Regulation of UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase by retinoic acid in P19 cells

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UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT) is the first enzyme in the dolichol pathway of protein N-glycosylation, and is implicated in the developmental programmes of a variety of eukaryotes. In the present study we describe the effects of all-*trans*-retinoic acid (RA) on the levels of GPT protein and enzymic activity, and on the transcription rate of the GPT gene, in mouse P19 teratocarcinoma cells. RA caused a dose-dependent and protein-synthesis-dependent induction of enzyme activity. The maximum induction of GPT activity (about 3-fold) required 2 days of exposure to 1 μM RA. Induced GPT activity also resulted in an increase in the rate of incorporation of [^{3}H]mannose into $Glc_3Man_9GlcNAc_2$. Enzymic activities paralleled GPT gene expression. The GPT gene was induced (2-fold) after 7 h of RA treatment. An approx. 3-fold increase in a 48 kDa GPT protein and approx. 4-fold increases in the levels of three GPT transcripts (1.8, 2.0 and 2.2 kb) were observed after 2 days of RA treatment. The enhanced levels of GPT protein and mRNAs began to decline 3 days after the initiation of differentiation, and GPT expression was down-regulated during cellular differentiation. GPT activity decreased about 2.8-fold to a constant level in differentiated P19 cells. The results indicate that the RA-induced enzyme activity was mainly determined by increased transcription of the GPT gene. RA-treated P19 cells were about 4-fold more resistant to tunicamycin, a fungal antibiotic which inhibits GPT, than were control cells. In addition, GPT activity in membranes from RA-treated P19 cells exhibited approx. 4-fold increased resistance to tunicamycin compared with activity in membranes from untreated control cells, demonstrating that resistance to tunicamycin is correlated with induced GPT activity. Furthermore, increased GPT activity had regulatory significance with regard to the rate of incorporation of [^{3}H]mannose into $Glc_3Man_9GlcNAc_2$-*P*-*P*-dolichol and into glycoproteins. Together, the data provide additional insights into the hormonal regulation of GPT and present evidence that the RA-mediated induction of GPT has a regulatory impact on the dolichol pathway.

Key words: differentiation, dolichol pathway, glycosylation, P19 teratocarcinoma cells, tunicamycin.

INTRODUCTION

The asparagine-linked glycosylation of proteins in eukaryotes is initiated by the stepwise assembly of $Glc_3Man_9GlcNAc_2$-*P*-*P*-dolichol and its transfer to the nascent polypeptide in the endoplasmatic reticulum, followed by post-translational modification of the oligosaccharides in the secretory pathway (for a review, see [1]). The enzyme UDP–*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT; EC 2.7.8.15) catalyses the first, committed step of the dolichol pathway. This enzyme is, therefore, a potential control point for the overall regulation of oligosaccharide assembly and protein N-glycosylation. GPT is a multispan hydrophobic protein in the endoplasmatic reticulum, with its catalytic site oriented towards the cytoplasm [2,3]. GPT activity is essential for cell function and viability, as has been demonstrated by disruption of its chromosomal locus [4] and genetic mutagenesis in yeast [5], and by inhibition with tunicamycin in higher eukaryotes [6]. The GPT gene belongs to the evolutionarily conserved set of housekeeping genes in eukaryotic cells [7,8]. GPT expression is involved in normal development and differentiation of mammalian cells, and the level of enzymic activity is rate-limiting for glycosylation in different developmentally regulated biological systems [9–11]. A high level of GPT gene expression and abundance of GPT protein is correlated with high proliferative activity during hamster submandibular gland biogenesis [12]. Down-regulation of GPT expression and a decrease in GPT activity parallels postnatal development and differentiation of the hamster submandibular gland [12]. In postnatally developing rat liver, GPT gene expression and GPT activity initially increase, and then decrease to constant levels [13]. Changes in GPT activity have also been demonstrated during the oestrogen-induced differentiation of the chick oviduct [10].

Retinoic acid (RA) is an endogenous metabolite of vitamin A and a potent regulator of growth and differentiation. The actions of RA are mediated by nuclear receptors (RA receptors and retinoid X receptors) that belong to the steroid hormone receptor superfamily. These receptors are expressed in various tissues and act as ligand-inducible transcription factors (for a review, see [14]). RA promotes the differentiation of several cell lines, including the mouse teratocarcinoma cell lines P19 [15] and F9 [16], which differentiate into a neuronal-like phenotype and into parietal endoderm respectively. The differentiation of teratocarcinoma cells provides a unique model system for studying the regulation of protein N-glycosylation by RA. Developmental increases in multiple glycosyltransferase activities that act later in carbohydrate synthesis and in protein glycosylation have been described in F9 cells [17–21], and the regulation of two glycosyltransferase genes is mediated at the transcriptional and post-transcriptional levels [22,23]. In P19 cells, RA has been shown

Abbreviations used: Dol-*P*, dolichyl phosphate; GlcNAc-TI, *N*-acetylglucosaminyltransferase I; GPT, UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase; mGPT, mouse GPT; RA, all-*trans*-retinoic acid.

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to increase the activity of *N*-acetylglucosaminyltransferase I (GlcNAc-TI), which initiates the synthesis of complex and hybrid N-linked carbohydrates, and induced GlcNAc-TI mRNA levels were detected early in differentiating P19 cells [24].

The activity of GPT in eukaryotic cells is inhibited by the antibiotic tunicamycin [6,25], with concomitant inhibition of N-linked glycosylation [26]. The structure of the antibiotic closely resembles the structures of the two substrates for GPT, UDP-GlcNAc and dolichyl phosphate (Dol-*P*), and tunicamycin appears to act at the catalytic site of GPT [27]. Diminished GPT activity and hypersensitivity to tunicamycin were observed in a mutant yeast strain expressing a mutant yeast homologue of the GPT gene [5]. Conversely, tunicamycin-resistant *Leishmania* and Chinese hamster ovary cells have been shown to overexpress the enzyme due to gene amplification [25,28–30], and overexpression of the GPT gene in *Schizosaccharomyces pombe* increased resistance of the cells to tunicamycin [31]. As a result, N-linked protein glycosylation increased in cells that overproduced the transferase [29]. We have found recently that inhibition of protein N-glycosylation in P19 cells by tunicamycin was diminished when cells were co-incubated with RA (W. H. Mueller, D. Kleefeld, B. Khattab, J. D. Meissner and R. J. Scheibe, unpublished work). Therefore we hypothesize that increased protein N-glycosylation, as well as resistance to tunicamycin, may result from increased levels of GPT activity, suggesting that RA induces GPT in P19 cells.

Here we report the induction of GPT activity by RA during differentiation of P19 cells. This induction was dose- and time-dependent. Additionally, RA increased the steady-state level of GPT mRNA, which was mainly due to transcriptional activation of the GPT gene. In terminally differentiated P19 cells, GPT expression was down-regulated and GPT activity decreased to a constant level. RA-induced GPT activity resulted in an increase in the rate of incorporation of [^{3}H]mannose into $Glc_3Man_9GlcNAc_2$ and into glycoproteins, and was correlated with increased tunicamycin-resistance of P19 cells. Thus the P19 cell line provides a useful system in which to investigate the RA-mediated regulation of GPT and the impact of induced GPT activity on the dolichol pathway during neuronal differentiation.

MATERIALS AND METHODS

Materials

UDP-[^{3}H]GlcNAc (36.5 Ci/mmol) and [^{3}H]mannose (25 Ci/mmol) were obtained from New England Nuclear (Köln, Germany). [^{32}P]dCTP (3000 Ci/mmol) was from Hartmann Analytic (Braunschweig, Germany). Desoxyribonucleotide triphosphates and random-primed DNA labelling kit were from Promega (Mannheim, Germany). An 18 S rRNA probe was from Clontech (Heidelberg, Germany). Rainbow® protein molecular mass markers were from Amersham (Braunschweig, Germany), RNA molecular size markers were from Gibco BRL (Eggenstein, Germany), and keyhole-limpet haemocyanin was from Calbiochem (Bad Soden, Germany). α-Minimal essential medium and newborn-calf serum were from Gibco (Karlsruhe, Germany). Bacteriological-grade Petri dishes were obtained from Greiner (Nuertingen, Germany), and tissue culture dishes were from Nunc (Roskilde, Denmark). Secondary goat anti-(rabbit IgG) antibodies were obtained from Janssen Life Science (Beerse, Belgium). Liquid scintillation cocktail was purchased from Zinsser Analytic (Frankfurt, Germany). all-*trans*-RA, tunicamycin B complex (no. T5643), calf thymus DNA, penicillin-G and streptomycin were from Sigma-Aldrich (Deisenhofen, Germany). All other products were analytical grade reagents from Sigma-Aldrich, Fluka AG (Neu-Ulm, Germany) or Bio-Rad (Munich, Germany).

Cells and culture conditions

The mouse teratocarcinoma line P19 [15] was grown in α-minimal essential medium supplemented with 2.5 % (v/v) fetal calf serum and 7.5 % (v/v) newborn calf serum. The cells were maintained at 37 °C in a 5 % CO_2 atmosphere. All culture media contained penicillin-G (100 units/ml) and streptomycin (100 μg/ml), and were replaced after 2 days.

Cells were grown in the presence or absence of RA at the concentrations indicated in the Figure legends. Differentiation of P19 cells was induced as follows. Cells were treated with PBS containing 0.125 % (w/v) trypsin for 15 min. The cell suspension was pelleted (5 min at 1000 ***g***), resuspended in medium with or without 1 μM RA, and plated at a density of 10^6 cells/ml into bacteriological-grade Petri dishes, where they aggregated spontaneously. The medium was replaced after 2 days, and the aggregates were transferred to tissue culture dishes (60 mm), where they grew in monolayers. For treated cultures, RA was added to fresh medium and cells were grown in the presence of RA for 8 days. In some experiments, cells were treated simultaneously with RA (1 μM) and tunicamycin (0.001–5.0 μg/ml). Tunicamycin and RA were present in the medium for the first 2 days. After 2 days, the medium was changed and fresh medium without drugs was added to each culture. At various time points after the initiation of treatment, as indicated in the Figure legends, cells were washed twice with ice-cold PBS and harvested with a rubber scraper in 2 ml of PBS. Cells were then homogenized and microsomal membranes were utilized for biochemical analysis. To test for the expression of cellular marker proteins of differentiation (see below), cells were counted in a haemocytometer 4 days after initiation of treatment.

Preparation of membranes

Mouse microsomes were prepared from tissue from a pool of 20 mouse mammary glands and from mouse brain as described previously [32]. Membranes from P19 cells were prepared by the method of Criscuolo and Krag [28], with minor modifications. Cells were homogenized in the presence of 100 μg/ml DNase I to reduce aggregation of microsomal material, and after homogenization membranes were adjusted to 0.25 M sucrose. Membranes were recovered by centrifugation at 100000 ***g*** for 1 h after removal of debris (1500 ***g*** for 5 min). Membrane protein was determined by the procedure of Oyama and Eagle [33].

GPT assay

Standard assay conditions consisted of 5 mM $MnCl_2$, 64 mM Tris/HCl, pH 7.4, 105 mM NaCl and 0.1 μCi of UDP-[^{3}H]GlcNAc in a final volume of 0.4 ml. Reactions were initiated by the addition of 20 μg of membrane protein, followed by a 30 min incubation at 37 °C with gentle shaking. AMP (1 mM) was also included as a protection against degradation of the substrate nucleotide sugar by contaminating enzymes. The dolichol sugar conjugates were then extracted into solvent as described previously [34]. The ^{3}H content was determined by evaporation of the solvent in glass vials, the addition of 5 ml of aqueous counting scintillant and counting in a liquid scintillation counter.

Analysis of lipid-linked oligosaccharides in cultured cells

Experiments were performed as described [35], with some modifications. Briefly, cells were grown in the presence or absence of

RA (1 μM) for 48 h. Medium was changed and cells were incubated in low-glucose medium containing 1 mCi/ml [^{3}H]mannose for 15 min at 37 °C. Then 1 ml of pre-warmed medium containing 10 mM mannose was added and cells were incubated for an additional 5 min. The mannose chase allows the conversion of biosynthetic intermediates that may remain after the labelling period into mature oligosaccharides. Cells were then harvested and collected by centrifugation at 4 °C, and washed once with ice-cold PBS. Lipid-linked oligosaccharides were then recovered by organic extraction [36], cleaved with weak acid, reduced and fractionated by silica-gel HPLC as described [37]. Approx. 100000 c.p.m. of lipid-linked oligosaccharides were recovered per dish.

Incorporation of [^{3}H]mannose into proteins

Incorporation of [^{3}H]mannose into proteins was performed as described in [35], with some modifications. Briefly, P19 cells were pre-incubated for 48 h in the presence of 1 μM RA. Cells were then washed and serum-free medium containing [^{3}H]mannose (1 mCi/ml) was added. After 2 h at 37 °C, incorporation was determined by washing twice with ice-cold PBS. Cells were harvested and homogenized, and protein was precipitated by the addition of perchloric acid. Precipitates were washed on filters and assayed for radioactivity in a Berthold CS 5000 liquid scintillation counter (Berthold GmbH, Bad Wildbad, Germany) using Quickzint 2000 as the scintillant. Total cell protein was determined as described [33].

RNA analysis and nuclear run-off assay

For RNA isolation, cells (1×10^8) were plated in 150 mm dishes. The medium was changed 2 days after the cells were seeded and 1 μM RA was added. Total cellular RNA was purified from P19 cells according to the method of Chirgwin et al. [38]. The RNA was size-fractionated on a 1.2% (w/v) agarose/formaldehyde gel and transferred to nitrocellulose. Mouse GPT (mGPT) cDNA [39] and 18 S rRNA were labelled with [^{32}P]dCTP using random hexamer as a primer [40]. The mGPT cDNA probe was a 1.6 kb *Hin*dIII–*Eco*RI fragment containing the entire coding region and portions of 5′- and 3′-non-coding sequences [39]. Filters were prehybridized at 42 °C for at least 5 h in solution containing 50% (v/v) formamide, $5 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 0.02% (w/v) Ficoll, 0.02% (w/v) BSA, 1% (w/v) SDS and 100 μg/ml denatured calf thymus DNA. Hybridization was performed for 12 h at 42 °C in the same solution containing 1×10^6 c.p.m./ml labelled DNA probes. Blots were washed with $0.2 \times$ SSC/0.5% (w/v) SDS at 65 °C for 2×30 min. Autoradiography was performed with intensifying screens at −70 °C. Nuclear run-off assays were performed as described [41].

Generation of anti-mGPT-(309–323) antibody

A peptide corresponding to amino acids 309–323 of the deduced amino acid sequence of mGPT [39] was synthesized and conjugated to keyhole-limpet haemocyanin in the presence of glutaraldehyde [42]. The conjugated peptide was mixed with complete Freund's adjuvant and injected subcutaneously into a rabbit. Multiple secondary injections of the conjugated peptide in incomplete Freund's adjuvant were given at 2–3-week intervals. The blood was collected 8–10 days later and tested for the production of antibodies by Western blot analysis. The IgG fraction was purified by DEAE-cellulose column chromatography as described by the manufacturer (Bio-Rad). The immunoadsorption assays with antiserum and mouse microsomes were performed using the procedure described previously [8]. Mouse microsomes were prepared from tissue from a pool of 20 mouse mammary glands as described [32].

Western blot analysis

Samples were electrophoresed on a 10% (w/v) polyacrylamide gel under reducing conditions and transferred to nitrocellulose as described previously [41]. The blots were incubated with primary anti-mGPT-(309–323) antibody and secondary goat anti-(rabbit IgG) antibodies, and the red signal was enhanced by silver staining [41].

RESULTS

RA-induced GPT activity

The kinetics of induction of GPT by RA were determined in P19 teratocarcinoma cells. The *in vitro* assay for GPT contained membranes and UDP-[^{3}H]GlcNAc, and detected the transfer of [^{3}H]GlcNAc to the endogenous Dol-*P* in membranes. Cells were grown in the presence or the absence of 1 μM RA on bacteriological-grade Petri dishes (as described in the Materials and methods section) for 2 days, where they aggregated spontaneously. Aggregates were then transferred to tissue culture dishes, where they grew in monolayers and differentiated preferentially into neuronal cells with long processes and glia-like cells [15]. Replicate cultures were harvested after various times. Membrane fractions were prepared from each sample, and GPT activity was measured. Figure 1 shows that an increase in activity was detected 8 h after the initiation of RA treatment. GPT activity had increased approx. 2.5-fold after 24 h of treatment with RA. The maximum increase in GPT activity was approx. 3-fold relative to that in untreated control cells, and occurred 2 days after RA (1 μM) treatment. Approx. 3 days after the initiation of treatment, the elevated enzyme activity began to decline, and had decreased to a constant level by 8 days after initiation of treatment. Between 8 h and 6 days, the mean values for GPT activity in each RA-treated group were significantly different from the values for the controls. Induction of GPT activity occurred at RA concentrations between 0.01 μM and

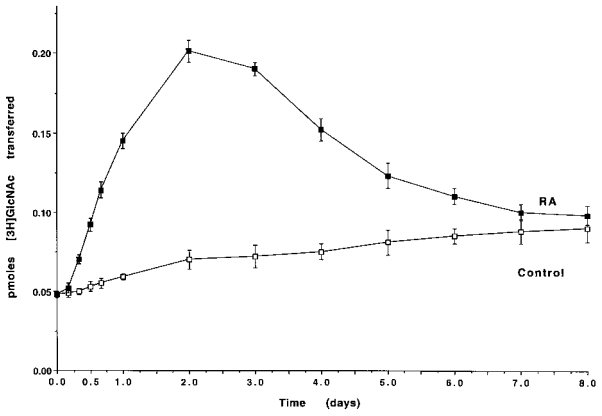

Figure 1 RA-induced GPT activity during P19 cell differentiation: time course

P19 cells were incubated in the absence or presence of RA (1 μM). Individual cultures were harvested at various times after the initiation of treatment, and membranes were isolated to determine GPT activity. Samples containing 20 μg of membrane protein were assayed as described in the Materials and methods section. Data represent means ± S.D. of four replicate cultures.

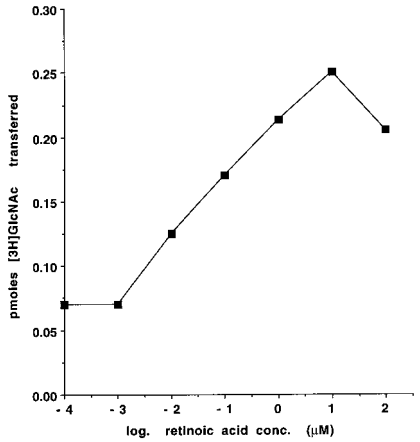

Figure 2 RA-induced GPT activity in P19 cells: dose–response curve

Cells were grown in the presence of RA at the concentrations indicated. At 2 days after the initiation of treatment, cells were harvested and membranes were isolated. Samples of 20 μg of membrane protein per assay were used for the determination of GPT activity.

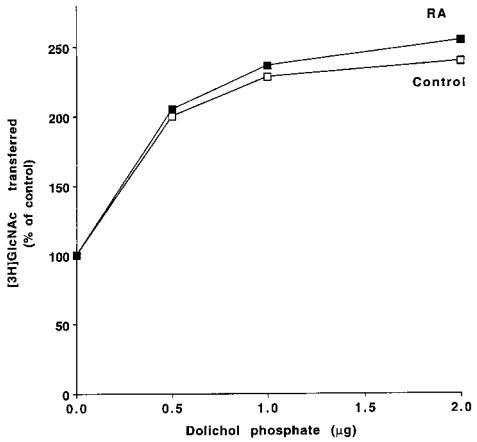

Figure 3 Proportional stimulation of GPT activity in membranes from untreated and RA-treated P19 cells

Membrane protein (20 μg) isolated from untreated or RA-treated (1 μM for 2 days) cells was assayed for GPT activity in the presence of 0.005% (w/v) Nonidet P-40 and exogenously added Dol-*P* (0–2 μg per assay). The values are plotted as percentages of the activity obtained in the presence of 0.005% (w/v) Nonidet P-40 without Dol-*P*, and are means of three independent experiments.

100 μM, and the most effective concentration was 10 μM RA (Figure 2). When P19 cells were treated with 0.01 μM RA, GPT activity increased about 1.8-fold, whereas treatment with 10 μM RA resulted in an increase in GPT activity of approx. 3.6-fold over the control level. We chose to perform experiments using the physiological concentration of 1 μM RA. Figure 3 shows that the control and elevated GPT activities were stimulated proportionally by exogenous Dol-*P*. The data suggest that membranes from RA-treated cells did not differ from membranes from control cells with regard to the level of endogenous Dol-*P*, but rather showed increased catalytic activity.

To characterize further the increased GPT activity, we studied the rate of incorporation of [^{3}H]mannose into $Glc_3Man_9GlcNAc_2$-*P*-*P*-dolichol. Figure 4 shows an HPLC profile of the lipid-linked oligosaccharides that accumulated

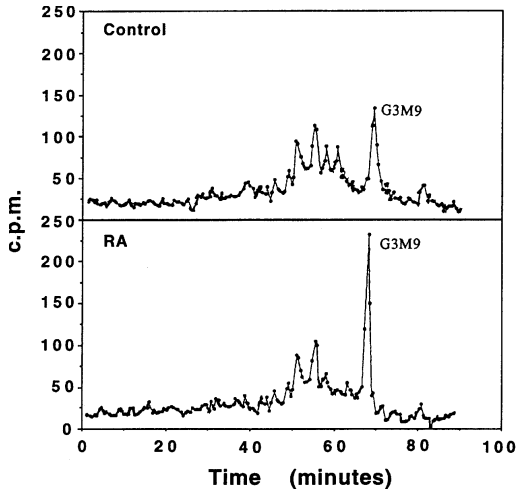

Figure 4 Lipid-linked oligosaccharides synthesized by untreated and RA-treated P19 cells

P19 cells were grown in the absence or presence of RA (1 μM). After 48 h, cells were labelled with [^{3}H]mannose, dolichol-linked oligosaccharides were isolated, and silica HPLC was performed as described in the Material and methods section. The peak eluting at 68 min is the $Glc_3Man_9GlcNAc_2$ oligosaccharide (G3M9).

in control and RA-treated cells. The predominant oligosaccharide in P19 cells eluted in the position characteristic of $Glc_3Man_9GlcNAc_2$. In the presence of RA, the rate of $Glc_3Man_9GlcNAc_2$-*P*-*P*-dolichol synthesis increased by approx. 85% compared with controls. Furthermore, we determined the effect of RA on protein glycosylation by metabolic labelling with [^{3}H]mannose. The mean ($\pm$S.D.) values from three independent experiments for [^{3}H]mannose incorporation into glycoprotein were 3100 ± 220 c.p.m./mg of protein in controls and 5900 ± 390 c.p.m./mg of protein in cells after 48 h of RA exposure, demonstrating an approx. 2-fold increase in mannosylation of proteins in the presence of RA. Together, the results suggest that RA-induced GPT activity has a regulatory impact on the dolichol pathway and on protein glycosylation.

RA induces the level of GPT protein

In order to examine the effect of RA on the level of GPT protein, antibodies were raised against a segment of the deduced amino acid sequence of mouse GPT [39]. The selected peptide (residues 309–323) is present in the large cytoplasmic loop in the model of the GPT structure proposed by Zhu and Lehrman [43]. Incubation of increasing amounts of anti-peptide antiserum with a constant amount of solubilized enzyme from mouse mammary gland microsomes progressively precipitated out the GPT activity. Incubation with pre-immune serum did not result in significant removal of enzyme activity. Pre-immune and immune sera were used to probe Western blots (Figure 5). The pre-immune serum failed to bind to a protein in solubilized mouse mammary gland microsomes (Figure 5, lane 1), whereas the anti-peptide antibody recognized a protein of approx. 48 kDa (lane 2). Similar results were obtained when microsomal membranes from mouse brain (lanes 3 and 4) and P19 cells (lanes 5–12) were used. The size of the detected protein is in good agreement with that predicted from the cDNA sequence [39] and with that reported previously in membranes from mouse brain [8]. Untreated cells were assayed at 0 h (Figure 5, lanes 5 and 6), 2 days (lane 9) and 8 days (lane 11). RA-treated (1 μM) P19 cells were assayed at 0 h (lane 7), 12 h (lane 8), 2 days (lane 10) and

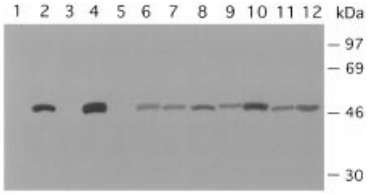

Figure 5 Western blot analysis of GPT in induced and uninduced P19 cells

Solubilized microsomes from mouse mammary gland (lanes 1 and 2), mouse brain (lanes 3 and 4), untreated P19 cells (0 h, lanes 5 and 6; 2 days, lane 9; 8 days, lane 11), and RA-treated (1 μM) P19 cells (0 h, lane 7; 12 h lane 8; 2 days, lane 10; 8 days, lane 12) were subjected to Western blot analysis and probed with pre-immune serum (lanes 1, 3 and 5) or anti-GPT-(309–323) antibody (lanes 2, 4 and 6–12). The positions of protein molecular mass standards (in kDa) are indicated.

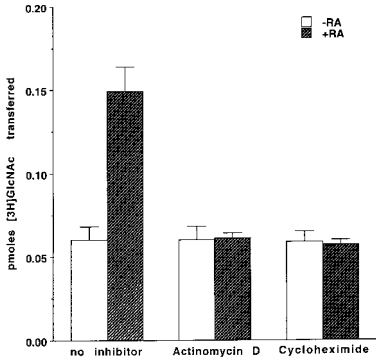

Figure 6 Effects of actinomycin D and cycloheximide on GPT activity

P19 cells were cultured for 24 h in the presence or absence of 1 μM RA in combination with 0.1 μg/ml actinomycin D or 1.0 μg/ml cycloheximide. GPT activity in membranes was determined as described in the Materials and methods section. Results are means $\pm$ S.D. of four replicate cultures.

8 days (lane 12). When P19 cells were treated with 1 μM RA for 2 days, an approx. 3-fold increase in the 48 kDa protein was detected (Figure 5, compare lanes 9 and 10). Thus the induction of GPT activity was accompanied by a similar increase in GPT protein. Microsomes prepared from P19 cells grown in the presence or absence of RA for 8 days were also examined for expression of GPT protein. In RA-treated cultures, the amount of GPT had declined almost to the level of control cultures (Figure 5, compare lanes 11 and 12). Thus the amount of GPT protein detected by Western blot analysis corresponds to the level of enzyme activity during P19 differentiation (see Figure 1).

RA induces expression of the GPT gene

The first set of experiments was performed to determine whether or not the addition of inhibitors of transcription or translation inhibits the induction of GPT activity by RA. Actinomycin D (0.1 μg/ml) or cycloheximide (1 μg/ml) was added simultaneously with RA to P19 cells, and GPT activity was determined 24 h after treatment. As shown in Figure 6, both inhibitors blocked the induction of GPT activity by RA. Actinomycin D and cycloheximide decreased the number of cells per dish by about 25%; however, all of the values of GPT activity were corrected for the protein content of the membrane fractions. These results indicate that induced enzyme activity is dependent on RNA and protein synthesis.

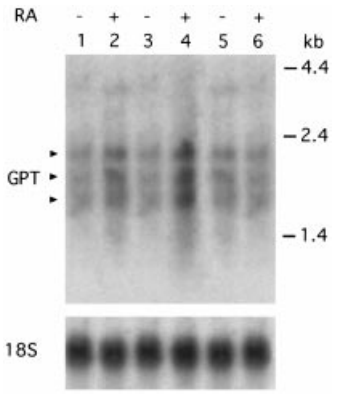

Figure 7 GPT mRNA expression during P19 cell differentiation

Cells were cultured in the presence or absence of 1 μM RA for 12 h (lanes 1 and 2), 2 days (lanes 3 and 4) or 8 days (lanes 5 and 6). Total RNA (20 μg) was isolated from each sample and fractionated by electrophoresis in a 1.2% (w/v) formaldehyde/agarose gel. After transfer of the RNA to a nitrocellulose membrane, the filter was hybridized with a ^{32}P-labelled GPT cDNA probe (1×10^6 c.p.m./ml) or an 18 S rRNA probe (1×10^6 c.p.m./ml), washed, and exposed to Kodak XAR-5 film for 2 days at -70 °C. Molecular size markers are ethidium bromide-stained RNA components derived from bacteriophage T7, yeast and bacteriophage λ DNA.

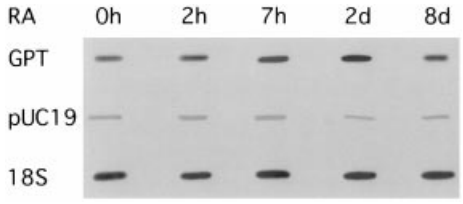

Figure 8 Rates of transcription of GPT mRNA in uninduced and RA-induced cells

Nuclei were isolated from untreated and RA-treated (1 μM) P19 cells, and nascent RNA was extended *in vitro* as described [41]. Radiolabelled RNAs were hybridized to 5 μg of linearized DNA that had been immobilized on nitrocellulose. Background was indicated by the level of hybridization to pUC19. The 18 S rRNA probe was used as internal control to normalize for RNA loading and transfer.

We then examined the expression of GPT mRNA over time in the presence and absence of RA (1 μM). The mGPT cDNA [39] probe was used to detect GPT mRNA by Northern blot analysis. The cDNA probe hybridized to three mRNA species of 1.8, 2.0 and 2.2 kb. The sizes of the transcripts in P19 cells are in agreement with those in mouse mammary gland [39] and Chinese hamster ovary cells [30]. A basal level of GPT transcript was detected in P19 control cells (Figure 7). This steady-state level of GPT mRNA remained unchanged in untreated cultures (Figure 7, lanes 1, 3, and 5). In contrast, the level of GPT mRNA was increased (2-fold; lane 2) 12 h after the addition of RA, and reached a maximum by 2 days (approx. 4-fold; lane 4). The elevated level of GPT mRNA began to decline 3 days after treatment of the cells with RA (results not shown), and transcript abundance was similar to that in untreated cells by 8 days (Figure 7, lane 6).

To investigate the GPT gene, we performed nuclear run-off assays. An increase in transcriptional activity (about 2-fold at 7 h) was observed in the nuclei of cells treated with RA (Figure 8). The greatest increase (about 3-fold) in GPT transcription was determined after 2 days of treatment. GPT gene expression was

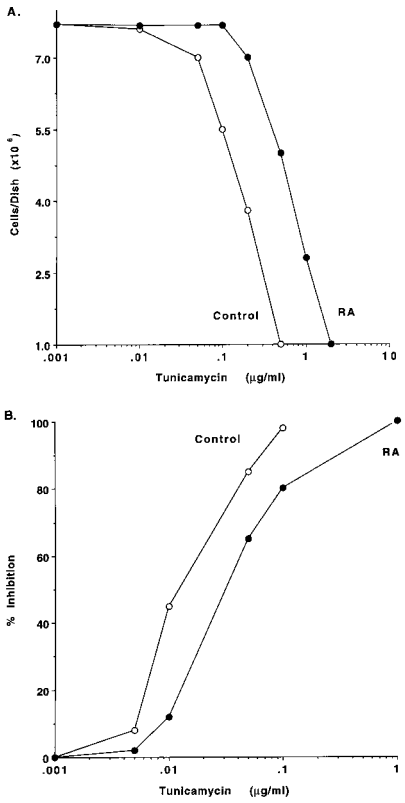

Figure 9 Effects of tunicamycin on the survival of P19 cells and on GPT activity in membranes

(**A**) Cells were seeded at low density (1×10^6 cells/ml) and grown in the presence or absence of RA (1 μM) and various concentrations of tunicamycin as indicated. The culture conditions are described in the Materials and methods section. At 6 days after plating, cells were counted in triplicate in a haemocytometer. The data shown are means of three independent experiments. (**B**) P19 cells were incubated in the absence or presence of RA (1 μM) for 2 days. Membranes were isolated and samples of 20 μg of protein from untreated controls and RA-treated cells were assayed for GPT activity in the presence of increasing concentrations of tunicamycin. Results are expressed as the percentage inhibition compared with activity in the absence of tunicamycin. Each data point represents the mean of three replicate cultures.

down-regulated by 8 days after initiation of RA treatment, by which time P19 cells were terminally differentiated [15]. Thus RA increased the steady-state level of GPT mRNA, which is mainly due to transcriptional activation of the GPT gene.

RA increases the tunicamycin-resistance of P19 cell growth and of GPT activity in membranes

Tunicamycin is a specific inhibitor of the N-linked glycosylation of proteins and of GPT activity *in vivo* and *in vitro* [26]. Recently we found that inhibition of protein N-glycosylation by tunicamycin was diminished when P19 cells were co-incubated with RA (W. H. Mueller, D. Kleefeld, B. Khattab, J. D. Meissner and R. J. Scheibe, unpublished work). In the present study, we have demonstrated that RA induced GPT expression and activity. To investigate whether RA also increases the resistance of P19 cells to tunicamycin, we treated cells with increasing concentrations of tunicamycin (0.001–5.0 μg/ml) in the presence or absence of 1 μM RA. Tunicamycin and RA were present in the medium for the first 2 days of the experiment. The number of cells per dish was determined 4 days after initiation of differentiation. As shown in Figure 9(A), little effect on cell growth was seen up to a concentration of 0.05 μg/ml tunicamycin, whereas no growth was seen with 0.5 μg/ml tunicamycin in the absence of RA (control) or with 2 μg/ml in the presence of RA. Thus RA-treated P19 cells were about 4-fold more resistant to the cytotoxic effect of tunicamycin than control cells. In addition, we added tunicamycin directly to membranes prepared from cells grown in the presence or absence of 1 μM RA for 2 days. As expected, GPT activity was inhibited by tunicamycin in membranes from both RA-treated and untreated cultures (Figure 9B). However, the concentration required for 50% inhibition differed between controls and RA-treated cells by about 4-fold. Thus resistance of enzymic activity in membranes corresponds to induced GPT mRNA levels in RA-treated cells, as indicated by Northern blot analysis (Figure 7). Together the results demonstrate that the fold increase in resistance to tunicamycin in RA-treated P19 cells correlates with the fold induction of GPT activity in cells grown in the presence of RA for 2 days (Figure 1). The data indicate that tunicamycin-resistance results from induced GPT activity and that RA has an impact on the dolichol pathway that affects the survival of cells.

DISCUSSION

The present study describes the induction of GPT activity by RA in mouse P19 teratocarcinoma cells; this is followed by a decrease in activity later during differentiation into neuronal and glia-like cells. The induction occurred at physiological concentrations (range 10 nM–1 μM RA). Increased GPT activity was detected within 8 h and the maximal induction, measured after 2 days of RA (1 μM) treatment, was approx. 3-fold. Induction of GPT activity paralleled increases in the rates of $Glc_3Man_9GlcNAc_2$-*P*-*P*-dolichol synthesis and of protein glycosylation in RA-treated cells, as determined by metabolic labelling with [^{3}H]mannose. At the same time, increases in GPT protein and mRNA levels were observed. The decrease in GPT induction at high concentrations of RA (100 μM) may be explained by the toxicity of the drug. The induced enzymic activity was blocked by the addition of actinomycin D to the culture medium at the time of RA treatment. These results indicate that induced GPT activity is dependent on transcriptional events. During differentiation, the elevated GPT activity and mRNA levels decreased nearly to those in control cultures.

The GPT gene is transcriptionally activated by RA, as demonstrated by nuclear run-off assays. The transcription rate was increased approx. 2-fold after 7 h and approx. 3-fold after 2 days of treatment, while Northern blot analysis showed that GPT mRNA levels were increased about 2-fold after 12 h and 4-fold after 4 days of treatment. The difference between transcription rate and amount of mRNA might be due to an additional effect of RA on the half-life of the GPT transcript. RA can induce enzyme activity via transcriptional and early post-transcriptional events, as shown for alkaline phosphatase in promyelocyte leukaemia [44]. Alternatively, the difference between the rate of transcription and accumulation of mRNA could be due to an unknown occurrence, e.g. increased transfer of GPT mRNA from the nucleus to the cytoplasm, in RA-treated P19 cells. Northern blot analysis of both untreated and RA-treated P19 cells with the mGPT probe showed three mRNA species (1.8, 2.0 and 2.2 kb). In Chinese hamster ovary cells, the GPT gene gives rise to a family of mRNAs of similar sizes [30,45], while in yeast two mRNA species (1.4 and 1.6 kb) have been found [4], and a single band (2 kb) was detected with RNA from mouse mammary gland [39]. In yeast, the size heterogeneity appears to be due to variations at the 5′ and 3′ ends of the mRNA [4,43]. RA treatment of P19 cells increased the levels of all three GPT

transcripts by approx. 4-fold. The biological significance of the expression of three transcripts requires further investigation.

Simultaneous treatment of P19 cells with RA and tunicamycin reduced the inhibition of GPT activity by tunicamycin. Tunicamycin is known to inhibit GPT activity (for a review, see [26]), and tunicamycin resistance in *Leishmania*, Chinese hamster ovary cells and yeast is characterized by an increase in GPT activity due to gene amplification [25,28–30,46]. We have now demonstrated that RA treatment of P19 cells for 2 days increased the transcriptional activity of the GPT gene by about 3-fold, GPT mRNA levels by approx. 4-fold, and resistance to tunicamycin by about 4-fold. It has been reported that GPT mRNAs were also enriched in tunicamycin-resistant Chinese hamster ovary cells [30], although to a much greater extent (40-fold), and cells became 80-fold more resistant to tunicamycin. In P19 cells, we found a smaller increase in GPT activity (3-fold at 2 days) in comparison with the effect on tunicamycin resistance (4-fold). Differences between the fold increases in GPT activity and tunicamycin resistance have also been reported by others [28,30], and may indicate that additional enzyme molecules are present in the cells that are not detectable in the assay.

Although changes in protein glycosylation, cell-surface carbohydrates and activities of several glycosyltransferases have long been established during RA-mediated differentiation of teratocarcinoma cells [17,19–21,47], only two reports exist on the regulation of the transferases in F9 cells [22,23]. Recently, RA has been suggested to induce GlcNAc-TI activity in P19 cells as early as 24 h due to transcriptional activation of the GlcNAc-TI gene [24]. GlcNAc-TI is a key enzyme for generating complex N-asparagine-linked carbohydrates [48]. In addition, regulation of two other glycosyltransferases has been found to occur at the transcriptional and post-transcriptional levels in F9 cells [22,23]. Controlled expression of genes encoding glycosyltransferases is expected to be critical for the synthesis of developmentally regulated carbohydrates. Both GPT and GlcNAc-TI have regulatory functions in the biosynthesis of N-linked glycoproteins. These enzymes exhibit regulated expression early in the differentiation of P19 cells and are evidently targets for the overall regulation of oligosaccharide assembly and protein N-glycosylation by RA in this system.

There have been some previous reports regarding the developmental and hormonal regulation of GPT activation. Regulated expression of the GPT gene has been demonstrated in the postnatally developing hamster submandibular gland [12] and the mouse mammary gland [8,49]. In postnatally developing rat liver, GPT activity initially increased and then decreased 2-fold to a constant level [13]. Similarly, we found that GPT activity increased in P19 cells during the first 2 days of RA-mediated differentiation. In terminally differentiated cells, enzymic activity declined almost to the level of controls. GPT activity is known to be elevated in different developmentally regulated biological systems, such as the sea urchin [11] and the oestrogen-induced differentiating chick oviduct [10]. The values for the increase in GPT activity in the oestrogen-stimulated chick oviduct and in RA-induced P19 cells are within a similar range. Although oestrogen, like RA, mediates its action via ligand-inducible nuclear receptors belonging to the steroid hormone receptor superfamily, increases in Dol-*P* concentrations following oestrogen stimulation have been implicated in the increase in GPT activity in the chick oviduct [10,50,51]. Therefore we investigated whether increased GPT activity in RA-treated P19 cells is a result of increased levels of Dol-*P* in P19 cells following RA treatment. Our data demonstrate that membranes from RA-treated cells did not differ from membranes of control cells with regard to the level of endogenous Dol-*P*. These data rule out an indirect effect of RA on GPT activity due to altered levels of Dol-*P*, and support results from Northern blot analysis and nuclear run-off assays indicating that RA-induced GPT activity in P19 cells is determined mainly by transcriptional events.

RA regulates the transcription of genes via RA receptors that act as ligand-inducible transcription factors. A portion of the mouse GPT promoter has been cloned [52], and it will be important to elucidate whether the GPT gene is the primary target for RA action, or whether its transcriptional activation is caused indirectly by other *trans*-acting factor(s) activated by RA. The increases in the rates of $Glc_3Man_9GlcNAc_2$-*P*-*P*-dolichol synthesis and of protein mannosylation argue for a regulatory significance for induced GPT with regard to the dolichol pathway and protein glycosylation. Thus the P19 cell line is a useful system in which to study the molecular mechanism(s) by which RA modulates GPT expression and regulates protein glycosylation during neuronal differentiation.

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