REPRODUCTIVE BIOLOGY

ORIGINAL RESEARCH

N-glycodiversity of the Pregnancy-Associated Glycoprotein family (PAG) produced *in vitro* by trophoblast and trophectoderm explants during implantation, placentation and advanced pregnancy in the pig[1]

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Received: 12 January 2004; accepted: 5 March 2004

SUMMARY

The PAG family is encoded by distinct genes expressed in extra-embryonic chorionic membranes (TR−trophoblast, TRD−trophectoderm) of various pregnant mammals. The objective of our study was to determine N-glycodiversity of porcine PAG protein family (pPAG) produced in vitro by TR or TRD explants of gilts (n=26) throughout pregnancy (16-77 dpc). Explants were cultured for over 1200h (TR, 16 dpc) or for 8h (TRD, 17-77 dpc). Released proteins were isolated from media by separating ultra-filtration (>10 kDa). A deglycosylation (removal of N-linked carbohydrate side chains) of proteins was performed by glycopeptidase F, and compared to

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[1] reported porcine sequences have been deposited in the GenBank (Acc. Nos.: L34360, L34361, AF355377, AF272734, AY188554, AF272735 and AY373029 for cDNAs of pPAG1-6, and pPAG8, respectively);

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non-deglycosylated forms by PAGE-Western blotting with anti-pPAG sera and additionally to polypeptide pPAG precursors, coded by ORF of their cloned cDNAs. We demonstrated gestation-stage dependent diversity of deglycosylated/glycosylated forms of the pPAG proteins produced in vitro in the pig. TR explants harvested on 16 dpc during long term culture released 43 kDa pPAG proteins. These proteins were deglycosylated to ~36.9 and ~39.6 kDa (16 dpc). Tissue harvested on 17 dpc *in vitro* secreted 65-68 kDa pPAG proteins which were reduced to three forms, 50.6, 58.7 and 63.5 kDa. In addition, ~73.3 kDa major pPAG proteins (77 dpc) were reduced to at least three forms: ~39.6, ~36.9 and ~33.4 kDa. Such N-deglycosylation was not detected on days 25-61. N-deglycosylation of native pPAG proteins clearly corresponded to three N-glycosylation sites of asparagines (N-x-S/T) found in ORF of the pPAG2-like precursors, identified by their in silico translated cDNAs. Thus, the pregnancy-stage dependent N-glycodiversity of the pPAG protein family, containing an average 9.66% of N-linked oligosaccharides, may play some role(s) in porcine conceptus attachment, successful implantation and during advanced pregnancy. *Reproductive Biology 2004 4 (1): 67-89.*

Key words: chorionic pPAG proteins, deglycosylation, glycoproteins, N-glycodiversity

INTRODUCTION

In the pig, a dialog occurs between the conceptus and mother during implantation and placental development that is believed to be crucial for early embryonic survival. About 40% of early embryonic mortality occurs during implantation, prior to Day 20 [37]. This corresponds to the period of rapid differentiation and intensive expansion (4.5 mm / h) of the conceptus' elongating trophoblastic membranes [18].

Estradiol, which is secreted by porcine conceptuses, is the major signal for early maternal recognition of pregnancy. Embryonic estradiol supports maternal maintenance of luteal secretion of progesterone. The latter stimulates endometrial production of various polypeptide growth factors and numerous proteins (histotroph) before implantation (for review see: [39 and 40]). After implantation, various embryonic origin cells of mammalian membranes secrete, across placental barrier, several glycoproteins, which maintain corpus luteum function and progesterone production. Placental gonadotrophins of primates - hCG [28] or equine species - eCG, with conserved glycosylation pattern of beta subunits [8], are the best known glycoproteins secreted by chorionic cells of mammalian conceptuses. However, in various eutherian ungulate mammals (*Placentalia: Artiodactyla, Perissodactyla, Carnivora* and *Rodentia*), chorionic cells (trophoblastic: TR and trophectodermal: TRD) secrete also numerous pregnancy-associated glycoproteins (PAGs). The PAGs were first identified as bovine placenta-specific antigens existing in peripheral blood of mother [9] and other ruminants [1, 35, 43, 44]. Recently, several cDNAs, PCR-amplified genomic fragments of the PAG family or proteins were identified in cattle, sheep [24, 56-61], pigs [45-49, 51], horses [23], goats [16, 17], elk, moose [29], cats [15] and mice [11]. All members of the PAG family belong to aspartic proteinase (AP) group (EC 3.4.23), which includes various identified mammalian proteolytic enzymes: pepsins [34], cathepsins D and E [12] or napsins [52]. The AP also family includes several plasmepsins [2] and two histo AP (HAP) genes [5] that have been identified in Plasmodium falciparum, the human malaria parasite. All members of the AP and the PAG protein family possess two-bilobe structure with a cleft capable of binding short peptides. Equine PAG and porcine PAG2 are the most proteolytically active among recently numerous identified PAGs [25, 45-47, 60]. In the pig, two cDNAs of pPAG1 and pPAG2 have been initially identified as the first members of two distinct pPAG1-like and pPAG2-like subfamilies, which coded either potentially inactive or enzymatically active polypeptide precursors, respectively [47]. Recently, novel distinct cDNAs of the pPAG2-like members (pPAG4, pPAG6 and pPAG8) have been cloned with sequences identical to pepsin within regions of two classical catalytic domains 1 and 2 [46]. Moreover, two novel members of the pPAG1-like subfamily have been identified (pPAG3 and pPAG5; [46]).

Our previous studies indicated that the PAG transcripts (mRNAs) are generally expressed in various chorionic cells. We have found that in the pig, pPAG mRNA expression appeared in pre-placental trophoblastic and persisted in the placental trophectoderm cells only [45-47]. Porcine [45-47] or equine [23] placental sections examined by *in situ* hybridization (ISH) with various PAG probes (anti-sense cDNA or cRNA) revealed that the PAG mRNAs were mainly expressed within mononucleate cells. In contrast, the ruminant PAG mRNA family exhibits spatially and temporally distinct expression patterns in mononucleate or binucleate cells during pregnancy [17, 24, 56, 60]. However, *in vitro* and *in situ* studies indicated strong pPAG mRNA expression in various porcine multinucleated cells [46].

Various numbers of the PAG genes can be expected in each species of previously examined eutherians. Our results of genomic DNA and cloning of distinct PAG cDNAs revealed that various PAG families include many genes. Among over 100 genes expected in ruminants (bovine PAG, ovine PAG, caprine PAG), 40-80 genes may belong to the bPAG1-like subfamily, 30-60 may belong to the bPAG2-like subfamily [60]. So far, only single cDNA of chorionic PAG genes in the horse (equine - ePAG), zebra (zPAG), cat (feline - fPAG) and mouse (mPAG) were identified [11, 15, 22, 23]. However, in the pig, at least eight genes belonging to the pPAG2-like subfamily are expected in porcine genome [45, 48-49]. Presently, a number of pPAG1-like genes remain unknown; however cDNAs of pPAG1, pPAG3 and pPAG5 genes, belonging to this subfamily, have been already cloned and identified [45-47]. This gene-diversity of the mammalian PAG family contributes to multiple products and their highly heterogenic expression [45-47, 50, 51]. Presently, this may be explained by coding sequences of identified cDNAs and the post-translational processing of polypeptide PAG precursors, examined on the basis of the open reading frames (ORF) translated *in silico*.

Our earlier *in silico* studies of polypeptide PAG precursors (coded by their cDNAs) proved that most PAG proteins are uniquely post-translationally modified in placentas of aforementioned numerous eutherian species [45-47]. The post-translational processing of the polypeptide PAG precursors may involve carbohydrate chain modifications, mainly N-glycosylation. Different numbers of N-glycosylation sites of asparagines (N-x-T/S) have been found in various sequences of the PAG precursors in ruminants [56-57, 60] and the pig [45-47]. Additional metabolic studies, performed with tunicamycin (inhibitor of N-glycosylation) or with L-[^{3}H]leucine, clearly demonstrated that the molecular mass of major immunoprecipitated products were strongly reduced [57]. Thus, the native heterogeneity/diversity of isolated PAG proteins in different species are linked with different N-glycosylation sites of asparagines (N-x-T/S) that were localized in various domains of polypeptide PAG precursors by *in silico* methods [45].

The objective of our present study was to identify a profile of N-glycodiversity (by *in vitro* enzymatic deglycosylation) of the pPAG proteins produced *in vitro* and released by various TR/TRD explants throughout pregnancy in the pig.

MATERIALS AND METHODS

Materials

Molecular markers (Mm) were from Bio-Rad (Richmond, Ca, USA), ICN (Costa Mesa, Ca, USA) or from Kucharczyk (Warsaw, Poland). Glycopeptidase F (PNGase F) was purchased from Sigma (St. Louis, MO, USA). A 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP), and nitro blue tetrazolium (NBT) were purchased from Promega (Madison, WI, USA). Nitrocellulose membranes (Optitran BA-S 83) were from Fisher Scientific GmbH (Germany). Alkaline phosphatase linked monoclonal mouse anti-rabbit antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and all other required reagents were from ICN (Costa Mesa, CA, USA).

Animals and tissue collection

Mature crossbred gilts were naturally bred during oestrus (day 0). Sixteen (n=4), 17 (n=2), 18 (n=2), 19 (n=2), 20 (n=2), 22 (n=2), 25 (n=2), 31 (n=2), 38 (n=2), 42 (n=2), 45 (n=2), 61 (n=1) and 77 (n=1) days post coitum (dpc) all gilts (n=26) were sacrificed and their uteri were recovered at a local slaughterhouse. Care and use of gilts were in full agreement with local ethical authorities.

Chorionic tissues were recovered from uteri and separated from the remaining embryonic tissues. Trophoblastic tissues collected during implantation periods were dissected from embryos. Trophectodermal tissues collected during advanced gestation have been dissected from the remaining embryonic membranes according to Szafranska et al [47]. The tissues were then placed in sterile PBS supplemented with penicillin (200 IU/ml) and streptomycin (200 IU/ml) and were transported to the laboratory.

Long- and short-term cultures of porcine trophoblastic and trophectodermal explants

Chorionic explants (TR, TRD) were cultured in short-term [47] and long-term experiments [45-46, 51]. Briefly, TR and TRD tissues were washed in PBS, minced into small pieces (1-3 mm^3), washed 2-3 times in PBS and then in Dulbecco`s Modified Eagle’s Medium (DMEM) serum free (ICN, USA) with antibiotics (100 IU/ml). Explants of TR (16dpc) or TRD (17-77 dpc) were placed in flasks on the rocking platform and cultured for 8 h (37°C, 5% CO_2:95% air). Cultures of pre-placental TR explants (16 dpc) were extended until ~52 days, (pigs P2 and P3: 1214 h; P1 and P4: 1238 h). During these long-term cultures, collected media were replaced (in 24-48 h intervals) by fresh serum free DMEM with antibiotics and nystatine (240 IU/ml). The long-term cultures were necessitated by the small amounts of TR explants collected from individual pigs. This approach enabled us to achieve TR protein production sufficient for PAGE/Western blotting.

Harvesting and fractionation of chorionic proteins

Chorionic proteins, released by TR or TRD explants into media, were harvested by methods previously described [45-47, 51] with some modifications. Media collected during long-term cultures of TR explants (16 dpc) were filtered (20 µm mesh) and stored at -70°C. Other secretory chorionic TR/TRD proteins (17-77 dpc) were harvested from media collected after 8 h of culture. Released TR/TRD proteins were separated by ultra-filtration (>10 kDa), dialysis of media components and concentrated by Centriprep-10 cartridges (MWCO 10 kDa; Amicon, Beverly, MA, USA).

The amounts of TRD proteins released *in vitro* by explants originated from gilts in more advanced stages of pregnancy (31-77 dpc) necessitated an introduction of three precipitation steps. Thus, bigger volumes of culture media were initially recovered by 20%, 40% and 75% saturation of $(NH_4)_2SO_4$ for 24 h at 4°C and subsequent centrifugation (3500 x g, 1h). Pellets containing TRD proteins were dissolved in 20 mM Tris-HCl/2 mM EDTA, dialyzed (MWCO 12-14 kDa) at 4°C for complete removal of remaining $(NH_4)_2SO_4$, and then similarly concentrated as described above. Total chorionic protein concentration was determined by the Bradford procedure with BSA used as the standard [41]. According to low TR protein content in media of 16 dpc explants, media collected in the course of the long-term experiments were combined in two fractions in a manner presented in Table 1. Next, all TR and TRD proteins were subjected to treatments described below.

Glycopeptidase F treatment

Deglycosylation of porcine TR and TRD proteins was performed using glycopeptidase F (PNGase F) treatments. Briefly, the porcine chorionic proteins, released by TR/TRD explants were deglycosylated with PNGase F (0.05 IU PNGF per 1 µg of protein) in potassium buffer (200 mM K_3PO_4; 20 mM EDTA, pH 7.2) for 12 h at 37°C [41]. Then, N-glycosylated (G) and deglycosylated (D) proteins were separated by 1D-SDS-PAGE and monitored by homologous Western immunoblotting.

PAGE and Western analysis

Porcine proteins (2.5 - 10 µg/sample), released *in vitro* by different TR and TRD explants were separated in denaturing 12.5% polyacrylamide gels and then blotted as described previously [41, 46-47, 50-51]. Briefly, non-specific binding of NC membranes was blocked in 4% NFDM (non fat dry milk, v/v) in TBST for 4-5 h at 4°C. Then, NC membranes were incubated for 24 h at 4°C with primary rabbit polyclonal/polyvalent anti-pPAG sera (titer 1:300), raised against semi-purified porcine native (produced *in vitro*) or recombinant antigens. The antisera were purified by specific

Table 1. Combining of fraction collected from various cultures of the TR explants.

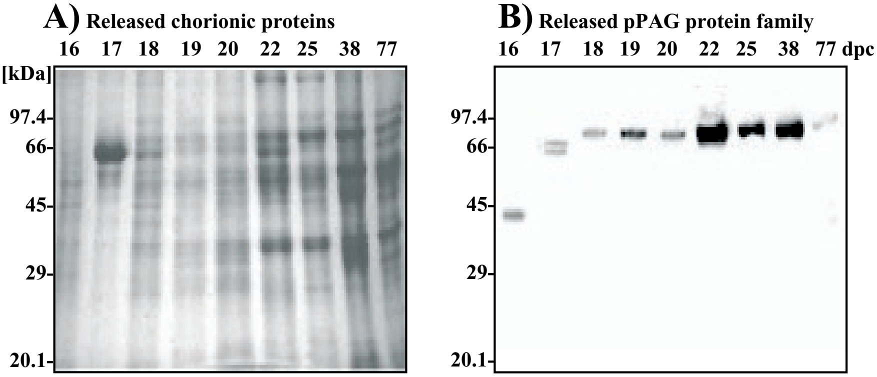
Code of the pig	Fraction 1	Fraction 2
P1	0-261h	261-1238h
P2	0-237h	237-1214h
P3	0-1214h	
P4	0-261h	261-1238h

adsorptions and characterized and standardized for binding of various chorionic native and recombinant pPAG antigens [50, 51]. Blotted NC membranes were washed three times in TBST and exposed to secondary mouse anti-rabbit monoclonal IgG-conjugated with alkaline phosphatase (titer: 1:150 000) for 1-1.5 h at room temperature. The membranes were washed again and pPAG proteins were visualized by alkaline phosphatase substrates, NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate; Promega, USA). Gels and blots were photographed and digitally archived by FOTO/Analyst Archiver (Fotodyne, Hartland, WI, USA). The percentage of carbohydrate content was calculated according to molecular mass reduction of immunodetected D-forms compared to their parallel G-forms.

RESULTS

Heterogeneity of the pPAG protein family with progress of pregnancy

Western blotting of the pPAG proteins, released *in vitro* by TR and TRD explants into media (examined accordingly to gestation stages), revealed heterogeneous (in M.W.) and pregnancy stage-dependent expression pattern of multiple products of the pPAG gene family. We demonstrated molecular mass-heterogeneity of the pPAG protein family (from ~43 to ~73 kDa). This pPAG family was chronologically examined throughout pregnancy during: a/ implantation (16-20 dpc), b/ early placentation (22-38 dpc) and c/ during later stages (40-77 dpc) of advanced placenta development. Different profiles (fig. 1) of multiple pPAG proteins (M.W.) were identified as


A) Released chorionic proteins

B) Released pPAG protein family

Fig. 1. The diversity of the porcine PAG protein family secreted *in vitro* by trophoblast (TR) or trophectoderm (TRD) explants of pigs on 16-77 *post coitum* days (dpc; 10 µg of protein/lane): A) SDS-PAGE of released chorionic proteins stained by CBB dye; B) Western blotting of the pPAG proteins released by chorionic explants during implantation, placentation and later stages of the placenta development: 43 kDa (16 dpc), 65-68 kDa (17 dpc), ~72 kDa (18-20 dpc) and ~73.3 kDa (22-77 dpc).

dominant immuno-reactive bands: ~43 kDa (16 dpc), 65-68 kDa (17 dpc), and ~72 kDa (18-20 dpc) or ~73.3 kDa (22-77 dpc).

Trophoblastic explants harvested during implantation (n= 4 gilts, 16 dpc), then cultured *in vitro* released higher or lower amounts of the uniquely N-glycosylated (G) pPAG proteins (fig. 2). Concentrations of TR proteins (2 or 4 µg per sample; gilts P3 and P4) in combined fractions (tab. 1) were too low and did not allow for proficient immuno-detection of the pPAG protein family (fig. 2 B). Higher production of released TR proteins (5-10 µg) was found in media of two cultured TR explants only (gilts P1 and P2; tab. 1). Our results indicated that at least 5-10 µg of TR proteins was required for proper Western blotting. Such differences in TR protein production may be related to individual pPAG-genotypes of embryos.

N-glycodiversity of the pPAG protein family during placenta development

Western blotting revealed various molecular range of post-transitionally modified native mature N-glycosylated pPAG (G) or deglycosylated (D)

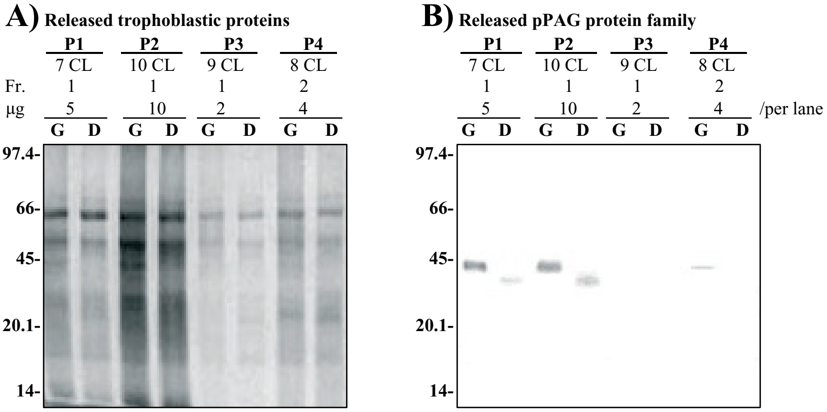


Fig. 2. N-glycosidase F digestion of pre-placental chorionic proteins released by TR explants of four (P1-P4) early pregnant pigs (16 dpc). A/ SDS-PAGE of released TR proteins stained by CBB dye. The proteins N-glycosylated (G) or deglycosylated (D) by PNG-ase F (-/+) treatment (0.05 IU/1μg) were compared to molecular markers. B/ Western blotting of released pPAG proteins (G- and D-forms) detected by polyvalent anti-pPAG sera before and after N-deglycosylation.

proteins released *in vitro* by TR/TRD explants on different days of porcine pregnancy (figs. 2-4). This diversity of N-glycosylated pPAG proteins has been caused by different carbohydrate side chains responsible for various reductions of molecular masses of TR/TRD proteins throughout gestation (fig. 5). Our results revealed post-transcriptional modifications of released native N-glycosylated pPAG protein family. The most N-glycosylated pPAG proteins (figs. 1-5) were specifically immunodetected in culture medium of TR tissue harvested on 16 and 17 dpc (implantation time). In addition, other forms of N-glycosylated pPAG forms were detected as pregnancy advanced. On 16 dpc, native mature G-forms of the pPAG proteins, 43 kDa, were deglycosylated (+PNGase F) and converted to faster migrating D-forms: ~39.6 and ~36.9 kDa (figs. 2 and 4). These two smaller D-forms represented the pPAG proteins after removal of at least two N-oligosaccharide side chains. In different gilts examined on 17 dpc, 65-68 kDa G-forms of secretory pPAG proteins were reduced to three immunodetected D-forms: 50.6, 58.7 and 63.5 kDa (figs. 1B and 4B). During this stage of early porcine placenta development, the highest level of N-linked carbohydrate content

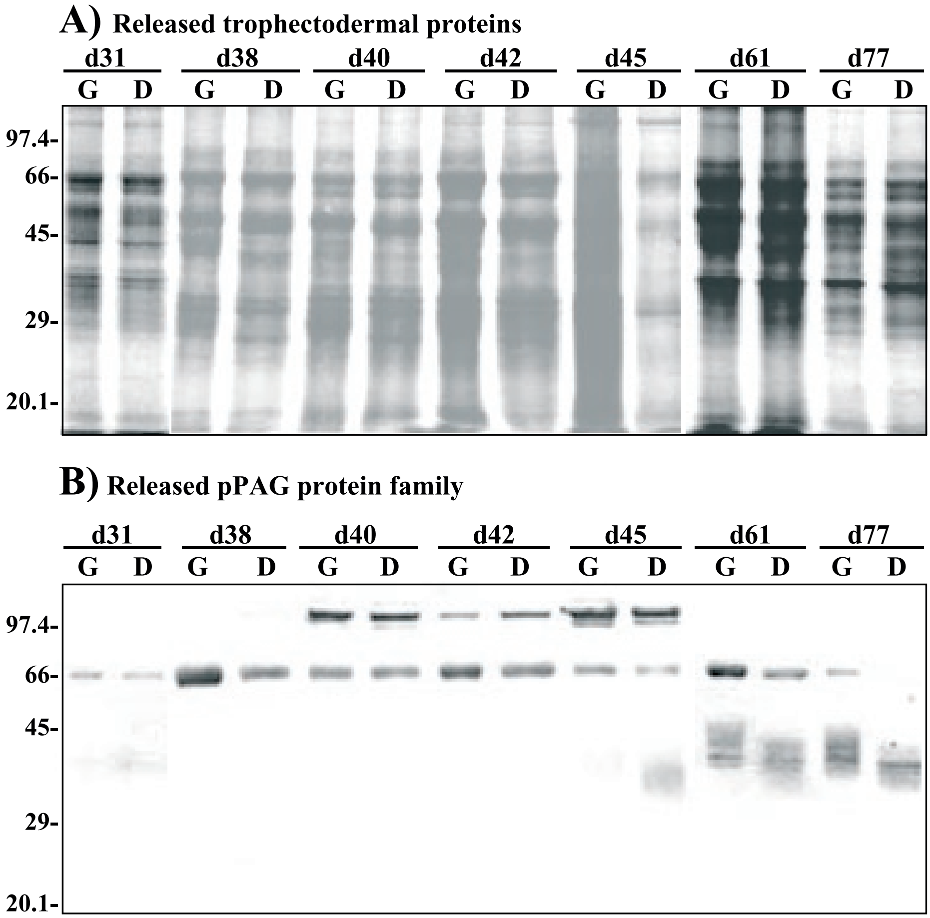


Fig. 3. Secretory chorionic proteins (10 µg/lane), produced *in vitro* by placental TRD explants of pigs on 31-77 dpc, and treated with PNG-ase F. A) SDS-PAGE of released TRD proteins stained by CBB dye. B) Western blotting of released glycosylated (G) and deglycosylated (D) forms of the pPAG proteins detected by polyvalent anti-pPAG sera.

was detected and it varied from 6.7 up to 25.7% (fig. 5). Deglycosylations were not observed on 31-42 dpc (figs. 3B and 5).

Some additional unexpected forms (~120 kDa) of the pPAG proteins were detected on 40-45 dpc (fig. 3B), presumably as a result of occurrence of some specific duo-domain forms of mature and/or processed polypeptide

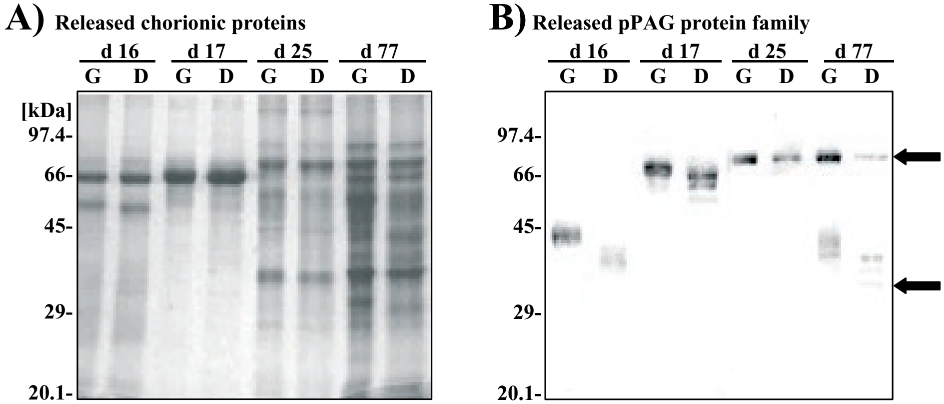


Fig. 4. The N-glycodiversity of porcine secretory chorionic proteins (10 μg/lane) produced by TR or TRD explants of pigs (16-77 dpc). A) SDS-PAGE of released proteins stained by CBB dye. B) Western blot of the pPAG protein family detected by polyvalent anti-pPAG sera. Arrows on the right indicate differences in molecular masses (Mr) of all released chorionic pPAG proteins during various periods of placenta development.

pPAG precursors. A relatively suppressed degree of the N-glycodiversity occurred in proteins released by various TRD explants recovered from individual gilts on 45 dpc (4-8%) or 61 dpc (~2%). As pregnancy advanced (77 dpc), higher N-glycodiversity (9.6-15.8%) of the pPAG proteins was found (figs. 3B, 4B and 5).

DISCUSSION

This is the first paper indicating the N-glycodiversity of released chorionic porcine PAG proteins produced during long-term *in vitro* studies. Previously, the N-glycodiversity of the PAG protein family was demonstrated in the sheep by Atkinson et al. [1] who examined SBU3/oPAG proteins for carbohydrate contents. In this paper, we showed the pPAG proteins examined *in vitro* during implantation, placentation and throughout advanced porcine pregnancy occurs as post-translationally G-glycosylated forms. Enzymatic N-deglycosylations of the pPAG proteins (D-forms) were identified by homologous Western-blotting.

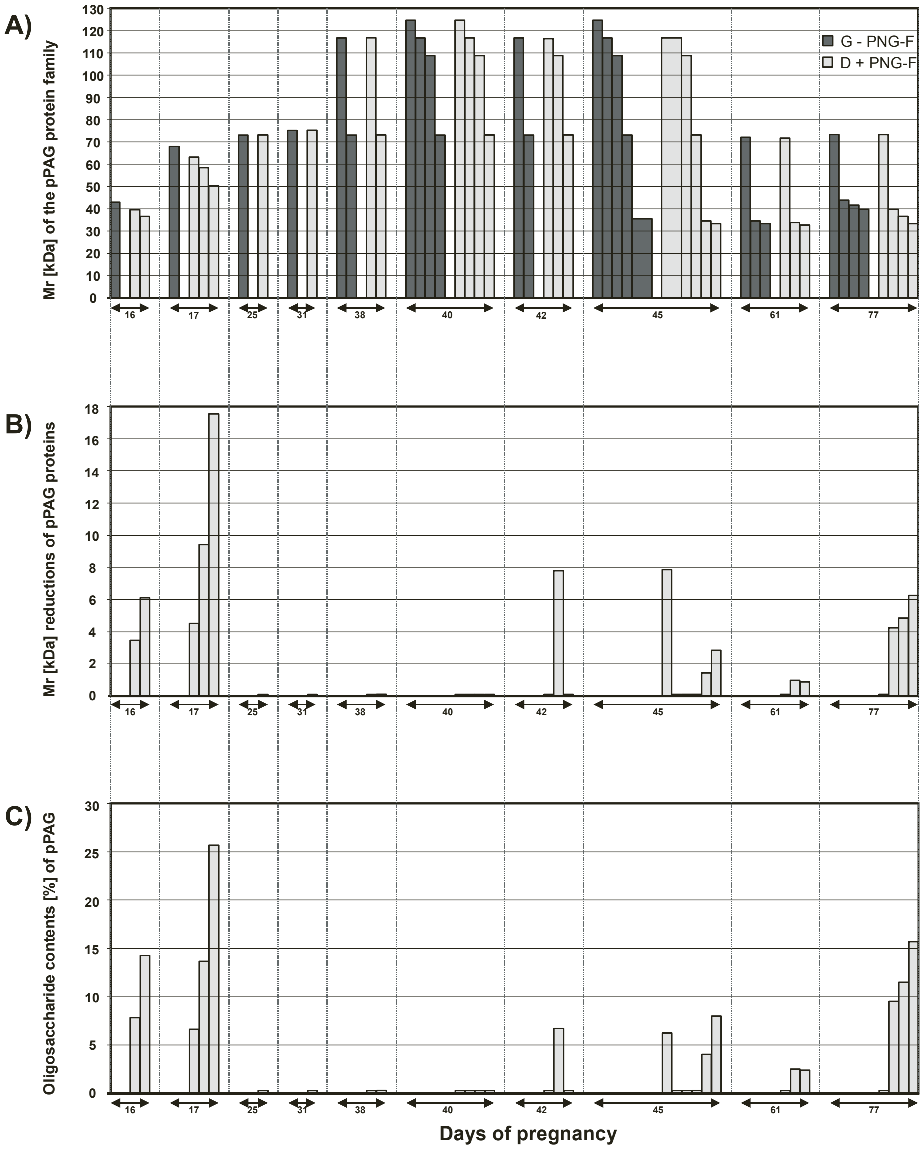


Fig. 5. Differences in molecular masses (Mr) of the pPAG protein family produced *in vitro* throughout pregnancy: A) N-glycosylated (G) or deglycosylated (D) proteins by PNG-ase F treatment (–/+); B) various reductions in Mr of the pPAG proteins by removal of N-linked oligosaccharides; C) oligosaccharide contents (%) in the pPAG protein family.

The present study reports the pregnancy stage-dependent N-glycodiversity of secretory pPAG protein family produced *in vitro* by various TR/TRD explants, individually harvested in cross-bred pregnant gilts. Such distinct profiles of the released pPAG protein glyco-heterogeneities have been demonstrated throughout pregnancy. In addition, we present a long-term culture model, as an improved source of pre-placental TR proteins, quantity of which is normally restricted by small amounts of the TR tissues available in the early pregnant pig. It is possible that different pPAG genotypes [49] of presently examined commercial cross-bred gilts may be responsible for demonstrated N-glycodiversity of released pPAG proteins and/or high early embryonic mortality in the pig.

Our present results concerning long term cultures of TR explants harvested on 16 dpc (implantation time) demonstrated that the major ~43 kDa G-form of released *in vitro* pPAG proteins were effectively deglycosylated generating two faster migrating D-forms (~39.6 and ~36.9 kDa) in Western blots. This N-glycodiversity of the porcine PAG protein family can be analyzed only in comparison to our previous data [45-51] or the results presented by one other laboratory [4, 13, 19, 20]. These authors found multiple small pBP (porcine basic proteins) released by TR explants i.e.: (i) 20-25 kDa (10–12 dpc); (ii) 35-50 kDa (14–17 dpc); (iii) ~45 kDa (18 dpc); and bigger forms released by advanced TRD placental explants (iv) 50-70 kDa (25–30 dpc). They demonstrated only the N-deglycosylation of the ~43 kDa pBP proteins (15 dpc) to D-forms 39.8 and 36.3 kDa that were detected by anti-pBP serum [4, 20]. This pBP proteins subjected to N-terminal micro-sequencing [13] appeared to be the internal fragment of mature pPAG2 proteins, identified previously as polypeptide precursor by ORF of its cDNA (13-17 dpc) cloning [45, 47]. However, we have found that this micro-sequence of one pBP protein is not identical to the pPAG2 precursor sequence due to the second asparagine of pBP that is replaced by valine in the pPAG2 precursor. This valine is also frequent in the entire pPAG2-like subfamily, which includes pPAG2, pPAG4, pPAG6 [45, 46] and pPAG8[1]. Thus, our comparison of similar micro-sequences of

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[1] Panasiewicz G., Szafranska B. (2003) Six novel identified complementary DNA (cDNA) sequences of the Pregnancy-Associated Glycoprotein genes transcribed within trophoblast cells during implantation in the pig (Sus scrofa). X National Cytogenetic Conference, 5-6 June, Poznań, p28-29.

the pBP/pPAG2-like sequences suggested the existence of several other as yet unidentified pre-placental TR proteins and/or their mRNAs. Our laboratory is currently investigating this possibility.

Our results can be compared to other studies regarding PSPB/PAG/SBU3 proteins examined mainly in domestic or wild ruminants. In the sheep, several native oPAG proteins (55, 60, 61, 65 kDa) have been harvested from media of cultured cotyledons (CT) on 100 dpc. Only one micro-sequence of isolated oPAG protein (65 kDa) was comparable to a 380 AA sequence of two oPAG3 and oPAG7 polypeptide precursors coded by their distinct ORF of cloned cDNAs [59, 60]. In this species, among multiple forms of 47-90 kDa produced *in vitro* by CT (15-25 and 100 dpc), only one prominent ~70 kDa ovine PAG protein (oPAG1) digested with PNGase F was reduced to 53-61 and ~47 kDa [58, 59]. Furthermore, incubations of ovine TRD explants (25 dpc) with tunicamycin (inhibitor of N-glycosylation) indicated that molecular mass of one major immuno-precipitated released product was reduced from 70 to about 50 kDa. In the same experiment, some other minor released protein forms were reduced only by 8-10 kDa [57]. Supplementary incubations of ovine CT explants (15, 25 dpc) in the absence of glucose resulted in secretion of 3 distinct forms (49-70 kDa) of the oPAG proteins [58]. In the sheep various multi-antennary oligosaccharide side chains increase the masses of the SBU3 proteins (PAG proteins identified on 2-3 month of pregnancy) by almost 18% [1]. In the cattle, a dominant 67 kDa form of the bPAG proteins isolated from CT tissues contained approximately 10% of oligosaccharides [61]. However, the PSPB/PAG proteins isolated from late placental tissues of game animals, the moose and the elk, contained only 3.15 and 4.98% of oligosaccharides, respectively [29].

Morphological changes of developing placentas are connected with structural and compositional changes of glycans that are differentially distributed either on the surface of chorionic microvillus or uterine epithelium [32, 33]. Specific glycan-glycan interactions influence early development of conceptuses and inhibit maternal immune response. Specific patterns of a variety of glucosaminoglycans on endometrial surface of luminal uterus are required for successful implantation in the pig [3]. Any disturbance of aforementioned interaction leads to high porcine embryonic mortality (30-

40%) during early pregnancy [37]. These glycans play favourable/unfavourable roles in attachment and adhesion of TR to uterine epithelium at the beginning of implantation in interbreeding and non-interbreeding species. This is strongly influenced by precise regulation of steroid and glycoprotein secretions in each species. Only compatible patterns of surface glycan expressions permit cross-breeding of horse with donkey. In contrast, distinct expression patterns indicated in camel, horse and donkey placentas prevent their cross-breeding [33]. Thus, non-specific profiles of protein glycosylation do not allow for inter-species implantation, and/or defend against birth of infertile/sterile chimeras.

In different mammals including primates, many pituitary/placental heterodimeric glycoproteins composed of non-covalently associated α and β subunits possess unique sites of glycosylations that can be involved in their specific functions during pregnancy. Particular oligosaccharide(s) linked to various asparagines of free α-subunit may prevent its combining with available free β-subunits of hCG coexisting in the same fetal-placental unit during early human pregnancy [6, 7]. The carbohydrate composition studies indicated that hCG molecules contain mono- and bi-antennary oligosaccharides, while fucosylated multi-antennary oligosaccharide structures were detected in free α-subunits [6]. However, carbohydrate modifications generated during the biosynthesis of both hCG subunits, examined in extraembryonic coelomic fluid during the first trimester of pregnancy, suggested that free α-subunits can exist in the presence of β-subunit [7]. In the horse, partially deglycosylated eCG ($\alpha N^{56}/\beta$) stimulated estradiol production, confirming that its stimulatory effect is resistant to the removal of these N-linked oligosaccharides [42]. In contrast, partially deglycosylated eFSH ($\alpha N^{56}/\beta$) did not show such stimulation, indicating that the oligosaccharides at N^{56} are require for eFSH activity [42]. However, O-glycosylation of C-terminus appears to be responsible for the β-subunit contribution to the substantial difference in LH receptor-binding activity observed between eLH or eCG [8]. In the sheep, two isoforms of free α subunits of oLH, isolated from ovine pituitaries (14 kDa-αA and 13.5 kDa-αB), contained only N-acetyl galactosamine residues, but none of them contained O-linked oligosaccharides [10]. However, both isoforms were able to recombine with the oLHβ-subunit and exhibited distinct efficiencies in stimulating prolactin release from ovine fetal pituitaries [10]. In this species, multi antennary N-linked carbohydrate chains (bi-, tri- and tetra-antennary oligosaccharides) of the SBU3 proteins are unique for placental tissues only [1]. It is possible that in the pig, the pPAG proteins secreted during implantation can possess very similar and unique multi-antennary structures (chains) of N-linked oligosaccharides, but such examinations require further carbohydrate composition studies.

The pregnancy stage-dependent glycodiversity of the pPAG proteins could also be induced by changes in specific activity of the pPAG gene family throughout pregnancy in the pig. The diversity of deglycosylated forms of the PAG proteins can be compared to transcriptional activity of the PAG gene family previously described in various species [16, 17, 22-24, 45-47, 51, 60]. In these studies, identifications of distinct N-terminus of various native PAG proteins revealed the post-translational processing (including proteolytical degradation) of polypeptide PAG precursors (coded by ORF of their cDNAs and translated *in silico*). These PAG precursors possess various numbers (3-7) of N-glycosylated asparagines (N-x-T or S) with different localization of their sites in sequences of polypeptide PAG precursors. Presently obtained deglycosylations of the pPAG proteins depicted as three faster migrating smaller forms (17 dpc, fig. 4) directly correspond to three potential N-x-T/S sites ($N^{56, 79, 119}$) of polypeptide pPAG2, pPAG4 and pPAG6 precursors. In contrast, distinct four potential N-x-T/S sites in the sequence of pPAG1, pPAG3 and pPAG5 have been identified [19, 22, 23]. Thus, our results provide convincing data of glyco-variability of released pPAG proteins due to their diverse N-glycosylation, confirmed by cDNAs cloning and their translation *in silico*. Another N-x-T/S site (N^{348}) has been found in the pPAG1, pPAG3 and pPAG5 also. Similarly localized glycosylation site found in equine PAG (N^{356}) was not identified in the sequence of zebra PAG (NNL^{356}) precursor. Single N-x-T/S sites have also been found in other APs: cathepsins D, E and pepsynogen precursors (34–52% of AA homology to sequences of various pPAG precursors).

In ruminants, pregnancy stage-dependent expression/diversity of distinct multiple PAG genes and heterogeneity of identified PAG proteins were indicated in various placentas during gestation. For example, Northern blotting revealed caprine PAG2 (cPAG2) mRNA expression only on 18 dpc, while cPAG3, 4, 7, 9-11 mRNAs were expressed throughout all examined days of pregnancy [17]. However, some transcribed bovine PAG genes (bPAG1, -6 and -7) start strong placental expression on 45 dpc, while other bPAGs (bPAG2, -4, -5 and 8-11) are already expressed during implantation time [24]. Similarly to other AP enzymes, most of well-known polypeptide PAG precursors (lately identified in pigs, ruminants, horses, cats and mice) are post-translationally proteolytically activated. The PAG precursors are at first reduced by 15 AA signal peptides and then by 33-38 AA peptide cleavage [11, 15, 17, 22-24, 45-47, 56, 60]. Thus, proteolytical degradation of polypeptide PAG precursors and presently observed post-translational gestation stage-dependent N-glycodiversity of released *in vitro* PAG protein family may play principal role(s) during implantation and pregnancy maintenance in various ungulate species, in which CG have not been identified. Degrees of glycosylation, glyco-diversities, unique oligosaccharide content and post-translational processing of the PAG family are probably fundamental for proper functioning of various placentas of domestic animals and appropriate embryo-maternal steroid/gonadotrophin exchange during pregnancy.

Aforementioned diversity of mRNAs of transcribed placental PAG genes results in high multiplicity of identified polypeptide PAG precursors. Moreover, numerous PAG proteins are secreted by chorion of different eutherian species. In ruminants, the PAG proteins, passing placental barriers in binuclear cells, are detectable (from 2-3 weeks of post-breeding) by radioimmunologic assays (RIA) in maternal peripheral blood circulation. In several domestic and wild ruminants [14, 26, 27, 30, 31, 36, 43, 44, 53, 55, 62], these PAG RIAs are useful for profitable and very accurate pregnancy diagnoses [21]. Similar RIA tests of PAG proteins were also beneficially used for recognition of early or late embryonic mortality, sex of fetus and single or twin pregnancies [35, 38, 54].

In summary, we described the immuno-monitoring of *in vitro* produced the pPAG proteins and their pregnancy-stage dependent N-glycodiversity. Molecular masses of N-glycosylated pPAG proteins were reduced (by ~4.68 kDa) by deglycosylation with PNGase F treatment. Our data indicate that carbohydrate content of the pPAG proteins may reach 9.66%. The results concerning the native pPAG proteins deglycosylation corresponded to potential N-glycosylation sites (N-x-S/T) that have been found in sequences of polypeptide pPAG2-like precursor subfamily, encoded by their cloned cDNAs and translated *in silico*. Diverse N-deglycosylation pattern of the pPAG protein family may indicate vital role(s) of the pPAG proteins during conceptus attachment, successful implantation and late pregnancy in the pig. However, aforementioned unique carbohydrate content should be identified in additional studies. Hopefully, future research on the PAG protein family will reveal some function(s) of these chorionic molecules. Our present study provides a background for future investigations clarifying the functional significance of differential glycosylations in the pig, and will be helpful for extended studies of the PAG family glycosylation as pregnancy advances in other eutherian species.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. Adam Ziecik (Polish Academy of Sciences in Olsztyn) and Dr. Anita Franczak (University of Warmia and Mazury) for providing part of porcine chorions. The study were supported by State Committee for Scientific Research (PBZ-KBN 084/P06/02/3.4), Poland.

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