Glycoprotein synthesis in explants of developing rabbit mammary gland in culture

Judith P. BRADSHAW and David A. WHITE

Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

(Received 23 April 1981/Accepted 8 June 1981)

1. Explants of mammary glands of pregnant rabbits cultured in the absence of insulin, prolactin and cortisol incorporated [2-³H]mannose into lipid-linked mono- and oligo-saccharide and protein. 2. Inclusion of the hormones in the culture medium stimulated the incorporation of [2-³H]mannose into lipid-linked monosaccharide 4-fold, into lipid-linked oligosaccharide 4-fold and into protein 13-fold after 24 h in culture. 3. Addition of tunicamycin to the incubation medium completely inhibited the incorporation of [2-³H]mannose into lipid-linked oligosaccharide and protein after an initial lag period of about 2 h. Incorporation of this radiolabel into lipid-linked monosaccharide was increased 4-fold under these conditions. 4. Incorporation of [4,5-³H]leucine into protein was unaffected by the presence of tunicamycin. 5. Analysis of mannose-labelled proteins by polyacrylamide-gel electrophoresis indicated that a major radiolabelled protein of apparent mol.wt. 65 000–70 000 was synthesized and approx. 70% of this protein appeared in the soluble fraction. 6. Glycosylation of the protein but not synthesis of its peptide backbone was sensitive to tunicamycin. 7. Possible origins of this glycoprotein synthesized when the tissue is stimulated to differentiate in culture are discussed.

Developmental changes in a number of tissues are often associated with increased rates of *N*-glycosylation, and sometimes with the appearance of novel glycoproteins. Since *N*-glycosylation of proteins utilizes dolichol-linked sugar intermediates (for reviews see Waechter & Lennarz, 1976; Gibson *et al.*, 1980) its regulation during development may be mediated through changes in activity of the enzymes associated with this pathway, e.g. mannosyltransferase, or the availability of the lipid carrier dolichol, or through a combination of these.

Lucas & Levin (1977) proposed that increased substrate pool size rather than increased activity of mannosyltransferase was responsible for the increased transfer of mannose from GDP-mannose to dolichyl phosphate seen in diethylstilboestrol-treated chicks compared with controls. Developmental changes in the rate of synthesis of dolichyl phosphate mannose in white-matter membranes from pig brain were due mainly to changes in the concentration of endogenous dolichyl phosphate

accessible to the mannosyltransferase, although experimental evidence also supported smaller changes in the activity of mannosyltransferase (Harford & Waechter, 1980). Thus the maximal activity of dolichyl phosphate mannose synthase occurred during active myelination when specific glycoproteins are laid down in the myelin of the central nervous system.

The mammary gland uses the dolichol-linked sugar pathway for biosynthesis of *N*-linked glycoproteins (Speake & White, 1978) and the lactating gland incorporates radiolabelled mannose into glycopeptides of apparent mol.wts. 40 000–80 000. Incorporation of mannose into these peptides and in particular into a glycopeptide of mol.wt. approx. 65 000–75 000 is sensitive to tunicamycin (Speake & White, 1979), indicating that this major radio-labelled glycoprotein is glycosylated via a dolichol-linked intermediate. The present paper describes the formation of lipid-linked sugars in explants of pregnant mammary gland stimulated to differentiate in culture with hormones and the appearance of novel glycoproteins whose synthesis is dependent on this pathway.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Animals

New Zealand White (albinos) rabbits were obtained from the Joint Animal Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. The animals were at least 6 months old at the time of mating.

Materials

D-[2-³H]Mannose and L-[4,5-³H]leucine were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Ox insulin was a gift from Mr. J. V. Birkinshaw, Boots Pure Drug Co., Nottingham, U.K. Cortisol 21-acetate, nystatin, benzyl penicillin and streptomycin sulphate were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Ovine prolactin (NIAMDD-OPRL-14) was a gift from Dr. Salvatore Raiti, Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolism and Digestive Diseases, National Pituitary Agency, Baltimore, MD, U.S.A. Medium 199 with and without glucose (Morgan *et al.*, 1950) and 4.4% NaHCO₃ solution were obtained from Wellcome Research Laboratories, Beckenham BR3 3BS, Kent, U.K. Tunicamycin was a gift from Dr. D. Cuthbertson, Glaxo Group Research, Greenford, Middx., U.K. Rabbit casein was a gift from Miss H. Hasan of this Department. All other chemicals and solvents were of A.R. grade and obtained from commercial suppliers.

Preparation and culture of mammary explants

Explants of lobuloalveolar mammary tissue were dissected from mid-pregnant rabbits (day 16 of pregnancy) as described by Forsyth & Myers (1971) and cultured in medium 199 in the presence or absence of insulin, prolactin and cortisol for 24, 48, 72 and 96 h as described by Al-Sarraj *et al.* (1979).

Measurement of incorporation of D-[2-³H]mannose and L-[4,5-³H]leucine into endogenous acceptors of mammary explants

Duplicate groups of 20 explants were washed with medium 199 (glucose-free) and incubated for periods of up to 5 h at 37°C under O₂/CO₂ (19:1) in medium 199 (glucose-free) (1 ml) with or without hormones. The incubation medium also contained sodium pyruvate (5 mM) and either 10 µCi of D-[2-³H]mannose (sp. radioactivity 16 Ci/mmol) or 10 µCi of L-[4,5-³H]leucine (sp. radioactivity 72 Ci/mmol). When the effects of tunicamycin were studied it was included in the incubation medium at a concentration of 20 µg/ml.

At the end of the incubation period each group of explants was washed with medium 199 (glucose-free), weighed and extracted for lipid-linked mono-

saccharide, lipid-linked oligosaccharide and protein fractions as described by Speake & White (1978).

Characterization of radiolabelled proteins

Groups of 50 explants were incubated as described above with 20 µCi of D-[2-³H]mannose or 20 µCi of L-[4,5-³H]leucine for 2 h periods in the presence or absence of tunicamycin. After incubation explants were washed in ice-cold medium and homogenized and sonicated for 5 min in 5 mM-EGTA, pH 7.0, containing 2 mM-phenylmethanesulphonyl fluoride. Soluble and pellet fractions were obtained by centrifugation of the resulting homogenate at 100 000 *g*_{av} for 1 h at 4°C. The portions of these fractions that were soluble after incubation for 5 min at 100°C in SDS (final concentration 2%) and dithiothreitol (final concentration 0.1 M) were analysed by polyacrylamide-gel electrophoresis. This was performed on 15% acrylamide slab gels in the presence of SDS by the method of Laemmli (1970). Between 50 and 250 µg of protein was applied to each gel lane. Protein standards used were bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 43 000), pepsin (mol.wt. 34 700), trypsinogen (mol.wt. 24 000), β-lactoglobulin (mol.wt. 18 400) and lysozyme (mol.wt. 14 500) present in the Dalton Mark VI TM Kit (Sigma Chemical Co.). Casein was also used as standard.

After electrophoresis at 40 V overnight, gels were fixed and stained for protein using PAGE Blue 83 (BDH). Each gel was then cut into 5 mm slices, and the radioactivity in each slice was determined by liquid-scintillation spectrometry.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Characterization of radiolabelled sugars

Portions of soluble and pellet fractions of explants previously incubated with D-[2-³H]mannose were subjected to strong acid hydrolysis as described by Speake & White (1978). Descending paper chromatography was performed on the hydrolysate using Whatman 3MM paper in ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.). The paper was then cut into 5 mm strips and the radioactivity present was determined by liquid-scintillation spectrometry.

Results

Incorporation of radioactivity from D-[2-³H]mannose into lipid and protein fractions

Figs. 1(a)–1(c) show the effects of culturing mid-pregnant mammary tissue for up to 96 h in the presence and absence of hormones on the incorporation of radioactivity from D-[2-³H]mannose

Fig. 1. Effect of culture conditions on incorporation of D-[2-³H]mannose into (a) lipid-linked monosaccharide, (b) lipid-linked oligosaccharide and (c) lipid-free protein by mammary explants

Duplicate groups of 20 explants were cultured for periods of up to 96 h at 37°C under O₂/CO₂ (19:1) in medium 199 in the presence (○) or absence (●) of insulin, prolactin and cortisol. Each group was then incubated for 2 h at 37°C under O₂/CO₂ (19:1) in medium 199 (glucose-free) (1 ml) containing 10 μCi of D-[2-³H]mannose. At the end of the incubation period each group of explants was fractionated into lipid and protein pools, and the radioactivity in each fraction was determined.

into lipid and protein fractions. The results shown are typical of six separate experiments. The weight of explants cultured in the presence of hormones was always approx. 30% greater than those cultured without hormones.

The radioactivity present in lipid-linked monosaccharide was always the lowest of the three fractions, but was increased approx. 5-fold after 24 h culture in the presence of hormones (Fig. 1a). This level of stimulation was only marginally increased after a further 72 h culture. Culture in the absence of hormones caused only a 2–3-fold increase in the amount of radioactivity present after 24 h, and this slowly reverted to basal level over a further 72 h in culture.

Incorporation of radioactivity into the lipid-linked oligosaccharide fraction was also increased nearly 2-fold after 24 h in culture in the absence of hormones (Fig. 1b). Addition of hormones to the culture medium produced an even greater increase in the radioactivity incorporated into this fraction, and this increase was maintained over a further 72 h.

The highest incorporation of [2-³H]mannose was into the residual protein fraction (Fig. 1c), both in the presence and absence of hormones. In the absence of hormones a 6-fold increase in incorporation compared with zero time was apparent after 24 h, but this gradually decreased so that after

96 h in culture the increase was of the order of only 2-fold. However, inclusion of hormones in the culture medium caused a 13-fold increase after 24 h compared with the zero time incorporation, and this decreased only slightly over the ensuing 72 h in culture.

Certain variations in the incorporation of radioactivity into this fraction were discernible when using different rabbits. The increased incorporation seen in the absence of hormones was frequently less significant than indicated in Fig. 1(c), and occasionally maximum incorporation did not occur until after 48 h in culture in the presence of hormones. However, a high level of stimulation in the presence of hormones was evident in all cases, and maintained for up to 96 h in culture.

Characterization of radiolabelled sugars

The only radiolabelled sugar detectable after strong acid hydrolysis of explant proteins was chromatographically identical with mannose.

Kinetics of incorporation of D-[2-³H]mannose into lipids and proteins and of L-[4,5-³H]leucine into proteins of cultured mammary explants

The incorporation of mannose into endogenous acceptors of explants cultured for 48 h in the presence of hormones is shown in Fig. 2. There was

Fig. 2. Effect of tunicamycin on the incorporation of D-[2-³H]mannose into (a) lipid-free protein, (b) lipid-linked oligosaccharide and (c) lipid-linked monosaccharide and of L-[4,5-³H]leucine into (d) lipid-free protein by mammary explants

Duplicate groups of 20 explants previously cultured for 48 h in the presence of hormone were incubated for up to 5 h at 37°C under O₂/CO₂ (19:1) in 1 ml of medium 199 (glucose-free) in the presence (□) or absence (■) of tunicamycin. At the end of the incubation period each group of explants was fractionated into lipid and protein pools, and the radioactivity in each fraction was determined.

a rapid incorporation into protein (Fig. 2a), which was linear over a 5 h time course.

Incorporation into the lipid-linked oligosaccharide fraction (Fig. 2b) was much lower, being less than 5% of that into protein after 3 h incubation. The incorporation of D-[2-³H]mannose into lipid-linked oligosaccharide was linear for between 30 and 60 min and reached a plateau after about 2 h of incubation. Only very small amounts of radioactivity were incorporated into the lipid-linked monosaccharide fraction (Fig. 2c), being maximal after 2 h when it was less than 0.5% of the radioactivity present in protein.

The incorporation of leucine into protein was linear over the 5 h incubation period (Fig. 2d).

Effects of tunicamycin on incorporation of D-[2-³H]mannose and L-[4,5-³H]leucine into protein and lipid fractions

Tunicamycin, added to the incubation system at the same time as radiolabelled substrate, had a marked inhibitory effect on the incorporation of mannose into both protein- and lipid-linked oligosaccharide, although there was a lag period of approx. 1–2 h before this became apparent. Further incubation caused almost complete inhibition of the incorporation of radiolabel into both fractions. In contrast, incorporation of mannose into lipid-linked monosaccharide (Fig. 2c) was stimulated 4-fold in the presence of tunicamycin, the lag phase being much decreased.

Protein synthesis, as measured by incorporation of L-[4,5- ^3H]leucine into protein, was unaffected by the inclusion of tunicamycin in the incubation medium (Fig. 2d).

Analysis of radiolabelled proteins

Highly radiolabelled soluble and pellet fractions were isolated after homogenization, sonication and centrifugation from explants incubated with D-[2- ^3H]mannose or L-[4,5- ^3H]leucine. Proteins were separated by polyacrylamide-gel electrophoresis and the levels of radioactivity present were determined by liquid-scintillation spectrometry. The Figures shown below are typical of at least six experiments.

(i) *Explants cultured in the absence of hormones.* Incorporation of radiolabelled mannose into either pellet (Fig. 3, Ia) or soluble (Fig. 3, IIa) proteins of explants cultured in the absence of hormones was low, and no obvious peaks of radioactivity were discernible. The same was true when leucine was used as radiolabelled substrate (Fig. 4, Ia and IIa).

(ii) *Explants cultured in the presence of hormones.* (a) Pellet. Culturing explants in the presence of hormones gave rise to a general increase in the incorporation of radioactivity from [^3H]mannose into peptides of molecular weight greater than approx. 30 000 (Fig. 3, Ib). In particular, at least three major peaks were discernible with molecular

weights greater than 50 000. Qualitative examination of the gels revealed little difference in banding pattern after protein staining with Coomassie Blue, many bands being present in this region of the gel in pellets from both control and hormone-stimulated explants.

Analysis of the gels of pellet fractions from leucine-labelled explants also showed a generally increased labelling of proteins. However, although at least one peak of radioactivity was apparent with a molecular weight greater than 50 000, the major peak was in peptides coincident with casein standard (Fig. 4, Ib).

Inclusion of tunicamycin in incubations of hormonally stimulated explants almost completely inhibited incorporation of mannose into protein (Fig. 3, Ic) and the level of radioactivity present in the gel was accordingly low. No peaks of radioactivity corresponding to those highly labelled in the absence of tunicamycin were seen (Fig. 4, Ib), although the banding pattern of the gel appeared qualitatively unaltered.

In contrast, the radioactivity profile of gels of pellet proteins from hormonally stimulated explants incubated with [^3H]leucine was not affected by the inclusion of tunicamycin in the incubation medium, the distribution pattern being essentially unchanged (Fig. 4, Ic).

Fig. 3. Polyacrylamide-gel electrophoresis of proteins labelled with D-[2- ^3H]mannose in the presence or absence of tunicamycin, from explants previously cultured in the presence or absence of hormones

Groups of 50 explants previously cultured in the absence (a) or presence (b and c) of hormones were incubated for 2 h in 1 ml of medium 199 (glucose-free) with 20 μCi of D-[2- ^3H]mannose in the absence (a and b) or presence (c) of tunicamycin (20 $\mu\text{g}/\text{ml}$). At the end of incubation explants were fractionated into pellet (I) and soluble (II) phases. Polyacrylamide-gel electrophoresis in the presence of SDS was carried out as described in the Materials and methods section. Pellet protein (250 μg) or soluble protein (100 μg) was applied to each gel lane. The mobilities of protein standards ($10^{-3} \times$ molecular weight) and rabbit casein are indicated, together with the buffer front (B.F.).

Fig. 4. Polyacrylamide-gel electrophoresis of protein labelled with L-[4,5- ^3H]leucine in the presence or absence of tunicamycin, from explants previously cultured in the presence or absence of hormones. Conditions are as described in Fig. 3, except that explants were incubated with 20 μCi of L-[4,5- ^3H]leucine.

(b) Soluble. Compared with control explants, when [^3H]mannose was the precursor there was again a general increase in the radioactivity associated with peptides of molecular weight greater than approx. 30000. Three apparent peaks of radioactivity were present at mol.wts. approx. 20000, 30000 and 50000 (Fig. 3, IIb). However, the major radiolabelled peptide was of mol.wt. approx. 70000, this peak being 2–3-fold greater than the others.

Inclusion of tunicamycin in the incubation medium completely abolished the labelling of these peptides (Fig. 3, IIc).

Analysis of soluble peptides labelled with [^3H]leucine showed two major peaks of radioactivity (Fig. 4, IIb). The one with an apparent mol.wt. of 70000 was coincident with the major mannose-labelled soluble peptide (Fig. 3, IIb), whereas the second was again coincident with casein standard. Several more minor peaks of radioactivity were also discernible, at least two of which were coincident with soluble mannose-labelled peptides.

None of the radioactive peaks appeared to be sensitive to the inclusion of tunicamycin in the incubation medium (Fig. 4, IIc).

Discussion

The culture of explants of mid-pregnant mammary tissue in the presence of insulin, prolactin and cortisol leads to differentiation of the tissue and the onset of lactogenesis. In the present series of experiments differentiation is associated with increased protein synthesis as measured by incorporation of [^3H]mannose and [4,5- ^3H]leucine into lipid-free protein (Figs. 1c and 2d).

An increase in the incorporation of [^3H]mannose into lipid-linked mono- and oligo-saccharides is also seen (Figs. 1a and 1b) in hormone-stimulated explants compared with controls. Incorporation into these intermediates was maximal after approx. 2–3 h incubation, suggesting that the concentration of carrier lipid was limiting. However, incorporation of [^3H]mannose into protein increased linearly with time during incubations of up to 5 h. This is in agreement with the work of Hubbard & Robbins (1980). These workers concluded the rate-limiting step of protein glycosylation to be the transfer of the oligosaccharide from its lipid carrier to the polypeptide chain. Thus availability of the carrier for further lipid-linked oligosaccharide

synthesis is determined directly by the rate of synthesis of polypeptide.

That incorporation of mannose into protein was dependent on the formation of lipid-linked oligosaccharide intermediates was demonstrated by the action of tunicamycin. This antibiotic inhibits the formation of dolichyl pyrophosphoryl *N*-acetylglucosamine, and completely blocked the synthesis of lipid-linked oligosaccharide and mannoprotein in the present system. The delay in action of the antibiotic may reflect the time taken for its entry into the cell, and has been noted previously (Struck & Lennarz, 1977; Speake & White, 1979). The increased labelling of monosaccharide lipid under these conditions suggests that the pool of dolichyl phosphate serving as carrier of mannose to the growing oligosaccharide lipid chain is not normally saturated. Only when mannose transfer is inhibited owing to decreased availability of the oligosaccharide lipid acceptor does this intermediate begin to accumulate.

The failure of tunicamycin to inhibit peptide synthesis is in agreement with the results of Speake & White (1979) in lactating mammary gland and of Firestone & Heath (1981) in mouse L-cells. However, inhibition of protein synthesis by tunicamycin has been described in mouse neuroblastoma cells by Milenkovic & Johnson (1980) and rat thyroid by Seager *et al.* (1980). Such differences in the effect of tunicamycin on protein synthesis may reflect differential tissue response or the source of the antibiotic. Mahoney & Duksin (1979) showed by high-pressure-liquid-chromatographic analysis that an enriched tunicamycin preparation was a mixture of at least 10 homologues and, whereas the two major components inhibited mannose incorporation into protein, only one had an inhibitory effect on protein synthesis. The tunicamycin preparation used in the present study, like that used previously (Speake & White, 1979), gave a single major peak on high-pressure-liquid-chromatographic analysis (F. W. Hemming, personal communication).

Increases in the rate of glycosylation of proteins in diethylstilboestrol-treated chicks were shown to be consistent with an increased concentration of dolichyl phosphate carrier lipid (Lucas & Levin, 1977). Recently Burton *et al.* (1981) have shown that this is likely to have been caused by an increase in the activity of dolichol kinase rather than to an increased supply of its substrate dolichol. Harford *et al.* (1977) and Harford & Waechter (1980) have also suggested that developmental changes in the initial rate of dolichyl phosphate mannose synthesis in porcine white matter is due to a modulation in the concentration of dolichyl phosphate in the central nervous system. The activity of dolichyl phosphate mannose synthase is maximal at a time when major glycoproteins are being laid down in myelin.

Glycoprotein synthesis in the mammary gland during pregnancy appears minimal, no major glycoprotein peaks being discernible on polyacrylamide-gel electrophoresis. The increased incorporation of mannose into protein seen on culturing pregnant mammary explants in the absence of hormone (Fig. 1c) is probably due to the action of residual endogenous mammatrophic hormones.

Stimulation of the tissue with hormones in culture gives rise to the appearance of at least three major leucine-containing polypeptides. Two of these have mobilities similar to casein and α -lactalbumin, and immunodiffusion studies using a sheep anti-(rabbit casein) antibody (Al-Sarraj *et al.*, 1978) confirm increased concentrations of casein in hormone-stimulated explants. The third major leucine-containing polypeptide has an apparent mol.wt. of approx. 60 000–70 000 and is identical in mobility with the major mannose-containing polypeptide. Preliminary results utilizing concanavalin A confirm that this is a glycoprotein.

The identity of this novel glycoprotein is not known, although its appearance on differentiation of the tissue suggests that it is either a whey protein or a plasma-membrane protein rapidly synthesized to replace that lost as part of the milk-fat-globule membrane. Its presence in the milk-fat-globule membrane would explain its distribution into the soluble fraction on disruption of the tissue. Increased synthesis of the protein would not occur until the tissue is stimulated to synthesize and secrete triacylglycerol. Present work is designed towards isolation of the major [2-³H]mannose-labelled glycoprotein in developing mammary gland, and its location in the cell.

We thank the Agricultural Research Council for a fellowship to J. P. B. and for financial support of this work.

References

- Al-Sarraj, K. A. R., White, D. A. & Mayer, R. J. (1978) *Biochem. J.* **173**, 877–883
- Al-Sarraj, K. A. R., Newbury, J., White, D. A. & Mayer, R. J. (1979) *Biochem. J.* **182**, 837–845
- Burton, W. A., Lucas, J. J. & Waechter, C. J. (1981) *J. Biol. Chem.* **256**, 632–635
- Firestone, G. C. & Heath, E. C. (1981) *J. Biol. Chem.* **256**, 1404–1411
- Forsyth, I. A. & Myers, R. P. (1971) *J. Endocrinol.* **51**, 157–168
- Gibson, R., Kornfeld, S. & Schlesinger, S. (1980) *Trends Biochem. Sci.* **5**, 290–293
- Harford, J. B. & Waechter, C. J. (1980) *Biochem. J.* **188**, 481–490
- Harford, J. B., Waechter, C. J. & Earl, F. R. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1036–1043
- Hubbard, S. C. & Robbins, P. W. (1980) *J. Biol. Chem.* **255**, 11782–11793
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lucas, J. J. & Levin, E. (1977) *J. Biol. Chem.* **252**, 4330–4336
- Mahoney, W. C. & Duksin, D. (1979) *J. Biol. Chem.* **254**, 6572–6576
- Milenkovic, A. G. & Johnson, T. C. (1980) *Biochem. J.* **191**, 21–28
- Morgan, J. F., Morton, H. J. & Parker, R. C. (1950) *Proc. R. Soc. Exp. Biol. Med.* **73**, 1–8
- Seager, M. J., Miquelis, R. D. & Simon, C. (1980) *Eur. J. Biochem.* **113**, 91–96
- Speake, B. K. & White, D. A. (1978) *Biochem. J.* **170**, 273–283
- Speake, B. K. & White, D. A. (1979) *Biochem. J.* **180**, 481–489
- Struck, D. K. & Lennarz, W. J. (1977) *J. Biol. Chem.* **252**, 1007–1013
- Waechter, C. J. & Lennarz, W. J. (1976) *Annu. Rev. Biochem.* **45**, 95–112