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Low expression of lipid-linked oligosaccharide due to a functionally altered Dol-P-Man synthase reduces protein glycosylation in cAMP-dependent protein kinase deficient Chinese hamster ovary cells

Dipak K. Banerjee, Eira Aponte and Jaime J. DaSilva

Department of Biochemistry, School of Medicine, University of Puerto Rico, San Juan, PR 00936-5067, USA

Chinese hamster ovary cells express a wide variety of glycoproteins with M_r ranging from 15,000 to 200,000 dalton and higher. Glycosylation of these proteins was much less in cAMP-dependent protein kinase (PKA)-deficient mutants which expressed either (i) a defective C-subunit with altered substrate specificity and having no detectable type II kinase (mutant 10215); or (ii) an altered RI subunit and having no detectable type II kinase (mutant 10248); or (iii) exhibited the lowest level of total kinase with no detectable type I kinase but having a small amount of type II kinase (mutant 10260). Addition of 8Br-cAMP enhanced protein glycosylation index in wild type cells 10001 by 120% but only 7 to 23% in the mutant cells. The rate of lipid-linked oligosaccharide (LLO) biosynthesis was linear for 1 h in all cell types, but the total amount of LLO expressed was much less in PKA-deficient mutants. Pulse-chase experiments indicated that the $t_{1/2}$ for LLO turnover was also twice as high in PKA-deficient cells as in the wild type. Size exclusion chromatography of the mild-acid released oligosaccharide confirmed that both wild type and the mutant cells synthesized $Glc_3Man_9GlcNAc_2$-PP-Dol as the most predominating species with no accumulation of $Man_5GlcNAc_2$-PP-Dol in the mutants. Kinetic studies exhibited a reduced mannosylphosphodolichol synthase (DPMS) activity in mutant cells with a K_m for GDP-mannose 160 to 400% higher than that of the wild type. In addition, the k_{cat} for DPMS was also reduced 2 to 4-fold in these mutant cells. Exogenously added Dol-P failed to rescue the k_{cat} for DPMS in CHO cell mutants; however, *in vitro* protein phosphorylation with a cAMP-dependent protein kinase restored their kinetic activity to the level of the wild type.
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Abbreviations: **PKA, cAMP-dependent protein kinase; cAMP, adenosine 3′,5′-cyclic monophosphate; CHO, Chinese hamster ovary cells; Dol-P, dolichyl monophosphate; Dol-P-Man/DPM, mannosylphosphodolichol; DPMS, mannosylphosphodolichol synthase; LLO, lipid-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$-PP-Dol); PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; alpha-MEM, alpha modified Eagle's medium without riboside and deoxyriboside; DMEM, Dulbecco's minimal essential medium; PBS, phosphate-buffered-saline; M_r, apparent molecular weight; Me_2SO, dimethyl sulfoxide.**

Introduction

It has been reported that ß-adrenoreceptor regulation of protein N-glycosylation in rat parotid acinar cells is mediated *via* cAMP [1]. The increased $Glc_3Man_9GlcNAc_2$-PP-Dol synthesis and its turnover following isoproterenol treatment in these cells [2] were primarily due to the activation of key glycosyltransferases {*i.e.*, mannosylphosphodolichol (Dol-P-Man) synthase, glucosylphosphodolichol (Glc-P-Dol) synthase, and N-acetylglucosaminyl 1-phosphate (GlcNAc-1P) transferase [3]}. This increase in enzyme activity was (i) independent of exogenous Dol-P levels, and (ii) observed under conditions in which the specific activities of donor sugar nucleotides were kept constant. Furthermore, activation of these enzymes was specific

To whom correspondence should be addressed: Dipak K. Banerjee, Ph.D., Department of Biochemistry, School of Medicine, University of Puerto Rico, San Juan, PR 00936-5067, USA. Tel.: (787) 758-7090; Fax: (787) 274-8724; E-mail: dbanerjee@rcm.upr.edu

because measurement of NADPH-cytochrome c reductase [4], a marker for endoplasmic reticulum (ER), indicated no differences between control and isoproterenol-treated membranes. Since, isoproterenol, a ß-aderenergic agonist, activates cAMP-dependent protein kinase (PKA) in these cells and other cell types [5–7], it was hypothesized that cAMP-dependent protein phosphorylation may be responsible for the activation of these enzymes. Phosphorylation *in vitro* of ER membrane proteins of acinar cells by PKA showed enhanced Dol-P-Man synthase activity to the same degree as observed in the isoproterenol-treated membranes [8,9], which supported the above hypothesis.

In this paper, we have used wild type CHO cells and their cAMP-dependent protein kinase (PKA) deficient mutants to establish that PKA is essential for up-regulating the Dol-P-Man synthase activity and consequently the $Glc_3Man_9GlcNAc_2$-PP-Dol biosynthesis and protein N-glycosylation in cAMP-responsive cells. Our results showing reduced $Glc_3Man_9GlcNAc_2$-PP-Dol synthesis, low Dol-P-Man synthase activity as well as low cAMP-response to protein glycosylation in PKA mutants supported the concept of a molecular coupling between protein phosphorylation signaling and modulation of protein glycosylation in eukaryotic cells.

Materials and methods

Alpha-EMEM, DMEM, Ham's F-12 medium, glutamine, and penicillin-streptomycin were purchased from Biofluids, Inc., Rockville, MD. Fetal bovine serum was a product of HyClone Laboratories, Logan, UT. 8Br-cAMP, Dol-P, AMP, ATP (Na salt), Me_2SO, catalytic subunit of cAMP-dependent protein kinase (bovine heart), and sodium azide were supplied by Sigma Chemical Co., St. Louis, MO. GDP-[U-^{14}C]-mannose (307 mCi/mmol; a Ci = 37 GBq), 2-[^{3}H]mannose (19 Ci/mmol), [U-^{14}C]leucine (302 mCi/mmol), Amplify, and [^{14}C]methylated protein mixture (M_r14,300–200,000) were obtained from Amersham Pharmacia Biotech, Newark, NJ. Gamma-[^{32}P]ATP (25 Ci/ mmol) was supplied by ICN. Electrophoresis reagents were obtained from Bio-Rad Laboratories, Hercules, CA. All other chemicals were of reagent quality.

Cell culture

The wild type CHO cells and their PKA-deficient mutants were grown in alpha-EMEM [10]. During isotopic labeling, the cells were washed with either serum-free DMEM or Ham's F-12 and incubated at 37°C with either 2-[^{3}H]mannose or U-[^{14}C]leucine in low-glucose or leucine-free medium for 60 min unless otherwise mentioned.

Mammalian Dol-P-Man synthase

ER membrane proteins were isolated from cultured cells by differential centrifugation [11] and kept frozen at −20°C in multiple aliquots until needed. Enzymatic formation of Dol-P-Man was assayed by incubating the ER membranes in 5 mM Tris-HCl, pH 7.0 containing 12.5 mM sucrose, 5 μM EDTA, 5 mM $MnCl_2$, 4 mM 5′AMP, and 2.5 μM GDP-[U-^{14}C]-mannose in 100 μl for 5 min at 37°C, unless otherwise mentioned. Each assay was initiated by the addition of GDP-[U-^{14}C]mannose and stopped at the desired time by the addition of 20 volumes of chloroform-methanol (2:1, v/v). After centrifugation at 1520 × g for 5 min at room temperature, the supernatant fluid was removed. The chloroform-methanol extracts were washed with 0.2 volume of 0.9% NaCl and the aqueous phase was discarded. The organic phase was washed twice more with chloroform-methanol-water (3:48:47, v/v/v), dried, and assayed for the radioactivity [3]. Protein concentration in the ER membranes was determined by Bradford Protein Assay using bovine serum albumin as standard [12]. Phosphorylation of either membrane-bound Dol-P-Man synthase or solubilized/partially purified enzyme was carried out by incubating at 30°C for 20 min in 10 mM Tris-HCl, pH 7.0 containing 25 mM sucrose, 10 μM EDTA, 10 mM $MgCl_2$, 10 mM KF, 1% Me_2SO, 0.2 mM ATP, and an appropriate amount of catalytic subunit from cAMP-dependent protein kinase in a total volume of 50 μl [9]. In some experiments, the reactions were started by the addition of 5 μCi of gamma-[^{32}P]ATP. The phosphorylated Dol-P-Man synthase was assayed for its activity by incubating from 0 to 30 min as described, in a shaking water bath.

SDS-PAGE of [^{3}H]mannose-labeled glycoproteins

Wild type and the PKA-deficient CHO cell mutants were labeled with 2-[^{3}H]mannose (50 μCi/ml) in low-glucose media at 37°C for 4 h. The cells were separated, washed and lysed in 20 mM Tris-HCl, pH 8.0 containing 150 mM NaCl and 1% NP-40 at 4°C for 20 min. The extracts were clarified by centrifuging at 100,000 × g for 60 min. Equal amounts of protein were separated on 7.5% SDS-polyacrylamide gels [13]. The gels were fixed for 30 min in methanol-water-acetic acid (5:5:1, v/v/v) and processed for autoradiography in Amplify [14].

Isolation and analysis of LLO

The lipid-linked oligosaccharide biosynthesis in wild type CHO cells and their PKA-deficient mutants were studied by labeling them from 30 min to 4 h at 37°C with 2-[^{3}H]mannose (25 μCi/ml) in 1 ml of serum-free low-glucose DMEM. At the end of each incubation, cells were washed with PBS, pH 7.4 and the monosaccharide-lipid was extracted with chloroform-methanol (2:1, v/v). The pellets were subsequently washed with 0.9% NaCl followed by deionized distilled water, and the LLOs were extracted with chloroform-methanol-water (10:10:3, v/v/v). The [^{3}H]mannose-oligosaccharides were made free of Dol-PP by mild-acid hydrolysis in 0.1 N HCl in 80% tetrahydrofuran at 50°C for 30 min [15]. Sizing of the mild-acid released [^{3}H]mannose-oligosaccharides was carried out over a Bio-Gel P-4 column (54 cm × 0.9 cm). The column was washed, equilibrated and eluted with 50 mM ammonium formate, pH 8.0 containing 0.02% NaN_3.

2-[^{3}H]deoxy-D-glucose uptake

Wild type CHO cells and its PKA-deficient mutants were seeded in 24 well clusters at a density of 5×10^4 cells/well in 1.5 ml of alpha-EMEM supplemented with 5% fetal bovine serum. Just before initiating the experiment, the media were removed by aspiration and the cells were washed with 2×1 ml of standard reaction mixture (SRM; 118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/2.2 mM $CaCl_2$/25 mM Hepes-NaOH/10 mM mannitol, pH 7.4). The cells were then incubated at 37°C for 15 min in the presence of 0.1 mM 2-deoxyglucose and 1.0 μCi/ml of 2-[^{3}H]deoxyglucose in SRM [16]. At the end of the incubation, cells were washed with PBS, pH 7.4, counted for the radioactivity and the results were computed. Incubation at ice-cold temperature served as 'zero time'.

Results

Protein glycosylation in PKA-deficient mutants

The physiological consequences of PKA-deficiency in CHO cells were addressed with respect to macromolecular synthesis in the wild type and its three mutants following the incorporation of ^{14}C-leucine and ^{3}H-mannose into proteins and glycoproteins, respectively. The results in Figure 1 explained that both wild type and its mutant cells synthesized glycoproteins of varying molecular sizes ranging from M_r 15,000 to M_r 200,000 or larger. It also explained that the extent of protein glycosylation was less in the mutants compared to the wild type when equal amounts of [^{3}H]mannose-labeled glycoproteins from each cell type were analyzed by a 7.5% SDS-PAGE. The least amount of glycosylation occurred in mutant 10248 which has the altered regulatory subunit for type I (RI) kinase.

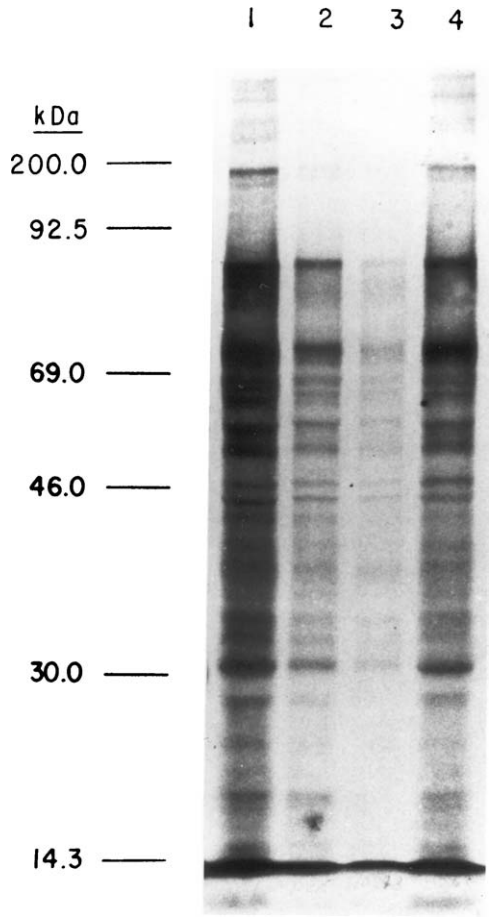

Figure 1. SDS-PAGE of [^{3}H]Man-glycoproteins from the wild type and PKA-deficient CHO cells. The cells were seeded at a density of 2×10^6 cells per 75 cm^2 flask. At 90% confluency the cells were washed with 2 ml of low-glucose medium and labeled with 2-[^{3}H]mannose 50 μCi/ml/flask; Sp. Act.18.5 Ci/mol) for 4 h. At the end of the incubation, the media were removed; the cells were washed with PBS, pH 7.4 and pelleted. 500 μg of protein from each cell type was processed by 7.5% SDS-polyacrylamide (16 cm $\times$ 16 cm) slab gel electrophoresis according to Laemmli's procedure [17], followed by autoradiography. Lane 1 = wild type 10001; lane 2 = mutant 10215; lane 3 = mutant 10248; lane 4 = mutant 10260. The migration of [^{14}C]methylated protein standard myosine (M_r 200,000), phosphorylase b (M_r 97,400), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and lysozyme (M_r 14,300) are shown at the far left.

Rate of LLO synthesis in wild type and PKA-deficient mutants

To address the obligatory requirement of cAMP-dependent protein kinase for increased Glc_3Man_9-$GlcNAc_2$-PP-Dol synthesis during ß-adrenoreceptor activation, we have examined the synthesis of [^{3}H]mannosylated oligosaccharide-PP-Dol in wild type and its PKA-deficient cells. As shown in Figure 2, mannosylated-oligosaccharide-PP-Dol biosynthesis in wild type cells was linear for 1 h. The PKA-deficient mutants followed a similar kinetic although the amounts of [^{3}H]mannosylated-oligosaccharide-PP-Dol synthesized in these mutants were much less than in the wild type cells. To examine it critically, the LLO synthesis was submitted to a pulse-chase protocol. The results in Figure 3 showed that the LLO in wild type cells turned over ($t_{1/2}$ = 15 min) at least twice as fast as in the PKA-deficient mutant 10248 ($t_{1/2} = 30$ min). Analysis of the mild-acid released [^{3}H]mannosylated-oligosaccharides by size exclusion chromatography on a Bio-Gel P-4 column (54 cm $\times$ 0.9 cm) indicated that both wild type and mutant cells synthesized oligosaccharide-PP-Dol of similar sizes (Figure 4a). In all cases $Glc_3Man_9GlcNAc_2$-PP-Dol was found to be the most predominating species; however, a small peak for $Man_9GlcNAc_2$-PP-Dol was also observed. It needs to be emphasized here that there was no accumulation of $Man_5GlcNAc_2$-PP-Dol in PKA-deficient mutants. Treatment of [^{3}H]mannosylated-oligosaccharide with α-mannosidase digested all mannose-labeled oligosaccharides except the $Glc_3Man_9GlcNAc_2$ species (Figure 4b).

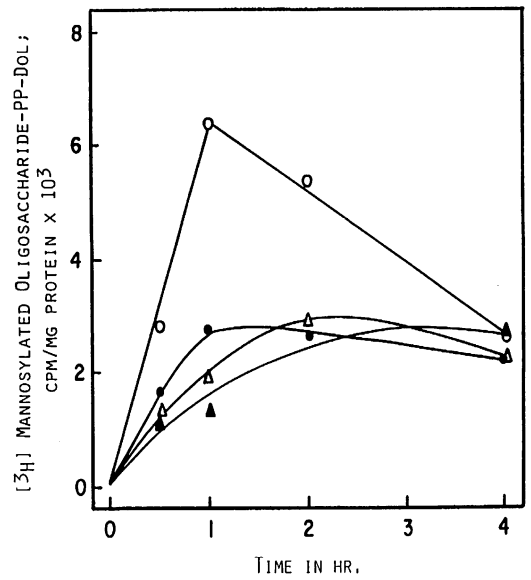

Figure 2. Time-course of [^{3}H]Man-oligosaccharide-PP-Dol biosynthesis in CHO cells. CHO cells (1×10^6) were seeded into 60 mm dishes. At 90% confluence, the cells were labeled with 25 μCi/ml of 2-[^{3}H]mannose (Sp. Act. 18.5 Ci/mol) from 30 min to 4 h. The oligosaccharide-PP-Dol was extracted and counted in a liquid scintillation spectrometer. ○—○, wild type 10001; ●—●, PKA mutant 10215; △—△, PKA mutant 10248; ▲—▲, PKA mutant 10260.

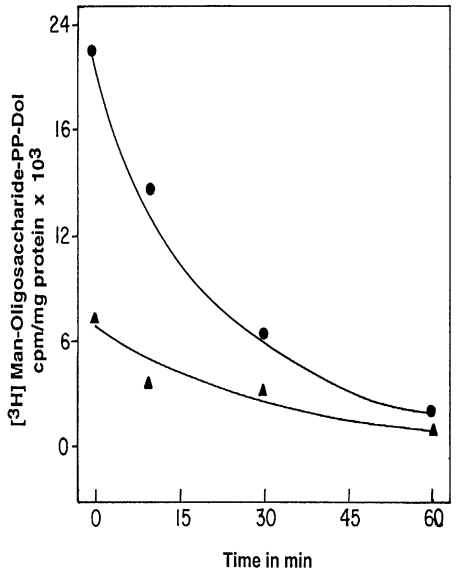

Figure 3. Turnover of [^{3}H]Man-oligosaccharide-PP-Dol in CHO Cells. CHO cells (3×10^6) were seeded into 60 mm dishes. At 90% confluence, the cells were labeled with 25 μCi/ml of 2-[^{3}H]-mannose (Sp. Act. 18.5 Ci/ml) for 1 h at 37°C. The cells were washed with low-glucose DMEM (2×1 ml) and incubated with 1 ml of low-glucose DMEM containing 20 mM mannose and chased for 0,5,15,30 and 60 min at 37°C. [^{3}H]Man-oligosaccharide-PP-Dol was isolated as described in materials and methods and counted in a liquid scintillation spectrometer. ○—○, wild type 10001; ●—●, PKA mutant 10248.

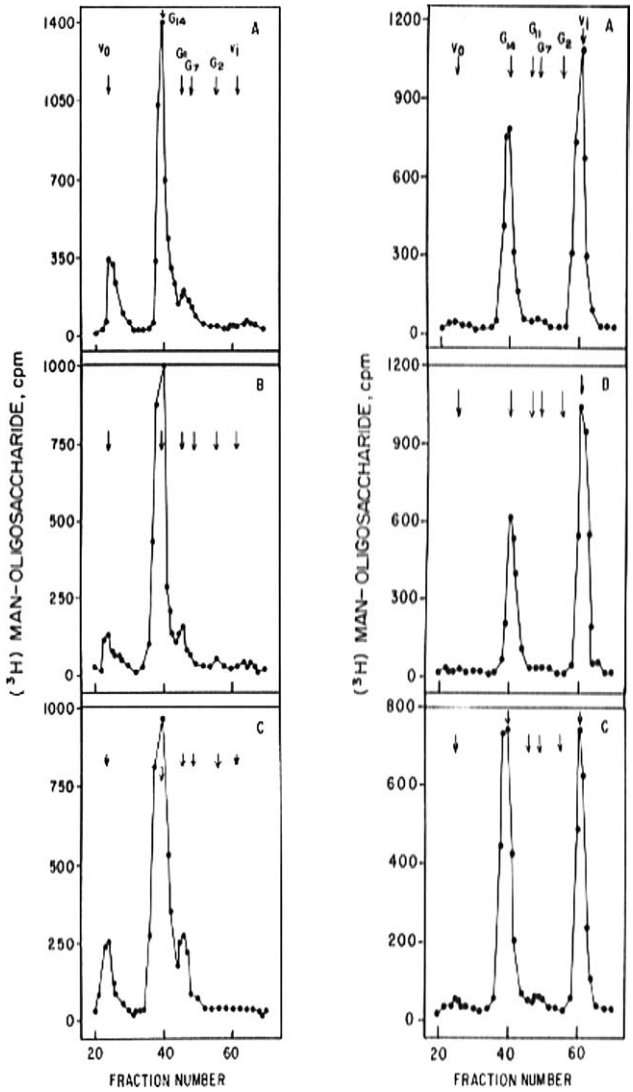

Figure 4. Bio-Gel P-4 column chromatography of [^{3}H]Man-oligosaccharide-PP-Dol before and after digestion with α-mannosidase. Wild type and PKA-deficient CHO cell mutants (1×10^6) were seeded in 100 mm dishes. At 90% confluency the cells were labeled with 75 μCi/ml of 2-[^{3}H]mannose (Sp. Act. 18.5 Ci/mol) in serum-free, low-glucose DMEM for 1 h at 37°C. The oligosaccharide-PP-Dol was extracted and made free from the lipid backbone. (a) Mild-acid released oligosaccharide were applied to a Bio-Gel P-4 column (54 cm $\times$ 0.9 cm). The column was equilibrated and washed with 50 mM ammonium formate, pH 8.0 containing 0.02% sodium azide. 0.5 ml fractions were collected at a flow rate of 6 ml/h and monitored by measuring the radioactivity in a scintillation spectrometer. (b) The oligosaccharides were digested with α-mannosidase at 37°C for 24 h over a drop of toluene and applied to the Bio-Gel P-4 column as mentioned in (a). A = wild type 10001; B = mutant 10215; C = mutant 10248; v_o = blue dextran; v_i = mannose/N-acetylglucosamine; G_2 = $GlcNAc_2$; G_7 = $Man_5GlcNAc_2$; G_{11} = $Man_9GlcNAc_2$; G_{14} = $Glc_3Man_9GlcNAc_2$.

Table 1. 2-deoxy-D-glucose uptake in wild type and PKA-deficient Chinese hamster ovary cells

	nmol/mg Protein	
Cell type	*Control*	*8Br-cAMP (2 mM)*
Wild type 10001	2.76 ± 0.20	3.01 ± 0.02
Mutant 10215	3.59 ± 0.27	3.25 ± 0.23
Mutant 10248	3.77 ± 0.14	4.03 ± 0.19
Mutant 10260	ND	ND

The cells were cultured in 24 well clusters. At 90% confluency, the media were removed and the cells were washed with 2 x 1 ml of standard reaction mixture (SRM; 118 mM NaCl/4.4 mM KCl/1.2 mM Mg_2SO_4/2.2 mM $CaCl_2$/25 mM Hepes-NaOH/10 mM mannitol, pH 7.4). The cells were incubated at 37°C for 15 min in the presence of 0.1 mM 2-deoxyglucose and 1.0 μCi/ml of 2-deoxy-[2,5-^{3}H]-D-glucose (Sp. Act. 35Ci/mmol) in 500 μl of SRM. The experimental group received 2 mM 8Br-cAMP. Following incubation, the cells were washed 3 × 1 ml with PBS, pH 7.4. 500 μl of PBS was added to each well and the radioactivity was counted. Incubation at ice-cold temperature served as 'zero time'. The results are the mean ± SEM from three experiments. ND = not determined.

Transport of 2-deoxy-D-glucose in wild type and PKA-deficient cells

In order to eliminate the possibility that the reduced level of [^{3}H]mannosylated-oligosaccharide-PP-Dol in these somatic cell mutants was not due to a decreased availability of sugars, transport of a non-metabolizable sugar, 2-[^{3}H]-deoxy-D-glucose into these cells was studied. A direct assessment of 2-[^{3}H]deoxy-D-glucose uptake in mutants 10215 and 10248 indicated that the sugar transport was not reduced in the mutant cells but elevated by 30 to 36% compared to that of the wild type cells (Table 1). Furthermore, 2 mM 8Br-cAMP did not affect the movement of 2-deoxy-D-glucose across their plasma membranes.

Activity of Dol-P-Man synthase in wild type and in PKA-deficient CHO cell mutants

Absence of $Man_5GlcNAc_2$-PP-Dol accumulation and reduction of a total oligosaccharide-PP-Dol in mutants suggested an altered Dol-P-Man synthase activity. To address it Dol-P-Man synthase activity was examined directly. The results showed that Dol-P-Man synthase in mutant cells was less active than that of the wild type cells. Kinetic measurements at saturating concentrations of GDP-mannose demonstrated that the K_m for GDP-mannose was increased by approximately 400% in mutants 10248 and 10260, and 160% in mutant 10215 over the wild type cells 10001 (Table 2). k_{cat} for DPMS in PKA-deficient mutants was reduced significantly (*i.e.*, 2 to 4-fold) whereas the catalytic efficiency (k_{cat}/K_m) was reduced by only 10 to 30%. Comparable results were obtained in the presence of exogenously added Dol-P (Table 3). If protein phosphorylation regulates DPMS activity *in situ* then the defects in these CHO cell mutants can be corrected by exogenous protein kinase. In fact, *in vitro* phosphorylation of the ER membrane protein from the mutants 10248 and 10260 restored the Dol-P-Man synthase activity to the level of the wild type enzyme (Table 4).

Discussion

A variety of CHO cell mutants (10215/10248/10260) defective in cAMP-dependent protein kinase have been used to elucidate the role of PKA in regulating protein glycosylation and more importantly, the Dol-P-Man synthase activity during ß-adrenoreceptor stimulation. The wild type CHO cell line 10001 is a derivative of the CHO Pro$^-$ [17] cell line. The cAMP-resistant mutant CHO cell lines 10215, 10248, and 10260 were generated from wild type CHO line 10001 and have three classes of defects in cAMP-dependent protein kinase [18–20]: (i) mutant 10215 has a defective C-subunit with altered substrate specificity and does not express any detectable type II kinase [18]; (ii) mutant 10248 has an altered R-subunit for type I kinase which binds cAMP poorly and also has no detectable type II kinase [21]; and (iii) mutant 10260 has the lowest level of total kinase activity with no detectable type I kinase, but does have a small amount of type II kinase [20]. In addition, mutant 10260 has a reduced amount of C-subunit (approximately 10% of wild type) [22]. Mutants 10215 and 10248 are also defective in phosphorylation of a 50 kDa cellular substrate [23], whereas mutant 10260 appears able to phosphorylate this substrate.

Table 2. Kinetic constants for Dol-P-Man synthase in wild type and PKA-deficient Chinese hamster ovary cells

	K_m *(μM)*		V_{max} *(pmol/mg protein/min)*	
Cell type	*−Dol-P*	*+Dol-P*	*−Dol-P*	*+Dol-P*
Wild type 10001	0.18	0.32	4.4 ($r = 0.99$)	5.3 ($r = 0.99$)
Mutant 10215	0.29	0.45	8.9 ($r = 0.99$)	8.9 ($r = 0.99$)
Mutant 10248	0.76	0.93	16.0 ($r = 0.96$)	17.1 ($r = 0.99$)
Mutant 10260	0.82	0.48	12.4 ($r = 0.99$)	11.1 ($r = 0.99$)

ER membranes (21.25–25.00 μg protein) from each cell type was incubated for 1 min at 37°C in 5 mM Tris-HCl, pH 7.0 buffer containing 12.5 mM sucrose, 5 μM EDTA, 5 mM $MnCl_2$, 4 mM 5′AMP, 0.5% Me_2SO, and 0.0–1.0 μM GDP-[U-^{14}C]-mannose (Sp. Act. 323 cpm/pmol) in a total volume of 100 μl in the presence and absence of 50 μg dolichyl monophosphate. Reactions were stopped and the mannolipid was extracted. The results are the mean from two separate experiments.

Table 3. Turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of Dol-P-Man synthase from the wild type and PKA-deficient Chinese hamster ovary cells

	k_{cat} (s^{-1})		k_{cat}/K_m ($M^{-1}s^{-1}$)	
Cell type	*−Dol-P*	*+Dol-P*	*−Dol-P*	*+Dol-P*
Wild type 10001	2.3×10^{-6}	2.9×10^{-6}	12.8	9.1
Mutant 10215	4.9×10^{-6}	4.9×10^{-6}	16.9	10.9
Mutant 10248	8.9×10^{-6}	9.5×10^{-6}	11.7	10.2
Mutant 10260	6.9×10^{-6}	6.3×10^{-6}	8.4	13.1

k_{cat} values were calculated using the K_m values from Table 2. The calculation was based on the molecular weight of Dol-P-Man synthase as 32,000 dalton.

Table 4. Dol-P-Man synthase activity in wild type and PKA-deficient Chinese hamster ovary cells after *in vitro* protein phosphorylation

	Dol-P-Man (pmol/mg protein/min)	
Cell type	*−Phosphorylation*	*+Phosphorylation*
Wild type 10001	7.4	7.9
Mutant 10248	5.6	6.8
Mutant 10260	5.7	7.7

ER membranes (30 μg protein) from each cell type were phosphorylated by incubating at 30°C for 20 min in a buffer containing 10 mM Tris-HCl, pH 7.0, 25 mM sucrose, 10 μM EDTA, 10 mM $MgCl_2$, 10 mM KF, 1% Me_2SO, 0.2 mM ATP, and 12.5 units of catalytic subunit of cAMP-dependent protein kinase in a total volume of 50 μl. Incubations containing no catalytic subunit served as controls. The phosphorylated membranes were then incubated with 2.5 μM GDP-[U-^{14}C]mannose (Sp. Act. 318 cpm/pmol) in a total volume of 100 μl for 5 min at 37°C, and the mannolipd was extracted as described in Materials and methods. The results are average from two experiments.

Our results suggest that glycosylation in PKA-deficient mutants is not due to impaired protein synthesis, because ^{14}C-leucine incorporation per milligram of protein was 291% higher in mutant 10215, 117% higher in mutant 10248 and 230% higher in mutant 10260 as compared to the wild type cells. The protein glycosylation index (a ratio of ^{3}H-mannose to ^{14}C-leucine incorporation into glycoproteins) [24] in wild type CHO cells was increased by 120% in the presence of 2 mM 8Br-cAMP, whereas it was only increased by 23% and 20% in mutants 10248 and 10260, respectively. Absence of significant differences in ^{14}C-leucine incorporation into cellular proteins between the wild type and the PKA-deficient mutants in the presence or absence of 8Br-cAMP supported the idea that increased glycosylation by the cAMP-pathway requires active participation from the cAMP-dependent protein kinase.

Reduced [^{3}H]mannosylated-oligosaccharide-PP-Dol biosynthesis in mutants was also a consequence of PKA deficiency. An observed decrease in [^{3}H]mannosylated-oligosaccharide-PP-Dol level after 1 h of incubation in wild type cells is due to its high turnover. Size exclusion chromatography of the mild-acid released mannose-labeled oligosaccharide on Bio-Gel P-4 registered no abnormality in the oligosaccharide profiles. The glycan structure of the mannosylated-oligosaccharide species eluted at the void volume (V_o) is currently unknown, but we can conclude that it contained oligomannosyl residues because of its sensitivity to α-mannosidase digestion. The mild-acid released [^{3}H]mannosylated-oligosaccharides from the wild type and its various mutants when analyzed on a Concanavalin A-Sepharose affinity column, the profile matched that of the Bio-Gel P-4 column, (data not shown).

In the synthesis of $Glc_3Man_9GlcNAc_2$-PP-Dol, $Man_9GlcNAc_2$-PP-Dol is an essential intermediate [25]. Conversion of $Man_5GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol requires a steady supply of Dol-P-Man, a product of the reaction GDP-mannose + Dol-P ⇔ Dol-P-Man catalyzed by DPMS in the rough endoplasmic reticulum [15,26]. In the present study neither an accumulation of $Man_5GlcNAc_2$-PP-Dol nor a reduction in 2-deoxy-D-glucose uptake was observed in PKA-deficient mutants. We, therefore, hypothesize that DPMS activity is present in these mutants but is down-regulated. Phosphorylation *in vitro* rescued the DPMS activity of the PKA-deficient cells almost to the level of the wild type and supported the idea that protein phosphorylation is one of the key elements regulating the synthase activity. This observation is analogous to the increased DPMS activity found in the rat acinar cells following ß-adrenoreceptor stimulation [3]. It should be noted that the synthase activity was enhanced 38% in isoproterenol-treated cells but only 33% after *in vitro* phosphorylation of the ER membrane protein [3,9]. PKA-deficient mutants 10248 and 10260 (deficiency primarily in type I kinase) exhibited 20–40% loss in DPMS activity as compared to the wild type cells. Increased K_m for GDP-mannose and decreased k_{cat} for DPMS in mutant cells suggested that the observed difference in mutant cells is due to a deficiency intrinsic to the synthase. Observed marginal changes in the catalytic efficiency of the enzyme from mutant cells suggested a compensatory mechanism in these mutants, since PKA deficiency is not lethal in the absence of cAMP [10]. It is then concluded that reduced DPMS activity may not be lethal for the PKA-deficient cells, but it may contribute to other metabolic consequences such as growth and differentiation [27–29] as well as the development of Congenital Defect in Glycoprotein Syndrome (CDG) [30]. Dpm1 gene has now been cloned from a number of species such as *Saccharomyces cerevisiae, Trypanosoma brucei, Ustilago maydis, Schizosaccharomyces pombe, Caenorhabditis briggsiae* as well as from human [31–34]. Most striking is that all Dol-P-Man synthases have a serine residue in the position corresponding to Ser141 in the *S. cerevisiae*. Serine-141, and the conserved residues at the NH_2-terminus meet the criteria for a consensus site for phosphorylation by cAMP-dependent protein kinase [35,36]. Further studies are therefore warranted to establish the role of the phosphorylation motif on DPMS structure-function.

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References

1 Kousvelari EE, Grant SR, Banerjee DK, Newby MJ, Baum BJ, Cyclic AMP mediates beta-adrenergic-induced increases in N-linked protein glycosylation in rat parotid acinar cells, *Biochem J (London)* **222**, 17–24 (1984).
2 Grant SR, Kousvelari EE, Banerjee DK, Baum BJ, beta-Adrenergic stimulation alters oligosaccharide pyrophosphoryl dolichol metabolism in rat parotid acinar cells, *Biochem J (London)* **231**, 431–8 (1985).
3 Banerjee DK, Kousvelari EE, Baum BJ, beta-Adrenergic activation of glycosyltransferases in the dolichylmonophosphate-linked pathway of protein N-glycosylation, *Biochem Biophys Res Commun* **126**, 123–9 (1985).
4 Hatefi Y, Rieske JS, The preparation and properties of DPNH-Cytochrome *c* reductase (complex I–III of the respiratory chain, *Methods Enzymol* **20**, 225–31 (1967).
5 Jahn R, Unger C, Soling HD, Specific protein phosphorylation during stimulation of amylase secretion by beta-agonists or dibutyryl adenosine 3′,5′-monophosphate in the rat parotid gland, *Eur J Biochem* **112**, 345–52 (1980).
6 Baum BJ, Freidberg JM, Ito H, Roth GS, Filburn CR, beta-Adrenergic regulation of protein phosphorylation and its relationship to exocrine secretion in dispersed rat parotid gland acinar cells, *J Biol Chem* **256**, 9731–6 (1981).
7 Freedman SD, Jamieson JD, Hormone-induced protein phosphorylation. II. Localization to the ribosomal fraction from rat exocrine pancreas and parotid of a 29,000-dalton protein phosphorylated *in situ* in response to secretagogues, *J Cell Biol* **95**, 909–17 (1982).
8 Banerjee DK, Regulation of mannosylphosphoryl dolichol synthase activity by cAMP-dependent protein phosphorylation. In *Highlights of Modern Biochemistry*, edited by Kotyk A, Skoda J, Paces V, Kostka V (VSP International Science Publishers, Zeist, The Netherlands, 1989), p. 379.
9 Banerjee DK, Kousvelari EE, Baum BJ, cAMP-mediated protein phosphorylation of microsomal membranes increases mannosylphosphodolichol synthase activity, *Proc Natl Acad Sci (USA)* **84**, 6389-93 (1987).
10 Banerjee DK, Baksi K, Gottesman MM, Genetic evidence that action of cAMP-dependent protein kinase is not an obligatory step for antiviral and antiproliferative effects of human interferon in Chinese hamster ovary cells, *Virology* **129**, 230–8 (1983).
11 Maheshwari RK, Banerjee DK, Waechter CJ, Olden K, Friedman RM, Interferon treatment inhibits glycosylation of a viral protein, *Nature* **287**, 454–6 (1980).
12 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analyt Biochem* **72**, 248–54 (1976).
13 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680–5 (1970).
14 Bonner WM, Laskey RA, A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels, *Eur J Biochem* **46**, 83–8 (1974).
15 Banerjee DK, Scher MG, Waechter CJ, Amphomycin: Effect of the lipopeptide antibiotic on the glycosylation and extraction of dolichyl monophosphate in calf brain membranes, *Biochemistry* **20**, 1561–8 (1981).
16 Tavárez–Pagán JJ, Oliveira CM, Banerjee DK, Insulin up-regulates a $Glc_3Man_9GlcNAc_2$-PP-Dol pool in capillary endothelial cells not essential for angiogenesis, *Glycoconjugate J*, **20**, 179–88 (2004).
17 Puck TT, Ciecuria SJ, Robison A, Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects, *J Exptl Med* **108**, 945–55 (1958).
18 Evain D, Gottesman MM, Pastan I, Anderson WB, A mutation affecting the catalytic subunit of cyclic AMP-dependent protein kinase in CHO cells, *J Biol Chem* **254**, 6931–7 (1979).
19 Gottesman MM, LeCam A, Bukowski M, Pastan I, Isolation of multiple classes of mutants of CHO cells resistant to cyclic AMP, *Somatic Cell Genetics* **6**, 45–61 (1980).
20 Singh TJ, Roth C, Gottesman MM, Pastan I, Characterization of cyclic AMP-resistant Chinese hamster ovary cell mutants lacking type I protein kinase, *J Biol Chem* **256**, 926–32 (1981).
21 Gottesman MM, Singh T, LeCam A, Roth C, Nicolas JC, Cabral F, Pastan I, Cyclic AMP-dependent phosphorylations in cultured fibroblasts: A genetic approach, *Cold Spring Harbor Conferences on Cell Proliferation* **8**, 195–209 (1981).
22 Murtaugh MP, Steiner ALK, Davies PJA, Localization of the catalytic subunit of cyclic AMP-dependent. Protein kinase in cultured cells using a specific antibody, *J Cell Biol* **95**, 64–72 (1982).
23 LeCam A, Nicolas JC, Singh TJ, Cabral F, Pastan I, Gottesman MM, Cyclic AMP-dependent phosphorylation in intact cells and in cell-free extracts from Chinese hamster ovary cells. Studies with wild type and cyclic AMP-resistant mutants, *J Biol Chem* **256**, 933–41 (1981).
24 Kousvelari EE, Grant SR, Baum BJ, beta-Adrenergic receptor regulation of N-linked protein glycosylation in rat parotid acinar cells, *Proc Natl Acad Sci (USA)* **80**, 7146–50 (1983).
25 Kornfeld, RA, Kornfeld S, Assembly of asparagine-linked oligosaccharides, *Annu Rev Biochem* **54**, 631–64 (1985).
26 Chapman A, Trowbridge IS, Hyman R, Kornfeld S, Structure of the lipid-linked oligosaccharides that accumulate in class E Thy-1-negative mutant lymphomas, *Cell* **17**, 509–15 (1979).
27 Banerjee DK, Vendrell-Ramos M, Is asparagine-linked protein glycosylation an obligatory requirement for angiogenesis? *Indian J Biochem Biophys* **30**, 389–94 (1993).
28 Oliveira CM, Banerjee, DK, Role of extracellular signaling on endothelial cell proliferation and protein N-glycosylation, *J Cellular Physiol* **144**, 467–72 (1990).
29 Banerjee DK, Microenvironment of endothelial cell growth and regulation of protein N-glycosylation, *Indian J Biochem Biophys* **25**, 8–13 (1988).
30 Marquardt T, Denecke J, Congenital disorders of glycosylation: Review of their molecular bases, clinical presentations and specific therapies, *Eur J Pediatr* **162**, 359–79 (2003).

31 Orlean P, Albright C, Robbins PW, Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein, *J Biol Chem* **263**, 17499–507 (1988).
32 Mazhari-Tabrizi R, Eckert V, Blank M, Muller R, Mumberg D, Funk M, Schwarz RT, Cloning and functional expression of glycosyltransferases from parasitic protozoans by heterologous complementation in yeast: The dolichol phosphate mannose synthase from Trypanosoma brucei brucei, *Biochem J* **316**, 853–8 (1996).
33 Zimmerman JW, Specht CA, Ceganes BX, Robbins PW, The isolation of a Dol-P-Man synthase from Ustilago maydis that functions in Saccharomyces cerevisiae, *Yeast* **12**, 765–71 (1996).
34 Colussi PA, Taron CH, Mack JC, Orlean P, Human and saccharomyces cerevisiae dolichol phosphate mannose synthases represent two classes of the enzyme, but both function in schizosaccharomyces pombe, *Proc Natl Acad Sci (USA)* **94**, 7873–8 (1997).
35 Krebs EG, Beavo J, Phosphorylation-dephosphorylation of enzymes *Annu Rev Biochem*, **48**, 923–59 (1975).
36 Taylor SS, Buechler JA, Yonemoto W, cAMP–dependent protein kinase: Framework for a diverse family of regulatory enzymes, *Annu Rev Biochem* **59**, 971–1005 (1990).