

Biodiversity of multiple Pregnancy-Associated Glycoprotein (PAG) family: gene cloning and chorionic protein purification in domestic and wild eutherians (Placentalia) – a review

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Abstract – This review presents a broad overview of chorionic glycoproteins encoded by the Pregnancy-Associated Glycoprotein (PAG) gene family and also serves to illustrate how the recent discovery of the PAG family has contributed to our general knowledge of genome evolution, placental transcription and placental protein expression. The complex and large PAG family is restricted to the Artiodactyla order, although single PAG-like genes have also been identified in species outside the Artiodactyla. The PAGs are members of the aspartic proteinase (AP) superfamily. Unexpectedly, however, some members of the PAG family possess amino acid substitutions within and around the active site that likely render them unable to act as proteinases. This paper summarises the available information regarding biodiversity of PAG gene expression based on cDNA cloning, mRNA localisation studies and the structural organisation of the PAG genes with a particular emphasis on PAG promoters. It also compares available data regarding PAG protein purifications, sequencing and their N-glycodiversity. Finally, it discusses the scientific relevance, possible functional roles of the PAGs and describes possible profitable applications related to the detection of PAG proteins in the blood of pregnant domestic and wild species.

chorionic glycoproteins / eutherian PAG family / diagnostic PAG markers

Abbreviations: AP: Aspartic Proteinases; cDNA: complementary DNA; cRNA: complementary RNA; CG: mammalian Chorionic Gonadotrophin; eCG: equine CG; hCG: human CG; LH: Luteinising Hormone; PAG: Pregnancy-Associated Glycoprotein family; bPAG: bovine PAG (*Bos taurus*); bubPAG: buffalo PAG (*Buffalo bubalis*); cPAG: caprine PAG; CdPAG: dromader PAG (*Camelus dromedarius*); CfPAG: two-humped camel PAG (*Camelus ferrus*); ePAG: equine PAG; EbPAG: European bison PAG (*Bison bonasus L.*); fPAG: feline PAG (*Felis catus*); LpPAG: alpaca PAG (*Lama pacos*); oPAG: ovine PAG (*Ovis aries*); pPAG: porcine PAG (*Sus scrofa domestica*); wtdPAG: white tail deer PAG (*Odocoileus virginianus*); zPAG: zebra PAG (*Equus zebra-mountain zebra*); zebuPAG (*Bos indicus*); pBP: porcine Basic Protein; PGF_{2 α} : prostaglandin F_{2 α} ; PSP60: 60 kDa Pregnancy Serum Protein; Prl: prolactin; PSPB: Pregnancy Specific Protein B family; bPSPB: bovine PSPB; ePSPB: elk PSPB; mPSPB: moose PSPB; SBU3: oPAG proteins with specific oligosaccharide epitopes; RPA: Ribonuclease Protection Assay; tsp: transcription start point.

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1. IDENTIFICATION HISTORY AND EVOLUTIONARY CONSERVATION OF THE PAG FAMILY

The PAGs, comprise a large group of placental AP superfamily that are expressed in pre-placental trophoblast (TR) and after implantation in the trophoctoderm (TRD) – the chorionic epithelium of eutherian mammals (Placentalia) with different placenta types. The first member of the family was described by Butler et al. [1] and was given initially the name pregnancy specific protein B (PSPB) in bovine placenta. Subsequent work by other research groups identified related proteins, which were given names such as PAGs [2,3], 60 kDa – pregnancy serum protein (PSP60) [4], or SBU3 [5] in ruminant ungulates (see Sect. 2.4, 2.5 and 2.7). These PAG/PSPB/PSP60/SBU3 proteins are detectable in maternal blood and were reported to be useful as prenatal markers for pregnancy diagnoses in various domestic and wild ruminants (see Sect. 2.8).

The first PAGs to be cloned were from cattle (bPAG1) and sheep (oPAG1) [2]. Later, related genes were described in pigs; these were named porcine (p) PAG1 and pPAG2 [6] and they represent two pPAG1- and 2-like subfamilies that have since been shown to include additional PAG members, including pPAG3-6, pPAG8 and pPAG10 [7, 8]. Ruminants and pigs are even-toed ungulates (Artiodactyla order), but they have strikingly different placenta types (synepitheliochorial cotyledonary and epitheliochorial diffuse, respectively). Not surprisingly, a range of PAG forms, unique to each placenta type, have been identified in artiodactyls, however, single PAG-like genes have also been reported in non-artiodactyls with another placenta type (Tab. I). Moreover, Southern analysis of genomic DNA (gDNA) provides the means to study the presence of

PAGs in the genomes of various wild ruminants: gaur, moose, mule deer, gnu, yak, buffalo, impala, goral, etc. [9]. Unfortunately, the cDNA of PAGs have yet to be cloned from most of these species.

Phylogenetic analyses of PAG and PAG-like genes indicated that the PAG family originates from an ancient PAG-like precursor (pro-gene) by duplication and positive selection approximately 86 million years ago [18–20], which is consistent with estimates for the split of the artiodactyls from other ungulate orders. The conservation and homology of the PAG in many eutherians (Artiodactyla, Perissodactyla, Carnivora and Rodentia) is suggestive of their importance in the reproductive physiology of ungulate species.

2. BIODIVERSITY OF IDENTIFIED MULTIPLE PAG GENES, TRANSCRIPTS, PROTEINS AND THEIR CLASSIFICATION

The PAGs belong to the AP superfamily (EC 3.4.23), which includes proteolytic enzymes present in vertebrates and non-vertebrates. Examples include the following: pepsin A, C, F (EC 3.4.23.1, EC 3.4.23.3, EC 3.4.23.-), aspergillopepsin (EC 3.4.23.18), candidopepsin (EC 3.4.23.24), chymosin-preprorenin (EC 3.4.23.4), renin (EC 3.4.23.15), cathepsin D (EC 3.4.23.5) and cathepsin E (EC 3.4.23.34). The AP superfamily also includes parasite plasmepsins (EC 3.4.23.38 and EC 3.4.23.39) as well as several other fungal and retroviral enzymes.

2.1. Cloned cDNAs and their *in silico* translation to full-length polypeptide PAG precursors

Multiple PAG genes have been cloned and identified in Artiodactyla: domestic, wild ruminants and pigs (Tab. I). All

Table I. Cloned cDNA of the PAG gene family, chronologically deposited in the international GenBank database.

Name	Accession numbers to deposited cDNA	Coded polypeptide precursors (aa)	Transcripts (day of pregnancy)	Species	Identified by
oPAG1	M73961	382	100	Sheep	
bPAG1*	M73962	380	260	Cattle	[2]
bPAG2	06151	376	260	Cattle	[10]
pPAG1	L34360	389	13-17	Pigs	[6]
pPAG2*	L34361	387	13-17		
oPAG2	U30251	376	13		
oPAG3*	U94789	380	100		
oPAG4	U94790	380	100		
oPAG5	U94791	379	100	Sheep	[9, 11]
oPAG6	U94792	380	100		
oPAG7*	U94793	380	100		
oPAG8	U94794	341	100		
oPAG9	U94795	346	100		
bPAGv-3	L06153	381	260		
bPAG4*	AF020506	380	25		
bPAG5	AF020507	377	25		
bPAG6*	AF020508	379	25		
bPAG7*	AF020509	341	25	Cattle	[9]
bPAG8	AF020510	387	25, 19		
bPAG9	AF020511	379	25, 19		
bPAG10	AF020512	380	25		
bPAG11	AF020513	376	25, 19		
bPAG12	AF020514	375	19		
fPAG	AF036953	388	30	Cat	[12]
ePAG	L38511	388	25	Horse	
zPAG	AF036952	388	term placenta	Zebra	[13]
bPAG13-16*, 17*-21	AF192330-8	370-381	19	Cattle	[14]
cPAG1-11	AF191326-336	341-386	45, 65, 87, 115	Goat	[15]
mPAG-like	AF240776	387	16	Mouse	[16]
wtdPAG1	AY509865	380			
wtdPAG2	AY509866	376			
wtdPAG2v	AY509867	376			
wtdPAG3	AY509868	381	85-90	White-tailed deer	[17]
wtdPAG4	AY509869	385			
wtdPAG5	AY509870	385			
wtdPAG6	AY509871	376			
wtdPAG7	AY509872	381			
wtdPAG8	AY509873	376			
wtdPAG9	AY509874	258			
pPAG3 (103C)	AF315377	389			
pPAG4*(A11A)	AF272734	389			
pPAG5 (101C)	AY188554	342			
pPAG6*(113B)	AF272735	389	13-17	Pigs	[8]
pPAG8* (109C)	AY373029	342			
pPAG10*(108B)	AY775784	389			

Explanation: Sequences of PAG cDNA can be accessed in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) under the indicated numbers (Acc. Nos.). Genes that are marked (*) code polypeptide chains of precursors with high homology to N-terminal micro-sequences of native PAG proteins, presented in Tables III and IV.

these identified cDNA sequences represent distinct transcripts encoding unique polypeptide PAG precursors. Some eutherian species outside the Artiodactyla, comprising the Perissodactyla, Carnivora and Rodentia orders, have been shown to possess only a single PAG-like member. These include equine PAG in the horse and zebra, feline and mouse PAG-like cDNA (Tab. I). Cloning of the PAG and PAG-like gene family (bPAG, cPAG, ePAG, fPAG, mPAG, oPAG, pPAG, wtdPAG and zPAG) revealed distinct chorionic transcripts. The number of the PAG mRNA is known to vary between species and their expression can sometimes change during the course of pregnancy (Tab. I). Presumably, distinct number and pregnancy-stage dependent expression of the PAG transcripts can be associated with specific requirements of foetal development in different species.

The identification of an open reading frame (ORF) for each cloned cDNA allowed for *in silico* translation of the full-length polypeptide PAG precursors (Tab. I). *In silico* translations also permitted a comparison of the homology/identity between family members and the detection of numerous amino acid (aa) substitutions. The pre-forms of the PAG polypeptide chains are generally 375-389 aa in length, however, shorter 258-342 aa forms were also identified in various species. The PAG family has several conserved regions and shares ~ 50% sequence identity with the pepsinogen family. One of the regions of greatest conservation among the PAGs [21] and other members of the AP superfamily [22, 23] is within those areas comprising the active site centre of these molecules. Catalytic activity of the AP depends on two aspartic acids located within the different active domains belonging to the amino- and carboxy-terminal lobes that, together, create the substrate-binding cleft. Some of the PAG family members possess multiple aa substitutions within the catalytic site and, therefore,

are predicted to be unable to function as classical proteinases [2, 6-9, 14, 15, 17]. In the pig, for example, the typical pepsin-like sequence within the catalytic domains is conserved in the pPAG2-like subfamily only (pPAG2, pPAG4, pPAG6, pPAG8 and pPAG10) [6, 8]. No classical erosion of the maternal tissues occurs during porcine placentation; therefore, *in silico* predicted proteolytic activity of the pPAG-2 group [7, 8] is likely directed toward specific substrates/targets at the placenta-uterine interface rather than a general activity that might normally have permitted erosion of maternal tissues and placental invasion. In contrast, the pPAG1-like subfamily (pPAG1, pPAG3 and pPAG5) was predicted to be potentially inactive, according to four aa substitutions: Gly → Ala⁸¹ in domain 1, and Thr → Ser²⁶³, Thr → Ser²⁶⁵, Ser → Ala²⁶⁶ in domain 2 [8]. Similar aa substitutions have been reported in other eutherians, e.g., Gly → Ala⁸¹ in domain 1 of bPAG1, or Asp → Gly²⁵⁷ in domain 2 of oPAG1 [2], and in several other ruminant PAG members [9, 14, 15, 17]. Although such mutations are predicted to render the PAGs incapable of proteolytic activity, the recent identification of enzyme activity in the histo-aspartic proteinase genes (HAP I and II) and several plasmepsins from the malarial parasite (*Plasmodium falciparum*) does suggest that proteolytic activity may still reside in PAGs with atypical active sites [24, 25].

2.2. Exon-intron structure of the PAG genes, promoters and gene number analyses

The identification of the structural organisation of the PAG genes or their promoters was performed on the basis of identified gDNA sequences (Tab. II) and required single gene isolation by genomic bovine or porcine library screenings (~ 1.2 kbp promoter and ~ 8 kbp gene).

Table IIA. Recognised structural organisation of two genes from the PAG family, with their DNA sequences that have been deposited in the GenBank database.

Gene (clone)	Accession number	gDNA sequences	Identified by
bPAG1 (bpg2000)	L27833	exons 1-6	[26]
	L22834	exons 7-8	
	L27832	exon 9	
	U41421	exon 1	
	U41422	exon 2	
pPAG2 (g5)	U39199	exon 3, intron C, exon 4	[27]
	U41423	exon 5	
	U41424	exon 6	
	U39762	exon 7, intron G, exon 8	
	U39763	exon 9	

Table IIB. Identified promoter sequences of the PAG gene family deposited in the GenBank database.

Gene	Accession number	Length of identified promoter regions (bp)	Identified by
bPAG1	L27833	1260	[26]
bPAG2	AY212886	955	[28]
ePAG	AF061188	389	[13]
pPAG2	U39198	1204	[27]

The entire exon-intron structures were identified for two PAG genes only, bovine PAG1 [26] and porcine PAG2 [27]. The gDNA sequencing of the PAG genes and comparison to the ORF of previously cloned cDNAs allowed for structural identification of 9 exons and 8 introns (A-H) in the bovine and porcine PAG genes. For example, in the pPAG2 gene, the exons are 99-200 bp long and introns contain from 85 bp up to ~ 1.8 kbp. The bPAG1 gene has a similar structure. However, intron H is ~ 1.8 kbp in the bPAG1 gene, but is much shorter (292 bp) in the pPAG2 gene. Exon 1 codes for signal peptides and propieces, exons 2-5 code for the N-terminal lobe and exons 6-9 code for the C-terminal lobe of the mature PAG polypeptide.

An examination of the functional activity of PAG genes begins with the cloning and sequencing of the promoter [13,26-28]. So far, only a few promoter sequences of the PAG genes

were identified and deposited in GenBank (Tab. IIB). The comparison of the PAG promoter sequences revealed that their proximal regions are similar to each other, but not to promoters of other AP or other trophoblast-expressed genes [27]. This study demonstrated ~ 65.8% identity between pPAG2 and bPAG1 promoters, and ~ 74% identity to 350 bp fragment of the ePAG promoter. In addition, unique tandem repeats TCTTATCAGG in the pPAG2 promoter were found. Shorter conserved repeats (7 bp) were also found in other PAG promoters. Presently, the significance of these unique repeats remains unknown. Short tandem repeats were also found in the proximal promoter region of the alpha subunit of human chorionic gonadotrophin [29]. Thus, it can be expected that short tandem repeats, identified in sequences of trophoblastic gene promoters, may play a crucial role in placental-specific transcription of the PAG genes.

An activation of the PAG genes is probably specifically regulated by many transcription factors. Indeed, consensus binding sites for GATA, STAT and Ets transcription factors were identified in the PAG precursors [26,27]. Five fragments (-1162, -974, -691, -357 and -149 bp upstream of tsp - transcription start point) of the pPAG2 promoter (containing these transcription factor sequences) were cloned and examined in various transfected stable cell lines. The highest activity was found for the shortest proximal fragment (-149 bp) of the pPAG2 promoter, when JAr or JEG3 (Human choriocarcinoma) and Rcho (Rat choriocarcinoma) were transfected. Such activity did not occur in non-trophoblastic cell lines, Cos-1 (monkey kidney), L-929 (mouse adipose tissue fibroblast), but lower activity was found in CHO-K1 (Chinese hamster fibroblasts). Presumably, Ets factors can play an important role in the activation of the PAG genes. The involvement of Ets factors in the control of IFN- τ has been shown in ruminants [30,31] or in specific control of human gene expression [32]. The activation of the PAG genes may also be controlled by other mechanisms or transcription factors that are indicated for cathepsin D promoter [33] or other placental promoters of hCG [34], Prl [35], Prl-like placental proteins [36,37] and lactogens [38]. Overall, the PAG genes have structural exon-intron organisation very similar to the pepsinogen family; however, the promoter sequences are very distinct. The promoters of the PAG genes and the involvement of various transcription factors in the mechanism of the PAG gene activation within chorionic cells require future investigation.

Evidence for a multiplicity of PAG genes has come from the screening results of various cDNA libraries (Tab. I). Other studies performed with restriction endonuclease-digested cellular gDNA by Southern hybridisation also demonstrated that numerous PAG genes are likely to

exist in artiodactyls [9, 26, 27, 39]. Indeed, as many as 100 PAG genes are predicted to exist in the genomes of ruminant ungulates. In the porcine genome, at least 8 pPAG2-like genes were determined. However, the number of the pPAG1-like genes is presently unknown, as well as in other eutherian species.

Recent studies of gDNA examined by PCR and autoradiographic Southern hybridisation demonstrated polymorphism of the PAG gene family, i.e. different number and length of the PAG amplicons in various species, including the pig, wild boar, cattle, sheep, goat, elk, beaver [40–43], Carnivora species [44] and European bison (Eb) – classified as an endangered species: EN A2ce, C2a, IUCN [45, 46]. These produced gDNA amplicons suggest that some PAG genes in these species can have shortened introns E, G and H, which are between exons 5-6, 7-8 and 8-9, respectively. A profile of produced gDNA amplicons indicated the PAG genotypes of various domestic and wild animals. Moreover, macroscopic examination of reproductive organs of eliminated Polish Eb showed that almost 80% of bulls possess a vestigial uterus [47]. Interestingly, examination of gDNA amplicons in the Eb suggests that those Polish males with these regressive uteri present distinct PAG genotypes than the Swedish male population [46]. It is possible that inbreeding between related animals influenced the genotypes of these Polish males. Thus, presumably, the PAG gene family can be used as a novel genetic PAG marker that can be advantageous for early pre-selection of young animals what can contribute for economically effective reproduction and better conservation of endangered eutherians.

2.3. Pregnancy-stage dependent and cell-specific mRNA expression of the PAG family

Spatial profiles and cell-specific localisation of PAG mRNA expression in

chorionic cells (TR, TRD) during different embryo/foetal development has been determined with numerous PAG cDNAs as probes (see Tab. I). Northern analyses revealed that PAG transcript expression starts in the TR at peri-implantation and persists in TRD until term. In ruminants, the mRNAs of the bovine and ovine PAG genes are detectable from 17 days post coitum (dpc) [2, 9, 10, 14] and caprine PAG mRNAs from 18 dpc [15]. In the pig, the pPAG mRNAs is detectable by Northern hybridisation from 15 dpc [6], but in the mare from 32 dpc [13]. However, more sensitive ribonuclease protection assays (RPA) of placental mRNA performed with antisense ovine riboprobes (cRNA) detected ovine PAG mRNA as early as 13 dpc [14]. RPA performed with porcine PAG cRNA revealed multiple pPAG2-like transcripts during early pregnancy [7]. In addition, hybridisations of pregnant endometrial samples with the PAG probes have been found to be beneficial for demonstrating TRD contamination of endometrial samples [6].

During early placenta development of several species, TR cells proliferate and differentiate into specialised giant cells. In ruminants, such cells are known as binuclear cells (BNC) that comprise approximately 20% of the TRD cell population [48–51]. Upon formation of a mature placenta, some BNC migrate from the TRD to fuse with maternal uterine epithelial cells, creating either a foetal-maternal syncytium or short-lived trinucleate cells, depending on the species [50].

Several PAG mRNAs are differentially transcribed in ruminant mononuclear (MNC) and/or BNC. Numerous PAGs are detectable at different stages of pregnancy [2, 9, 10, 14, 51]. Bovine PAG1 is expressed from 45 dpc till 250 dpc, but is not expressed as early as 25 dpc [14]. In bovine BNC, bPAG1, 3, 4, 5, 6, 7 and 9 are expressed, while bPAG2, 8, 10–13 are expressed in both MNC and BNC [14]. In sheep, oPAG1 and oPAG3–9

are transcribed in BNC, but oPAG2 is expressed throughout TRD [9, 14]. In goats, cPAG2 has been detected only during early pregnancy (18–19 dpc) throughout TRD, cPAG8 is expressed during the entire pregnancy, but 9 distinct transcripts (cPAG1, cPAG3–7 and cPAG9–11) start expression from 45 dpc [15]. The PAG mRNA family expression was also identified in European bison and camelids by hybridisation with the use of porcine PAG probes [45, 52].

In the equine placenta, the ePAG mRNA was found in the allantochorion and avascular chorion [13]. In the equine girdle cell layer, multinucleate chorionic cells (30–32 dpc) strongly proliferate [53, 54] into invasive chorionic girdle cells that are involved in the formation of maternal endometrial cups. Chorionic girdle cells produce a placental gonadotrophin (eCG), previously known as PMSG (Pregnant Mare Serum Gonadotrophin), which is detectable in maternal blood circulation after 40 dpc [55].

In the pig, surprisingly, reverse transcription-PCR indicated that the pPAG1-like and pPAG2-like transcripts are present as early as the morula stage and 4-cell stage, respectively [56]. However, the porcine genome is activated during the 4-cell stage [57], then, on day 6–7 dpc blastocysts hatch from the zona pelucida and embryonic TR cells start intensive proliferation and morphological differentiation [58, 59]. After implantation, an early placenta is developed by intensive growth of TRD and an expansion within maternal endometrium [49, 60, 61]. Interesting questions arise about the expression of the PAG2-like family (with conserved pepsin-like sequences) within the foeto-maternal interface of the porcine placenta [6–8], which was anatomically classified as the epitheliochorial diffuse non-invasive type [49, 61]. Moreover, porcine TR cells removed from the uterus and transplanted into an ectopic site indicated invasive properties, creating structures similar to the

cytotrophoblast and syncytium containing an increased number of lysosomes [62]. Our *in situ* hybridisation (ISH) studies of the PAG mRNA expression demonstrated remodelled regions of placental surface, where endometrial cells occurring within villous folds exhibit changed shape, which is similar to conditions of digested cells [7]. In these regions, much stronger mRNA expression was determined for the pPAG2-like than for the pPAG1-like subfamily. This ISH study also revealed direct contact of TRD cells with maternal endometrial cells and several porcine binuclear cells. In addition, cultures of TR cells demonstrated their differentiation to multinucleated forms (from 3 to 9 nucleuses) that were observed *in vitro* only [7]. It seems that some porcine TR/TRD cells cultured *in vitro* (removed from inhibitory influence of maternal uterine secretion) can be more invasive as suggested previously.

2.4. Identification of the PAG proteins

Generally, initial cDNA cloning permits to identify full-length polypeptide precursors that can allow for sequence comparison to short micro-sequences of purified proteins, thus giving final native PAG protein identification.

Immunological identification of the PAG proteins requires various anti-PAG sera that will recognise specific antigenic determinants (epitopes) of these molecules. However, the production of polyclonal and monoclonal antisera requires species-specific antigens, thus harvesting various placental PAG proteins. Initially, anti-PAG sera were produced against semi-purified chorionic antigens, then the obtained semi-specific antisera were purified by adsorption with various bovine tissues harvested from non-pregnant animals [1]. Later, a few potential protein sources were utilised for produc-

tion of placental antigens (Tab. III). As a first source, native antigens were isolated by multistep extraction from tissues, mainly ruminant placentas. As a second source, ultra-fractionated proteins from media were collected during long- or short-term *in vitro* cultures of chorionic tissues [63, 80–82]. Removal of non-pregnancy specific immunoglobulins from anti-PAG sera was performed by use of *in vitro* produced adsorbents of non-pregnant animals [63, 81, 82]. As a third source, recombinant PAG proteins were produced on the basis of previously cloned cDNAs of the PAG genes [8, 13, 63]. However, recombinant PAG proteins, produced so far in bacterial systems, were useful antigens for immunological detection in some species, in which the native PAG proteins are not highly glycosylated during post-translational modifications [13]. Several polyclonal potentially monovalent antisera were produced against singular PAG antigens, electrophoretically homogenic (identical kDa). Polyclonal polyvalent antisera were produced against multiple antigens that allowed for diagnostic immunological detection of different PAG proteins during the same blotting [63]. Finally, a specificity of some anti-PAG sera was directly confirmed by beneficial immunoscreening of cDNA clones isolated from trophoblastic libraries and bounding of fusion PAG proteins [2, 63].

These anti-PAG sera allowed for species-specific (homologous) dot- or PAGE Western analyses and immunohistochemistry of the PAG proteins on the placental sections. In addition, relatively high homology of the PAG epitopes allowed also for heterologous (cross-species) immunological detection, very useful especially in domestic, wild and endangered animals, in which the PAG family has not been identified previously. For example, anti-porcine PAG sera were useful for identification of EbPAG proteins in placental sections

Table III. The native PAG proteins, isolated from placental tissues of different species, in comparison to the family of PAG precursors (* see Tab. I). The theoretical molecular masses of pPAGs (***) calculated on the basis of polypeptides coded by their cDNA were compared to masses (M_r) of processed glycosylated products of the pPAG family (****). Some forms of ovine and bovine PAG protein (****) that N-terminus corresponded to ovine and bovine polypeptide PAG precursors translated from their cDNA (see Tab. I).

Name	M_r , kDa	Ip variants pH	Source	Day or month of pregnancy	Identified by
Porcine PAGs					
pPAG-like	35.4-72.3****		Medium	15-77 d	[7, 63, 64]
pPAG1	42.79**				[6]
pPAG2	42.57**				
pPAG3	43.48**				
pPAG4	43.58**		mRNA	13-17 d	
pPAG5	37.99**				
pPAG6	43.59**				[8]
pPAG8	38.17**				
pPAG10	43.59**				
pBP	20-25	5.6-6.2	Medium	10.5-12 d	
	35-50	7		14-16 d	
	45			18 d	[65, 66]
	50-70	6-7.6		25-30 d	
pBP	42.8-43.1		Medium	14-17 d	[67]
pBP	43	9.08	Medium	15 d	[68]
Equine PAGs					
ePAG	37, 41	Activity at	Medium	25 d	[13]
R-ePAG	41	pH 4.5		recombinant	
Ruminant PAGs					
bPSPB	47-53	4.0-4.4	Tissues	20-45 d	[1]
bPSPB	37, 48, 65, 85, 90	4.0-4.4	Medium	90 d	[69]
	78		Tissues		
oPSPB	43, 59, 66	4.06-4.65	Tissues	mid- and late	[70]
oPAG1	47-90		Medium	15-25, 100 d	
R-oPAG1	46			recombinant	[2, 71]
bPAG1			Medium	90 d	
bPAG	67	4.4-5.4	Tissues	2-6 m	[3]
bPAG2	31-70		Medium	90 d	[10]
oSBU-3	69, 62, 57		Tissues	~ 2-3 m	[5]
oPAG3/7	42.49 / 42.93		mRNA	100 d	[9]
oPAG	65****	5.54, 5.19,			
oPAG	61	4.06, 4.0,			
oPAG	60	3.56	Medium	100 d	[11]
oPAG	55	4.0-5.68			
cPAG	62, 59, 55	4.9-6.2	Tissues	48-69 d	[72]
mPSPB	58, 31	4.8-6.7	Tissues	?	[73]
ePSPB	57, 45, 31	4.8-6.2			
bubPAG	52-77		Tissues		[74]
zebuPAG	51-69	4.4-6.7	Tissues	10-31 weeks	[75]
oPAG	59, 57, 55	4.1-5.9	Tissues	≥ 100 d	[76, 77]
oPAG	55-66	4.0-6.8	Tissues	60-100 d	[78]
wtPAG	30, 50, 55, 60, 90		Tissues	85-90 d	[17]
EbPAG	65, 67, 78		Medium	60-150 d	[45]
bPAG6/7,1,17	75, 66, 56****		Tissues	100-180 d	[79]
bPAG4,6,7,16	75, 65, 55****		Medium	24-150 d	[80]

of endangered European bison PAG (*Bison bonasus* L.) and camelids, including CdPAG–dromader PAG (*Camelus dromedarius*), CfPAG–two-humped camel (*Camelus ferrus*) and LpPAG–alpaca PAG (*Lama pacos*) [83, 84].

2.5. Purification and N-terminal microsequencing of native PAG proteins

The PAG family has been identified as a huge group of placental proteins specifically produced and secreted by the chorion of several eutherian mammals. The PAG proteins (Tab. III) were harvested on different days of pregnancy from the pig (pPAG, pBP), cattle (*Bos taurus* – bPAG or bPSPB), sheep (oPAG, SBU3), equidae (ePAG, zebraPAG), moose and elk (mPBSB, ePBSB), white-tailed deer (wtd-PAG) and European bison (EbPAG). Moreover, the PAG proteins (Tab. III) were also purified and identified in the goat (cPAG), zebu (*Bos indicus* – zebuPAG) and buffalo (*Buffalo bubalis* – bubPAG). Initial heterologous and later homologous immunodetection of the PAG proteins demonstrated a high diversity of secretory forms and pregnancy-stage dependent spatial chorionic expression. Purified PAGs possess a variety of molecular masses (varying from 30 to 90 kDa) and electrostatic properties (Ip; 4.0–9.08).

The PAG proteins contain some relatively similar epitopes that allowed for cross-species immunodetection. Relatively high homology of the PAG proteins in each species caused extreme difficulties during isolation and purification of singular forms of the PAG proteins in various mammals. Because of these identification difficulties, several purified PAG proteins were initially characterised by biochemical procedures and Western blotting (Tab. III), but only a small number of them were characterised by microsequencing of the N-terminal ends (Tab. IV).

These N-terminal micro-sequences of native PAG proteins were identical or very similar to the sequences of the polypeptide PAG precursors coded by cloned cDNAs in various animals (Tab. IV). For example, in the pig, the sequence of native pBP43 protein [68] was similar, but not identical, to the sequences of the pPAG2, 4, 6, 8 and 10 polypeptide precursors [6, 8]. In sheep, oPAG65 protein revealed a similar micro-sequence to the sequences of two distinct precursors, oPAG3 and oPAG7 [9, 11]. Moreover, the N-terminus of some lately identified native oPAG proteins (55–60 kDa) revealed high homology to the sequences of the polypeptide oPAG1, 4 and 6 precursors [76–78].

All purified native PAG protein identification provides contributive data about placental proteomes of the examined animals. The exemplary comparisons demonstrate very high diversity and multiplicity of the PAG family. This diversity of the PAG proteins is the result of the aforementioned heterogeneity of the PAG gene family. Thus, complete identification of the entire PAG family requires several chorionic protein purifications, examinations of their multiple isoforms, and cloning of cDNA that code polypeptide PAG precursors.

2.6. Activation of polypeptide PAG precursors

In mammals, the most important stage in the activation process of various enzymatic proteins is the transformation of their zymogens (polypeptide precursors) to mature secretory forms. The post-translational activation of the PAG is probably similar to that of other members of the AP family. The above mentioned comparison of N-terminal micro-sequences and in silico analyses (Tab. IV) revealed that the PAGs precursors are specifically catalytically activated. Primarily, the pre-pro-polypeptide

Table IV. The NH₂-terminal micro-sequences of the native PAG proteins (kDa) isolated from the placenta of different species in comparison to PAG precursors and rabbit pepsinogen F sequences (*-coded by their cDNA). The sequences of porcine PAG2, 4, 6, 8, 10, ovine PAG1, 4, 6 and oPAG3/oPAG7 precursors (***) corresponded to native proteins (***) of pBP(43) and several oPAGs. The potential sites of N-glycosylation (N-x-T/S) of PAG precursors corresponded to empty cycles during micro-sequencing (marked by ?) of native PAG proteins. Conserved fragments are boxed (consensus), possible sites of propeptide cleavage in precursors are marked (↓), gaps (–) were inserted for maximal sequences alignment and identical amino acids of r-pepsinogen F and pPAG2-like precursors are in bold letters.

Name (kDa)	Micro-sequences				Identified by
	consensus				
pPAG1*	KF GLKGS L CSPKIS L	RLWN	YLD M VVYVGNITIG T P		[6]
pPAG2*,**	NL LSK N SS HVQ K F S YQ	PLRN	YLDMVVYVGNISIGTP		
pPAG3/5*	KF GLKGS L CSPKIS L	RLWN	YLD M VVYVGNITIG T P		[8]
pPAG4/6/8/10*,**	NL LSK N SS HVQ K F S YQ	PLRN	YLDMVVYVGNISIGTP		
pBP(43)***	L S K ? SS HVQ K F S YQ	PLRN	YLD M VY ? G		[68]
rPepsinogen F*	TY KLLSGQ QDPDV S F E	PLRN	YLDLAYIGIISIGTP		[22]
ePAG*	TH KLLHKH AD S GV A F E	PMRN	YLD I AYMG I ISV G T P		[13]
bPAG1*	YS LSQISF RGS N L T TI H	PLRN	IKDLVYMG N ITIG T P		[2]
oPAG1*	YR LSQISF RAS N L T TI H	PLRN	IMD M LYVGNITIG T P		
bPAG(67)		PLRN	FLDLVYMG E F T ↓ ? K D E		[3]
bPAG2*	YR LSK N -- -DSK I TI H	PLRN	YLD T AYVGNITIG T P		[10]
zebuPAG(67)		PLRN	IKDLVYMG		[75]
oSBU-3(69)		PLRN	MKDIFYV G ↓ ? ITID T P		[5]
oSBU-3(62)		PLRN	M↓ ? DIFYRG↓ ? ITIG G P		
oSBU-3(57)		PLRN	TLHLVYL↓ ? NIH↓ ? GN P		
oPAG1*,**	YR LSQISF RAS N L T TI H	PLRN	IMD M LYVGNITIG T P		[9]
oPAG4*,**	YR LPQISF RGS N L I TI H	PLRN	IRD T FFVGNITIG T P		
oPAG3/7*,**	YR LPQISF RDS N V T IL	PLRN	MKDIFYVGNITIG T P		
oPAG6*,**	YR LSQISF RGS N L T TI H	PLRN	TKDLVYLG N ITIG T P		
oPAG(65)***		PLRN	MKDIFYV G ↓ ?		[11]
oPAG(61)		PLRN	VKD V VYL↓ ? G		
oPAG(60)		PLRN	HRD		
oPAG(55)	SQ I ↓ ? LTVS↓ ? L T I↓ ?	NL			
oPAG(59)		PLRN	IRD		[76, 77]
oPAG(57)	ISSRV S N L TI H	PLRN	IMD M L		
oPAG(55)		PLRN	MRD I VY		
oPAG(66a)		PLRN	MKD		[78]
oPAG(66b)		PL			
oPAG(61)		PLRN	TND I DY		
oPAG(60)***		PLRN	TKDRVY I G		
oPAG(59)***		PLRN	IKDLVYLG N MG I G		
oPAG(58a)***		PLRN	TKDLVYLG		
oPAG(58b)***		PLRN	IRD T FFVGNIT		
oPAG(55)***		PLRN	I I DLRYV G		
cPAG(62)		PLRN	MRD I VYV G ↓ ? ITIG T P		[72]
cPAG(59)		PLRN	IMD M L H MG↓ ? ITIG T P		
cPAG(55)	ISS PVS↓ ? L T TI H	PLRN	IMD M LYV G ↓ ? IT I		

chains of the PAGs precursors are degraded by approximately 1.6 kDa (15 aa signal peptide removal) and later by a 4–5 kDa decrease, caused by the removal of the pro-peptide, which varies in length somewhat across the family (38 aa in ruminants, 37 aa in pigs or 33 aa in horses) [2, 6–9, 13–15]. However, so far, an auto-activation (inhibited by pepstatin A) has only been indicated for the recombinant PAG-like proteins in mice and horses [13, 16]. Presumably, the autoregulation of the placental proteins is associated with morphological and functional differentiation of the chorionic cells during placenta development.

It seems that in N-terminal ends of ruminant PAG native proteins the sequence of 3 amino acids, \downarrow R-D/G-S dominates mainly (Tab. IV). However, similar sequence R-D/G-S does not occur in pepsinogen precursors and in porcine and equine PAG precursors. The N-terminus of mature secretory porcine PAG2-like proteins begins with sequence \downarrow LSK [7, 8, 68], or with sequence \downarrow THK in the equine PAG [13]. In addition, almost all identified micro-sequences of ruminant PAG proteins contain conserved sequence PLRN (Tab. IV). This sequence is changed to P_MMRN in the ePAG precursor [13], or it is modified to RLWN in the pPAG1, 3 and 5 precursors [6, 8]. The physiological significance of these changes is presently unknown and requires future investigation.

2.7. Glyco-diversity of the PAG family

Generally it is known that various intracellular and membrane glyco-conjugates are involved in several important processes: cell-cell adhesion, masking of surface antigens during embryonic development and removal of glycoproteins from blood circulation. The diversity of glycoprotein receptors, variable content, secretion profile and yield of glycosylation play

important roles in the regulation of foeto-maternal interactions during early human pregnancy [85]. In the pig, a heterogenic group of glucosaminoglycans (GAGs) are produced by uterine tissues that influence several changes in secretion profiles required for morphological differentiation of endometrial epithelium and successful implantation [86]. Moreover, culture medium supplementation with GAGs (heparin and hyaluronic acid) affected embryonic GAG receptors and generally improved the developmental competence of porcine pre-implantation embryos in terms of increased blastocyst formation rate or total trophoblast cell number [87].

The native PAG proteins, either purified from placental tissues or produced *in vitro* by chorionic explants and released into media, are characterised by extremely high Mr in comparison to the predicted masses of the polypeptide PAG precursors, as calculated from the cloned cDNA (Tab. III). Several PAG proteins possess masses of ~ 70 kDa, approximately two times larger than their calculated masses (42.5 kDa). First evidence of high glycosylation was found during sequencing of cloned cDNA of the PAG genes. Various numbers of potential N-glycosylation sites of asparagine (N-x-S/T in precursors presented in Tab. IV) were identified, at least three in porcine PAG precursors [6, 8, 64], up to seven glycosylation sites in ruminant PAGs [9, 15], and only one glycosylation site in ePAG, which is not present in the zebra PAG precursor [13]. More evidence for N-glycodiversity come from N-terminal microsequencing of purified PAG proteins (marked by “?” in Tab. IV). In sheep and cattle, only some of these N-glycosylation sites are identical in the polypeptide PAG precursors and in the PAG proteins. In the pig, a second empty cycle (?) during microsequencing of the pBP (43 kDa) protein [68], did not confirm N-x-S/T site in the pPAG2-like precursors (Tab. IV), in which Val was identified [6, 8].

This suggests additional expression products within the pPAG2-like subfamily that dominate during early pregnancy in this species. The post-translational modifications of native PAG proteins i.e. specific glycosylation within the placenta lead to a large variability of the PAG proteins purified and identified from placental extracts or culture media.

The post-translational glycosylation of the PAG proteins were identified in several domestic and wild animals during various stages of pregnancy (see Tab. III for gestation stage). In these studies, the PAG proteins were enzymatically deglycosylated by glycopeptidase F treatment. In the pig, the oligosaccharide content is an average 9.66% throughout pregnancy, but can be from 7 to 25% during implantation [64]. In cattle, the content of oligosaccharides can account for 10% of total mass of native bPAG (67 kDa) proteins [3]. However, other studies indicated that the content of oligosaccharides can be much higher (up to 49%), because the mass of three partially purified bPAG proteins (75, 66 and 56 kDa) were reduced to approximately 37 kDa [79,88]. Also, native 67 kDa and recombinant 54/52 kDa bPAG proteins were also reduced to 37 kDa [89]. In wild ruminants, the PAG/PSPB proteins contain 3.15 and 4.98% of oligosaccharides in the moose and the elk, respectively [73]. In sheep, the glycosylation of SBU3 proteins, belonging to the oPAG family, may increase the mass of polypeptides by 18% [5]. During this study, the content of various monosaccharides was also estimated: fucose 0.34%, mannose 1.81%, galactose 1.94%, glucose 0.61%, N-acetylgalactosamine 3.62%, N-acetylglucosamine 5.26% and sialic acid 4.25%. Moreover, some experiments performed with an inhibitor of glycosylation (tunicamycin) revealed decreased forms (by 8–10 kDa) of the oPAG proteins [71]. The oPAG/SBU3 proteins were examined for structures of these oligosaccharides and

multi-antennae chains (bi-, tri- and tetra-antennae oligosaccharides) were found in this species [5]. These structures are probably unique for the glycosylation occurring during post-translational modification of the PAG proteins within the placenta.

2.8. Pregnancy diagnostic PAG tests

The highest embryonic mortality occurs during maternal recognition of pregnancy and during early implantation in different species [90]. In ruminants, over 35% of foetuses are lost during the first trimester of pregnancy [91]. In the pig, embryonic mortality is comparable and can reach 30–40% [92]. Such losses have been attributed to asynchronic development of conceptuses that are created during the 96 h ovulation period [92]. Embryonic mortality decreases during early placentation, before 30 dpc, and is the lowest during middle and late pregnancy [93]. These periods of greatest embryonic mortality are correlated with intensive proliferation of trophoblastic cells. Binucleate chorionic cells migrate in the direction of maternal uterine vessels, where granules containing the PAG proteins are released [3,94].

Purified native or recombinant PAG proteins and specific anti-PAG sera has led to the establishment of several diagnostic tests of pregnancy based on the detection of PAGs in maternal blood plasma or serum. The major significance of PAG assays in ruminants, similar to urine or plasma hCG tests in primates, is the direct identification of a placental product present in the maternal system that can be used as a marker of a viable pregnancy. In ruminants, numerous forms of the PAG proteins are detectable in peripheral maternal blood during the third week of pregnancy. The presence of PAGs can be measured by radioimmunologic (RIA) and immunoenzymatic (ELISA) tests. These PAG tests allow for early

pregnancy diagnoses in domestic cattle [69, 80, 91, 95–98], sheep [70, 99–104] and goats [105, 106]. Moreover, heterologous PAG tests were also useful for pregnancy diagnoses in wild ruminants, i.e. wood bison [107], moose [108], rocky mountain elk [109], white-tailed deer [110, 111], mule deer [111], red deer [112], sika deer [113], fallow deer [114, 115], mountain goat [116] and Alpine goats [117–119]. The measurement of PAG protein concentrations in maternal blood or milk of ruminants allow for 76.6–100% accuracy rates for early pregnancy diagnoses [120, 121] and provide an alternative method to ultrasonography for determining late embryonic mortality [96, 122].

The concentration of circulating PAGs depends on the number of well-being embryos/foetuses, and is higher in females with multiple than single pregnancies, according to the greater mass of the chorionic membranes in animals bearing twins [4, 70, 98, 99, 117, 118]. The secretion of PAGs can also differ based on the sex and breed of the foetus. Higher PAG concentration was determined in cows with male foetuses of the same breed, however in Hereford cows with transferred Holstein embryos the PAG concentration was higher in mothers with female foetuses [97].

A profile of circulating PAG is also pregnancy-stage dependent. In cattle and goats, the PAG concentration increases as pregnancy progresses [80, 97, 119]. In sheep, the concentration pattern of the PAGs is biphasic [99, 102, 104], increases (300–400 ng.mL⁻¹) during the second month of pregnancy, when chorionic cells have intensively proliferated and the placenta was formed. During mid-pregnancy, the concentration of ovine PAGs is somewhat decreased (< 100 ng.mL⁻¹), but increases again towards term, 300 ng.mL⁻¹ in Merino, 600 ng.mL⁻¹ in Churra [102] and over 1000 ng.mL⁻¹ during multiple pregnancies in Berrichone breeds [99].

Thus, it can be expected that the PAG protein families are more diverged in various breeds of different animals.

3. POTENTIAL FUNCTION(S) OF THE PAG FAMILY

Bovine PAG proteins were hypothesised as local immunosuppressive factors that can be involved in the maintenance of the histoincompatible foeto-maternal unit [123]. Diverse localisation of two PAG groups suggests different possible functions [51]. One ancient PAG group, mainly expressed at the foeto-maternal interface (microvillar junction), can be involved in binding the surfaces together and/or in the establishment of an immunological barrier. The expression of the more recently evolved second PAG group is restricted to bovine BNCs located mainly in the maternal villi of the placentomes, thus they are ideally positioned to modulate the maternal immune system [51].

High homology of the residues within domains creating an active site (substrate-binding cleft) of the PAG proteins are probably important for some function of these molecules. Only a few studies suggested protein-protein interactions or some targets/receptors of the PAG/PSPB molecules. In cyclic ruminants, endometrial explants (harvested on day 16) perfused with bPSPB alone or together with oxytocin increased the secretion of PGF_{2α} and PGE₂ [124]. In addition, luteal cells treated with bPSPB increased production of PGF_{2α} and PGE₂, without influence on progesterone (P₄) secretion [125]. In contrast, another study demonstrated that PSPB treatment increased P₄ secretion by mixed small and large (days 17–18) bovine luteal cells [126]. The regulation of PGE secretion by ovine CL (88–90 dpc) may also be via PSPB, which increased PGE and P₄ secretion [127]. During *in vivo* studies, plasma PSPB and

oestradiol 17β concentration was increased in ovariectomised (OVX) pregnant ewes (90 dpc), but oestradiol increased before PSPB [128]. In vitro studies with placenta slices collected seven days later from the same OVX ewes revealed that secretion of P_4 , PGE, oestradiol 17β and PSPB was increased by 2.7-, 3.6-, 2.2- and 2.4-fold, respectively, when compared to secretion by placenta slices of intact pregnant ewes [128]. The authors concluded that oestrogens regulate chorionic secretion of PSPB, which can influence placental secretion of PGE_2 that affect placental secretion of P_4 during mid-pregnancy in sheep. Moreover, endometrial (END) secretion in vitro of PGE_2 was increased by PSPB treatment (on 60 and 90 dpc), thus the authors concluded that PGE_2 secretion by caruncular/placental tissues after 50 dpc appears to be regulated by PSPB, not LH [129]. In another study, END from day 14 non-pregnant cows treated with the PSPB/PAG proteins increases secretion of 8 kDa uterine protein that was identified to be an alpha chemokine – granulocyte chemotactic protein-2 (GCP-2) by Western blotting [130]. Possibly, starting expression of trophoblastic PSPB/PAG can stimulate the GCP-2 during implantation time. However, during this time the uterine GCP-2 is also induced by short-time expression of trophoblastic antiluteolytic IFN- τ in ruminants [131].

In the pig, secretory pPAG proteins produced in vitro competed with ^{125}I -hCG in a concentration-dependent and pregnancy stage-dependent manner for binding by luteal (CL) and uterine gonadotropin receptors (myometrial and endometrial) of cyclic and pregnant pigs [82, 132, 133]. These studies suggest that the pPAG proteins may play a role as potential antiluteolytic or luteoprotective chorionic-origin signalling ligands mainly during the peri-implantation period. The PAG proteins may also act locally within the uterus and influence placenta development. Recently

obtained ligand-target/receptor binding results with the use of ^{125}I -hCG suggest that the identification of gonadal and extragonadal receptors require further studies with the use of purified and ^{125}I -labelled PAG standards – as the positive control chorionic ligands (commercially not-available at the present).

4. CONCLUSIONS

The recent cloning of the PAG genes has increased our knowledge of similarities and differences in the genomes and placental transcriptomes of eutherian animals. Presumably, the identification of the entire PAG protein family, coded by distinct genes in particular species, will allow for preparation of various commercial diagnostic tests: protein markers – for embryonic mortality, and genetic markers – for early evaluation of young animals for their effective reproduction. Moreover, the inclusion of PAG probes in microarray platforms may provide a means to ascertain selective genetic tests, based on PAG polymorphisms between individuals and to correlate different PAG genotypes to reproductive performance. In addition, the availability of numerous PAG cDNAs provides an important source of templates required for recombinant PAG protein production. Each cloned cDNA also permits an accurate guide for the identification of the structural organisation of PAG genes and their promoters.

The function(s) of multiple PAG protein family (active and inactive as proteinases) may be complex and combined with specific expression during pregnancy stages. Some PAG members can affect maternal recognition of pregnancy, adhesion of trophoblast and implantation, while other PAG members may be involved in remodelling of the foeto-maternal unit during placenta development in various mammalian species.

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