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Synthesis of Lipid-Linked Oligosaccharides Is Dependent on the Cell Cycle in Rat 3Y1 Cells[1]

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Synchronized cultures of rat 3Y1 cells, prepared by the density-arrested method, were used to investigate the relationship of *N*-glycosylation to the cell cycle. Although total cellular proteins were synthesized independently of the cell cycle, the synthesis of membrane-bound proteins was found to be dependent on the cell cycle, the time of maximum synthesis being just before that of maximum DNA synthesis. The synthesis of lipid-linked oligosaccharides (LLO), which are utilized as intermediates in *N*-glycosylation, increased in the S phase to a level at least ten times higher than in the G_1, G_2, and M periods. Moreover, the activities of dehydrodolichyl diphosphate synthase and farnesyl diphosphate synthase, which synthesize the precursors of dolichol, also increased in the S phase concomitantly with LLO synthesis. However, since the cell cycle dependency curves of these two enzyme activities were somewhat broader than that of LLO synthesis, the rate-limiting enzyme in the regulation of LLO synthesis might be another one such as dehydrodolichol reductase. These results suggest that LLO synthesis is regulated by multiple synthetic enzymes which are activated in a cell cycle-dependent manner.

Key words: cell-cycle dependency, dehydrodolichyldiphosphate synthase, farnesyl diphosphate synthase, lipid-linked oligosaccharides, rat 3Y1 cell.

The well-regulated *N*-glycosylation of appropriate sites of various glycoproteins is indispensable for the controlled biological functions of various secreted and membrane-bound glycoproteins. For example, (1) human transferrin receptor, if lacking one of its three *N*-linked sugar chains, cannot form a dimer and is easily degraded by proteases in the endoplasmic reticulum (*1*). (2) Carbohydrate-deficient glycoprotein (CDG) syndrome type I, characterized by a partial deficiency of *N*-linked sugar chain transfer (*2-4*), shows clinical pleiotropic abnormalities (*5-7*). (3) γ-Glutamyltranspeptidase isoforms, which have different numbers of *N*-linked sugar chains (*8*), tend to become highly *N*-glycosylated isoforms in the course of tumorigenesis (*9, 10*). (4) Normal human microtubule-associated protein tau is non-glycosylated, while *N*-glycosylated and hyperphosphorylated tau appears in Alzheimer disease and is involved in the helicity of paired helical filaments (PHFs) (*11*).

One of the mechanisms for regulation of *N*-glycosylation depends on the existence of potential *N*-glycosylation sites, Asn-X-Thr(Ser), in the nascent polypeptides. Another interesting aspect of the regulation is the relationship of *N*-glycosylation activity to the cell cycle, since membrane constituents including *N*-linked glycoproteins are synthesized prior to cell division. In the case of yeasts, the relationship between *N*-glycosylation and the cell cycle has been well studied, and several related enzymes and the genes encoding them have been isolated and characterized (*12, 13*). In mammalian cells, it has been reported that *N*-glycosylation is indispensable for progression of the cell cycle, as indicated by studies involving tunicamycin, which inhibits the biosynthesis of lipid-linked oligosaccharides (LLO) (*14, 15*). Furthermore, we recently found that LLO synthesis is dependent on the cell cycle and that the level of LLO increases substantially in the S phase in human fibroblasts (*16*). However, it is very difficult to assay the rate-limiting enzymes involved in the cell cycle dependency of LLO synthesis, because quiescent cells increase in accordance with the population doubling level of human fibroblasts. In this study, we examined LLO formation in relation to *N*-linked glycoprotein synthesis throughout the cell cycle using synchronized cultures of rat 3Y1 cells, because 3Y1 cells can be reproducibly synchronized, and over 95% of the cells are constantly mitotic in the M period, even though the population doubling level increases.

Synchronized cultures of rat 3Y1 cells were prepared by the density-arrested method (*17*). The rat 3Y1 cells were kindly provided by Dr. K. Ohno (Tottori University). The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum until confluency. The confluent cells were refed with fresh medium and then cultured for an additional 3 days. Then, the density-arrested cells were stimulated by replating in fresh medium at a density of 1×10^6 cells per 90 mm plastic dish or 1.5×10^5 cells per 35 mm dish. Synchronization was checked by measuring [^{3}H]thymidine incorporation into DNA, as shown by the dotted lines in Figs. 1, 2, and 4. The cells in 35 mm dishes were labeled with [^{3}H]thymidine (26.0 Ci/mmol, Du Pont-NEN) (1.5 μCi/ml) for 2 h inter-

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Abbreviations: CDG, carbohydrate-deficient glycoprotein; LLO, lipid-linked oligosaccharides; PHFs, paired helical filaments.

vals. The cells were lysed in 5% sodium dodecyl sulfate-5% trichloroacetic acid, the [^{3}H]thymidine-labeled insoluble DNA was trapped on a glass filter (Whatman GF/C), and then the radioactivity was measured with a liquid scintillation counter.

At first, we investigated whether or not the synthesis of membrane-bound and total cellular proteins is dependent on the cell cycle by metabolic labeling with [^{35}S]methionine. [^{35}S]Methionine-labeled total cellular proteins were assayed by measuring the radioactivity of the TCA-insoluble fraction. The incorporation of [^{35}S]methionine into total cellular proteins was independent of the cell cycle (Fig. 1A, solid line). The synthesis of membrane-bound (glyco-)proteins was measured as follows. The synchronized cell cultures were incubated with [^{35}S]methionine (40 Ci/mmol, Amersham) (60 μCi/ml) in 35 mm dishes for 2 h intervals. After the medium had been removed, 1 ml of 5 mM Tris-HCl, pH 8.0, containing 1 mM EDTA was added and the cells were collected. After centrifugation at $10^5 \times g$, the membranes precipitated were lysed in 10% sodium dodecyl sulfate and then ice-cold 10% trichloroacetic acid was added. The insoluble materials were trapped on a glass filter and the radioactivity was measured. It was found that the synthesis of membrane-bound proteins is dependent on the cell cycle (Fig. 1B, solid line). These results might reflect a biosynthetic regulation mechanism which ensures that membrane-bound glycoproteins are synthesized before the M period.

When *N*-linked glycoproteins are synthesized, oligosaccharides constituting the glycan moiety of LLO, $Glc_3 \cdot Man_9 \cdot GlcNAc_2$, are transferred *en bloc* to the protein moiety by oligosaccharyltransferase. In order to determine whether or not the synthesis of LLO is dependent on the cell cycle, as seen in the case of membrane-bound glycoproteins, we measured the amount of ^{3}H-labeled LLO by pulse-labeling with [^{3}H]glucosamine at different periods of the cell cycle. [^{3}H]Glucosamine·HCl (54 Ci/mmol, Du Pont-NEN) (125 μCi/ml) was added to the cultures in 90 mm dishes at the indicated times and the cultures were incubated for a further 2 h. Since it takes only several minutes for completion of the conversion of dolichol to $Glc_3 \cdot Man_9 \cdot GlcNAc_2 \cdot PP \cdot$dolichol (*21*), labeling for 2 h should be sufficient to obtain the steady state level of LLO. Thereafter, the medium containing [^{3}H]glucosamine was immediately replaced with methanol : 5 mM Tris-HCl, pH 8.0, containing 1 mM EDTA(=1 : 1) on ice, and then the cells were collected. Other lipids were removed by extraction with CM (chloroform : methanol=2 : 1). Then, LLO was extracted with CMW (chloroform:methanol : water= 10 : 10 : 3) (*18*). After mild acid treatment (0.5 ml of *n*-propanol-1 ml of 0.01 N HCl at 100°C for 25 min), the oligosaccharides released from LLO were passed through an AG-3 (OH^- form) column, and then applied to a Bio-Gel P-4 (extra fine) column (2×120 cm). The radioactive oligosaccharides eluted from the Bio-Gel P-4 column were taken in calculation as being derived from LLO. As shown by the solid line in Fig. 2, the radioactivity incorporated into LLO increased sharply at the period just before the peak of DNA synthesis. The sizes of the oligosaccharides eluted from the Bio-Gel P-4 column corresponded to those of $Glc_{1-3} \cdot Man_9 \cdot GlcNAc_2$ (Fig. 3), which are transferred *en bloc* to the nascent polypeptide chain to initiate *N*-glycosylation. The same results as shown in Figs. 2 and 3 were obtained with the [^{3}H]mannose metabolic labeling method (data not shown). As described in the previous paper (*16*), when metabolic labeling with [^{3}H]mannose was performed using synchronized cultures of human fibroblasts, the radioactivity incorporated into GDP-[^{3}H]mannose was

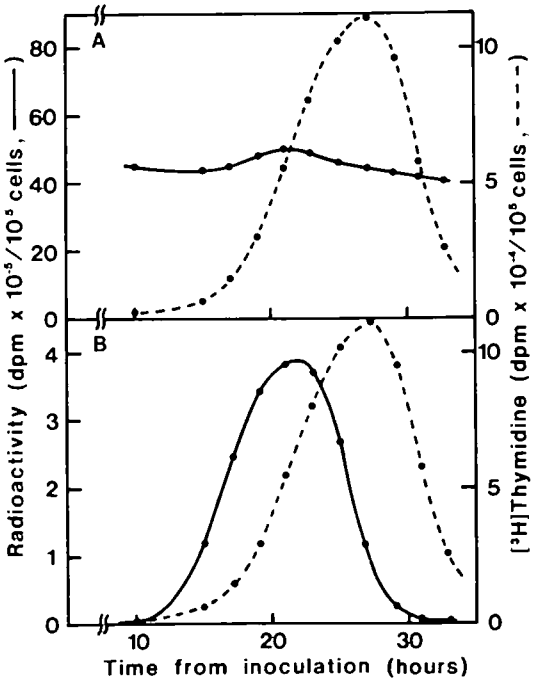

Fig. 1. **The cell cycle-dependent incorporation of [^{35}S]methionine into total cellular proteins (A) and membrane-bound proteins (B) in rat 3Y1 cells.** Density-arrested cells were replated and metabolically labeled with [^{35}S]methionine (60 μCi/ml) for 2 h intervals. Synchronization was checked by assaying [^{3}H]thymidine incorporation into DNA (dotted line).

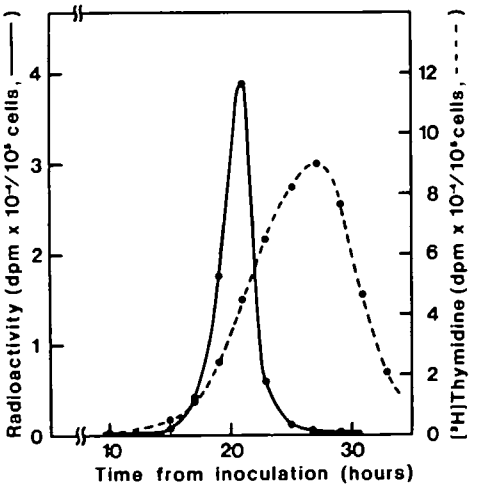

Fig. 2. **The cell-cycle dependency of LLO synthesis.** Density-arrested cells were replated and metabolically labeled with [^{3}H]-glucosamine-HCl (125 μCi/ml) for 2 h intervals. After CMW (chloroform : methanol : water=10 : 10 : 3) extraction and mild acid treatment, the radioactive oligosaccharides eluted from Bio-Gel P-4 columns were taken in calculations as being derived from LLO. Synchronization was checked by assaying [^{3}H]thymidine incorporation into DNA (dotted line).

constant throughout from the G_1 to the S phase. Accordingly, the incorporation of [^{3}H]glucosamine or [^{3}H]mannose into LLO should reflect the synthetic activity of LLO. These results suggeted that the synthesis of LLO increased in the S phase to a level at least ten times higher than that in the G_1, G_2, and M periods (see Fig. 2).

The earliest steps of LLO synthesis are the rate limiting ones, as described in the previous paper (*16*). Because the dolichol biosynthetic pathway is proposed to be as follows; mevalonate→isopentenyl diphosphate→geranyl diphosphate→farnesyl diphosphate→dehydrodolichyl diphosphate→dehydrodolichyl monophosphate→dehydrodolichol →dolichol (*22, 23*), we measured the activities of two enzymes which synthesize precursors of dolichol, dehydrodolichyl diphosphate synthase and farnesyl diphosphate synthase, at the respective periods of the cell cycle by the methods previously described (*19, 20*). As shown in Fig. 4, A and B, the activities of both enzymes increased concomitantly with the increase in the level of LLO in synchronized cultures of 3Y1 cells. Because the time required for mature LLO formation from dolichol is only several minutes (*21*), the cell cycle dependency of these enzyme activities might be necessary for sharp change of LLO synthesis, and these two enzymes might regulate the synthesis of dolichol, which is the earliest precursor in LLO synthesis. Because the cell cycle dependency curves of these two enzyme activities were somewhat broader than that of LLO synthesis, the rate-limiting enzyme in the regulation of LLO synthesis might be another one such as dehydrodolichol reductase. It has been reported by Adair and Cafmeyer (*24*) that the levels of dolichol kinase and *cis*-prenyltransferase activity increase in the S phase. In contrast, as reported by Maltese and Sheridan (*25*), the level of the activity of 3-hydroxy-3-methylglutaryl coenzyme A synthase, one of the enzymes involved in mevalonate synthesis, is similar in all phases of the cell cycle. Therefore, the various enzymes related to LLO synthesis can be categorized into two types, one group is dependent on the cell cycle and the other group is independent of the cell cycle. The cell cycle dependent enzymes may be related directly to the synthesis of membrane-bound glycoproteins, the levels of which increase sharply in the S phase. Because of the insufficient data accumulated for a complete view of *N*-glycosylation throughout the cell cycle, we are further investigating the enzymes dependent on the cell cycle in relation to the regulatory mechanisms involved in glycoprotein synthesis in 3Y1 cells.

Since there have been reports that *N*-glycosylation is indispensable for progression of the cell cycle (*14, 15*) and we previously found that the transfer of *N*-linked sugar chains to γ-glutamyltranspeptidase is increased in malignant tumor cells (*8-10*), we are investigating the regulation mechanism underlying the cell cycle dependency of *N*-glycosylation activity in cancer cells, which are able to

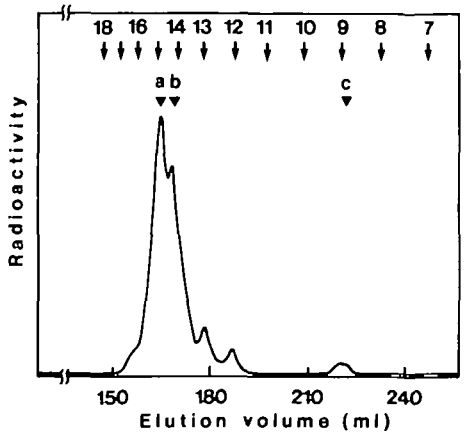

Fig. 3. **The elution patterns obtained on Bio-Gel P-4 (extra fine) column chromatography of [^{3}H]glucosamine-labeled oligosaccharides released from LLO in Fig. 2.** [^{3}H]Glucosamine-labeled oligosaccharides were released from LLO (2 h pulse labeling, 20-24 h after replating) by mild acid hydrolysis (0.01 N HCl : *n*-propanol = 2 : 1, at 100°C for 25 min). The arrows at the top indicate the elution positions of glucose oligomers (the numbers indicate those of glucose units). The black triangles indicate the elution positions of authentic $Glc_3 \cdot Man_9 \cdot GlcNAc \cdot GlcNAc$ (a), $Glc_1 \cdot Man_9 \cdot GlcNAc \cdot GlcNAc$ (b), and $Man_5 \cdot GlcNAc \cdot GlcNAc$ (c).

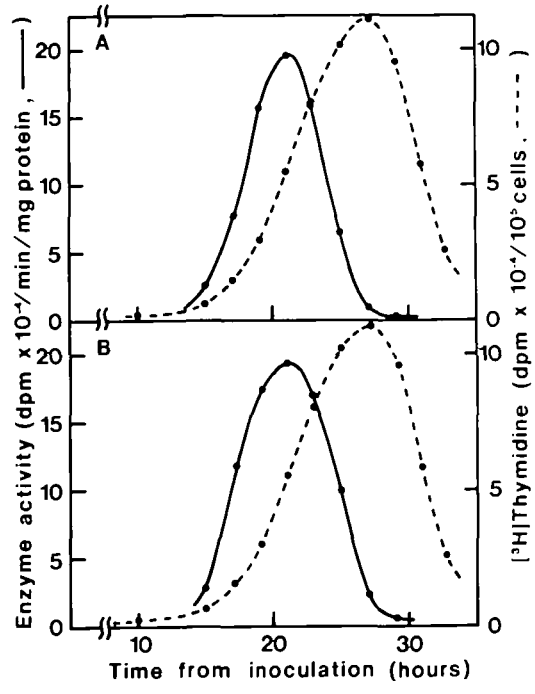

Fig. 4. **The relationship of dehydrodolichyl diphosphate synthase activity (A) and farnesyl diphosphate synthase activity (B) to the cell cycle.** Dehydrodolichyl diphosphate synthase activity was measured as follows (*19*). Reaction mixtures (100 μl) comprising 50 mM Tris-HCl (pH 8.5), 1 mM dithiothreitol, 50 mM potassium fluoride, 1 mM $MgCl_2$, 20% glycerol, 2.0% Triton X-100, 20 μM farnesyl diphosphate, 0.25 μM [1-^{3}H]isopentenyl diphosphate (20 Ci/mmol), and cell homogenates (3 μg of protein) were incubated at 37°C. Then, 100 μl of a 2 M NaCl solution was added, and the radioactive products were extracted with 200 μl of *n*-butanol, digested with potato acid phosphatase, and then separated on reverse-phase thin-layer chromatographic plates of LKC 18 (Merck) using acetone : methanol (= 19 : 1, v/v). The levels of tritium-labeled farnesol were determined using a Raytest radiochromatogram scanner, Model RITA-90. Farnesyl diphosphate synthase activity was assayed by essentially the same method as that in a previous report (*20*). Reaction mixtures (100 μl) comprising 50 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 50 μM geranyl diphosphate, 0.1 μM [1-^{3}H]isopentenyl diphosphate (20 Ci/mmol), and cell homogenates (3 μg of protein) were incubated at 37°C. The radioactive products were purified by the same methods as employed for the products of dehydrodolichyl diphosphate synthase.

proliferate in an uncontrollable manner.

Furthermore, in normal cells, the potential *N*-glycosylation sites in the respective proteins are not necessarily glycosylated, but the specific sites in them are constantly *N*-glycosylated. When the balance between *N*-glycosylation and polypeptide synthesis has been disrupted in some diseases, abnormal glycoproteins are synthesized. When microtubule-associated protein tau is *N*-glycosylated and hyperphosphorylated in Alzheimer disease, PHFs are formed (*11*). Congenital CDG syndrome type I is a partial deficiency of the conversion from dehydrodolichol to dolichol, and the resulting loss of dolichol leads directly to underglycosylation of various *N*-glycosylated glycoproteins (*2, 16*), and induces clinical pleiotropic abnormalities (*5-7*). Another characteristic of this disorder is that various abnormalities can often be observed in the liver and nervous system, although the cells in these are usually arrested at the G_0 period. Because these cells continuously synthesize a lot of glycoproteins, it is easy to detect abnormal glycoproteins. In contrast, skin fibroblasts are usually non-proliferating quiescent cells *in vivo* and synthesize lower levels of glycoproteins than ones in the liver and nervous system except in the S phase. So, the defect in this disorder could be detected in the S phase of synchronized fibroblasts, in which a lot of membrane-bound glycoproteins are synthesized (*16*).

Since 3Y1 cells are classified as fibroblasts, the like human skin fibroblasts, and the levels of LLO at the G_0, G_1, G_2, and M periods in 3Y1 cells are extremely lower than those in liver and nervous system cells, 3Y1 cells are a good model for investigating the regulation mechanism underlying the cell cycle dependency of *N*-glycosylation, although it is unclear at this stage whether or not the regulation mechanisms for *N*-glycosylation in the G_0 period in liver cells and the S phase in 3Y1 cells are different.

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