Pregnancy-Associated Glycoprotein (PAG) family: transcripts and gene amplicons in camelids

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SUMMARY

In this study, the placental localization of PAG-like transcripts and genomic existence of PAG-like amplicons in new-world (Lp, Lama pacos, alpaca) and old-world camelids (Cb, Camelus bactrianus, bactrian; Cd, Camelus dromedarius; dromedary) are reported for the first time. Sections of Lp (150–347 days post coitum), Cd (43–90 cm crown-rump length) and Cb (term) placentas were used for heterologous (ht; cross-species) autoradiographic in situ hybridization (aISH) with single-stranded diagnostic (antisense) or control (sense) [α-³⁵S]dATP-labeled 323 nt porcine PAG8 (pPAG8) cDNA probes produced by asymmetric PCRs. The aISH with antisense ³⁵S-pPAG8 probe identified camelid PAG-like (LpPAG, CbPAG and CdPAG) mRNA expression restricted to chorionic epithelium cells within placentas of camelids.

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In addition, genomic DNA (gDNA), isolated from placental sections were used as templates for camelid PAG-like gene amplicon production by PCR. Specificity of the obtained multiple camelid gDNA PAG-like amplicons was confirmed by double ht-Southern hybridizations with $[\alpha^{-32}P]dATP$-labeled 611 bp pPAG5 and pPAG10 double-stranded cDNA probes. The double ht-Southern hybridizations of camelid gDNA amplicons (with pPAG5 and -10 probes) allowed the identification of length-polymorphism of LpPAG, CbPAG and CdPAG genes, coding catalytically active and potentially inactive forms. Such an application of porcine PAG probes may be advantageous for future identification of still undiscovered PAG-like families in other eutherian species. Reproductive Biology 2009 9 2: 127-150.

**Key words:** camelids, alpaca, camels, chorion, mRNA, gDNA, PAG, pregnancy

**INTRODUCTION**

Many distinct PAG transcripts have been found in pre-placental trophoblast (TRF) and chorionic epithelium (TRD) after placentation in several eutherians [see 40]. Most numerous complementary DNAs (at least 66 cDNAs) of the PAG genes (aspartic proteinases – AP) have been cloned in the Artiodactyla order, but only single PAG orthologous genes have been cloned in other ungulate orders (Perissodactyla, Carnivora and Rodentia). Cloned cDNAs permit the identification of distinct placental PAG transcripts that are localized in mono- or/and bi-nucleate chorionic cells of the eutherians developing non-invasive or invasive placenta types. Transcripts of the PAG genes have been identified in various artiodactylous species including cattle and sheep [12, 46], goats [9], pigs [27, 33, 34, 37] and white-tail deer [6]. Other studies have indicated a unique placental glycosylation at the fetomaternal interface in camels [16] and distinct highly-glycosylated PAG forms in pigs [23, 28, 38], domestic cattle and two bison subspecies [18, 19, 22, 24, 39]. These studies have indicated the PAG family conservation during evolutionary changes of the eutherian species examined so far. In several domestic and wild ruminant species, accurate measurement of the
members of the PAG family in maternal blood or milk samples (by RIA or ELISA) may be used for routine prenatal diagnoses: early pregnancy, embryo/fetus well-being or mortality [40].

Previous comparative morphological or phylogenetical studies [3, 7] did not take into account the placenta classification of camelids that, similar to pigs, develop a diffuse epitheliochorial placenta type [47]. Such placenta develop during pregnancy that is three times longer in most camels (342–348, 350–404 and 360–440 days in the alpaca, dromedary and bactrian, respectively) than in pigs (115 days). Camelid placenta morphology has already been examined [1, 2, 8, 25, 26, 31, 32]. However, expected placental mRNA expression of the PAG family and its genomic existence has not yet been identified in the Camelidae species. In this study, we decided to use a porcine PAG2-like (pPAG) probe as a representatively expressed gene for the dominating pPAG2-like subfamily since a morphological homology of pig and camelid placenta types exists during early pregnancy. The choice of cross-species hybridization of the selected pPAG2-like probe was based on a lack of camelid placental mRNA and genomic sequences.

Most past discoveries of eutherian PAGs were initiated with ht-Southern hybridization during cDNA library screenings [6, 9–12, 34, 46]. However, cellular placental expression, functional significance and genomic diversity of the PAG family has not been recognized in many domesticated or wild eutherians. Therefore, in this study performed with cameld tissues, we used the most recently identified porcine PAGs (pPAGs). The pPAGs are members of two sub-families (pPAG1-like and pPAG2-like) that are classified as catalytically active (pPAG8 and -10) or potentially inactive (pPAG5) forms identified in pigs [27, 33, 40]. Conversely to inactive pPAG1-like members (pPAG1, 3 and 5 with 99.0–99.7% homology), the active pPAG2-like subfamily members (pPAG2, -4, -6, -8 and -10 with 87.7–99.6% sequence homology) are dominantly expressed in diffuse porcine placenta [27, 33, 34, 40]. Thus, we expected that the catalytically active PAG forms would also dominate in placenta of camelids. Previously, cross-species hybridization was successfully used for the identification of cellular localization of bison PAG-like transcripts [39]. This allowed the protein localization of a PAG-like family [24] that led to successful bison protein sequencing [18, 19].
The bison studies should be mentioned because late/term camelid placenta morphology (micro-cotyledonary) is also similar to the synepithelial chorionic cotyledonary structure of the ruminant placenta.

The objectives of this study were: 1) to localize the cellular expression of PAG-like transcripts in placental sections, and 2) to identify gene existence and length-polymorphism of PAG-like amplicons in three Camelidae taxa including one new-world (Lama pacos, alpaca, Lp) and two old-world camelids (Camelus bactrianus, bactrian, Cb; Camelus dromedarius; dromedary, Cd).

MATERIALS AND METHODS

Placental tissues

Placental tissues were harvested from alpacas (n=5) and camels (Cb, n=2; Cd, n=5). The pregnancy stage (days post coitum – dpc) was determined by ultrasonography and estimated according to crown-rump length (crl) of camelid fetuses: Lp (150–347 dpc), Cd (43–90 cm crl) and Cb (term) as previously described [1, 2, 25, 26].

Autoradiographic in situ hybridization (aISH)

Preparation of placental section. Placental tissues of alpaca were fixed in 4% paraformaldehyde and both camelid tissues (Cd and Cb) were fixed in Bouin’s solution for 24–48 h. Tissues were washed in 0.1 M phosphate buffer pH 7.4, dehydrated in a graded series of ethanol (EtOH), embedded in paraffin Paraplast Plus® (Sheerwood Medical, St Louis, MO, USA) and sectioned (5 µm).

Cellular expression of the PAG family in various placental transcriptomes was examined by histological autoradiography [11, 33, 34, 39]. Before aISH, placental sections were deparaffinized in xylene (2×10 min/4°C), rehydrated in EtOH (100, 95, 70 and 50%) and washed (10 min/room temperature – RT) in RNAse-free water (DEPC-H₂O). Deparaffinized specimens required addi-
tional short fixation in 4% paraformaldehyde/ PBS (5 min/RT), and washing three times in PBS for 3 min each, and two times in saline-sodium citrate buffer (SSC) for 2 min. Next, specimen permeability was achieved by four sequential 10-min washings in DEPC-\(\text{H}_2\text{O}\), 0.1 M triethanolamine/0.25% acetic anhydride, 2×SSC and DEPC-\(\text{H}_2\text{O}\). Then the sections were dehydrated consecutively in 30, 50, 70, 80, 95 and 100% EtOH (1 min), dried (RT) and subjected to the next aISH steps.

Production of antisense and sense \(^{[35}\text{S}]\)-labeled pPAG8 probes. Prepared placental sections of camelids were examined with heterologous (ht; cross species) antisense (diagnostic) or sense (control) probes, produced on a plasmid of cloned pPAG8 cDNA (GenBank: AY373029) used as a template inserted to pBluescript SK vector and amplified in transformed \(E. \text{coli}\) XL1-blue strain [27]. Both radioactive ht-pPAG8 probes were produced as previously [34], modified and validated for homologous [11, 33] or ht-aISH [39]. Briefly, both antisense and sense ht-probes were produced by double-rounds asymmetric PCR: 94°C (15 s), 60°C (15 s) and 72°C (20 s). In the first non-radioactive round (“cold”) with the use of internal PAG primers (ex7S and pagCas; 5’-CCGGGACGTCGATGCTGCA-3’ and 5’-CAGGCCAATCCTGTTCTGTCCT-3’, respectively), 323 bp long cDNA amplicons were generated. These amplicons correspond to bases 692–1014 of the pPAG8 ORF coding exons 7–9 [27]. This region was selected since it contained a conserved sequence, typical for the pPAG2-like gene subfamily. The selected region represents also the highest sequence dissimilarity (31.4–32.3%) compared to the pPAG1-like gene subfamily. The PCR-generated pPAG8 gene amplicons were purified from 1% agarose gels by electrophoretic elution with the use of GeneCapsule™ (Geno Technology Inc., USA), and then the amplicons were used as non-radioactive 323 bp templates for the next PCR. The second (radioactive – “hot”) round of asymmetric PCR (20 µl) for antisense or sense probe generation was completed by incorporation of \([\alpha-^{35}\text{S}]\)-deoxy ATP of 3000 Ci/mmol (NEN Life Science Products, USA) and with the use of non-radioactive pPAG8 amplicon templates (20 ng) from the first PCR round. For this radioactive PCR, the same conditions/solutions were used except a concentration of selected eutherian PAG primers. Antisense (diagnostic) probe
was generated with a tripled concentration of antisense pagCas–primer, but for a sense probe (negative control) only a regular concentration of sense ex7S–primer was used. Both $\alpha^{35}S$-pPAG8 cDNA probes (323 nt) were chromatographically purified (Sephadex G50; Pharmacia Biosciences, Sweden). The specific activity of antisense and sense pPAG probes was approximately $0.53 \times 10^7$ and $0.68 \times 10^5$ cpm/20 ng of pPAG8 amplicon template used for PCR, respectively.

**Heterologous in situ hybridization.** Both antisense and sense ht-$\alpha^{35}S$-pPAG8 cDNA probes were hybridized with the camelid placental sections and with porcine specimens – used as positive controls. The pPAG8 probes were denatured in a hybridization buffer (50% formamide, 4×SSC, 1×Denhardt, 10 mM dithiothreitol, 10% dextran sulphate, 100 µg/ml fragmented salmon sperm DNA). Then, 323 nt antisense and sense probes were applied to the centre of each slide (220 000 and 60 200 cpm, respectively). Placental specimens were immediately covered by parafilm and hybridized in a humidified autoradiographic chamber (approx. 20 h/42°C). After hybridization the parafilm was removed in a pre-warmed washing buffer (1×SSC/1 min/37°C). Non-bounded pPAG probes were washed out (5–6×/3 min/37°C) in a post-hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.1% β-mercaptoethanol). Then, placental specimens were dehydrated (50, 70, 80 and 95% EtOH in 0.1×SSC/3 min each/RT), washed (in deionized H₂O and 70% EtOH, 3 min/each), dried and subjected to histological autoradiography.

**$^{35}$S-autoradiography and post-aISH staining.** Placental sections were coated with autoradiographic NTB-2 emulsion, dried (24 h) in a dark chamber, and then exposed for one to three weeks at 4°C in light-protected boxes. The NTB-2 emulsion was visualized by sequential use of Dectol D-19 developer and fixer solutions (Kodak, USA). After autoradiography, placental specimens were counterstained with Harris’s modified hematoxylin and eosin, dehydrated and cover-slipped with the use of Entellan (Merck, Germany). Placental specimens were evaluated by both brightfield and darkfield microscopy (CH40 and Camera DP10; Olympus, Japan).
PAG-like amplicon production with genomic templates isolated from morphological sections

Genomic DNA (gDNA) of the *Camelid* species was isolated from placental sections, recovered from slides (approx. 0.1 mg/animal) by “a macro-dissection” separating maternal and embryonic tissues. The gDNA samples were purified (GenomicMini kit; Kucharczyk TE, Poland) and the obtained gDNA was used for amplification PAG-like gene fragments.

Genomic PAG-like amplicons were produced by PCR (GeneAmp 2400; Perkin Elmer, USA) with the use of camelid gDNA templates (30–150 ng/40µl reactions) and internal sense (ex5Se; 5’-TCCTGGGCTGGCCTATCCC-3’) and antisense (pagC; 5’-CAGGCCAATCCTGTTCTGTCTCCT-3’) PAG primers, complementary to exon 5 and 9 sequences, respectively. The primers were specific for eight distinct pPAG cDNAs and also for PAG-like amplicons previously identified in other ungulate species [27, 33–36]. For effective PCR conditions, 40 following cycles were used: 94°C (30 s) for the denaturation of gDNA templates, 60°C decreased to 46°C (by 2°C/4–6 cycles) for primer annealing (120 s), and 72°C (120 s) for amplicon synthesis. Obtained camelid PAG-like amplicons, parallel to a pKO3 marker, negative (without template) and pPAG10 cDNA – used as positive control, were electrophoresed, visualized by UV (Fotodyne, USA), and then vacuum-transferred onto positively charged nylon membranes (0.2 µm Nytran; Schleicher & Schuell, Germany). The specificity of PCR-produced camelid PAG-like amplicons (Lp, Cb and Cd) was examined by double ht-Southern hybridizations.

*Production and specificity of [32P]-labeled porcine PAG5 and PAG10 probes.* Two [32P]-labeled double stranded pPAG probes (complementary to ORF of exons 5–9) were produced with the use of cloned pPAG5 and pPAG10 cDNAs (GenBank: AY188554 and AY775784, respectively) as templates. The first pPAG5 probe was specific for the pPAG1-like gene subfamily. The second specific pPAG10 probe allows the identification of the entire pPAG2-like gene subfamily. Both pPAG probes were produced by double round PCR with the use of internal pPAG primers (ex5SE and pagC). In the first PCR round with non-radioactive dNTP, 611 bp pPAG amplicons were produced for the most different cDNA fragment (34.6% of dissimilarity)
of the pPAG5 or pPAG10 cDNA templates (encompassing 404–1014 and 545–1155 bp ORF, respectively). Electrophoretically purified pPAG amplicons were used as specific templates required for second radioactive PCR round with $[\alpha-^{32}P]$ deoxy ATP of 3000 Ci/mmol activity (NEN, USA). Both pPAG probes were purified by a column chromatography (Sephadex G 50). Specific radioactivity of the pPAG5 ($1.05 \times 10^6$ cpm) and pPAG10 ($6.55 \times 10^6$ cpm) probes was measured (1470 Wizard™, Wallac, USA) before Southern analyses. Both appropriately diluted pPAG probes (5 250 cpm/ml and 18 700 cpm/ml of buffer) were used for double membrane hybridization of camelid PAG-like amplicons.

**Southern hybridization of camelid gDNA amplicons with $[^{32}P]$-labeled pPAG5 and pPAG10 probes.** Double ht-Southern analyses of camelid gDNA amplicons were performed with $^{32}P$-labeled pPAG5 and pPAG10 cDNA probes. The use of ht-Southern analyses permitted the verification of the specificity and length-polymorphism of diverse LpPAG, CbPAG and CdPAG gene amplicons. An amplicon, produced on the cloned pPAG10 cDNA was used as a positive control in the Southern hybridization.

The non-specific binding of nylon membranes, containing bounded camelid gDNA amplicons, was blocked (2–4 h/42°C) in pre-hybridization buffer (50% formamide, 5×SSC, 5×Denhardt, 0.5% SDS, 0.1 mg/ml fragmented salmon sperm DNA). Blocked nylon membranes were hybridized (24 h/42°C) with both denatured (5 min/95°C) pPAG probes. Then the nylon membranes were washed (3×5 min) in post-hybridization buffer (0.1×SSC, 0.5% SDS or 2×SSC, 0.5% SDS) and used for signal detection. After autoradiographic visualization of one probe, the nylon membranes were stripped (3×3 min/95°C) and subjected to the next Southern hybridization with the second probe. Removal of the first probe (from membranes) was monitored by a $\beta$–radiometer ECO-C (Polon Ekolab, Poland) and controlled by an additional autoradiography.

All membranes were adequately exposed (−70°C) to radiographic films (Retina X-ray XBM 100 NIF, Fotochemische Werke GmbH, Germany; or MXB 100 NIF, Estman Kodak, Rochester, USA) in cassettes (Rego, Germany; or Hypercassette, Amersham Biosciences, Sweden) supplied with intensifying screens (Perlux Extrarapid 200, Germany). After the required
nylon exposures (from 1 h to 1–3 days), the radiographic films were developed (Developer X-OMAT M43 Processor; Kodak, USA), photographed (CCD camera, Sony, Japan) and finally digitally archived (FOTO/Analyst® Archiver; Fotodyne, USA).

RESULTS

Distribution of camelid PAG-like mRNA

The ht-aISH with the diagnostic antisense $^{35}$S-labeled porcine PAG8 cDNA probe permitted the cellular localization of PAG transcripts within the placental sections of all three examined camelid species (figs. 1 and 2). Porcine placental sections used as positive controls and aISH with the antisense $^{35}$S-pPAG8 probe confirmed the specificity by very strong hybridization signals (data not shown). The use of sense probe as a negative control, did not show any ht-aISH signals in camelid placental sections (fig. 1L–O).

Cellular LpPAG mRNA expression in the alpaca placenta. We demonstrated the specific cellular localization of the LpPAG transcripts within diffuse epitheliochorial alpaca placenta. The strongest signals were detected in the fetal festoon-like chorionic epithelium layer (fig. 1A–H’). The specific localization of the LpPAG transcript expression (fig. 1A–C’) was restricted to the TRD cells forming intensive placenta folding (257 dpc). The TRD layer was mechanically separated from the neighboring maternal endometrial (END) epithelium layer during multi-step procedures. Despite such embryonic and maternal tissue separation, an adequate interdigitation of the TRD within the uterine END structure is still visible.

Within the TRD layer, frequent enlarged or giant cells exhibited the highest LpPAG mRNA expression. These LpPAG-positive cells were located at the apex (fig. 1E and G) and at the base of the TRD folds (fig. 1F and H). In contrast, the maternal epithelium, the END gland and stroma cells did not show any positive signals with the antisense $^{35}$S-pPAG8 probe (fig. 1A’–H’). However, within some placental sections (244 dpc), singular cells with much weaker signals were occasionally observed (fig. 1I–K). These dispersed
Figure 1. Localization of the alpaca PAG transcripts with antisense $^{35}$S-pPAG8 probe (A–K) in chorionic cells of alpaca placenta (244 and 257 days post coitum, dpc) comparing to negative control (neg) with sense probe (L–O). Darkfield views (silver grains appearing white; A’–I’ and O) reveal the same sections in brightfield views (A–I and N); Magnification: 125× (A–D), 250× (L–O), 500× (E–I) and 1250 × (J–K).
cells (probably immunologic) were detected within the maternal placenta unit, but they were found mainly in the vicinity of maternal vessels in the endometrium.

**Cellular CbPAG- and CdPAG mRNA expression in camel placenta.** The cellular localization of PAG transcripts was also identified in the dromedary and the bactrian camel placenta. Due to a relative similarity and a quality of examined placental sections only selected results of both camel species are presented (fig. 2). In bactrian placental sections, the strongest PAG-like signals were identified in developed mushroom- and balloon-like villus structures of the TRD layer (Cb term) that resembles spatial morphology of the micro-cotyledonary structure (fig. 2A, B, D–F). Moreover, we found that some enlarged or giant TRD cells (existing at the apex of embryonic villus) revealed the strongest hybridization signals indicating the CbPAG mRNA localization (fig. 2B–F).

Similar, in dromedary placental sections, dominating signals of the CdPAG transcripts were identified mainly within the developed secondary

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**Figure 2.** Localization of the bactrian camel PAG transcripts with antisense $^{35}$S-pPAG8 probe in chorionic cells of bactrian placenta (term). Darkfield views (silver grains appearing white; $A'$–$F'$) reveal the same sections in brightfield views ($A$–$F$). Magnification: $125\times$ ($A$, $E$) and $250\times$ ($B$–$D$, $F$).
folds of the TRD layer (data not shown). Spatial singular cells (probably immunologic) surrounding blood vessels in the END glands, with a relatively lower signal, were also identified within the dromedary placental sections. However, PAG-like signal intensity in the dromedary placental sections was lowered in comparison to fully developed bactrian placenta.

Identification of the PAG-like family in camelid genomes

Identification of LpPAG, CbPAG and CdPAG gene amplicons was possible due to effective isolation of gDNA templates from placental sections. This identification was impeded by the quality of some histological fragments. Optimized ht-amplification allowed the use of stringent conditions (60°C) for cross-species primer (ex5S/pagC) annealing to relatively homologic sequences existing in gDNA templates of the camelids.

Isolated gDNA templates allowed the generation of PAG-like amplicons (LpPAG, CbPAG and CdPAG), whose specificity was confirmed by ht-Southern hybridization (fig. 3). The quality of gDNA templates and effectiveness of the LpPAG, CbPAG and CdPAG gene amplicon production differed among samples and was section-quality and pregnancy stage-dependent (fig. 3A–C). Some histological sections of bactrians (Cb#A and Cb#B), dromedary (Cd#90) or alpaca (Lp#347) were sufficiently digested (3 h) by proteinase K (fig. 1A). In contrast, tissue fragments of other animals (Cd: #43, #3P, #22P and Lp: #150, #257) required double amounts of proteinase K and extended digestion (24h). Decreased quality of some fragmented gDNA templates resulted in relatively lower effectiveness of the LpPAG, CbPAG and CdPAG gene amplicon productions (fig. 3A–C). Due to a low quality of gDNA template (i.e. Lp#257) during the first isolation (fig. 3A), the gDNA purification was repeated with increased tissue quantity required for the effective production of the LpPAG amplicons identified by standard EtBr staining (fig. 3B–C).

Autoradiographic Southern analysis with 32P-labeled pPAG5 and pPAG10 probes confirmed the existence and specificity of LpPAG, CbPAG and CdPAG amplicons in genomes of the examined camelids (fig. 3D–E).
Figure 3. Exemplary electrophoreograms of genomic amplicons of camelid PAGs (alpaca, Lp; bactrian, Cb; and dromedary, Cd) stained with ethidium bromide (A–C) and Southern autoradiograms with $^{32}$P-pPAG5 probe (D) or $^{32}$P-pPAG10 probe (E). Mm: molecular marker, Cp: positive control (amplicons of pPAG10 cDNA), Cn: negative control with omitted template.
The relatively high sequence homology (65.4%) of pPAG5 probe to control pPAG10 cDNA (Cp; 10 ng of plasmids) was reflected by the strongest autoradiographic signal for Cp hybrids in comparison to LpPAG, CbPAG and CdPAG amplicons produced with an increased amount of gDNA templates. Cross-species Southern hybridization in stringent conditions (50% FA/5×SSC/0.5% SDS/42°C) proved the specificity of dominating LpPAG, CbPAG and CdPAG amplicons. The profile of hybridized PAG-like amplicons was similar in both camel species, but varied in all examined alpacas (Lp#150–Lp#347). The first hybridization with the 32P-pPAG5 probe (pPAG1-like subfamily) revealed dominant 611 bp camelid PAG-like amplicons that resembled pPAG10 cDNA (545–1155 bp) used as positive control. In addition, shorter, ~500 bp gDNA amplicons, were identified (strongly hybridizing especially in Lp#150), and miscellaneous smaller pPAG1-like gene amplicons with lower hybridizing specificity were also detected in some animals (mainly in alpacas; fig. 3D). Moreover, the Southern analysis permitted the identification of some LpPAG amplicons that were not detectable during standard UV visualization in gels (Lp#347). In contrast, after the second hybridization with the 32P-pPAG10 probe, few LpPAG amplicons were found to be less specific or completely non-specific (Lp#150 and Lp#347, respectively) for the pPAG2-like subfamily (fig. 3E).

**DISCUSSION**

This is the first report which describes the cellular localization of the PAG-like mRNA in the camelid placenta and the existence of orthologous PAG-like gene family members in the genomes of camels. We examined three selected Camelidae species: the alpaca, bactrian camel and dromedary which, including pigs, ruminants and whales, belong to the Cetartiodactyla [30]. Similarity in blastocyst development, implantation and placentation have been observed in many even-toed hoofed ungulate mammals, but so far, the PAGs were identified only in pigs and some domestic and wild ruminants [40].
Identification of cellular localization of the PAG mRNA expression within camelid placenta

In camelids, cDNA of the PAG genes have not been cloned yet. Thus, the study of the localization of LpPAG, CbPAG and CdPAG mRNA expression was performed by aISH with the use of $^{35}$S-labeled porcine PAG8 cDNA probe. The obtained results of the PAG-like mRNA expression in camelids are difficult to discuss because analogous data are not available. However, our data may be compared to some similarities and also species-specific differences in placenta structure of other species. In a pioneering morphological study [4], camelid placenta was classified as a non-invasive diffuse, epitheliochorial type. In the lama, placenta non-villous regions were observed between intense ancillary TRD villi regions [8]. Later morphological analysis of camelid placenta emphasized its higher similarity to the pig placenta [31, 49]. The placenta structure of alpacas is particularly similar to that of pigs because of an increased placental surface folding observed with the progress of pregnancy in both species [25, 26]. The camelid placenta is described as a unusual diffuse type, often named as a micro-cotyledonary in regard to strong branching of TRD folds and structural similarity to cotyledonary morphology within ruminant placentomes [1, 2, 21]. Like in pigs [17] and cattle [48, 50], camelid chorionic epithelial cells are covered by numerous microvilli that firmly interdigitate with microvilli on the surface of the luminal uterine endometrial epithelium [43]. The microvilli on adhesive surfaces of both epithelial layers assemble a complete microvillar junction between fetal and maternal tissues [8, 25, 26, 31].

Despite many morphological similarities of placental development in alpacas, bactrian camels, dromedaries, pigs and cattle, presumably, some homologous placental gene expression should be expected. We found expression of the LpPAG, CbPAG and CdPAG mRNA restricted to the chorionic cell layer of fully developed placental folds. The strongest PAG-like expression (indicated by porcine PAG8 probe) dominated in abundant enlarged or giant chorionic cells, frequently located close to the apex of TRD folds. The most frequent camelid cells expressing PAG-like mRNA formed a certain layer of balloon-like structure of chorionic folds. Expression
pattern of PAG-like transcripts in the alpaca placenta was morphologically more similar to less-branched TRD folds of pigs, but in the bactrian and the dromedary it resembled the cotyledonary placentomal organization of ruminants. Moreover, intriguing immunological cells (with PAG-signals) surrounding endometrial blood vessels were rarely observed. Our findings of placental expression of camelid PAG-like transcripts suggest that the PAG family may be involved in crucial feto-maternal interactions during successful placenta development in camelids. Such involvement was observed in other artiodactyls [29, 41, 42, 50].

The cellular localization of the camelid PAG-like transcripts (by aISH) may be compared only to the PAG mRNA expression in other ungulate species including cattle and sheep [12, 44, 46], pigs [33, 34], horses [11], goats [9], white-tail deer [6] and the European bison [39]. Most of these studies demonstrated that within synepitheliochorial ruminant placentomal units (cotyledons/caruncules), PAG-transcripts are expressed in mono-(MNC), as well as in bi-nucleated cells (BNC). Some differences among the examined species were identified in placental PAG expression. For example, in epitheliochorial horse placenta, equine PAG (ePAG) mRNA expression exists within diversified TRD cell layers, localized mainly in chorionic areas adjacent to END cups [11]. In pigs, various pPAG mRNA groups representing two subfamilies (catalytically active and potentially non-active) were identified. They were detected mainly in MNC within secondary intensively branched TRD folds deeply interdigitating maternal END tissue [34]. The pPAG mRNA was also localized in multi-nucleated TRD cells during in vitro experiments [33].

Evolutionary analysis of the PAGs implied that phylogenetical diversification of one PAG group starts from ancestral pro-gene duplication approximately 85–87 MYA (million years ago), but the diversification of the second group starts 52–55 MYA [12, 46]. The long-term evolutionary survival of the ruminant “younger” group is restricted to BNC expression, whereas the “older” group is expressed in MNC and BNC. The older PAG group originated from the duplication of a pepsinogen F-like gene and positive selection [14, 15]. This period corresponds to evolutionary diversification of the Artiodactyla and Perissodactyla orders [20]. The formation of younger PAG genes may correspond to
the separation of the lines (approx. 55 MYA) leading to the evolution of presently surviving ruminant and swine species [14, 15]. The older PAG group may participate in the adhesion of TRD and END epithelial layers and tightening of the tissue connections, while the younger group is implicated in chorionic secretion directed into maternal blood circulation [50]. Both PAG groups varied in affinity to pepstatin (pH 5 or 7), but mainly the older group exhibited some properties more typical for active aspartic proteinases [11–13, 46]. Recently, the expected diversity of the camelid PAG family was demonstrated by heterologous immunodetection (with anti-bovine PAG-2 polyclonals, R#435) of semi-purified multiple protein forms extracted from the epitheliochorial placenta of alpaca and dromedary [5]. Moreover, in the pig, secretory pPAGs produced in vitro competed with $^{125}$I-hCG in a concentration-dependent and pregnancy stage-dependent manner for binding by luteal and uterine receptors of cyclic and pregnant pigs [29, 42]. Presumably, in the camelid placenta, various distinct members of the PAG-like family may also be expected. However, at present, it is not possible to distinguish and characterize various products of the camelid PAG family and its functional significance. Such identification requires cDNA cloning with the use of well preserved mRNA samples isolated from deep-frozen camelid placentas harvested during various pregnancy stages.

**PAG-like family in camelid genomes**

Examination of camelid genomic templates permitted the identification of a PAG-like gene family in three species. Alpacas, bactrian camels and dromedaries are the first *Camelidae* species in which the LpPAG, CbPAG and CdPAG-like gene amplicons were identified. The amplicons of PAG-like gene fragments were obtained with gDNA templates isolated from histological sections and by ht-amplification with specific primers for the PAG genes. The specificity of the LpPAG, CbPAG and CdPAG-like gene amplicons was verified by ht-Southern hybridization with two $^{32}$P-labeled pPAG probes. This confirmed the nucleotide specificity and also revealed a length-polymorphism, especially for the LpPAGs. Our ht-amplification in rigorous condition of primer annealing (60°C) and ht-Southern analysis performed also in stringent hybridization conditions (42°C) permitted spe-
pecific formation of distinct camelid PAG-like amplicons. The restrictive PCR conditions revealed the existence of dominating 611 bp LpPAG, CbPAG and CdPAG or shorter amplicons. High length-similarity of dominating camelid gDNA amplicons compared to cDNA amplicons of the pPAG genes may suggest that camelid PAG amplicons could be formed on mRNA templates (as a potential gDNA contamination) isolated from histological sections. However, PAG amplicons (611–616 bp), similar in length, were formed on various gDNA templates (isolated from leukocytes, hair roots or semen) of many ungulate species [36]. These results should eliminate the suggestion concerning gDNA template contamination by placental LpPAG, CbPAG and CdPAG transcripts. A better explanation for such short PAG gDNA amplicons could be a primer-homology to sequence expected within approximately 0.85 kbp intron E (between exons 5 and 6) in respect to the structural organization of PAG genes [35, 45]. Moreover, in camelid PAG genes, some missing or shortened exons can be expected, similar to porcine PAG5 and PAG8 cDNA [27] as well as to shorter PAGs in other eutherians [6, 9–12, 40, 46].

All LpPAG, CbPAG and CdPAG amplicons generated with gDNA templates represent various PAG genes existing in camelids. Ht-Southern hybridization with pPAG5 cDNA probe confirmed the high homology of the LpPAG, CbPAG and CdPAG amplicons to porcine PAG genes and revealed the presence of additional camelid amplicons that were non-visible during standard UV identification. Similar profiles of the CbPAG and CdPAG amplicons (611 bp) and the diversified profiles of the LpPAG amplicons (611 bp and shorter), indicated length-polymorphism of the camelid PAG gene family. The pPAG5 probe allowed the identification of some LpPAG, CbPAG and CdPAG genes corresponding to the pPAG1-like subfamily that code potentially inactive polypeptides. The pPAG10 probe identified camelid PAGs corresponding to the pPAG2-like subfamily that code active pepsin-

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like polypeptides. It is important to remark that the sequences of exons 5–6 and 8–9 of the PAG genes are more conserved than exon 7, in which nucleotide sequence is the most variable between the pPAG1- and pPAG2-like gene subfamilies [27, 35, 36]. High sequence homology within regions of primer annealing (in exons 5 and 9) suggests that the produced LpPAG, CbPAG and CdPAG amplicons represent both gene subfamilies.

The diversity of multiple PAG genes was demonstrated by Southern hybridization of digested gDNA templates in many wild eutherians [46]. Higher sequence homology resulted in the strongest bPAG1 signals detection in the genomes of species belonging to the hollow-horned – Bovidae family (e.g. gaur and yak). Weaker bPAG1-like signals were found in some species belonging to the Cervidae family (i.e. white-tail deer), in which ten distinct wtdPAG cDNAs were recently cloned and fully identified [6]. Although positive signals were almost undetectable with the bPAG1 probe in horse and rhinoceros (Perissodactyla) or panda (Carnivora) during the Southern hybridization of digested gDNA [46], the single PAG cDNA was cloned from placental transcriptomes of horses and zebra [11] or cats [10], respectively. Thus, great heterogeneity among multiple orthologous PAG genes in many species may confirm permanent evolutionary diversification of the PAG pro-gene.

Our results revealed higher heterogeneity and length-polymorphism of PAG-like genes in alpaca genome than bactrian or dromedary genomes. Diversified profiles and length-polymorphism of the LpPAG amplicons suggest a higher number or/and a greater heterogeneity of the PAG-like gene family in the alpaca genome. Based on a relatively high homology of used pPAG5 and pPAG10 cDNA templates and resulting Southern hybridization, it can be expected that some groups of LpPAG, CbPAG and CdPAG genes may be comparable with the sequence homology of the porcine PAG1- and 2-like gene subfamilies.

In conclusion, our results provided novel data and increased the general knowledge about pregnancy stage-dependent LpPAG, CdPAG and CbPAG mRNA expression in the placenta of three camelid species. This is also the first study that describes the PAG-like family in the genomes of selected camelids. The present results suggest that there is a conservation of sev-
eral developmental properties in *Camelidae*, *Suidae* and *Bovidae* families during diversified placental evolution of the *Artiodactyla* order. Our study proves that further work on PAG-expression in the camelid placenta is definitely required. Future cloning and sequencing of camelid PAG-like cDNAs as well as sequential characterization of selected PAG-like gene structures will finally permit the determination of the precise PAG gene number in various camelids.

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**REFERENCE**


36. 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.