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REQUIREMENT OF PROTEIN KINASE TYPE I FOR CAMP-MEDIATED UP-REGULATION OF LIPID-LINKED OLIGOSACCHARIDE FOR ASPARAGINE-LINKED PROTEIN GLYCOSYLATION

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Abstract–Glycan chains of asparagine-linked (N-linked) glycoproteins play a significant role in protein structure and function, as well as in angiogenesis an essential process for breast or other solid tumor growth. Non-availability of these chains causes incorrect folding of glycoproteins and leads to programmed cell death (i.e., apoptosis) through *unfolded protein response* (*UPR*) signaling. Cells actively processing cAMP signals modulate the glycan chain biosynthesis by PKA. Glycosylation of cellular proteins in a PKA type I-deficient CHO mutant 10248 was much reduced when compared with the wild type CHO 10001. The rate of LLO biosynthesis is similar in both cell types but quantitatively it is low in the mutant. Pulse-chase experiments indicated that the $t_{½}$ for LLO-turnover in CHO 10248 was twice as high as that of the wild type. This correlated with the reduced DPMS activity. The K_m for GDP-mannose for the DPMS activity was 3-4 folds higher in the mutant than that of the wild type with or without exogenously added Dol-P. The k_{cat} of DPMS was also reduced in the mutant. *In vitro* phosphorylation of microsomes from the CHO 10248 by PKA, on the other hand, restored the DPMS activity to the normal level. The LLO biosynthesis also improved significantly in MR1, a revertant of the CHO 10248. The turnover of LLO in MR1 and the glycoprotein profile were also at par with the wild type. Therefore, we conclude that PKA type I plays an important role in modulating the protein N-glycosylation in cAMP responsive cells.

Key words: cAMP-dependent protein kinase type 1; Lipid-Linked oligosaccharide; Chinese hamster ovary cells; Mannosylphospho dolichol synthase; Asparagine-linked protein glycosylation; Dolichyl monophosphate

INTRODUCTION

The process of quality control for nascent proteins in the lumen of the endoplasmic reticulum (ER) is an excellent example of oligosaccharide-based information. Specifically, information-carrying N-linked oligosaccharides on newly synthesized ER glycoproteins are continuously altered during folding and assembly to reflect the status of the glycoproteins and to promote interaction(s) with appropriate components of the quality control machinery (18). Lipid-linked oligosaccharide (LLO; $Glc_3Man_9GlcNAc_2$-PP-Dol) is transferred cotranslationally by the multi-subunit enzyme oligosaccharyl transferase to sterically accessible asparaginyl residues in Asn-Xaa-Ser/Thr on nascent proteins in the ER lumen but does not complete the translation. Triglucosyl sequence of $Glc_3Man_9GlcNAc_2$ is important for recognition by oligosaccharyl transferase (51, 53) but, although its hydrophilic character promotes protein folding, no specific function or informational content has been reported. Conversely, cells have several strategies for maintaining the quantity of LLO.

LLO synthesis occurs in a stepwise manner from dolichol-P (Dol-P), requiring four donor substrates in the order: UDP-GlcNAc, GDP-mannose, dolichol-P-mannose (Dol-P-Man), and dolichol-P-glucose (Dol-P-Glc). Dol-P-Man and Dol-P-Glc are formed by transfer of mannose or glucose from GDP-mannose or UDP-glucose, respectively, to dolichol-P. Elongation of

Abbreviations: **AMP**, adenosine monophosphate; **ATP**, adenosine triphosphate; **CHO**, Chinese hamster ovary cells; **Dol-P**, dolichyl monophosphate; **Dol-P-Glc**, glucosylphospho dolichol; **Dol-P-Man**, mannosylphospho dolichol; **DPMS**, mannosylphospho dolichol synthase; **EDTA**, ethyelenediamine tetra-acetic acid; **ER**, endoplasmic reticulum; **GDP**, guanosine diphosphate; **LLO**, lipid-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$-PP-Dol); **Me_2SO**, dimethtyl sulfoxide; **NaCl**, sodium chloride; **PKA**, cAMP-dependent protein kinase; **SDS**, sodium dodecyl sulfate.

$Man_5GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol prior to acquisition of three glucose residues is completed by transferring mannose residues from Dol-P-Man. Dol-P-Man donates the sixth and possibly the remaining three mannose residues in N-glycan precursors, all three (four in yeast) mannose residues in glycophosphatidylinositol (GPI), the first mannose in O-mannosylation of many yeast proteins (1,27,28,36,50), and a mannose in C-mannosylation of Trp-7 ribonuclease 2 (16). Mannosylphospho dolichol synthase (DPMS) catalyzes the biochemical reaction GDP-Mannose + Dol-P <= Mn^{2+} => Dol-P-Man + GDP on the cytosplasmic site of the ER membrane. In the absence of Dol-P-Man, protein N-glycosylation would be impaired and the glycosylphosphatidyl inositol (GPI)-anchored proteins would not be synthesized. O- and C-mannosylation of proteins would also be arrested. DPMS deficiency has been observed earlier in a Class E *Thy*-1 lymphoma patient (13) but, it has been reported recently that partially deficient Dol-P-Man biosynthesis causes congenital disorder of glycosylation (CDG; 30, 35). Patients deficient in Dol-P-Man synthesis lacking either N-glycan and/or GPI-biosynthesis causes developmental delay, seizures, hypotonia, and dysmorphic features.

Dol-P-Man has been reported to stimulate UDP-GlcNAc:Dol-P GlcNAc-1-phosphate transferase involved in the first step of LLO biosynthesis (32,34). Exogenous addition of Dol-P-Man to microsomes from various cell types stimulated the GlcNAc-1-phosphate transferase activity seven to eight times (32). It was also reported that microsomes derived from BW5147 class *Thy*-1 negative cells that lack Dol-P-Man synthase had GlcNAc-1-phosphate transferase and was stimulated when Dol-P-Man was added exogenously (33).

DPMS activity is widely distributed and has been detected in higher eukaryotes including human cells and tissues, yeast, protozoan parasites, plant fungi, nematodes, as well as in archaebacteria (61). Bacterial homolog for DPMS has also been identified. Dol-P-Man synthase in various organisms are grouped into two: one is a single-component enzyme represented by *Saccharomyces cerevisiae* Dpm1p (14), and the other is a multi-component enzyme represented by human Dol-P-Man synthase, which contains a catalytic subunit DPM1 and two accessory proteins DPM2 and DPM3 (45,46). The former group includes the enzymes of *S.cerevisiae* (52), the plant fungus *Ustilago maydis* (62), the protozoa *Trypanosoma brucei* (49), and *Leishmanaia mexicana* (29). These DPMS can be expressed in *Escherichia coli* as functional enzyme (62,60). In contrast, human DPM1 cannot be stably expressed in *E.coli* (14) and requires the presence of DPM2 for stable expression in the ER (46). Members of the former group share 50%-60% amino acid identity and have a transmembrane domain near the C-terminus, whereas human DPM1 has only 30% amino acid identity to the former group members and does not have a transmembrane domain (14,46). *Schizosaccharomyces pombe* and *Cenerohabditis briggsiae* have DPM1 homologs that lack a transmembrane domain, and have 65% and 64% amino acid identity to human DPM1, respectively (14). Clearly, the enzymes of the two groups have different structural and functional characteristics.

It is not clear, why DPMS has diverged into two groups, except that the multiple-component system should be suitable for regulation. For example, DPMS was activated almost identically whether a microsomal membrane preparation; or a detergent solubilized, antibody-affinity purified enzyme, free from other membrane components was phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA); or the activity was measured in the microsomal membranes from cells pretreated with the β-agonist, isoproterenol (5,6,8). No change in synthase activity in the presence of actinomycin D (an inhibitor of DNA-dependent RNA polymerase activity) downplayed the possibility of enhanced DPMS transcription because of isoproterenol treatment. Sequence alignment of cloned DPMS identified the presence of a conserved PKA-mediated phosphorylation site in all DPMS sequences equivalent to that of the serine-141 in *S.cerevisiae*. *In vitro* phosphorylation of serine-141 of the recombinant *S.cerevisiae* DPMS by PKA exhibited (i) a 6-fold increase in the V_{max}, and (ii) higher enzyme turnover (k_{cat}) as well as enzyme efficiency (k_{catr}/K_m). When serine-141 was replaced with alanine by polymerase chain reaction (PCR) site-directed mutagenesis, the S141A mutant exhibited more than half-a-fold reduction in the DPMS activity upon phosphorylation (10).

Functions and importance of the PKA have been studied extensively in the past (19, 26, 39, 40, 44, 58). Most cells have two main types of PKAs (i.e., type I and type II) that differ from each other in their regulatory subunits. However, studies of the protein structure (17, 53) and

isolation of cDNA clones for the subunits (31, 42, 54, 56) suggest that additional forms of both regulatory and catalytic subunits are present. To understand the involvement of PKA subtypes in regulating the LLO biosynthesis and consequently that of the protein N-glycosylation, we have recently used a series of Chinese hamster ovary (CHO) cell mutants defective in PKA. Our observation indicated that the CHO cell mutant 10248 conferred the growth inhibitory effect of cAMP due to an alteration in the R-subunit for type I kinase (i.e., RI) and binds cAMP poorly, expressed low level of LLO due to a reduced DPMS activity. The K_m for GDP-mannose was increased by 3-4 fold compared to that of the wild type. A comparable down-regulation of the k_{cat} was also observed (9). To further evaluate the role of type I kinase on LLO biosynthesis we have used a revertant of CHO 10248, i.e., MR1 (2) that appeared to have identical PKA activity as that of the wild type. In this article we present evidence that the amounts of both LLO and the glycoproteins in the revertant MR1 are comparable to that of the wild type.

MATERIALS AND METHODS

Alpha-minimal essential medium with Earl's salt (alpha-EMEM), Dulbecco's minimal essential medium (DMEM), Ham's F-12 medium, glutamine, and penicillin-streptomycin were purchased from BioSource, Rockville, MD, USA. Fetal bovine serum was a product of HyClone Laboratories, Logan, UT, USA. 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 Aldrich, St. Louis, MO, USA. 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_r 14,300 - 200,000) were obtained from GE Healthcare, Newark, NJ, USA. Gamma-[^{32}P]ATP (25 Ci/mmol) was supplied by ICN, Costa Mesa, CA, USA. Electrophoresis reagents were obtained from Bio-Rad Laboratories, Hercules, CA, USA. All other chemicals were of reagent quality.

Cell Culture

The wild type CHO cells, their PKA-deficient mutant 10248, and the revertant MR1 were grown in alpha-EMEM (4). During isotopic labeling, the cells were washed with either serum-free DMEM or Ham's F-12 and incubated at 37^{o}C with either 2-[^{3}H]mannose or U-[^{14}C]leucine in low-glucose or leucine-free medium for 60 minutes unless otherwise mentioned.

Isolation and Analysis of LLO

The lipid-linked oligosaccharide biosynthesis in wild type CHO cells, their PKA-deficient 10248 mutant, and the revertrant MR1 were studied by labeling them for 1 hour at 37^{o}C with 2-[^{3}H]mannose (25 µCi/ml) in 1 ml of serum-free low-glucose Dulbecco's minimal essential medium. At the end of the incubation, cells were washed with phosphate buffered saline (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% sodium chloride (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 0.1N HCl in 80% tetrahydrofuran at 50^{o}C for 30 minutes (3). Sizing of the mild-acid released [^{3}H]mannose-oligosaccharides was carried out over a Bio-Gel P-4 column (54 cm x 0.9 cm). The column was washed, equilibrated, and eluted with 50 mM ammonium formate, pH 8.0 containing 0.02% sodium azide (NaN_3).

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

Wild type, the PKA-deficient CHO cell mutant 10248 and the revertant MR1 were labeled with 2-[^{3}H]mannose (50 µCi/ml) in low-glucose media at 37^{o}C for 4 hours. 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^{o}C for 20 min. The extracts were clarified by centrifuging at 100,000 xg for 60 minutes. Equal amounts of protein were separated on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (38). The gels were fixed for 30 minutes in methanol-acetic acid-water (5:5:1, v/v/v) and processed for autoradiography in Amplify™ (11).

Dol-P-Man Synthase Assay

ER membrane proteins were isolated from cultured cells by differential centrifugation (47) and kept frozen at -20^{o}C in multiple aliquots until needed. Enzymatic formation of Dol-P-Man was assayed by incubating the ER membranes in 5mM Tris-HCl, pH 7.0 containing 12.5 mM sucrose, 5 µM ethylene diamine tetra-acetic acid (EDTA), 5 mM $MnCl_2$, 4 mM 5'AMP, and 2.5 µM GDP-[U-^{14}C]- mannose in 100 µl for 5 minutes at 37^{o}C, unless otherwise mentioned. Each assay was initiated by the addition of GDP-[U-^{14}C]mannose and stopped at desired time by the addition of 20 volumes of chloroform-methanol (2:1, v/v). After centrifugation at 1520 xg for 5 minutes 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 (5). Protein concentration in the ER membrane was determined by Bradford Protein Assay (12) using bovine serum albumin as standard. Phosphorylation of either membrane-bound Dol-P-Man synthase or solubilized/partially purified enzyme was carried out by incubating at 30^{o}C for 20 minutes 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 (6).

RESULTS

LLO biosynthesis in wild type, PKA-deficient mutant 10248, and the revertant MR1

To address the obligatory requirement of cAMP-dependent protein kinase type I for $Glc_3Man_9GlcNAc_2$-PP-Dol synthesis, we have examined the synthesis of [^{3}H]mannosylated-oligosaccharide-PP-Dol in wild type CHO cells,

their PKA-deficient mutant 10248 and in the revertant MR1. As shown in Figure 1, mannosylated – oligosaccharide – PP - Dol biosynthesis in wild type cells was linear for 1 hour. The PKA-deficient mutant 10248 also followed a similar kinetic, but the amount of [3H]mannosylated – oligosaccharide – PP - Dol synthesized in this mutant was much less. When a similar experiment was conducted with the revertant MR1, the [^{3}H]mannosylated-oligosaccharide-PP-Dol synthesis was significantly improved. It fact, the revertant MR1 had expressed a comparable amount of [^{3}H]mannosylated-oligosaccharide-PP-Dol as that of the wild type (Figure 2).

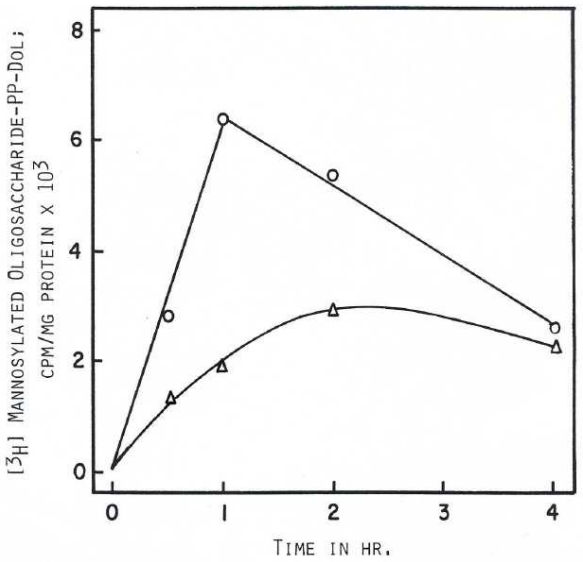

Figure1. Time-course of [^{3}H]Man-oligosaccharide-PP-Dol Biosynthesis in Wild Type and PKA-deficient CHO Cell Mutant: CHO cells (1x10^6) were seeded into 60 mm dishes. At 90% confluence, the cells were labeled with 25µCi/ml of 2-[^{3}H]mannose (Sp. Act. 18.5 Ci/mol) from 30 minutes to 4 hours. The oligosaccharide-PP-Dol was extracted and counted in a liquid scintillation spectrometer. o---o, Wild type 10001; Δ---Δ, PKA mutant 10248.

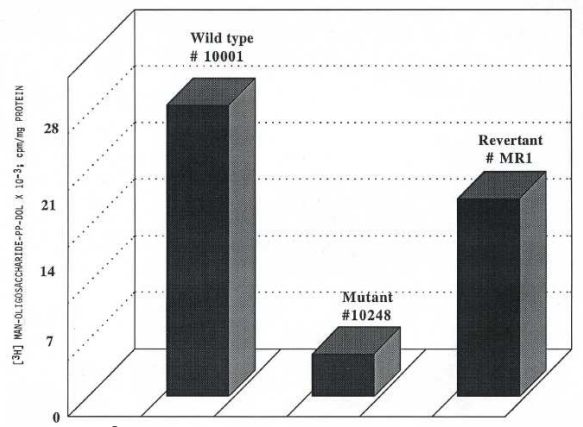

Figure 2. [^{3}H]Man-oligosaccharide-PP-Dol Biosynthesis in the Revertant MR1: Cells (1x10^6) were seeded into 60 mm dishes. At 90% confluence, the cells were labeled with 25µCi/ml of 2-[^{3}H]mannose (Sp. Act. 18.5 Ci/mol) for 1 hour. The oligosaccharide-PP-Dol was extracted and counted in a liquid scintillation spectrometer.

Activity of Dol-P-Man synthase in wild type and PKA-deficient mutant 10248

Elongation of $Man_5GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol requires active participation of DPMS. Therefore, reduction of oligosaccharide-PP-Dol level in the mutant 10248 suggested an altered Dol-P-Man synthase activity in the mutant. To address such a possibility, we analyzed the DPMS activity in mutant 10248. The results showed that DPMS in the mutant 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 the mutant 10248 over the wild type cells 10001 (Table 1). k_{cat} for DPMS in the mutant 10248 was also reduced significantly (i.e., 3 to 4-fold) whereas the catalytic efficiency (k_{cat}/K_m) remained largely unaffected. A comparable result was obtained in the presence of exogenously added Dol-P (Table 2). If protein phosphorylation regulates the DPMS activity *in situ* then the deficiency in the mutant 10248 can be corrected by phosphorylating the DPMS with exogenous protein kinase. In fact, *in vitro* phosphorylation of the ER membrane protein from the mutant 10248 restored the Dol-P-Man synthase activity to the level of the wild type enzyme (Table 3).

Table 1. Kinetic Constants for Dol-P-Man Synthase in Wild Type and PKA-deficient Chinese Hamster Ovary Cell Mutant 10248

ER membrane (21.25 -25.00 µg protein) from each cell type was incubated for 1 minute 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.

Cell Type		K_m (µM)		V_{max} (pmol/mg protein/min)	
		- 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	10248	0.76	0.93	16.0 (r=0.96)	17.1 (r=0.99)

Table 2. 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 Cell Mutant 10248

k_{cat} values were calculated using the K_m values from **Table 1**. The calculation was based on the molecular weight of Dol-P-Man synthase as 32,000 dalton.

Cell Type		$k_{cat}(s^{-1})$		$k_{cat}/K_m(M^{-1}s^{-1})$	
		- Dol-P	+ Dol-P	- Dol-P	+ Dol-P
Wild Type	10001	2.3×10^{-6}	2.9×10^{-6}	12.8	9.1
Mutant	10248	8.9×10^{-6}	9.5×10^{-6}	11.7	10.2

Table 3. Dol-P-Man Synthase Activity in Wild Type and PKA-deficient Chinese Hamster Ovary Cell Mutant 10248 after *in vitro* Protein Phosphorylation

ER membranes (30 µg protein) from each cell type were phosphorylated by incubating at 30°C for 20 minutes 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 minutes at 37°C, and the mannolipid was extracted as described in **Materials and Methods**. The results are average from two experiments.

Cell Type		Dol-P-Man (pmol/mg protein/min)	
		- Phosphorylation	+ Phosphorylation
Wild Type	10001	7.4	7.9
Mutant	10248	5.6	6.8

Rate of LLO biosynthesis in the revertant MR1 and correcting protein glycosylation deficiency

The above observations have made it clear that any deficiency of PKA type I due to a loss in the regulatory subunit RI will make the DPMS less active and this will ultimately lead to a reduction in the LLO level, and finally the protein N-glycosylation. To establish it beyond any doubt we have used the revertant MR1 in which the RI is fully restored (2) to critically evaluate the LLO biosynthesis and the protein glycosylation, and compare them with that of the wild type and the PKA-deficient mutant 10248. The results in Figure 3 show that the turnover ($t_{½}$) of LLO in the wild type 10001 and the revertant MR1 was twice as fast as in the mutant 10248. For example, the $t_{½}$ for LLO in the wild type and the reverant MR1 was 10.8 min and that of the mutant 10248 was 18.5 min, respectively. When this is followed by the incorporation of ^{3}H-mannose into glycoproteins and analyzed by SDS-PAGE, the wild type, the mutant 10248 as well as the revertant MR1, all synthesized glycoproteins of molecular sizes ranging from M_r 15,000 to M_r 200,000 dalton or larger. However, the extent of glycosylation of these glycoproteins was much lower in the mutant 10248. On the other hand, the glycoprotein profile in the revertant MR1 was comparable to that of the wild type (Figure 4).

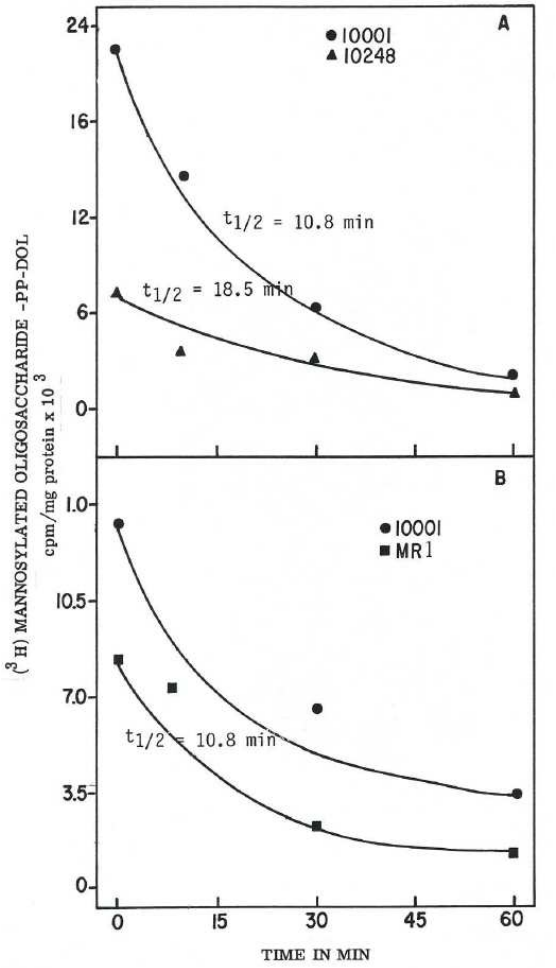

Figure 3. Turnover of [^{3}H]Man-oligosaccharide-PP-Dol in Wild Type, PKA-deficient 10248 CHO Cell Mutant and in the Revertant MR1: CHO cells ($3x10^6$) were seeded into 60 mm dishes. At 90% confluence, the cells were labeled with 25 µCi/ml of 2-[^{3}H]-mannose (Sp. Act. 18.5 Ci/ml) for 1 hour at 37°C. The cells were washed with low-glucose DMEM (2x1 ml) and incubated with 1 ml of low-glucose DMEM containing 20 mM mannose and chased for 0, 5, 15, 30, and 60 minutes 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; ♦----♦ MR1.

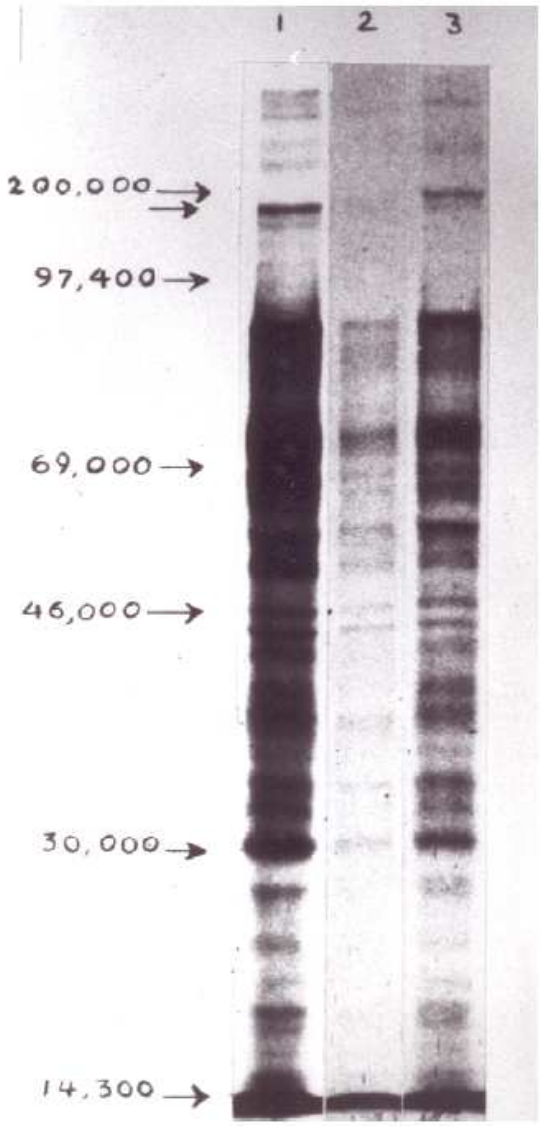

Figure 4. SDS-PAGE of [^{3}H]Man-glycoproteins from the Wild Type, PKA-deficient CHO Cell Mutant and the Revertant MR1: The cells were seeded at a density of 2x10^6 cells per 75 cm^2 flask. At 90% confluency the cells were washed with 2 ml of low-glucose medium and labeled with 2-[^{3}H]mannose 50 μCi/ml/flask; Sp. Act.18.5 Ci/mol) for 4 hours. 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 gel electrophoresis according to Laemmli's procedure [38], followed by autoradiography. Lane 1 = wild type 10001; lane 2 = mutant 10248; lane 3 = MR1. 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.

DISCUSSION

Cells undergo striking changes after they are cultured in cAMP (22), including a decrease in membrane blebbing (21), stimulation of ornithine decarboxylase (43) and transglutaminase (51), formation of gap junctions (59), changes in cell morphology and arrangement, growth inhibition (21) and stimulation (7,15), as well as capillary lumen formation (15). We have also seen up-regulation of protein N-glycosylation in rat parotid acinar cells (37), and in bovine adrenal medullary capillary endothelial cells (7) following β-adrenoreceptor stimulation with isoproterenol. The degree of up-regulation however, was reduced with aging (38). The mechanistic details indicated that increased $Glc_3Man_9GlcNAc_2$-PP-Dol (LLO) synthesis and turnover (23) of LLO is due to PKA-mediated activation of DPMS (5,6,9). Recent reports from our laboratory using a series of PKA-deficient CHO cells indicated that PKA type I plays a critical role in up-regulating LLO biosynthesis following phosphorylation activation of DPMS (9).

We have used here CHO mutant 10248 and the reverant MR1 in which the deficiency of RI subunit for PKA type I in CHO mutant 10248 has been corrected following the differential sensitivity of the mutant cells to colcemid (2). MR1 appears to have PKA activity similar to that of the wild type, based on the PKA activation profile, diethylaminoethyl (DEAE)-cellulose separation of PKA, as well as cAMP-dependent phosphorylation of a 52 kDa protein. Like the wild type, the revertant has lost its colcemed sensitivity and cAMP resistance as well as the additional RI gene(s) that was (were) present in the parental cell (2). The MR1 is therefore a pseudo wild type, which exhibits a normal level of LLO biosynthesis and turnover as well as protein glycosylation. Thus, we surmise that PKA type I plays a critical role in regulating the LLO biosynthesis and protein N-glycosylation in cells by modulating the DPMS activity. It is worth noting that studies have been planned to establish if PKA type II plays an analogous role in this process.

Neovascularization is a ‘key step’ in tumor progression and invasion, and is formed by angiogenesis through remodeling of the pre-existing blood vessels. Angiogenesis perfuse the growing tumors, while the endothelial cells release important “paracrine” growth factors for tumor cells (20, 24, 25). For example, in malignant breast tissue the endothelial cells of blood capillaries proliferate 45 times faster than that of the surrounding benign breast. Furthermore, in invasive breast carcinoma patients with metastasis, the mean microvessel

count is 101 ± 49.3 per 200x whereas those without metastasis are 45 ± 21.1 per 200x field. We have shown earlier that cAMP induces capillary lumen formation *in vitro* (15). Based on the information provided in this article, we have become more interested in studying the molecular mechanism of this important process. When treated with 8Br-cAMP the capillary endothelial cells exhibited increased LLO biosynthesis and the cellular proliferation. Mechanistic details indicated no change in the expression of total Bcl-2 or the activity of caspase-3 under the experimental condition. However, HSP-70 and HSP-90 (cytosolic chaperones) expression was up-regulated with a down-regulation of GRP-78/Bip and GRP-94 (ER chaperones) expression. It was thus concluded that a differential regulation of HSP-70/HSP-90 and GRP-78(Bip)/GRP-94 group of chaperone expression in the presence of 8Br-cAMP is associated with the switching to a new angiogenic phenotype (48).

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