Long-term effect of cyclic AMP on N-glycosylation is caused by an increase in the activity of the *cis*-prenyltransferase

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Previously we have shown that long-term pretreatment of JEG-3 choriocarcinoma cells with 8-bromo-cAMP increases the capacity for N-glycosylation that was caused by an 8–10-fold enlargement of the dolichol pyrophosphoryl oligosaccharide (Dol-PP-oligosaccharide) pool [Konrad and Merz (1994) J. Biol. Chem. **269**, 8659–8666]. The factors involved in the effect of cAMP on synthesis of Dol-PP-oligosaccharide are investigated here. The GlcNAc transfer to dolichol phosphate (Dol-P) was found to be unaffected by pretreatment with 8-bromo-cAMP. By measuring the uptake of [^{3}H]mevalonate, a 20-fold increase in the incorporation of the label into Dol-P was observed in the cells treated with 8-bromo-cAMP. Under the same conditions, the synthesis of dolichol was enhanced 60-fold. However, the incorporation of the radioactivity into cholesterol was not increased in the JEG-3 cells pretreated with 8-bromo-cAMP, which suggests a specific stimulation of the dolichol/Dol-P pathway by cAMP. The *cis*-prenyltransferase activity was found to be increased 10-fold in cells pretreated with 8-bromo-cAMP. Dolichol kinase activity was unaffected by stimulation with 8-bromo-cAMP. The present study suggests that the larger glycosylation capacity in JEG-3 cells treated with 8-bromo-cAMP is caused by an increase in the microsomal *cis*-prenyltransferase activity.

INTRODUCTION

N-Glycosylation of proteins in the endoplasmic reticulum (ER) involves the transfer *en bloc* of the lipid-linked core-oligosaccharide $Glc_3Man_9(GlcNAc)_2$ to the nascent polypeptide chain. Specific glycosyltransferases catalyse the multi-step synthesis of this oligosaccharide, anchored in the ER membrane by coupling to dolichol pyrophosphate (Dol-PP) [1–3].

Although the formation of dolichol pyrophosphoryl oligosaccharide (Dol-PP-oligosaccharide) has been studied extensively, the regulation of this pathway is still unclear. Changes in glycosylation metabolism have been observed during cellular development. In the mouse mammary gland, the activities of glycosyltransferases are increased during lactation [4]; in sea urchin embryos, cellular development involves a change of the activities of enzymes of Dol-PP-oligosaccharide synthesis [5–7]. Besides regulation by internal factors of cellular metabolism [3], N-glycosylation seems also to be regulated by hormonal stimulation. Oestrogens induce dolichol kinase [8] and glycosyltransferases [9–11] in chick oviduct, as well as the synthesis of Dol-P-mannose in mouse uterus [12–14]. Probably, no common principle is followed in controlling N-glycosylation with regard to the activation of a single step or a group of enzymes being concerned in Dol-PP-oligosaccharide synthesis.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of four N-linked and four O-linked carbohydrate moieties (approx. 30 % of the molecular mass). Previously we investigated the influence of cAMP on the N-glycosylation of hCG and of a synthetic peptide containing the consensus sequence for N-glycosylation (Asn-Xaa-Ser/Thr) in JEG-3 choriocarcinoma cells that produce hCG [15]. cAMP induced the stimulation of hCG synthesis as well as an increase of the capacity of N-glycosylation. The effect of cAMP on N-glycosylation was probably caused not by the acute action of cAMP-dependent protein kinases, but by gene activation. Exposure of the cells to an elevated cAMP concentration for more than 16 h resulted in an increased formation of the Dol-PP-oligosaccharide. The present study demonstrates that the long-term incubation of JEG-3 cells with cAMP increases the activity of the *cis*-prenyltransferase, which causes an intensified synthesis of the lipid-carrier Dol-P that seems to be the key factor for the up-regulation of Dol-PP-oligosaccharide synthesis. Therefore we present evidence that a pleiotropic stimulator such as cAMP, which affects a great number of cellular functions, up-regulates Dol-PP-oligosaccharide synthesis by increasing the activity of a single enzyme.

EXPERIMENTAL

Cell culture

JEG-3 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Monolayer cultures were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., Deisenhofen, Germany) containing 10 % (v/v) fetal calf serum (Biochrom KG, Berlin, Germany). The medium was supplemented with 3.7 g/l sodium bicarbonate, 100 i.u./ml penicillin and 100 μg/ml streptomycin (Biochrom KG). Confluent cell monolayers in 25 cm^2 plastic flasks were used. Unless otherwise stated, treatment of cells with 250 μM 8-bromo-cAMP (sodium salt; Sigma Chemical Co.) or forskolin (Sigma Chemical Co.) and 3-isobutyl-1-methylxanthine (IBMX; Serva, Heidelberg, Germany) was started 40 h before cells were

Abbreviations used: Dol-P, dolichol phosphate; Dol-PP, dolichol pyrophosphate; Dol-PP-oligosaccharide, dolicholpyrophosphoryl oligosaccharide; ER, endoplasmic reticulum; GPT, UDP-GlcNAc:dolichol phosphate *N*-acetylglucosaminyl-1-phosphate transferase; hCG, human chorionic gonadotropin; IBMX, 3-isobutyl-1-methylxanthine.

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prepared for measuring of enzyme activities or used for labelling experiments. Control cells received fresh culture medium at the same time as cultures were treated with 8-bromo-cAMP. The numbers of cells in the cultures were counted as described [16]. Cellular protein content was determined after washing of the cells with PBS as described [17].

Labelling and extraction of lipid-linked oligosaccharides and of protein

Cultures were washed twice with PBS before addition of the labelling medium (glucose-free medium supplemented with 10 μCi/ml D-[2,6-^{3}H]mannose, 52 Ci/mmol (Amersham Buchler, Braunschweig, Germany). Inhibition of protein biosynthesis was performed by incubation with cycloheximide (180 μM; Serva) or puromycin (400 μM; Sigma Chemical Co.) when indicated below. After the addition of the inhibitors to the culture medium and mixing, each flask received 2 ml of the medium. The cells were incubated for the indicated times at 37 °C, followed by removal of the radioactive media. Labelling was stopped by washing the cells with ice-cold PBS. After layering with 0.6 ml of ice-cold water, the cells were removed from the plate by a cell scraper (Costar, Cambridge, MA, U.S.A.) and the radioactivity associated with Dol-PP-oligosaccharide and glycoprotein was determined by procedures including stepwise extraction with organic solvents as described [15]. Protein synthesis was measured under the same conditions, with a mixture of 0.1 μCi/ml L-[U-^{14}C]lysine monohydrochloride (150 mCi/mmol; Amersham Buchler) and 0.1 μCi/ml L-[U-^{14}C]tyrosine (225 mCi/mmol; Amersham Buchler) as radioactive label. The proteins were precipitated by the addition of equal volumes of ice-cold 20 % (w/v) trichloroacetic acid. Precipitated protein was collected by centrifugation, washed with 10 % (w/v) trichloroacetic acid, dissolved in Protosol (NEN, Dreieich, Germany), and counted in a liquid-scintillation counter (Tricarb 2450; Packard Instrument, Frankfurt/Main, Germany).

Labelling and extraction of dolichol phosphate, dolichol and cholesterol

Cultures were incubated for 16 h with 20 μCi/ml *RS*-[5-^{3}H]mevalonate (60 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO, U.S.A.). An influence of medium change was excluded by adding [^{3}H]mevalonate directly to an aliquot of the culture supernatant and transferring this back to the cultures. Labelling was terminated and cells were harvested as described above. The cell suspension was transferred into a reaction vial (2 ml total volume) containing 1.2 ml of chloroform/methanol (1:1, v/v) and mixed thoroughly. The organic phase was removed, and the aqueous phase and the interphase were re-extracted with 0.6 ml of chloroform. Both organic phases were pooled and washed with 0.6 ml of chloroform/methanol/water (3:48:47, by vol.) to obtain the lipid fraction. Briefly, the following procedure was adopted as described [18]. The lipids were separated on DEAE columns [1 ml pipette tips filled with 200 μl of DEAE-cellulose (Sigma Chemical Co.)] into neutral and negatively charged substances. The anionic compounds were retained on the column and eluted with 2 ml of chloroform/methanol (2:1, v/v) containing 100 mM ammonium acetate. Dol-P was quantified by HPLC analysis. The samples were dried in a vacuum concentrator (Bachhofer, Reutlingen, Germany) and subsequently dissolved in 0.03 ml of n-hexane/propan-2-ol/1.4 M phosphoric acid (965:35:0.5, by vol.) [13] and injected onto a Hypersil (5 μm) column (250 mm × 4.0 mm; Knauer GmbH, Berlin, Germany). The column was developed at a flow rate of 0.5 ml/min and fractions of 0.15 ml were collected. The radioactivity of each fraction was measured by scintillation counting. Non-radioactive dolichol, Dol-P and cholesterol standards were run on the same Hypersil column and their retention time was used to identify the labelled products. The flow-through from the DEAE columns, containing cholesterol and dolichol, was dried in a vacuum concentrator and dissolved in 0.5 ml of methanol. A further separation of sterols and prenols was performed on reverse-phase columns [1 ml pipette tips filled with 100 μl of Lichroprep RP-18 (Merck, Darmstadt, Germany)]. Cholesterol (flow through) and dolichol [eluted with 3 ml of methanol/ethanol/2-propanol (5:90:5, by vol.)] were analysed by HPLC as described before.

UDP-GlcNAc:Dol-P *N*-acetylglucosaminyl-1-phosphate transferase (GPT) assay

Cell extracts were prepared by sonication (Branson sonifier Model 250, power setting 3, microtip, 10 s at 20 % charge) in 50 mM Tris/HCl, pH 7.6, containing 250 mM sucrose, 10 mM 2-mercaptoethanol and 1 mM EDTA. After cellular debris had been removed by centrifugation (10 min at 1000 ***g***, 4 °C), the membrane fraction was sedimented at 100000 ***g*** (90 min at 4 °C). Membranes were homogenized in 0.25 M sucrose in a glass microtube equipped with a loosely fitting Teflon piston. Assay mixtures contained 30–70 μg of membrane protein, and 100 mM Tris/HCl, pH 9.2, containing 125 mM sucrose, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 % Triton X-100, 50 μM Dol-P (Sigma Chemical Co.) and 50 μM [6-^{3}H]UDP-GlcNAc (25 Ci/mmol; American Radiolabeled Chemicals Inc.). The mixture was incubated for 10 min at 37 °C. Glycolipids were extracted three times with 0.6 ml of chloroform/methanol (2:1, v/v). The organic phases were washed four times with 0.3 ml of 0.9 % NaCl and the radioactivity of the aliquots was determined in a liquid-scintillation counter. GlcNAc-lipids were subjected to mild acid hydrolysis and analysed by gel filtration as described [15].

Dolichol kinase assay

[γ-^{32}P]CTP was prepared enzymically by the procedure of Bauer and Várady [19] ([^{32}P]phosphate was obtained from ICN Biomedicals Inc., Irvine, CA, U.S.A.). Membranes were prepared as described above (see GPT assay). The activity of the dolichol kinase was determined by the method of Sumbilla and Waechter [20] with the following modifications. The reaction mixture with a total volume of 50 μl contained 0.2–0.3 mg of membrane protein in 50 mM Tris/HCl, pH 7.2, containing 125 mM sucrose, 0.5 mM EDTA, 20 mM UTP, 30 mM $CaCl_2$, 5 mM 2-mercaptoethanol, 0.04 % Nonidet P40, 60 μM dolichol (Sigma Chemical Co.) and 80 μM [γ-^{32}P]CTP (400–700 c.p.m./pmol). Incubations at 37 °C for 15 min were terminated by addition of chloroform/methanol (2:1, v/v). After extraction, the product of the enzyme assay was analysed by HPLC as described for Dol-P.

cis-Prenyltransferase assay

The enzyme activity was measured by following the procedure described by Ericsson et al. [21]. The incubation mixture (total volume 60 μl) consisted of 25 mM imidazole/HCl, pH 7.0, containing 1 mM $MgCl_2$, 100 mM KF, 1 mM dithiothreitol, 1.0 % (w/v) Triton X-100, 10 μM farnesyl-PP (Sigma Chemical Co.) and 40 μM [1-^{14}C]isopentenyl-PP (54 mCi/mmol; Amersham Buchler). The reaction was initiated by the addition of 0.2–0.3 mg of membrane protein and the incubation was continued for 60 min at 37 °C. The incubation was terminated by the addition of 60 μl of 0.87 M KOH, hydrolysis was performed at 100 °C for

30 min, and the samples were supplemented with 300 μl of 2 M KCl and 60 μl of 1 M HCl. The lipids were extracted three times with 0.6 ml of a mixture of diethyl ether and light petroleum (1:1, v/v). The organic phases were pooled and washed three times with 0.3 ml of 0.9 % NaCl. The radioactivity of an aliquot was measured in the scintillation counter. The composition was determined by HPLC as described for the analysis of Dol-P. Prenol 18-phosphate (Sigma Chemical Co.) and prenol C_{80}–C_{110} (Sigma Chemical Co.) were used as non-radioactive standards.

Density-gradient centrifugation

Microsomal membranes and peroxisomes were obtained by homogenization of JEG-3 cells in a medium containing 10 mM glycylglycine, pH 7.4, 0.1 % (v/v) ethanol and 250 mM sucrose (motor-driven glass–Teflon homogenizer, 40 up-and-down strokes at 1000 rev./min). The homogenate was centrifuged for 5 min at 1000 ***g***, 4 °C. The pellet was discarded. After centrifugation of the supernatant at 100000 ***g***, 4 °C, the re-homogenized pellet was layered on to a density gradient ranging from 14 to 45 % (w/v) Nycodenz on a cushion of 70 % (w/v) Nycodenz. Centrifugation was performed in a Kontron Centrikon T-1065 centrifuge equipped with a Sorvall TV-865 rotor (r_{av} 7.83 cm). The fractions were obtained as described [22]. The activity of the peroxisomal marker enzyme catalase was determined by the method of Baudhuin et al. [23]. Esterase activity as a microsomal marker enzyme was measured as described by Beaufay et al. [24].

RESULTS

GPT

Recently we showed that long-term preincubation with 8-bromo-cAMP increases the incorporation of [^{3}H]mannose into the Dol-PP-oligosaccharide fraction [15]. The mechanism underlying this phenomenon is unknown. The kinetics of the cAMP effect (requiring a preincubation of at least 16 h) strongly suggests that the gene activities of key enzymes involved in the synthesis of Dol-PP-oligosaccharide are up-regulated. This led us to investigate the assembly of the dolichol-linked core-oligosaccharide at the level of the transfer of GlcNAc-1-P to Dol-P, which is catalysed by GPT. This reaction was described as a key step of Dol-PP-oligosaccharide synthesis [3]. After preincubation of JEG-3 cells for various times with 250 μM 8-bromo-cAMP, the enzyme activity (coupling of [6-^{3}H]UDP-GlcNAc to Dol-P) remained unchanged in comparison with control cells (Table 1). The specificity of GlcNAc transfer was assessed by addition of the GPT inhibitor tunicamycin (5 μg/ml) to the assay mixture. Tunicamycin treatment of control and 8-bromo-cAMP-stimulated cells caused a decrease in the GlcNAc transfer into the glycolipid fraction by 99 % (results not shown). The extracted glycolipids were hydrolysed and separated on a Bio-Gel P4 column to identify the products. The major part (approx. 84 % of the eluted radioactivity) of the label was detected at the position of GlcNAc, and a minor part (approx. 14 %) was identified as $(GlcNAc)_2$ (results not shown). The cells treated with 8-bromo-cAMP also showed a similar ratio of GlcNAc to $(GlcNAc)_2$ compared with the control cells. This demonstrates that neither the activity of the GPT nor the transfer of the second GlcNAc onto Dol-PP-GlcNAc was stimulated in cells treated with 8-bromo-cAMP.

Table 1 Absence of a stimulation of the activity of GPT in JEG-3 cells in response to long-term preincubation with 250 μM 8-bromo-cAMP

Membranes of control cultures and cells treated with 8-bromo-cAMP were incubated with [6-^{3}H]UDP-GlcNAc and Dol-P as substrates. Each value represents the mean ± S.D. for four independent experiments. The units of GPT activity are pmol of GlcNAc incorporated/10 min per mg of protein.

Preincubation period with 8-bromo-cAMP (h)	GPT activity
0 (Control)	141 ± 41
16	137 ± 17
24	116 ± 37
39	153 ± 20

Table 2 Incorporation of [^{3}H]mevalonate into the Dol-P fraction in control cultures and cells pretreated with 250 μM 8-bromo-cAMP

Monolayers of JEG-3 cells were labelled for 16 h with 20 μCi/ml [^{3}H]mevalonate. Lipids were extracted as described in the Experimental section. The radioactivity associated with the Dol-P fraction is given as means ± S.D. ($n = 4$) on the basis of the amount of substance obtained from 10^6 cells. Similar results were obtained in three other experiments.

Preincubation period with 8-bromo cAMP (h)	[^{3}H]Mevalonate incorporated into Dol-P (c.p.m.)
0 (Control)	150 ± 17
16	329 ± 34
25	508 ± 27
40	3359 ± 162

Dol-P

The lack of effects mediated by 8-bromo-cAMP on the GPT activity led us to investigate the availability of the lipid carrier Dol-P, which serves as a substrate for the GPT reaction. Dol-P is formed by synthesis *de novo* or is re-used for Dol-PP-oligosaccharide synthesis after its release during protein N-glycosylation. The formation of Dol-P was examined under basal conditions and in cultures pretreated with 8-bromo-cAMP by measuring the incorporation of radioactive mevalonate. Cells were incubated for 16 h with [^{3}H]mevalonate (20 μCi/ml). The lipids were extracted, the anionic compounds were separated on DEAE-cellulose, and the labelled Dol-P was determined by HPLC analysis. Table 2 clearly shows the stimulation of Dol-P synthesis in response to the duration of preincubation with 250 μM 8-bromo-cAMP. An approximately 20-fold higher [^{3}H]mevalonate incorporation into Dol-P was observed in the cells preincubated for 40 h with 8-bromo-cAMP.

Free Dol-P is also formed from Dol-PP by dephosphorylation after the transfer of the core-oligosaccharide on to the polypeptide chain. We addressed the question whether the stimulation of protein synthesis by 8-bromo-cAMP itself might cause a stimulation of Dol-PP-oligosaccharide synthesis by increasing the rate of released Dol-P. Therefore the stimulatory effect of cAMP on Dol-PP-oligosaccharide synthesis is expected to be nullified in response to inhibition of protein synthesis. Control cells and cells pretreated with 8-bromo-cAMP (250 μM, 40 h) were incubated for 45 min with puromycin (400 μM) or cycloheximide (180 μM) and during the last 15 min of incubation the cultures were labelled with 10 μCi/ml [^{3}H]mannose or a mixture of 0.1 μCi/ml [^{14}C]tyrosine and 0.1 μCi/ml [^{14}C]lysine. Analysis of the lipid-linked oligosaccharides synthesized under these

Table 3 cAMP-induced stimulation of [^{3}H]mannose incorporation into Dol-PP-oligosaccharide is not driven by the increase in protein synthesis

Monolayers of cells were preincubated for 40 h with 250 μM 8-bromo-cAMP or control medium before [^{3}H]mannose was added for 15 min. Protein synthesis was inhibited by treating the cells with puromycin (0.4 mM) 30 min before and during labelling with [^{3}H]mannose. The effect of puromycin on protein synthesis was assayed by incorporation of [^{14}C]tyrosine and [^{14}C]lysine under the same conditions. The means $\pm$ S.D. ($n = 4$) of the incorporated radioactivity into the total fractions obtained from 10^6 cells are shown.

Condition	[^{3}H]mannose label in Dol-PP-oligosaccharide		[^{14}C]tyrosine, [^{14}C]lysine in protein	
	− Puromycin	+ Puromycin	− Puromycin	+ Puromycin
Control (c.p.m.)	13949 $\pm$ 2414	376 $\pm$ 69	2805 $\pm$ 338	35 $\pm$ 2
8-Bromo-cAMP (c.p.m.)	64208 $\pm$ 4205	5039 $\pm$ 1308	10025 $\pm$ 590	54 $\pm$ 4
Fold stimulation (8-bromo-cAMP/control)	4.6	13.4	3.6	1.5

Table 4 Specificity of cAMP-induced increase in [^{3}H]mevalonate into dolichol

Monolayers of cells were labelled for 16 h with 20 μCi/ml [^{3}H]mevalonate. Lipids were extracted as described in the Experimental section. Results were obtained in the same representative experiment. The means $\pm$ S.D. ($n = 4$) obtained from 10^6 cells are given. Similar results were obtained in three other experiments.

Preincubation with 8-bromo-cAMP (h)	[^{3}H]Mevalonate incorporated into cholesterol (c.p.m.)	[^{3}H]Mevalonate incorporated into dolichol (c.p.m.)
0 (Control)	16369 $\pm$ 1100	63 $\pm$ 7
16	20015 $\pm$ 4125	127 $\pm$ 15
25	18417 $\pm$ 2040	408 $\pm$ 45
40	24090 $\pm$ 5026	4424 $\pm$ 805

conditions showed that [^{3}H]mannose was incorporated predominantly (over 90%) into the core-oligosaccharide $Glc_3Man_9(GlcNAc)_2$ (results not shown). The presence of puromycin inhibited protein synthesis (by approx. 99%) in control as well as in stimulated cells, as determined by uptake of labelled amino acids (Table 3). Moreover, [^{3}H]mannose incorporation into Dol-PP-oligosaccharide was also decreased. However, the stimulatory effect of 8-bromo-cAMP was not nullified but increased almost 3-fold by inhibition of protein synthesis (Table 3). Similar results were observed when cycloheximide was used as inhibitor (results not shown).

Synthesis of cholesterol and dolichol *de novo*

The above results clearly suggest that cAMP affects Dol-P synthesis directly. We therefore investigated the synthesis of cholesterol and dolichol to see whether this is a specific event or the result of an overall stimulation of isoprenoid synthesis. After various periods of preincubation with 250 μM 8-bromo-cAMP, the uptake of [^{3}H]mevalonate into cholesterol and dolichol was measured. Table 4 clearly indicates a stimulation of the synthesis of dolichol, whereas the labelling of cholesterol remained unchanged. This suggests a specific stimulation of the dolichol pathway by 8-bromo-cAMP without any effect on the formation of cholesterol, which is a major product of isoprenoid metabolism. The syntheses of Dol-P and cholesterol diverge after the formation of farnesyl-PP. Therefore the stimulatory effect of 8-bromo-cAMP was not expected to occur in earlier steps.

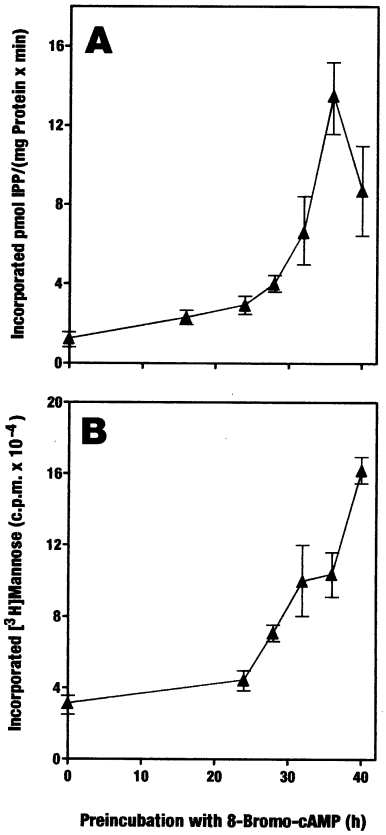

Figure 1 Influence of pretreatment time with 250 μM 8-bromo-cAMP on the activity of *cis*-prenyltransferase (A) and synthesis of Dol-PP-oligosaccharide (B)

(**A**) Membranes were incubated for 60 min with [^{14}C]isopentenyl-PP (IPP) and farnesyl-PP. Labelling of the prenyl phosphates extracted with a mixture of ether and light petroleum (boiling range 40–60 °C) was measured. Each value shown here represents the mean $\pm$ S.D. for four independent experiments. (**B**) JEG-3 cells were incubated for 30 min with 10 μCi/ml [^{3}H]mannose. Incorporation of radioactivity into the Dol-PP-oligosaccharide fraction of 10^6 cells is given. Each value shows the mean $\pm$ S.D. for three determinations.

cis-Prenyltransferase

The synthesis of Dol-P from farnesyl-PP is initiated by *cis*-prenyltransferase. This enzyme builds up the polyprenyl chain by adding 14–17 isopentenyl residues to farnesyl-PP. The effect of

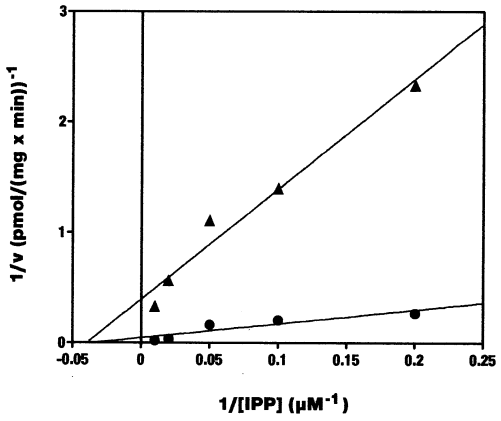

Figure 2 Kinetic analysis of the *cis*-prenyltransferase activity in membrane preparations of control cells (▲) and cells preincubated with 8-bromo-cAMP (250 μM for 40 h; ●)

The enzyme activity was measured in the presence of various concentrations of isopentenyl-PP (IPP). The values are presented in double-reciprocal form.

cAMP on this key step was investigated by incubating membranes with farnesyl-PP and [^{14}C]isopentenyl-PP as substrates. After 60 min of incubation, the products were hydrolysed and extracted as described in the Experimental section. Identification of the reaction products by HPLC analysis showed that all the radioactivity was incorporated into prenyl phosphates. After long-term preincubation with 8-bromo-cAMP, the activity of *cis*-prenyltransferase was increased by up to 10-fold in comparison with the control cells (Figure 1A). The induction of *cis*-prenyltransferase activity seems to occur concurrently with the increase of the synthesis of Dol-PP-oligosaccharide (Figure 1). The maximum enzyme activity was obtained after 36 h of preincubation with 8-bromo-cAMP, whereas the Dol-PP-oligosaccharide synthesis kept on increasing until 40 h (Figures 1A and 1B). Kinetic analysis of the *cis*-prenyltransferase suggests that the cells treated with 8-bromo-cAMP contained a higher enzyme activity of equal affinity for isopentenyl-PP (Figure 2).

cis-Prenyltransferase activity has been described as occurring in the microsomal and peroxisomal fractions [25]. We therefore investigated which cellular compartment contributes to the increase in the *cis*-prenyltransferase activity of JEG-3 cells induced by 8-bromo-cAMP. The microsomal and peroxisomal fractions were separated by density-gradient centrifugation and the esterase and catalase activities were assayed. The peroxisomes were found mainly in fractions 4–6 (69% of total catalase activity) whereas the microsomes were predominantly collected in the light fractions, 7–10 (54% of total esterase activity) (Figure 3); 51% of total *cis*-prenyltransferase activity was found in the microsomal fractions of control cells (compared with 23% in the peroxisomal fractions). A similar distribution was found (50% microsomal *cis*-prenyltransferase compared with 24% peroxisomal enzyme activity) in cells pretreated with 8-bromo-cAMP. The specific enzyme activity in the microsomal fractions was increased 4.4-fold after preincubation with 8-bromo-cAMP (11.5 pmol/min per mg in cells treated with 8-bromo-cAMP, compared with 2.6 pmol/min per mg in control cells). Under these conditions only a 2-fold stimulation of the specific *cis*-prenyltransferase activity was detected in the peroxisomal fractions (2.3 pmol/min per mg in control cells and 4.9 pmol/min per mg in cells pretreated with 8-bromo-cAMP). Possibly the *cis*-prenyltransferase activity found in the peroxisomal fractions was

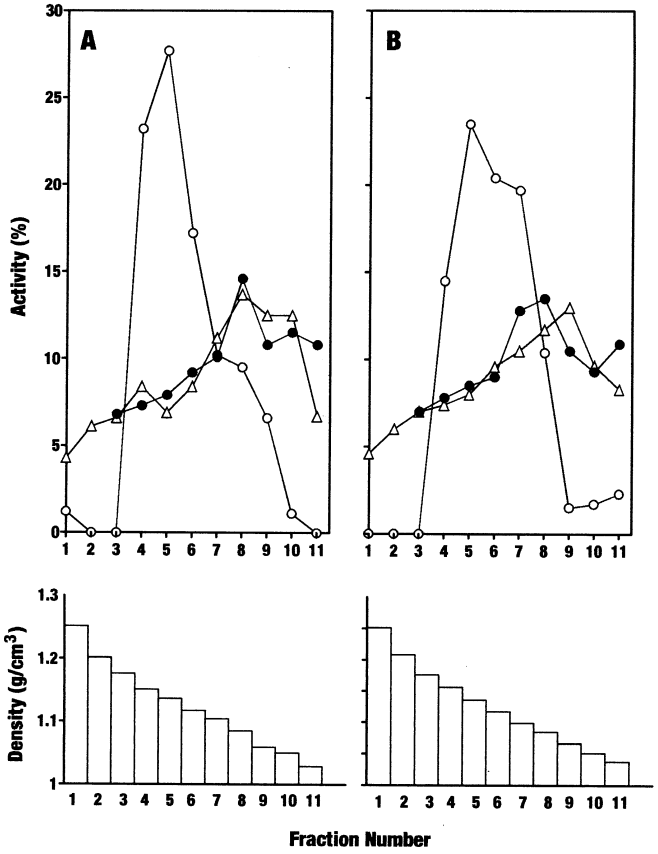

Figure 3 Density-gradient centrifugation of membranes of (A) control cultures and (B) cells treated with 8-bromo-cAMP (250 μM for 40 h)

cis-Prenyltransferase (●) and the marker enzymes esterase (△) for microsomes and catalase (○) for peroxisomes were measured. The values are given as the percentage of the total activities loaded at the top of the gradient. The density of the fractions is shown in the panel below.

due to contamination with microsomes. These results suggest that the effect of 8-bromo-cAMP on *cis*-prenyltransferase activity was predominantly contributed by the microsomal enzyme.

Dolichol kinase

Because pretreatment of JEG-3 cells with 8-bromo-cAMP increases the syntheses of Dol-P and dolichol, the question arises whether the phosphorylation of dolichol is also stimulated or whether it is large enough for the greater demand. To address this, the membranes of control cultures and cells pretreated with 250 μM 8-bromo-cAMP for various periods of time were incubated for 15 min with [γ-^{32}P]CTP and dolichol as substrates. The radioactivity in Dol-P was counted in a chloroform/methanol (2:1, v/v) extract and Dol-P was identified as the reaction product by HPLC as described in the Experimental section. The activity of the dolichol kinase in response to preincubation of the cells with 8-bromo-cAMP was found to be unchanged (Table 5).

Stimulation of *cis*-prenyltransferase by forskolin

To compare the effect of endogenously stimulated cAMP with exogenously applied 8-bromo-cAMP, the JEG-3 cells were treated with different concentrations of forskolin (20–100 μM) in

Table 5 The stimulatory effect of cAMP on dolichol phosphate concentration is not caused by an increase in dolichol kinase activity

Membranes of JEG-3 cells of controls and cultures treated with 8-bromo-cAMP were incubated for 15 min with [γ-^{32}P]CTP and dolichol. The uptake of ^{32}P into Dol-P in the lipid fraction was measured. The units of dolichol kinase activity are pmol of Dol-P formed per 15 min per mg of protein. Means $\pm$ S.D. ($n = 3$) are shown. Similar results were observed in three other independent experiments.

Preincubation period with 8-bromo-cAMP (h)	Dolichol kinase activity
0 (Control)	2.7 ± 0.2
15	3.2 ± 0.8
25	2.3 ± 0.5
40	2.7 ± 0.3

Table 6 Effect of forskolin on the activity of *cis*-prenyltransferase

JEG-3 cells were grown at basal conditions or were incubated with forskolin in the presence of 100 μM IBMX for 40 h. Membranes were collected, and an enzyme assay based on the incorporation of [^{14}C]isopentenyl-PP (IPP) into prenyl phosphate was performed as described in the Experimental section. The units of CPT activity are pmol of IPP incorporated/60 min per mg of protein. Means $\pm$ S.D. ($n = 4$) are given.

[Forskolin] (μM)	CPT activity
0	102 ± 18
20	232 ± 33
50	219 ± 72
100	188 ± 26

the presence of the phosphodiesterase inhibitor IBMX for 40 h, which increases intracellular cAMP levels. An incubation with 20 μM forskolin increased *cis*-prenyltransferase activity 2.3-fold (Table 6).

DISCUSSION

We have previously demonstrated that long-term preincubation with cAMP stimulates N-glycosylation in choriocarcinoma cells [15]. This was caused by an increased synthesis of Dol-PP-oligosaccharide and not by a change in the activity of oligosaccharyltransferase [15]. In other cases, such as rat liver [26,27] or rat parotid acinar cells [28], an increase in cAMP levels (from several minutes up to a few hours) also showed stimulation of N-glycosylation. These short-term effects were explained by activation of enzymes of the glycosylation pathway by cAMP-dependent phosphorylation [26–30]. The stimulation by cAMP of N-glycosylation, described in the present study, is different both qualitatively and quantitatively, because it needs a preincubation with cAMP for 16 h or longer, which results in a stronger effect [15]. This suggests a mechanism that is mediated by gene activation rather than by acute protein phosphorylation. We therefore investigated this long-term effect of cAMP on N-glycosylation with regard to the key steps of the synthesis of Dol-PP-oligosaccharide. The first step of the synthesis is the addition of GlcNAc-1-P to the lipid carrier Dol-P by GPT. Changes in the activity of this enzyme have been reported during development in the mouse mammary gland [4], in the diethylstilboestrol-induced hen oviduct [10] and in B-lymphocytes in response to bacterial lipopolysaccharide [31]. Although GPT is regarded as a key enzyme in Dol-PP-oligosaccharide synthesis in many systems [3], our experiments clearly demonstrate that long-term treatment of the cells with 8-bromo-cAMP did not affect the activity of GPT, which suggests that GPT was not the target of the cAMP effect (Table 1). Analysis of the products of the enzyme incubation by gel filtration indicates that the transfer of the second GlcNAc on to Dol-PP-GlcNAc by UDP-GlcNAc:Dol-PP-GlcNAc *N*-acetylglucosaminyl transferase was also unaffected by cAMP.

The glycosyl acceptor for the GPT reaction is Dol-P, which serves as the lipid carrier for the whole core-oligosaccharide assembly. In addition Dol-P serves as carrier for the transport of Man and Glc into the lumen of the ER as Dol-P-Man and Dol-P-Glc respectively. The availability of Dol-P seems to be a limiting factor in the rate of glycoprotein synthesis under certain conditions [9,32]. The Dol-P pool is fed from synthesis *de novo* as well as from the release of Dol-P in the course of oligosaccharyl transfer to the protein. In response to the elevated cAMP concentration, the biosynthesis of several proteins, as well as Dol-PP-oligosaccharide synthesis, is increased in JEG-3 cells. This raises the question of whether the enhanced release of Dol-P in consequence of augmented protein glycosylation may automatically drive the amplification of newly formed Dol-PP-oligosaccharide. The experiments presented here show that this seems not to be the only reason. In the presence of inhibitors of protein biosynthesis, the formation of Dol-PP-oligosaccharide is quantitatively diminished (Table 3), which is in agreement with others [33–35]. Although it has been proposed that this inhibition might be caused by a feedback mechanism [33], neither the inhibited step nor the intermediate(s) involved in the inhibition were identified [35,36]. The cAMP-induced stimulation of [^{3}H]mannose incorporation into Dol-PP-oligosaccharide was observed to be higher than in control cells (treated with protein synthesis inhibitors only), suggesting that the enhancement of the synthesis of Dol-PP-oligosaccharide by 8-bromo-cAMP was not substantially influenced by the Dol-P released during glycoprotein synthesis (Table 3).

We have clearly shown here that 8-bromo-cAMP exerted an effect on synthesis of Dol-P *de novo* (Table 2). *cis*-Prenyltransferase catalyses the formation of the Dol-P polyprenyl chain by adding activated isoprene units to *trans*,*trans*-farnesyl-PP. In B-cells [37] and in embryonic rat brain [38], enzyme activity increases in response to developmental changes. Similarly the enzyme activity in JEG-3 cells treated with 8-bromo-cAMP (as determined by the incorporation of [^{14}C]isopentenyl-PP into the polyprenyl fraction) was increased by up to 10-fold in comparison with the control cells (Figure 1A). The optimum *cis*-prenyltransferase activity was measured when the cells were preincubated for 36 h. Prolongation of treatment with 8-bromo-cAMP (more than 36 h) led to a diminution of the effect. This might be due to down-regulation of the enzyme triggered by a feedback mechanism. The *cis*-prenyltransferase activity and the synthesis of Dol-PP-oligosaccharide depend very similarly on the preincubation time with 8-bromo-cAMP (Figure 1). The induction of *cis*-prenyltransferase activity precedes the increase in Dol-PP-oligosaccharide synthesis slightly. Therefore these results suggest that the increase in Dol-P supply was the result of the induction of *cis*-prenyltransferase and that this directly caused the increased formation of the dolichol-linked core oligosaccharide.

cis-Prenyltransferase is predominantly a membrane-bound enzyme [39,40] and has shown significant activity in the soluble cellular fraction [41]. Because *cis*-prenyltransferase activity was also reported to be present in peroxisomes [25,42], we separated both compartments by density-gradient centrifugation (Figure

3). Most of the enzyme activity in control cells as well as in cells treated with 8-bromo-cAMP was associated with the microsomal fraction.

When intracellular cAMP levels were elevated by irreversible activation of adenylate cyclase with forskolin in the presence of IBMX (an inhibitor of phosphodiesterases), the activity of *cis*-prenyltransferase was doubled (Table 6). This augmentation is comparable with the effect of a preincubation with 250 μM 8-bromo-cAMP for 24 h. These results indicate that both the exogenously applied 8-bromo-cAMP and an endogenously elevated intracellular cAMP level have the same qualitative effect on *cis*-prenyltransferase activity.

It has been proposed that the primary end product of synthesis of Dol-P *de novo* is dolichol, which is phosphorylated afterwards by dolichol kinase [43]. We therefore measured the enzyme activity in response to an elevated cAMP concentration. Long-term pretreatment of the JEG-3 cells with 8-bromo-cAMP, however, did not induce any change in the activity of this enzyme (Table 5). Possibly its activity is sufficient for the greater demand in cAMP-stimulated cells or this step is not required for the synthesis of Dol-P *de novo* in JEG-3 cells. The low specific activity might indicate that the situation is similar to that in rat hepatocytes, where dolichol kinase is only of limited functional importance for Dol-P synthesis [44].

In conclusion, 8-bromo-cAMP stimulates the formation of Dol-PP-oligosaccharide, the glycosylation of octanoyl tripeptide [15] and the incorporation of [^{3}H]mevalonate into dolichol and Dol-P with the same kinetics, whereas the activity of key enzymes of the glycosylation pathway such as GPT, dolichol kinase and oligosaccharyltransferase are unaffected. The stimulatory effect of cAMP on N-glycosylation is caused by an enhanced expression of microsomal *cis*-prenyltransferase, resulting in an increase in the Dol-P pool. In JEG-3 cells the size of the endogenous Dol-P pool seems to be a limiting factor for the formation of Dol-PP-oligosaccharide, which is necessary to fulfil the greater demand of increased glycoprotein synthesis in cAMP-stimulated cells.

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