Early dolichyl sugar synthesis during differentiation of non-growing brine-shrimp (*Artemia salina*) embryos

Luis A. QUESADA-ALLUE

Instituto de Investigaciones Bioquímicas, 'Fundación Campomar', Obligado 2490, 1428 Buenos Aires, Argentina

(Received 15 May 1981/Accepted 5 June 1981)

Incubation of cell-free extracts of *Artemia salina* with sugar nucleotides resulted in the formation of dolichyl derivatives. These enzymic activities were detected early in the development of the encysted *Artemia* embryos. Dolichyl sugars seemed to act as intermediates in the glycosylation of proteins, which may be involved in the initiation of cellular differentiation.

Dolichol-linked sugars are intermediates in the glycosylation of some eukaryotic and viral proteins (Schachter, 1978; Parodi & Leloir, 1979). We previously reported biosynthesis both *in vivo* and *in vitro* of some insect dolichyl derivatives (Quesada-Allue *et al.*, 1976a,b; Quesada-Allue & Belocopitow, 1978), which also seemed to be involved in the glycosylation of insect proteins (Quesada-Allue, 1978, 1980; Butters & Hughes, 1980), thus confirming that this mechanism is a general feature of eukaryotic organisms.

Because of its unusual biology, the brine shrimp (*Artemia salina*) has become a useful system for studies on developmental biochemistry (Finamore & Clegg, 1969; Hentschel & Tata, 1976). In conditions of extreme salinity its development stops and the embryos (mainly gastrulae) are encysted, entering a dormant, ametabolic stage. After the cysts are desiccated and then rehydrated, development continues. In the initial postgastrular development of encysted *Artemia* embryos, cellular differentiation and morphogenesis take place without the occurrence of cell division, DNA synthesis or growth (Hentschel & Tata, 1976).

Since I recently obtained evidence for the biosynthesis of dolichol-linked sugars by crustacean enzymes (Quesada-Allue, 1978), the *Artemia* system offers a unique opportunity to study the activation of the dolichol pathway and its relations with the process of differentiation. When *A. salina* cryptobiotic embryos are activated, a number of rapid molecular changes take place. The present paper describes the early biosynthesis of dolichyl phosphate mannose and dolichyl phosphate glucose. The present evidence is the first to support a relationship between dolichol pathways and differentiation in the complete absence of growth and cell division.

Experimental

The analytical procedures used in the present paper have been described previously (Quesada-Allue & Belocopitow, 1978; Quesada-Allue, 1980). The same results were obtained with different batches of *Artemia* cysts.

After treatment with 5% NaClO, to remove the chorion layer and contaminants (Finamore & Clegg, 1969), encysted embryos of *Artemia* were immersed in cold 4% NaCl containing streptomycin and penicillin. Viable cysts were activated by incubation at 20°C for the desired period. The activated cysts were extensively washed and divided into two identical aliquots. Those in one of them were carefully disaggregated in a large-clearance glass/glass Kontes homogenizer to obtain entire cells. The washed cells remained viable for at least 8 h (Trypan Blue test) and showed permeability to labelled amino acids. The other portion of cysts was transferred to a pre-cooled mortar, suspended in liquid N₂ and homogenized in 80 mM-Tris/HCl buffer, pH 7.5, containing 4 mM-NaCl, 15 mM-KCl, 5 mM-MgCl₂, 2 mM-EDTA, 2 mM-2-mercaptoethanol, 100 mM-sucrose, 1 mM-butylated hydroxytoluene and 1% glycerol. The broken material was further fractionated as described by Quesada-Allue & Belocopitow (1978) by successive centrifugations to obtain a microsome-enriched fraction. The latter fraction showed an appreciable synthesis of polyprenyl sugar-like substances, whereas other fractions have only trace activities.

The incubation mixtures contained, in a final volume of 60 µl: 6 mg of microsomal protein, 80 mM-Tris/maleate buffer, pH 8.0, 30 mM-2-mercaptoethanol, 13 mM-MgCl₂, 6 mM-KCl, 1.5 mM-EDTA and GDP-[¹⁴C]mannose (2 × 10⁵ c.p.m.) or

Fig. 1. Anion-exchange and thin-layer chromatography of *Artemia* lipid-linked monosaccharides

(a) *Artemia* lipid-linked [^{14}C]mannose (●) or lipid-linked [^{14}C]glucose (○) (see the Experimental section) were dried under an N_2 stream and dissolved in 0.2 ml of chloroform/methanol (3:2, v/v) containing purified liver dolichyl phosphate [^3H]glucose (▽). The samples were applied to a DEAE-cellulose (acetate form) column (0.6 cm $\times$ 5.0 cm) equilibrated with chloroform/methanol (3:2, v/v). The columns were washed with the same solvent and then eluted with chloroform/methanol (1:1, v/v) (arrow 1) followed by a stepwise gradient of 0, 5 mM- and 25 mM-ammonium formate in chloroform/methanol/water (10:10:3, by vol.) as indicated by arrows 2, 3 and 4 respectively. Fractions (1.0 ml) were desalted, dried in glass vials and counted for radioactivity in a gas flow counter. (b) Desalted fractions from identical columns as described in (a) were pooled, dried under an N_2 stream, dissolved in chloroform/methanol (3:2, v/v) and spotted on silica-gel Eastman (6061) sheets. Thin-layer sheets were developed with chloroform/propan-2-ol/ethanol/1 M-acetic acid (2:2:3:1, by vol.) (Quesada-Allue *et al.*, 1976a). Bands (1 cm) were cut off and counted for radioactivity in a scintillator. ■, Endogenous *Artemia* lipid-linked [^{14}C]glucose; ○, *Artemia* lipid-linked [^{14}C]glucose from dolichyl phosphate-supplemented (10 μg) incubation mixture; ▽, liver dolichyl phosphate [^3H]glucose. The arrows indicate the following: 5, origin;

UDP-[^{14}C]glucose (3×10^5 c.p.m.). The mixtures were incubated for 30 min at 25°C, stopped, processed and counted for radioactivity as described by Quesada-Allue *et al.* (1976b). The lower phases of partitions of the incubation mixtures (Folch *et al.*, 1957) were used as a source of lipid-linked monosaccharides (Quesada-Allue *et al.*, 1976b).

Ornithine decarboxylase activity was measured by the method of Jänne & Williams-Ashman (1971).

Results and discussion

When GDP-[^{14}C]mannose or UDP-[^{14}C]glucose were used as sugar donors in cell-free experiments, the main acid-labile lipidic substances biosynthesized behaved respectively as dolichyl phosphate [^{14}C]mannose and dolichyl phosphate [^{14}C]glucose. The identity was assigned by the following criteria.

(a) Anion-exchange chromatography on DEAE-cellulose columns (Quesada-Allue & Belocopitow, 1978) showed that both mannosylated and glucosylated *Artemia* substances were retained by the resin and were co-eluted by salts together with ^3H -labelled liver dolichyl phosphate glucose (Fig. 1a).

(b) The biosynthesis was stimulated by exogenous addition to incubation mixtures of insect or liver dolichyl phosphate (Fig. 1b).

(c) The putative dolichyl phosphate [^{14}C]mannose and dolichyl phosphate [^{14}C]glucose chromatographed like authentic samples in silica-gel G thin-layer plates (Figs. 1b and 1c) (Quesada-Allue *et al.*, 1976a,b).

(d) The substances were broken down by mild acid hydrolysis and the liberated products co-chromatographed in several systems with mannose and glucose (Fig. 1c).

The labelled mannosylated and glucosylated protein-like material obtained after complete delipidation of the incubation mixture (Quesada-Allue, 1980) was insoluble in hot 10% trichloroacetic acid (Fig. 2a). When treated with *Streptomyces griseus* proteinase for 3 days at 37°C, 80% of the radioactivity became soluble in trichloroacetic acid and migrated in paper electrophoresis with both acid and basic buffers. As judged by gel filtration in

6, glucose; 7, mannose; 8, solvent front. (c) The same as (b) but *Artemia* lipid-linked [^{14}C]mannose (●) was chromatographed. An identical sample of the latter was dried and treated with 200 μl of 0.01 M-HCl for 30 min at 98°C. After HCl evaporation, labelled material was dissolved both with chloroform/methanol (3:2, v/v) and water, mixed and chromatographed in a parallel lane (□). A standard of liver dolichyl phosphate [^3H]glucose was also present (▽).

Fig. 2. Relationship between (a) biosynthesis of dolichol-linked sugars and glycoproteins in cell-free preparations of *Artemia* developing embryos and (b) rate of $[^3\text{H}]$ leucine incorporation into proteins by unbroken embryonic cells

(a) *Artemia* dolichyl phosphate $[^{14}\text{C}]$ mannose and dolichyl phosphate $[^{14}\text{C}]$ glucose were obtained and identified as indicated in the legend to Fig. 1. After acid hydrolysis (see Fig. 1c) the liberated water-soluble labelled material was paper chromatographed with butan-1-ol/pyridine/water (6:4:3, by vol.). To detect radioactivity in mannose (●) or glucose (○) bands were cut off and counted in a scintillator. Mannosylated (▲) and glucosylated (△) protein-like materials were suspended in 10% trichloroacetic acid, heated for 7 min at 99°C , processed and counted for radioactivity as described by Quesada-Allue (1980). (b) Cells (1 mg of protein) from embryos immersed for the indicated times were incubated in 50% artificial sea water containing 25% lobster haemolymph (Grand Island Biol. Co.), 50 mM-Tris/HCl buffer, pH 7.6, and $10\ \mu\text{Ci}$ of $[4,3\text{-}^3\text{H}]$ leucine (sp. radioactivity 67.6 Ci/mmol) or $10\ \mu\text{Ci}$ of $[2\text{-}^3\text{H}]$ mannose (sp. radioactivity 12 Ci/mmol) in a final volume of $200\ \mu\text{l}$. Incubations were carried out in the flat-bottom (2 cm² area) sterile tissue-culture multi-well plates for 30 min at 30°C . Incorporation of $[^3\text{H}]$ leucine into hot trichloroacetic acid-soluble material (see above) was measured either in the absence (■) or the presence (□) of 1 mM-cycloheximide. No incorporation of $[2\text{-}^3\text{H}]$ mannose into glycoproteins could be detected (▲).

biosynthesis becomes detectable in the cell-free system 2 h after immersion of cysts (Fig. 2a), preceding the glycosylation of microsomal protein-like material. Ornithine decarboxylase, an enzyme whose activity increases markedly when certain undifferentiated cells are activated (Jänne & Williams-Ashman, 1971), was not detected until 6.5 h after immersion. In the undisturbed-cells experiment (Fig. 2b), the incorporation of $[2\text{-}^3\text{H}]$ leucine into hot 10% trichloroacetic acid-insoluble material is detectable 1 h after immersion of the cysts. Cycloheximide diminished the incorporation of radioactivity, probably indicating that polypeptide elongation had been inhibited. When $[^3\text{H}]$ mannose was used as tracer it was taken up by the cells but, under the conditions of the assay, glycosylation of proteins could not be detected.

The data on leucine incorporation into polypeptide-like material using the intact cell system is in agreement with those obtained in cell-free systems by others (Clegg, 1966; Hentschel & Tata, 1976). Thus our evidence strongly suggests that dolichyl phosphate derivative synthesis was activated early and could be detected simultaneously with the re-assumption of polypeptide elongation. In addition, these pre-programmed (Muthukrishnan *et al.*, 1975) polypeptides might be some of the ones that became glycosylated in cell-free experiments. Since the embryos of *Artemia salina* undergo some morphogenetic events in the complete absence of growth and cell division (Finamore & Clegg, 1969; Hentschel & Tata, 1976), it appears that dolichol-linked sugars are biosynthesized early, probably to act as intermediates in the glycosylation of proteins (Clegg, 1966; Osuna *et al.*, 1977; Nagainis & Warner, 1979), which, in turn, are somehow related to the initiation of cellular differentiation (James & Tata, 1980).

I am a Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina). This work was partially supported by the Organization of American States. I am indebted to Professor Dr. Luis F. Leloir for his continuous interest and support, and to the other members of the Instituto de Investigaciones Bioquímicas for many helpful discussions.

References

- Butters, T. D. & Hughes, R. C. (1980) *Biochem. Soc. Trans.* **8**, 170–172
- Clegg, J. S. (1966) *Nature (London)* **212**, 517–519
- Finamore, F. J. & Clegg, J. S. (1969) in *The Cell Cycle: Gene-Enzyme Interactions* (Padilla, G. M., Whitson, G. L. & Cameron, I. L., eds.), pp. 249–278, Academic Press, New York and London
- Folch, J. M., Lees, M. & Sloane, S. G. (1957) *J. Biol. Chem.* **226**, 497–509

Sephadex G-15 the putative glycopeptides diminished in size during the treatment with proteinase.

The *Artemia* metabolic machinery remains quiescent up to a complete hydration, 40–60 min after immersion of encysted gastrulae (Finamore & Clegg, 1969). Dolichyl phosphate monosaccharide

- Hentschel, C. C. & Tata, J. R. (1976) *Trends Biochem. Sci.* **1**, 97–100
- James, T. C. & Tata, J. R. (1980) *Differentiation* **16**, 9–21
- Jänne, J. & Williams-Ashman, H. G. (1971) *J. Biol. Chem.* **246**, 1725–1732
- Muthukrishnan, S., Filipowicz, W., Sierra, J. M., Both, G. W., Shatkin, A. J. & Ochoa, S. (1975) *J. Biol. Chem.* **250**, 9336–9341
- Nagainis, P. A. & Warner, A. H. (1979) *Dev. Biol.* **68**, 259–270
- Osuna, C., Olalla, A., Sillero, A., Gunther, M. A. & Sebastian, J. (1977) *Dev. Biol.* **61**, 94–103
- Parodi, A. J. & Leloir, L. F. (1979) *Biochim. Biophys. Acta* **559**, 1–37
- Quesada-Allue, L. A. (1978) Ph.D. Thesis, University of Buenos Aires
- Quesada-Allue, L. A. (1980) *Mol. Cell. Biochem.* **33**, 149–155
- Quesada-Allue, L. A. & Belocopitow, E. (1978) *Eur. J. Biochem.* **88**, 529–541
- Quesada-Allue, L. A., Marechal, L. R. & Belocopitow, E. (1976a) *Acta Physiol. Lat. Am.* **26**, 349–363
- Quesada-Allue, L. A., Marechal, L. R. & Belocopitow, E. (1976b) *FEBS Lett.* **67**, 243–247
- Schachter, H. (1978) in *The Glyconjugates* (Horowitz, M. & Pigman, W., eds.), vol. 2, pp. 87–181, Academic Press, New York