In Vitro Phosphorylation by cAMP-dependent Protein Kinase
Up-regulates Recombinant Saccharomyces cerevisiae
Mannosylphosphodolichol Synthase*

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DPM1 is the structural gene for mannosylphosphodolichol synthase (i.e. Dol-P-Man synthase, DPMS) in Saccharomyces cerevisiae. Earlier studies with cDNA cloning and sequence analysis have established that 31-kDa DPMS of S. cerevisiae contains a consensus sequence (YRRVIS) that can be phosphorylated by cAMP-dependent protein kinase (PKA). We have been studying the up-regulation of DPMS activity by protein kinase A-mediated phosphorylation in higher eukaryotes, and used the recombinant DPMS from S. cerevisiae in this study to advance our knowledge further. DPMS catalytic activity was indeed enhanced severalfold when the recombinant protein was phosphorylated in vitro. The rate as well as the magnitude of catalysis was higher with the phosphorylated enzyme. A similar increase in the catalytic activity was also observed when the in vitro phosphorylated recombinant DPMS was assayed as a function of increasing concentrations of exogenous dolichylmonophosphate (Dol-P). Kinetic studies indicated that there was no change in the $K_m$ for GDP-mannose between the in vitro phosphorylated and control recombinant DPMS, but the $V_{max}$ was increased by 6-fold with the phosphorylated enzyme. In vitro phosphorylated recombinant DPMS also exhibited higher enzyme turnover ($k_{cat}$) and enzyme efficiency ($k_{cat}/K_m$). SDS-PAGE followed by autoradiography of the $^{32}$P-labeled DPMS detected a 31-kDa phosphoprotein, and immunoblotting with anti-phosphoserine antibody established the presence of a phosphoserine residue in in vitro phosphorylated recombinant DPMS. To confirm the phosphorylation activation of recombinant DPMS, serine 141 in the consensus sequence was replaced with alanine by PCR site-directed mutagenesis. The S141A DPMS mutant exhibited more than half-a-fold reduction in catalytic activity compared with the wild type when both were analyzed after in vitro phosphorylation. Thus, confirming that S. cerevisiae DPMS activity is indeed regulated by the cAMP-dependent protein phosphorylation signal, and the phosphorylation target is serine 141.

Mannosylphosphodolichol (Dol-P-Man), a mannosyl donor in the assembly of the precursor oligosaccharide-lipid Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol in N-glycosylation of proteins, in the synthesis of glycosylphosphatidylinositol (GPI) anchors, in O-glycosylation of proteins in yeast, and in C-mannosylation of Trp-7 in human ribonuclease 2 (RNase 2) is formed by the transfer of mannose from GDP-mannose to the polyisoprenoid-lipid, dolichylmonophosphate (Dol-P; 1–7). Dol-P-Man is synthesized at the cytoplasmic face of the endoplasmic reticulum (ER) membrane (8–10), and catalyzed by mannosylphosphodolichol synthase (DPMS; Dol-P-Man synthase, EC 2.4.1.83). Dol-P-Man synthase deficiency has been observed in a Class E Thy-1 lymphoma patient and is unable to elongate Man$_n$GlcNAc$_n$-PP-Dol to Man$_n$GlcNAc$_n$-PP-Dol, a pre-requisite for Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol synthesis (11). We have also made a similar observation using in vitro studies with amphotericin, a lipopeptide antibiotic from Streptomyces canas, which forms a complex with Dol-P in a calcium-dependent manner, and inhibiting the Dol-P-Man synthase activity (12, 13). Recent reports indicate that partial deficiency of Dol-P-Man synthase causes congenital disorder of glycosylation (14, 15). DPMS deficiency in these patients is associated with developmental delay, seizures, hypertonia, and dysmorphic function (16). The DP1 gene is essential for viability in yeast because disruption of the gene is lethal (17).

Dol-P-Man synthase has been partially purified from mammalian tissue (18, 19) and highly purified from the budding yeast, Saccharomyces cerevisiae (20). Cloning of the Dol-P-Man synthase gene DPM1p from S. cerevisiae has shown DPMS to be a structural gene in yeast (17) expressing active protein both in Escherichia coli and in mammalian cells (21). Information on the primary structure of Dol-P-Man synthase obtained from cDNA analysis has indicated that (i) it codes for a protein of 267 amino acids with an apparent mass of 30.36 kDa; (ii) there is a potential membrane spanning domain of 25 amino acids at its carboxyl terminus; (iii) a highly conserved amino acid sequence in the membrane spanning domain originally suggested a potential dolichol and/or polyisoprenoid substrate recognition site; and (iv) the predicted sequence contains one positive site for phosphorylation by cAMP-dependent protein kinase (i.e. PKA) while the activity of the synthase is dependent on phosphorylation by cAMP-dependent protein kinase (PKA).

1 The abbreviations used are: Dol-P-Man mannosylphosphodolichol; DPMS, Dol-P-Man synthase; PKA, cAMP-dependent protein kinase; Me$_2$SO, dimethyl sulfoxide; IPTG, isopropyl β-D-thiogalactopyranoside; TPCK, 1-1-tosylamido-2-phenylethyl chloromethyl ketone.

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Phosphorylation Regulation of Yeast DPMS

Phosphorylation of Yeast Dol-P-Man synthase (DPMS) has been studied extensively, revealing critical roles in its regulation and function. DPMS is a key enzyme in the biosynthesis of dolichyl phosphomannose, which serves as a primer for N-linked glycosylation. The regulation of DPMS activity is crucial for proper glycosylation and cellular functions.

Materials and Methods

The yeast DPMS mutant was constructed by site-directed mutagenesis to replace serine 141 with an alanine residue. This mutant, S141A, was used to study the effects of phosphorylation at serine 141 on DPMS activity.

Phosphorylation Target Identification

To identify the phosphorylation target in DPMS, the catalytic subunit of the cAMP-dependent protein kinase (PKA) was used to phosphorylate the wild-type and S141A mutant enzymes. Phosphorylation was detected using autoradiography of the 32P-labeled Dol-P-Man synthase.

Results and Discussion

Phosphorylation of serine 141 was confirmed by autoradiography, suggesting that this residue is a critical phosphorylation target in DPMS. This finding is supported by the fact that the phosphorylation of serine 141 is important for the regulation of DPMS activity.

Regulation of DPMS Activity

Phosphorylation of serine 141 is critical for the regulation of DPMS activity. In vitro phosphorylation experiments showed that phosphorylation increases the activity of DPMS, indicating that phosphorylation plays a role in the activation of this enzyme.

The Role of Serine 141

Serine 141 is a critical residue for the regulation of DPMS activity. The S141A mutant exhibited reduced activity compared to the wild-type enzyme, indicating that serine 141 is essential for optimal DPMS function.

Conclusion

The phosphorylation of serine 141 is a critical event in the regulation of DPMS activity. This phosphorylation target is essential for the regulation of DPMS, and its recognition and binding of Dol-P are critically important for the recognition and binding of Dol-P. This study highlights the importance of phosphorylation in the regulation of DPMS activity and suggests potential targets for future therapeutic interventions.
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RESULTS

Effect of in Vitro Phosphorylation on the Dol-P-Man Synthase Activity—Mannos-6-phosphate-dolichol synthesis was monitored with in vitro phosphorylated and control recombinant DPMS from S. cerevisiae. The time course of the synthase activity, when examined for a period of 0–5 min under the conditions described above, indicated that the specific activity of Dol-P-Man synthase was severalfold higher in the in vitro phosphorylated enzyme compared with control (Fig. 1). In addition, Dol-P-Man synthase remained high at all time points with the in vitro phosphorylated enzyme.

Dependence of GDP-Mannose and Dol-P Concentrations on Synthase Activity—Based on a single point assay, the Dol-P-Man synthase activity was found to be nearly 3-fold higher (p < 0.05) in the in vitro phosphorylated enzyme compared with control (Table I). However, the kinetic analysis at the initial...
rate (Figs. 2, a and b) of the recombinant DPMS indicated that the $K_m$ for GDP-mannose for in vitro phosphorylated and control enzymes were $1.1 \times 10^{-6}$ M and $1.2 \times 10^{-6}$ M, respectively, whereas the corresponding $V_{max}$ values were 135.8 and 23.6 nmol/min/mg of protein, respectively. The in vitro phosphorylated enzyme also exhibited almost 6-fold higher turnover ($k_{cat} = 70.9$) and enzyme efficiency ($k_{cat}/K_m = 6.4 \times 10^3$) as compared with the control enzyme ($k_{cat} = 12.1$ and $k_{cat}/K_m = 1 \times 10^3$) (Table II). The apparent $K_m$ for Dol-P was approximately a magnitude higher than that of GDP-mannose but the values did not differ between the in vitro phosphorylated and control enzymes (data not shown).

Characterization of Dol-P-Man Synthase as a Phosphoprotein—Recombinant Dol-P-Man synthase was phosphorylated in vitro in the presence of [γ-32P]ATP according to the conditions described under “Materials and Methods.” The phosphorylated enzyme was immunoprecipitated with a mouse monoclonal antibody to yeast Dol-P-Man synthase, and subjected to a 12% SDS-PAGE followed by autoradiography on X-AR films. It detected Dol-P-Man synthase as a 31-kDa phosphoprotein (Fig. 3). To identify that serine 141 is the target for PKA-mediated phosphorylation, Dol-P-Man synthase was separated on a 12% SDS-PAGE after in vitro phosphorylation and transferred onto a nitrocellulose membrane. One-half of the membrane was processed with anti-DPMS antibody, and the other half with anti-phosphoserine antibody. Both anti-DPMS antibody and the anti-phosphoserine antibody detected a 31-kDa protein on the blots (Fig. 4, a and b) supporting that Dol-P-Man synthase was indeed phosphorylated at serine 141 by the PKA. As a control, cell extracts from IPTG-uninduced and -induced E. coli carrying the wild type Dpm1 gene or its S141A mutant were examined by Western blot analysis against anti-phosphoserine antibody. These results (Fig. 4c) were negative, suggesting the presence of no other phosphorylated protein equivalent to the 31-kDa Dol-P-Man E. coli cell extracts.

Dol-P-Man Synthase Activity of the Phosphorylation Site-deficient (S141A) Mutant after in Vitro Phosphorylation by cAMP-dependent Protein Kinase—To establish further that serine 141 of the wild type Dol-P-Man synthase gene is indeed the target for PKA-mediated phosphorylation, serine 141 was replaced by alanine by site-directed mutagenesis. Alanine substitution at serine 141 of the Dol-P-Man synthase was confirmed by (a) restriction analysis of the DNA constructs; and (b) DNA sequencing. The restriction enzyme analysis demonstrated that the wild type Dol-P-Man synthase gene contained only the BstU1 site but not SalI site. On the other hand, the S141A Dol-P-Man synthase mutant gene contained both a BstU1 and a SalI site. DNA sequencing of the wild type and mutant DPMS genes demonstrated the presence of the TCC sequence (a codon for serine) at amino acid residue 141 of the wild type gene but the corresponding sequence in the mutant was GCC (a codon for alanine; data not shown). It should be noted here that there were no other sequence differences among the wild type, S141A mutant, and published S. cerevisiae DPMS sequence.

Analysis of the expressed protein by 12% SDS-PAGE as well as by immunoblotting with a yeast anti-DPMS monoclonal antibody indicated that IPTG-induced cultures expressed high levels of S141A mutant DPMS as a 31-kDa protein (Fig. 5, a and b). DPMS activity of the mutant enzyme was determined and compared with that of the wild type before and after in vitro phosphorylation. Basal DPMS activity in S141A mutant extracts was ~1.5-fold higher than in control extracts. Wild type DPMS was activated by 1.5-fold as opposed to 1-fold in the S141A mutant. This means if the wild type DPMS was activated by 2-fold after phosphorylation, the S141A mutant DPMS was activated less...
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Fig. 2. Dependence of GDP-mannose concentration on the recombinant Dol-P-Man synthase activity. Control and in vitro phosphorylated Dol-P-Man synthase activity were assayed by incubating at 37 °C for 30 s as described under “Materials and Methods” but in the presence of 0.125–5.0 μM GDP-[14C]mannose (specific activity 318 cpm/pmol). a, V versus [S] plot; b, Lineweaver-Burk plot (1/V versus 1/[S]). ○, control; ●, in vitro phosphorylated.

Table II

| Experimental condition | $K_m$ (μM) | $V_{max}$ (μmol/min/mg protein) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$s$^{-1}$) |
|-----------------------|------------|---------------------------------|--------------------|-------------------------------|
| Control               | 1.2 × 10$^{-6}$ | 25.1                           | 12.1               | 1 × 10$^7$                   |
| After in vitro phosphorylation | 1.1 × 10$^{-6}$ | 146.7                          | 70.9               | 6.4 × 10$^7$                 |

Fig. 3. Autoradiographic detection of 32P-labeled Dol-P-Man synthase. Recombinant Dol-P-Man synthase was phosphorylated in vitro by incubating with a catalytic subunit from cAMP-dependent protein kinase (12 units per 30 μg of DPMS protein) at 30 °C for 20 min in the presence of 30 mM Hepes, pH 7.0, buffer containing 10 mM NaF, 10 mM MgCl$_2$, and 5 μCi of [γ-32P]ATP. Control had everything except the cAMP-dependent protein kinase. 32P-Labeled Dol-P-Man synthase was immunoprecipitated with a mouse monoclonal antibody to yeast Man synthase from E. coli and was exposed to an x-ray film. Lane 1, 32P-methylated protein markers; lane 2, [32P]Dol-P-Man synthase.

than one-half of 2-fold (Fig. 6, a and b). E. coli cells transfected with vector alone had no DPMS activity.

DISCUSSION

Using biochemical parameters we have proposed earlier that mammalian Dol-P-Man synthase is up-regulated by a cAMP-dependent protein kinase-mediated phosphorylation event (39). In addition, we have also shown that the mammalian Dol-P-Man synthase activity is associated with a 32-kDa phosphoprotein (40). We have now shown using somatic cell genetics that Chinese hamster ovary cells deficient in cAMP-depend-
rylated DPMS, we have substituted the serine residue with alanine by PCR site-directed mutagenesis. Restriction enzyme analysis as well as DNA sequencing have confirmed the S141A substitution in the DPM1 gene. It is important to note that the S141A mutant exhibits a small increase in basal DPMS activity compared with that of the wild type. This may be because of the in vitro assay condition, or because of a change in the protein conformation in this mutant, or it may be a combination of the two and can only be explained by x-ray crystallographic studies in the future. In vitro phospho-
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Phosphorylation of DPMS while responding to cAMP signaling. This valuable information is a step toward understanding the fundamentals of the closely related DPMS family of proteins, and the development of congenital disorder of glycosylation.

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