Two free isoforms of ovine glycoprotein hormone α-subunit strongly differ in their ability to stimulate prolactin release from foetal pituitaries

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Abstract

α-Subunit dissociated from glycoprotein hormones has been previously shown to stimulate rat pituitary lactotroph differentiation and proliferation. However, whether the free form of the α-subunit (free α) can also play such a role is not known. To test whether free α may act on prolactin (PRL) release from ovine foetal pituitaries, this molecule was purified and two major isoforms, αA and αB were isolated. Free αA was found to be more acidic and more hydrophobic than both free αB and ovine LH α-subunit (oLHα). Free αA and oLHα exhibited a molecular mass of 14 kDa as determined by mass spectrometry, whereas free αB displayed a molecular mass of only 13·5 kDa because of its truncated N-terminus. All three α molecules bear mature-type N-linked saccharide chains including N-acetyl galactosamine residues but none of them contains O-linked oligosaccharide.

The free αA isoform, more than the oLHα, was able to stimulate PRL release from ovine foetal pituitary explants in culture, whereas the free αB isoform displayed no activity. Moreover, the free αA and αB isoforms were able to recombine with the ovine LH β-subunit (oLHβ). The free αB/oLHβ, and the oLHα/oLHβ dimer were 4-fold more active than the free αA/oLHβ dimer in a specific LH radioreceptor assay and in the stimulation of testosterone release from rat Leydig cells.

The present study demonstrates that the two free α isoforms of ovine glycoprotein hormones exhibit distinct efficiencies in stimulating PRL release from ovine foetal pituitaries. Moreover, despite their identical ability to recombine with the oLHβ, the free α isoform, which is the most efficient on PRL release, is the least efficient in conferring LH activity on the α/β dimer.

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Introduction

The glycoprotein hormones, luteinising hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and chorionic gonadotrophin (CG) consist of two non-covalently linked subunits named α and β (Pierce & Parsons 1981). The combination between the common α-subunit and the hormone specific β-subunit confers its biological activity to the α/β dimer (Combarnous 1992). In addition to the combined forms, a large amount of a free form of the α-subunit (free α) has been found in the adult pituitary of different species (Hagen & McNeilly 1975, Prentice & Ryan 1975, Bloomfield *et al.* 1978, Hoshina & Boime 1982, Bousfield & Ward 1984), as well as in human placenta (Vaitukaitis 1974) and extraembryonic coelomic fluid (Iles *et al.* 1992).

Little is known about the specific biological function of the free α and more particularly concerning the relationships between structural differences and biological activity. The effect of free α has only been studied in human placenta where it is capable of stimulating the secretion of prolactin (PRL) from human decidual cells in primary cultures with the same efficiency as the human CG (hCG) α-subunit (Blithe *et al.* 1991). In women, during the menstrual cycle, α-subunit acts synergistically with progesterone to induce decidualisation of human endometrial stromal cells (Moy *et al.* 1996). Moreover, some studies have reported that dissociated LH α-subunit from glycoprotein hormones stimulates lactotroph differentiation and proliferation in rat pituitary, either in the foetus or during the postnatal period (Bégeot *et al.* 1984, Van Bael & Denef 1996). Whether free α, which may exhibit structural differences compared with dissociated LH α-subunit, has a similar effect is not known.

Indeed, free α and α-subunit display different biochemical features mainly resulting from their distinct carbohydrate compositions (Kourides *et al.* 1980*a*, Parsons *et al.* 1983, Blithe & Nisula 1985, Kawano *et al.* 1988,

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Blithe 1990*a*, Bousfield & Ward 1992). Hence, human placental free α possesses two more complex N-linked oligosaccharides and occasionally an additional O-linked oligosaccharide (Cole *et al.* 1984, Corless *et al.* 1987, Peters *et al.* 1989, Blithe 1990*a*). However, differences in the glycosylation between placental free α and dissociated hCG α-subunit do not seem to be important for their ability to stimulate PRL. Bovine and equine pituitary free α bear an additional O-linked oligosaccharide on threonine 43 (Parsons *et al.* 1983, Ward *et al.* 1989). In all cases, these differences in carbohydrate structures prevent the free α molecule from combining *in vitro* with the β-subunit (Parsons & Pierce 1984, Saccuzzo-Beebe *et al.* 1989, Blithe 1990*b*, Blithe & Iles 1995). To our knowledge, no data are available concerning the structure and/or the function of ovine pituitary free α.

The aim of the present work was first to determine whether ovine free α molecules exhibit a specific function in ovine foetal pituitary compared with the ovine LH α-subunit (oLHα). The second objective was to establish whether free α molecules and oLHα display different structural features that may be correlated to their respective functions

Materials and Methods

Purification of ovine free α isoforms

One kilogram of frozen pituitaries from adult sheep was finely ground in cold acetone (− 20 °C) using an Ultra Turax mixer and the resulting material was filtered through filter paper. Acetone from the retentate was evaporated for 24–36 h under air circulation. The dry 'cake' was then reduced to fine powder using a coffee grinder. The yield was 182 g acetone powder per kg fresh pituitaries. The acetone powder was vigorously dispersed in 1 litre 0·15 M ammonium sulphate pH 4·4 at 4 °C, using an Ultra Turax mixer for 10–15 s and was then agitated for 90 min in a cold-room. After centrifugation (3000 ***g***, 35 min, 4 °C) the supernatant (S1) was recovered and the pellet was re-extracted in 1 litre 0·15 M ammonium sulphate pH 5·7 at 4 °C for 1 h. The supernatant recovered after centrifugation as above (S′1) was added to S1. Metaphosphoric acid was then added to the supernatant S1+S′1 at 4 °C under efficient agitation until pH 3·2 was reached. After immediate centrifugation, the supernatant was recovered (S2; 1·7 l) and solid ammonium sulphate was added up to 1·2 M final concentration. After 1 h at 4 °C and centrifugation, the resulting pellet was dialysed and lyophilised (precipitate at 1·2 M: 1·626 g) whereas the supernatant (1·8 l) had ammonium sulphate added up to 2·0 M final concentration. After 1 h at 4 °C and centrifugation, the resulting pellet was dialysed and lyophilised (precipitate at 2·0 M: 1·471 g) whereas the supernatant (1·95 l) was brought to 3·6 M ammonium sulphate. After overnight incubation at 4 °C, the pellet (precipitate at 3·6 M) was recovered by centrifugation. The fractions containing free α isoforms were determined at each step of purification by an enzyme-linked immunosorbent assay (ELISA) specific for the oLHα. Free α was found only in the 3·6 M precipitate, which was dissolved in 220 ml 1 M ammonium sulphate and loaded onto a phenyl-Sepharose column (volume 200 ml; Pharmacia, Orsay, France) equilibrated with 1 M ammonium sulphate in 50 mM ammonium bicarbonate. After elution of the unretained fraction, the column was eluted stepwise with 0·5 and 0·2 M ammonium sulphate in 50 mM ammonium bicarbonate, then by 50 mM ammonium bicarbonate alone and finally with 50% ethylene glycol in 50 mM ammonium bicarbonate. Free α was mainly found in the fraction eluted with 0·5 M ammonium sulphate (also containing FSH) and in the fraction eluted with 0·2 M ammonium sulphate (containing very low amounts of FSH and LH). Free α from these fractions was isolated by low pressure chromatography on a Sephacryl S-200 column (2 × 85 cm, Pharmacia) and then by a final size-exclusion HPLC (SE-HPLC) on a Waters (St Quentin en Yvelines, France) chromatograph (625 LC system) using two Superdex 75 high resolution columns (Pharmacia) in tandem equilibrated with 100 mM ammonium bicarbonate, at a flow rate of 0·4 ml/min.

Purification of dissociated oLHα and β subunits

Ovine LH CY 1083 purified in our laboratory (3·5 × NIH LH S1) was dissociated into its constituent subunits by incubation in 0·1 M sodium phosphate/citrate buffer pH 4·0, for 5 min at 55 °C at a final concentration of 1 mg/ml. The ovine LHβ-subunit (oLHβ) was precipitated by 3 M NaCl final concentration for 1 h at 4 °C (Sairam & Li 1974). After centrifugation (3000 ***g***, 20 min), the supernatant was recovered and the pellet was re-precipitated by 3 M NaCl, for 1 h at 4 °C. The supernatant recovered after centrifugation as above was added to the first supernatant, dialysed against water and the oLHα was recovered after lyophilisation. The pellet (containing oLHβ) was re-suspended in 0·1 M sodium phosphate/citrate buffer pH 4·0, dialysed and lyophilised. To remove eventual oLH contaminant, oLHα and oLHβ were purified by SE-HPLC (as above). LH contamination, estimated by an *in vitro* bioassay specific for LH activity (see below), was less than 1·5% and 1% for oLHα and oLHβ respectively.

oLHα ELISA

The fractions containing free α were determined at each step of purification by a competitive ELISA specific for the dissociated oLHα. Microtitration plates (96 wells; CML, Angers, France) were coated with purified oLHα (10 ng/100 µl in 0·1 M carbonate buffer, pH 9·6) for 1 h at 37 °C and then overnight at 4 °C. After washing and saturation

with 0·2% BSA in 0·1 M PBS/0·1% Tween 20, an antibody against dissociated porcine LHα (1/10 000) was added to the wells together with the fraction at varying concentrations, for 1 h at 37 °C. Antibody excess was then washed off with PBS/Tween and the bound antibody fraction was detected with horseradish peroxidase-labelled goat anti-rabbit IgG (1/1000) (Sanofi/Pasteur Diagnostics, Marnes-la-Coquette, France). After 1 h at 37 °C and washing, peroxidase activity was measured with hydrogen peroxide and tetramethylbenzidine (Sigma, St Quentin Fallavier, France) as substrates (Bos *et al.* 1981). After stopping the reaction with 1 M H_2SO_4, the signal was measured with an ELISA reader (Multiskan Plus; Labsystemsx, Helsinki, Finland) at 450 nm. The minimum detectable concentration of oLHα was 10 ng/ml. Mean intra- and inter-assay coefficients of variation (CV) were less than 8·3 and 7·8% respectively. Cross-reaction with highly purified oLH CY1085 (3·45 × NIH LH S1), oFSH CY1767 (32 × NIH FSH S1) and oTSH (NIADDK oTSH-12) was 3·5, 0·2 and 8·8% respectively.

SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE under reducing conditions was performed in 15% polyacrylamide gel (acrylamide/bis 29:1; Bio-Rad, Richmond, CA, USA), pH 8·8 according to the method of Laemmli (1970). The samples were dissolved in a solution containing 4% SDS and 10% β-mercaptoethanol and heated to 100 °C for 5 min. Proteins were stained with Coomassie blue.

Electrospray ionisation mass spectrometry (ES/MS)

ES/MS was used to compare the masses of free α and oLHα. Lyophilised purified free α was dissolved in water/acetonitrile (50/50, v/v) containing 0·2% formic acid. Sample volumes of 3 μl (0·3 μg free α) were introduced by flow injection into the electrospray source at a flow rate of 3 μl/min. The calibration of the mass/charge scale was carried out by prior analysis of erythropoietin.

Polyacrylamide gel electrophoresis (PAGE)

The charge heterogeneity of the purified material was estimated by PAGE on 7% polyacrylamide gel, pH 9·5 according to the method of Liao *et al.* (1969) with minor modifications.

Reverse-phase HPLC (RP-HPLC)

Differences in hydrophobic interactions were determined using a reverse-phase C18 column (5 μ Deltapack; Waters) with a linear 80 min gradient from 0 to 90% acetonitrile in 0·1% trifluoroacetic acid (TFA) at a flow rate of 0·5 ml/min, at 40 °C.

N-terminal amino acid sequence

N-terminal amino acid sequence was determined with a Beckman (Villepinte, France) automated Edman protein sequencer after SDS-PAGE in 15% polyacrylamide gel with β-mercaptoethanol and transfer to a polyvinylidene difluoride (PVDF) membrane.

Carbohydrate analysis

After methanolysis of oligosaccharides of free α isoforms with 0·5 M methanol/HCl for 24 h at 80 °C and peracetylation, neutral and amino sugars were analysed on a gas chromatograph (Delsi, DI 700; Rueil Malmaison, France) equipped with a flame ionisation detector.

Neuraminidase treatment

To study the role of sialic acids in charge heterogeneity, the purified free α and oLHα (20 μg/10 μl) were treated with 5 mU/mg protein of neuraminidase from *Clostridium perfringens* (Type X; Sigma) in 20 mM sodium phosphate buffer, pH 6·0, for 4 h at 37 °C. Fetuin (Sigma) was used as a control of desialylation. Samples were analysed by PAGE in 7% polyacrylamide gel.

Peptide-N-glycosidase F (PNGase F) and O-glycosidase treatments

For characterisation of the free α isoforms and oLHα oligosaccharides, the three α molecules were incubated with PNGase F (Boehringer Mannheim, Meylan, France) following neuraminidase and O-glycosidase (endo-α-N-acetylgalactosaminidase; France Biochem, Meudon, France) treatments. The proteins were dissolved in 0·1 M sodium phosphate, 1 mM EDTA, 10 mM dithiothreitol, 0·5% SDS, pH 6·0 at a final concentration of 5 μg/μl and boiled for 3 min. After cooling, Nonidet P-40 and phenylmethylsulphonylfluoride were added at final concentrations of 0·7% and 1 mM respectively. The denatured α molecules were then digested with 50 mU PNGase F/μg protein at 37 °C for 24 h at a concentration of 2 μg/μl. Samples were analysed by SDS-PAGE in 15% polyacrylamide gel under reducing conditions as above. For O-glycosidase treatment, half of the PNGase F-treated samples were first incubated with 5 mU neuraminidase/mg protein for 4 h at 37 °C. Then, the pH was adjusted to 4·5 with 0·1 M phosphate/citrate buffer pH 3·5 and the samples were incubated with 0·05 mU O-glycosidase/μg protein at 37 °C for 24 h and analysed by SDS-PAGE.

Recombination with oLHβ

The reactions were performed by incubating equimolar amounts of either oLHα, free αA or αB with oLHβ in

0·1 M ammonium bicarbonate, for 24 h at 37 °C, at a final concentration of 3·3 mg/ml. The percentage of reconstituted dimers was estimated by SE-HPLC on two Superdex 75HR columns in tandem by measuring the ratio between the heterodimer peak area and the total peak area (PDA software; Waters).

LH radioligand assay

Receptor binding activity of reconstituted dimers was determined on rat testicular membranes as previously described (Guillou *et al.* 1985). Rat testicular membranes were incubated overnight with a fixed amount of ovine ^{125}I-LH (30 000 c.p.m.) and increasing amounts of reconstituted dimers at room temperature. After incubation, bound and free hormone were separated by centrifugation (2000 ***g***, 45 min at 4 °C), the supernatant was discarded and the radioactivity of the pellet was counted in a gamma counter (Multidetector RIA system 95424; Packard, Rungis, France).

In vitro *bioassay for LH activity*

Reconstituted dimers bioactivity was assayed *in vitro* by determining testosterone release from cultured rat Leydig cells. Rat Leydig cells were isolated as previously described (Guillou *et al.* 1985) and incubated for 4 h at 34 °C with increasing concentrations of reconstituted dimers. Testosterone concentration in the culture medium was estimated by a specific RIA (Guillou *et al.* 1985).

Culture of ovine foetal pituitary explants

Sheep foetuses were obtained by Caesarean section at 50 days of gestation from crossbred Ile de France × Romanov ewes. Pituitaries were removed after decapitation from female foetuses, weighed and collected in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing transferrin (10 μg/ml), ascorbic acid (17·6 μg/ml) and antibiotics. Each pituitary was cut in half in the sagittal plane under a microscope and the posterior pituitary was removed. Cultures of explants were established according to the method described by Watanabe *et al.* (1973) with minor modifications. Briefly, each half pituitary was placed on a round piece of cellulose acetate (13 mm diameter) (Millipore, St Quentin en Yvelines, France) on stainless mesh platforms in a culture dish. The culture dishes were placed at 37 °C in a 5% CO_2 atmosphere. Explants were maintained in culture over 8 days in DMEM containing 0·2% human serum albumin alone or with free αA, free αB or oLHα at a concentration of 10^{-7} M. The oLHβ and oLH (10^{-7} M) were used as negative controls. The medium was changed every day and assayed for PRL by a specific enzyme immunoassay. Each condition was run in duplicate.

Competitive enzyme immunoassay for PRL

PRL released into the culture medium was assayed using a specific competitive enzyme immunoassay. Microtitration plates (96 wells, CML) were coated with 100 ng/100 μl oPRL CY 1101 III purified in our laboratory (22 IU/mg). The anti-bovine PRL serum (Dubois 1971) at 1/160 000 initial dilution was preincubated with increasing concentrations of standard oPRL (oPRL CY 1101 III) or the samples for 2 h at 37 °C. One hundred microlitres of the mixture were added to the wells for 1 h at 37 °C and, thereafter, the procedure was similar to that used for oLHα ELISA (see above).

The minimum detectable concentration of oPRL was 3 ng/ml. Mean intra- and inter-assay CVs were less than 8·0 and 11·5% respectively. Cross-reaction with ovine growth hormone (NIADDK oGH-I-4) was 0·2%.

Statistical analysis

Values are expressed as the mean ± S.E.M. of three to five independent experiments. After $\log_{10}$ transformation of the data, the effect of the treatment throughout the culture was determined by ANOVA with repeated measures followed by two-factor ANOVA for individual comparisons. Experiment effect was taken into account and no interaction between treatment and experiments was observed.

Results

Purification of the free αA and αB isoforms

Ovine free α molecules were detected by a specific immunoassay in fractions II and III of the hydrophobic interaction chromatography, suggesting that free α molecules with different hydrophobicity (III>II) were separated.

After the final step of SE-HPLC, 9·2 mg free αA (from fraction III) and 16 mg free αB (from fraction II) isoforms were obtained from 1 kg pituitaries.

Structural characterisation of the free αA and αB isoforms

A single band was revealed in SDS-PAGE (data not shown) as well as a single peak in SE-HPLC (Fig. 1a and b) for both free αA and αB suggesting that they consisted of highly purified free α isoforms.

Size In both SE-HPLC (Fig. 1a) and SDS-PAGE (data not shown), free αA and oLHα displayed similar size whereas free αB exhibited a slightly smaller apparent size (Fig. 1b). This result was confirmed by ES/MS analysis (Fig. 2) since the oLHα, free αA and free αB molecular masses were respectively in the range of 13 200–14 500 Da (maximum 13 937), 13 600–14 500 Da (maximum 14 019) and 13 017–14 147 Da (maximum 13 514).

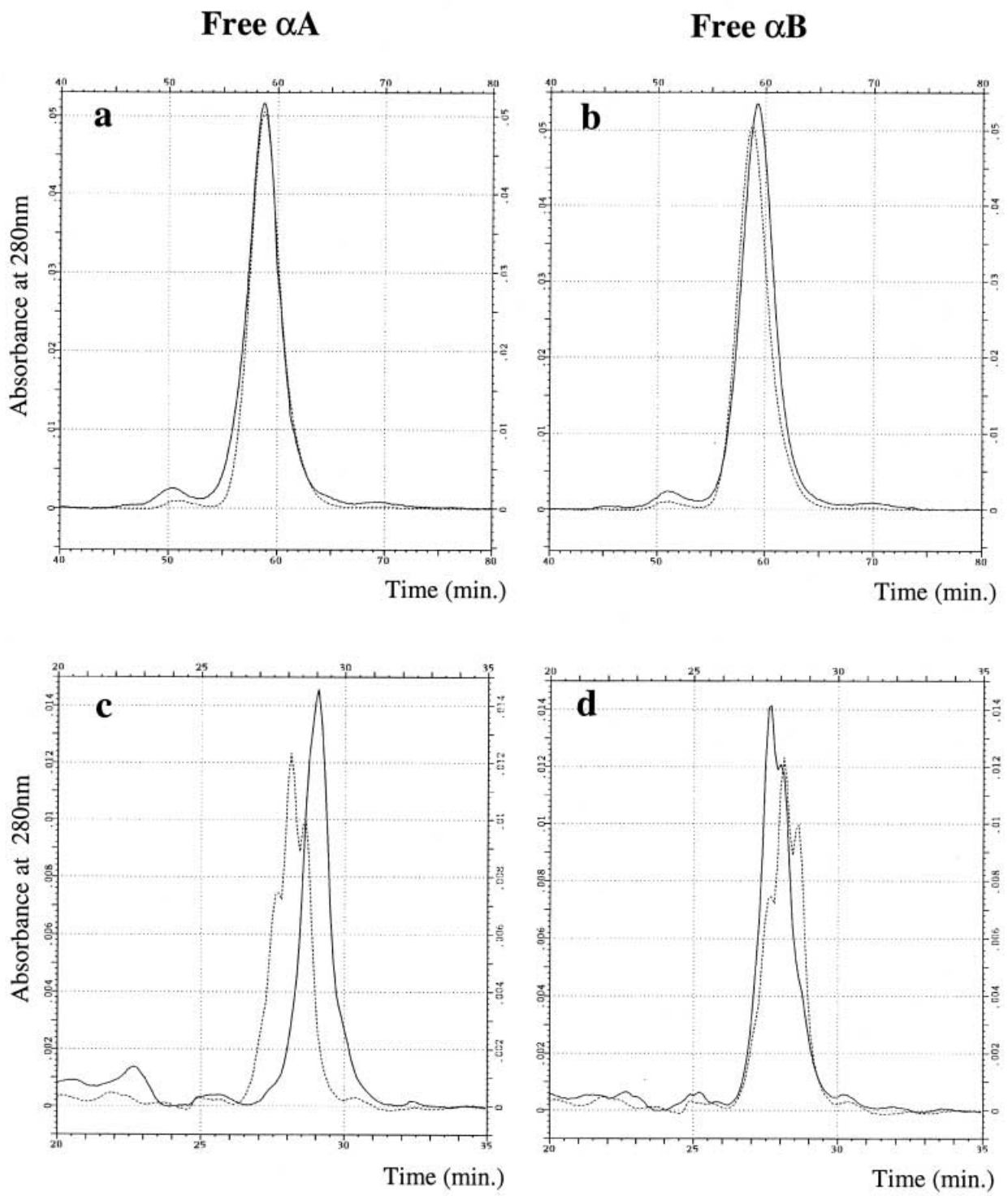
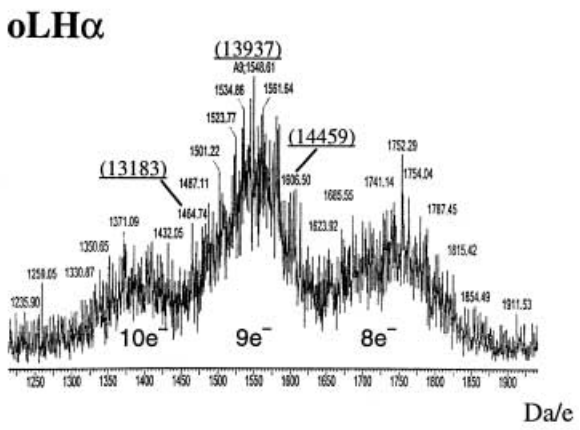
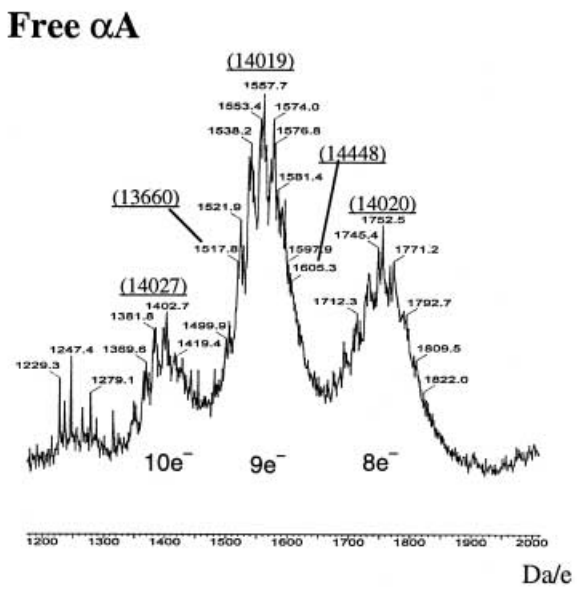


Figure 1 (a, b) SE-HPLC of the two free α isoforms (solid line) and oLHα (broken line) on two Superdex 75 HR columns in series. The elution buffer was 100 mM ammonium bicarbonate at a flow rate of 0·4 ml/min. (c, d) RP-HPLC of the two ovine free α isoforms (solid line) and oLHα (broken line) on a C18 column using a linear gradient from 0 to 90% acetonitrile in 0·1% TFA, with a flow rate of 0·5 ml/min at 40 °C.





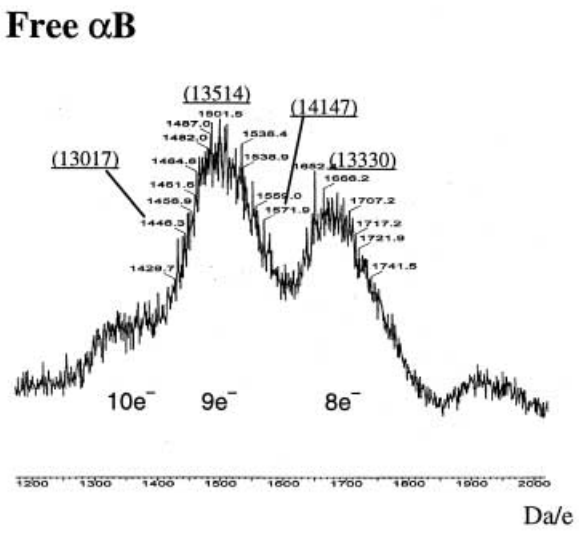


Figure 2 ES/MS spectra of oLHα, free αA and free αB. Da/e represents mass/charge (m/z) scale and underlined numbers show the molecular mass (Da) of the major form and the range of masses for the population of molecules.

	1 5
oLHα	Phe – Pro – Asp – Gly – Glu – Phe – Thr – Met – Gln – Gly...
Free αA	Phe – Pro – Asp – Gly – Glu – Phe – Thr – Met – Gln – Gly...
Free αB	Phe – Pro – Asp – Gly – Glu – Phe – Thr – Met – Gln – Gly... Gly – Glu – Phe – Thr – Met – Gln – Gly... Met – Gln – Gly...

Figure 3 N-terminal amino acid sequence of the two free α isoforms compared with oLHα determined after SDS-PAGE in 15% polyacrylamide gel under reducing conditions and transfer to a PVDF membrane.

This represents a difference of 423 Da between free αB and oLHα on the one hand and of 505 Da between free αB and free αA in the other hand.

Hydrophobicity In keeping with its behaviour in hydrophobic interaction chromatography, free αA was more retained in RP-HPLC on a C18 column than free αB (Fig. 1c and d). The oLHα was found to exhibit retention times similar to that of free αB (Fig. 1d).

N-terminal heterogeneity of free α subunits The N-terminal amino acid sequences of free αA and αB were determined and compared with that of the oLHα. As shown in Fig. 3, free αA displayed a complete homogeneous N-terminal sequence similar to that of oLHα whereas free αB appeared to be a mixture of three polypeptides with different N-terminal regions starting either at Phe[1], Gly[4] or Met[8]. The N-terminal isoforms starting at Gly[4] and Met[8] were the prominent forms in free αB. No contaminating sequence was detected by this technique, confirming that the preparations consisted of highly purified free α isoforms.

Carbohydrate analyses and charge heterogeneity Carbohydrate compositions established by gas-phase chromatography (data not shown) revealed that oligosaccharide chains in the two free α isoforms were of mature type with the detection of sialic acid, N-acetyl galactosamine and N-acetyl glucosamine residues but not polymannose. Carbohydrate compositions of the two free α isoforms did not exhibit significant differences. Figure 4 shows that free αA displayed more acidic charge isoforms than free αB and oLHα with two major bands. Free αA

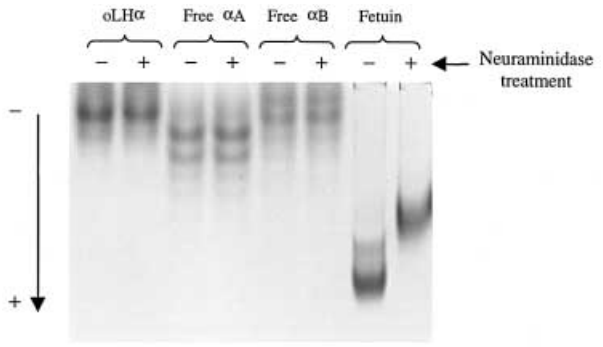


Figure 4 Native PAGE (7% acrylamide, pH 9·5) of oLHα, free αA and free αB (20 µg) without (−) or with (+) neuraminidase treatment (4 h , 37 °C). Fetuin was used as control of desialylation. The proteins were stained with Coomassie blue.

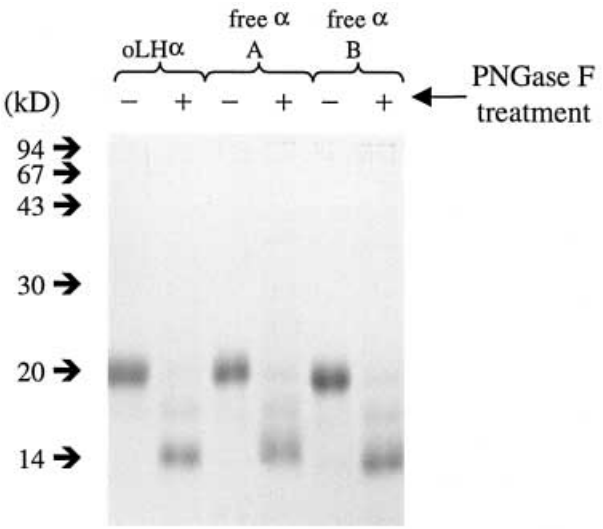


Figure 5 SDS-PAGE (15% polyacrylamide gel) of oLHα, free αA and free αB (3 µg) without (−) or with (+) PNGase F treatment (24 h, 37 °C). The proteins were stained with Coomassie blue.

and αB were more heterogeneous in charge than the oLHα. There was no difference in migration between neuraminidase-treated and non-treated free α molecules (Fig. 4).

Deglycosylation After PNGase treatment (Fig. 5), the majority of the three α molecules were converted to faster migrating forms in SDS-PAGE. A major band, corresponding to an apparent molecular mass of approximately 15 kDa, probably represented α molecules after removal of their two N-oligosaccharide chains. The faint band with intermediate mobility on the SDS gel, corresponding to a molecular mass of approximately 18 kDa, probably represented α molecules after removal of only one N-oligosaccharide chain. The difference in apparent molecular mass between the free αB isoform and both free αA isoform and oLHα persisted after PNGase F treatment.

A subsequent treatment with O-glycosidase (data not shown) had no effect at all on the mobility of any three α molecules suggesting that none of them contains O-linked oligosaccharide.

Functional characterisation of free αA and αB isoforms

Receptor binding and *in vitro* bioactivity of α/β heterodimers Table 1 shows the percentage of recombined dimer after 24 h at 37 °C using SE-HPLC. The two free α isoforms were able to recombine with oLHβ with a slightly lower efficiency than that of the oLHα. Both oLHα/oLHβ and free αB/oLHβ dimers were able to bind to rat testis LH receptors with a similar affinity (ED_{50}: 3·0 ng/100 µl) whereas the free αA/oLHβ dimer was 3·5-fold less potent (ED_{50}: 10·5 ng/100 µl) (Fig. 6).

Recombined oLHα/oLHβ and free α/oLHβ were examined for their ability to stimulate *in vitro* secretion of testosterone in cultured rat testicular Leydig cells. oLHα/oLHβ and free αB/oLHβ dimers stimulated testosterone release to the same extent (Fig. 7) whereas the free αA/oLHβ dimer was 4-fold less effective. The bioactivity/binding ratios (Table 1) were similar for the three recombined molecules indicating that the differences in the α

Table 1 Characteristics of relative combination, binding and stimulating activities of α/β dimers resulting from 24 h incubation of either oLHα, free αA or αB with equimolar amounts of oLHβ. The percentage of associated dimer was estimated by SE-HPLC as described in Materials and Methods. The oLHα/oLHβ dimer was given as reference (*). The binding and stimulating activities of free αA/oLHβ and free αB/oLHβ were calculated according to their ED_{50} compared with oLHα/oLHβ. The experiment was repeated three times. LH receptor binding activity was determined by radioreceptor assay using ^{125}I-labelled oLH and rat testicular membranes. *In vitro* LH bioactivity was determined by stimulation of testosterone release from cultured rat testicular Leydig cells

	Associated dimer	Binding (B)	Stimulation (S)	S/B
oLHα/oLHβ	100*	100*	100*	1
Free αA/oLHβ	87	29	24	0·83
Free αB/oLHβ	87	92	120	1·3

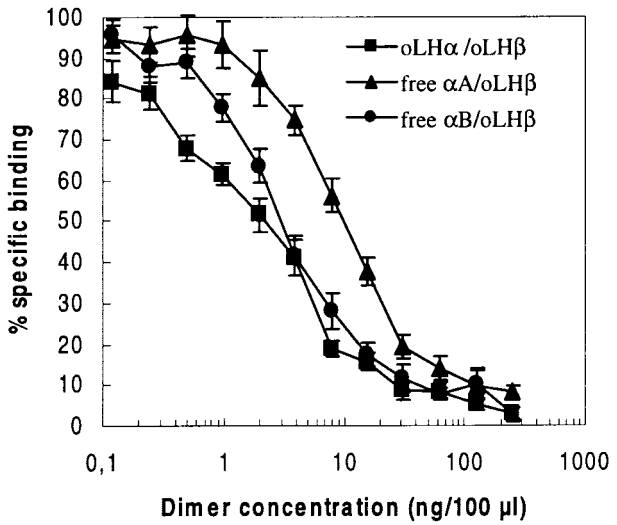


Figure 6 Binding activity of oLHα/oLHβ and free α/oLHβ dimers to membranes isolated from rat testis. The membranes were incubated overnight at room temperature with ^{125}I-labelled oLH in the absence or presence of varying concentrations of either unlabelled recombined oLHα/oLHβ or free α/oLHβ dimers. Values are mean ± S.E.M. of three independent experiments.

moieties do not interfere with signal transduction of the heterodimers upon binding.

Effects of free αA, αB isoforms and oLHα on PRL release in foetal pituitary explants Figure 8a shows that continuous treatment of ovine female foetal pituitary explants with free αA for 8 days leads to stimulation of PRL release ($P<0{\cdot}001$ compared with the control). When the pituitary explants were chronically treated with free αB, PRL release was not different from the control (Fig. 8b). A slight stimulatory effect of the oLHα (Fig. 8c) was observed at the beginning of the culture ($P<0{\cdot}05$) but was not maintained. The oLHβ (data not shown) and oLH (Fig. 8d) have no effect on PRL release compared with the control.

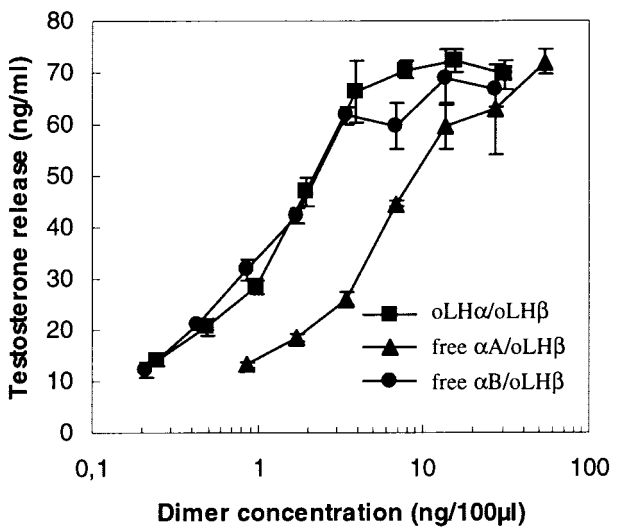


Figure 7 *In vitro* bioactivity of oLHα/oLHβ and free α/oLHβ dimers in rat Leydig cell assay (4 h, 34 °C). Testosterone released into the culture medium was measured by a specific RIA. Each point is the mean ± S.E.M. of one representative experiment.

Discussion

Our results demonstrate that differences in the stimulatory effect on PRL release of free α isoforms are related to biochemical features. Moreover, these features do not prevent the recombination of the free α isoforms with oLHβ. However, the reconstituted dimers have differential LH activity depending on the free α considered.

Two free α isoforms were isolated from ovine pituitaries on the basis of their hydrophobic properties, suggesting that the ovine free α molecule exhibits polymorphism as previously described for the α-subunit of glycoprotein hormones (Pierce & Parsons 1981). Although large amounts of free α molecules were obtained, no free β-subunit was found, in agreement with studies in other species, indicating that the α-subunit is synthesised in large excess over the β-subunit (Prentice & Ryan 1975, Kourides *et al.* 1980*b*, Hoshina & Boime 1982). This excess therefore favours the non-covalent association with β-subunit. Hence, the amount of α-subunit does not limit the assembly with the β-subunit. However, an additional physiological role of this pituitary free α may be hypothesised. Indeed, one recent study suggests that free α from human pituitary might play a role during the menstrual cycle in women since α-subunit from dissociated hCG acts synergistically with progesterone to induce decidualisation of endometrial cells (Moy *et al.* 1996). Moreover, dissociated TSH α-subunit stimulates PRL release from myometrium of post-menopausal women (Stewart *et al.* 1995) and α-subunit from dissociated FSH increases PRL levels released from bullfrog pituitary cells in culture (Oguchi *et al.* 1996).

Our study demonstrates that one of the two purified ovine free α isoforms (free αA) has a biological function of its own in stimulating PRL release from ovine foetal pituitary explants. This result reinforces the view that free α might play a paracrine role in the lactotroph function during pituitary development as previously suggested with heterologous α-subunits from dissociated LH in rat pituitary explants (Bégeot *et al.* 1984) or reaggregate cells (Van Bael & Denef 1996). In addition, our results reveal that the three α molecules (oLHα and the two free α isoforms) exhibit largely different potencies in stimulating PRL release from foetal pituitary explants. Indeed, free αB appears to be devoid of activity, oLHα shows a transient effect, whereas the free αA isoform exhibits significant activity over the 8 days of stimulation. In cultures of placental decidual cells, the only previous study which

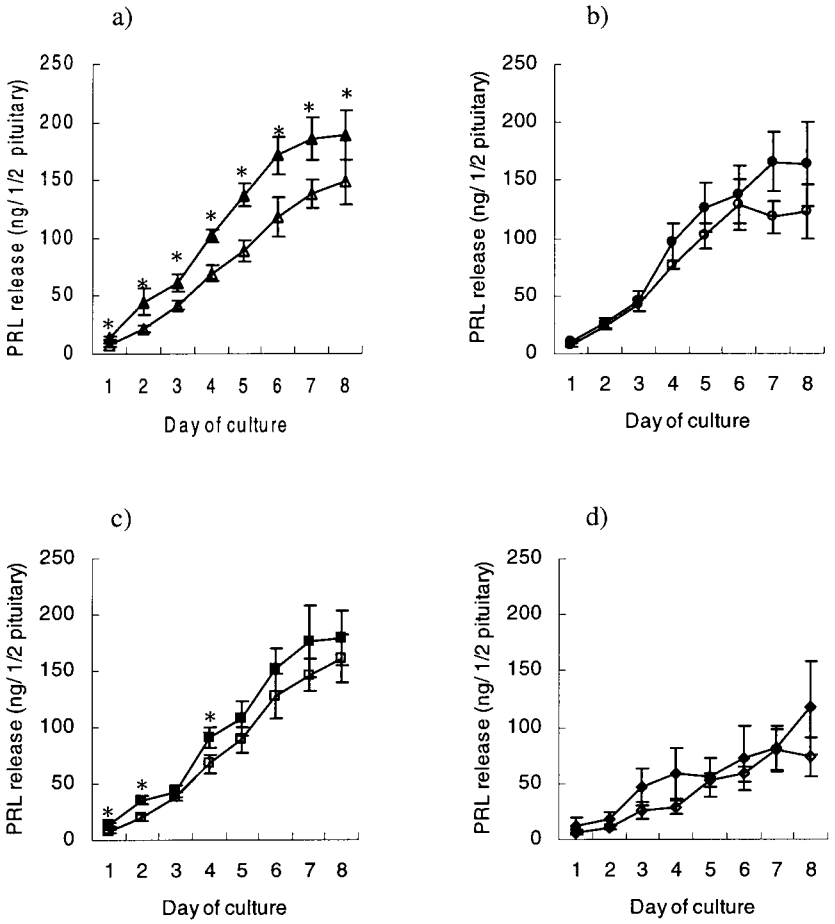


Figure 8 Effects of continuous treatments with free αA, free αB, oLHα or oLH on PRL release from ovine foetal pituitary explants in culture. Female pituitaries were collected at 50 days of gestation and cultured for 8 days without (open symbols) or with (closed symbols) free αA (a), free αB (b), oLHα (c) or oLH (d) at 10^{-7} M. Culture media were changed everyday and assayed for PRL by a competitive enzymatic immunoassay. Values are means ± S.E.M. of three to five experiments. The effect of the treatments during the 8 days of the culture was estimated by ANOVA with repeated measures. *P<0·05 (compared with the control at each day, by two-factor ANOVA).

compared the effects of hCG α-subunit and placental free α showed that both of them display the same efficiency in stimulating PRL release (Blithe *et al.* 1991). Similar to human placenta, treatment of foetal pituitary explants with oLHβ or oLH has no or little effect on PRL release (Blithe *et al.* 1991). These results suggest that the stimulation of PRL release is specific for the α molecule in a free state, since oLH displays no activity. Furthermore, comparison of the activity of the three α molecules as part of reconstituted α/β heterodimers clearly shows that ovine free α molecules are capable of recombination with β-subunit in contrast to bovine and equine pituitary free α (Parsons *et al.* 1983, Bousfield & Ward 1992). However, the dimers have different potencies. Indeed, the reconstituted free αB/oLHβ dimer was as potent as the oLHα/oLHβ dimer and displayed a 4-fold higher efficiency than the reconstituted free αA/oLHβ dimer in binding to the LH receptor and in stimulating testosterone secretion.

Taken together, these results reveal that the free α isoform, which is the most efficient on PRL release, is the least efficient at conferring LH activity on the α/β dimer. The fact that only one of the two free α molecules had an effect on PRL release strongly suggests the existence of some specific post-translational processing leading to α molecules with different activities either in the free state (PRL release stimulation) or in α/β heterodimeric state (gonadotrophic/thyrotrophic activity).

Characterisation by ES/MS, SE-HPLC and SDS-PAGE clearly shows that ovine pituitary free α molecules

are not of higher molecular mass than the oLHα, in contrast to other species (Parsons *et al.* 1983, Bousfield & Ward 1992). The free αB isoform is even of smaller molecular mass than both the oLHα and the free αA probably because of its truncated N-terminus. Indeed, the lack of an average of three N-terminal amino acid is in keeping with the difference of 400–500 mass units between the major component of the free αB (13 514 Da) and both the free αA isoform (14 019 Da) and oLHα (13 937 Da). This was not specific for the ovine free α, since similar heterogeneities in the N-terminal sequences of the LH or FSH α-subunits of ovine, porcine and equine species have also been previously reported (Bousfield & Ward 1984, Nomura *et al.* 1988, 1989) and attributed to proteolytic degradation.

After the removal of the two N-linked oligosaccharides by PNGase F treatment, SDS-PAGE analysis revealed that free αA still exhibited a diffuse band despite its identical N-terminus with oLHα. Moreover, O-glycanase treatment did not reveal any O-linked oligosaccharides. This result is consistent with the observation that both free αA and αB fully recombine with oLHβ since the presence of an O-linked carbohydrate in bovine free α has been shown to inhibit recombination with a β-subunit (Parsons & Pierce 1984). Furthermore, small amounts of sialic acids were detected in free αA and αB in contrast to pituitary (Parsons *et al.* 1983, Bousfield & Ward 1992) and secreted free α in other species (Kourides *et al.* 1980*a*, Blithe 1990*a*). Sialic acids therefore were not involved in charge heterogeneity of the two ovine free α isoforms. Taken together, these results demonstrate that N-terminal amino acid sequence and oligosaccharide chains are not responsible for the more acidic charge and more hydrophobic feature of the free αA isoform.

In spite of equal combination with oLHβ, the two free α isoforms confer very different potencies to the αβ dimers in the stimulation of testosterone secretion in rat Leydig cells. For the time being, the structural differences leading to inverse potencies of free αA and αB as PRL-releasing factors in the free state or as LH subunit remain elusive.

In conclusion, we have isolated two free forms of the glycoprotein α-subunit in the ovine species which display divergent activities related to their charge and hydrophobicity. These results suggest that some, still unknown, post-translational modification(s) must control the fate of the α molecules either as potential subunits for glycoprotein hormones or as free molecules with intrinsic paracrine activity. The elucidation of the precise structural features of the α molecule either in dimers or in the free state, leading respectively to lower potency in the stimulation of testosterone secretion and in higher stimulation of PRL release, should provide important clues concerning the role of post-translational modification(s) on the structure–function relationships in this molecule. In this respect, the search for a receptor of free α responsible for its action in lactotrophs would be of utmost interest.

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