Bovine prolactin-related protein-I is anchored to the extracellular matrix through interactions with type IV collagen

Toru Takahashi, Osamu Yamada¹, Michael J Soares² and Kazuyoshi Hashizume³

Reproductive Biology Research Unit, National Institute of Agrobiological Sciences, Tsukuba City, Ibaraki 305-8602, Japan

¹Miyagi Prefectural Livestock Hygiene Center, Sendai City, Miyagi 983-0832, Japan

²Division of Cancer and Developmental Biology, Department of Pathology and Laboratory Medicine, Institute of Maternal-Fetal Biology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA

³Faculty of Veterinary Medical Science, Iwate University, Morioka City, Iwate 020-8550, Japan

(Correspondence should be addressed to K Hashizume; Email: kazuha@iwate-u.ac.jp)

Abstract

The bovine placenta produces an array of proteins structurally similar to pituitary prolactin (PRL). At least ten genes of the bovine placental PRL family, including bovine placental lactogen (bPL) and ten bovine PRL-related protein-I to -X (bPRP-I to -X), encode hormones/cytokines predicted to be involved in the establishment and maintenance of pregnancy. Targets and biological roles for most members of the bovine PRL family have yet to be specified. This study focused on three members of bovine PRL family, bPL, bPRP-I, and bPRP-VI. An alkaline phosphatase (AP) tagging strategy was used to monitor interactions of the ligands with their targets. AP-bPRP-I and AP-bPRP-VI specifically bound to tissue sections of the bovine placentome. AP-bPRP-I and AP-bPRP-VI binding within the placentome mimicked the distribution of the extracellular matrix (ECM). Consequently,

AP fusion protein binding to individual ECM components (heparin, laminin, fibronectin, type I collagen, and type IV collagen) was evaluated. AP-bPRP-I specifically bound to type IV collagen, but not to the other ECM components. AP-bPRP-VI exhibited weak interactions with ECM components, while AP-bPL and AP did not show significant binding to any of the ECM components. Binding of AP-bPRP-I to type IV collagen was concentration-dependent, influenced by salt concentrations, and specific to the N-terminal cross-linking domain (7S) of type IV collagen but not its triple-helical domain. The interaction of bPRP-I with type IV collagen suggests that bPRP-I accumulates in the ECM where it likely acts on cells traversing the bovine placentome.

Journal of Endocrinology (2008) **196**, 225–234

Introduction

The bovine placenta produces an array of proteins structurally and functionally similar to pituitary prolactin (PRL; Soares 2004). Bovine placental lactogen (bPL) and ten PRL-related protein (bPRP) genes have been identified (Schuler & Hurley 1987, Yamakawa *et al.* 1990, Tanaka *et al.* 1991, Ushizawa *et al.* 2005b, Larson *et al.* 2006). The genes in the PRL family are presumed to originate from an ancestral gene by means of gene duplication. Some have been localized to chromosome 23 of the bovine genome (Dietz *et al.* 1992), where they are likely clustered similar to that observed for the expanded mouse and rat PRL families (Wiemers *et al.* 2003, Alam *et al.* 2006). Nucleotide sequences for member of the bovine placental PRL family of genes more closely resemble bovine PRL than bovine growth hormone (GH), suggesting that they arose from duplication events of the ancestral PRL gene (Milosavljevic *et al.* 1989, Wallis 1993).

Bovine PL and at least ten bPRPs are expressed in the bovine placental cotyledon. In particular, bPL, bPRP-I, -VII, -VIII,

and -IX have been shown to be expressed in trophoblast binucleate cells (Milosavljevic *et al.* 1989, Kessler *et al.* 1991, Yamada *et al.* 2002a, Ushizawa *et al.* 2005a,b). The mRNA encoding bPRP-I is first detected in the placenta around day 20 of gestation, correlating with the first the appearance of binucleate cells (Yamada *et al.* 2002a). Bovine PRP-I expression peaks around day 60 of gestation and then declines, while bPL transcripts increase with progression of gestation until term (Patel *et al.* 2004).

Bovine PRP-I is a glycoprotein with 35% amino acid sequence identity to bPL (Kessler *et al.* 1989). However, unlike bPL, which possesses mammatropic activities and effectively interacts with PRL receptors (Josimovich & Maclaren 1962, Buttle & Forsyth 1976), bPRP-I does not use the PRL or GH signaling pathways (Kessler & Schuler 1997). The identity of cellular targets or a specific receptor for bPRP-I is unknown.

Binding of ligands to extracellular matrices modulates biological activity and availability of the ligand *in situ* and plays an important role in the ligand-mediated biology. Binding to

extracellular matrix (ECM) components has been reported for several cytokines and growth factors, such as oncostatin M (Somasundaram *et al.* 2002), basic fibroblast growth factor (Faham *et al.* 1998), platelet-derived growth factor (Somasundaram & Schuppan 1996), hepatocyte growth factor (Lyon *et al.* 1994), transforming growth factor- β 1 (Paralkar *et al.* 1991), tumor necrosis factor- α and interleukin-2 (Somasundaram *et al.* 2000), interleukin-7 (Ariel *et al.* 1997), and a member of the rat PRL family, decidual/trophoblast PRP (d/t PRP; Rasmussen *et al.* 1996, Wang *et al.* 2000). The purpose of the present study was to explore the target sites of bPL, bPRP-I, and bPRP-VI *in situ* and to dissect the target-binding characteristics of bPRP-I. In this report, tissue targets for bPRP-I are investigated and bPRP-I is shown to specifically interact with components of the ECM, including type IV collagen.

Materials and Methods

Reagents

Matrix-immobilized microtiter plates (Biocoat; fibronectin, laminin, collagen I, collagen IV and poly-L-lysine) were purchased from BD Biosciences (Bedford, MA, USA). Engelbreth-Holm-Swarm (EHS) tumor-derived type IV collagen was precoated on Biocoat matrix-immobilized plates. For in house fabrication of collagen IV-immobilized plates, EHS collagen IV (purchased from BD Biosciences) was also utilized. Fibronectin, laminin, and collagen I of Biocoat plates were derived from human serum, mouse EHS tumor, and rat tail respectively. The 7S domain of type IV collagen was purified from bovine ophthalmic lens capsule and kindly supplied by Dr Billy Hudson (Vanderbilt University, Nashville, TN, USA). Antisera against bovine collagens I and IV were purchased from LSL (Tokyo, Japan). Fluorescein isothiocyanate-conjugated anti-rabbit IgG, used for the secondary antibody, was purchased from Leinco (St Louis, MO, USA). Cell culture media and fetal bovine serum (FBS) were purchased from Invitrogen Corp. Gly-Pro-Hyp tripeptide was purchased from BACHEM (Budendorf, Switzerland). Other reagents were purchased from Sigma, unless stated otherwise.

Cell culture

The 293 cell line of human fetal kidney origin was obtained from American Type Culture Collection (Rockville, MD, USA) and was used as the host for recombinant expression vectors. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F12 (F12) supplemented with 10% FBS, 100 units/ml penicillin G, and 100 μ g/ml streptomycin in an atmosphere of 5% CO₂-95% air at 37 °C in a humidified incubator.

Generation of alkaline phosphatase (AP)-fusion proteins

The generation of AP-fusion protein was performed as described previously (Berger *et al.* 1988, Muller & Soares 2006). The expression vector pCMV3-SEAP (Tropix, Bedford, MA, USA) was used for recombinant expression of AP-fusion proteins. This vector contains the human placental AP gene and generates AP at the amino terminus of the recombinant protein. cDNAs encoding mature protein regions of bPL, bPRP-I, and bPRP-VI (GenBank accession numbers J02840, J02944, and X59504 respectively) were amplified by PCR with *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and oligonucleotide primers containing *Hind*III (forward primer) and *Xba*I (reverse primer) restriction sites. Bovine PL, bPRP-I, and bPRP-VI templates were kindly supplied by Dr Linda Schuler (University of Wisconsin, Madison, WI, USA). Amplified products and the vector were digested with *Hind*III/*Xba*I, ligated with T4 ligase (Promega) and transformed into *Escherichia coli* XL-1 Blue supercompetent cells (Stratagene). Ampicillin-resistant transformants were collected and subcultured in LB broth in the presence of ampicillin. Plasmids were isolated from broth culture with Mini Prep kit (Qiagen). Isolated plasmids were screened for the presence of the genes of interest by restriction digestion and nucleotide sequencing. Plasmids containing the bPL, bPRP-I, and bPRP-VI cDNAs with the correct nucleotide sequence were designated as AP-bPL, AP-bPRP-I, and AP-bPRP-VI respectively.

The AP-bPL, AP-bPRP-I, and AP-bPRP-VI plasmids were linearized with *Bgl*II prior to liposome-mediated transfection into 293 cells, as described previously (Ushizawa *et al.* 2006). A non-engineered pCMV-SEAP vector was similarly transfected into 293 cells and served as a negative control. The transfected cells were selected with G418 (0.5 mg/ml) for 2 weeks. The G418-resistant cell population was expanded. Confluent cell cultures were switched to serum-free DMEM/F12, incubated for 3 days and, conditioned medium harvested and stored at -20 °C. The expressions of AP-fusion proteins were monitored by colorimetric assay (Muller *et al.* 1998, Muller & Soares 2006) and ligand blot analysis. For colorimetric assay, conditioned media of AP-fusion protein were initially heated for 30 min at 65 °C to inactivate endogenous APs. The samples were then incubated in a glycine buffer (50 mM glycine, 0.5 mM MgCl₂, 0.5 mM ZnCl₂, pH 10.5) at room temperature in the presence of *p*-nitrophenylphosphate (0.5 mg/ml) as a substrate for AP. After 15 min incubation, the absorbance was measured at 405 nm. In AP-titration, purified human placental AP (Sigma) was used for reference standards. One unit of AP is defined as the amount of enzyme that hydrolyzes 1 μ mol *p*-nitrophenylphosphate to *p*-nitrophenol in 1 min at 37 °C in a volume of 1 ml. In the latter assay, the samples were separated by sodium dodecyl sulfate PAGE under non-reducing conditions, electrophoretically transferred to nitrocellulose membranes, and the AP activity detected by incubation with nitroblue tetrazolium and bromochloro-indolyl phosphate (NBT/BCIP).

Generation of recombinant bPRP-I

A modified pFLAG CMV 3 expression vector (Sigma) was used for the recombinant expression of bPRP-I. This modified vector results in the inclusion of FLAG-6xHis-FLAG epitope tags at the amino terminus of the recombinant protein. A 0.7 kb cDNA fragment encoding the mature protein region of bPRP-I was amplified by PCR with *Pfu* polymerase (Stratagene) and oligonucleotide primers containing *Hind*III (forward primer) and *Xba*I (reverse primer) restriction sites. A bPRP-I cDNA, kindly supplied by Dr Linda Schuler (University of Wisconsin) was used as a template for the PCR. The amplified product and vector were digested with *Hind*III/*Xba*I, ligated with T4 ligase (Promega) and transformed into *E. coli* XL-1 Blue super-competent cells (Stratagene). The ampicillin-resistant transformants were collected and subcultured in LB broth in the presence of ampicillin. Plasmids were isolated from broth culture with Mini Prep kit (Qiagen). Isolated plasmids were screened for the presence of bPRP-I by restriction digestion and nucleotide sequencing. A plasmid containing the bPRP-I cDNA with the correct nucleotide sequence was designated as mFLAG-bPRP-I.

The mFLAG-bPRP-I plasmid was linearized with *Apa* I prior to liposome-mediated transfection into 293 cells, as described previously (Ushizawa *et al.* 2006). The transfected cells were selected with G418 (0.5 mg/ml) for 2 weeks. Clonal lines were isolated by limiting dilution from the G418-resistant cell population. The expression of recombinant bPRP-I protein was monitored by western blot analysis with anti-FLAG M2 antibody (Sigma). After cell cloning, highly expressing clones were seeded into culture flasks and propagated up to 1×10^8 cells in DMEM/F12 containing 10% FBS and antibiotics. The cells were washed with serum-free DMEM/F12, adapted to a serum-free culture in spinner flasks with 200 ml of 293 SFM (Invitrogen), and cultured for 2 days at 37 °C, 8% CO₂ in air with gentle stirring (60 r.p.m.). Once adapted, the cells were suspended in 1 l of the same medium at 1×10^5 cells/ml and further incubated up to 1×10^6 cells/ml to express recombinant bPRP-I (8–9 days). Ten liters of conditioned medium were generated and utilized to purify recombinant bPRP-I. The conditioned medium was centrifuged at 1600 g for 60 min to remove particulate material and concentrated up to 180 ml with a tangential flow ultrafiltration apparatus (Amicon, Millipore, Bedford, MA, USA) and a regenerated cellulose filter with a molecular mass cutoff of 10 kDa (YM-10, Millipore). The concentrated conditioned medium was combined with 20 ml of 10×binding buffer (1 M phosphate (pH 8.0), 3 M NaCl, 200 mM imidazole) and incubated with 3 ml Ni-NTA agarose gel (Qiagen) at 4 °C overnight with gentle stirring. The following day, the medium-gel mixture was transferred to a chromatography column, and the column was washed with a 1× binding buffer to remove unbound proteins. Recombinant bPRP-I was eluted with elution buffer (0.1 M phosphate (pH 7.0), 0.15 M NaCl, 0.25 M imidazole). The presence of recombinant bPRP-I protein in each fraction was determined by western blot analysis with an anti-FLAG M2

monoclonal antibody (Sigma). Immunopositive fractions were pooled and incubated with 1 ml anti-FLAG M2 affinity gel (Sigma) at 4 °C overnight under gentle agitation. The following day, the medium-gel mixture was transferred to a chromatography column and washed extensively with Tris-buffered saline (TBS) to remove unbound proteins. Recombinant bPRP-I was eluted with TBS containing 200 µg/ml FLAG peptide (Sigma). The presence of the target protein in each fraction was determined by western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma), anti-His polyclonal antibody (Sigma), and anti-bPRP-I polyclonal antibody generously provided by Dr Linda Schuler (University of Wisconsin). Immunopositive fractions were pooled and subjected to desalting by Econo-Pack 10DG column (Bio-Rad) and concentrated by ultrafiltration (Amicon Ultra, Millipore). The protein concentration of the purified product was assayed with a Bradford dye binding protein assay kit (Bio-Rad).

Microplate-binding assay

Matrix-coated 96-well microplates were obtained from BD Biosciences or prepared by our laboratory. The latter were used to assess concentration dependence of the AP-fusion protein interactions with components of the ECM. Briefly, different concentrations of type IV collagen were dispensed into 96-well microplates, incubated at 4 °C overnight, and then washed. For binding assays, the microplates were blocked with 5% nonfat dry milk in TBS for 1 h at room temperature (RT) and then washed three times with TBS containing 0.1% Tween 20 (TBST). Fifty microliters of conditioned medium containing the equivalent of 60 mU of AP activity were dispensed into microplates and incubated for 2 h at RT. Microplates were then washed three times with TBST. Specific binding of AP or AP-fusion proteins was measured by colorimetric assay with *p*-nitrophenylphosphate as reported previously (Muller *et al.* 1998, Muller & Soares 2006).

In situ AP-ligand-binding assay

Bovine liver and uteroplacental tissues were collected at a local slaughterhouse, embedded in OCT compound (Sakura Finetech, Tokyo, Japan), frozen in liquid nitrogen vapor, and stored at −85 °C until sectioned. Tissue sections were prepared with the aid of a cryostat (Mikrom, Walldorf, Germany). An *in situ* AP-binding assay was carried out as described previously (Muller *et al.* 1998). The sections were mounted onto glass slides and washed with modified Hank's balanced salt solution (mHBSS; containing 20 mM HEPES, 0.5 mg/ml BSA and 0.1% sodium azide) for 5 min at RT, incubated with AP-fusion protein-conditioned medium for 75 min at RT, and then washed six times (5 min each) with mHBSS supplemented by 0.1% Tween 20. The washed sections were fixed for 2 min with the acetone–formaldehyde fixative (60% acetone, 3% formaldehyde, and 20 mM HEPES (pH 7.0)) and washed three times (5 min each) with HEPES-buffered saline (HBS).

The sections were subsequently incubated in HBS at 65 °C for 30 min to inactivate the endogenous tissue AP activity. AP-PRP binding was detected by incubation with NBT/BCIP.

Immunofluorescence

Immunofluorescence detection was carried out as previously described (Yamada *et al.* 2002b). Placental tissue was fixed with 4% paraformaldehyde, embedded in paraffin wax, and sectioned 5 µm thickness. The deparaffinized sections were treated with 0.05% protease (P6911, Sigma) for 30 min at 37 °C and then rinsed. Nonspecific antibody binding was blocked with 10% normal goat serum for 20 min. Sections were incubated with primary antibody overnight at 4 °C and then rinsed. They were further incubated with secondary antibody for 60 min at RT. After subsequent rinsing, they were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA USA) and assessed using epifluorescent microscope (ECLIPSE E800, Nikon, Tokyo, Japan).

Results

Expression of AP-fusion and mFLAG- bPRP-I proteins

AP-bPL, AP-bPRP-I, and AP-bPRP-VI expression vector constructs were transfected into 293 cells, which successfully produced AP-fusion proteins (Fig. 1). Unmodified AP electrophoretically migrated as a protein with an apparent molecular size ranging from 110 to 135 kDa. AP-bPL, AP-bPRP-I, and AP-bPRP-VI electrophoretically migrated as proteins with apparent molecular sizes ranging from 130 to 145 kDa. Untransfected 293 cells did not produce specific proteins possessing heat stable AP activity (data not shown).

Approximately 0.7 mg mFLAG-bPRP-I-recombinant protein was purified from 10 l culture medium conditioned with transfected 293 cells. The mFLAG-bPRP-I-recombinant

Figure 1 Electrophoretic analysis of AP-fusion proteins. Serum-free conditioned media were separated by sodium dodecyl sulfate PAGE (SDS-PAGE) in 7.5% gels under non-reducing conditions. Samples were electrophoretically transferred to nitrocellulose membrane. Filters were incubated with the AP substrate (NBT/BCIP). Lane 1, AP-vector as negative control. Lane 2, AP-bPL. Lane 3, AP-bPRP-I. Lane 4, AP-bPRP-VI. Molecular size markers are shown in the lane labeled M.

protein was specifically recognized by anti-FLAG, anti-6xHis, and anti-bPRP-I antibodies. Recombinant mFLAG-bPRP-I protein was expressed primarily as a monomer with molecular size of 32 kDa (Fig. 2). A little immunopositive protein was migrated at apparent molecular size of 64 kDa that was coincided to the dimer of mFLAG-bPRP-I.

AP-bPL and AP-bPRPs specifically bind to bovine tissues

The AP-tagging strategy for identifying specific targets for bPRPs was evaluated using bovine tissue sections. AP-bPL and AP-bPRP-I specifically bound to liver tissue sections (Fig. 3). Spatial distributions of AP-bPL, AP-bPRP-I, and AP-bPRP-VI bindings were respectively distinct. AP-bPRP-I binding was localized at the gap between the hepatocellular laminae, whereas AP-bPL binding was relatively diffused in the section. The binding signal of AP-bPRP-VI was focally observed in the section. Unmodified AP failed to bind hepatic sections. In uteroplacental tissues, AP-bPRP-I bound to the lamina propria of cotyledonary villi and the septum of maternal caruncle (Fig. 4). AP-bPRP-I binding within the placenta mimicked the distribution of the type IV collagen (Figs 4 and 5). Binding of AP-bPRP-I could be competed with 200 µg/ml mFLAG-bPRP-I (Fig. 6). Both AP-bPL and AP failed to bind bovine uteroplacental sections. AP-PRP-I appeared to specifically associate with ECMs in liver and uteroplacental tissues.

AP-bPRP-I interacts with type IV collagen

Given the apparent interaction of AP-bPRP-I with the ECM, we investigated AP-bPRP-I binding to components of ECMs. AP-bPRP-I bound specifically to type IV collagen immobilized on polystyrene microtiter wells but not to type I

Figure 2 Sodium dodecyl sulfate PAGE (A) and western blot analysis (B) of purified mFLAG-bPRP-I. (A) Sample was separated by SDS-PAGE in 15% gel under reducing conditions and stained with Coomassie brilliant blue. (B) Samples were separated in the same way as (A), blotted to nitrocellulose membranes and blocked with skim milk (5% skim milk in Tris-buffered saline, TBS). Blots were incubated with anti-FLAG M2 antibody (lane 1), anti-6xHis antibody (lane 2) and anti-bPRP-I antibody (lane 3), washed with TBS containing 0.2% Tween 20 (TBST), incubated with AP-coupled secondary antibodies and then washed with TBST. Blots were incubated with NBT/BCIP as substrates for AP.

Figure 3 *In situ* binding of AP-fusion proteins to bovine liver tissue. Liver tissue was cryosectioned and incubated with AP-bPL (A), AP-bPRP-I (B), AP-bPRP-VI (C), or AP (D) for 75 min at RT. After incubation, tissue sections were washed, fixed, and heated at 65 °C for 30 min to inactivate the endogenous AP activity. AP activities were visualized following incubation with NBT/BCIP. Bar = 100 μm.

collagen, laminin, fibronectin, or poly-D-lysine (Fig. 7). AP-bPRP-VI exhibited weak interaction with collagen IV when higher dose of the matrix was immobilized to the plate (Fig. 8). Unmodified AP and AP-bPL did not bind to any of the immobilized matrices tested. All AP-fusion proteins failed to interact with immobilized heparin (data not shown). The binding of AP-bPRP-I to immobilized type IV collagen was dependent on concentrations of both the AP-fusion protein and the immobilized type IV collagen (Fig. 8).

AP-bPRP-I binding for type IV collagen was increased when the reactions were performed in buffers containing increased NaCl concentrations. The colorimetric absorbance (A405) of collagen IV–AP-bPRP-I binding increased approximately threefold at 4096 mosm compared with that at physiological osmolarity. In contrast, the bindings of AP-bPL and AP-bPRP-I to type IV collagen were not affected by the ionic strength of the incubation buffer. These results suggest that hydrophobic interactions are a primary factor in the interaction between AP-bPRP-I and type IV collagen.

AP-bPRP-I interactions with immobilized type IV collagen were effectively competed with 7S domain of type IV collagen (Fig. 9), whereas the collagenous peptide was ineffective. The interactions between AP-bPRP-I and the 7S domain of type IV collagen were also demonstrated by ligand

blot analysis (Fig. 10). Under reducing conditions, the 7S domain peptide migrated at apparent molecular sizes of 34 and 60 kDa, which specifically bound AP-bPRP-I.

Discussion

In this study, we generated AP-fusion proteins for bPL, bPRP-I, and bPRP-VI and investigated target tissues for these bovine PRL family ligands. A similar strategy was previously used to identify cellular targets for other PRL family ligands (Muller *et al.* 1998, Lin & Linzer 1999, Wang *et al.* 2000, Noel *et al.* 2003).

In many mammals, PLs are one of the principal molecules in classical members of PRL gene family. Also, in bovine, bPL is typical of most members of placental PRL family genes and capable of binding to PRL receptors (Schellenberg & Friesen 1982). In the present study, AP-bPL exhibited binding to the liver, in spite of no binding to the placenta. The expression of bovine PRL receptor transcripts is abundant in the liver but little in both cotyledon and caruncle (Scott *et al.* 1992). The different binding of AP-bPL could be attributed to the relative abundance of PRL receptor expression. Bovine PRP-I and bPRP-VI did not activate the PRL receptor-signaling pathway but instead

Figure 4 *In situ* binding of AP-fusion proteins to bovine placenta (gestation day 65). Placental tissue was cryosectioned and incubated with AP-bPL (A), AP-bPRP-I (B), AP-bPRP-VI (C), or AP (D) for 75 min at RT. After incubation, the tissue sections were washed, fixed, and heated at 65 °C for 30 min to inactivate endogenous AP activity. AP activities were visualized following incubation with NBT/BCIP. Lamina propria of cotyledonary villi (arrow) and septum of maternal caruncle (S) are shown. Bar=100 μm.

exhibited specific binding to target tissues (including the liver and placentome). The association of bPRP-I with the ECM suggests that it acts within a short distance of where it is produced. Consistent with this observation, Kessler & Schuler

(1997) reported that bPRP-I could be detected in uterine fluid but not in serum samples. Within the ECM, bPRP-I specifically bound to type IV collagen, and similar but weak interaction was observed in AP-bPRP-VI. Other cytokines and growth factors

Figure 5 Immunofluorescence detection of collagen I and IV in bovine placenta (day 65 gestation) Tissue sections were stained with hematoxylin-eosin (A). Immunofluorescence detection of collagens I (B) and IV (C). Lamina propria of cotyledonary villi (arrow) and septum of maternal caruncle (S) are shown.

Figure 6 *In situ* AP-bPRP-I binding to the bovine uterus. Cryosectioned bovine uterine tissue was incubated with AP-bPRP-I (A), AP-bPRP-I and mFLAG-PRP-I (B) or AP (C). Positive staining in (A) (arrow) is competed with 200 μ g/ml mFLAG-bPRP-I (B). Hematoxylin staining of an adjacent section is shown in (D). Bar = 100 μ m.

bind to type IV collagen, including the transforming growth factor- β 1 (Paralkar *et al.* 1991), bone morphogenetic protein 4 (Paralkar *et al.* 1992), platelet-derived growth factor (Somasundaram & Schuppan 1996), hepatocyte growth factor (Schuppan *et al.* 1998), interleukin 2 (Somasundaram *et al.* 2000), keratinocyte growth factor (Ruehl *et al.* 2002), and oncostatin M (Somasundaram *et al.* 2002). These molecules that are secreted by the cell and adhere to specific extracellular structures have been proposed the term 'crinopectin' (Feige *et al.* 1998). This crinopectin paradigm leads to the idea that biological signal of crinopectin is triggered by their liberation from storage sites. Growth factors and cytokines interacting with collagens retain their biological activities. Although the biological actions of bPRP-I are unknown, it would be expected that interactions with type IV collagen would serve as a reservoir of biologically active bPRP-I in a critical location, where it could interact with a variety of cell types traversing the vasculature.

In rodents, d/tPRP has been reported to be capable of binding heparin-containing molecules (Rasmussen *et al.* 1996). Despite the abundant accumulation of d/tPRP in decidual tissues, d/tPRP does not present in maternal serum at a detectable level. These data suggest that ECM plays as an anchor molecule to restrict and maintain biological activities.

Figure 7 AP-bPRP-I binds to immobilized type IV collagen. AP-fusion proteins (60 mU/well each) were incubated in 96-well microtiter plates coated with ECM components. After five washes, bound AP-fusion proteins were measured by measuring AP activity with a colorimetric assay. mHBSS solution served as a negative control. Shown are means $\pm$ S.E.M. of quadruplicate measurements.

Figure 8 AP-fusion protein interactions with type IV collagen. Left panel: concentration-dependent binding of AP-fusion proteins (10–300 mU/well) to immobilized type IV collagen. AP-bPRP-I (■), AP-bPRP-VI (▲), AP-bPL (×), and AP (◆) were incubated with immobilized type IV collagen. Shown are means $\pm$ S.E.M. of quadruplicate measurements. Middle panel: concentration-dependent binding of immobilized type IV collagen (5 or 20 µg/well) to AP-fusion proteins (300 mU/well). AP-bPRP-I (■), AP-bPRP-VI (▲), AP-bPL (×), and AP (◆) were incubated with immobilized type IV collagen. Right panel: effect of osmolarity on AP-fusion protein-type IV collagen interactions. AP-fusion proteins AP-PRP-I (■), AP-PRP-VI (▲) and AP (●; 60 mU/ml) were incubated with immobilized type IV collagen under different osmolalities. Shown are means $\pm$ S.E.M. of quadruplicate measurements.

Binding of bPRP-I to type IV collagen was enhanced by increasing the ionic strength of the incubation buffer, indicating the potential involvement of hydrophobic domains in the protein–protein interaction. Of the growth factors and cytokines known to interact with type IV collagen, keratinocyte growth factor, and oncostatin M, have been shown to bind to the consensus collagen sequence glycine–proline–hydroxyproline (Ruehl *et al.* 2002, Somasundaram *et al.* 2002). The interaction between ligands and the triple-helical domain of collagens is inhibited by higher ionic strength indicating the major contribution of ionic forces to the interaction.

Type IV collagen consists of a 7S domain at the amino terminus, a centrally located triple-helical region and an NC1 domain at the carboxyl terminus (Hudson *et al.* 1995). Type

IV collagen forms a supramolecular structure by associating in a head-to-head manner to form dimers (hexamer of NC1 peptide) and a tail-to-tail manner to form tetramers (dodecamer of 7S peptide), contributing to the lattice-like structure of the basement membrane. Binding experiments with purified fragments of type IV collagen indicated that bPRP-I specifically recognized the 7S domain. The 7S domain of type IV collagen has previously been implicated in mediating interactions with usherin, a basement membrane protein involved in retinal and inner ear development (Bhattacharya *et al.* 2004), and as a target for *Candida albicans* interactions with basement membranes (Alonso *et al.* 2001). The biological significance of bPRP-I interactions with the 7S domain of type IV collagen is unknown.

Figure 9 Effect of the components of the collagen molecule on interactions of AP-bPRP-I with immobilized type IV collagen. Left panel: AP-bPRP-I (■) and AP (◆) were incubated at 60 mU/well with immobilized type IV collagen in the presence of collagenous portion of type IV collagen. Shown are means $\pm$ S.E.M. of quadruplicate measurements. Right panel: AP-bPRP-I in the presence or absence of 7S domain of type IV collagen (40 µg) or AP was incubated with immobilized type IV collagen. AP-bPRP-I and AP concentrations were 60 mU/well. Shown are means $\pm$ S.E.M. of quadruplicate measurements.

Figure 10 Ligand blot analysis of the interaction of AP-bPRP-I with the 7S domain of type IV collagen. The 7S domain of type IV collagen (approximately 3 µg) were separated by SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. Blots were incubated with AP-vector (lane 1), AP-bPL (lane 2), AP-bPRP-I (lane 3), and AP-bPRP-VI (lane 4). The concentration of every fusion protein was adjusted in terms of AP activity to 1–2 U/ml. After extensive washing, specific binding of AP-fusion protein to the 7S domain was visualized with NBT/BCIP.

Bovine PRP-I was previously shown to interact with a modulator of the ECM, α_2 -macroglobulin (Kessler & Schuler 1997). α_2 -Macroglobulin inhibits proteinases, including matrix metalloproteinases, and modulates the activities of growth factors and cytokines (Chu & Pizzo 1994). In the mouse and rat, α_2 -macroglobulin is abundantly expressed at the placentation site (Gu *et al.* 1994, He *et al.* 2005), where it modulates trophoblast–uterine stromal cell interactions (Esadeg *et al.* 2003, Tayade *et al.* 2005). It is logical that bPRP-I– α_2 -macroglobulin interactions may affect proteolytic events in utero–placental ECMs.

Collectively, these observations are consistent with bPRP-I associating with the uteroplacental ECM. Bovine PRP-I is produced by trophoblast binucleate cells of the bovine placenta and interacts with type IV collagen, but not with type I collagen, laminin, fibronectin, or heparin containing molecules. Although biological activities of bPRP-I were not determined in this study, hypothetic action of bPRP-I could be restricted to near the secreted source. Deposited bPRP-I may participate in the formation of cotyledonary placenta at its interface with the uterine caruncle.

Acknowledgements

The authors thank Drs Billy Hudson (Vanderbilt University, Nashville, TN, USA) and Linda Schuler (University of

Wisconsin, Madison, WI, USA) for reagents used in these experiments. This work was supported in part by grants from the National Institutes of Health (HD039878, HD048861, and HD055523), and by grants from the Japan Society for the Promotion of Science (16580235 and 17580284). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 3 October 2007

Accepted 30 October 2007

Made available online as an Accepted Preprint
30 October 2007