cAMP-mediated protein phosphorylation of microsomal membranes increases mannosylphosphodolichol synthase activity*

(N-linked glycoprotein/dolichol monophosphate/glycosyltransferase/neurotransmitter regulation)

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ABSTRACT We have investigated the possible role of a cAMP-mediated protein-phosphorylation event(s) as the key regulatory mechanism in β-adrenoreceptor-stimulated activation of mannosylphosphodolichol (Man-P-Dol) synthase (GDP-mannose:dolichyl-phosphate O-β-n-mannosyltransferase, EC 2.4.1.83) in rat parotid acinar cells. Microsomal membranes isolated from these cells pretreated with 10 μM isoproterenol for 60 min showed ~40–80% enhanced Man-P-Dol synthase activity compared to the untreated controls. This change in enzyme activity was not associated with a significant alteration in apparent Km for GDP-mannose, but the Vmax was enhanced 2-fold. When microsomal membranes isolated from control cells were phosphorylated in vitro by a cAMP-dependent protein kinase, an increase in Man-P-Dol synthase activity, similar to that with membranes from isoproterenol-treated cells, was observed (i.e., a moderate change in Km for GDP-mannose but a 2-fold higher Vmax). Furthermore, treatment of in vitro phosphorylated microsomal membranes by alkaline phosphatase led to a substantial reduction in Man-P-Dol synthase activity. Increased Man-P-Dol synthesis (~30–40%) was also observed in bovine brain and hen oviduct microsomal membranes after in vitro protein phosphorylation. In aggregate, these results strongly suggest that agents that increase cAMP in cells may modulate protein N-glycosylation in those cells by activating the only glycosyltransferase of the dolichol pathway by a cAMP-dependent protein kinase-mediated protein phosphorylation/dephosphorylation cycle.

The biosynthesis of oligosaccharides and their attachment to protein asparagine residues is a multistep process that has received considerable attention in the last decade (1). The individual enzymes in this cascade pathway have been documented (2–6), but information concerning mechanism(s) by which cells may regulate the extent of N-linked protein glycosylation is relatively limited. However, several reports have suggested that extracellular signaling may contribute to the regulation of events leading to protein N-glycosylation (7–11).

Earlier we demonstrated (12) that β-adrenoreceptor stimulation markedly enhances protein N-glycosylation through a cAMP-mediated mechanism in rat parotid acinar cells. This appears to be due to increased enzymatic transfer of N-acetylgalactosamine 1-phosphate, mannose (Man), and glucose from their respective nucleotides to dolichol monophosphate (Dol-P) (13). Since isoproterenol, a β-adrenergic agonist, activates cAMP-dependent protein kinases (14–16), it was hypothesized that enhancement of glycosyltransferase activities seen in microsomal membranes from cells pretreated with isoproterenol may be due in part to a cyclic AMP-dependent protein kinase-mediated phosphorylation of these enzymes.

In work reported here, we tested this hypothesis by utilizing an in vitro phosphorylating system and found evidence supporting protein phosphorylation as a likely mechanistic event leading to enhanced mannosylphosphodolichol (Man-P-Dol) synthase (GDP-mannose:dolichyl-phosphate O-β-n-mannosyltransferase, EC 2.4.1.83) activity as a consequence of β-adrenoreceptor stimulation.

MATERIALS AND METHODS

Three-month-old male Wistar rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Collagenase (CLSPLA, lot S2C481) was purchased from Worthington. Bovine testicular hyaluronidase (type I-S), bovine serum albumin (fraction V), isoproterenol, dithiothreitol, dimethylsulfoxide (Me2SO), ATP (Na salt), catalytic subunit of cAMP-dependent protein kinase (bovine heart), protein kinase inhibitor (beef heart, type II), and alkaline phosphatase were from Sigma. GDP-[U-14C]mannose (307 mCi/mmol; 1 Ci = 37 GBq) was purchased from Amersham.

Preparation of Microsomal Membranes. Dispersed parotid acinar cells, prepared as described (9, 12), were incubated at 37°C in a shaking water bath in the presence and absence of 10 μM isoproterenol in Ham’s F-12 medium for 1 hr with gassing (95% O2/5% CO2) at 20-min intervals. At the end of incubations, cells were separated by centrifugation (15 sec at 40 x g), resuspended in 0.1 M Tris-HCl (pH 7.0) containing 0.25 M sucrose and 0.1 mM EDTA, and homogenized with a Brinkmann Polytron (setting 5 for 10 sec). The microsomal membranes were prepared following a procedure described for calf brain (17) with a minor modification (13). Two separate preparations of microsomal membranes were used here (from cells of 10 and 15 rats, respectively). Similar results were obtained with each preparation. The membranes were stored frozen in multiple aliquots in the homogenization buffer until used as enzyme source.

Phosphorylation of Microsomes. Phosphorylation was carried out in a buffer containing 10 mM Tris-HCl (pH 7.0), 25 mM sucrose, 1.0 μM EDTA, 10 mM MgCl2, 10 mM KF, 1% Me2SO, 0.2 mM ATP, and 12.5 units (an optimum amount, determined separately) of the catalytic subunit of cAMP-dependent protein kinase (18, 19). The reactions were started with the addition of catalytic subunit, and were carried out at 30°C for 20 min in a total volume of 50 μl. At the end of this incubation time, an appropriate amount of sugar-conjugated

Abbreviations: Man-P-Dol, mannosylphosphodolichol; Dol-P, dolichol monophosphate; Me2SO, dimethyl sulfoxide.

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nucleotide was added, and the mixture was assayed for glycosyltransferase activity.

Assay for the Transfer of [14C]Mannose from GDP-[14C]Manose into Endogenous Microsomal Acceptor. The enzymatic transfer of [14C]mannose to Dol-P to form [14C]mannosylphosphodolichol ([14C]Man-P-Dol) was carried out by the procedure described earlier (13). The reaction mixture was incubated at 37°C for 0–10 min in a shaking water bath and stopped by addition of 20 volumes of chloroform/methanol, 2:1 (vol/vol). After centrifugation at 1520 × g for 5 min, the supernatant was removed. The chloroform/methanol extract was washed with 0.2 volume of 0.9% NaCl. The aqueous phase was discarded. The organic phase was washed twice with chloroform/methanol/H2O, 3:48:47 (vol/vol), dried, and assayed for radioactivity. Mannolipid synthesis was linear between 0 and 137 μg of membrane protein and was linear as well for approximately 1 min. Therefore, experiments determining kinetic constants assayed mannolipid synthesis at 1 min. All other experiments presented here were assayed at 5 min. While these data may not quantitatively reflect initial reaction rates, they do reflect, however, the qualitative differences observed for control and experimental treatments measured at 1 min.

Characterization of [14C]Man-P-Dol Synthesized in Rat Parotid Microsomes. The [14C]-labeled mannolipid synthesized as a single product was characterized by the following four criteria: (i) mild acid hydrolysis, (ii) SG-81 paper chromatography, (iii) stimulation of mannolipid synthesis by exogenous Dol-P, and (iv) the effect of amphomycin on [14C]-labeled mannolipid synthesis.

In mild acid hydrolysis, the lower phase containing the [14C]-labeled mannolipid (5000 cpm) was incubated for 60 min at 50°C in the presence of 0.1 M HCl/50% 1-propanol (0.5 ml). At the end of the incubation period, 2 ml of chloroform/methanol, 2:1 (vol/vol), was added and partitioned with 0.5 ml of H2O. The upper aqueous phase and lower organic phase were collected separately, dried, and assayed for radioactivity (20). Nearly 80% of the starting material was hydrolyzed in 60 min and recovered as a water-soluble product. In SG-81 paper chromatography, extracted [14C]-labeled mannolipid was chromatographed on EDTA-treated (21) SG-81 paper with the solvent system chloroform/methanol/H2O, 65:25:4 (vol/vol). The presence of radioactivity was monitored by autoradiography on XAR-2 film. The intact mannolipid migrated as a single component when chromatographed on SG-81 paper. The migration pattern and the relative mobility (Rf) were comparable to those observed earlier for a similar lipid from rat parotid gland (22).

To observe stimulated mannolipid synthesis by exogenous Dol-P, rat parotid microsomes were incubated in the presence of exogenously added Dol-P in 0.5% Me2SO (13), and the transfer of [14C]mannose from GDP-[14C]mannose into the lipid to form the [14C]-labeled mannolipid was monitored as described above. Enhanced mannolipid synthesis was always observed in the presence of exogenously added Dol-P. In a typical experiment, ~70% enhancement of mannolipid synthesis was observed in presence of 34 nmol of Dol-P (13).

To observe the effect of amphomycin on [14C]-labeled mannolipid synthesis, the synthesis was followed in rat parotid microsomes both in the presence and absence of amphomycin at 50 μg/ml (4). The extracted mannolipid from both incubated samples was quantitated by radioactivity. This lipopeptide antibiotic inhibited the formation of mannolipid profoundly (~70%) in these membranes (23).

All of these observations strongly imply that the mannolipid synthesized by rat parotid acinar cell microsomal membranes is indeed β-mannosylphosphodolichol.

Miscellaneous Procedures. Protein was measured by Bradford's procedure (24) with bovine serum albumin as a standard. Radioactivity incorporated into various fractions was assayed by liquid scintillation spectrometry.

RESULTS

Characteristics of Man-P-Dol Synthesis in Membranes Isolated from Cells Pretreated with Isoproterenol. We have presented evidence (13) that microsomal membranes isolated from rat parotid acinar cells pretreated with isoproterenol show ~40% higher Man-P-Dol synthase activity than do membranes from control cells. To understand the basis of this increase, detailed kinetic studies were performed. First, Man-P-Dol synthase activity was followed as a function of time with membranes from both control and isoproterenol-treated cells (Fig. 1). Under the conditions used here, Man-P-Dol synthase initially proceeded quite rapidly in both membrane preparations, being linear with time only for about 1 min, and reached a steady state within 2–5 min. Enzymatic activity in membranes from isoproterenol-treated cells was higher at each time point of incubation. Man-P-Dol synthase activity was then examined as a function of GDP-mannose concentration. All activity was measured after an incubation time of 1 min at 37°C (Fig. 2). Man-P-Dol synthase in both membrane preparations followed Michaelis–Menten kinetics with little difference in the apparent Km for GDP-mannose (0.12 μM and 0.16 μM for membranes from control and isoproterenol-treated cells, respectively). Conversely, a significant increase in Vmax values was observed. The Vmax values were found to be 9.4 and 19.1 pmol/minute per mg of protein for membranes from control and isoproterenol-treated cells, respectively.

Effect of In Vitro Phosphorylation of Microsomal Membranes on Man-P-Dol Synthase Activity. The above experiments showed that isoproterenol treatment of rat parotid acinar cells results in increased Man-P-Dol synthase activity in microsomal membranes. Since isoproterenol generates cAMP and activates cAMP-dependent protein kinases in parotid acinar cells (14-16), we examined whether a cAMP-mediated protein phosphorylation event was a mechanistic step in activating Man-P-Dol synthase. To address this
possibility, isolated microsomes from rat parotid acinar cells were phosphorylated in vitro in the presence of the catalytic subunit from cAMP-dependent protein kinase and ATP. To be certain that the in vitro conditions used here could result in fact in cAMP-dependent protein kinase-mediated phosphorylation of microsomal membranes, we first incubated membranes in the presence of [γ-32P]ATP. The incorporation of 32P into 10% CC13COOH-insoluble material was utilized as a measure of protein phosphorylation. Results (Table 1) show that protein phosphorylation occurred in these membranes because of the enzymatic transfer of 32P from [γ-32P]ATP by cAMP-dependent protein kinase. Without the catalytic subunit being present, there was 60% less incorporation of 32P into microsomal membranes. Furthermore, when KF (a phosphatase inhibitor) was removed from the incubation mixture, there was also a substantial reduction in 32P incorporation.

Separate experiments investigated the ability of the heat-stable inhibitor for cAMP-dependent protein kinase (25) to block 32P incorporation into membranes. Protein kinase inhibitor (5 μg) was able to reduce membrane protein phosphorylation to the extent of 46% under the phosphorylation conditions followed here (data not shown). We then analyzed the ability of such in vitro phosphorylated microsomes to synthesize Man-P-Dol compared to control membranes, which were incubated in parallel but without the catalytic subunit. These results are shown in Table 2. There was ∼32% increase in Man-P-Dol synthase activity in microsomal membranes subjected to in vitro phosphorylation conditions, and these data are quite comparable to the data obtained with microsomal membranes isolated from cells treated with isoproterenol (Figs. 1 and 2).

Next we performed a kinetic study of GDP-mannose-dependent Man-P-Dol synthase in microsomal membranes that had been phosphorylated in vitro. Man-P-Dol synthase activity of in vitro phosphorylated microsomal membranes was assayed at a 1-min time point and followed Michaelis-Menten kinetics (not shown). These membranes showed an apparent Km for GDP-mannose of 0.23 μM as compared to control (incubated in parallel without the catalytic unit) membranes (0.14 μM), a 60% increase. However, the corresponding Vmax values were 29.1 and 14.5 pmol/min per mg of protein for phosphorylated and control membranes, respectively.

**Effect of Divalent Cations in the in Vitro Protein Phosphorylation Mixture on Man-P-Dol Synthase Activity.** It is well known that cAMP-dependent protein kinase has a requirement for divalent cations with a strong preference for Mg2+ (18, 26, 27), while Man-P-Dol synthase requires either Mn2+ or Mg2+ for optimal enzyme activity (4, 28). As an additional criterion to show that the changes occurring in parotid microsomal membranes are relevant to protein phosphorylation by cAMP-dependent protein kinase, we evaluated the effect of different divalent ions present during the phosphorylation of microsomal membranes by the catalytic subunit on subsequently measured Man-P-Dol synthase activity (Table 3). Man-P-Dol synthase was highest in membranes phosphorylated in the presence of Mg2+ (almost 50% higher than in control membranes—i.e., when no catalytic subunit was present). When the phosphorylation mixture contained Mn2+, Man-P-Dol synthase was subsequently reduced compared to Mg2+.

![Graph](https://example.com/graph.png)

**Table 1.** Transfer of 32P from [γ-32P]ATP to endogenous protein acceptor(s) in the presence and absence of the catalytic subunit of cAMP-dependent protein kinase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity incorporated, cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>17,635 ± 1,483 (n = 8)</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>7,132 ± 917 (n = 8)</td>
</tr>
<tr>
<td>Without KF</td>
<td>11,055 ± 1,537 (n = 6)</td>
</tr>
</tbody>
</table>

Protein phosphorylation was carried out by incubating 30 μg of microsomal membrane proteins in a buffer containing 10 mM Tris-HCl (pH 7.0), 25 mM sucrose, 10 mM EDTA, 10 mM MgCl2, 10 mM KF, 1% MeSO, 0.2 mM ATP, and 12.5 units of the catalytic subunit from cAMP-dependent protein kinase. The reactions were started with the addition of 5 μCi of [γ-32P]ATP, and the reaction mixture (total volume, 50 μl) was incubated at 30°C for 20 min (18). Proteins were precipitated in 10% CC13COOH, filtered, and assayed for radioactivity. The results presented above are means ± SEM from the number of determinations indicated in the parentheses.

**Table 2.** Effect of in vitro phosphorylation on Man-P-Dol synthase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Man-P-Dol synthase activity, pmol/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>16.09 ± 0.25 (n = 18)</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>12.22 ± 0.19 (n = 12)</td>
</tr>
</tbody>
</table>

Protein phosphorylation of the microsomal membranes (30 μg) was carried out as described. At the end of the 20-min period, 2.5 μM GDP-[U-14C]mannose was added, and the reaction mixture was reincubated at 37°C for 5 min in a total volume of 100 μl. Mannolipid synthesized was measured as described in the text. The results presented above are means ± SEM for the number of determinations indicated in the parentheses.
Table 3. Effect of divalent ions present during in vitro phosphorylation on Man-P-Dol synthase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Divalent ions</th>
<th>Man-P-Dol synthase activity, pmol/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>17.45</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>11.69</td>
</tr>
<tr>
<td>Complete</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>13.23</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>10.64</td>
</tr>
<tr>
<td>Complete</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>11.83</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12.33</td>
</tr>
<tr>
<td>Complete</td>
<td>EDTA</td>
<td>0.16</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>EDTA</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Protein phosphorylation, followed by mannolipid synthesis, was measured as described in Table 2 except for varying the divalent ions. The final concentration of divalent metal ions was kept at 5 mM. The concentration of EDTA was 10-fold higher than that of Mg<sup>2+</sup>. The results are the means of two determinations, which were within 10% of average values.

pared to results with Mg<sup>2+</sup>. Incubation of membranes with Ca<sup>2+</sup> during in vitro protein phosphorylation resulted in no cAMP-dependent protein kinase-related increase in Man-P-Dol synthase activity. Furthermore, inclusion of EDTA in phosphorylation mixtures abolished all Man-P-Dol synthase activity.

**Treatmen of in Vitro Phosphorylated Microsomal Membranes with Alkaline Phosphatase and Its Relationship to Man-P-Dol Synthase Activity.** The above studies strongly supported the notion that protein phosphorylation mediated by cAMP-dependent protein kinase enhanced the Man-P-Dol synthase activity in parotid microsomal membranes. Therefore, it seemed reasonable to assume that removal of phosphate from in vitro phosphorylated microsomal membranes should lower the Man-P-Dol synthase activity. To test this hypothesis, the microsomal membranes first were phosphorylated in vitro, washed, and reincubated at 37°C in the presence of alkaline phosphatase at pH 7.0. After this incubation, the membranes were further washed and then assayed for Man-P-Dol synthase activity. The results (Table 4) show that there was a substantial loss in Man-P-Dol synthase activity of in vitro phosphorylated membranes after alkaline phosphatase treatment.

**Effect of in Vitro Phosphorylation of Microsomal Membranes from Different Sources on Man-P-Dol Synthase Activity.** All of the above studies were conducted with microsomal membranes from rat parotid acinar cells. To assess if subjecting microsomal membranes to in vitro phosphorylation generally leads to increased Man-P-Dol synthase activity, we examined membranes from two other well-studied sources, calf brain and hen oviduct. The results (Table 5) obtained in paired experiments suggested that Man-P-Dol synthase in both of these membrane preparations could be modulated by a cAMP-dependent protein phosphorylation mechanism similar to that observed with parotid gland. Approximately 30–40% enhancement of Man-P-Dol synthase activity was seen in phosphorylated membranes.

**DISCUSSION**

The purpose of the present study was to evaluate the molecular mechanism responsible for isoproterenol-mediated enhancement of Man-P-Dol synthase activity in rat parotid acinar cells (13). There are two likely possible reasons for this increase in enzymatic activity: (i) enhanced de novo enzymic protein synthesis and/or (ii) activation of the endogenous enzyme due to a covalent modification. Since our previous studies showed that treatment of cells with isoproterenol for a relatively short period of time led to enhanced Man-P-Dol formation and enhanced Man-P-Dol synthase activity (13, 23), we surmised that the possibility of increased enzyme synthesis was very remote. Moreover, it has been reported that activities of more than 30 different enzymes involved in carbohydrate, protein, lipid, and nucleic acid metabolism can be modulated by a phosphorylation/dephosphorylation cycle (18). In addition, smooth muscle contraction (29), cellular resistance to some viruses (30), submission to viral transformation (31, 32), and the cellular response to adrenergic hormones, insulin, and epidermal growth factor are modulated as well by a reversible phosphorylation of specific proteins (33). Since changes in protein N-glycosylation caused by isoproterenol are primarily mediated by cAMP, we hypothesized that cAMP-dependent protein phosphorylation events might be responsible for enhanced glycosyltransferase activity in rat parotid acinar cells.

To examine this possibility, it was necessary first to characterize Man-P-Dol synthase activation as occurring in intact cells following β-adrenoreceptor stimulation. Man-P-Dol synthesis was very rapid, being linear for only about 1 min and reaching saturation between 2 and 5 min. Not only was the apparent rate of the reaction much faster in membranes from agonist-treated cells but also there was an apparent increase in the total amount of Man-P-Dol formed. When kinase studies were performed with these preparations, we observed a modest change in apparent $K_M$ for GDP-mannose, but $V_{max}$ was increased by >2-fold. There are several factors to consider in evaluating this apparent difference in total product formation. First, in intact cells we have shown (23) an increased rate of Man-P-Dol formation after

Table 4. Effect of alkaline phosphatase treatment of phosphorylated microsomal membranes on Man-P-Dol synthase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Man-P-Dol synthase activity, pmol/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>9.79</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>5.63</td>
</tr>
<tr>
<td>With alkaline phosphatase (30 min)</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Microsomal membranes (30 μg) were phosphorylated as described, and membranes were washed with 0.1 M Tris-HCl (pH 7.0) buffer containing 0.25 M sucrose and 0.1 mM EDTA. The phosphorylated membranes were then incubated with 5 μg of alkaline phosphatase in 50 mM Tris-HCl (pH 7.0) containing 125 mM sucrose, 50 μM EDTA, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, and 1% MeSO<sub>4</sub> for 30 min. At the end of the incubation, the membranes were washed with 0.1 M Tris-HCl (pH 7.0) buffer containing 0.25 M sucrose and 0.1 mM EDTA. Mannolipid synthesis was assayed as described in Table 2. The results are the means of two determinations, which were within 10% of average values.

Table 5. The effect of in vitro phosphorylation of microsomal membranes from different sources on Man-P-Dol synthase activity

<table>
<thead>
<tr>
<th>Source</th>
<th>Man-P-Dol synthase activity, pmol/min per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat parotid</td>
<td>4.41</td>
</tr>
<tr>
<td>Complete</td>
<td>3.19</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>4.33</td>
</tr>
<tr>
<td>Bovine brain</td>
<td>5.62</td>
</tr>
<tr>
<td>Complete</td>
<td>4.33</td>
</tr>
<tr>
<td>Without catalytic subunit</td>
<td>20.09</td>
</tr>
<tr>
<td>Hen oviduct</td>
<td>14.99</td>
</tr>
</tbody>
</table>

The conditions for protein phosphorylation of microsomal membranes and Man-P-Dol synthase assay were the same as described in Table 2. The results presented above are the mean of two determinations, which were within 10% of average values.
treatment with the β-adrenergic agonist isoproterenol, but at 30 min comparable levels of mannolipid were present in control and agonist-treated cells. Second, Man-P-Dol synthase catalyzes a bisubstrate reaction, utilizing both GDP-mannose and dolichol monophosphate. We know from the data presented in Figs. 1 and 2 that we have ample levels of GDP-mannose present in reaction tubes (i.e., the maximum amount of Man-P-Dol formed is 22.5 pmol, whereas reaction mixtures for time-course experiments shown in Fig. 1 contained 25 pmol of GDP-mannose). Thus, it is unlikely that the difference in product formation reflects depletion of GDP-mannose. Thirdly, these reaction mixtures contain only endogenous dolichol monophosphate. Though we have not measured the actual level of the lipid in these membrane preparations, it is quite possible that the levels of dolichol monophosphate would be inadequate to sustain maximum product formation. However, it is unlikely that this occurrence would compromise any data interpretations presented herein because we have demonstrated (13) that addition of exogenous dolichol monophosphate to membrane incubations increases the level of Man-P-Dol formed but does not alter the relationship between enzyme activity found in control and agonist-treated membranes. It is also worth recalling that Man-P-Dol formed in the enzymatic reaction being studied here is itself a substrate for subsequent reactions (4), making the interpretation of quantitative differences in apparent product formation in microsomal membranes at the later time points examined here difficult. Finally, we have used crude microsomal membranes as our source of Man-P-Dol synthase. Considering all these factors, it is likely that the apparent differences in total Man-P-Dol synthase shown in Fig. 1 do not reflect actual differences in product formation between the two membrane preparations.

We then compared these results with the behavior of the enzyme in microsomal membranes subjected to in vitro conditions conducive to protein phosphorylation. Direct demonstration of microsomal membrane protein phosphorylation was obtained after transfer of 32P from [γ-32P]ATP. That it was indeed mediated by a cAMP-dependent protein kinase was supported by (i) the requirement of catalytic subunit for maximum activity, (ii) metal ion requirements for phosphorylation, and (iii) effects of the heat-stable inhibitor of cAMP-dependent protein kinase. Furthermore, in vitro phosphorylation of microsomal membranes and the resultant increase in Man-P-Dol synthase activity appeared to be reversible. Thus, when phosphorylation was carried out in the absence of KF, 32P incorporation into membrane protein was markedly reduced. Similarly, when phosphorylated membranes were treated with alkaline phosphatase at neutral pH, Man-P-Dol synthase activity was diminished. It should be noted that under the conditions used here, the alkaline phosphatase treatment reduced Man-P-Dol synthase activity to 23% of control (i.e., minus the catalytic (subunit) values). The explanation for this observation is not clear and, indeed, is difficult to address without a pure enzyme. The results might indicate that a significant proportion of Man-P-Dol synthase is present in a phosphorylated form. However, the removal of phosphate from microsomal membranes corresponds with the reduced Man-P-Dol synthase activity. Kinetic studies of Man-P-Dol synthase activity in in vitro phosphorylated membranes showed quite similar behavior (≈60% increase in K_m for GDP-mannose and 2-fold increase in V_max) to that seen with the enzyme in membranes isolated from agonist-treated cells.

Taken together, the present findings provide the first evidence supporting the idea that Man-P-Dol synthase activity in mammalian cell microsomal membranes can be regulated by a cAMP-dependent protein phosphorylation/dephosphorylation cycle. In addition, we also have presented evidence that suggests this conclusion is not limited to the excocrine rat parotid gland but that similar regulation of this enzyme may occur in such diverse tissues as hen oviduct and bovine brain. These observations are particularly important because the latter two tissues are most often utilized in mechanistic studies of dolichol-linked glycosyltransferase and oligosaccharide assembly for protein N-glycosylation.

It is important to reemphasize here that our studies have been performed with microsomal membranes as a primary source of Man-P-Dol synthase activity. It is, therefore, difficult to meet the stringent criteria for unequivocally demonstrating that an enzyme undergoes a physiologically significant phosphorylation/dephosphorylation process (18, 19). Therefore, additional work is needed with a solubilized enzyme preparation not only to support unequivocally a phosphorylation/dephosphorylation cycle as a physiologically relevant mechanism regulating Man-P-Dol synthase activity in situ but also to appreciate more clearly the kinetic characteristics associated with such changes.