Presence of growth hormone-binding proteins in cattle plasma and milk

A. Devolder*, R. Renaville*, M. Sneyers*, I. Callebaut*, S. Massard*, A. Goffinet*, A. Burny* and D. Portetelle†

*Molecular Biology and Animal Physiology Unit and †Microbiology Unit, Faculty of Agronomy, 13 Avenue Maréchal Juin, B-5030 Gembloux, Belgium

RECEIVED 16 November 1992

ABSTRACT

The presence of GH-binding proteins (GHBP)s in the plasma of adult cattle was investigated using Sephadex G-200 filtration, Western ligand blotting and Western blotting. The changes in the concentration of GHBP in the plasma of dairy half-sister heifers during the first year of life as well as the presence of GHBP in milk were also investigated. When analytical chromatography (on a 1.6 × 100 cm column) was performed, five peaks of recombinant bovine GH (rbGH)-associated radioactivity were revealed in cattle plasma; the first peak, which appeared near the void volume, was presumed to represent aggregates, the second (Mr, 290 kDa) and the third peaks (Mr, 75 kDa) corresponded to specific rbGH-GHBP complexes; the last two peaks representing free 125I-labelled rbGH and NaF[125I]. Western ligand blotting revealed multiple GHBP.s. Three major bands were observed at approximately 190, 58 and 31 kDa; an excess of unlabelled hormone blocked the binding of 125I-labelled rbGH. Minor non-specific binding bands were also detected in cattle plasma with molecular weights between 40 and 136 kDa. One monoclonal antibody (8H7) produced against synthetic peptide (amino acids 54–63 of the extracellular domain of the bovine GH receptor) specifically interacted with 190 and 58 kDa bands while the 31 kDa band was not recognized. Finally, Western ligand blots were performed to evaluate the changes in plasma GHBP during the first year of life in 55 dairy half-sister heifers and to identify GHBP in milk. In plasma, the intensity of the 31 kDa band varied greatly between animals while the other specific bands remained stable. In milk, all specific GHBP bands observed by Western ligand blotting disappeared a few days after parturition. In conclusion, these results demonstrate the presence of GHBP.s in cattle plasma and milk. There were important variations in GHBP between dairy half-sister heifers during the first year of life but the potential role of these binding proteins in the regulation of the biological activity of GH in ruminants and their functions in milk remain to be determined.


INTRODUCTION

The physiological role of growth hormone (GH) in supporting growth and lactation in animals, particularly in cattle, is well documented (Moseley et al. 1992; West et al. 1990).

A high-affinity GH-binding protein (GHBP) (50–60 kDa) has been identified and characterized in human sera (Baumann et al. 1986), as well as in rabbit (Spencer et al. 1988), mouse (Smith et al. 1989), rat (Massa et al. 1990) and poultry (Vasilatos-Youunke et al. 1990) sera. Many reports have also demonstrated that the amino acid sequence of GHBP is identical to the extracellular domain of the liver membrane GH receptor (Leung et al. 1987; Spencer et al. 1988; Baumbach et al. 1989; Smith et al. 1989). More recently, a second GHBP of >120 kDa molecular weight, with low affinity to GH but high capacity to complex the hormone, has been described in man (Baumann & Shaw, 1990), dog and pig (Daughaday et al. 1988). This low-affinity GHBP is not related to the high-affinity binding protein or GH receptor (see review by Baumann, 1991).

The presence of GHBP has also been observed in rabbit (Postel-Vinay et al. 1991) and human (Mercado & Baumann, 1992) milk. Mercado & Baumann (1992) have reported that the high-affinity GHBP identified in human milk differs from the serum GHBP/GH receptor in its hormone specificity, molecular size and solid-phase binding characteristics.
A controversy has recently appeared on the existence of these GHBP s in ruminants. Using gel filtration on AcA 44 columns, Gavin et al. (1991) have concluded that GHBP is absent in ruminants, including cattle, sheep, goat, deer, bison and elk, while Davis et al. (1992), using a dextran-coated charcoal separation assay, have demonstrated the presence of specific GHBP s in bovine and ovine serum. The use of different techniques may help to explain the differences observed between the two studies and the origin of the radiolabelled GH may also influence the molecular integrity and the binding characteristics of the hormone (Davis et al. 1992).

We here provide evidence for GHBP s in cattle plasma, using exclusion chromatography, a Western ligand blotting technique and specific monoclonal antibodies. The changes in plasma binding protein in young dairy heifers and the presence of GHBP in milk were also evaluated by Western ligand blots.

**MATERIALS AND METHODS**

**Materials**

Methionyl bovine GH (rbGH; batch M-91–07226) used as an iodinated tracer and excess hormone was kindly supplied by Monsanto (St Louis, MO, U.S.A.). Sephadex G-200 was obtained from Pharmacia (Uppsala, Sweden). Molecular weight markers used in chromatographic exclusion were blue dextran 2000, thyroglobulin (670 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13-7 kDa) and were purchased from Pharmacia. Pre-stained 14C-labelled molecular weight markers were employed in the Western ligand blotting technique (rainbow markers from Amersham International plc, Amersham, Bucks, U.K.). Immobilon sheets were obtained from Millipore (Bedford, MA, U.S.A.). Gelatin and bovine serum albumin (A 7638) used for saturation in Western ligand blotting and Western blotting respectively were obtained from Merck (Darmstadt, Germany) and Sigma (St Louis, MO, U.S.A.).

**Plasma samples**

Bovine plasma pools were obtained from five 3-year-old Normandy cows. Jugular blood was withdrawn by venepuncture into heparinized tubes and was immediately refrigerated at 4 °C and centrifuged at 3000 g for 15 min. The plasma was collected, pooled and stored at −20 °C until tested.

**Milk samples**

The bovine milk pools were obtained from five 5-year-old Holstein–Friesian cows. Milk was collected from each animal twice a day in the middle of milking during 1 week after parturition. Milk samples were refrigerated at 4 °C and used in Western ligand blot analysis.

**Iodination procedure**

Methionyl bovine GH was labelled with Na[125I] using a modified chloramine-T procedure (Hintz, 1987). 125I-labelled rbGH was separated from free iodine on Sephadex G-25 columns (25 cm; Pharmacia) followed by purification using fast-protein liquid chromatography (FPLC; Pharmacie). The specific activity averaged 120 μCi/μg.

**Exclusion chromatography**

Plasma proteins were separated according to their size by exclusion chromatography. The Sephadex G-200 column (200 ml column, C16/100; Pharmacia) was calibrated using blue dextran (V0), a set of molecular weight markers and free Na[125I] (Vc). Chromatography was performed at 4 °C under neutral conditions (Tris–HCl (50 mmol/l), pH 7-5, 0-02% (w/v) NaN3) at a flow rate of 2 ml/h. Fractions (1 ml) were collected and the radioactivity was determined in a gamma counter. Plasma pool samples (400 μl) were incubated with 125I-labelled rbGH (240 000 c.p.m., 1 ng) for 90 min at 39 °C before the chromatographic run.

**Western ligand blotting**

Western ligand blotting was performed using 125I-labelled rbGH to characterize GHBP in cattle plasma and milk. The method of Hossenlopp et al. (1986) with slight modifications was used. Briefly, 0-5 μl pooled plasma (or 1 μl crude milk) was denatured using sodium dodecyl sulphate and reducing agents and applied to a 4% stacking gel and electrophoresed through a 12.5% polyacrylamide gel. Afterwards, gels were soaked in Towbin buffer (Tris (25 mmol/l), pH 8.3, glycine (192 mmol/l), 20% (v/v) methanol) and proteins were blotted onto Immobilon sheets (Millipore, Bedford, MA, U.S.A.).

Electrophoresis and electrotransfer were performed using the Mini-Protean II system (Bio-Rad, Richmond, CA, U.S.A.). Membranes were washed with Tris-gelatin buffer (Tris–HCl (50 mmol/l), pH 7-6, NaCl (0-1 mol/l), MgCl2 (10 mmol/l), 0-02% (w/v) NaN3, 0-1% (v/v) Triton X-100, 0-5% (w/v) gelatin). The incubation with 125I-labelled rbGH (1000

c.p.m/cm² blot was carried out overnight at room temperature in Tris-gelatin buffer. Thenceforth, the membranes were washed twice with Tris-gelatin buffer, twice with Tris buffer (Tris-HCl (0-1 mol/l), pH 7-5, NaCl (1-5 mol/l)), MgCl₂ (0-02% (w/v) Na₃), twice with Tris-HCl (10 mmol/l), pH 7-6 and once in distilled water (all washings were carried out for 30 min). They were then air dried and exposed to Kodak X-Omat AR5 films for 1-2 days at -70 °C. Molecular weight markers were run in parallel lanes.

Anti-GHBP monoclonal antibody production

To predict surface located regions, the amino acid sequence of the extracellular domain of the bovine GH receptor (Hauser et al. 1990; Sneyes et al. 1992) was submitted to hydrophobic cluster analysis (Lemesle-Verloot et al. 1990) and to the methods described by Chou & Fasman (1974), Eisenberg et al. (1984) and Garnier et al. (1978). For the synthesis of peptide (RGH2; TKCRSPELET; amino acids 54-63), we used a semiautomated procedure based on a tBoc/Bzl strategy as recently described for other peptides (Callebaut et al. 1991). The purity of the peptide used for coupling and immunization was above 95%. The peptide was conjugated to keyhole limpet hemocyanin (KLH; Sigma) using as coupling agent maleimidocaproic-N-succinimide ester (Sigma) or glutaraldehyde (Merck) (Callebaut et al. 1991).

Ten-week-old female Balb/c mice were each immunized with 100 µg KLH conjugate in complete Freund's adjuvant (0-1 ml total volume) equally distributed into the rear footpads. On day 14, six mice were bled, as recommended by Mirza et al. (1987), and the draining lymph nodes (popliteal and inguinal) were removed and pooled for fusion. Single cell suspensions were prepared by tearing pooled lymph nodes through 100-gauge stainless steel mesh. Cells were washed, mixed with myeloma cells, fused and cloned according to procedures previously described (Brück et al. 1982) except that the non-secreting mouse Balb/c myeloma were used as the fusion partner. Specific antibody-producing hybridomas were identified by indirect enzyme-linked immunosorbent assay using peptides adsorbed onto the wells (Callebaut et al. 1991). The monoclonal reagent (8H7) used in this study was obtained from ascitic fluid.

Western blotting

After electrotransfer, Immobilon sheets were incubated overnight with a 1/250 dilution of anti-RGH2 monoclonal antibody (ascitic fluid diluted in Tris-HCl (50 mmol/l), NaCl (200 mmol/l), pH 7-4 (TBS) containing 1% bovine serum albumin (BSA)) then for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG. Sheets were first washed for 30 min with TBS containing 0.1% Nonidet P-40, secondly for 1-5 h with TBS containing 1% BSA and finally for 15 min with TBS containing 0·1% Tween 20. A non-related monoclonal antibody (against the bovine leukemia virus) was also used as negative control.

Colour development from NBT-BCIP substrates (NBT, Sigma; BCIP, Boehringer, Mannheim GmbH, Germany) were used as recommended by the manufacturers to detect alkaline phosphatase activity.

Changes in GHBP in the plasma of dairy heifers

To determine the changes in GHBP during the first year of life, 55 daughters of a Holstein bull were sampled by venepuncture at 4, 6, 9 and 12 months of life. These 55 half-sister dairy heifers were being bred in various farms (n=25). The plasma was separated from 10 ml blood as described above and stored at 20 °C until assayed.

GHBP were evaluated by Western ligand blotting. Autoradiograms were scanned using densitometry (GS-300; Hoefer, San Francisco, CA, U.S.A.) and band intensities were measured for each lane using the GS-365W Hoefer program.

RESULTS

Exclusion chromatography

Figure 1a shows the radioactive elution profile on a Sephadex G-200-calibrated column of pooled cattle plasma preincubated with 125I-labelled rbGH under non-denaturing conditions. Five peaks of radioactivity were observed. The first peak appeared near the void volume, the second peak (approximately 290 kDa) probably corresponded to the low-affinity GHBP of high molecular weight while the third peak (approximately 75 kDa) contained predominantly the high-affinity GHBP found in human plasma. Free 125I-labelled rbGH and Na[125I] eluted with peaks 4 and 5 respectively.

Western ligand blotting

Incubation with 125I-labelled rbGH of proteins blotted onto Immobilon sheets after electrophoresis under denaturing and reducing conditions revealed three distinct major binding sites with apparent molecular weights of 190, 58 and 31 kDa by autoradiographic visualization (Fig. 2). Specificity studies indicated that an excess of unlabelled rbGH was able to displace the total 125I-labelled rbGH bound to these bands (Fig. 3a). Other non-specific minor bands were also observed with molecular weights of 136, 97, 46 and 40 kDa.
To assess the binding specificity of ¹²⁵I-labelled rbGH in the exclusion chromatography experiment, Western ligand blots were performed on fractions corresponding to peaks 1, 2 and 3 (Fig. 1b). After autoradiography, peak 2 contained predominantly GHBP probably corresponding to low-affinity GHBP affinity (190 kDa band) and also contained a 58 kDa protein in smaller quantities, while peak 3 was essentially made up of 58 and 31 kDa proteins. Other minor bands were not significantly evident in the fractions of the two elution chromatographic peaks. No specific GHBP bands were detected in fractions from peaks 1, 4 and 5.

**Western blotting**

Western blot analysis performed with a monoclonal antibody (8H7) directed against RGH2 peptide identified two specific bands with an estimated molecular weight of 190 and 58 kDa respectively (Fig. 3b, lane 3). No 31 kDa specific additional major or minor bands were recognized by our monoclonal antibody.

**Changes in GHBP in the plasma of dairy heifers**

After autoradiography, the major GHBP bands at 190 and 58 kDa described above showed similar intensities in plasma samples of half-sister heifers throughout the experimental period. However, the 31 kDa GHBP form varied in intensity between animals (Fig. 2). Indeed, at each blood-collecting period, the amount in this band fluctuated between being absent to high intensity. The differences between animals remained similar throughout the experimental period. Data also indicated that the intensity of this band remained relatively stable with age in the majority of animals (Fig. 2).

**Identification of GHBP in milk**

 Autoradiographic visualization after Western ligand blotting of milk samples revealed GHBP bands similar to those identified in plasma. However, the intensity of the 190 kDa band was less substantial than in plasma. Immediately after parturition the intensity of all bands observed in milk reduced rapidly and remained low from day 3 to day 5 of lactation (Fig. 4).

**DISCUSSION**

In contrast to the studies performed by Gavin et al. (1991), the present data clearly demonstrate that bovine plasma and milk proteins are able to bind GH. These observations confirm and extend previous reports on the existence of specific plasma GHBP in cattle (Shaw & Baumann, 1988; Davis et al. 1992).

Exclusion chromatography revealed two peaks of bound ¹²⁵I-labelled rbGH in the plasma of cattle as have been described in human (Baumann, 1991) and rat (Massa et al. 1990) plasma. However, the molecular weight of the first rbGH–GHBP chromatography complex (290 kDa) that we identified in cattle was much higher than that reported in other species (120–190 kDa) (Massa et al. 1990; Baumann, 1991). This reason for this is unclear but may be due to differences between species, to variations in the methods or the resins used to isolate the GHBP or to the different origins of GH (recombinant or native) used as radioligands (Davis et al. 1992). Another possible reason for the differences is the presence of anti-GH receptor antibodies which constitute a large complex in the plasma, as has recently been reported in human acromegaly (Campino et al. 1992). This suggestion requires further investigation. The additional peak which eluted near the void volume may be an artefact because non-specific GHBP bands were seen in Western ligand blots. As suggested by Daughaday et al. (1987, 1988), this peak may have resulted from an aggregation of the labelled hormone.

Because of controversy about the identification of GH-binding activity in the plasma of cattle by exclusion chromatography we also used Western ligand blotting for the identification of GHBP. This technique revealed multiple bands and thus confirmed the ability of bovine plasma to bind GH. As in rats (Baumbach et al. 1989), mice (Smith et al. 1989) and poultry (Vasilatos-Youken et al. 1990), major binding sites were observed at approximately 58 and 31 kDa; this latter one was described by Vasilatos-Youken et al. (1990) as a protein lacking N-linked carbohydrates. These proteins appeared to bind rbGH specifically because an excess of unlabelled GH prevents binding of the corresponding labelled hormone. Attention must be drawn to the fact that iodination by the lactoperoxidase method prevents saturation assays under our experimental conditions, even when large quantities of unlabelled hormone have been used. The 58 kDa band observed in the plasma of cattle could be identified as the high-affinity GHBP described in human plasma and as the extra-cellular domain of the GH receptor found in the plasma membrane of hepatocytes described in other species (rabbit, human, rat, mouse) (see review by Baumann, 1991). Indeed, using the Western blotting technique, our monoclonal antibody directed against amino acid 54–63 of the external domain of the bovine GH receptor clearly recognized this band. Purification, characterization and amino acid sequence determination of this plasma bovine GHBP are needed before the ultimate relationship between this and the
GH receptor (Hauser et al. 1990) can be discerned in cattle.

Non-specific minor bands with apparent molecular weights between 40 and 136 kDa also appeared in Western ligand blotting as reported by the authors quoted above in poultry, mice and rats, and we have also shown an additional major band at 190 kDa. This band at 190 kDa identified in cattle plasma and saturable by an excess of unlabelled hormone could correspond to the low-affinity 120 kDa GHBP described in man (Baumann, 1991). The cross-reactivity found by Western blotting between this band and our monoclonal antibody directed against 10 amino acids of the extracellular domain of the bovine GH receptor has not been clear until now; indeed the low-affinity binding protein of 120 kDa has been described in man as structurally different from the high-affinity binding protein (Baumann, 1991). The purification and determination of the amino acid sequence of this binding protein might confirm or refute the fact that there may be some similarities between the 58 and 190 kDa bands.

Finally, the lack of cross-reactivity of the 31 kDa band with our monoclonal anti-peptide antibody in Western blotting may first suggest that this band could be a truncated form of the 58 kDa GHBP

FIGURE 2. Western ligand blotting of plasma GH binding proteins from representative half-sister dairy heifers (numbers 11, 12 and 15) at 4, 6, 9 and 12 months of age. The positions of molecular weights (kDa) are indicated.

FIGURE 3. (a) Western ligand blots where $^{125}$I-labelled recombinant bovine GH (rbGH) was incubated in the presence (lane 1, 1 mg rbGH; lane 2, 500 µg rbGH + 500 µg bovine serum albumin (BSA)) or absence (lane 3, 1 mg BSA) of unlabelled rbGH. Molecular weights (kDa) are indicated. (b) Western blot analysis of GH-binding proteins after electrophoresis under denaturing conditions. Immunoblotting was performed on bovine pool plasma using phosphate buffer (lane 1), a non-related monoclonal antibody (lane 2) or monoclonal antibody directed against the synthetic peptide representing amino acids 54–63 of the extracellular domain of the bovine GH receptor (lane 3). Molecular weights (kDa) are shown.

without the binding site of the anti-peptide antibody or secondly that, by analogy with results obtained in poultry (Vasilatos-Younken et al. 1990), the 31 kDa band could be a deglycosylated form of the 58 kDa GHBP where the epitope is not available to the anti-peptide antibody even after Western ligand blotting. In fact, we have recently shown that the reactive potential of anti-peptide antibodies against a retrovirus glycoprotein may required an additional acetylation of the sulphhydryl group of the blotted protein (Callebaut et al. 1991b). However, inhibition of labelled hormone binding by an excess of unlabelled
hormone demonstrated the specificity of this 31 kDa band in binding GH. Further investigations are needed to explain the nature of this band.

The Western ligand blotting performed on elution chromatography fractions of plasma confirmed the presence of GHBP in cattle plasma; peak 2 was predominantly made up of the 190 kDa band and partially by the 58 kDa band while peak 3 contained the 58 and 31 kDa bands.

Our studies also show that GHBP activity in 55 half-sister dairy heifers, determined by Western ligand blotting, revealed a great variability in the intensity of the 31 kDa band without being affected by the farm from which the heifers were taken. This observation has not been reported elsewhere to our knowledge and determination of the precise origin and structure of the 31 kDa band is necessary to examine its biological significance; for example, it might be studied when the experimental animals are lactating. Moreover, an increase in plasma GHBP's determined by the same technique was also observed in a minority of half-sister heifers during the first year of life, and more precisely against the 31 kDa band.

Confirming the observations in the rabbit (Postel-Vinay et al. 1991) and man (Mercado & Baumann, 1992), Western ligand blot performed on the milk of cattle revealed similar specific GHBP bands to those in plasma. However, the intensity of these bands decreased quickly during the first days of lactation. The reason for this is unclear and should be investigated further.

In conclusion, the present studies clearly identified GH-binding proteins in cattle plasma. Using Western ligand blotting, we demonstrated three major specific bands that bind GH. Further investigations on the role of the circulating binding proteins in GH regulation and their biological action in cattle remain to be carried out with regard to the variability of the 31 kDa band observed between half-sisters.

ACKNOWLEDGEMENTS

This work was supported by grants 5293A and 5402A from the Institute for the Encouragement of Scientific Research in Industry and Agriculture (IRSIA; Belgium). The authors wish to thank Mrs. R.-M. Maistriaux and M. Nuttinck and Mr Y. Baret for their technical assistance.

REFERENCES


