

Multiple forms of Pregnancy-Associated Glycoproteins released *in vitro* by porcine chorion or placentomal and interplacentomal explants of wild and domestic ruminants

Marta Majewska², Grzegorz Panasiewicz², Maciej Dabrowski², Zygmunt Gizejewski³, Jean-François Beckers⁴, Bozena Szafranska^{1,2}
²Department of Animal Physiology, University of Warmia and Mazury, Olsztyn, ³Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland, ⁴Department of Reproductive Physiology, Faculty of Veterinary Medicine, University of Liege, Sart-Tilman, Belgium

Received: 20 January 2005; accepted: 12 May 2005

SUMMARY

Characterization of the Pregnancy-Associated Glycoproteins (PAG) is important for studies of reproduction of various eutherian domestic, wild and endangered mammals. Distinct chorionic PAG genes are expressed in embryo-origin cells: pre-placental trophoblast (TR) and in placental trophoctoderm (TRD) of various eutherians. This study demonstrates *in vitro* production of the PAG proteins during long-term cultures of various chorionic explants: porcine TR or TRD, cotyledonary (CT) of European bison (Eb), and CT or

¹ Corresponding author: Department of Animal Physiology, University of Warmia and Mazury, 10-719 Olsztyn-Kortowo, Oczapowskiego Str 1A/222, Poland, email: szafran@uwm.edu.pl

intercotyledonary (intCT)-TRD of the cattle. Chorionic proteins isolated from media were analyzed by homologous or heterologous Western immunoblotting with anti-PAG sera, raised against cellular bovine or secretory porcine antigens. Used anti-PAG sera identified diverse molecular forms of released PAG proteins: 43-69 kDa for EbPAG proteins, 40-85 kDa for bovine PAG (bPAG), and 43-73 kDa for porcine PAG (pPAG). Immunoblotting revealed also that both CT and intCT-TRD explants secreted equivalent amounts of bPAG proteins. This useful system of *in vitro* protein production can provide native chorionic PAG proteins with placental unique carbohydrate chains. The PAG proteins are required as standard markers for diagnostic tests of pregnancy in domestic and wild mammals, in which seasonal reproductive processes are relatively difficult to control. *Reproductive Biology* 2005 5 (2):185-203.

Key words: chorion, glycoproteins, PAG, placenta, pregnancy

INTRODUCTION

Chorionic proteins are crucial components of a molecular complex responsible for appropriate early embryonic development and advanced pregnancy maintenance in mammals with different placentation types [1, 9, 10, 41]. Pregnancy-associated glycoproteins (PAG) are expressed in extra-embryonic cells of peri-placental trophoblast (TR), and after implantation/placentation, in trophoblast (TRD; chorionic epithelium) of various ungulate species. The PAG family was identified according to sequences of distinct complementary DNA (cDNA) of the PAG mRNA in various chorionic transcriptomes (TR/TRD). The PAG cDNAs have been cloned in several eutherians (*Placentalia*), including *Artiodactyla*: pig [38, 45], cattle, sheep [21, 60], goat [18], white-tail deer¹; *Perissodactyla*: horse, zebra [20], or *Rodentia* (mouse) [8] and *Carnivora* (cat) species². Genomic

¹ Vawter GA Parks TE Killian G Ealy AD Green JA 2004 Cloning and preliminary characterization of Pregnancy-Associated Glycoproteins expressed in the placenta of white-tailed deer. *Biology of Reproduction* 70 153.

² Gan X Xie S Green J Roberts RM 1997 Identification of transcripts for pregnancy-associated glycoprotein (PAG) in *Carnivora* and *Perissodactyla*. *Biology of Reproduction* 56 431 190

studies indicated that the PAG genes originated from duplicated ancestral conserved progene [27, 28], and multiple PAG genes are expected in different genomes: over one hundred genes in ruminants [57, 60] or at least eight pPAG2-like genes in pigs [46, 47]. However, a number of the PAG genes or proteins in other mammals remain still unknown.

Amino acid sequences suggest [22] that the PAG family includes catalytically active and inactive placental aspartic proteinases (AP). This AP family (EC 3.4.23) includes also pepsins [32], cathepsins D and E [11] and napsins [51] of various vertebrates; two histo-APs (HAP I and II) and plasmepsins of malaria parasites [3, 6], as well as aspergillopepsin II [26] and other fungal and retroviral AP enzymes. Most of these AP members are secreted as zymogens, and presumably, a specific control of their catalytic activation is strongly associated with still unknown functions of these molecules [34, 44, 49, 50].

Identified cDNAs of the PAG family can be used as genetic markers for pre-selection of young animals [7, 47], comparative microarray mapping [30, 52] or as the major templates for recombinant PAG proteins (recPAG) required for commercial application of prenatal diagnostic tests. *In silico* cDNA translation to amino acid sequences of polypeptide PAG precursors revealed their very high homology to N-terminal micro-sequences of purified native PAG proteins. In ruminants, the PAG protein family is also known as a pregnancy-specific protein B (PSPB), SBU3 proteins or 60 kDa pregnancy-specific protein (PSP60). Numerous native PAG proteins have been purified mainly from ruminant cotyledons: cattle [61], zebu [43], sheep [2, 13, 15, 59], goats [17], elk and moose [25]. In the pig, a single native pPAG2-like protein purified from culture media and sequenced was named basic protein (BP, 43 kDa) previously [12].

Only a few of the aforementioned purified native PAG proteins have been used as markers for plasmatic pregnancy diagnoses in domestic ruminants [19, 36, 62] and for immunodiffusion tests in non-ruminant species [14]. These PAG proteins of domestic species have also been beneficially used as standards and tracers during radioimmunologic tests of early pregnancy and embryonic mortality in wild related ruminants [16, 23, 24, 29, 37, 53, 55]. The PAG proteins were also useful as prenatal markers for diagnosis of multiple pregnancies [5, 40, 54].

European bison (*Bison bonasus L.*) has been classified to international Red List of endangered species (EN A2ce, C2a, IUCN). The European bison is relative to a wood bison (*Bison bison athabascae*), in which the PAG-like proteins were found in peripheral blood plasma during cross-species pregnancy diagnoses with use of bovine PAG standards [23]. Thus, characterization of chorionic molecules requires suitable method of native protein harvesting, especially in wild animals, in which the reproductive processes are difficult to control. The objectives of this study were: 1) to produce *in vitro* secretory forms of native PAG proteins by various chorionic explants of domestic or wild eutherians (*Sus scrofa*, *Bos taurus* and *Bison bonasus L.*); and 2) to compare the PAG protein production by ruminant placental and interplacental chorionic explants.

MATERIALS AND METHODS

Animals and placental tissue collection

Pregnant gilts (n=18) were slaughtered on day 16 (n=5) *post coitum* (dpc), 17-19 dpc (n=6), 20-25 dpc (n=4) and 30-39 dpc (n=3). Placentomes (6-8 cm) were collected *post mortem* from a middle-pregnant (~5 month) female of European bison (Eb; *Bison bonasus L.*) eliminated in the forest of the Bialowieza National Park (Poland). Uteri of pregnant cows (n=3) were harvested at a local slaughterhouse and pregnancy status (30, 45 and 90 dpc) was estimated according to crown-rump length of the fetuses (0.5-18 cm).

Porcine chorionic tissues, pre-placental TR and post-implantational TRD, were recovered from uteri and dissected from embryos and remaining membranes. Bovine cotyledons (CT) were dissected from the maternal caruncles (0.5-2 cm) and from inter-cotyledonary TRD (intCT-TRD), an interplacental chorionic epithelium. Collected chorionic tissues were placed in sterile PBS supplemented with penicillin (100 I.U./ml) and streptomycin (100 µl/ml), then all chorionic tissues were immediately used for *in vitro* studies. Protocols for procedures used in this study and for animal care were approved by the local animal authorities.

Cultures of chorionic explants

Explants of the extra-embryonic chorionic membranes (TR, TRD and CT or intCT-TRD) were long-term cultured as previously described for porcine explants [39, 44, 45, 49, 50]. Briefly, collected TR, TRD and CT or intCT-TRD explants (from 0.32 up to 529 g) were minced into small pieces (1-3 mm³), washed in PBS and harvested as pre-culture protein fraction (0 h) for each explant type. These fractions represented chorionic proteins leaking from damaged cells during slicing (cellular proteins). All explants were cultured in serum free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics (ICN, Costa Mesa, CA, USA). The explants (approx. 5 g/l medium) were placed in flasks on the rocking platform and initially cultured for 8 h (37°C, 5% CO₂:95% air; IG 150, Jouan, France). During extended periods of the long-term experiments, collected media were replaced (in 24-48 h intervals) by fresh serum-free DMEM with antibiotics and nystatine (240 I.U./ml). Cotyledonary explants of European bison (Eb) were cultured for 72 h. Bovine CT or intCT explants were cultured up to 480 h. Porcine TR or TRD explant cultures were extended up to 768 h. The variable incubation times were necessitated by the quality and conditions of chorionic explants harvested in the forest or slaughterhouses.

Harvesting of chorionic proteins

Chorionic proteins, obtained during mincing of chorionic tissues in PBS (cellular proteins, fraction 0 h) or released by long-term cultured explants into media (for 768 h), were harvested according to a modified method described previously for porcine placental tissues [44, 49, 50]. During the current study, released TR, TRD, CT and intCT proteins were isolated from media (total 20.88 liters) by ultrafiltration (>10 kDa) until each protein reached approximately 1 µg/ 1µl. Ultrafiltration, dialysis and concentration of chorionic proteins were performed in Centriprep-10 cartridges (MWCO 10 kDa; Amicon, Beverly, MA, USA). Final protein concentration was determined by the Bradford procedure [42].

Western blotting

Pre-placental TR, placental TRD and CT or intCT proteins (10 µg / sample) were separated by SDS-PAGE parallel to molecular markers (ICN, Costa Mesa, Ca, USA; or Kucharczyk T.E., Warsaw, Poland) and subjected to Western blotting [39, 44, 45, 49, 50]. Briefly, chorionic proteins were stained with Coomassie Brilliant Blue dye (CBB). Duplicates of PAGE-separated proteins were analyzed by homologous or heterologous (cross-species) Western analysis by alkaline phosphatase with use of NBT and BCIP as standard substrates. Blotting was performed with rabbit (titer 1:300) anti-bovine (code: 497) PAG sera [62] or polyclonal/polyvalent anti-porcine PAG sera [44], purified by adsorption and characterized for binding specificity with negative and positive controls [48, 49]. Specific binding of the anti-PAG sera was confirmed by using recombinant fusion PAG proteins and/or by immunoscreening of PAG clones isolated from cDNA libraries [20, 49, 60]. Gels and blots were photographed and archived by FOTO/Analyst Archiver (Fotodyne, Hartland, WI, USA).

RESULTS

***In vitro* production of porcine and ruminant chorionic proteins**

Mammalian TR, TRD or CT and intCT explants produced various amounts of chorionic proteins released into the media harvested during long-term *in vitro* cultures (tab. 1). Among the porcine TR and TRD as well as ruminant CT and intCT proteins separated by SDS-PAGE, the family of the PAG proteins (with various M_r) was detected by double homologous or cross-species heterologous Western blottings (figs. 1-4). The anti-bovine PAG serum (fig. 1 B) detected two major groups of the EbPAG proteins: 67-69 kDa and 43-45 kDa. This anti-bPAG serum also detected 67-69 kDa forms of bovine and porcine PAG proteins. The anti-porcine PAG sera (fig. 1 C) mainly detected 67-69 kDa major forms of the EbPAG, bovine PAG (~69 kDa) and porcine PAG (70-72 kDa) proteins.

Table 1. The effectiveness of chorionic protein production during long-term *in vitro* cultures of various explants harvested from eutherian mammals on different day (dpc) or month (m) of pregnancy

Code of animals	dpc	Average protein production		Total production [mg/h of culture]
		[mg/24h]	[µg/100 mg of tissue]	
Domestic pig (<i>Sus scrofa</i>)				
Z 1	16	0.075	16.49	2.400 / 768
Z 30		0.550	-	1.100 / 48
Z 32		0.029	46.40	0.464 / 384
Z 35		0.313	1852.07	6.260 / 480
Z 36		0.139	952.17	3.066 / 528
Z 2	17	0.070	70.97	2.200 / 744
Z 31		0.933	169.09	1.860 / 48
Z 29	18	1.010	572.38	22.38 / 528
Z 07p		0.596	234.09	9.551 / 384
Z 09p		7.580	49.22	7.580 / 24
Z 28	19	0.037	8.98	0.265 / 168
Z 3	20	0.070	51.64	2.200 / 672
Z 33	22	0.200	33.37	2.800 / 336
Z 34		5.729	104.17	11.459 / 48
Z 38	30	25.33	79.11	157.270 / 149
SL I	33	31.23	969.52	486.700 / 374
SL II	39	64.93	164.08	562.730 / 208
Ruminants (<i>Bos taurus</i> and <i>Bison bonasus</i> L.)				
Cow I (CT)	30	4.00	-	64.3 / 384
Cow I (intCT-TRD)		23.01	-	46.02 / 48
Cow II (CT)	45	0.72	-	14.370 / 480
Cow III (CT)	90	20.84	298.49	180.615 / 208
Cow III (intCT-TRD)		17.33	250.94	150.190 / 208
European bison	5 m	204.1	-	612.313 / 72

(-) not determined

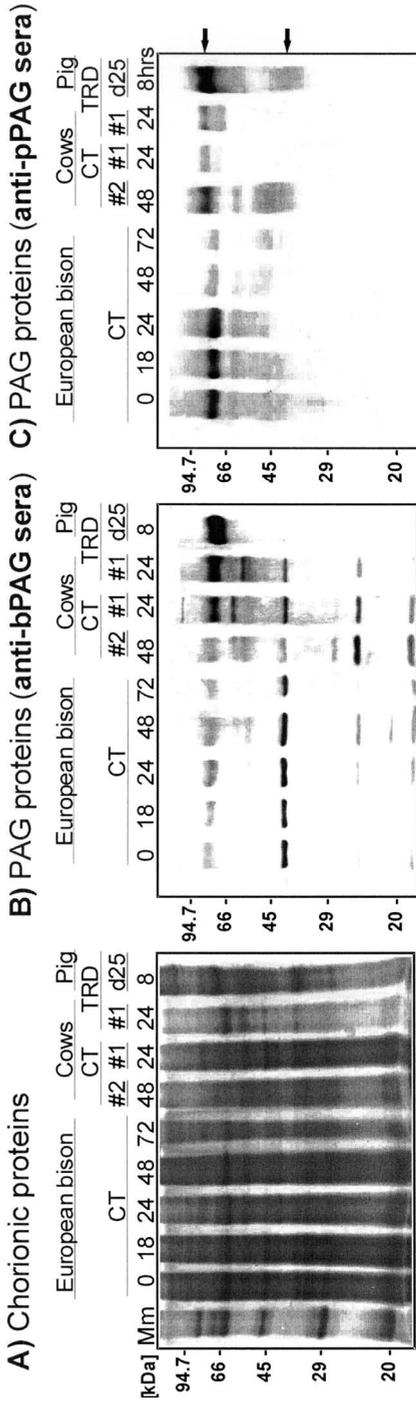


Figure 1. *In vitro* production of mammalian chorionic proteins during long-term cultures of cotyledonary (CT) explants of European bison, CT or interplacentomal trophoctoderm (TRD) of two cows (#1 and #2) and TRD of the pig (d25). **A)** SDS-PAGE of various chorionic proteins (10 µg/lane) stained with CBB dye. **B)** Heterologous and homologous Western blotting of the PAG protein family performed with anti-bovine PAG (anti-bPAG) or **C)** anti-porcine PAG (anti-pPAG) sera. Molecular marker (Mm) is on the left. Arrows on the right indicate molecular range of the pPAG proteins.

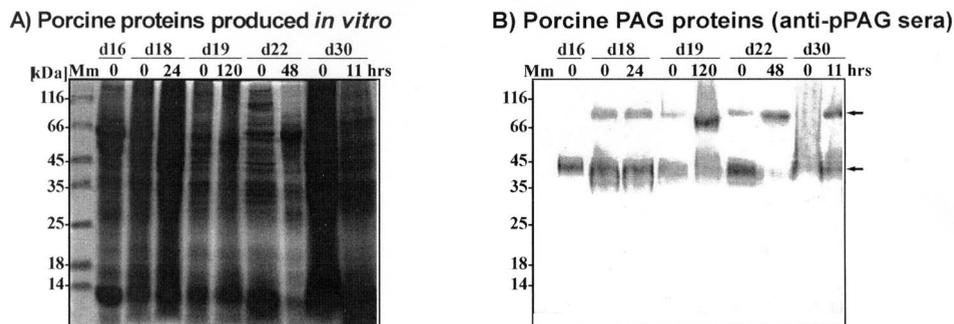


Figure 2. Porcine trophoblastic and trophoctodermal proteins released *in vitro* by placental explants harvested on various days (d) of pregnancy (16-30 dpc). **A)** SDS-PAGE of cellular (0 hrs) or secretory (11-120 hrs) chorionic proteins (10 μ g/lane) stained with CBB dye. **B)** Homologous Western blotting of porcine PAG protein family with anti-porcine PAG sera (anti-pPAG). Molecular marker (Mm) is on the left. Arrows on the right indicate molecular range of the pPAG proteins.

Heterogeneity of the PAG proteins as pregnancy progresses

In the pig, homologous Western immunoblotting of the pPAG protein family revealed similar molecular forms (M.W.) harvested on 18-30 dpc as cellular proteins (0 fraction) and proteins released *in vitro* by TR/TRD explants (11-120 hrs; fig. 2). The anti-porcine PAG serum detected mostly two major groups of ultra-filtrated (>10 kDa MWCO) cellular (0 h) and/or secretory forms: ~43 kDa and 70-72 kDa pPAG proteins (fig. 2 B). During this comparative study, relatively strong cellular 43 kDa pPAG protein expression (0 h) was detected on 16 dpc. However, this smaller form was also determined in all protein samples examined during other early placentation periods (18 - 22 dpc). After placentation (33 or 39 dpc), homologous Western blotting (fig. 3) revealed heterogeneity of the pPAG proteins produced *in vitro* by TRD explants harvested from various pigs on different days of pregnancy. The M.W.-heterogeneity (from ~45 to ~73 kDa) of the pPAG proteins was detected throughout period of the 208-374 h long-term cultures (fig. 3 A and B). Moreover, during extended periods (88-184 h) of porcine TRD culture (39 dpc),

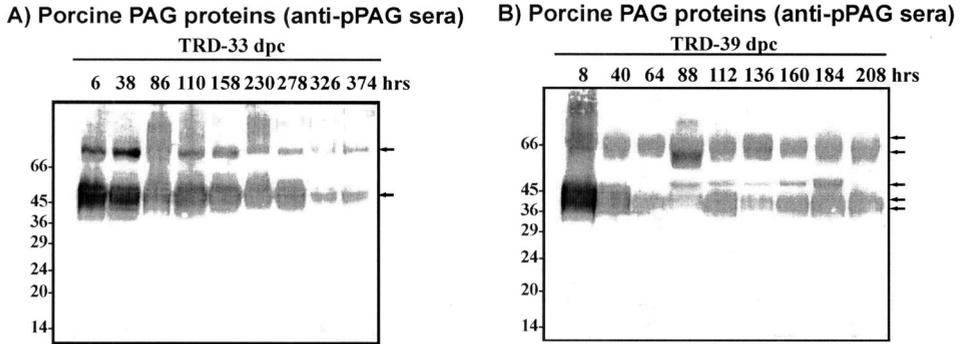


Figure 3. *In vitro* production of porcine trophodermal (TRD) proteins during long-term cultures of explants harvested on days 33 and 39 of pregnancy (dpc). **A, B** Homologous SDS-PAGE Western blotting of pPAG proteins (10 µg/lane) with anti-porcine PAG sera (anti-pPAG). Arrows on the right indicate molecular range of the pPAG proteins.

additional ~47 kDa forms of the pPAG proteins were immunodetected as culture time progressed.

In the cattle, double Western immunoblotting (fig. 4) revealed various forms of the bPAG proteins (45-85 kDa) released by bovine CT or by intCT-TRD explants collected on 90 dpc. During 208-h long cultures, almost equal amounts of the bPAG proteins were produced by both types of tissues, CT and intCT-TRD explants. The homologous Western blotting of bovine PAG proteins with anti-bovine PAG sera (fig. 4 B and E) or cross-species heterologous blotting with anti-porcine PAG sera (fig. 4 C and F) demonstrated diverse profiles of secretory bPAG proteins. The anti-bovine PAG sera detected two major groups of bPAG proteins (67 and 53 kDa) produced by CT explants collected on 90 dpc (fig. 4 B), and only one group of the dominant bPAG proteins (67 kDa) produced by intCT-TRD explants (fig. 4 E). The anti-porcine PAG sera, in turn, recognized two major groups of dominant bPAG protein forms (67 and 65 kDa) released by CT explants on 90 dpc (fig. 4 C). This anti-pPAG serum detected also two major groups of dominant bPAG protein forms (67 and 59 kDa) released by intCT-TRD explants collected on 90 dpc (fig. 4 F) and expressed during the entire 208 h of culture. In addition, weak immunoreactive and smaller secretory

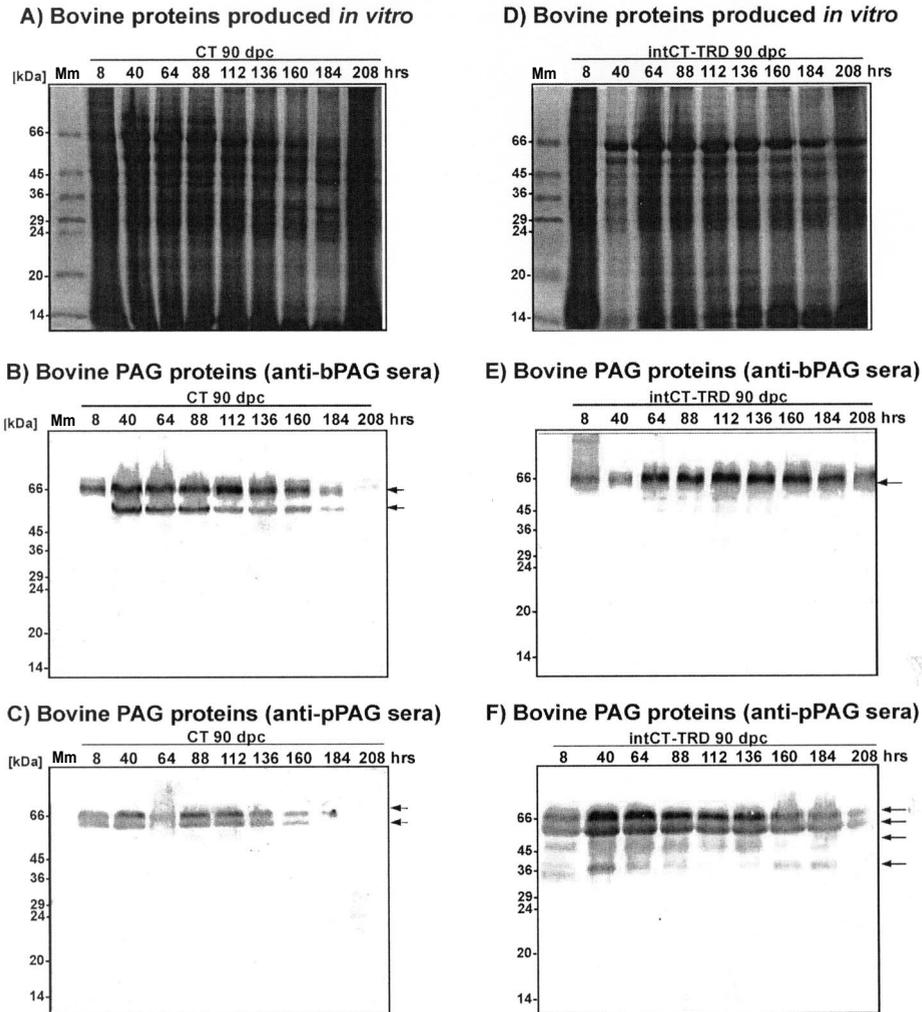


Figure 4. *In vitro* production of the bovine PAG protein family during long-term cultures of various placental explants of CT or intCT-TRD collected on Day 90 of pregnancy (90 dpc). **A, D**) SDS-PAGE of released CT and intCT-TRD proteins (10 μ g/lane) stained with CBB dye. **B, E**) Homologous Western blotting with polyclonal anti-bovine PAG serum (anti-bPAG). **C, F**) Heterologous Western blotting with anti-porcine PAG serum (anti-pPAG). Molecular marker (Mm) is on the left. Arrows on the right indicate molecular range of the bPAG proteins.

forms (49 and 36 kDa) were also detected, mainly during the early culture periods (up to 136 h).

DISCUSSION

This paper demonstrates efficient *in vitro* production of multiple native PAG proteins released by various chorionic explants of three mammalian *Artiodactyla* taxons: domestic pigs and cattle as well as wild endangered European bison. We compared molecular masses of multiple immunoreactive forms of native PAG protein families (pPAG, bPAG and EbPAG) as pregnancy advanced: 1/ cellular or secretory pPAG proteins released by porcine TR and TRD explants; and 2/ various secretory bPAG protein forms released by two dissected bovine placental tissue types, CT or intCT-TRD explants. Such comparison of the PAG family expression was not previously performed.

The proposed long-term *in vitro* procedure provides a few advantages over protein purification by multistep extractions from placental tissues. First, these secreted *in vitro* chorionic proteins do not require purification steps to separate and remove numerous cellular or membrane proteins which do not belong to the PAG protein family. Second, the long-term cultures of pre-placental trophoblast explants provides efficient method of chorionic protein harvesting from limited amounts of small pre-placental trophoblastic tissues available during very early stages of the pregnancy in wild and domestic mammals. Third, the harvesting of chorionic tissues during advanced pregnancy in endangered mammals is limited to occasionally eliminated wild females. Thus, the presented long-term cultures of chorionic explants enable production of the secretory PAG proteins without a requirement of multiple pregnant females. Moreover, stable chorionic cell lines of endangered mammals are not available.

Previous morphologic studies demonstrated abundant chorionic expression of multiple PAG transcripts and proteins in domestic pigs, horses and cattle possessing various placenta types: epitheliochorial diffuse, epitheliochorial with girdle cells and synepitheliochorial cotyledonaria,

respectively [20, 44, 45, 49, 60]. However, available reports concerning the PAG family are very limited for non-domesticated mammals with seasonal reproduction. Only a few studies reported multiplicity of the PAG family in wild pregnant females. Green et al. [20] has cloned only one cDNA in zebra (zPAG). Recently, nine distinct cDNAs of the PAG genes have been also cloned in white-tailed deer (wtdPAG)¹. Numerous wtdPAG proteins (30-90 kDa) were detected by cross-species Western blotting with anti-bovine PAG sera¹. Another earlier study [25] also reported the PAG protein extraction from placental tissues harvested from three cotyledons of hunter-killed elk (ePSPB: 31, 45, 57 kDa) and from two moose cotyledons (mPSPB: 31, 58 kDa). Unfortunately, NH₂-terminus of native purified PAG proteins has not been micro-sequenced yet in any wild species.

In domesticated pecoran mammals, distinct members of the PAG protein family have been recently identified and spatially/temporally specific placental expressions were detected during early or advanced gestation. In sheep, four oPAG proteins (55, 60, 61 and 65 kDa) were purified on 100 dpc [59]. Recently, three novel oPAG proteins (55, 57 and 59 kDa) with different N-terminal ends were extracted from more advanced (>100 dpc) ewe placenta [13]. These studies revealed that the 55 kDa oPAG proteins (identical in kDa) obtained during two different purification procedures represented various forms with electrostatic properties [Ip: 5.54; 59] or [Ip: 4.1-5.9; 13]. However, N-terminal micro-sequence of the 65 kDa oPAG protein was identical to sequences of two distinct oPAG3 and oPAG7 polypeptide precursors, coded by open reading frame (ORF) of cloned cDNAs [59]. Moreover, the 59 kDa oPAG protein sequence differs from previously identified sequence of the oPAG4 polypeptide precursor (coded by ORF) only in one position among the 15 identified N-terminal protein residues [13, 15, 59]. These studies suggest that the oPAG family require more investigation for complete identification of the entire multiple ovine PAG family.

Recently, novel PAG proteins were also purified by multiple step extraction from placental tissues of zebu (*Bos indicus*; zebuPAG) and buffalo (*Bubalus*

¹ Vawter GA Parks TE Killian G Ealy AD Green JA 2004 Cloning and preliminary characterization of Pregnancy-Associated Glycoproteins expressed in the placenta of white-tailed deer. *Biology of Reproduction* 70 153.

bubalis; bubPAG). Numerous forms of 51-69 kDa zebuPAG and 52-77 kDa bubPAG proteins were detected by heterologous Western blottings performed with different anti-PAG sera raised against extracted bovine, ovine and caprine PAG cellular antigens [4, 43]. These studies revealed multiple forms of the zebuPAG and bubPAG proteins during pregnancy stage-dependent chorionic development in both examined species. Moreover, Sousa et al. [43] reported a low efficiency of the zebuPAG-like protein extraction, from 0.61% up to 1.81% of total crude proteins, which were extracted from 10-31 week gestational age of placentas. Similarly, bubPAG-like proteins represented only 0.33% of the total soluble proteins extracted from cotyledonary tissues [4]. These studies proved that multiple step extraction procedures allow for PAG protein purifications from various placental tissues, but with very low extraction yield. Thus, complete purifications of various PAG proteins by tissue extractions require large amounts of placental materials. In contrast, during the long-term cultures, secretory chorionic proteins (without cellular and membrane proteins) can be effectively produced by smaller amounts of placental tissues.

The PAG proteins are differentially post-translationally glycosylated and may contain 3-18% of oligosaccharides in pigs [12, 50], sheep [2, 58], cattle [33, 56]. Carbohydrate contents of the PAG proteins were examined in only two wild taxons [25], the elk (3.15%) and the moose (4.98%). Similarly, high glycodiversity has been found in near-term epitheliochorial placentas of other examined, interbreeding and non-interbreeding, eutherian species including the horse, donkey and camel [31]. Presumably, high glycodiversity of the PAG proteins found during pregnancy in close related interbreeding wild and domestic taxons, is mainly associated with abnormally induced high plasma concentration of PAG proteins measured in peripheral blood circulation [16].

The plasmatic PAG protein concentration measurement is a very useful tool for fetal well-being monitoring, because the PAG concentration is usually pathologically changed in the cases of pregnancy difficulties [35]. Previously, a few cross-species diagnostic PAG tests were used in wild ruminants, including wood bison (*Bison bison athabasca*) [23], moose [24], French Alpine goats [29], white-tailed deer [37], sika deer [55] and

rocky mountain elk [53]. These pregnancy tests were performed using bovine PAG standards and iodinated tracers, mainly bPAG (R-37) or bPSPB - cellular proteins extracted from cotyledonary tissues. However, the pregnancy tests were not continued. The PAG/PSPB tests require purified standard proteins and specific anti-PAG sera, produced against various cellular or secretory antigens. Thus, obtaining huge amounts of purified native PAG proteins, with their unique carbohydrate chains, specific for various placentas during each period of gestation, would allow further plasmatic RIA/ELISA pregnancy diagnostic tests.

In conclusion, we demonstrated very high M.W.-diversity of secretory and cellular PAG forms and provided direct proteomical confirmation of multiplicity of the PAG family in three domestic and wild species. The described method provides the most effective harvesting system of glycosylated PAG proteins. Our study demonstrates an effective long-term culture system of native secretory and glycosylated PAG protein production in different domestic and wild species. Native PAG protein harvesting is procedure equally important to biotechnological production of recombinant PAG (recPAG) proteins. The importance of the proposed *in vitro* system of native protein harvesting is due to a general lack of carbohydrate side chains in the recPAG proteins produced in very popular and effective bacterial systems. The post-translational modifications of native PAG proteins i.e. specific glycosylation within placenta lead to a large variability of PAG proteins purified from placental extracts or culture media. Complete characterization of particular placental proteins requires suitable sources of placentas and/or useful method of placental protein harvesting. This is especially important in wild endangered animals, in which seasonal reproductive processes are relatively difficult to control.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Zbigniew Krasinski (Bialowieza National Park, Poland) for opportunity of European bison placenta collection. This study was supported by the State Committee for the Scientific Research (PBZ-KBN 084/P06/02/3.4, Poland) granted to B. Szafranska.

REFERENCES

1. Allen WR, Carter AM, Chavatte-Palmer P, Dantzer V, Enders AC, Freyer C, Leiser R, Mglino MA **2003** Comparative placentation – a workshop report. *Placenta* **24** 100-103.
2. Atkinson YH, Gogolin-Ewens KJ, Hounsell EF, Davies MJ, Brandon MR, Seamark RF **1993** Characterization of placentation-specific binucleate cell glycoproteins possessing a novel carbohydrate. *Journal of Biological Chemistry* **268** 26679-26685.
3. Banerjee R, Liu J, Beatty W, Pelosof L, Klemba M, Goldberg DE **2002** Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *Proceedings of the National Academy of Sciences of the United States of America* **99** 990-995.
4. Barbato O, Sousa NM, Malfatti A, Beckers JF, De Santis G, Debenetti A **2003** Purification of Pregnancy-associated glycoproteins (PAGs) in buffalo (*Bubalus bubalis*): preliminary results. In: Atti II Congresso Nazionale sull'Allevamento del Bufalo. Roma, Italy. 279-283.
5. Batalha ES, Sulon J, Figueiredo JR, Beckers JF, Martins GA, Silva LDM **2001** Relationship between maternal concentrations of caprine pregnancy-associated glycoprotein in Alpine goats and the number of fetuses using a homologous radioimmunoassay. *Small Ruminant Research* **42** 105-109.
6. Berry C, Humphreys MJ, Matharu P, Granger R, Horrocks P, Moon RP, Certa U, Ridley RG, Bur D, Kay J **1999** A distinct member of the aspartic proteinase gene family from the human malaria parasite *Plasmodium falciparum*. *FEBS Letters* **447** 149-154.
7. Bishop MD, Hawkins GA, Keefer CL **1995** Use of DNA markers in animal selection. *Theriogenology* **43** 61-70.
8. Chen X, Rosenfeld CS, Roberts RM, Green J **2001** An aspartic proteinase expressed in the yolk sac and neonatal stomach of the mouse. *Biology of Reproduction* **65** 1092-1101.
9. Cross JC **2001** Genes regulating embryonic and fetal survival. *Theriogenology* **55** 193-207.
10. Cross JC, Baczyk D, Dobric N, Hemberger M, Hedges M, Simmons DG, Yamamoto H, Kingdom JCP **2003** Genes, development and evolution of the placenta. *Placenta* **24** 123-130.
11. Davies DR **1990** The structure and function of the aspartic proteinases. *Annual Review of Biophysics and Biophysical Chemistry* **19** 189-215.
12. Dore JJ Jr, Kattesh HG, Godkin JD **1996** Isolation and identification of porcine embryonic basic protein as a fragment of pregnancy-associated glycoprotein-2. *The International Journal of Biochemistry and Cell Biology* **28** 1249-1255.
13. El Amiri B, Remy B, Sousa NM, Joris B, Ottiers NG, Perenyi Z, Banga-Mboko H, Beckers JF **2003** Isolation and partial characterization of three pregnancy-associated glycoproteins from the ewe placenta. *Molecular Reproduction and Development* **64** 199-206.
14. El Amiri B, Sousa NM, Mecif K, Desbuleux H, Banga-Mboko H, Beckers JF **2003** Double radial immunodiffusion as a tool to identify pregnancy-associated glycoproteins in ruminant and non-ruminant placentae. *Theriogenology* **59** 1291-301.
15. El Amiri B, Remy B, Sousa NM, Beckers JF **2004** Isolation and characterization of eight pregnancy-associated glycoproteins present at high levels in the ovine placenta between day 60 and day 100 of gestation. *Reproduction Nutrition Development* **44** 169-181.
16. Fernandez-Arias A, Alabart JL, Folch J, Beckers JF **1999** Interspecies pregnancy of spanish ibex (*Capra pyrenaica*) fetus in domestic goat (*Capra hircus*) recipients induces abnormally high plasmatic levels of Pregnancy-Associated Glycoprotein. *Theriogenology* **51** 1419-1430.
17. Garbayo JM, Remy B, Alabart JL, Folch J, Wattiez R, Falmagne P, Beckers JF **1998** Isolation and partial characterization of a pregnancy-associated glycoprotein family from the goat placenta. *Biology of Reproduction* **58** 109-115.

18. Garbayo JM, Green J, Manikkam M, Beckers JF, Kiesling DO, Early AD, Roberts RM **2000** Caprine pregnancy-associated glycoproteins (PAG): their cloning, expression, and evolutionary relationship to other PAG. *Molecular Reproduction and Development* **57** 311-322.
19. Gonzalez F, Sulon J, Garbayo JM, Batista M, Cabrera F, Calero P, Gracia A, Beckers JF **1999** Early pregnancy diagnosis in goats by determination of pregnancy-associated glycoprotein concentrations in plasma samples. *Theriogenology* **52** 717-725.
20. Green J, Xie S, Szafranska B, Newman A, Gan X, McDowell K, Roberts RM **1999** Identification of a new aspartic proteinase expressed by the outer chorionic cell layer of the equine placenta. *Biology of Reproduction* **60** 1069-1077.
21. Green J, Xie S, Quan X, Bao B, Gan X, Mathialagan N, Roberts RM **2000** Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. *Biology of Reproduction* **62** 1624-1631.
22. Guruprasad K, Blundell TL, Xie S, Green J, Szafranska B, Nagel RJ, McDowell K, Baker CB, Roberts RM **1996** Comparative modelling and analysis of amino acid substitutions suggest that the family of pregnancy-associated glycoproteins includes both active and inactive aspartic proteinases. *Protein Engineering* **9** 849-856.
23. Haigh JC, Gates C, Ruder CA, Sasser RG **1991** Diagnosis of pregnancy in wood bison using a bovine assay for pregnancy-specific protein B. *Theriogenology* **26** 749-754.
24. Haigh JC, Dalton WJ, Ruder CA, Sasser RG **1993** Diagnosis of pregnancy in moose using a bovine assay for pregnancy-specific protein B. *Theriogenology* **40** 905-911.
25. Huang F, Cockrell DC, Stephenson TR, Noyes JH, Sasser RG **1999** Isolation, purification, and characterization of pregnancy-specific protein B from elk and moose placenta. *Biology of Reproduction* **61** 1056-1061.
26. Huang XP, Kagami N, Inoue H, Kojima M, Kimura T, Makabe O, Suzuki K, Takahashi K **2000** Identification of a glutamic acid and an aspartic acid residue essential for catalytic activity of aspergillopepsin II, a non-pepsin type of acid proteinase. *Journal of Biological Chemistry* **275** 26607-26614.
27. Hughes AL, Green JA, Garbayo JM, Roberts RM **2000** Adaptive diversification within a large family of recently duplicated, placentally expressed genes. *Proceedings of the National Academy of Sciences of the United States of America* **97** 3319-3323.
28. Hughes AL, Green JA, Piontkivska H, Roberts RM **2003** Aspartic proteinase phylogeny and the origin of Pregnancy-Associated Glycoproteins. *Molecular Biology and Evolution* **20** 1940-1945.
29. Humblot P, De Montigny G, Jeanguyot N, Tetedoie F, Payen B, Thibier M, Sasser RG **1990** Pregnancy-specific protein B and progesterone concentrations in French Alpine goats throughout gestation. *Journal of Reproduction and Fertility* **89** 205-212.
30. Ishiwata H, Katsuma S, Kizaki K, Patel OV, Nakano H, Takahashi T, Imai K, Hirasawa A, Shiojima S, Ikawa H, Suzuki Y, Tsujimoto G, Izaike Y, Todoroki J, Hashizume K **2003** Characterization of gene expression profiles in early bovine pregnancy using a custom cDNA microarray. *Molecular Reproduction and Development* **65** 9-18.
31. Jones CJP, Wooding FBP, Abd-Elnaeim MM, Leiser R, Stoddart RW **2000** Glycosylation in the near-term epitheliochorial placenta of the horse, donkey and camel: a comparative study of interbreeding and non-interbreeding species. *Journal of Reproduction and Fertility* **118** 397-405.
32. Kageyama T, Ichinose M, Tsukada-Kato S, Omata M, Narita Y, Moriyama A, Yonezawa S **2000** Molecular cloning of neonate/infant-specific pepsinogens from rat stomach mucosa and their expressional change during development. *Biochemical and Biophysical Research Communications* **267** 806-812.

33. Klisch K, Leiser R **2003** In bovine binucleate trophoblast giant cells, pregnancy-associated glycoproteins and placental prolactin-related protein-1 are conjugated to asparagine-linked N-acetylgalactosaminyl glycans. *Histochemistry and Cell Biology* **119** 211-217.
34. Koelsch G, Mares M, Metcalf P, Fusek M **1994** Multiple functions of pro-parts of aspartic proteinase zymogens. *FEBS Letters* **343** 6-10.
35. Kornmatitsuk B, Veronesi MC, Madej A, Dahl E, Ropstad E, Beckers JF, Forsberg M, Gustafsson H, Kindahl H **2002** Hormonal measurements in late pregnancy and parturition in dairy cows - possible tools to monitor fetal well being. *Animal Reproduction Science* **72** 153-164.
36. Mialon MM, Camous S, Renand G, Martal J, Menissier F **1993** Peripheral concentrations of a 60-kDa pregnancy serum protein during gestation and after calving and in relationship to embryonic mortality in cattle. *Reproduction Nutrition Development* **33** 269-282.
37. Osborn DA, Beckers JF, Sulon J, Gassett JW, Muller LI, Murphy BP, Miller KV, Marchinton RL **1996** Use of glycoprotein assays for pregnancy diagnosis in white-tailed deer. *Journal Wildlife Management* **60** 388-393.
38. Panasiewicz G, Majewska M, Szafranska B **2004** Trophoblastic cDNA cloning of porcine Pregnancy-Associated Glycoprotein gene family (pPAG) and *in silico* analysis of coded polypeptide precursors. *Reproductive Biology* **4** 131-141.
39. Panasiewicz G, Majewska M, Szafranska B **2004** The involvement of luteinizing hormone (LH) and Pregnancy-Associated Glycoprotein family (PAG) in pregnancy maintenance in the pig. *Reproductive Biology* **4** 143-163.
40. Ranilla MJ, Sulton J, Carro MD, Mantecon AR, Beckers JF **1994** Plasma profiles of pregnancy-associated glycoprotein and progesterone levels during gestation in Churra and Merino sheep. *Theriogenology* **42** 537-545.
41. Roberts RM, Xie S, Mathialagan N **1996** Maternal recognition of pregnancy. *Biology of Reproduction* **54** 294-302.
42. Sambrook J, Fritsch EF, Maniatis T **1989** Molecular Cloning. A Laboratory Manual. *Cold Spring Harbor Laboratory Press, New York*.
43. Sousa NM, Remy B, El Amiri B, De Figueiredo JR, Banga-Mboko H, Dias Goncalves PB, Beckers JF **2002** Characterization of pregnancy-associated glycoproteins extracted from zebu (*Bos indicus*) placentas removed at different gestational periods. *Reproduction Nutrition Development* **42** 227-241.
44. Szafranska B, Panasiewicz G **2002** The placental expression of the porcine pregnancy-associated glycoprotein (pPAG) gene family examined *in situ* and *in vitro*. *Animal Reproduction Science* **72** 95-113.
45. Szafranska B, Xie S, Green J, Roberts RM **1995** Porcine Pregnancy-Associated Glycoproteins: new members of the aspartic proteinase gene family expressed in the trophectoderm. *Biology of Reproduction* **53** 21-28.
46. Szafranska B, Miura R, Ghosh D, Ezashi T, Xie S, Roberts RM, Green J **2001** Gene for porcine Pregnancy-Associated Glycoprotein 2 (poPAG2): its structural organization and analysis of its promoter. *Molecular Reproduction and Development* **60** 137-146.
47. Szafranska B, Panasiewicz G, Waclawik A **2001** Length polymorphism of PCR-amplified genomic fragments of the Pregnancy-Associated Glycoprotein (PAG) gene family in the pig and some other domestic or wild mammals. *Journal of Applied Genetics* **42** 335-349.
48. Szafranska B, Ziecik AJ, Okrasa S **2002** Primary antisera against selected steroids or proteins and secondary antisera against γ -globulins - an available tool for studies of reproductive processes. *Reproductive Biology* **2** 187-204.

49. Szafranska B, Panasiewicz G, Majewska M, Beckers J-F **2003** Chorionic expression of heterogeneous products of the PAG (Pregnancy-Associated Glycoprotein) gene family secreted *in vitro* throughout embryonic and foetal development in the pig. *Reproduction Nutrition Development* **46** 497-519.
50. Szafranska B, Majewska M, Panasiewicz G **2004** N-glycodiversity of the Pregnancy-Associated Glycoprotein family (PAG) produced *in vitro* by trophoblast and trophoctoderm explants during implantation, placentation and advanced pregnancy in the pig. *Reproductive Biology* **4** 67-89.
51. Tatnell PJ, Cook M, Peters C Kay J **2000** Molecular organization, expression and chromosomal localization of the mouse pronapsin gene. *European Journal of Biochemistry* **267** 6921-6930.
52. Tuggle CK, Green JA, Fitzsimmons C, Woods R, Prather RS, Malchenko S, Soares BM, Kucaba T, Crouch K, Smith C, Tack D, Robinson N, O'Leary B, Scheetz T, Casavant T, Pomp D, Edeal BJ, Zhang Y, Rothschild MF, Garwood K, Beavis W **2003** EST-based gene discovery in pig: virtual expression patterns and comparative mapping to human. *Mammalian Genome* **14** 565-579.
53. Willard ST, Sasser RG, Gillespie JC, Jaques JT, Welsh THJr, Randel RD **1994** Methods for pregnancy determination and the effects of body condition on pregnancy status in rocky mountain elk (*Cervus elaphus nelsoni*). *Theriogenology* **42** 1095-1102.
54. Willard ST, White DR, Wesson CAR, Stellflug J, Sasser RG **1995** Detection of fetal twins in sheep using radioimmunoassay for pregnancy-specific protein B. *Journal of Animal Science* **73** 960-966.
55. Willard ST, Hughes DM, Bringans M, Sasser RG, White DR, Jaques JT, Godfrey RW, Welsh THJr, Randel RD **1996** Artificial insemination, hybridization and pregnancy detection in sika deer. *Theriogenology* **46** 779-789.
56. Xie S, Low BG, Nagel RJ, Beckers JF, Roberts RM **1994** A novel glycoprotein of the aspartic proteinase gene family expressed in bovine placental trophoctoderm. *Biology of Reproduction* **51** 1145-1153.
57. Xie S, Green J, Beckers JF, Roberts RM **1995** The gene encoding bovine pregnancy-associated glycoprotein-1, an inactive member of the aspartic proteinase family. *Gene* **159** 193-197.
58. Xie S, Nagel RJ, Green J, Beckers JF, Roberts RM **1996** Trophoblast-specific processing and phosphorylation of pregnancy-associated glycoprotein-1 in Day 15 to 25 sheep placenta. *Biology of Reproduction* **54** 122-129.
59. Xie S, Green J, Bao B, Beckers JF, Valdez KE, Hakami L, Roberts RM **1997** Multiple pregnancy-associated glycoproteins are secreted by day 100 ovine placental tissue. *Biology of Reproduction* **57** 1384-1393.
60. Xie S, Green J, Bixby JB, Szafranska B, DeMartini JC, Hecht S, Roberts RM **1997** The diversity and evolutionary relationships of the pregnancy-associated glycoproteins, an aspartic proteinase subfamily consisting of many trophoblast-expressed genes. *Proceedings of the National Academy of Sciences of the United States of America* **94** 12809-12816.
61. Zoli AP, Beckers JF, Woutters-Ballman P, Falmagne P, Ectors F **1991** Purification and characterization of a bovine pregnancy-associated glycoprotein. *Biology of Reproduction* **45** 1-10.
62. Zoli AP, Guilbault LA, Delabaut P, Orates WEB, Beckers JF **1992** Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. *Biology of Reproduction* **46** 83-92.