltransferase.

Preparation of Periodate-Oxidized GDP–Adipic Acid Hydrazide–Sepharose. Cyanogen bromide activated Sepharose 4B was coupled with adipic acid dihydrazide as described (Wilcheck & Lamed, 1974). Dihydrazide bound by the Sepharose was tested with the trinitrobenzenesulfonic acid reagent (Habeeb, 1966). Periodate-oxidized GDP (dial-GDP) was then coupled to the dihydrazide–Sepharose. The amount of GDP bound to the Sepharose was about 5 μmol/g of Sepharose as determined by the absorbency at 262 nm. The affinity column was washed with 500 mM NaCl before being equilibrated with buffer.

Analytical Methods. Protein was measured by the method of Bradford with bovine serum albumin as the standard (Bradford, 1976). Lipid-linked saccharides were subjected to mild acid hydrolysis in 0.02 N HCl in 20% methanol at 100 °C for 20 min. The reaction mixture was washed with $CHCl_3$, and the aqueous layer was removed, concentrated to dryness, and chromatographed on Bio-Gel P-4 columns to identify the oligosaccharides. These columns were run in 0.5% acetic acid and were standardized with the following oligosaccharides: $Glc_3Man_9(GlcNAc)_2$, $Man_9(GlcNAc)_2$, $Man_7(GlcNAc)_2$, $Man_5(GlcNAc)_2$, $Man_3(GlcNAc)_2$, $Man_2(GlcNAc)_2$, Man-$(GlcNAc)_2$, GlcNAc-GlcNAc, and GlcNAc.

RESULTS

Purification of β-Mannosyltransferase. The solubilized enzyme fraction (72 mL) was applied to a DE-52 column as described in Figure 1. The β-mannosyltransferase emerged in the 100 mM NaCl elution in fractions 42–60. A peak of α-mannosyltransferase that added α-linked mannose residues from GDP-mannose to Man-β-$(GlcNAc)_2$-PP-dolichol and larger lipid-linked oligosaccharides emerged in the 50 mM NaCl fraction. Although much of this activity was clearly separated from the β-mannosyltransferase, there was some overlap as seen in the figure.

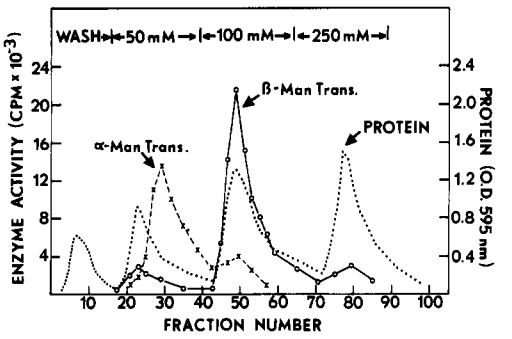

FIGURE 1: Chromatography of β-mannosyltransferase on DEAE-cellulose. The solubilized enzyme was applied to a 2 × 20 cm column of DE-52 that had been equilibrated with 20 mM Tris buffer, pH 7.0, containing 20% glycerol, 0.1% Triton X-100, and 0.5 mM dithiothreitol. The column was washed with this buffer, and enzyme was eluted batchwise with 150 mL of a solution of 50, 100, 250, and 400 mM NaCl in the same buffer. Six-milliliter fractions were collected, and 0.1-mL aliquots were assayed for β-mannosyltransferase (O) and α-mannosyltransferase (×) as described in the text. Protein (···) was measured by the method of Bradford.

The fractions containing the β-mannosyltransferase activity were pooled, concentrated to about 20 mL on an Amicon filtration apparatus using a UM-10 filter, and dialyzed overnight against 1 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 0.1% Triton X-100, and 0.5 mM dithiothreitol. This enzyme preparation was then applied to a 2.5 × 10 cm column of hydroxylapatite that had been equilibrated with 1 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 0.1% Triton X-100, and 0.5 mM dithiothreitol. The column was washed with 100 mL of buffer and eluted with 200 mL of a linear gradient of 0–0.2 M potassium phosphate buffer containing the glycerol and other components. The β-mannosyltransferase activity emerged at 80–100 mM concentration of buffer (data not shown). The active fractions were pooled, concentrated to about 15 mL on the Amicon apparatus, and dialyzed overnight against 1 mM potassium phosphate buffer containing glycerol and other components as described above.

The above enzyme fraction was applied to another hydroxylapatite column (2 × 10 cm) that had been equilibrated with the potassium phosphate buffer that contained glycerol, Triton X-100, and dithiothreitol. After the column was washed with the same buffer, the β-mannosyltransferase activity was eluted with 200 mL of a linear gradient of 1–100 mM potassium phosphate in the same buffer solution. The enzyme emerged at about 60–80 mM concentration of potassium phosphate (data not shown). Active fractions were pooled, concentrated to about 15 mL with the Amicon apparatus, and dialyzed overnight against 10 mM Tris buffer, pH 7.0, containing 20% glycerol, 0.1% Triton X-100, and 0.5 mM dithiothreitol.

The concentrated enzyme from the second hydroxylapatite column was adjusted to 5 mM with respect to $MgCl_2$ and applied to the dial-GDP–adipic acid hydrazide–Sepharose column that had been equilibrated with 10 mM Tris buffer, pH 7.0, containing 20% glycerol, 5 mM $MgCl_2$, 0.1% Triton X-100, and 0.5 mM dithiothreitol. Figure 2 shows the elution profile of this column. After the column was washed with 50 mL of the buffer, the enzyme was eluted with a linear gradient (0–0.5 M) of NaCl in the same buffer. The enzyme eluted at about 0.3–0.35 M NaCl. Active fractions were pooled, dialyzed, and kept in ice for further use.

By the use of the above procedures, the β-mannosyltransferase was purified about 730-fold with a recovery of about 53%. The summary of the purification data is presented

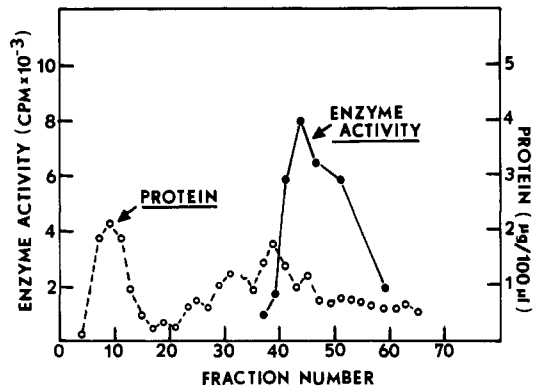

FIGURE 2: Purification of β-mannosyltransferase on GDP affinity columns. The dialyzed hydroxylapatite fraction was applied to a 1 × 10 cm column of dial-GDP–adipic acid dihydrazide–Sepharose that had been equilibrated with 10 mM Tris buffer, pH 7.0, containing 20% glycerol, 0.1% Triton X-100, 0.5 mM dithiotreitol, and 5 mM $MgCl_2$. The column was washed with this buffer and eluted with 200 mL of 0–0.5 M NaCl in the same buffer. Three-milliliter fractions were collected and assayed for enzyme activity (●) and protein (○) as described.

Table I: Purification of Mannosyltransferase

purification step[a]	total protein (mg)	total activity (units)[b]	sp act. (units/mg)	yield (%)
microsomes	457	5481	11.9	100
solubilized	273	5391	19.7	98
DEAE-cellulose	14.2	6312	443	115
hydroxylapatite I	3.8	4776	1243	87
hydroxylapatite II	0.84	3408	4104	62
dial-GDP–Sepharose	0.34	2936	8764	54

[a] In the reaction mixture of microsomal and solubilized enzyme preparations, 0.2 mM AMP was added to prevent the degradation of GDP-mannose. The other incubations were as described in the text. [b] A unit of enzyme is defined as that amount of enzyme that catalyzes the transfer of 1000 cpm of [^{14}C]mannose from GDP-[^{14}C]mannose into Man-β-GlcNAc-GlcNAC-PP-dolichol in a 30-min incubation.

in Table I. During the purification, especially at the DEAE-cellulose step, the β-mannosyltransferase was separated from α-mannosyltransferase, which catalyzed the formation of $Man_2(GlcNAc)_2$-PP-dolichol and $Man_3(GlcNAc)_2$-PP-dolichol from $Man(GlcNAc)_2$-PP-dolichol. Thus, the purified β-mannosyltransferase appeared to be free of α-mannosyltransferases, as well as the enzyme that catalyzes the synthesis of dolichyl-P-mannose. The β-mannosyltransferase, at the final stage of purity, was stable in ice for at least 1 week when kept in Tris buffer, pH 7.0, containing 20% glycerol, 0.1% detergent, and 0.5 mM dithiothreitol. It was also stable for at least 1 month when kept in the frozen state in this same buffer. The β-mannosyltransferase was examined by native gel electrophoresis to determine its state of purity. The most purified fraction showed two or three bands on these gels, but it is not known whether any of these bands represent the β-mannosyltransferase.

Properties of β-Mannosyltransferase. The formation of Man-β-$(GlcNAc)_2$-PP-dolichol from GDP-[^{14}C]mannose and dolichyl-PP-*N*,*N*′-diacetylchitobiose by the purified enzyme was linear with time of incubation for at least 20 min and was also proportional to enzyme concentration up to about 5 μg of protein (data not shown). As shown in Figure 3, the enzyme showed an almost absolute requirement for divalent cation. The best divalent metal ion was Mg^{2+}, and optimum activity with this metal was observed at a concentration of 6 mM. Other metal ions, such as Mn^{2+}, Ca^{2+}, and Co^{2+}, were also effective but considerably less so than Mg^{2+}. Zn^{2+} and Hg^{2+}

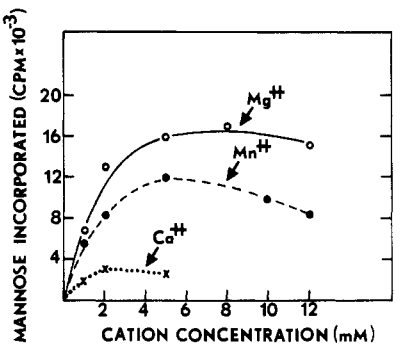

FIGURE 3: Effect of divalent cations on β-mannosyltransferase activity. Incubations were as described in the text except that various amounts of the divalent cations shown in the figure (i.e., Mg^{2+}, Mn^{2+}, or Ca^{2+}) were added. Other reaction components were added, and 6 μg of the hydroxylapatite enzyme was used. The formation of Man-GlcNAc-GlcNAc-PP-dolichol was determined as described in the text.

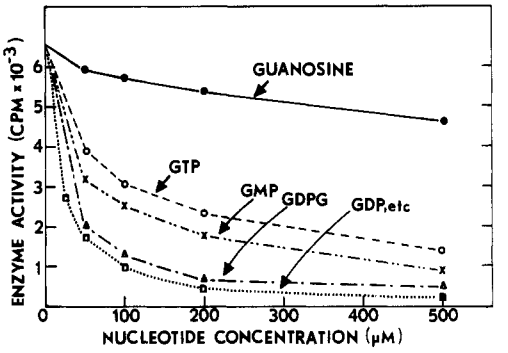

FIGURE 4: Effect of various guanosine nucleotides on β-mannosyltransferase activity. Incubations were as described in the text, except that increasing amounts of the guanosine nucleotides shown in the figure were added. The reactions were started by the addition of 6 μg of the hydroxylapatite enzyme and GDP-[^{14}C]mannose, and the product was measured as described in the text.

were strongly inhibitory at concentrations of 100 μM, even in the presence of optimum concentrations of Mg^{2+}. The enzyme was quite sensitive to high ionic strength such that the activity was diminished by 50% at 200 mM NaCl, KCl, or Tris.

The effect of pH of the reaction mixture on the formation of Man-β-$(GlcNAc)_2$-PP-dolichol was examined over the range of 5.5–8.0 with both 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer and Tris–acetate buffer. With either buffer, the enzyme showed a sharp optimum at pH 6.9–7.0, and activity fell off rapidly at higher and lower pH values (data not shown). The enzyme was found to be quite unstable at pH values far removed from this optimum. Thus, all experiments were done at pH values of 7.0.

The effect of substrate concentration on the reaction was also examined. The rate of the reaction increased with increasing concentration of GDP-mannose to about 1.8 or 2 μM, and when the data were plotted according to the method of Lineweaver and Burk, the apparent K_m was estimated to be 1.7×10^{-6} M (data not shown). The effect of concentration of dolichyl-PP-GlcNAc-GlcNAc was also examined. This reaction was run at 2 μM concentrations of GDP-mannose. The formation of product increased with increasing concentrations of GlcNAc–lipid to about 8 μM, and the apparent K_m was estimated to be 9×10^{-6} M (data not shown).

Effect of Guanosine Nucleotides on β-Mannosyltransferase. A number of nucleotides and sugar nucleotides were examined for their ability to inhibit the β-mannosyltransferase. Among the nucleotides examined, only guanosine nucleotides showed any effect as demonstrated in Figure 4. Enzyme activity was

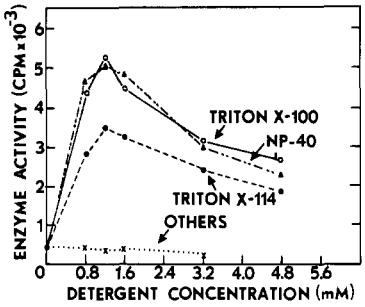

FIGURE 5: Effect of detergent concentration on activity of β-mannosyltransferase. The enzyme was freed of detergent by gentle shaking with Bio-Beads. Various amounts of the detergents shown were added to assay tubes, followed by the other reaction ingredients. The detergent-free hydroxylapatite enzyme (6 μg) was used, and assays were as described.

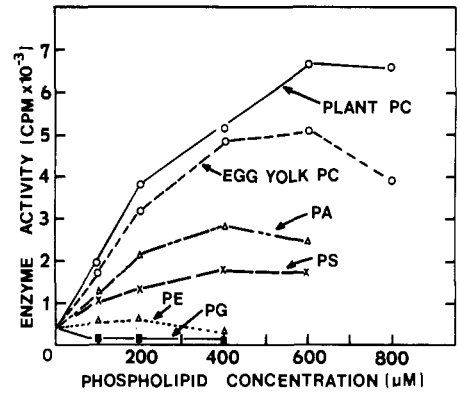

FIGURE 6: Effects of phospholipids on activity of detergent-free β-mannosyltransferase. Incubation mixtures were as described in the text except that various amounts of the phospholipids shown in the figure were added. The phospholipids were sonically dispersed before being added to the incubation tubes. The other reaction components were added, followed by 6 μg of detergent-free hydroxylapatite enzyme. The reaction was initiated by the addition of GDP-[^{14}C]mannose, and the formation of product was measured as described before. Phospholipids are as follows: PC = phosphatidylcholine; PA = phosphatidic acid; PS = phosphatidylserine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol.

maximally inhibited by periodate-oxidized GDP with 50% inhibition occurring at 20–40 μM concentrations of this nucleotide. GDP-glucose and GDP were equally effective, whereas other nucleotides such as GTP and GMP also inhibited but required higher concentrations to achieve 50% inhibition. Guanosine was only slightly effective, whereas UDP-glucose, UDP-mannose, ADP-glucose, ADP-mannose, ATP, ADP, CTP, CDP, CMP, UMP, UDP, and TTP were without effect.

The inhibition caused by GDP-glucose was examined in more detail to determine the type of inhibition. Thus, concentration curves of one of the substrates, GDP-mannose, were run at three different concentrations of the inhibitor, GDP-glucose. When the data were plotted by the method of Lineweaver and Burk, the inhibition was seen to be of the competitive type (data not shown). Similar results were obtained with GDP.

Requirements for Detergent by β-Mannosyltransferase. Since the partially purified enzyme appeared to be strongly stimulated by detergent, it was of interest to determine which detergents would stimulate or inhibit the enzymatic activity. The Triton X-100 that was present in the enzyme preparation was removed by shaking the enzyme gently with Bio-Beads as previously described (Holloway, 1973). This treatment lowered the Triton concentration to about 0.018%, which is near the critical micelle concentration of about 0.015%. In addition, the Triton concentration was diluted 10-fold more in the reaction mixtures, and therefore, the final detergent concentration was probably not more than 0.002% before the addition of other detergents.

Various detergents were tested for their effect on the enzymatic activity as shown in Figure 5. Little or no enzymatic activity was observed in the absence of detergent (or upon removal of most of the detergent as described above). However, most of the activity could be recovered by the addition of certain detergents as shown in the figure. Thus, Triton X-100 showed a strong stimulatory effect with optimum activity being observed at about 0.08–0.1% concentrations (1.2–1.6 mM). NP-40, another nonionic detergent, was equally as good as was Triton X-100, while Triton X-114 was not nearly as effective. A number of other nonionic detergents were found to be without effect, including octyl β-glucoside and dodecyl maltoside. Sodium dodecyl sulfate was also ineffective at stimulating the β-mannosyltransferase. We are not certain whether this stimulation by detergent is due to a direct effect on the enzyme or because detergent alters the physical state of the acceptor substrate, or both. When detergent is removed from the enzyme, the enzyme gradually aggregates, and after 3–4 h, all of the activity is found in the precipitate (after centrifugation). More than 80% of this activity can be recovered when the precipitate is extracted with 0.1% Triton X-100 containing 20%g glycerol, 20 mM Tris buffer, and 0.5 mM dithiothreitol.

Phospholipid Stimulation of β-Mannosyltransferase. More than 95% of the activity of the β-mannosyltransferase was lost when the Triton X-100 was removed from the purified enzyme. Although this activity could be restored by the addition of Triton X-100, we were interested in knowing whether phospholipids could completely replace this detergent requirement. Thus, the effect of various concentrations of a number of phospholipids on restoring enzymatic activity was examined as shown in Figure 6. In these experiments, the detergent-free enzyme was used, and the phospholipids were dispersed by sonication in the assay mixture before the addition of enzyme and GDP-mannose. It can be seen that both plant and egg yolk phosphatidylcholines were quite effective at stimulating the enzymatic activity, although the plant phospholipid appeared to be slightly more effective. Phosphatidylserine and phosphatidic acid also showed some stimulatory effect, but they were much less effective than the phosphatidylcholines. On the other hand, no stimulation was seen with various concentrations of phosphatidylethanolamine or phosphatidylglycerol. The product of the reaction catalyzed by the phosphatidylcholine-stimulated enzyme was also examined by gel filtration and found to be Man-β-GlcNAc-GlcNAc-PP-dolichol.

The specificity of the phospholipid stimulation was further examined by use of a number of synthetic phosphatidylcholines that contained fatty acids of different lengths and/or different degrees of unsaturation. The effects of these various phosphatidylcholines are shown in Figure 7. As seen previously, the naturally occurring plant phosphatidylcholine was quite effective at stimulating the enzymatic activity. However, the synthetic phosphatidylcholines having 18-carbon-length fatty acids with one or two unsaturations were more effective than the natural phospholipid. On the other hand, the phosphatidylcholine that had saturated 18-carbon fatty acids was not effective at stimulating mannosyltransferase, nor were those molecules that had 14-carbon- or 16-carbon saturated fatty acids. These results indicate that both the polar head group and the fatty acid chains of the phospholipid have a distinct

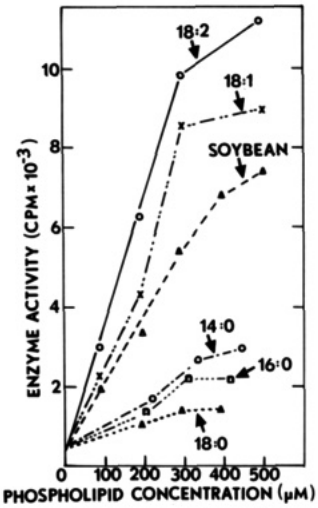

FIGURE 7: Effect of synthetic phosphatidylcholines on activity of β-mannosyltransferase. In order to determine whether fatty acid structure played any role in activity, various synthetic phosphatidylcholines having the fatty acid compositions shown were added to incubation mixtures and tested with the detergent-free mannosyltransferase. Incubation mixtures and assays were as described in Figure 8.

influence on the enzyme activity.

Since the activation by phospholipids seemed fairly specific, the stimulation may be due to lipid–protein interactions. We attempted to show such an interaction between phosphatidylcholine vesicles and the β-mannosyltransferase by gel filtration on Sephracryl S-500 columns or Sephadex G-200 columns. Unfortunately, we were not able to detect enzymatic activity on these columns and are therefore unable to state whether phospholipids interact with enzyme, with substrate, or with both. However, we did extract the most highly purified enzyme preparation with $CHCl_3$–CH_3OH to determine whether this preparation contained phospholipids. Upon thin-layer chromatography in $CHCl_3$–CH_3OH–20% methylamine (65:36:10), two spots were observed corresponding to phosphatidylcholine and phosphatidic acid standards. However, there was not sufficient material available for quantitation. Since the mannosyltransferase was not homogeneous, we cannot be certain that phospholipid is associated with this protein nor of the number of phospholipid molecules associated with each protein molecule.

Characterization of Reaction Product and Identification of the Mannosyl Donor. In order to identify the product formed by the purified mannosyltransferase, the enzyme was incubated with dolichyl-PP-GlcNAc-GlcNAc and GDP-[^{14}C]mannose, and lipid-linked saccharides were extracted with $CHCl_3$–CH_3OH as described above. Chromatography of the $CHCl_3$–CH_3OH extract on thin-layer plates as shown in Figure 8 (lane B) revealed a single radioactive spot that corresponded in mobility to authentic Man-β-GlcNAc-GlcNAc-PP-dolichol. On the other hand, when GDP-[^{14}C]-mannose was replaced with dolichyl-P-[^{14}C]mannose as the mannosyl donor, no radioactivity was seen in the Man-$(GlcNAc)_2$-PP-dolichol area (lane A), and the only radioactive spot corresponded to the dolichyl-P-mannose. These data indicate that the mannosyl donor for the β-linked mannose is GDP-mannose rather than dolichyl-P-mannose.

In order to further characterize the product formed in this reaction, the lipid-linked saccharides extracted from the above incubation mixtures (i.e., either with GDP-mannose or dolichyl-P-mannose as mannosyl donor) were subjected to mild acid hydrolysis to release the sugars or oligosaccharides, and the aqueous phase was chromatographed on columns of Bio-Gel P-4. The results are shown in Figure 9. As shown by profile A, when GDP-[^{14}C]mannose was used as the mannosyl donor, only a single radioactive oligosaccharide was detected,

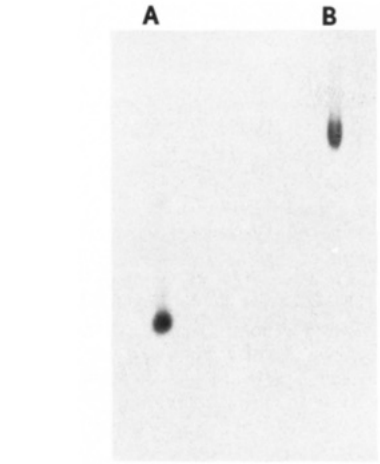

FIGURE 8: Thin-layer chromatographic identification of product produced by purified β-mannosyltransferase. The enzyme from hydroxylapatite or the more purified fraction from GDP–Sepharose columns was incubated with dolichyl-PP-$(GlcNAc)_2$ and GDP-[^{14}C]mannose, and the radioactive product was isolated by solvent extraction. This product was spotted on plates of Kieselgel 60F_{254} (0.5 mm thick) and run in $CHCl_3$–CH_3OH–NH_4OH–H_2O. Lane B shows the single labeled product when this plate was exposed to Agfa film for 3 days. Lane A shows the results obtained when dolichyl-P-[^{14}C]mannose was used in place of GDP-mannose. The spot in lane A corresponds to authentic dolichyl-P-mannose while that in lane B corresponds to Man-GlcNAc-GlcNAc-PP-dolichol.

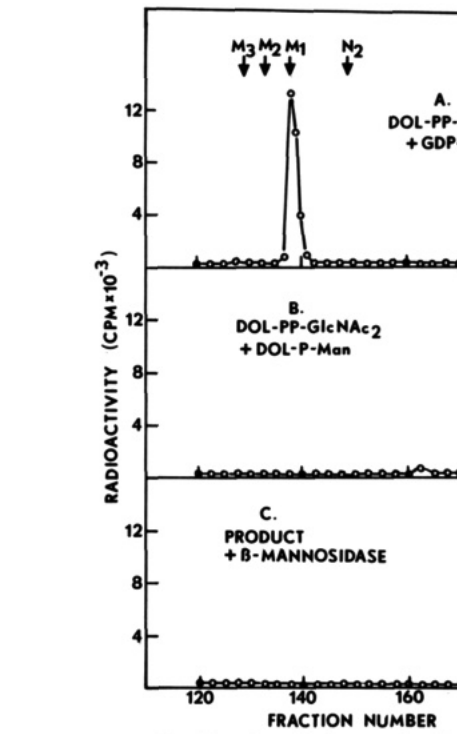

FIGURE 9: Identification of oligosaccharide products by chromatography on columns of Bio-Gel P-4. The lipid produced by the hydroxylapatite enzyme from GDP-mannose and dolichyl-PP-$(GlcNAc)_2$ was hydrolyzed in acid, and the aqueous phase was identified on Bio-Gel P-4 (profile A). In profile B, the lipid that resulted when GDP-mannose was replaced by dolichyl-P-mannose was hydrolyzed and run on the same column. In profile C, the oligosaccharide obtained in profile A was incubated with fungal β-mannosidase, and the product was identified on the column. Standards shown at the top are as follows: M_3 = $Man_3GlcNAc$-GlcNAc; M_2 = $Man_2GlcNAc$-GlcNAc; M_1 = Man-GlcNAc-GlcNAc; N_2 = GlcNAc-GlcNAc; M = mannose.

and this corresponded in migration to that of Man-β-GlcNAc-GlcNAc. On the other hand, when dolichyl-P-mannose was used as the mannosyl donor, no radioactivity appeared in the Man-β-GlcNAc-GlcNAc, and instead, all of the radioactivity was in free mannose (profile B). The product obtained from GDP-mannose and dolichyl-PP-$(GlcNAc)_2$ was susceptible to digestion by fungal β-mannosidase with the release of all of the radioactivity as free mannose (profile C). However, the trisaccharide (M_1) was not hydrolyzed by treatment with jack bean α-mannosidase, indicating that the mannose was attached in a β-glycosidic linkage. The fact that no larger sized oligosaccharides were produced in these incubation indicates that the β-mannosyltransferase is free of the α-mannosyltransferase that adds the next mannose residue (presumably in 1,3-linkage) to form $Man_2(GlcNAc)_2$-PP-dolichol.

Discussion

The synthesis of trisaccharide–lipid has previously been reported in particulate preparations from cotton fibers (Forsee & Elbein, 1975), mung bean seedlings (Lehle et al., 1976; Forsee et al., 1976; Hori & Elbein, 1982), and developing cotyledons of peas (Beevers & Mense, 1977). However, that enzyme has not previously been solubilized, purified, or characterized from any plant source, nor has its mannosyl donor been identified. On the other hand, the β-mannosyltransferase was partially purified from yeast (Sharma et al., 1982) and also from pig aorta (Kaushal & Elbein, 1986). In this paper, we describe the solubilization and 700-fold purification of the β-mannosyltransferase from suspension-cultured soybean cells. This purified enzyme preparation catalyzed the formation of a single product identified as Man-β-GlcNAc-GlcNAc-PP-dolichol from dolichyl-PP-GlcNAc-GlcNAc and GDP-[^{14}C]mannose. These data indicate that this enzyme requires GDP-mannose as the mannosyl donor rather than dolichyl-P-mannose. Earlier studies (Levy et al., 1974; Chen & Lennarz, 1976) using microsomal enzyme fractions had suggested that the mannosyl donor for this β-linked mannose was GDP-mannose. In addition, the mannosyl donor for the addition of the first four α-linked mannose residues has been shown to be GDP-mannose (Chambers et al., 1977; Chapman et al., 1980; Jensen et al., 1980).

In general, the problem with purifying the glycosyltransferases that participate in the lipid-linked saccharide pathway has been one of stability of these enzymes. The β-mannosyltransferase described here could be stabilized by the addition of 20% glycerol and 0.5 mM dithiothreitol to the solutions. Under these conditions, the enzyme could be kept in ice for at least a week with almost no loss in activity and could also be stored for some time in the frozen state. Thus, these conditions rendered the purification of the enzyme feasible. Similar conditions were found to be effective for the β-mannosyltransferase from aorta, although that enzyme was only purified about 100-fold. Also like the aorta enzyme, the soybean mannosyltransferase showed an almost absolute requirement for divalent cation, with Mg^{2+} being by far the best metal ion. However, Mn^{2+} was almost without effect on the aorta enzyme, whereas the plant enzyme is still strongly stimulated by Mn^{2+}.

During the purification of the β-mannosyltransferase, Triton X-100 was included in the elution buffers and was present during storage of the enzyme, usually at about 0.1%. Removal of the detergent with Bio-Beads resulted in the loss of most of the activity. However, this activity could be regained by the addition of the detergents Triton X-100 or NP-40, as well as by the addition of the phospholipid phosphatidylcholine. Interestingly enough, the stimulation or activation by phospholipid was not additive over that with detergent, and in fact, enzyme activity was inhibited when the detergent to phospholipid ratio was about 1:2, but was almost unaffected when the ratio was 4:1. These effects on activity may be the result of lipid–protein interactions that lead to changes in enzyme conformation, or they may be due to gross changes in membrane fluidity that affect activity.

The stimulation of activity by phospholipid was fairly specific since only egg yolk and plant phosphatidylcholine gave a significant stimulatory effect, while phosphatidic acid and phosphatidylserine were slightly stimulatory and phosphatidylethanolamine and phosphatidylglycerol were without effect. Other glycosyltransferases such as UDP-glucuronosyltransferase (Mitranic et al., 1983) also showed relatively specific preference for phosphatidylcholine. In addition, we found that not only was the polar head group important for activation of the β-mannosyltransferase but the nature of the acyl group was also of some significance. Thus, synthetic phosphatidylcholines having 18-carbon fatty acids with one or two unsaturations were the most effective stimulators, whereas those molecules with 18-, 16-, or 14-carbon saturated fatty acids were considerably less effective or inactive. Studies with the bovine milk galactosyltransferase also indicated that the nature of the acyl chains of the lipid played a secondary, but definite, role on the activity.

At any rate, these observations suggest that the β-mannosyltransferase is somewhat similar to other glycosyltransferases such as GlcNAc-1-P transferase (Plouhar & Bretthauer, 1982; Kaushal & Elbein, 1986a,b) and α-1,3-mannosyltransferase (Jensen & Schutzbach, 1982), whose activities are modulated by specific phospholipids. However, the GlcNAc-1-P transferase was stimulated by phosphatidylglycerol or a mixture of phosphatidylglycerol and phosphatidylcholine, whereas α-1,3-mannosyltransferase was stimulated by phosphatidylethanolamine. Such results indicate that these enzymes are anchored in membranes by a specific lipid-binding domain, but whether such a binding domain plays any role in regulation remains to be established.

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Characterization of Maturation-Activated Histone H1 and Ribosomal S6 Kinases in Sea Star Oocytes†

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ABSTRACT: DEAE-Sephacel chromatography of cytosolic extracts from sea star oocytes resolved at least two distinct peaks of maturation-activated protein kinase activity, each of which catalyzed the phosphorylation of histone H1, ribosomal protein S6, and Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA), a synthetic peptide based on the sequence of a phosphorylation site in the latter protein. The first peak (elution conductivity $\simeq$ 6 mmho) contained the major activated kinase with respect to the phosphorylation of histone H1, and the second peak (elution conductivity $\simeq$ 10.5 mmho) contained the major activated kinase with respect to the phosphorylation of S6 and RRLSSLRA. These kinase activities were barely detectable in extracts from immature oocytes. The major stimulated histone H1 kinase exhibited an apparent M_r of $\sim$90 000 on Sephacryl S-300 but eluted from TSK-400 with an apparent M_r of $\sim$10 000. After DEAE-Sephacel fractionation, this kinase was shown to utilize both ATP (apparent $K_m \simeq 45$ μM) and GTP (apparent $K_m \simeq 10$ μM), although the V_{max} was 8-fold higher with ATP than with GTP. The enzyme phosphorylated histone H1 with an apparent $K_m \simeq 50$ μg/mL. Its properties resembled those of the growth-associated histone kinase. The major stimulated RRLSSLRA kinase had an apparent M_r of $\sim$84 000 on Sephacryl S-300 and $\sim$40 000 on TSK-400. After DEAE-Sephacel chromatography, this kinase selectively utilized ATP (apparent $K_m \simeq 25$ μM). The kinase activities in both DEAE-Sephacel peaks were inhibited by NaF, $MnCl_2$, $CaCl_2$, zinc acetate, *N*-ethylmaleimide, trifluoperazine, and chlorpromazine, but the major stimulated RRLSSLRA kinase (peak at $\sim$10.5 mmho) was less sensitive than the major stimulated histone H1 kinase (peak at $\sim$6 mmho) to inhibition by β-glycerol phosphate, NaCl, pyrophosphate, quercetin, and heparin. The RRLSSLRA kinase (peak at $\sim$10.5 mmho) may be related to the mitogen-activated S6 kinase detected in mammalian and avian cells.

Protein phosphorylation may be instrumental in the orchestration of molecular events that facilitate progression through the cell cycle. In particular, chromosomal condensation and other nuclear changes during mitosis have been temporally linked with extensive phosphorylation of histone H1 subtypes (3–6 mol of phosphate/mol of H1) and histone H3. A chromatin-associated, cAMP-independent protein kinase (or kinases) detected in nuclear extracts of proliferating cells may be responsible for the cell cycle dependent phosphorylation of histone H1 (Lake & Salzman, 1972; Schlepper & Knippers, 1975; Langan, 1978b; Paulson & Taylor, 1982;

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