Changes in insulin-like growth factor binding protein (IGFBP) isoforms during bovine follicular development

B. Nicholas^{1,2}, R. K. Scougall^{1,2}, D. G. Armstrong² and R. Webb¹

¹Division of Agricultural Sciences, School of Biosciences, University of Nottingham, Sutton Bonington, Loughborough LE12 5RD, UK; and ²Department of Integrative Biology, Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS, UK

The insulin-like growth factor binding proteins (IGFBPs) bind IGFs with high affinity and so regulate their access to the type 1 and 2 IGF receptors. This is the principal mechanism involved in regulating IGF bioavailability during folliculogenesis. IGFBPs undergo a number of post-translational modifications, including proteolytic cleavage, phosphorylation and glycosylation, which can regulate the affinity of IGFBPs for IGFs. However, the post-translational changes to IGFBPs that occur during folliculogenesis have not been fully characterized. The charge and size variants of the IGFBPs in bovine follicular fluid were examined by two-dimensional non-reducing SDS-PAGE followed by non-isotopic western ligand blot analysis, and

immunoblot analysis during follicular development. The results demonstrate the presence of at least 51 IGFBP isoforms corresponding to IGFBP-1 to -6 in bovine follicular fluid from subordinate follicles, many of which were phosphorylated. The total number of IGFBPs was reduced in dominant follicles, whereas no gross changes in isoforms were observed during follicular development. These results demonstrate the high degree of conservation of IGFBP post-translational modifications between species, and from the *in vitro* dephosphorylation of these proteins it is hypothesized that these modifications may result in changes to IGF binding or susceptibility to proteolytic cleavage.

Introduction

The insulin-like growth factors (IGFs) are peptides that are structurally related to proinsulin and regulate cell differentiation, growth and survival (Bach and Rechler, 1995; Jones and Clemmons, 1995). The bioavailability of IGFs is regulated by a family of IGF binding proteins (IGFBPs) that bind IGFs with high affinity and so regulate their access to the type 1 and 2 IGF receptors through which their effects are mediated. The IGFBP family consists of six structurally related proteins (IGFBP-1–6) with high but different affinities for IGF-I and IGF-II (Shimasaki *et al.*, 1991; Armstrong and Webb, 1999), as well as a number of IGFBP-related proteins with lower affinities for IGFs (Kim *et al.*, 1997).

The affinity of IGFBPs for IGFs is regulated by a number of post-translational modifications, including phosphorylation, glycosylation and proteolytic cleavage (Firth, 1998). For example, phosphorylation of IGFBP-1 increases its affinity for IGFs (Jones *et al.*, 1991; Coverley and Baxter, 1997) and proteolytic cleavage of IGFBPs generally results in reduced affinity of the fragments for IGFs (Cwyfan-Hughes *et al.*, 1997). The effects of glycosylation have been less well studied (LaTour *et al.*, 1990; Cheung *et al.*, 1991).

The IGF system has been well characterized during folliculogenesis (Armstrong *et al.*, 1998, 2000; Spicer *et al.*, 2001). In cattle and other species, including sheep (Monget *et al.*, 1993; Armstrong *et al.*, 1996; Besnard *et al.*, 1996),

IGFBP profiles are altered during development, reflecting follicular status. In this regard, IGFBP-3 concentrations are essentially unchanged in dominant follicles when compared with healthy subordinate follicles; however, IGFBP-2, -4 and -5 concentrations are significantly lower in dominant follicles than in subordinate follicles (Monget *et al.*, 1993; Cwyfan-Hughes *et al.*, 1997; Armstrong *et al.*, 1998; Mihm *et al.*, 2000; Spicer *et al.*, 2001). Conversely, it has been reported that IGFBP-4 and -5 concentrations are high in follicular fluid from atretic follicles (Monget *et al.*, 1993). These changes in the steady-state concentration of IGFBPs in follicular fluid occur through a combination of changes in gene expression (Armstrong *et al.*, 1998) and proteolysis (Rivera *et al.*, 2001; Spicer *et al.*, 2001).

The classical method by which IGFBP profiles have been examined has been by non-reducing one-dimensional SDS-PAGE followed by western ligand blot analysis (Hossenlopp *et al.*, 1986), although this procedure cannot detect the charge variants of the various IGFBPs. Thus, recent work has emphasized the advantage of two-dimensional non-reducing SDS-PAGE followed by western ligand blot analysis of IGFBP profiles in several species, including rats and humans (Chan and Nicoll, 1994; Weber *et al.*, 1999).

With the exception of proteolytic degradation, limited information is available on the IGFBP post-translational modifications that occur during bovine follicular development. These changes could control the affinity of the IGFBPs for their ligand as well as regulating their susceptibility to proteolytic degradation and, hence, could potentially

Email: benjamin.nicholas@nottingham.ac.uk

be key mechanisms in regulating IGF bioavailability during follicle growth.

As a first step toward analysing the role of IGFBP post-translational modification in the control of follicle growth we have used two-dimensional SDS-PAGE to describe, for the first time, the various IGFBP size and charge variants present in follicular fluid from dominant and subordinate bovine ovarian follicles.

Materials and Methods

Experimental animals

The oestrous cycles of 20 Hereford $\times$ Holstein heifers were synchronized using two injections of a PGF_{2 α} analogue (Estrumate; Schering Health Care Limited, Burgess Hill) at 12 day intervals. After the second injection, the animals were observed twice a day for signs of behavioural oestrus (oestrus = day 0) and were killed on day 5 after oestrus.

Materials

The follicles were dissected and graded on the basis of size into groups of < 4 mm (subordinate) and > 8 mm (dominant) in diameter. These sizes were selected as both 4 mm and 8 mm are key stages in bovine follicular development (Webb *et al.*, 1999). Follicular fluid was collected immediately by aspiration, centrifuged at 13 000 *g* for 5 min and stored in aliquots at -80°C until use. A protease inhibitor cocktail was added (Sigma P-2714; Sigma-Aldrich Company Ltd, Poole) to half of the samples before freezing.

Rabbit antisera raised against bovine IGFBP-2 and human IGFBP-4 were purchased from TCS Biologicals (Upstate Biotechnology, Lake Placid, NY). The antiserum against IGFBP-4 has been reported to have 50% cross-reactivity with bovine IGFBP-2. PVDF membranes were obtained from Amersham Pharmacia Biotech (Little Chalfont), SDS molecular weight standards (Precision protein standards, Broad range) were purchased from Bio-Rad (Hemel Hempstead), extravidin-peroxidase, BSA (radioimmunoassay grade) and anti-rabbit peroxidase conjugate were obtained from Sigma. IGF-II was purchased from Gropep Pty Ltd (Adelaide) and D-biotinoyl- ϵ -amino-caproic acid-*N*-hydroxysuccinimide ester (Biotin-7-NHS) was purchased from Boehringer-Mannheim (Mannheim).

Preparation of biotinylated IGF-II

Biotinylated IGF-II was prepared by diluting IGF-II (50 μg) in 500 μl PBS (pH 7.4). Biotin-7-NHS was dissolved in dimethylsulphoxide to a concentration of 20 mg ml^{-1} ; 10 μl of this solution was added to the IGF-II solution and incubated for 2 h at room temperature with agitation. After the labelling reaction, free biotin ester was removed by PBS buffer exchange using centricon 3 microconcentrating units (Millipore (UK) Limited, Watford), concentrating to the dead-stop volume of 10 μl three times. The final solution

was made up in 200 μl PBS and the labelled IGF-II was stored at -80°C in 20 μl aliquots.

Non-reducing SDS-PAGE

The procedure followed was a modification of that of Hossenlopp *et al.* (1986). Samples of follicular fluid (0.75 μl) were diluted with 10 μl of non-reducing sample buffer (50 mmol Tris-HCl l^{-1} , pH 8.3, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) and fractionated on 4% stacking/12% resolving SDS-PAGE gels (8.6 cm $\times$ 6.8 cm Mini-Protein III system; Bio-Rad). The proteins were electroblotted on to a PVDF membrane for 1.5 h at room temperature (45 m per gel using an LKB Novablot semi-dry system and Towbin buffer) and were visualized by staining with 0.1% (w/v) Ponceau red in 0.5% (v/v) acetic acid.

Non-reducing two-dimensional SDS-PAGE

The procedure followed was a modification of that of Weber *et al.* (1999). Follicular fluid samples (125 μg protein per strip) were diluted in and made up to 185 μl with rehydration buffer containing 8 mol urea l^{-1} , 0.2% (w/v) Ampholine, pH 3–10 (Amersham Biosciences, Little Chalfont), and 1% (w/v) CHAPS detergent. The prepared sample solutions were used to rehydrate 11 cm long Bio-Rad ReadyStrip IPG (immobilized pH gradient) strips (pH 3–10 linear) in a Bio-Rad isoelectric focussing/rehydration cell at 50 V for 12 h. Isoelectric focusing was performed in a Bio-Rad Protean IEF cell for a total of 35 000 V hours at a maximum of 8000 V at 20°C by a preset programme according to the manufacturer's instructions. The gel strips were stored at -80°C until use, when they were equilibrated for 15 min in 50 mmol Tris-HCl l^{-1} , pH 8.3, 6 mol urea l^{-1} , 30% (v/v) glycerol, 2% (w/v) SDS and a trace of bromophenol blue before overlay on 12% SDS-PAGE gels (13.3 cm $\times$ 8.7 cm Criterion gel system; Bio-Rad) in molten 1% (w/v) SeaPlaque agarose in SDS-PAGE running buffer. The isoelectrically focused proteins were fractionated and electroblotted as described for the single dimension gels. Transfer of proteins was checked in each case by staining the membrane with Ponceau red solution.

Total protein staining using biotinylation

Non-reducing two-dimensional gels of bovine follicular fluid were electroblotted on to PVDF membrane as described previously and stained with Ponceau red to determine transfer efficiency. The Ponceau stain was removed by washing twice for 10 min with PBS (pH 7.4) containing 0.1% (v/v) Tween 20, followed by two washings with PBS. The membrane was incubated at room temperature (18– 24°C) with agitation in the presence of labelling solution (20 ml PBS containing 10 μl of 20 mg biotin-7-NHS ml^{-1}) for 1 h. After the labelling reaction, the blot was washed three times with PBS–0.1% (v/v) Tween 20 and blocked with 3% (w/v) BSA in PBS–1.0% (v/v) Tween 20 for 1 h. The biotinylated proteins were detected using extravidin-peroxidase (Sigma; 1 in 3000 dilution in blocking solution)

at room temperature for 45 min and the membrane was washed three times for 5 min each with PBS–0.1% (v/v) Tween 20. Horseradish peroxidase label was detected using ECL-plus kit (Amersham), with a typical exposure time of 30 s.

Western ligand blot

The blotted membrane was rinsed twice with PBS–0.1% (v/v) Tween 20 and blocked for 2 h at room temperature with 3% (w/v) BSA in PBS–0.1% (v/v) Tween 20 with agitation. The blocking solution was replaced with 15 ml fresh blocking solution containing 2 µl biotinylated IGF-II per minigel and the membrane was incubated overnight at 4°C with agitation. The next day the membrane was washed three times for 5 min each with PBS–0.1% (v/v) Tween 20 and the solution was replaced with 20 ml of a 1 in 4000 dilution of extravidin-peroxidase in blocking solution. The membrane was incubated with agitation for 2 h at room temperature and washed five times with PBS–0.1% (v/v) Tween 20 before detection of signal using the ECL-plus system on hyperfilm-ECL film, and developed using an automated system (X-Ograph). Typical exposure times were between 10 s and 5 min.

Immunoblots

PVDF membranes from two-dimensional separations of follicular fluid were probed for IGFBP-2 and -4 by first blocking in 3% (w/v) BSA in PBS containing 0.1% (v/v) Tween 20 for 1 h at room temperature. The blots were incubated overnight in blocking solution containing a 1 in 2000 dilution of either rabbit anti-bovine IGFBP-2 or rabbit anti-human IGFBP-4. The membranes were washed in PBS–0.1% (v/v) Tween 20 and primary antibodies were detected using a 1 in 5000 dilution of goat anti-rabbit IgG peroxidase conjugate for 2 h at room temperature. Peroxidase signal was detected using the ECL-plus system.

Phosphatase reaction

Follicular fluid samples (3 µl (180 µg) per tube), snap frozen in liquid nitrogen without added protease inhibitor cocktail, were incubated in 5 µl of 50 mmol Tris–HCl I⁻¹ (pH 7.4) for 3 h at 37°C, containing 5 U calf intestinal alkaline phosphatase (Promega, Madison, WI). The control tube contained no alkaline phosphatase.

On the next day, all samples were diluted in 185 µl non-reducing two-dimensional rehydration buffer and analysed by two-dimensional SDS-PAGE as described previously.

Results

One-dimensional western ligand blot analysis

The results of a one-dimensional non-isotopic western ligand blot analysis performed on follicular fluid samples from four separate cows to be tested by two-dimensional SDS-PAGE are shown (Fig. 1). These results demonstrate

Fig. 1. One-dimensional western ligand blot analysis of bovine follicular fluid samples from four day 5 oestrous-synchronized cattle detected using biotinylated recombinant bovine insulin-like growth factor II (IGF-II). Follicular fluid samples collected in the presence of protease inhibitor from four dominant follicles (9, 10, 12 and 18 mm in diameter) and pooled samples from four subordinate follicle populations (1–4 mm in diameter) are shown. Molecular masses (kDa) are shown.

that the IGFBP profile alters depending on follicular developmental status. The dominant follicle (> 8 mm in diameter on day 5) contained only IGFBP-3, observed as a doublet of between 40 kDa and 45 kDa, and occasionally a 33 kDa IGFBP (identified by immunoblot as IGFBP-2, results not shown). Pooled follicular fluid from small follicles from the same ovary (< 4 mm in diameter) contained IGFBPs with molecular masses of approximately 29 and 33 kDa, corresponding to IGFBP-4 (glycosylated form) and IGFBP-2, respectively. Occasionally an increase in an IGFBP species at a slightly higher molecular mass than the glycosylated form of IGFBP-4, which is known to contain IGFBP-5 (approximately 31 kDa) by western immunoblot, was observed in these small follicles; however, the size and irregularity of the band indicate that there may be an increase in other IGFBPs of similar molecular mass. Exposure times were very low (generally less than 5 min) and background signals were limited, demonstrating that this non-isotopic method is ideally suited for western ligand blot analysis. The identities of IGFBP-2 and -4 were confirmed by immunoblot analysis (results not shown).

Two-dimensional SDS-PAGE

The two-dimensional analysis of IGFBP profiles was initially performed on a pooled follicular fluid sample from small (< 4 mm in diameter) follicles, as this was likely to demonstrate all the major species present in bovine follicles. A representative total protein profile obtained after two-dimensional SDS-PAGE of pooled bovine follicular fluid from subordinate (< 4 mm) follicles (cow number 2 in Fig. 1), in which comparatively few proteins were observed in the lower molecular mass region (< 50 kDa), is shown (Fig. 2a). The IGFBP western ligand blot profile of this follicular fluid sample, with a major doublet at 40–45 kDa

Fig. 2. Two-dimensional electrophoresis of bovine follicular fluid from a subordinate follicle (collected in the presence of protease inhibitor) from a pooled sample of 1–4 mm bovine follicles on day 5 after induced oestrus transferred on to PVDF membrane and stained either for (a) total protein or probed with biotin-labelled insulin-like growth factor II (IGF-II) in the (b) absence or (c) presence of excess unlabelled IGF-II. pI: isoelectric point. Molecular masses (kDa) are shown.

with a broad isoelectric point (pI) range, is shown (Fig. 2b). A second series of spots was observed at a molecular mass of 33 kDa; a third series was observed at 29 kDa and a fourth series was observed at 24 kDa.

The detection of all the IGFBP spots was inhibited in the presence of an excess of unlabelled IGF-II, thereby demonstrating the specificity of this method for the detection of IGFBPs (Fig. 2c).

In duplicate blots to those used in Fig. 2, western immunoblot analysis was performed to confirm the identity of some of the spots observed by western ligand blot analysis (Fig. 3). IGFBP-2 was identified as the series of five major spots (and two fainter spots only visible after increased exposure of the film) occurring at approximately 33 kDa, between a pI value of 6.2 and 7.7 (Fig. 3a). IGFBP-4 was identified as a doublet of spots occurring at approximately 29 and 24 kDa (Fig. 3b), with four spots visible at

Fig. 3. Western blots of bovine follicular fluid (collected in the presence of protease inhibitors) from a pooled population of subordinate follicles (1–4 mm in diameter) on day 5 after induced oestrus, probed with (a) an insulin-like growth factor binding protein 2 (IGFBP-2) antibody and (b) an IGFBP-4 antibody. pI: isoelectric point. Molecular masses (kDa) are shown.

Fig. 4. Two-dimensional western ligand blots probed with biotin-labelled insulin-like growth factor II (IGF-II) of bovine follicular fluid samples on day 5 after induced oestrus (collected in the presence of protease inhibitors). Follicular fluid samples were collected from (a) a dominant follicle (> 8 mm in diameter) and (b,c) from two pools of subordinate (1–4 mm) follicles, the latter of which showed increased IGF-II binding at a 29–32 kDa band by one-dimensional western ligand blot analysis. pI: isoelectric point. Molecular masses (kDa) are shown.

29 kDa (pI value between 6.2 and 6.8), and three at 24 kDa (pI value between 6.4 and 7.4).

As a result of the lack of specific antibodies, the other IGFBP species were identified by molecular mass and

Fig. 5. Diagrammatic representation of the insulin-like growth factor binding protein (IGFBP) profiles detected in bovine follicular fluid samples by two-dimensional electrophoresis and western ligand blot analysis. These results represent the total number of spots identified from five oestrous-synchronized animals including dominant and pooled 1–4 mm subordinate follicle samples from each animal. Green: putative IGFBP-3; red: IGFBP-2; yellow: putative IGFBP-6; orange: putative IGFBP-5; blue: IGFBP-4; purple: IGFBP-1; black: unknown.

patterns from two-dimensional western ligand blots from five different animals (representative examples can be seen in Figs 2 and 4). For example, IGFBP-3 with a molecular mass of 40 and 44 kDa had a pI value of between 5.3 and 7.9, and 4.9 and 7.9, respectively. It was observed that in the samples which contained increased IGF-II binding to a band at the 31 kDa region by one-dimensional western ligand blot analysis (Fig. 1; subordinate follicle from cow 1), several extra sets of spots occurred about the 31 kDa region (Fig. 4c); however, these spots occurred in distinct pI value regions. For example, the spot tentatively identified as IGFBP-1, with a molecular mass of approximately 31 kDa, had a pI value of approximately 5.3, which was only faintly visible in most cases, whereas another of a similar molecular mass was observed at a pI value of 6.1, in a similar pI value and molecular mass position to that of human IGFBP-6. Furthermore, a series of spots between 30 kDa and 32 kDa and at a pI value of 6.6–8.6 and 7.5–8.6 was observed in a very similar molecular mass and pI value distribution to that of human IGFBP-5, although this identification could not be confirmed by immunoblotting.

A diagrammatic map of the occurrence of each IGFBP species was constructed using the results from five individual pairs of dominant and subordinate follicular fluid samples to map the position of each IGFBP species (Fig. 5), and a summary of pI value ranges for IGFBPs observed consistently at each molecular mass is shown (Table 1).

Phosphatase treatment

Removal of phosphorylated sites by alkaline phosphatase treatment resulted in the loss of almost all IGFBP spots detected by ligand blot analysis (Fig. 6b) when compared with controls (Fig. 6a), although single spots at 43 kDa (pI value 6.1), 41 kDa (pI value 6.0) 33 kDa (pI value 6.5) and

Table 1. Molecular mass and pI values for each insulin-like growth factor binding protein (IGFBP) isoform detected in bovine follicular fluid

IGFBP	Molecular mass (kDa)	Number of charge variants	pI value range
Putative IGFBP-3	44	13	4.9–7.9
Putative IGFBP-3	40	10	5.3–7.9
IGFBP-2	33	7	6.2–7.7
Putative IGFBP-6	32	1	6.1
Putative IGFBP-5	31	4	7.5–8.6
Putative IGFBP-5	30	7	6.6–8.6
Putative IGFBP-1	30	1	5.3
IGFBP-4	29	4	6.2–6.8
IGFBP-4	24	3	6.4–7.4
Unknown	24	1	8.2

pI: isoelectric point.

24 kDa (pI value 7.2) were visible, probably representing the dephosphorylated forms of IGFBP-3, IGFBP-2 and non-glycosylated IGFBP-4, respectively. Protein loadings for these gels were increased to maximize detection of the faint spots in the phosphatase-treated samples, resulting in overloading of the control samples.

Developmental changes in IGFBP profiles

Follicular fluid from dominant (> 8 mm in diameter) follicles contained only IGFBP-3 (Fig. 4a), whereas in the subordinate follicles (< 4 mm in diameter), IGFBP-2, -3 and -4 were detected (Fig. 4b). In the follicular fluid samples of subordinate follicles that contained increased bands on one-dimensional western ligand blot analysis at 29–31 kDa, extra spots were detected which corresponded to the

Fig. 6. Representative two-dimensional western ligand blots probed with biotin-labelled insulin-like growth factor II (IGF-II) of follicular fluid samples pooled from 1–4 mm subordinate bovine follicles (without protease inhibitors) on day 5 after induced oestrus, incubated in the (a) absence or (b) presence of calf intestinal alkaline phosphatase (control sample contained buffer only). The results shown are typical of three individual experiments. pI: isoelectric point. Molecular masses (kDa) are shown.

expected molecular masses and pI values for IGFBP-6 and IGFBP-1 (Fig. 4c), as observed for human IGFBP isoforms. Spots in the pI value and molecular mass region corresponding to human IGFBP-5 and an unknown spot at 24 kDa (pI value 8.2) were only faintly visible on these blots; however, they were clearly visible after prolonged exposure of the film to the chemiluminescent signal.

No obvious differences in the post-translational modification of IGFBP-3 were observed between small and large follicles.

Discussion

In the present study the first analysis of IGFBPs in bovine follicular fluid by two-dimensional SDS-PAGE is presented. The charge and size variants of individual IGFBPs present during the final stages of follicle development in both dominant and subordinate follicles are described – information that is not available when conventional one-dimensional ligand blot analysis is performed. The results also confirm the efficacy of the non-radioactive western ligand blot method used in these studies, which gave reliable ligand blot results with low exposure times (typically less than 1 min).

Two-dimensional analysis revealed an array of at least 51 spots corresponding to binding proteins, many of which are seen in other species, but other spots that appear to be novel. Although there are few publications on the expected

binding protein arrays, the pattern obtained was similar to that observed in rat (Chan and Nicoll, 1994) and human (Weber *et al.*, 1999) serum binding protein arrays. However, the method of analysis was different in each case and for the first time a two-dimensional western ligand blot procedure is described that combines the use of immobilized IEF strips with non-isotopic western ligand blot detection.

The binding protein spots detected using biotinylated IGF-II were completely removed in the presence of saturating concentrations of unlabelled IGF-II, indicating that all the spots detected were indeed genuine IGFBPs. The proteins detected by this method were different in dominant compared with subordinate follicles and corresponded to the changes observed using conventional one-dimensional western ligand blot analysis, which aided the identification of the spots on the two-dimensional gels.

The analysis of bovine follicular fluid samples by one-dimensional SDS-PAGE, followed by western ligand blot analysis, has revealed an IGFBP array essentially the same as that shown previously in sheep (Armstrong *et al.*, 1996), humans (Cataldo and Giudice, 1992) and cows (Mihm *et al.*, 2000), with the loss of low molecular mass species during selection of the dominant follicle. In some subordinate follicles there was an increase in intensity at a band at 29–32 kDa (cow number 1 subordinate follicle). This band was in fact irregular in shape and may have been due to changes in IGFBPs of slightly higher molecular mass; however, this contention could not be resolved by conventional analysis, but it was confirmed by western immunoblot analysis that this band contained IGFBP-5 (molecular mass = 31 kDa, results not shown), an IGFBP that is thought to be upregulated during follicular atresia (Monget *et al.*, 1993).

As observed by one-dimensional analysis, IGFBP-3 was present in follicular fluid from dominant and subordinate follicles, and was present as a doublet of multiple spots at two molecular masses, probably due to changes in glycosylation states. A number of charge variants for each of the size isoforms was present, which covered a wide range of pI values. In total, 23 different size and charge variants of IGFBP-3 were identified.

IGFBP-2 was observed only in follicular fluid from subordinate follicles and was positively identified using an anti-IGFBP-2 antibody. Six IGFBP-2 isoforms were detected in both western ligand and immunoblots that differed in charge but not molecular mass, indicating that this protein is not glycosylated, and thereby confirming the results of other studies (Chan and Nicoll, 1994). However, the existence of a seventh isoform as a pI value doublet at two slightly different molecular masses indicates that there may be other post-translational modifications that have not yet been described.

IGFBP-4 was detected in both ligand and immunoblots as two arrays of spots at 29 and 24 kDa. Immunoblot analysis proved difficult as the antibodies used were raised against the human protein and had low crossreactivity with

the bovine binding proteins. However, four charge variants of the glycosylated IGFBP-4 isoform and three charge variants of the non-glycosylated isoform were detected. A fourth faint spot at 24 kDa was observed using the western ligand blot analysis that may have been a further IGFBP-4 isoform; however, this spot was not observed when using the antibody, possibly due to the low quantity of this isoform, although it could also be a degradation product of another IGFBP. These protein arrays were almost identical to those identified in other species; however, a greater number of spots was observed than reported in other studies, with an increase in the number of charge variants for the glycosylated form of IGFBP-4 (an extra spot visible at approximately pI value 6.8) and an increase in the number of spots for IGFBP-2 (extra spot visible at pI value 6.2 and doublet at pI value 7.0).

Analysis of follicular fluid demonstrated increased IGFBP-4 concentrations in some samples. It also demonstrated the presence of other binding proteins at a slightly higher molecular mass, which may be due to the presence of IGFBP-1, -5 and -6, although no antibody is currently available for these IGFBPs in cattle. The molecular masses and pI values of the detected spots correspond to the expected size and pI values of these lower molecular mass binding proteins as determined in humans and rats (Chan and Nicoll, 1994; Weber *et al.*, 1999). Although the molecular masses of IGFBP-1, -2, -5 and -6 and glycosylated IGFBP-4 are similar (29–32 kDa), their wide range of pI values enables the precise resolution of these proteins, and the use of larger gels may help to characterize them even further. IGFBP-1 and -6 have not previously been observed in bovine follicular fluid samples. This may be due to very low expression or these isoforms may not be synthesized within the follicle compartment, entering instead as a result of changes in basal membrane leakage due to the onset of atresia. As their expression appears to be associated with follicular atresia, which occurs in only selected subordinate follicles at any one time, the expression of mRNA for IGFBP-1 and -6 may have been overlooked.

The phosphorylation state of IGFBPs was analysed using alkaline phosphatase. Treatment of follicular fluid resulted in the loss of all pI value arrays and the appearance of single spots at molecular masses of 33 kDa (pI value 6.7), 29 kDa (pI value 6.5) and 24 kDa (pI value 7.1), corresponding to IGFBP-2 and the glycosylated and non-glycosylated forms of IGFBP-4, respectively. The lack of detection of the expected IGFBP-3 doublet at a pI value of 4.9–7.9 may have been due to changes in IGF binding capability after dephosphorylation or to increased susceptibility to proteolytic cleavage. Seven IGFBP-2 charge variants were identified in the present study and all except one (the fully dephosphorylated form) were deleted after alkaline phosphatase treatment, indicating that all the potential phosphorylation sites are phosphorylated in IGFBP-2 in follicular fluid.

The significance of these post-translational modifications on the structure and function of the IGFBPs remains unknown, although phosphorylation of IGFBP-1 alters IGF

binding capability (Jones *et al.*, 1991; Webb *et al.*, 1999). These modifications may also have implications for the susceptibility of these proteins to proteolysis, which is one way in which IGF bioavailability may be altered. The detection of the same number of charge variants in either IGFBP-2 or IGFBP-4 immunoblots as in the ligand blots indicates that these modifications do not alter IGF binding capacity. In the future it may be possible to identify and purify each of the different isoforms of the binding proteins and to assess their differing biological activities.

In conclusion, we have demonstrated two-dimensional IGFBP profiles using a novel non-radioactive western ligand blot method, which has shown the presence of a greater number of IGFBP-2 and -4 isoforms than previous methods have detected in either human or rat serum. However, there has been a remarkable conservation in the general pattern of post-translational modification of the IGFBPs between species, which may indicate that the biological effect of these modifications is essential to the bioactivity of the IGFBPs.

This work was supported by the BBSRC.

References

- Armstrong DG, Hogg CO, Campbell BK and Webb R (1996) Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells: the effects of IGF-I, gonadotropin, and follicle size *Biology of Reproduction* **55** 1163–1171
- Armstrong DG, Baxter G, Gutierrez CG, Hogg CO, Glazyrin AL, Campbell BK, Bramley TA and Webb R (1998) Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status *Endocrinology* **139** 2146–2154
- Armstrong DG, Gutierrez CG, Baxter G, Glazyrin AL, Mann GE, Woad KJ, Hogg CO and Webb R (2000) Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles *Journal of Endocrinology* **165** 101–113
- Bach LA and Rechler MM (1995) Insulin-like growth factor binding proteins *Diabetes Reviews* **3** 38–61
- Besnard N, Pisselet C, Monniaux D, Locatelli A, Benne F, Gasser F, Hately F and Monget P (1996) Expression of messenger ribonucleic acids of insulin-like growth factor binding protein-2, -4 and -5 in the ovine ovary: localization and changes during growth and atresia of antral follicles *Biology of Reproduction* **55** 1356–1367
- Cataldo NA and Giudice LC (1992) Insulin-like growth factor binding profiles in human ovarian follicular fluid correlate with follicular functional status *Journal of Clinical Endocrinology and Metabolism* **74** 821–829
- Chan KC and Nicoll CS (1994) Characterization of rat serum insulin-like growth factor-binding proteins by two-dimensional gel electrophoresis: identification of a potentially novel form *Endocrinology* **134** 2574–2580
- Cheung PT, Smith EP, Shimasaki S, Ling N and Chernauek SD (1991) Characterization of an insulin-like growth factor binding protein (IGFBP-4) produced by the B104 rat neuronal cell line: chemical and biological properties and differential synthesis by sublines *Endocrinology* **129** 1006–1015
- Coverley JA and Baxter RC (1997) Phosphorylation of insulin-like growth factor binding proteins *Molecular and Cellular Immunology* **128** 1–5
- Cwyfan-Hughes SC, Mason HD, Franks S and Holly JM (1997) Modulation of the IGFBPs by follicle size in the human ovary *Journal of Endocrinology* **154** 35–43
- Firth SM (1998) Methods to detect and analyze modified insulin-like growth factor binding proteins. In *Molecular Mechanisms to Regulate the*

- Activities of Insulin-Like Growth Factors* pp 79–87 Eds K Takan *et al.* Elsevier, Amsterdam
- Hossenlopp P, Seurin KD, Segovia-Quinson B, Hardouin S and Binoux M** (1986) Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies *Analytical Biochemistry* **154** 138–143
- Jones JI and Clemmons DR** (1995) Insulin-like growth factors and their binding proteins: biological actions *Endocrine Reviews* **16** 3–34
- Jones JL, D'Ercole AJ, Camacho-Hubner C and Clemmons DR** (1991) Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and *in vivo*: effects on affinity for IGF-I *Proceedings National Academy of Sciences USA* **88** 7481–7485
- Kim HS, Nagalla SR, Oh Y, Wilson E, Roberts CT, Jr and Rosenfeld RG** (1997) Identification of a family of low affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily *Proceedings National Academy of Sciences USA* **94** 12 981–12 986
- LaTour D, Nohan S, Linkhart TA, Baylink DJ and Strong DD** (1990) Inhibitory insulin-like growth factor binding protein: cloning, complete sequence, and physiological regulation *Molecular Endocrinology* **4** 1806–1814
- Mihm M, Austin EJ, Good TEM, Ireland JLH, Knight PG, Roche JF and Ireland JJ** (2000) Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers *Biology of Reproduction* **63** 811–819
- Monget P, Monniaux D, Pisselet C and Durand P** (1993) Changes in insulin-like growth factor-I (IGF-I), IGF-II and their binding proteins during growth and atresia of ovine ovarian follicles *Endocrinology* **132** 1438–1446
- Rivera GM, Chandrasekher YA, Evans ACO, Giudice LC and Fortune JE** (2001) A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle *Biology of Reproduction* **65** 102–111
- Shimasaki S, Gao L, Shimonaka M and Ling N** (1991) Isolation and molecular cloning of insulin-like growth factor-binding protein-4 *Molecular Endocrinology* **5** 938–942
- Spicer LJ, Chamberlain CS and Morgan GL** (2001) Proteolytic activity degrading insulin-like growth factor-binding protein (IGFBP)-4 and -5 but not IGFBP-2 and -3 in preovulatory bovine follicles *Domestic Animal Endocrinology* **21** 1–15
- Webb R, Campbell BK, Garverick HA, Gong JG, Gutierrez CG and Armstrong DG** (1999) Molecular mechanisms regulating follicular recruitment and selection *Journal of Reproduction and Fertility Supplement* **54** 33–48
- Weber MM, Spottl G, Gossl C and Engelhardt D** (1999) Characterization of human insulin-like growth factor binding proteins by two-dimensional polyacrylamide gel electrophoresis and western ligand blot analysis *Journal of Clinical Endocrinology and Metabolism* **84** 1679–1684

Received 18 December 2001.

First decision 22 February 2002.

Revised manuscript received 21 May 2002.

Accepted 10 June 2002.