Phosphorylated dolichols in aging

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The age-associated changes in the levels and synthesis of dolichyl phosphate and dolichyl diphosphate derivatives were investigated in brain and liver of 057B1/NNia mice. The total chloroform/methanol (2:1, v/v)-extractable phosphorylated dolichols of brain increased from 1.01 $\mu\text{g/g}$ at 3 months to 5.22 $\mu\text{g/g}$ at 28 months of age. The long-chain dolichyl diphosphate oligosaccharide (Dol-PP-oligo) levels of brain increased from 0.82 $\mu\text{g/g}$ in 3 months to 2.8 $\mu\text{g/g}$ in 28-month-old animals. However, in liver and in kidney, the levels of these components were unaffected by age. Incorporation of labelled glucose from UDP-glucose into dolichyl phosphate glucose and Dol-PP-oligo in brain microsomes was unaffected by age, whereas, in liver microsomes, the rates of synthesis of both components increased by 50–150%. The increased rate of synthesis and lack of accumulation of Dol-PP-oligo in liver suggest an active utilization and/or catabolism of these glycoprotein precursors. The accumulation of Dol-PP-oligo in aging brain may reflect its decreased utilization for *N*-glycosylation and/or reduced catabolism.

INTRODUCTION

Dolichyl phosphate (Dol-P) is an obligatory intermediate in the *N*-glycosylation process (Struck & Lenarz, 1980; Hubbard & Ivatt, 1981). Besides free Dol-PP, tissues contain dolichyl phosphate glucose (Dol-P-Glc), dolichyl phosphate mannose (Dol-P-Man) and various oligosaccharide derivatives of Dol-PP that are involved in the synthesis of the completed oligosaccharide core, which contains three glucose, nine mannose and two *N*-acetylglucosamine residues. A number of studies (Lucas, 1979; Harford & Waechter, 1980; Rossignol *et al.*, 1981) have suggested that the level of free Dol-P regulates the level of *N*-glycosylation.

Previous studies from our laboratory (Pullarkat & Reha, 1982; Pullarkat *et al.*, 1984) and others (Ng Ying Kin *et al.*, 1983; Sakakihara & Volpe, 1985) have shown that free dolichol is increased in aging brain and in other tissues. A fairly recent report (Andersson *et al.*, 1987) showed moderate age-associated increases in the levels of total phosphorylated dolichols in human brain grey matter. In the present study we have examined this phenomenon in mouse liver, brain and kidney, and have found that there is an age-associated increase in phosphorylated dolichol obtained in chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3) extracts only in brain, but not in liver and kidney. Furthermore, the integrated synthesis of dolichyl phosphate glucose and Dol-PP-oligo in microsomal preparations of liver and brain are very active during aging.

EXPERIMENTAL

Materials

Mice (C57/BINNia) were obtained from Charles River laboratories. UDP-[^{14}C]glucose was obtained from

American Radiochemicals, St. Louis, MO, U.S.A. All solvents were either h.p.l.c. or residue-analysed grades and obtained from J. T. Baker, Phillipsburg, NJ, U.S.A. All cofactors were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Reversed-phase cartridge columns (SPICE) and h.p.l.c. columns were obtained from Rainin Instrument Co., Woburn, MA, U.S.A. Dolichol C-125 (125-carbon-long) was purchased from Polish Academy of Sciences, Warsaw, Poland, and converted into Dol-P by the method of Danilov & Chojnacki (1981).

Methods

Isolation of Dol-P. About 200 mg of brain or liver from mice was extracted twice with two 2.5 ml portions of chloroform/methanol (2:1). The combined extract was decreased in volume under a stream of N_2 . The residue after extraction with chloroform/methanol was extracted twice with 2.5 ml portions of chloroform/methanol/water (10:10:3) and the combined extract was decreased in volume under a stream of N_2 . Both extracts were treated with enough HCl to give a final concentration of 2 M, at 75 °C for 90 min, after adding 100 ng of internal-standard C-125 Dol-P. Dol-P was extracted with chloroform and isolated on a reversed-phase cartridge column (Pullarkat *et al.*, 1988). Briefly, the samples were applied in 200 μl of chloroform, washed with 15 ml of methanol, and 15 ml of acetone (dolichol), and Dol-P was eluted with 15 ml of acetone containing 5 mM-phosphoric acid. The solvent was evaporated, and a sample was analysed by h.p.l.c.

The h.p.l.c. system consisted of a Spectra Physics 8700 solvent-delivery system, a Rheodyne model 7125 sample injector and a Beckman model 160 fixed-wavelength u.v. detector (214 nm, zinc lamp), a Rainin reversed-phase C₁₈ column (Microsorb; 3 μm particle size; 0.46 cm $\times$ 10 cm) and a Spectra Physics 4100 computing integrator. All runs were made isocratically with a mobile

Abbreviations used: Dol-P, dolichyl phosphate; Dol-PP-oligo, dolichyl diphosphate oligosaccharide; Dol-P-Glc (Man), dolichyl phosphate glucose (mannose); C-125 etc., 125-carbon-long etc.

phase consisting of methanol/propan-2-ol (containing 10 mM-phosphoric acid)/hexane (40:37:23, v/v). All solvents were degassed continuously with helium. The detector sensitivity was kept at 0.01 a.u.f.s. (absorbance unit full scale).

Preparation of microsomal fractions. Brain was homogenized in 9 vol. of 0.32 M-sucrose containing 2 mM-EDTA and 2 mM-mercaptoethanol, and buffered with phosphate buffer at pH.7.0. Liver was homogenized in 3 vol. of 0.25 M-sucrose containing 2 mM-EDTA and 2 mM-mercaptoethanol, and buffered to pH.7.0 with 20 mM-phosphate buffer. The homogenates were centrifuged at 250 *g* for 10 min and the supernatant so obtained was centrifuged at 6800 *g* for 20 min. The mitochondria-free supernatant was centrifuged at 100000 *g* for 60 min to pellet the microsomes. Microsomal preparations were resuspended in 0.1 M-Tris/acetate buffer, pH.7.0, at a concentration of about 1 mg of protein/ml. Protein was measured by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard.

Biosynthesis of Dol-*P*-Glc and Dol-*PP*-oligo. Incorporation of glucose from UDP-[¹⁴C]glucose into Dol-*P*-Glc and Dol-*PP*-oligo were performed essentially as described by Spiro & Spiro (1986), except that a microsomal fraction was used as the enzyme source. Microsomal preparations (0.25–1 mg of protein) were incubated with 0.3 μ Ci of UDP-[¹⁴C]glucose (sp. radioactivity 300 mCi/mmol) at 27 °C for 10 min in 160 μ l of 50 mM-Tris/acetate buffer, pH.7.0, containing 25 mM-potassium acetate, 10 mM-magnesium acetate, 0.1 mM-sucrose, 0.1 mM-ATP, 0.5 mM-phosphocreatine and 25 μ g of creatinine phosphokinase. At the end of the incubation period, 3 ml of chloroform/methanol (2:1) was added and shaken well in a bath-type sonicator. The extract was partitioned after adding 500 μ l of water and 1 μ g of unlabelled UDP-glucose. After centrifugation the upper phase was discarded. The chloroform layer was washed twice with theoretical upper phase (chloroform/methanol/water, 3:47:48, by vol.). A portion of the chloroform layer was taken for radioactivity determination. The residue left after chloroform/methanol treatment was extracted twice with 3 ml of chloroform/methanol/water (10:10:3) and a portion was taken for radioactivity determination. The residue after extraction with chloroform/methanol/water was taken to determine the incorporation of glucose into proteins. Time-course study showed that the reaction was linear for at least 10 min.

T.l.c. The labelled compounds in the chloroform/methanol extract were separated on silica-gel G plates (chloroform/methanol/water, 13:7:1, by vol.) and radioactivities were determined in 0.5 cm bands. The acid-hydrolysed (0.02 M-HCl at 100 °C for 20 min) products from chloroform/methanol/water extract were separated on aluminum-backed silica-gel 60 plates (propanol/water/acetic acid, 3:2:2) and radioactivities were determined in 0.5 cm bands.

Measurement of radioactivity. Radioactivity of samples was determined in 10 ml of Hydrofluor (National Diagnostics, Sommerville, NJ, U.S.A.) in a Packard liquid-scintillation spectrometer. Portions of the chloro-

form and chloroform/methanol/water extracts were first dried under a stream of N₂, then suspended in 250 μ l of water, to which the Hydrofluor was added.

RESULTS

Fig. 1 shows the h.p.l.c. analysis of mouse brain Dol-*P* obtained from the chloroform/methanol extract. Under the conditions employed, all the major homologues of Dol-*P* were separated, and the internal standard was well separated from the sample. The method allows the analysis of nanogram levels of Dol-*P*, and hence it is

Fig. 1. Reversed-phase h.p.l.c. of Dol-*P* isolated after hydrolysis of the chloroform/methanol/water extract of mouse liver

Details of the procedure and h.p.l.c. conditions are given in the Experimental section. The internal-standard C-125 Dol-*P* represents 20 ng.

Table 1. Age-associated changes in the levels of phosphorylated dolichols in mouse tissues

Values are expressed as means $\pm$ S.D. for four animals. The method by which Dol-*P* was obtained from chloroform/methanol (C/M) and chloroform/methanol/water (C/M/W) extracts is given in the Experimental section.

Tissue	Age (months)	Dol- <i>P</i> (μ g/g wet wt.)	
		C/M	C/M/W
Brain	3	1.0 $\pm$ 0.1	0.8 $\pm$ 0.1
	28	5.2 $\pm$ 0.7	2.8 $\pm$ 0.5
Liver	3	1.9 $\pm$ 0.5	1.5 $\pm$ 0.5
	28	2.3 $\pm$ 0.5	1.1 $\pm$ 0.2
Kidney	3	3.6 $\pm$ 0.1	4.4 $\pm$ 0.4
	28	4.4 $\pm$ 0.9	5.0 $\pm$ 1.4

Fig. 2. Age-associated synthesis of Glc-P-Dol and Dol-PP-oligo in mouse brain microsomes

The values are expressed as pmol of glucose incorporated.
Ages: ●, 3 months; ■, 12 months; ▲, 28 months.

Fig. 3. Age-associated synthesis of Glc-P-Dol, Dol-PP-oligo and glycoproteins in mouse liver microsomes

The values are given as pmol of glucose incorporated.
Ages: ●, 3 months; ■, 12 months; ▲, 28 months.

useful in cases where amounts of samples are limited. The distributions of Dol-P homologues in chloroform/methanol and chloroform/methanol/water extracts were the same as that of the free dolichol for the given tissue.

From 3 to 28 months of age there was a 5-fold increase in the Dol-P obtained after acid hydrolysis of the chloroform/methanol extract, whereas the chloroform/methanol/water extract showed a 3-fold increase within the same age range (Table 1). By contrast, liver and kidney Dol-P levels were virtually unaffected by age.

The rates of synthesis of Dol-P-Glc and long-chain Dol-PP-oligo, as measured by the incorporation of labelled glucose from UDP-glucose in brain microsomal preparations, showed no change between 3 and 28 months of age (Fig. 2). The rates of synthesis of these compounds were unchanged (results not shown) when 1-

Fig. 4. T.l.c. analyses of the labelled compounds produced from UDP-[¹⁴C]glucose on incubation with mouse liver microsomes

(a) Chloroform/methanol extract on silica-gel G thin-layer plates developed in chloroform/methanol/water (13:7:1, by vol.). The mobility of glucosylcerebrosides are indicated by the arrows. (b) Oligosaccharides released on mild acid hydrolysis of the chloroform/methanol/water extract on silica-gel G plates developed in propanol/water/acetic acid (3:2:2, by vol.). The mobility of dodecasaccharide from GM₁ gangliosidosis urine is indicated by the arrow.

deoxynojirimycin (2 mM) was added to the incubation mixture, suggesting that there was no appreciable hydrolysis of the biosynthesized glucose-containing Dol-PP-oligo during the period of incubation. The level of synthesis of Dol-PP-oligo in the liver microsomes was increased from 0.5 pmol of glucose incorporated/min per mg of protein in 3-month-old mice to 1.55 pmol in 28-month-old mice (Fig. 3). In liver, the incorporation of labelled glucose into protein was decreased by about 40% at 28 months of age. In brain microsomes we were unable to obtain consistent values for the incorporation of glucose into proteins.

The labelled products extracted with chloroform/methanol and chloroform/methanol/water were examined by t.l.c. The chloroform/methanol-extractable products showed predominantly one major radioactive band with an *R_F* value of 0.25 (Fig. 4a) and a minor component of *R_F* value 0.45. No radioactivity was detected in glucocerebrosides. The oligosaccharide residues after mild acid hydrolysis of the chloroform/methanol/water extract on t.l.c. showed components with *R_F* values lower than dodecasaccharide from GM₁-gangliosidosis urine (Wolfe *et al.*, 1974) (Fig. 4b), suggesting that the glucose-containing compounds in this extract may be Dol-PP-oligo with 13 and 14 saccharide residues.

DISCUSSION

The total phosphorylated dolichol levels in liver, brain and kidney of 3-month-old animals represent 9.4, 8 and 7% respectively of that of free dolichol. The increase in the levels of phosphorylated dolichols with age is not as great as that observed for free dolichol. In mouse brain there was a 7-fold increase in the level of free dolichol from 3 months to 30 months of age (Pullarkat *et al.*, 1984), whereas the increase in the level of brain phosphorylated dolichols is in the range of 3–5-fold between the ages of 3 and 28 months. The chloroform/methanol extract is expected to contain free Dol-*P*, Dol-*PP*, Dol-*P*-Glc, Dol-*P*-Man and oligosaccharide derivatives of Dol-*PP* containing up to four sugar residues (Spiro *et al.*, 1976). The chloroform/methanol/water extract is expected to contain Dol-*PP*-oligo with five or more saccharide residues. In the present study, since only the Dol-*P* was estimated, it is not clear whether, in aging, any of the particular components are increased or whether there is an overall increase of all phosphorylated dolichol compounds. A previous study by one of us (Pullarkat, 1985) has shown that mouse brain Dol-*P*-Man, measured by the release of free dolichol on treatment with hot dilute alkali, is also increased with age.

Dol-*P* and various saccharide and oligosaccharides derivatives of Dol-*PP* are obligatory intermediates in the synthesis of asparagine-linked glycoproteins. Dol-*PP*-oligo that contains two *N*-acetylglucosamine, nine mannose and three glucose residues is a precursor of asparagine-linked glycoprotein (Kornfeld & Kornfeld, 1985). Once the oligosaccharide residue is transferred to the protein, the Dol-*PP* released could conceivably be converted into Dol-*P* and reutilized for Dol-*PP*-oligo synthesis. If this occurs efficiently it appears that, once the cycle is primed, there is no additional need for Dol-*P*. The age-associated accumulation of phosphorylated dolichols in brain suggest that there is a continued synthesis of these compounds in aging brain.

In the present study, synthesis of Glc-*P*-Dol and Dol-*PP*-oligo were measured at different protein concentrations in order to gain insight into the possible contribution from endogenous UDP-glucose. In almost all cases (Figs. 2 and 3), linear relationships were obtained, suggesting that the levels of endogenous UDP-glucose are insignificant compared with the added labelled substrate. Studies on the incorporation of labelled glucose from UDP-glucose in brain microsomes show active synthesis of Dol-*PP*-oligo that is unaffected by age, whereas in liver the synthesis is increased about 3-fold between the ages of 3 and 28 months. However, in liver, in spite of the increased synthesis, no accumulation of Dol-*PP*-oligo occurs (Table 1), suggesting active utilization or catabolism of these compounds. The incorporation of labelled glucose into proteins in liver microsomes was decreased by about 40% from 3 to 28 months of age, suggesting decreased utilization of Dol-*PP*-oligo for glycoprotein synthesis in aging. It is not clear why there is an increased synthesis of Dol-*PP*-oligo under conditions when its utilization is decreased. Lack of accumulation of Dol-*PP*-oligo in liver thus suggests an active catabolism of these compounds.

Even though the various steps in the biosynthesis of

Dol-*PP*-oligo are well worked out, the catabolism of these compounds is less well understood. Cacan *et al.* (1987) have shown that the deglycosylated Dol-*PP*-oligo species are cleaved by a phosphodiesterase to produce Dol-*P* and oligosaccharide phosphate. If the dol-*PP*-oligo is degraded as described by Cacan *et al.* (1987), then this should contribute to increased level of Dol-*P*. However, it is possible that Dol-*P* is converted into free dolichol by the Dol-*P* phosphatase, which is known to be increased in aging (Scher *et al.*, 1985). Indirect evidence from studies on inhibitors of processing enzymes show that the glucosidases I and II not only cleave the glucose residues of the protein-bound oligosaccharides, but also the dol-*PP*-bound oligosaccharides (Romero & Herscovics, 1986; Plantner & Kean, 1986). How these enzyme activities are affected in aging brain will be of interest in understanding the mechanism of accumulation of Dol-*PP*-oligo.

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