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DEVELOPMENTAL REGULATION AND TISSUE-SPECIFIC EXPRESSION OF HAMSTER DOLICHOL-P-DEPENDENT N-ACETYLGLUCOSAMINE-1-P TRANSFERASE (GPT)

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SUMMARY: The first enzyme in the dolichol pathway of protein N-glycosylation, dolichol-P-dependent N-acetylglucosamine-1-phosphate transferase, GPT, has been implicated in the development of a wide variety of eukaryotes. GPT is encoded by ALG7, an early growth-response gene, whose expression has been shown to affect the extent of N-glycosylation and secretion of proteins. To initiate the molecular characterization of ALG7 involvement in mammalian growth and differentiation, we have used the postnatally developing hamster submandibular gland (SMG) as an experimental paradigm. In this study we report that the ALG7 gene was differentially expressed during postnatal development and in terminally differentiated adult tissues. Throughout development, GPT activity paralleled ALG7 mRNA levels, suggesting that the amount of functional enzyme was determined by modulation of transcript abundance.

INTRODUCTION: Dolichol-P-dependent N-acetylglucosamine-1-phosphate transferase, GPT, catalyzes the first step in the dolichol pathway, and is the key enzyme involved in protein N-glycosylation (1,2). The gene encoding GPT, ALG7, belongs to a class of evolutionarily conserved housekeeping genes. The critical role of GPT to cell function has been demonstrated by disruption of its chromosomal locus in yeast (3), and by inhibition of the enzyme with tunicamycin in yeast (4) and in higher eukaryotes (5).

Increasing evidence indicates that modulation of ALG7 expression might be required for normal development and differentiation. Changes in the activity of the early dolichol pathway enzymes have been demonstrated in different developmentally regulated biological

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systems, such as the sea urchin (6) and the estrogen-induced differentiating chick oviduct (7). In postnatally developing rat liver, GPT activity was shown to increase initially, and then decrease two-fold to a constant level (8). Moreover, unwarranted changes in GPT activity in yeast interfered with its developmental program (9; Kukuruzinska, unpublished).

Recently, the yeast ALG7 gene, along with two other early dolichol pathway genes, ALG1 and ALG2, has been shown to be an early growth-response gene (10). Growth stimulation of G_0-arrested cells resulted in a rapid and dramatic increase in ALG7 mRNA levels in the absence of *de novo* protein synthesis. Regulation of ALG7 transcript abundance at the G_0/G_1 transition point in cell cycle suggested that increased ALG7 transcript levels were required for proliferation, while their downregulation participated in the acquisition of quiescence.

The cloning of hamster ALG7 cDNAs (11,12) has facilitated the accessibility of probes for characterization of developmental expression of ALG7 on a molecular level. The objective of the current study was to align previously observed developmental changes in GPT activity with ALG7 transcript abundance, using the postnatally differentiating hamster submandibular gland, SMG, as a model. Although SMG originates in the last third of the fetal period, the major aspects of its functional development occur postnatally (13). There is significant glandular growth, involving regulated cell proliferation and differentiation, leading to the establishment of the branching pattern typical of the adult SMG several weeks after birth. In this report, we present evidence that the acquisition of a differentiated SMG phenotype correlates with downregulation of ALG7 expression. We show that both ALG7 mRNA levels and GPT activity became progressively attenuated throughout the postnatal development, suggesting that developmental changes in GPT activity result from modulation of ALG7 transcript abundance. We also show that ALG7 expression is tissue-specific, most likely reflecting different N-glycosylation capacities of specialized tissues.

MATERIALS AND METHODS

Tissue isolation. Twenty timed 12-day pregnant hamsters were obtained from the Charles River Research Laboratories. Four days after birth, 110 newborns were sacrificed by cervical dislocation, their submandibular glands were removed and immediately placed in liquid nitrogen, followed by storage at -80°C. The remaining litters were sacrificed at the following days postpartum: 14 (30 animals), 28 (13 animals), 48 (8 animals), and 72 (5 animals). In addition to SMGs, the following tissues were removed from adult hamsters: parotid, liver, brain, kidney, spleen, heart and lung.

RNA isolation and analyses. Total RNAs were isolated from SMGs at different stages of postnatal development using the low temperature guanidinium thiocyanate extraction method (14). Approximately 1g of tissue was used for each extraction. For isolation of RNA from CHO cells, $1x10^8$ cells were used. The integrity of RNAs was assessed by ethidium bromide (0.5 μg/ml) staining of ribosomal RNAs on 1% agarose/6M formaldehyde gels.

RNA blotting assays were performed with 2% agarose/6M formaldehyde gels, as described (3). All hybridizations were at high stringency (42°C). The probe consisted of the BamHI-EcoRI fragment (981 bp) of ALG7 cDNA cloned into the SmaI site of the pBluescript vector (pGH3'981) (15). This cDNA spanned the C-terminal coding region and the entire 3'-UTR of the 1.9 kb mRNAs (nt 889-1843 plus 27 poly(dT) residues) (Fig. 1). The probe was labeled with [α-^{32}P]dCTP (10^9 cpm/μg) using the random primer method (Promega). Quantification of ALG7 mRNA levels was carried out by densitometry using the Gel Scan™ software (Pharmacia).

GPT activity assays. Microsomal membrane fractions from different stages of postnatally developing SMGs were isolated using a standard procedure (16). Protein concentration was determined by the BioRad assay. GPT activity was measured by incorporation of [^{14}C]UDP-GlcNAc into the upper phase of the two-phase scintillation cocktail, as described (17).

RESULTS

ALG7 expression in postnatally developing hamster SMG. In our studies, we used postnatally developing hamster SMGs at several distinct stages of cyto-morphodifferentiation (13): 4-6 days after birth, when the undifferentiated cells of the terminal tubules become terminal buds (these later give rise to the acini); 14-28 days, when the acini become progressively differentiated, some acquiring fully differentiated phenotypes; 42-56 days, when the acini almost completely replace the terminal buds and the cells are filled with secretory granules; and 72 days, representing the terminally differentiated adult SMG.

ALG7 mRNA levels. Total RNAs from the distinct stages of SMG's postnatal development were analyzed by RNA blotting assays. The ALG7-specific transcripts were detected using the EcoRI-BamHI fragment of hamster ALG7 cDNA (Fig. 1). The results showed that the amount of the accumulated ALG7 transcripts was 3-fold higher at 4 days postpartum than in the adult tissue (Fig. 2). This decrease in ALG7 mRNA steady-state levels occurred gradually throughout the postnatal period. Our results also show that, in contrast to CHO cells, in which three ALG7 mRNAs are produced (15), only the 1.9 kb species were detected, both throughout development and in the adult SMG.

GPT activity in postnatally developing SMG. To examine directly the relationship between the levels of mRNA and enzyme activity, microsomal membrane fractions from different

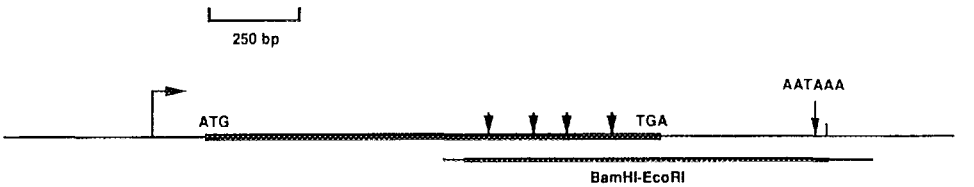

Fig. 1. Physical map of ALG7 1.9 kb cDNA. The BamHI-EcoRI probe used for RNA blotting assays is shown. Four arrows in the open reading frame represent intron locations (11). The BamHI-EcoRI fragment contains some plasmid sequences indicated by the thinner line.

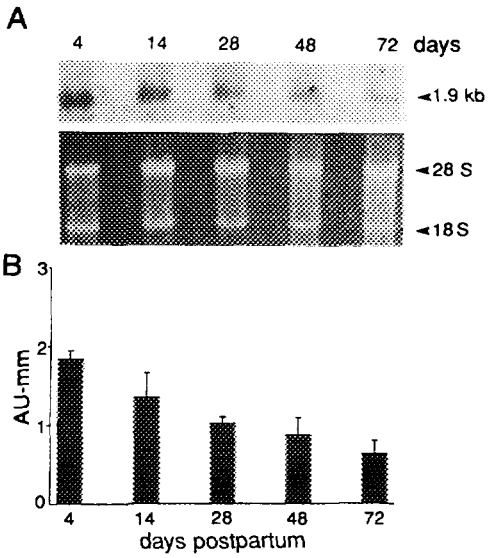

Fig. 2. Developmental expression of ALG7 1.9 kb mRNA. (A) *Upper panel.* Total RNAs (20 μg), isolated from 4, 14, 28, 48 and 72 days of postpartum, were analyzed by RNA blotting assays. The probe was as described in Fig. 1. *Lower panel.* The integrity and loading consistencies were assessed by ethidium bromide staining of ribosomal RNAs (28S and 18S). The experiments represent one of three independent determinations. (B) Quantification of the ALG7 mRNAs shown in (A). Autoradiograms used for analyses were in the linear range of film sensitivity, and they were quantified with densitometry using Gel Scan software (Pharmacia). The bars indicate standard error of the mean.

cyto-morphogenic stages of postnatally developing SMGs were assayed for GPT activity. The results, summarized in Table I, indicate that the specific activity of GPT was 2.8-fold higher in neonatal SMG compared to the terminally differentiated tissue. In fact, the level of GPT activity was progressively attenuated with tissue differentiation and followed the pattern for postnatal days: 4 > 12 > 24 > 72. These data indicate that GPT activity paralleled ALG7 mRNA abundance throughout postnatal development and differentiation, suggesting that it was modulated by regulation of the steady-state levels of ALG7 mRNA.

Tissue-specific expression of the hamster ALG7 transcripts. A comparison of the steady-state levels and patterns of ALG7 transcripts in different adult hamster tissues was carried out by RNA blotting assays. Three distinct ALG7 mRNAs were detected in CHO cells, which migrated as 1.5, 1.9 and 2.2 kb species (Fig. 3A), in agreement with the previously proposed average size of 2 kb (11) and the recently characterized species (15). Similar to CHO cells, three ALG7 mRNAs, 1.5, 1.9 and 2.2 kb, were also detected in brain (Fig. 3C). In contrast, the 2.2 kb species were not clearly discernable in such tissues as kidney, heart, lung and spleen (Fig. 3C). Furthermore, only the 1.9 kb mRNAs were evident in liver,

Table I. GPT activity at different stages of postnatal development[a]

Days postpartum	GPT activity [^{14}C]GlcNAc Incorporated pmol/h/mg	Relative activity
4	163 ± 31	2.8
12	136 ± 29	2.3
24	104 ± 25	1.8
72	59 ± 16	1.0

[a]Microsomal membrane fractions, isolated from 4-, 12-, 24- and 72-day old SMGs, were assayed for GPT activity. The experiments represent an average of three independent determinations.

submandibular, and parotid tissues (Fig. 3A). In all cases, the 1.9 kb transcripts were preferentially expressed. The overall abundance of the ALG7 transcripts varied depending on the tissue, and followed the order: brain > kidney > spleen ≥ heart ≥ lung (Fig. 3D). The steady-state levels of ALG7 mRNAs in liver were between those in the kidney and the spleen (Fig. 3B and D). Exocrine tissues, submandibular and parotid glands, displayed the lowest levels of ALG7 transcripts, being more than 3-fold lower compared to liver (Fig. 3B).

DISCUSSION

Evidence presented here indicates that downregulation of ALG7 expression correlates with postnatal development and differentiation of the hamster SMG. The observed changes in ALG7 expression entail a progressive decrease in ALG7 mRNA levels and an attenuation of GPT activity, suggesting that developmental regulation of ALG7 is likely to involve transcriptional and/or posttranscriptional controls. Since ALG7 mRNA belongs to a category of rare transcripts, and the abundance of housekeeping (18) and rare (19) mRNAs has been shown to be regulated mainly by posttranscriptonal controls, it is possible that changes in ALG7 mRNA stability account for the differences in ALG7 steady-state levels. In yeast, growth-dependent changes in ALG7 mRNA abundance have been shown to be regulated by the utilization of multiple control mechanisms (10).

It is generally accepted that the phenotype of the tissue is determined by the composition of its mRNA population. Hence, selective downregulation of ALG7 expression during development is likely to represent an important aspect of tissue differentiation and

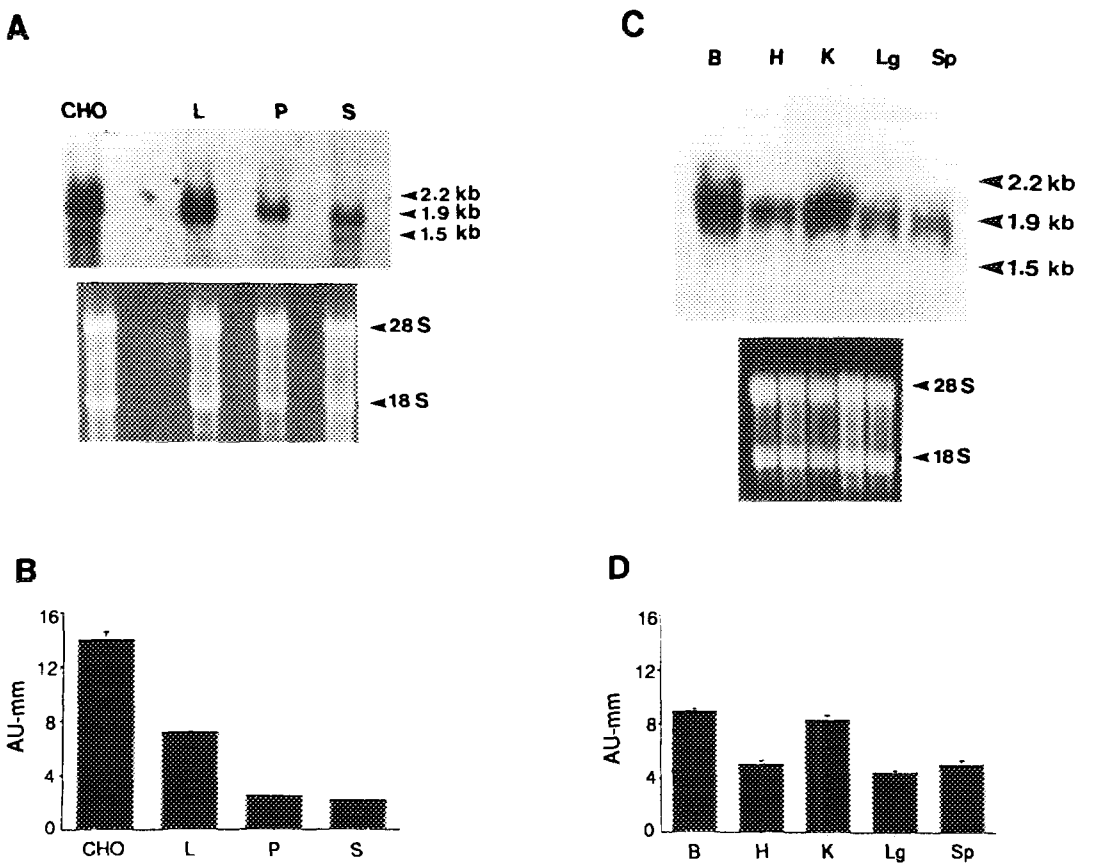

Fig. 3. Tissue distribution of ALG7 mRNAs. (A) and (C) *Upper panels.* Total RNAs (20 μg), isolated from CHO cells and adult hamster tissues, were analyzed by RNA blotting assays. The probe was as described in Fig. 1. L, liver; P, parotid gland; S, submandibular gland; B, brain; H, heart; K, kidney; Lg, lung; Sp, spleen. *Lower panels.* The integrity and loading consistencies were assessed by ethidium bromide staining of ribosomal RNAs (28S and 18S). The experiments represent one of three independent determinations. (B) and (D) Quantification of the ALG7 mRNAs shown in (A) and (C), respectively. Autoradiograms used for analyses were in the linear range of film sensitivity, and they were quantified with densitometry using Gel Scan software (Pharmacia). The bars indicate standard error of the mean.

acquisition of specialized function. The development of the SMG's branching pattern has been shown to be controlled by a complex network of interactions between the epithelial cells and the surrounding extracellular matrix (13,20). Since N-glycoproteins of the developing SMG basement membranes have been implicated in mediating epithelial-mesenchymal interactions (20), variations in the pools of N-glycoproteins' glycoforms may be involved in the SMG's branching morphogenesis. Modulation of ALG7 expression may affect the pools of N-glycoproteins' glycoforms that may be required for cellular control of recognition events

mediated by carbohydrate groups. This hypothesis is likely in view of the fact that the partial inhibition of ALG7 gene expression, using antisense RNA inhibitory strategy, has been shown to lower N-glycosylation and secretion of proteins (21).

Our studies also show that ALG7 is expressed in a tissue-specific manner. This may reflect different N-glycosylation "capacities" of specialized tissues. Similar tissue-specific expression has been reported for some terminal glycosyltransferases, such as α2,6 sialyltransferase (22). While differences in α2,6 sialyltransferase expression may reflect alterations in the types of terminal sequences on the carbohydrate groups, selective changes in ALG7 expression may affect the number of N-glycosylation sequons on N-glycoproteins. Since tissues have been shown to synthesize relatively few glycoforms in common (23), it is tempting to speculate that tissue-specific expression of some N-glycoproteins may be determined, at least in part, by the level of expression of the ALG7 gene.

It has been reported previously that activities of other early N-glycosylation enzymes are modulated with development, although not always in a coordinate manner (6,8). Our recent studies indicate that majority of the dolichol pathway genes in yeast are growth-regulated (Kukuruzinska, unpublished). Thus, it is possible that most of the dolichol pathway genes are downregulated with development and differentiation, although the precise mechanisms of their developmental regulation may differ. Further knowledge of developmental stage- and tissue-specific transcriptional and posttranscriptonal control steps involved in the regulation of N-glycosylation genes' expression will be necessary to reveal the common regulatory mechanisms utilized in control of N-glycosylation events.

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