Secretion and glycosylation of α -foetoprotein by the mouse yolk sac

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Secretion and glycosylation of α -foetoprotein (AFP) by mouse yolk sac were studied by using yolk-sac explants cultured *in vitro*. Yolk-sac explants rapidly incorporated [35 S]methionine into AFP, whereas radioactively labelled AFP was not found in the medium until 30 min after incubation was initiated. Electrophoretic analysis revealed that microheterogeneity of AFP synthesized in explants increased in parallel with the gestational age of the yolk sacs. The change in microheterogeneity was noted by the formation of increasingly acidic forms. Only the most acidic forms of AFP were found to be present in the medium on each gestational day studied. Tunicamycin reduced the incorporation of glucosamine into AFP with a concomitant decrease in molecular weight and microheterogeneity. However, the relative amount of AFP released into the medium was not altered by the presence of tunicamycin. The presence of underglycosylated AFP in the medium indicates that glycosylation of AFP is not essential for its secretion from the yolk sac. In light of these and previous findings, it is suggested that the glycosylation of AFP may be important for the turnover of this glycoprotein in serum.

The glycosylation of α -foetoprotein (AFP), a glycoprotein synthesized by the mammalian foetal liver and yolk sac (Gitlin & Boesman, 1967; Gitlin & Pericelli, 1970), may be important for its secretion, function or turnover. The mouse yolk sac, which surrounds the foetus during the latter half of gestation, is a simple structure consisting of a layer of endodermal cells, which synthesize AFP (Dziadek & Adamson, 1978), a mesenchymal-cell layer and a mesodermal-cell layer (Padykula *et al.*, 1966). These three cell layers are well separated from each other by basement membranes (Padykula *et al.*, 1966). The yolk sac produces a significant portion of AFP found in foetal serum and amniotic fluid (Ruoslahti & Adamson, 1978). For these reasons, the yolk sac may be a unique experimental system for studying the secretion and glycosylation of AFP.

In yolk-sac explant cultures, AFP constitutes 25% of the total protein synthesis at day 15.5 of gestation (Janzen *et al.*, 1982). Electrophoretic analysis of proteins secreted by the yolk sac reveals that AFP is the major member of a group of proteins that are expressed co-ordinately during gestation (Janzen

et al., 1982). To characterize the secretion of AFP, we examined, in the first phase of our study, the kinetics of AFP secretion and changes in post-translational modifications of newly synthesized and secreted AFP in relation to the time of gestation.

The carbohydrate portion of glycoproteins has been implicated to be important in protein secretion. Previous studies utilizing tunicamycin, an inhibitor of asparagine-linked glycosylation, have indicated that the reduction in glycosylation of glycoproteins parallels a reduction in their secretion (Hickman *et al.*, 1977; Matthews *et al.*, 1982). In the second phase of our study, yolk-sac explants were treated with tunicamycin and the synthesis and release into the medium of AFP were determined both qualitatively and quantitatively to ascertain whether glycosylation is a prerequisite for AFP secretion in mouse yolk-sac explants.

The findings indicate not only that the mouse yolk-sac explant is a useful experimental system to study the regulation of AFP secretion, but also that glycosylation of AFP is not a prerequisite for its secretion.

Materials and methods

Collection of yolk sacs

Swiss-Webster mice were allowed to mate overnight and the morning that a vaginal plug was

Abbreviations used: AFP, α -foetoprotein; SDS, sodium dodecyl sulphate.

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detected was designated day 0.5 of gestation. The yolk sacs were removed aseptically and rinsed several times with phosphate-buffered saline (15 mM-sodium phosphate/150 mM-NaCl, pH 7.2) before being placed in incubation medium.

Assay of protein synthesis and release

Whole yolk-sac explants were incubated in Eagle's Minimal Essential Medium containing 15 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.2, at 37°C for 30 min. The medium was then replaced with prewarmed methionine-free medium and 50 μ Ci of [³⁵S]methionine/ml (935 Ci/mmol; New England Nuclear) and the incubation continued for various times. Yolk-sac explants were removed, rinsed twice with phosphate-buffered saline and lysed as described by Janzen *et al.* (1982). The 'medium' fraction was prepared by adding phenylmethanesulphonyl fluoride and methionine to the incubation medium to a final concentration of 1 mM each, centrifuging in an Eppendorf microfuge at 12800 *g* for 15 min, and freezing the supernatant. The amount of radioactivity incorporated into protein was determined by adding 2.0 ml of 10% trichloroacetic acid to 10–25 μ l of sample to which 150 μ g of bovine serum albumin had been added as carrier. Each sample was placed in a boiling-water bath for 15 min, then cooled in ice. The precipitate was collected in glass-fibre filters, which were dried and the radioactivity determined. Total protein content was determined by the Bio-Rad Protein Assay Kit (Bradford, 1976).

Assay of AFP synthesis and release

The amount of radioactive AFP was determined by immunoprecipitation with AFP-specific antibodies. Rabbits were immunized with mouse AFP and the immunoglobulin G fraction prepared as described by Miura *et al.* (1979). Quantitative precipitation of AFP was achieved by the addition of 40 μ l of this antibody preparation plus 3 μ g of carrier AFP to 200 μ l samples. Reaction mixtures were prepared by adding antibodies and carrier AFP to lysates or medium and incubated at 28°C for 1 h and at 4°C overnight. The precipitates were washed extensively to remove any free isotope and the pellet dissolved in SDS-containing sample buffer (Laemmli, 1970).

Effect of tunicamycin on protein synthesis, release and glycosylation

Day-15.5 yolk-sac explants were incubated in the absence and presence (2 μ g/ml) of tunicamycin (Lilly Research Laboratories) for periods of 2 and 6 h. [³⁵S]Methionine (50 μ Ci/ml, 935 Ci/mmol; New England Nuclear) or D-[1,6(n)-³H]glucosamine hydrochloride (80 μ Ci/ml, 39.6 Ci/mmol; New Eng-

land Nuclear) was added to each explant culture during the last 1.5 h of incubation. Radioactively labelled proteins in the explants and medium were assayed as described above.

Electrophoresis

Alkaline gel electrophoresis was conducted in a slab gel consisting of 7% acrylamide at pH 8.9, prepared as described by Davis (1964). Samples were electrophoresed at 12 mA/gel for the 2 cm stacking gel and 20 mA/gel for the 30 cm separating gel.

SDS/polyacrylamide-gel electrophoresis was performed in a 10% acrylamide slab gel (10 cm $\times$ 1.5 mm) with a 3% acrylamide stacking gel (2 cm) prepared as described by Laemmli (1970). Samples containing an equal amount of radioactivity were adjusted to 3% SDS, 10% glycerol, 5% 2-mercaptoethanol and 62.5 mM-Tris, pH 6.8 (SDS sample buffer) and heated at 90°C for 10 min. Electrophoresis was at 10 mA/gel until the sample had passed through the stacking gel then the current was increased to 20 mA/gel.

Two-dimensional gel electrophoresis was performed as described by Janzen *et al.* (1982). Briefly, samples were made 0.5% in SDS, heated at 70°C for 20 min and urea, Nonidet P40, 2-mercaptoethanol and Ampholines (pH 4–6/pH 6–8/pH 3–10, 2:2:1; Bio-Rad) were added to a final concentration of 9 M, 2%, 5% and 2% respectively. The samples were placed on the isoelectric-focusing gel (0.3 cm $\times$ 15 cm), which consisted of 4% acrylamide and the urea-containing solution described above. Isoelectric focusing was at 350 V for 19 h. The gels were equilibrated for 1 h in SDS sample buffer, layered on to 8–16% linear-gradient-polyacrylamide/SDS slab gels and electrophoresed as described for SDS/polyacrylamide-gel electrophoresis. The radioactive proteins, analysed by each type of electrophoresis, were detected by fluorography (Bonner & Laskey, 1974).

Results

Synthesis and release of AFP by yolk-sac explants

Approx. 100 radioactive proteins were detected from day-15.5 yolk-sac explants that had been incubated in the presence of [³⁵S]methionine for 1 h, lysed and analysed by two-dimensional gel electrophoresis (Fig. 1a). A parallel assay of the medium from which the yolk-sac explants were removed revealed about 15 radioactive proteins (Fig. 1b). A comparison of these fluorograms indicated that proteins found in the medium were present in yolk-sac explants and in similar proportions. Immunoprecipitation with anti-AFP followed by two-dimensional electrophoretic analysis showed that AFP was the predominant protein in the medium (Fig. 1c).

Fig. 1. Two-dimensional polyacrylamide-gel analysis of proteins synthesized and released by day-15.5 yolk-sac explants

Yolk-sac explants were incubated in the presence of [^{35}S]methionine for 1 h and radioactive proteins in (a) the yolk sac and (b) the medium were analysed by two-dimensional polyacrylamide-gel electrophoresis. (c) Two-dimensional gel analysis of AFP immunoprecipitated from the medium.

Kinetics of AFP synthesis and release

The incorporation of [^{35}S]methionine into total protein and AFP was assayed as a function of the duration of incubation. There was a rapid increase in radioactivity in total protein and AFP in the yolk-sac explants during the first hour of incubation (Fig. 2a). In contrast, the amount of radioactivity in total protein and AFP in the medium remained at background levels during the first 30 min of incubation, and increased rapidly thereafter (Fig. 2b). In

the explants the radioactivity in the total protein doubled between 1 and 3 h, whereas the labelling of AFP did not increase appreciably during this period (Fig. 2a). In contrast, the radioactivity in both total protein and AFP in the medium increased 3–4 fold between 1 and 3 h of incubation (Fig. 2b).

Gestational changes in AFP microheterogeneity

The changes in microheterogeneity of AFP in the yolk-sac explants and medium were analysed in relation to the time of gestation by alkaline gel electrophoresis. At day 10.5 of gestation, one major band of AFP (Fp1) was observed. With the advance of gestation, increasingly acidic forms of AFP appeared (Fp2, Fp3, Fp4, Fp5) (Fig. 3b). Analysis of AFP found in the medium was less conclusive, because the AFP bands were not well defined. However, at day 11.5, Fp1 is the major form of AFP detected in the medium, whereas at each of the remaining gestational stages, the most acidic form of AFP found in explants was detected in the medium (Fig. 3c).

Effect of tunicamycin on the synthesis, release and glycosylation of AFP

The effect of tunicamycin on the incorporation of [^{35}S]methionine and [^3H]glucosamine into total protein and AFP of yolk-sac explants was determined. The incorporation of [^{35}S]methionine into total protein in yolk-sac explants was decreased to 79 and 64% of the control values after 2 and 6 h of incubation respectively (Table 1a). However, AFP synthesis, as a percentage of the total protein synthesis, was not significantly changed. The lack of change indicates that tunicamycin exerts a moderate inhibition on the synthesis of all proteins. This result is consistent with those obtained in previous studies (Struck *et al.*, 1978). In contrast, the incorporation of [^3H]glucosamine was inhibited severely by tunicamycin. The radioactivity in total protein decreased to 27 and 11%, and in AFP to 40 and 14% of the control at 2 and 6 h respectively (Table 1b).

The effect of tunicamycin on proteins found in the medium is shown in Table 2. Tunicamycin decreased [^{35}S]methionine incorporation into total protein to 65 and 54% at 2 and 6 h of incubation respectively, but did not significantly affect the relative amount of AFP in the medium (Table 2a). The decrease in total medium protein due to tunicamycin is most likely a reflection of the decreased protein synthesis seen above (Table 1a). Analysis of [^3H]glucosamine-labelled proteins showed decreases in radioactivity in total protein and AFP found in the medium (Table 2b), reflecting the inhibition of glycosylation of these proteins by tunicamycin (cf. Tables 1b and 2b).

Consistent with the molecular effects of tunica-

Fig. 2. Kinetics of AFP synthesis and release

Day-15.5 yolk-sac explants were incubated in medium containing 25 μ Ci of [35 S]methionine/ml for periods up to 3 h. Total protein content and radioactivity in total protein and AFP were determined for the tissue and medium fractions. (a) Yolk-sac explants; (b) medium.

Table 1. Effect of tunicamycin on the synthesis and glycosylation of proteins in mouse yolk sac

Day-15.5 yolk sacs were incubated with [35 S]methionine or [3 H]glucosamine in the absence or presence (2 μ g/ml) of tunicamycin, and radioactivity in total protein and AFP in the yolk-sac explants was measured.

(a) Incorporation of [35 S]methionine

Incubation time (h)	Control		Treated		Percentage of control	
	Total (c.p.m./mg of protein)	AFP (% of total)	Total (c.p.m./mg of protein)	AFP (% of total)	Total	AFP
2	2.35×10^6	16.3	1.85×10^6	19.0	79	116
6	4.30×10^6	18.8	2.77×10^6	17.7	64	94

(b) Incorporation of [3 H]glucosamine

Incubation time (h)	Control		Treated		Percentage of control	
	Total (c.p.m./mg of protein)	AFP (% of total)	Total (c.p.m./mg of protein)	AFP (% of total)	Total	AFP
2	94 300	15.4	25 500	6.2	27	40
6	63 300	19.4	7000	2.9	11	14

mycin on the inhibition of protein glycosylation, AFP recovered from yolk-sac explants and medium after tunicamycin treatment showed a decrease in molecular weight (Fig. 4). Two-dimensional gel analysis of AFP in the medium revealed that the microheterogeneity of AFP also was decreased by tunicamycin treatment (Fig. 5).

Discussion

The present study showed that the release of AFP from mouse yolk-sac explants had characteristics consistent with a secretory mechanism. These characteristics are: (i) the delay of AFP release into the medium, suggesting a requirement for pro-

Fig. 3. *Developmental changes in the microheterogeneity of AFP analysed by alkaline polyacrylamide-gel electrophoresis* Yolk-sac explants from day 10.5 to 17.5 of gestation were incubated in medium containing [35 S]methionine for 1 h, lysed and analysed for radioactive proteins by alkaline polyacrylamide-gel electrophoresis followed by fluorography. (a) The complete height of the 30cm gel in which radioactive proteins synthesized in day-15.5 yolk sac were analysed. (b) Radioactive AFP in yolk sac explants. (c) Radioactive AFP in the medium. For (b), total AFP synthesis, samples containing equal amounts of radioactivity were layered into each slot, whereas for (c), medium AFP, samples containing equal radioactivity in AFP were layered into each slot. The AFP bands are designated as Fp1–Fp5 (Wilson & Zimmerman, 1976), where Fp1 represents the non-sialylated AFP and Fp2–Fp5 contain increasing amounts of sialic acid residues. Abbreviations used: Tf, transferrin; Alb, albumin.

Table 2. *Effect of tunicamycin on the release of total protein and AFP by mouse yolk sac*

These results are derived from the 'medium' fractions of yolk-sac cultures described in Table 1. These fractions were analysed for radioactivity in total protein and in AFP.

		Control		Treated		Percentage of control	
Incubation time (h)		Total (c.p.m./100 μ l of medium)	AFP (% of total)	Total (c.p.m./100 μ l of medium)	AFP (% of total)	Total	AFP
2		108 000	53	70 000	48	65	91
6		144 000	50	78 000	51	54	102

(a) [35 S]Methionine-labelled protein

		Control		Treated		Percentage of control	
Incubation time (h)		Total (c.p.m./100 μ l of medium)	AFP (% of total)	Total (c.p.m./100 μ l of medium)	AFP (% of total)	Total	AFP
2		9000	40	5000	22	56	55
6		7400	30	1000	10	14	33

(b) [3 H]Glucosamine-labelled protein

Fig. 4. *Effect of tunicamycin on the molecular weight of AFP synthesized and released by yolk-sac explants* Yolk-sac explants were incubated in the presence ($2\mu\text{g/ml}$) and absence of tunicamycin for 6 h with [^{35}S]methionine present during the last 1.5 h of incubation. Radioactive proteins were analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography. (a) Proteins detected in the medium. (b) Proteins detected in the yolk-sac explants. 'Total' refers to the total protein and IPP refers to proteins precipitated by antibodies to AFP.

cessing time; (ii) a delay of appearance of total proteins into the medium, suggesting the appearance is not the result of cell lysis; (iii) the qualitative decrease in proteins released from explants, suggesting the release is selective; and (iv) the selective release of the more acidic forms of AFP, forms consistent with those observed in the serum and amniotic fluid. Because there is a progressive increase in the acidic microheterogeneous forms of AFP in the serum and amniotic fluid with increasing periods of gestation (Zimmerman *et al.*, 1976; Wilson & Zimmerman, 1976), it is likely that AFP secretion by yolk-sac explants parallels AFP secretion by this tissue *in vivo*.

The role of the carbohydrate portion of AFP has not been determined; however, it may be important in the secretion, function or turnover of this molecule. To investigate the effects of deleting the carbohydrate moiety on the secretion of AFP, yolk-sac explants were incubated in the presence of

tunicamycin. This antibiotic inhibits the formation of the dolichol intermediate in asparagine-linked glycosylation and produces a protein completely free of carbohydrate (Takahashi & Tamura, 1971; Struck & Lennarz, 1977). In the present experiments, tunicamycin inhibited the incorporation of glucosamine into glycoproteins and specifically reduced the incorporation of glucosamine into AFP.

Electrophoretic analysis of the carbohydrate-free AFP showed that the molecular weight was reduced by 4%. This reduction is consistent with a previous determination of the proportion of carbohydrate to the total molecular weight (Ruoslahti & Seppala, 1979). When analysed by two-dimensional electrophoresis, this low-molecular-weight species consisted primarily of one major isoelectric form, indicating that the sialic acid residues that give rise to the heterogeneity of AFP were not present. These electrophoretic results verify the reduction in glycosylation of AFP by tunicamycin. Two-dimensional

Fig. 5. Effect of tunicamycin on the microheterogeneity of AFP released by yolk-sac explants

Media in which yolk-sac explants were incubated with [35 S]methionine in the presence (b) or absence (a) of tunicamycin were analysed for AFP by using two-dimensional gel electrophoresis. The AFP region of the fluorogram is shown. The pH gradient is from right to left.

analysis of the secreted fraction also showed the presence of carbohydrate-free AFP, indicating that glycosylation of AFP is not an essential requirement for secretion. Furthermore, analysis of the secreted fraction revealed few quantitative differences in the secretion of AFP. We conclude that the carbohydrate portion of AFP is not required for the secretion of this molecule from the developing yolk sac.

Tunicamycin has been widely used to study the role of the carbohydrate portion on glycoprotein secretion. The results have been contradictory. In some cases, such as immunoglobulins A and E and complement factors C2, C4 and factor B, secretion is reduced (Hickman *et al.*, 1977; Matthews *et al.*, 1982), whereas in other studies, such as with transferrin and ovalbumin, there is no effect on secretion (Struck *et al.*, 1978; Keller & Swank, 1978). AFP secretion by mouse yolk-sac explants does not require the presence of carbohydrate and therefore corroborates evidence from those studies implying that the presence of carbohydrate is not necessary for glycoprotein secretion. However, two classes of secreted glycoproteins may exist in which their secretion is either dependent on or independent of glycosylation. Different classes of glycoproteins may exist as a consequence of the different functions of the carbohydrate moiety for each glycoprotein species. In the case of AFP, the carbohydrate portion may be important in either the function of AFP or its turnover in the serum.

Although the function of AFP is not completely defined, one popular and well-studied hypothesis is that AFP acts as a foetal albumin used for the transport of small hydrophobic molecules, mainly fatty acids and bilirubin, in the serum (Carlsson *et al.*, 1980; Vallette *et al.*, 1980). The hydrophobic sites to which these molecules bind were thought to be oestrogen-binding sites (Uriel *et al.*, 1972); however, recent evidence indicates that there is no oestrogen bound to AFP *in vivo* (Vallette *et al.*, 1980; Parmelee *et al.*, 1978), and in competitive binding assays the endogenously bound fatty acid fraction can compete with exogenously added oestrogen (Benassayag *et al.*, 1979). Early studies on oestrogen binding to these hydrophobic sites concluded that the carbohydrate may modulate, but was not necessary for, hormone binding (Aussel *et al.*, 1978). Recent studies have shown the hydrophobic binding sites to be an integral part of the protein portion of AFP (Berde *et al.*, 1979). These studies suggest that the carbohydrate portion of AFP is not essential for its binding function *in vivo*.

The carbohydrate portions of glycoproteins may play an important role in the degradation of serum proteins. The liver contains asialoprotein receptors that bind glycoproteins that have lost their terminal sialic acid residues (Ashwell & Hartford, 1982). These receptor-bound molecules are endocytosed and degraded. Because AFP is present in the serum only during foetal development and decreases to very low levels in the adult, a mechanism must be present which would remove AFP relatively quickly from the serum. Albumin, its counterpart, which is present in the serum throughout the foetal and adult stages, is not glycosylated and may therefore not require so rapid a degradatory mechanism. Consistent with this hypothesis is the difference in serum half-life between human albumin, which is 19 days (Peters, 1975), and human AFP, which is 4–6 days (Ruoslahti & Seppala, 1979). Furthermore, in the mouse, desialylated AFP has been shown to bind to the asialoprotein receptors, and there is an inverse relationship between the presence of serum AFP and the number of these receptors (Wilson & Zimmerman, 1976). The carbohydrate portion of AFP may therefore be required for the precise regulation of serum concentration of AFP.

In conclusion, mouse yolk-sac explants were used to study the secretion of AFP and the role of the carbohydrate moiety in AFP secretion. Mouse yolk-sac explants appear to secrete AFP in a manner similar to that found *in vivo*. The carbohydrate portion is not an essential requirement for AFP secretion by these tissues. This finding, along with previous evidence, suggests that the carbohydrate portion of AFP may be important in the regulation of AFP levels in the serum.

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