Purification and characterization of a uterine retinol-binding protein in the bitch

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A major canine endometrial secreted protein (cP6, 23000- M_r) was purified by ion-exchange and gel-filtration chromatography and characterized by two-dimensional gel electrophoresis. Anti-[human retinol-binding protein (hRBP)] serum identified cP6 on immunoblot analysis and immunoprecipitated cP6 from culture medium. This major protein was also shown to bind [3 H]retinol. N-terminal and internal amino acid sequences were determined and compared with previously identified protein, RNA, or DNA sequences. N-terminal analysis revealed that cP6 had high identity and similarity to serum retinol-binding proteins (RBPs), while internal sequence analysis showed a strong similarity to rat androgen-dependent epididymal protein and β -lactoglobulins.

Amino acid analysis, however, showed significant differences between these proteins and cP6 in both total amino acid content and certain selected amino acids. Immunohistochemical analysis showed staining for RBP only in the uterine luminal epithelium. These studies suggest that bitch endometrium secretes a family of proteins (cP6), some of which bind [3 H]retinol, are immunologically related to the RBP family, and have N-terminal and internal sequences with a high similarity to RBP, β -lactoglobulins and other members of the lipocalin family. This family of proteins may be important in early development for supplying retinol or derivatives to the developing embryo.

INTRODUCTION

Many species, including the sheep, cow, horse, pig, mouse, dog, cat, baboon and human [1–9], have been shown to secrete a complex array of oestrogen- and progesterone-regulated proteins into the uterine lumen during the oestrous or menstrual cycle and early pregnancy. During the peri-implantation and early implantation period, the embryo and conceptus are believed to require uterine secretory products, vitamins, growth factors, various proteins and other agents for a variety of roles such as nutrition, pre-implantation growth and development, immunoprotection and other implantation events that ensure establishment and maintenance of pregnancy.

In both cyclic and early pregnant bitches (between dioestrus days 3 and 10) circulatory levels of oestrogen are basal, whereas progesterone increases toward peak levels [6,10,11]. In pregnant animals, embryos enter the uterus after dioestrus day 3 [6] and are present by day 4–5. Embryos enter the uterus as morulae and rapidly become free-floating blastocysts, which grow and migrate throughout the uterus for 7–10 days [12] until implantation is initiated at some time after dioestrus day 10 [6,12,13].

In the dog, as in other species, progesterone is thought to stimulate or maintain this uterine secretory activity. Previous studies in our laboratory have shown that a complex array of proteins are synthesized and secreted by the endometrium from cyclic and pregnant bitches between days 3 and 10 [6], prior to the onset of implantation. Between days 10 and 26 after the onset of implantation, synthesis continues at sites where implantation has not yet begun, as well as at, and inbetween, implantation sites [6,13]. Two major groups of proteins, cP5 (M_r 54686) and cP6 (M_r 23010), appear to be differentially regulated during dioestrus days 3–10, suggesting control of protein expression by progesterone. A recent study has further shown that progesterone given to oestrogen-primed ovariectomized bitches for 7 or 14

days markedly increased *de novo* synthesis and secretion of both cP5 and cP6 by the endometrium [6]. Neither oestrogen nor progesterone alone had an effect. Protein cP6 appears, by relative molecular mass (M_r), pI and electrophoretic distribution, to be similar to a progesterone-induced family of retinol-binding proteins (RBPs) found in uterine secretions of the pig [14,15]. In addition, immunoprecipitation studies have suggested that cP6 proteins may be RBP-like proteins [13]. In the pig, these proteins form part of the histotroph thought to be essential for nourishment of the pre-implantation conceptus during early pregnancy [4].

The objectives of this study were: (1) to purify cP6 polypeptides, (2) to characterize cP6 by determining the N-terminal amino acid sequence and portions of the internal sequence obtained from fragments generated by cyanogen bromide (CNBr)-cleavage, (3) to compare these sequences to previously identified sequences in protein, RNA and DNA databases in order to identify the proteins, and (4) to localize immunohistochemically these proteins within the uterus.

MATERIALS AND METHODS

Materials

Acrylamide, *N,N'*-diallyltartardiamide, urea and SDS were obtained from Gallard-Schlesinger, Carle Place, NY, U.S.A.; X-Omat AR (XAR-5) was a product of Eastman-Kodak, Rochester, NY, U.S.A.; amino acids, protein standards and Protein A-Sepharose (CL-4B) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; ampholines, gel-filtration medium and DEAE-Sepharose CL-6B were from Pharmacia-LKB, Piscataway, NJ, U.S.A.; goat anti-(rabbit IgG)-horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.; all supplies and reagents for gel electrophoresis were purchased from either Bio-Rad Laboratories or

Abbreviations used: cP6, a major canine endometrial secreted protein; hRBP, human RBP; i.m., intramuscularly; [3 H]Leu, L-[4,5- 3 H]leucine; MEM, minimum essential medium; [35 S]Met, Tran[35 S]methionine; PVDF, poly(vinylidene difluoride); RBP, retinol-binding protein.

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Fisher Scientific, Orlando, FL, U.S.A.; L-[4,5-³H]leucine (specific radioactivity 120 Ci/mmol) was obtained from Amersham Corporation, Arlington Heights, IL, U.S.A.; Tran[³⁵S]methionine ([³⁵S]Met; specific radioactivity 1045 Ci/mmol) was purchased from ICN Radiochemicals, Irvine, CA, U.S.A.; rabbit anti-(human RBP) was purchased from DAKO Corporation, Santa Barbara, CA, U.S.A.; Immobilon-P and -PSQ membranes and poly(vinylidene difluoride) (PVDF) were purchased from Millipore Corporation, Bedford, MA, U.S.A.; Vectastain ABC Kit was obtained from Vector Laboratories, Burlingame, CA, U.S.A.; and all medium and culture supplies were purchased from Life Technologies, Grand Island, NY, U.S.A.

Animals

Adult mixed-breed bitches were housed at the University of Florida Animal Resource Facility in approved indoor/outdoor runs and observed for behavioural oestrus. Bitches were randomly assigned to oestrous cycle or pregnancy study groups, and the latter group was bred daily as long as they were receptive. During proestrus and until the end of oestrus, daily vaginal smears were taken and monitored for changes in microscopic appearance of exfoliated vaginal epithelium. The cornification index was determined as previously described [16] and was used to establish the beginning of dioestrus, defined as the initial appearance of > 20% of non-cornified epithelial cells (day 1). Day 1 of dioestrus is known to be correlated with the luteinizing hormone peak, which precedes it by 8–10 days. Concentrations of oestrogen, progesterone and luteinizing hormone were determined in daily samples of jugular vein blood collected into heparinized tubes after the onset of proestrus as previously described [6]. On days 3, 7, or 10 of dioestrus, pregnant or cyclic bitches were anaesthetized with thiamylal sodium (3.64 mg/kg body weight, given intravenously) and maintained on Halothane while the reproductive tract was exposed by a mid-line laparotomy, and one uterine horn, oviduct and ovary were surgically removed. Each uterine horn was flushed with 20 ml of sterile medium to collect uterine secretions and then the secretions were stored at –20 °C until analysed.

Medium preparation and explant culture

Eagle's leucine-deficient (modified) minimal essential medium (MEM) was prepared as described previously [17]. The aseptically collected tissue was trimmed free of peritoneal attachment and the uterine horn was opened longitudinally. The endometrium was removed, transferred to a sterile Petri dish, rinsed in MEM and subsequently cultured as previously described [6]. Briefly, 500 mg of endometrium was added to 15 ml of modified MEM containing 100 µCi of L-[4,5-³H]leucine ([³H]Leu) or 40 µCi of [³⁵S]Met and incubated at 37 °C in a controlled atmosphere of N₂/O₂/CO₂ (20:19:1, by vol.). After 24 h, medium and tissue were separated and stored at –20 °C until analysed.

Ion-exchange and gel-filtration chromatography

The major endometrial protein cP6 was purified by the procedure of Harney et al. [18] with some modification. Explant culture medium conditioned by endometrium collected from non-pregnant bitches on dioestrus days 7 (67 ml) and 10 (40 ml) was pooled and extensively dialysed (*M_r* cut-off 3500) against 10 mM Tris/HCl (pH 8.2) (two changes of 4 litres each/24 h) to remove low-*M_r* compounds and unincorporated label and then dialysed against deionized water (two changes of 4 litres each/24 h) to remove salts. Dialysed medium, diluted with 100 mM Tris/HCl (pH 7.5) to give a final concentration of 10 mM Tris/HCl, was

adsorbed on to a DEAE-Sepharose column (1 cm × 3 cm) equilibrated with 10 mM Tris/HCl, pH 7.5. The column was washed (8–10 bed vol. of 10 mM Tris/HCl, pH 7.5) and the protein eluted with 3 M NaCl in the loading buffer. Aliquots (2 ml) of 3 M NaCl eluent were subjected to gel-filtration chromatography on a Sephadex G-100 column (1.5 cm × 90 cm) equilibrated in 10 mM Tris/HCl, 0.4 M NaCl, 0.02% (w/v) NaN₃, pH 7.5. Fractions (2 ml) were collected, and radioactivity in 100 µl aliquots was determined by liquid scintillation spectrometry to establish elution profiles. Standards included Blue Dextran, ovalbumin, soybean trypsin inhibitor, cytochrome *c* and riboflavin.

Electrophoresis and Western blotting

Dialysed conditioned culture medium, uterine flush, gel-filtration fractions, or immunoprecipitates were lyophilized for one- or two-dimensional SDS/PAGE. For one-dimensional SDS/PAGE, lyophilized samples were dissolved in solubilization buffer containing 10 mM Tris/HCl, 10% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 15% (w/v) sucrose and 0.001% Bromophenol Blue. For two-dimensional SDS/PAGE, samples were dissolved in 5 mM K₂CO₃ containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol. Two-dimensional SDS/PAGE resolves acidic proteins (pI < 8) by isoelectric focusing in the first dimension and by molecular mass using SDS/10%-polyacrylamide separating gels in the second dimension [19]. Gels were fixed, stained with Coomassie Blue, destained, photographed, washed in water, soaked in 1 M sodium salicylate and dried. Fluorographs were prepared by exposing dried gels at –70 °C to Kodak XAR-5 film.

Alternatively, two-dimensional SDS/PAGE gels were equilibrated for 30 min in electrophoretic transfer buffer [0.025 M Tris/HCl, 0.192 M glycine, 20% (v/v) methanol, pH 8.3], and then proteins were transferred to PVDF membranes at 200 mA for 3 h at room temperature [20]. For N-terminal amino acid microsequencing, proteins were electrophoretically transferred to PVDF membranes using 10 mM 2-(*N*-morpholino)ethanesulphonic acid, 20% (v/v) methanol, 0.1% (w/v) EDTA, pH 6.0, at 200 mA for 4 h at room temperature and were stained with 0.01% Coomassie Blue in 50% (v/v) methanol, destained in 50% (v/v) methanol, and stored in deionized water containing 0.02% (w/v) NaN₃.

Immunoblot analysis

PVDF membranes, previously rinsed and stored in deionized water containing 0.02% (w/v) NaN₃, were incubated in TBS blocking buffer [20 mM Tris/HCl, 3% (w/v) gelatin, 0.5 M NaCl, pH 7.5] and washed in TBS containing 0.05% (v/v) Tween-20 (TTBS). Binding of rabbit anti-hRBP was detected using either ¹²⁵I-labelled Protein G [18] or goat anti-(rabbit IgG)-horseradish peroxidase conjugate as previously described [21]. For Protein G, blots were immersed in incubation buffer [10 mM Tris/HCl, 0.1% (w/v) ovalbumin, 0.15 M NaCl, 0.05% (v/v) Tween-20, 0.02% (w/v) NaN₃, pH 7.4] containing either rabbit anti-hRBP (1:200 dilution) or non-immune normal rabbit serum (1:200 dilution) and incubated overnight at room temperature. Blots were washed with three changes of wash buffer (1 h), incubated with 1 × 10⁶ c.p.m./ml ¹²⁵I-labelled Protein G in incubation buffer (1 h), washed with wash buffer (four changes/1.5 h), rinsed in deionized water, and dried. Dried blots were wrapped in plastic wrap and exposed to XAR-5 film at –70 °C.

Immunoprecipitation

Aliquots of [^3H]Leu-labelled endometrial culture medium or DEAE-Sephadex 3 M NaCl eluates were dialysed, lyophilized and dissolved in immunoprecipitation buffer [22]. Protein solutions were incubated with rabbit anti-hRBP serum (1:16 dilution) or normal rabbit serum (undiluted) and precipitated with 100–200 μl of a 10% (w/v) Protein A–Sephadex (CL-4B) suspension. After washing, immunoprecipitates were solubilized and analysed by one-dimensional SDS/PAGE and fluorography. Alternatively, immunoprecipitates were electrophoretically transferred to PVDF membranes after two-dimensional SDS/PAGE and subjected to microsequencing analysis.

Amino acid microsequencing of N-terminal and CNBr-cleaved peptides

PVDF membranes containing cP6 proteins from Sephadex G-100 pooled peak 3 or rabbit anti-hRBP immunoprecipitate from uterine explant culture medium were dried and stored at -20°C until subjected to N-terminal amino acid microsequencing analysis. For internal amino acid microsequencing analysis, PVDF membranes containing G-100 pooled peak 3 material (cP6) were cut into small strips and incubated overnight in 70% (v/v) formic acid and 1 M CNBr. Membranes were washed with deionized water and peptide extracted with propan-2-ol (70%, v/v)/trifluoroacetic acid (5%, v/v), followed by solubilization in one-dimensional PAGE buffer at room temperature and concentration in a Speed Vac apparatus. After washing with deionized water, CNBr-cleaved fragments were separated on a 15% Tris/Tricine gel [23] and transferred to Immobilon PSQ membrane using 10 mM 2-(*N*-morpholino)ethanesulphonic acid, pH 6.0, 0.1% (w/v) SDS, 20% (v/v) methanol at 70 V for 30 min. Blots were stained with Coomassie Blue and destained as described above.

Intact proteins and CNBr-cleaved peptides were microsequenced by the Protein Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida, using an Applied Biosystems Model 470A gas-phase protein sequencer with an on-line analytical HPLC system. Sequence data were analysed and compared with the National Biomedical Research Foundation (Washington, DC, U.S.A.) protein database and the GenBank Genetic Sequence Database (Intelligenetics, Mountain View, CA, U.S.A.) using FASTA [24], TFASTA [24] and BLAST [27] programs.

Retinol binding assay

Endometrial explant culture medium was pooled from bitches ovariectomized and treated with oestradiol cypionate (Upjohn Corp., Kalamazoo, MI, U.S.A.) at 5.4 $\mu\text{g/kg}$ body weight intramuscularly (i.m.) daily on days 1–3 and 10.8 $\mu\text{g/kg}$ on days 4–7 for a total of 1 week. This was followed by progesterone (progesterone in oil; Carter–Glogon Lab, Glendale, AZ, U.S.A.) at 5 mg/kg body weight i.m. daily for a total of 1 week or 2 weeks [6]. Medium was dialysed and protein cP6 purified by ion-exchange and gel-filtration chromatography as described. Pooled peak 3 protein (1 mg) in 2 ml of 20 mM sodium phosphate, pH 7.1, containing 0.3 M NaCl, was incubated for 4 h at 4°C in the dark with one of the following: 75 pmol (2 μCi) of [^{11}C]retinol (DuPont NEN Research Products, Wilmington, DE, U.S.A.) or 75 pmol (2 μCi) of [^3H]retinol plus 770 nmol of unlabelled retinol (all-*trans*; Sigma, St. Louis, MO, U.S.A.) [25]. After incubation the reaction mixture was loaded on to a Sephadex G-100 column (1 cm $\times$ 90 cm) and run in the dark at 4°C , and 2 ml fractions were collected. The elution profile was

determined by counting (0.4 ml) in a liquid scintillation spectrometer. Protein standards included Blue Dextran, ovalbumin, soybean trypsin inhibitor, cytochrome *c* and riboflavin.

Immunocytochemical localization of RBP

Bitch endometrial tissue was collected at surgery and placed in Bouin's fixative solution for 48 h, dehydrated through a graded series of ethanol solutions (50–100%), embedded in paraffin, and mounted on glass slides. Sections were deparaffinized and stained using the Vectastain ABC Kit and rabbit anti-hRBP antibody. Adjacent tissue sections were incubated with normal rabbit serum.

RESULTS

Electrophoretic analysis of uterine secretions and culture medium

Distribution of endometrial proteins synthesized *de novo* and secreted into explant culture by tissue from a pregnant bitch or found to be present in uterine flushes from pregnant bitches on dioestrus day 10 are shown in a representative two-dimensional SDS/PAGE gel and fluorograph (Figure 1). A similar distribution of proteins synthesized *de novo* was found in medium from cyclic bitches on day 10 (results not shown). A group of five acidic proteins at M_r 23010 were found in explant culture medium when labelled with [^{35}S]Met rather than with [^3H]Leu and exposed for short time periods (results not shown). When tissues were labelled with [^3H]Leu, only three proteins were clearly seen (Figure 1a, cP6b). Two other minor groups of proteins (cP6a, M_r 24904; cP6c, M_r 21506; Figure 1a), differed from cP6b by M_r 1500–1900, and each appeared to contain at least three species. Each minor protein appeared to correspond to a similar isoelectric species of the cP6b form. Although three isoelectric species of cP6b, shown in Figure 1(a), appear to be of equal intensity, spots 2 or 2 and 3 together were usually the most intense. The proteins synthesized *de novo* by cultured endometrial tissue can also be identified by two-dimensional SDS/PAGE in uterine secretions of pregnant or cyclic bitches during early dioestrus (Figure 1b). A group of low- M_r acidic proteins,

Figure 1 Electrophoretic analysis of proteins synthesized *de novo* and secreted into culture medium or present in uterine flushings from a pregnant bitch

(a) Representative fluorograph from a two-dimensional SDS/PAGE analysis of explant culture medium (200 000 c.p.m. [^3H]Leu) conditioned by endometrium taken on dioestrus day 10 from a pregnant bitch. The major 23010- M_r proteins (cP6b) are indicated by an arrow. Higher- and lower- M_r species (cP6a, 24904- M_r ; cP6c, 21506- M_r) and isoelectric variants are labelled with an a and b and numbers 1, 2 and 3, respectively. (b) Uterine flush (500 μg of protein) from a pregnant bitch on dioestrus day 11 was analysed by two-dimensional SDS/PAGE and Coomassie Blue staining. A major protein, which corresponds to labelled secreted proteins cP6b in culture medium in (a), is identified by an arrow. Relative molecular masses are indicated on the left.

Figure 2 Sephadex G-100 elution profile of acidic proteins (DEAE-positive) from pooled explant culture medium conditioned by endometrium from non-pregnant bitches

Endometrium samples were collected on dioestrus days 7 and 10. Three labelled peaks were eluted with 10 mM Tris/HCl, 0.4 mM NaCl, pH 7.5. Radioactivity (10^{-3} c.p.m./100 μ l) is indicated on the left. Standards included Blue Dextran (M_r 2×10^6), ovalbumin (M_r 45000), soybean trypsin inhibitor (M_r 20100), cytochrome *c* (M_r 12500), and riboflavin (M_r 376).

Figure 3 Representative fluorographs of a two-dimensional SDS/PAGE analysis of dog endometrial proteins present in the DEAE-positive fraction subsequently separated on Sephadex G-100

(a) Pooled peak 1 contained labelled proteins in the void volume. (b) Pooled peak 2 contained proteins in the included volume but with M_r greater than 45000. Protein identification corresponds to those canine proteins (cP) previously identified in pregnant and cyclic bitches [6]. Relative molecular masses are indicated on the left.

Figure 4 Western-blot analysis of cP6 with anti-hRBP serum

(a) Analysis of peak 3 from the Sephadex G-100 separation of explant culture medium with rabbit anti-hRBP serum treatment. Specific binding was detected by incubation with 125 I-Protein G, followed by radiography. (b) Same sample as in (a), but after treatment with normal rabbit serum. Arrows 6b and 6c refer to protein cP6 with M_r values of 23500 and 21000 respectively. (c) Analysis of cP6 from a dioestrus-day-11 uterine flush with rabbit anti-hRBP serum.

determined by Coomassie Blue staining, corresponded to the 23000- M_r group of proteins (cP6b) in Figure 1(a).

Purification

Explant culture medium (110 ml) was applied to DEAE-Sepharose to remove basic proteins, and the bound acidic fraction (DEAE positive; pH < 7.5) was recovered by a one-step elution with 3 M NaCl. The elution profile of the DEAE-positive endometrial proteins separated by gel-filtration chromatography on Sephadex G-100 is shown in Figure 2. Proteins were eluted in three radiolabelled peaks, designated 1, 2 and 3. As shown in Figure 3(c), peak 3, which eluted between protein standards ovalbumin (45000- M_r) and soybean trypsin inhibitor (20100- M_r), contained the three to five isoelectric species of the 23010- M_r proteins, the only radiolabelled proteins present. Only small amounts of non-radiolabelled proteins were present (results not shown). A recovery of about 15% of total labelled protein from pooled conditioned culture medium on days 7 and 10 was achieved.

Other proteins synthesized by early dioestrus endometrium [13] were localized in peaks 1 and 2 (Figures 3a and 3b). Proteins cP1 and cP8 were concentrated in peak 1, while proteins cP2,

Figure 5 Representative PVDF membrane containing Coomassie Blue-stained electrophoretically transferred peak 3 (cP6) protein after purification by ion-exchange and gel-filtration chromatography

Numbers 1, 2 and 3 indicate the most relatively acidic, neutral, and basic variants of cP6 respectively. These spots were subjected to N-terminal microsequencing analysis, amino acid analysis, and CNBr cleavage followed by N-terminal microsequencing analysis of electrophoretically separated major fragments. Relative molecular masses are indicated on the left.

cP4, cP7 and, the second major protein, cP5, were concentrated in peak 2.

Immunoprecipitation and Western blot analysis of uterine proteins

Endometrial explant culture medium containing radiolabelled proteins synthesized *de novo* and purified by ion-exchange and gel-filtration chromatography (peak 3) was treated with normal rabbit serum or rabbit anti-hRBP serum, and the precipitate was examined by one-dimensional SDS/PAGE and fluorography. Similarly to recent one-dimensional and two-dimensional SDS/PAGE studies [13], these results indicated that hRBP antiserum will immunoprecipitate the 23000- M_r cP6 synthesized *de novo* and secreted by uterine endometrium during early dioestrus.

To characterize further the cP6 proteins as an RBP, peak 3 protein, [^3H]Leu-labelled from explant culture medium, and uterine flush proteins were separated by two-dimensional SDS/PAGE and transferred to PVDF membranes. Western blots of peak 3 protein were incubated with either anti-hRBP or normal rabbit serum (results not shown), treated with ^{125}I -Protein G and examined by autoradiography (Figure 4a). Proteins with two M_r values and three isoelectric species were identified as corresponding to cP6. Similar proteins were identified on companion fluorographs after separation of peak 3 on two-dimensional SDS/PAGE. Blots of uterine flush protein were similarly treated with primary antiserum, and binding was detected by goat anti-(rabbit IgG)-conjugated horseradish peroxidase (Figure 4b).

These studies indicated that proteins with different M_r values (M_r 23 500; M_r 21 000) but with at least three isoelectric variants immunoreacted with anti-hRBP. Protein (cP6) synthesized *de novo* (Figure 1a), uterine flush protein (Figure 1b), immunoblotted purified cP6 (Figure 4a), and immunoblotted uterine flush protein (Figure 4b) all showed the same distribution of isoelectric and M_r variants.

Amino acid microsequencing of N-terminal and CNBr-cleaved fragments

Protein cP6 was immunoprecipitated from endometrial explant culture medium using anti-hRBP and Protein A-Sepharose, solubilized and separated by two-dimensional SDS/PAGE as described above, and transferred to Immobilon PVDF membranes. Coomassie Blue-stained PVDF membranes showed transfer of at least two isoelectric variants of the cP6b form (23000 M_r), and N-terminal amino acid analysis was performed on each. Twenty-two amino acids of isoelectric variants 1 and 2 (see Figure 5) were positively identified (Table 1). These acidic and basic variants showed 100 % identity to each other. Analysis of the N-terminal amino acid sequence showed a 68 % identity and a 95 % similarity to dog RBP in the first 22 amino acids [26]. Computer databases for protein and nucleic acids, using FASTA, TFASTA and BLAST programs, revealed a 77–81 % identity and 95 % similarity between cP6 and human, mouse, rat, pig, rabbit and cow RBPs.

Blots containing chromatographically purified isoelectric variants 1, 2 and 3 from protein cP6b (Figure 5) were treated with CNBr to generate internal fragments for N-terminal amino acid analysis. The major fragment from isoelectric variant 1 (most acidic) was sequenced through 20 amino acids (Table 2), while variant 3 (most basic) was sequenced through 10 amino acids and was found to be 100 % identical to variant 1 (results not shown). Two residues, 13 and 19, were not identified. While database searches did not indicate a similarity between internal cP6 fragments and dog RBP, there was a 54 % and 43 % identity in a 13- and 14-amino-acid overlap, respectively, between cP6b variant 1 and rat androgen-dependent epididymal 18.5 K protein and sheep, water buffalo, goat and bovine β -lactoglobulins. These overlaps began at residues 4 and 6 (CNBr-generated fragment 1) and residues 86 (β -lactoglobulins) and 83 (androgen-dependent epididymal protein) respectively. When conservative amino acid replacements were considered, variant 1 and the epididymal protein and the β -lactoglobulins shared 92 % and 86 % sequence similarity within the 13-/14-amino-acid overlap respectively.

Table 1 N-terminal amino acid sequence of bitch uterine secretory protein cP6 (acidic and basic variants) and dog and human plasma retinol-binding protein (dRBP and hRBP)

Protein	Amino acid position																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
cP6 ^a	Glu	Ser	Asp	Cys	Arg	Val	Ser	Asn	Phe	Gln	Val	Lys	Lys	Asn	Phe	Asp	Lys	Ala	Arg	Phe	Ala	Gly
dRBP ^b	Glu/ Gln	Ser/ Cys	Asn/ Asp	.	.	.	.	Asn/ Asp	.	Gln/ Glu	.	.	.	Asn/ Asp	.	Asn/ Asp	.	.	.	.	.	.
hRBP	.	Arg	.	.	.	.	.	Ser	.	Arg	.	.	Glu	.	.	.	.	.	.	.	Ser	.

^aIsoelectric variants 1 and 2 are identical; only one sequence is shown.

^bIdentical amino acids are indicated by a dot and substitutions are indicated.

Table 2 The N-terminal amino acid sequence of the major CNBr-generated fragment of protein cP6 isoelectric variant 1 (cP6b-1) compared with the androgen-dependent epididymal 18.5 K protein (ADEP) and sheep β -lactoglobulin (β LG)

(X) denotes unidentified amino acids.
Lines between two amino acids denotes identity and a dot indicates a conservative replacement.

Protein	Amino acid residues																	
			85					90					95				100	
β LG	Lys	Ile	Asp	Ala	Leu	Asn	Glu	Asn	Lys	Val	Leu	Val	Leu	Asp	Thr	Asp	Tyr	Lys
						.	.	.	.		.		.	.	.			.
cP6-1	Met	Pro	Pro	Ala	Leu	Gly	Lys	Asp	Thr	Val	Ala	Val	(Xaa)	Gly	Gly	Asp	Tyr	Leu
								.	.		.		.	.	.			
ADEP	Val	Thr	Arg	Leu	Ser	Gly	Lys	Lys	Glu	Val	Val	Val	Glu	Ala	Thr	Asp	Tyr	Leu
			80					85					90					95

Table 3 Amino acid composition of dog uterine secretory protein cP6, dog urinary RBP, human serum RBP, dog milk β -lactoglobulin (d β LG), and bovine β -lactoglobulin (b β LG)

Abbreviation used: ND, not determined.

Amino acid	Nearest integer of residues found					
	cP6 isoform		dRBP ^a	hRBP ^b	d β LG ^c	b β LG ^c
	Acidic	Basic				
Asx	18	17	27	27	20	15
Thr	12	11	9	9	10	8
Ser	10	9	11	11	4	7
Glx	42	43	18	18	29	25
Pro	14	16	7	5	8	8
Gly	19	19	12	11	2	4
Ala	16	15	14	13	13	15
Cys	ND	ND	7	6	4	5
Val	10	10	12	13	10	9
Met	3	3	4	4	6	4
Ile	10	10	4	4	6	10
Leu	22	22	13	14	21	22
Tyr	7	8	8	8	4	4
Phe	4	5	10	10	5	4
His	3	3	3	2	2	2
Lys	6	6	11	10	7	15
Arg	11	12	12	14	8	3
Trp	ND	ND	6	4	ND	ND
Total	207	209	188	183	159	160

^aDog urinary RBP amino acid composition according to Poulik et al. [26].
^bHuman RBP amino acid composition according to protein NBRF databank.
^cDog and bovine β -lactoglobulin amino acid composition according to Pervaiz and Brew [39].

Amino acid composition

Isoelectric variants 1 (acidic) and 2 (basic) of chromatographically purified cP6b (Figure 5) were analysed for amino acid composition and compared with amino acid compositions of dog and human RBP and dog and bovine β -lactoglobulin (Table 3). The basic isoform, while larger by two amino acids, appeared to be identical to the acidic form, and the calculated M_r (22800–23000), without inclusion of cysteine and tryptophan residues, was comparable to that determined by two-dimensional SDS/PAGE and fluorographic studies.

Comparison of the amino acid composition of cP6 relative to dog and human RBP suggested that there were similarities but

Figure 6 Elution profile of cP6 after incubation with [³H]retinol

(a) Sephadex G-100 elution profile (2 ml/tube) of cP6 protein (1 mg) after a 4 h incubation at 4 °C with 75 pmol (2 μ Ci) of [³H]retinol in the dark. Radioactivity (c.p.m./400 μ l) was determined by scintillation spectrometry. Peak 1, the void volume; peak 2, the fraction containing cP6; and peak 3, the salt volume; are shown. (b) Elution profile of cP6 protein (1 mg) from a Sephadex G-100 column after a 4 h incubation at 4 °C with 75 pmol (2 μ Ci) of [³H]retinol and 770 nmol of unlabelled retinol (all-trans) in the dark. The majority of the radioactive label eluted in the void volume and the salt volume.

also major differences (Table 3). These include significant differences in the amino acids Asx, Glx, Pro, Gly, Ile, Leu, Phe and Lys. The total amino acid content of cP6, not including Cys and Trp, was increased about 12 % relative to serum RBP, which

Figure 7 Immunocytochemical localization of RBP in a section of pre-implantation uterine tissue from a dioestrus day 10 pregnant bitch

Positive staining for RBP was detected in surface epithelium (a) (arrows) but not glandular epithelium (arrowheads). Staining was not found in the normal rabbit serum control (b).

reflected the increased M_r of cP6. Similarly, the amino acid composition of cP6 relative to dog and bovine β -lactoglobulin suggested a parallel distribution of some amino acids but a striking dissimilarity in the distribution of others, including Glx, Pro, Gly, Tyr and Arg. The total amino acid content of cP6 was increased about 30% relative to β -lactoglobulin, and again reflected the increased M_r of cP6.

Retinol binding assay

The Sephadex G-100 column was previously characterized using dog endometrial secreted proteins and cP6. After incubation of G-100 peak 3 protein and [3 H]retinol, three peaks of radioactivity were determined. Proteins in the range of 20000- M_r , appropriate for an RBP, bound [3 H]retinol (57% of total counts) as shown in Figure 6(a), peak 2. Radiolabel not eluting at the 20000- M_r range eluted either in the void volume (14% of total counts) or in the salt volume (29% of total counts), representing free ligand. Binding associated within the range of the 20000- M_r protein appeared to be specific, as addition of excess non-radioactive ligand reduced the radioactivity in peak 2 to 15% of the total radioactivity and increased elution of radiolabel in the void volume and salt volume (Figure 6b). Although the total counts in elution of the mixture of unlabelled retinol, [3 H]retinol, and peak 3 protein were slightly increased (about 35%) compared with elution of [3 H]retinol and peak 3 protein (Figure 6a), this

added radioactivity was determined to be [3 H]retinol stripped from the column matrix by unlabelled retinol.

Immunocytochemical localization

Paraffin-embedded sections of pre-implantation endometrial tissue collected from early dioestrus pregnant bitches were immunostained with anti-hRBP to determine the presence of RBP. Endometrial tissue from a dioestrus day 10 pregnant bitch is shown in Figure 7. Uterine tissue was observed to stain strongly in the apical regions of all luminal epithelium, while immunostaining at uterine gland openings was apparent but did not extend beyond the gland mouth. Staining in the glandular epithelium was either poor or absent, and no staining was observed in the tissue treated with normal rabbit serum.

DISCUSSION

Using a two-step procedure of ion-exchange and gel-filtration chromatography modified from Harney et al. [18], the endometrial protein cP6 was highly purified and appears to consist of several isoforms as well as M_r species. The major form of cP6, however, appears to be a 23000- M_r protein with at least five isoelectric species, as detected with high-specific-activity [35 S]Met labelling.

N-terminal amino acid sequence analysis of different isoelectric variants of cP6 yields a positive identical sequence of 22 amino acids, suggesting a shared N-terminal domain through 22 amino acids. This sequence has a significant identity and homology in the first 22 amino acids with dog RBP [26], rabbit, bovine, human, mouse, rat and pig serum RBP, with pig [18] and cat [28] conceptus RBP, with pig [15,29] and bovine [30] uterine RBP, and with bovine placental and allantoic fluid RBP [31], all of which are members of the lipocalin family. This protein also shares a 46% and 52% identity in the N-terminal region with rat androgen-dependent secretory protein and purpurin respectively, two other members of this superfamily [32]. The most highly conserved sequence in this family of proteins is the region beginning with residue 12, which contributes to the formation of the retinol-containing β -barrel and of particular importance is the glycine at residue 22 [32]. This sequence, including the glycine residue at position 22, appears to be well conserved in protein cP6. Furthermore, a cysteine residue (residue 4), which forms one of three disulphide bridges in RBP, appears to be conserved in cP6 also.

Internal fragments from the different isoelectric variants examined by microsequence analysis indicate that the two isoforms of cP6 are 100% identical through 10 amino acids and share a common sequence. When protein and nucleic acid databases were searched using FASTA and TFASTA programs, sequence similarity to serum RBPs of dog and other species was not found. However, a similarity to rat androgen-dependent epididymal protein and several β -lactoglobulins was demonstrated. These molecules also belong to the lipocalin superfamily and appear to be transporters of small hydrophobic ligands in aqueous environments [32–35]. Lipocalins, as a group, show limited primary and secondary structural similarities, with very few amino acids conserved in all members; however, where available, three-dimensional structural studies show significant similarities in their tertiary structure across this diverse group [32,34–37]. Pairwise comparisons among members of this family show only a 25–30% amino acid sequence similarity [38]. Further, one of several regions that are highly conserved, the amino acid triplet (Thr-Asp-Tyr) [32–34,38], is present in a majority of

members of the lipocalins, with some substitutions occurring in all three positions. In protein cP6, the triplet sequence is Gly-Asp-Tyr.

While N-terminal and internal amino acid sequences suggest identity between the different isoelectric variants of cP6, RBP and β -lactoglobulin, a comparison of the amino acid compositions suggests some dissimilarity. Select amino acids in cP6 appear to be dramatically different to those in the RBP and β -lactoglobulin families, with some amino acids present at levels twofold greater or smaller.

Results from a retinol binding experiment indicate that cP6 is capable of binding retinol *in vitro*, as has been reported for pig uterine [14,15] and conceptus RBP [25]. Similarly to these studies, excess retinol can almost completely displace radiolabelled retinol, suggesting specific binding. Since RBP prefers to bind retinol *in vivo* [40], it may therefore be the natural ligand for uterine cP6. Whether this occurs in the bitch uterine lumen is not known. One possible explanation for multiple isoforms of cP6 is that some isoforms may contain retinol while others may be able to accommodate a variety of other retinol analogues with different binding affinities [15,25,32]. As an example, one experiment in our laboratory suggested that [^3H]retinoic acid could also bind to cP6. Another possibility for these multiple forms is that cP6 may be chemically processed or modified as it accumulates within the uterine lumen or culture medium, changing its isoelectric point.

Immunolocalization experiments in this study demonstrated an RBP-like molecule in the apical portion of luminal epithelium from non-implantation sites of early pregnant bitches. This antigen was either present at very low levels or absent from glandular epithelium. This is in agreement with other studies in the pregnant and cyclic bitch as well as in ovariectomized bitches primed with oestrogen and followed by progesterone treatment [13]. These studies suggest that an RBP-like molecule (cP6) is secreted into the uterine lumen from the luminal epithelium but not from glandular epithelium. These results, however, are in contrast to studies in the pig [18] and cow [30] in which RBP was localized in both luminal and glandular epithelium, suggesting contributions by both epithelia.

Studies related to control of RBP synthesis and secretion have shown that secretion of uterine RBP occurred in ovariectomized gilts in response to progesterone or a combination of oestrogen plus progesterone [14]. Induction of RBP mRNA in endometrial tissue after ovariectomy and oestrogen priming followed by progesterone occurred in nearly half of the gilts examined [41]. However, in gilts treated with a second dose of oestrogen following the progesterone, a treatment which mimics conceptus oestrogen release, RBP mRNA expression was markedly enhanced. In the ovariectomized bitch, similar studies demonstrated that synthesis and secretion of cP6 by endometrium was clearly dependent upon oestrogen priming followed by progesterone [6] since secretion was not detected with oestrogen or progesterone alone.

Overall, these data suggest that bitch endometrium synthesizes and secretes a protein that has N-terminal and internal amino acid sequences and antigenic properties that are highly correlated with RBPs in particular, as well as with other members of the lipocalin family of low- M_r carriers of hydrophobic molecules. Based on sequence data alone, cP6 must be considered an RBP-like protein and a member of this family. Further, preliminary retinol binding studies suggest that cP6 has the capability to bind retinol *in vitro*. Unlike pig uterine RBP, however, it does not appear to be totally identical to serum RBP, as suggested by the internal sequencing and the amino acid composition studies. Further studies are required to determine the relationship of cP6

to serum RBP, β -lactoglobulin and other lipocalins and its role in early pregnancy and embryo/conceptus development.

During early pregnancy at the peri-implantation and implantation period, uterine secretions under conceptus or maternal steroid hormonal control are believed to be necessary to provide a milieu critical for embryonic growth, development and subsequent implantation. Vitamin A or retinoids have been suggested to have a number of important functions during early pregnancy [42]. These include modulating cellular differentiation and proliferation [43] through specific growth factors and receptors, gene transcription [44], epithelial cell integrity and function, haematopoiesis and immune cell function [45], and morphogenesis [46]. Recent studies [47,48] have suggested a beneficial effect of vitamin A and/or β -carotene, a precursor of retinol, in early pregnancy to increase litter size and decrease embryonic mortality in pigs. Prior to implantation the manner by which retinol is transferred from plasma to the embryo/conceptus is unclear, but the presence of a potential retinol-binding (lipophile) protein to transport retinol or other hydrophobic molecules from maternal tissues may be important in regulating embryo/conceptus development, differentiation and proliferation. These results suggest a common and conserved role for uterine RBP or RBP-like molecules during rapid growth and development. Thus, cP6 may fulfil a critical role in the early reproductive process in the bitch.

This work was supported in part by NIH grant HD20553 (W.C.B.) and the Florida Association of Kennel Clubs and Greyhound Racing Educational Foundation (V.M.S.).

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