A glycogen precursor associated with membrane in retina

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The incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into proteoglycogen fractions of a retinal microsomal preparation was studied. From the rate of labelling of acid-insoluble and -soluble proteoglycogen at different sugar-donor concentrations, and from the conversion of the labelled acid-insoluble into an acid-soluble form measured by a 'chase' with unlabelled UDP-glucose, it was concluded that acid-insoluble 42 kDa protein (p42)-bound glycogen of weight-average M_r 4.7×10^5 and acid-soluble p42-bound glycogen of weight-average M_r 7.0×10^5 [Miozzo, Lacoste & Curtino (1989) *Biochem. J.* **260**, 287–289] are related as precursor and product respectively. About one-third of the acid-insoluble proteoglycogen was excluded from a Sephacryl S-500 column and was associated with large membrane vesicles. Proteoglycogen was not dissociated from the membranes by treatment with saline solutions or with SDS at a low detergent-to-protein ratio. It was dissociated by treatment with detergents under conditions which were shown to solubilize integral membrane sialoglycoconjugates of retina. These results lead us to postulate that the biogenesis of retina glycogen starts on membrane-associated p42 to form acid-insoluble proteoglycogen, which is then dissociated from membranes and converted into acid-soluble proteoglycogen by the 'growth' of its polysaccharide moiety.

INTRODUCTION

The original report concerning protein-bound α 1,4-glucans was made by Krisman (1972), who proposed that the binding of the glucan moiety to a protein was responsible for the insolubility in trichloroacetic acid of α 1,4-glucans glucosylated *in vitro*. The same assumption about the insolubility of α -glucans in the acid was made later by others (Barengo *et al.*, 1975; Tandeczak *et al.*, 1975; Takahara & Matsuda, 1977; Curtino *et al.*, 1979; Blumenfeld *et al.*, 1983). The first evidence for protein covalently bound to α 1,4-glucan was shown by workers studying a trichloroacetic acid-soluble glycogen fraction (Aon & Curtino, 1984). Since then, glycogen fractions were isolated from several tissues with or without the use of trichloroacetic acid and studies were performed to characterize both the type of protein-polysaccharide linkage (Aon & Curtino, 1985; Smythe *et al.*, 1988) and the protein-bound moiety (Rodríguez & Whelan, 1985; Pitcher *et al.*, 1987; Rodríguez & Fliesler, 1988; Miozzo *et al.*, 1989).

Recently the trichloroacetic acid-insoluble glucan fraction in retina was characterized as a proteoglycogen whose 42 kDa protein (p42) moiety was similar in size to the protein moiety of the acid-soluble proteoglycogen fraction (Miozzo *et al.*, 1989).

In the present work we show that the trichloroacetic acid-insoluble proteoglycogen of retina is a biosynthetic precursor of glycogen. About one-third of the total acid-insoluble proteoglycogen precursor is found firmly associated with membrane vesicles. In the light of these results we postulate that p42 is bound to membranes when its glucosylation starts. We propose that the membrane-bound proteoglycogen dissociates from these membranes during the growth of its polysaccharide moiety. It is also proposed that, at least partially, the building of the glucan moiety is a channelling process (Srere, 1987), conferring on glycogen synthase the behaviour characteristic of an 'ambiquitous' protein (Wilson, 1980).

MATERIALS AND METHODS

Rabbit liver glycogen (type III), UDP-glucose, phenylmethanesulphonyl fluoride (PMSF), tosyl-L-lysylchloromethane (Tos-Lys-CH₂Cl; 'TLCK'), SDS, Emulphogene (polyoxyethylene 10-tridecyl ether), Sephacryl S-500 and glucoamylase from the mould *Rhizopus* (9.6 units/mg dry wt.) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). UDP-[^{14}C]glucose (285 Ci/mol) was from Instituto de Investigaciones Bioquímicas 'Fundación Campomar' (Buenos Aires, Argentina) and CMP-[^3H]N-acetylneuraminic acid (18.9 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.).

Incubation of the microsomal fraction with UDP-[^{14}C]glucose and measurement of labelled products

The microsomal fraction of bovine retina was prepared as described previously (Miozzo *et al.*, 1989). The membrane preparation (1.2 mg of protein) was incubated at 7 °C in a final volume of 80 μl with the amount of UDP-[^{14}C]glucose and for the time indicated in each case. Aliquots (10 μl) of the incubation mixture were precipitated with cold 10% (w/v) trichloroacetic acid (0.2 ml), cooled in ice for 10 min and centrifuged at 1200 *g* for 15 min at 4 °C. For the measurement of [^{14}C]glucose incorporated into the acid-soluble proteoglycogen fraction, the acid supernatant was mixed with carrier glycogen (0.4 mg) followed by 1.5 vol. of ethanol, cooled at –10 °C for 20 min and centrifuged at 1200 *g* for 15 min. The precipitate was dissolved in 0.2 ml of 1% KCl, the precipitation repeated by addition of 1.5 vol. of ethanol, and the final precipitate was solubilized in 0.2 ml of water and counted for radioactivity in a liquid-scintillation spectrometer after mixing with 2.5 ml of 2% (w/v) 2,5-diphenyloxazole and 10% (w/v) naphthalene in dioxan. For the measurement of the labelled acid-insoluble proteoglycogen, the material precipitated by trichloroacetic acid was washed twice by sonication in 0.5 ml of cold 5% (w/v)

Abbreviations used: Tos-Lys-CH₂Cl, tosyl-L-lysylchloromethane ('TLCK'); PMSF, phenylmethanesulphonyl fluoride.

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trichloroacetic acid containing 0.5% phosphotungstic acid and centrifuged. The washed residue was dispersed by sonication in 0.2 ml of 1 M-Tris containing 20% (w/v) SDS, heated at 100 °C for 3 min, mixed with the scintillation solution and counted for radioactivity as described above.

Solubilization of microsomal gangliosides and sialoglycoproteins by treatment with detergents

Endogenous gangliosides and sialoglycoproteins were labelled by incubation of the microsomal fraction of retina (4 mg of protein) at 37 °C for 30 min in a total volume of 300 μ l with 10 mM-MnCl₂ and 0.3 μ M-CMP-[³H]N-acetylneuraminic acid (Curtino *et al.*, 1977). Aliquots of 50 μ l were then incubated for 15 min in a total volume of 100 μ l with SDS or Emulphogene at 7 or 2 °C respectively, at the detergent-to-protein ratio indicated in each case, and centrifuged at 100 000 *g* for 90 min after mixing with 0.9 ml of water. The pellets were dispersed by sonication in 0.65 ml of water containing 1 mg of carrier albumin and precipitated by the addition of 0.1 vol. of cold 50% (w/v) trichloroacetic acid containing 5% (w/v) phosphotungstic acid. The precipitates were collected by centrifugation, washed once with 0.5 ml of the acid mixture and once with 0.5 ml of water, and extracted with 0.8 ml of chloroform/methanol (2:1, v/v). Labelled gangliosides and sialoglycoproteins were measured in the chloroform/methanol extracts and residues respectively (Curtino *et al.*, 1977).

Fractionation of the retina homogenate incubated with UDP-[¹⁴C]glucose

Total homogenate was prepared with four bovine retinas homogenized in 0.88 ml of 80% (w/v) sucrose containing 130 mM-NaCl, 2 mM-Tris/acetate buffer, pH 7.5, 100 μ M-PMSF and 100 μ M-Tos-Lys-CH₂Cl (final vol. 2.2 ml). The homogenate (1 ml) was incubated with 4 μ M-UDP-[¹⁴C]glucose at 7 °C for 90 min. After this period of incubation the incorporation of [¹⁴C]glucose into the endogenous proteoglycogen fractions reached a plateau. When the incubated homogenate was subjected to fractionation, an equal volume (1 ml) of 1 mM-Tris/acetate buffer, pH 7.5, containing 53 mM-EDTA, was added at the end of the incubation, mixed and centrifuged at 1100 *g* for 15 min at 1 °C. The pellet was resuspended in 0.35 ml of 8% sucrose. The 1100 *g* supernatant was centrifuged at 100 000 *g* for 60 min at 1 °C, and the pellet was resuspended in 0.35 ml of 8% sucrose. The incorporation of [¹⁴C]glucose into the acid-insoluble and -soluble glucans was measured as indicated for the microsomal fraction.

Gel filtration in Sephacryl S-500

The microsomal fraction (1 mg of protein) was incubated with 2 μ M-UDP-[¹⁴C]glucose for 20 min in a total vol of 80 μ l, mixed with 0.22 ml of 12 mM-Tris/acetate buffer, pH 7.5, containing 6% sucrose, 0.35% liver glycogen, 50 μ M-Tos-Lys-CH₂Cl and 0.02% NaN₃ (SGT solution) and passed through a Sephacryl S-500 column (10 ml), equilibrated and eluted with the SGT solution. Fractions (0.4 ml) were collected and monitored for protein (Lowry *et al.*, 1951) and for labelled acid-insoluble and -soluble proteoglycogen as indicated for the microsomal fraction, except that 0.6 mg of carrier albumin was added to the sample before precipitation with trichloroacetic acid.

The labelled peak excluded from the column (0.26 ml) was rechromatographed in another Sephacryl column before and after incubation for 15 min at 7 °C with 60 μ l of one of the following glycogen-containing (1.7%) solutions, as indicated in each case: (a) 2.4 M-NaCl; (b) 5.33 M-KCl; (c) 0.05% SDS; (d) 0.5% SDS; (e) 0.7% Emulphogene. Fractions (0.4 ml) were collected, and the labelled acid-insoluble proteoglycogen was

measured as described above, except that washing of the acid-insoluble residue with the acid mixture was omitted.

Electron microscopy

Electron-microscopy examination of the microsomal fractions eluted from the Sephacryl column were made on pellets obtained by centrifugation at 100 000 *g* for 60 min. The pellets were fixed in glutaraldehyde, post-fixed in OsO₄, dehydrated in graded ethanols and acetone and embedded in Araldite. Sections were stained with lead citrate and uranyl acetate and observed in a Hitachi HU-11B electron microscope.

RESULTS

Trichloroacetic acid-insoluble precursor of acid-soluble proteoglycogen

When the glycogen-containing microsomal fraction of bovine retina was incubated with 4 μ M-UDP-[¹⁴C]glucose, about 80% of the total [¹⁴C]glucose incorporated into endogenous glycogen was found in the trichloroacetic acid-insoluble proteoglycogen fraction (Fig. 1a). This fraction was present in the incubation

Fig. 1. Time course of the incorporation of [¹⁴C]glucose into proteoglycogen fractions at different concentrations of UDP-[¹⁴C]glucose

The microsomal preparation (1.2 mg of protein) was incubated at 7 °C with 4 μ M (a), 20 μ M (b), 40 μ M (c), 80 μ M (d) and 200 μ M (e) -UDP-[¹⁴C]glucose in a total volume of 80 μ l, and the incorporation of [¹⁴C]glucose into acid-insoluble (●) and -soluble (○) proteoglycogen was measured as indicated in the Materials and methods section.

Table 1. Incorporation of [¹⁴C]glucose into proteoglycogen precursor of subcellular fractions isolated after incubation of retina homogenate with UDP-[¹⁴C]glucose

The retina homogenate was incubated with 4 μ M-UDP-[¹⁴C]glucose for 90 min, mixed with an equal volume of Tris/acetate buffer containing EDTA, centrifuged at 1100 g, and the 1100 g supernatant was centrifuged at 100000 g. Labelled acid-insoluble proteoglycogen was measured in the pellets and supernatant. For further details, see the Materials and methods section.

Retina fraction	[¹⁴ C]Glucose incorporated	
	(c.p.m./mg of protein)	(c.p.m./retina)
1100 g Pellet	1860	61000
100000 g Pellet	27400	82200
100000 g Supernatant	2940	36800

mixture at a concentration of 0.28 mg of glycogen/ml, whereas acid-soluble proteoglycogen was at 0.96 mg of glycogen/ml (Miozzo *et al.*, 1989). The acid-insoluble fraction was thus labelled with a specific radioactivity (c.p.m./mg of glycogen) about 13-fold higher than the acid-soluble fraction. Increasing the concentration of UDP-[¹⁴C]glucose in the incubation mixture resulted in a decrease in the labelling ratio of acid-insoluble to acid-soluble proteoglycogen (Figs. 1*b-e*) and at 80 and 200 μ M-UDP-[¹⁴C]glucose, after an incubation of 15–20 min, the incorporation into the acid-soluble fraction was greater than into the insoluble fraction. The time course of [¹⁴C]glucose incorporation into the proteoglycogen fractions is characteristic of a precursor–product relationship. Accordingly, a ‘chase’ made by incubation for a period of 25 min with 10 mM-UDP-glucose after 15 min of incubation with 2 μ M-UDP-[¹⁴C]glucose resulted

in the conversion of nearly all of the labelled acid-insoluble into acid-soluble proteoglycogen (results not shown). The addition of glycogen (6 mg/ml of incubation mixture) affected neither the incorporation of [¹⁴C]glucose into acid-insoluble proteoglycogen before the chase nor the conversion of acid-insoluble proteoglycogen into acid-soluble proteoglycogen after the chase.

Distribution of proteoglycogen precursor in retina membrane and soluble fractions after incubation with UDP-[¹⁴C]glucose

When the retina homogenate was incubated with 4 μ M-UDP-[¹⁴C]glucose (for details see the Materials and methods section), the labelling ratio of acid-insoluble to acid-soluble proteoglycogen was similar to that shown for the microsomal fraction at the same sugar-nucleotide concentration (results not shown). Fractionation of the homogenate into a 1100 g pellet, a 100000 g pellet and a supernatant after incubation with UDP-[¹⁴C]glucose resulted in recovery of most of the labelled acid-insoluble proteoglycogen in the membranous fractions, that is, the 1100 g and 100000 g pellets (Table 1). The labelled precursor was concentrated in the microsomal 100000 g fraction.

Fig. 2. Gel filtration on Sephacryl S-500 of the microsomal fraction incubated with UDP-[¹⁴C]glucose

The microsomal fraction (1 mg of protein) was incubated at 7 °C with 2 μ M-UDP-[¹⁴C]glucose for 20 min and passed through the Sephacryl column (0.8 cm × 20 cm) equilibrated and eluted with Tris/acetate buffer containing sucrose, glycogen, Tos-Lys-CH₂Cl and NaN₃. Fractions (0.4 ml) were collected and monitored for labelled acid-insoluble (●) and -soluble (○) proteoglycogen. For full details, see the Materials and methods section.

Fig. 3. Electron micrographs of the microsomal fractions excluded and included in the Sephacryl column

The microsomal preparation (300 μ l) was incubated for 20 min with 2 μ M-UDP-[¹⁴C]glucose, passed through the Sephacryl column as described in Fig. 2 and the labelled peaks excluded (a) and included (b) in the column were examined as indicated in the Materials and methods section. The bars represent 1 μ m.

Fig. 4. Rechromatography in Sephacryl S-500 of the excluded proteoglycogen precursor subjected to treatment with detergent solutions

(a) and (b) Solubilization of ³H-labelled gangliosides (Δ) and sialoglycoproteins (▲) with SDS (a) or Emulphogene (b) at the indicated detergent/protein ratios. (c) and (d) The peak of labelled proteoglycogen precursor excluded from the Sephacryl column (Fig. 2) was rechromatographed in a similar column before (c, ●) and after treatment with SDS in a detergent/protein ratio of 0.24 (d, ■) or 2.4 (c, □) and after treatment with Emulphogene in a detergent/protein ratio of 3.4 (d, ○). For procedures see the Materials and methods section.

The proteoglycogen precursor is associated to membrane

When the microsomal fraction was passed through a Sephacryl S-500 column after incubation with 4 μM-UDP-[¹⁴C]glucose, 27–37% of the total labelled proteoglycogen precursor was excluded (Fig. 2). The excluded material contained large membrane vesicles composed of mitochondria, Golgi and rough

endoplasmic reticulum, whereas the included peak of labelled precursor contained smaller smooth vesicles (Fig. 3). The acid-soluble proteoglycogen fraction was included in the column.

Since the acid-insoluble proteoglycogen did not aggregate after purification (Miozzo *et al.*, 1989), the exclusion of this fraction together with large membrane vesicles might be due to an association with membranes or to its localization inside the vesicles. The latter possibility was discarded, since the membrane-associated proteoglycogen was accessible to the amylolytic action of exogenous glucoamylase (results not shown). In order to investigate the former possibility, the labelled material excluded from the Sephacryl column was subjected to treatments with saline solutions or detergents and analysed again on a similar column. Conditions for the treatments with detergents were chosen from studies of solubilization of gangliosides and sialoglycoproteins from the microsomal fraction (Figs. 4a and 4b). The treatment with SDS in a detergent-to-protein ratio of 0.24, which did not solubilize the labelled membrane glycolipid, and glycoprotein constituents had no effect on the association state of the proteoglycogen precursor (Fig. 4d). However, treatment with SDS under conditions that solubilized the integral membrane sialoglycoconjugates released the proteoglycogen precursor, which was now included in the column and was eluted at a volume slightly greater than that of the acid-soluble proteoglycogen (Fig. 4c). Emulphogene, used in a concentration which caused the release of about 40% of the retina sialo compounds, also released the precursor (Fig. 4d). On the other hand, treatment with 0.45 M-NaCl or 1 M-KCl did not change the elution volume of proteoglycogen (results not shown).

DISCUSSION

The present studies on the incorporation of [¹⁴C]glucose from UDP-[¹⁴C]glucose into the endogenous proteoglycogen fractions of retina were carried out at 7 °C in order to decrease reaction rates. This allowed us to measure early events in the time course of proteoglycogen labelling. The results shown in Fig. 1, and the chase made with unlabelled UDP-glucose, indicate that acid-insoluble proteoglycogen is the biosynthetic precursor of acid-soluble proteoglycogen. This conclusion is supported by previous work showing that both proteoglycogen fractions have a similarly sized protein moiety (p42) and differently sized polysaccharide moieties, the lower weight-average *M_r* corresponding to the acid-insoluble fraction (Miozzo *et al.*, 1989).

The acid-insoluble and -soluble proteoglycogens were present in the incubations in the ratio 1:4 (respectively). However, the acid-insoluble proteoglycogen was the preferred substrate for glycogen synthase (Fig. 1a). Glycogen, added in excess with respect to acid-insoluble proteoglycogen, did not compete for the incorporation of glucose. This could indicate that the enzyme did not dissociate from the glucan moiety of proteoglycogen precursor during the glucose-transfer activity. Thus glycogen biosynthesis would be a type of channelling process (Srere, 1987) in which glycogen synthase moves from the last-transferred glucose unit to the next without equilibration with the bulk cellular fluid, at least before a number of glucose units had been transferred. Since precursor proteoglycogen was found associated with membrane, the behaviour of glycogen synthase would be that of an 'ambiquitous' enzyme, the term 'ambiquitous' being proposed by Wilson (1980) to describe enzymes either bound to a structure or free in solution.

Studies on the distribution of proteoglycogen precursor in retina homogenate indicated that it was concentrated in the microsomal fraction (Table 1). About one-third of the acid-insoluble proteoglycogen was associated with large membrane vesicles (Figs. 2 and 3) and was dissociated by treatment with

detergents under conditions in which integral membrane constituents were solubilized (Fig. 4). These results lead us to postulate that p42 is bound to membrane at the moment at which the synthesis of glycogen *de novo* begins. After its saccharide moiety reaches a suitable size, the proteoglycogen molecule is released from membranes. This proteoglycogen is then converted into the acid-soluble form by further growth of the polysaccharide.

Further work will be needed to define the type of association between the proteoglycogen precursor and membranes.

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