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Original article

Chorionic expression of heterogeneous products of the PAG (Pregnancy-Associated Glycoprotein) gene family secreted in vitro throughout embryonic and foetal development in the pig

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Abstract — Porcine PAG (pPAG) are placental products of a multigene family that is strongly expressed in the chorionic epithelium (trophoblast and trophectoderm). The objective of this study was to define a pattern of the pPAG proteins, secreted in vitro by chorionic explants harvested on 16–77 days of pregnancy. Trophoblastic and trophectodermal explants were collected from pregnant (PR) gilts ($n = 27$) and used for protein in vitro production (8–261 h). Endometrial explants of luteal-phase gilts (E10, $n = 4$) and pseudopregnant gilts (PsE, $n = 2$) were used as negative controls for protein immunoblotting. Proteins (PR, E10, PsE) were isolated mainly from incubation media, fractionated, dialysed and separated by SDS-PAGE. Heterogeneous Western blotting with various polyclonal anti-PAG sera raised against bovine or ovine antigens (anti-bPAG, or anti-oPAG) initially identified the pPAG proteins. Such blotting of fractionated chorionic proteins allowed for the isolation of porcine antigens that were employed as immunogens to raise several homologous antisera (anti-pPAG). Crude antisera were adsorbed on endometrial extracts or proteins of non-PR pigs, to remove non-relevant antibodies. The patterns of pPAG proteins secreted in vitro varied throughout pregnancy (35–72 kDa). During implantation, ~43 kDa (Day 16) or ~68.1 kDa (Days 17–25) pPAG proteins were detected. During placentation and as pregnancy advanced (Days 31–77), ~72.3 kDa pPAG proteins were observed. The secretions of parallel multiple smaller proteins (35.4–47.2 kDa), presumably, as forms of processed pPAG precursors, increased with the progress of gestation. In conclusion, the pPAG protein family plays a very important role during implantation, placenta formation and embryonic/foetal development in the pig.

chorionic glycoproteins / placenta / pPAG gene family / pig / pregnancy / trophectoderm

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1. INTRODUCTION

Porcine pregnancy-associated glycoproteins (pPAG) are members of the aspartyl proteinase family, strongly expressed in the chorionic epithelium [1–5]. The PAG cDNA family had been initially identified in ruminants: cattle and sheep [6]. Several distinct cDNA among diverse PAG families have been characterised in different eutherian mammals, domestic even-toed ungulates (*Artiodactyla*): 21 bovine [7–10], 9 ovine [7], 11 caprine [11], 6 porcine [1, 5, 12–14]. So far, a single cDNA has been cloned in odd-toed ungulates (*Perissodactyla*): the horse and zebra [15, 16], and in non-hoofed mammals (*Carnivora* and *Rodentia*): the cat [17] and mouse [18], respectively. The ruminant PAG family (~100 genes) exhibits spatially and temporally distinct patterns of expression during pregnancy [19], within the entire outer epithelial layer of the placenta (trophectoderm), or in bi-nucleate cells of cattle and sheep [10] and goats [11]. In the pig, at least 8 genes related only to the pPAG2 gene are expected in the genome [3, 4]. The first two cDNA, pPAG1 and pPAG2 (GenBank Acc. No.: L34360 and L34361, respectively), which encode distinct 389 and 387 amino acid (aa) precursors, represent the first members of two distinct subfamilies of PAG genes [1]. Recently, additional two cDNA of pPAG4 and pPAG6, encoding distinct 389 aa polypeptides have been characterised (GenBank Acc. No.: AF272734 and AF272735, respectively), belonging to the pPAG2-like subfamily [12], or cDNA of pPAG3 (AF315377) and pPAG5 (AY188554) as novel members of the pPAG1-like subfamily [13, 14]. All aforementioned members of the PAG family are closely related in their sequences to the aspartyl proteinase (AP) gene family. The AP family includes pepsinogens A, C, F, cathepsins D, E, chymosin and renin [20, 21], and lately plasmepsins or histo-AP [22]. A catalytic activity of the PAG and AP family depends upon the presence of two aspartic acid residues that are present within a hydrogen-binding distance of each other on opposite sites of the cleft, created by two domains [23].

In all investigated species, the PAG proteins appear as multiple products of this large and diverged gene family. The ruminant PAG protein family [6, 8, 10, 11] was also previously known as the bPSPB-bovine pregnancy-specific protein B [24, 25], or ovine SBU-3 proteins [26]. In ruminants, the PAG proteins have been identified as placenta-specific antigens that enter the blood circulation of the mother and are used as markers for pregnancy diagnosis [27, 28].

In the pig, conditioned media of cultured trophoblast tissues, recovered from pregnant animals during implantation time, contain several forms of BP - basic proteins that have been identified as major multiple secretory products of conceptuses [29, 30]. These BP proteins vary in their M.W. (35–70 kDa) and are localised within the trophoblastic cells of elongating porcine blastocysts [31, 32]. A micro-sequence of the N-terminal fragment of one purified member (~43 kDa) of the native BP family appears to be very similar [33] to an internal fragment of the pPAG2 precursor coded by its cDNA [1]. On the basis of the sequence comparison of the BP and pPAG2 proteins and their high homology, it can be expected that mature BP proteins are products of a post-translational modification of the pPAG2-like precursors. The mature pPAG2 protein is coded by sequences of exons 2–9, according to the identified structural organisation of the pPAG2 gene, encompassing ~9.2 kbp [3, 4].

The present experiments were conducted to identify the diversity of the pPAG protein family, secreted in vitro by various explants of the trophoblast and trophectoderm of the pig. The objectives of our studies were: (1) the in vitro production of the pPAG proteins - monitored with polyclonal antisera raised against purified single native bovine or ovine PAG proteins, as heterologous

antigens; (2) the production of polyclonal antisera against various porcine PAG proteins, as homologous immunogens, and (3) to define a pattern of immunoreactive pPAG-like proteins secreted in vitro throughout embryonic and foetal development in the pig.

2. MATERIALS AND METHODS

2.1. Materials

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

2.2. Animals and in vitro cultures of porcine trophoblastic/ trophectodermal explants

Mature crossbred gilts were either naturally bred at oestrus (Day 0) or injected with estradiol benzoate (1 mg·d) on Days 11–15 of the oestrous cycle to induce a pseudopregnant state [34]. At Days 16 (4), 17–18 (4), 19–20 (4), 23–25 (5), 31–38 (3), 40–45 (5), 61 (1) and 77 (1) of pregnancy ($n = 27$), or at Day 17 of the pseudopregnancy ($n = 2$) the pigs were routinely sacrificed at a local slaughterhouse. The care and use of the animals were in full agreement with local ethical authorities. For bioethical reasons, a limited number of late pregnant gilts were used. The uteri from luteal-phase gilts ($n = 4$) were also collected at a local slaughterhouse.

Cultures of chorionic explants were performed as previously described [1]. Briefly, extra-embryonic membranes dissected from embryos, then trophoblast or trophectodermal membranes were harvested and placed in sterile PBS with antibiotics (100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin). Chorionic membranes were washed in PBS, minced into small (1–3 mm^3) pieces, washed in Dulbecco Modified Eagle Medium (DMEM) and then routinely cultured in DMEM serum-free media, in flasks on the rocking platform, for 8 h at 37 ºC under 5% CO_2:95% air. De novo synthesis of chorionic proteins (released by explants) was elucidated in some cultures (Day 16) that were extended until 261 hours, in which media containing secreted proteins were collected every 9–12 hours, and all harvested proteins during 261 h were combined for PAGE.

2.3. In vitro production of porcine chorionic antigens for immunisation

The secretory chorionic proteins were harvested from the media, which were filtered through four layers of cheesecloth and additionally separated from tissues by centrifugation (500 × *g* for 5 min at 4 ºC). The media containing released chorionic proteins or/and separated tissue explants were collected and immediately stored at –70 ºC for further isolation of total proteins.

Chorionic proteins of early pregnant gilts (Days 17, 18, 19, 20, 25) were recovered from the media by fractionation and concentration in Centriprep-10 and Centriprep-30 cartridges (MWCO 10 and 30 kDa; Amicon, Beverly, MA, USA), according to the molecular mass of pPAG proteins identified previously [1, 4, 5]. Component salts of culture medium were removed from the protein fractions by dialysis.

Media with secretory chorionic proteins of middle and late pregnant gilts (Days 31–77) were harvested as described above.

According to the increased mass of placental explants and subsequent bigger volumes of the obtained culture media, chorionic proteins were first recovered by precipitation with 20%, 40% and 75% saturation of $(NH_4)_2SO_4$ for 24 h at 4 °C and centrifugation (3.500 × *g* for 1 h). The pellets containing trophectodermal proteins were dissolved in 20 mM Tris HCl/2 mM EDTA, dialysed (MWCO 12–14 kDa) to remove $(NH_4)_2SO_4$, then the proteins were fractionated and concentrated in Amicon cartridges (MWCO 30 kDa), according to previously identified properties of the highly glycosylated forms of pPAG/BP proteins [1, 4, 5, 29–33]. The separated porcine proteins were analysed by 1D-SDS-PAGE and monitored by heterogeneous Western blotting with well-defined various anti-ruminant PAG sera, raised either against single purified bovine or ovine native PAG proteins, as immunogens [6, 27, 35]. According to the molecular masses of polypeptide pPAG precursors, calculated on the basis of their cDNA [1, 4, 5], presently obtained chorionic proteins were differentially fractionated. Finally, selected fractions of proteins obtained from pigs on Day 17 (> 10 kDa - all pPAG precursors), on Days 18–77 of pregnancy (> 30 kDa - high M_r forms possessing unique post-translational glycosylation), or recombinant pPAG2 proteins produced in the bacterial system (> 30 kDa - proteins without carbohydrates - unpublished data) – have been used as antigens to immunise crossbred rabbits ($n = 9$). Multiple intradermal injections were performed according to Vaitukaitis et al. [36] with modifications applied for single purified antigen [37] or preparation of conjugated protein and various steroid antigens described previously [38, 39]. Briefly, in the first injection, each rabbit received 200 μg of different porcine antigen supplemented with complete Freund adjuvant. One month later, boosters were repeated four times in 2–3 week intervals with lower doses of antigens (100 μg·booster^{-1}) and incomplete Freund adjuvant.

2.4. In vitro production of porcine endometrial adsorbents

Total endometrial proteins (used as negative controls for antisera adsorption, see below) were prepared by cultures of endometrial explants collected from gilts on Day 17 of pseudopregnancy (PsE) or from gilts on Day 10 of the oestrous cycle (E10). After the cultures, the media were filtered, separated from endometrial explants by centrifugation, similarly as described above for chorionic explants. The endometrial proteins (non-specific for pregnancy) were dialysed to remove the salt components of the medium, concentrated in Centriprep-10 cartridges (MWCO 10 kDa) and stored at –70 °C.

Both porcine chorionic and endometrial protein concentrations were determined by the Bradford procedure, with BSA used as the standard [40].

2.5. Selection of anti-pPAG sera

Two weeks following the fourth booster, blood samples were taken from rabbits by cannulas inserted into the neck artery [37] and allowed to clot overnight at 4 °C. The sera were collected and stored at –20 °C until use. Additional tenth "diagnostic" polyvalent antiserum was created by equal mixture of nine obtained antisera. Crude antisera were initially tested for their titer and bounding of selected antigens (specific for Days 17, 22, 25, 61 and 77 of pregnancy). Next, selected antisera were purified by several adsorptions to remove non-specific (for pregnancy) immunoglobulins (Igs). Non-specific Igs were adsorbed with sliced endometrial tissues (2:1, vol/vol) of luteal-phase gilts, similarly to a profitable method described previously [24, 27]. Moreover, non-specific Ig (for gestation) were adsorbed by endometrial proteins (200–5 000 μg·mL^{-1}) obtained from the culture media. All of the non-specific immunocomplexes for gestation (non-specific Ig with endometrial

proteins of luteal-phase cyclic pigs) were removed from purified antisera by centrifugation and the remaining pellets of immunocomplexes were discarded. Crude antisera (titer 1:300) and purified antisera (some titer was verified up to 1:500) were initially examined by Western dot blotting with lower doses of some chorionic antigens (5 μg·sample^{-1}) and double doses of negative controls – BSA and endometrial proteins (10 μg·sample^{-1}). The adsorption of each antiserum was performed several times, until non-specific Ig (binding of endometrial proteins of non-pregnant animals) had been removed. The adsorption efficiency of each antisera was monitored by Western dot blotting for testing of their non-specific binding to endometrial proteins (10.0, 1.0 and 0.1 μg·sample^{-1}) produced in vitro. Adsorption was completed when the antisera did not show any cross-reactions with endometrial proteins (non-specific for gestation) and then such purified antisera were subjected to immunoblotting of chorionic proteins separated by PAGE analysis. A specificity of anti-pPAG sera was examined by immunoscreening of fusion proteins released by distinct pPAG clones, isolated from a cDNA library, plaque-purified, sequenced and deposited in the GenBank database, as pPAG1, pPAG2, pPAG3, pPAG4 and pPAG6 [1, 4, 5, 12, 13].

2.6. SDS-PAGE and Western analysis of chorionic proteins

Porcine trophectodermal secretory proteins (10 μg·sample^{-1}), produced by different chorionic explants, were denatured in Laemmli buffer in SDS presence, then separated in 12.5% polyacrylamide gels (1D-PAGE) and stained with Coomassie Brilliant Blue dye [40].

Duplicates of separated porcine chorionic proteins were transferred onto nitrocellulose membranes Optitran BA-S 83 (NC). A non-specific binding of the NC membranes was blocked in 5% (wt/vol) non-fat dry milk dissolved in TBST buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20, vol/vol) and then the NC membranes were subjected to immunoblotting. The membranes were initially incubated with polyclonal heterologous antisera (titer 1:300) directed against native bovine PAG (bCG) or ovine PAG [6, 27, 35], which definitely recognised porcine PAG proteins due to high sequence homology of their cDNA and variable epitopes on the surface of the PAG molecules [23]. Finally, duplicates of separated porcine chorionic proteins were blotted with homologous antisera produced against various porcine antigens, and pre-selected during the aforementioned Western dot blotting. After overnight incubation with primary anti-PAG sera at 4 °C, all NC membranes were washed 3 times in TBST buffer and then incubated with a secondary alkaline phosphatase-conjugated anti-rabbit monoclonal IgG (titer 1:150 000) for 1 h at room temperature. The blots were washed again and immunoreactive pPAG proteins were visualised by developing with NBT and BCIP mixture, according to the manufacture's protocol (Promega). Non-specific binding (for gestational proteins) was determined by incubation of the NC membranes with normal rabbit pre-immune serum, as described previously [1].

3. RESULTS

3.1. Heterologous immunodetection of porcine PAG proteins

The high homology of the PAG family of ruminants and pigs allows for the cross-hybridisation of their cDNA [1, 4], thus admitted for the initial heterologous (cross-species) Western immunodetection of porcine PAG proteins with the use of anti-bovine PAG2 and anti-ovine PAG1 sera. These antisera allowed for the helpful

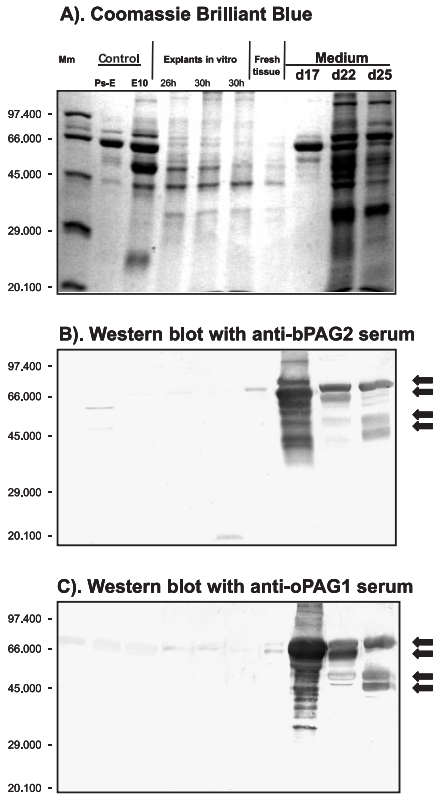

Figure 1. The effectiveness of the pPAG protein isolation monitored by cross-species Western analyses. Trophectodermal proteins isolated from fresh (Day 21 of pregnancy) or cultured tissues (Days 20 and 21) were compared to proteins released in vitro into the media by cultured (8 h) different explants of the trophoblast (Days 17, 22 and 25 of pregnancy) or endometrium of cyclic (E10) and pseudopregnant gilts (Ps-E). (**A**) Stained porcine proteins (10 μg·sample^{-1}) separated in 12.5% gel. Molecular markers (Mm) are shown on the left. Duplicates of proteins transferred onto the membranes were exposed to (**B**) polyclonal antisera raised against bPAG2, or (**C**) to polyclonal antisera raised against oPAG1. The arrows on the right indicate immunoreactive bands of the pPAG-like proteins detected by antisera produced against single purified ruminant PAG proteins.

monitoring of porcine antigens (pPAG) during their isolation (Figs. 1 and 2). Heterologous Western blotting with anti-bovine or ovine PAG sera compared the efficiency of porcine PAG protein recovery from both sources, tissues and various culture media. The higher efficiency of immunoreactive pPAG protein recovery was obtained for proteins secreted by chorionic explants into the culture media, when compared to total cellular proteins isolated from fresh or cultured porcine trophectodermal tissues (Fig. 1). According to this efficiency, various trophectodermal proteins, as porcine antigens required for immunisation, were produced in vitro. Several chorionic proteins (including pPAG) were harvested from the media of cultured trophectodermal explants that had been recovered from the placenta of gilts at different stages of pregnancy (Days 17–77). De novo-synthesised porcine trophectodermal proteins, released by cultured explants into the medium, were the most effectively precipitated by 75% saturation with ammonium sulphate (data not shown). Fractionated pPAG proteins (> 30 kDa) were satisfactorily detected by heterologous Western analysis (Fig. 2). Multiple porcine PAG-related proteins identified as major positive bands of apparent M_r 60–72 kDa were generally immunoreactive to bPAG2 antiserum (Fig. 2B) and were dominantly detected during periods of implantation (Day 17), early placentation (Days 22–25) and the second half of pregnancy (Days 61, 77). Some lower M_r forms of trophectodermal proteins (~45 and ~43 kDa) were immunopositive to oPAG1 antiserum (fewer visible) and were often present during the middle of pregnancy (Days 40, 42 and 61). Additionally, immunoreactive porcine PAG proteins were compared to endometrial proteins produced in vitro and isolated from the media of cultured explants obtained from luteal-phase gilts (E10) and pseudopregnant gilts (PsE). This comparison indicated that during extended exposure to alkaline phosphate substrates (NBT and BCIP), antisera against ruminant PAG antigens exhibited non-specific (for chorionic proteins) binding of several immunoreactive porcine

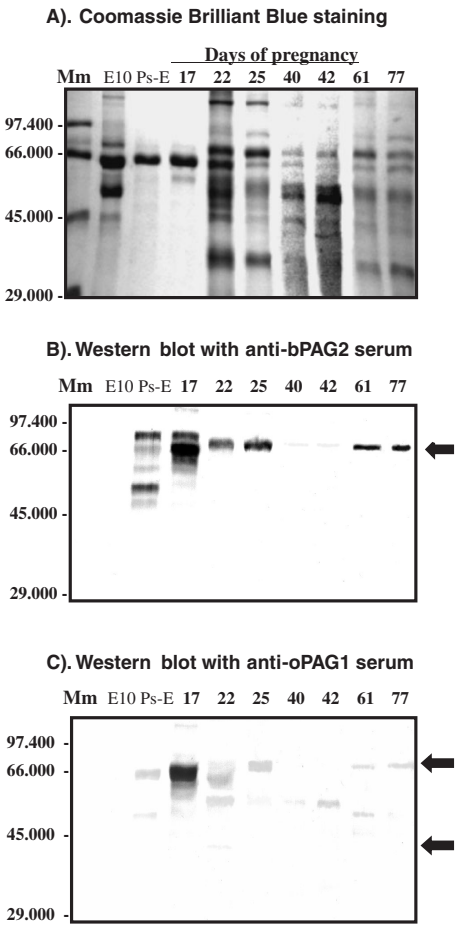

Figure 2. The cross-species immunodetection of the pPAG protein family examined throughout pregnancy in the pig. The SDS-PAGE-Western analysis of pPAG proteins (10 μg·sample^{-1}) released in vitro by trophectodermal explants (Days 17–77 of pregnancy) or by control endometrial explants of cyclic (E10) and pseudopregnant gilts (PsE), in the presence of a molecular marker (Mm). (**A**) CBB-stained proteins separated in 12.5% gel. Duplicates of proteins transferred onto membranes were exposed to polyclonal antisera raised against bPAG2 and oPAG1 proteins (**B** and **C**, respectively). Arrows on the right indicate M_r of the pPAG-like proteins.

endometrial proteins (PsE) obtained from pigs on Day 17 of pseudopregnancy (especially in Fig. 2B).

3.2. Cross-reactions and purification of antisera raised against various porcine antigens

Several porcine trophectodermal secretory proteins produced in vitro and separated according to their masses (> 10 or > 30 kDa) were profitable as native chorionic antigens for the generation of homologous antisera (Fig. 3). Obtained crude antisera were initially diluted 1:300 and examined by Western dot-blot analysis for primary titers and their specificity with different antigens specific for pregnancy, or non-specificity with other (BSA, endometrial proteins) antigens. To remove non-specific antibodies, anti-pPAG sera were purified by multiple adsorption with endometrial tissues or endometrial proteins produced in vitro by tissue explants of cyclic gilts (both used as adsorbents). These non-specific immunocomplexes were removed from anti-pPAG sera and their verified titers were increased up to 1:500 (Fig. 4). Their titer was equal or slightly higher then anti-bPAG sera (1:300) used during heterologous Western blotting (Figs. 1 and 2). However, antisera raised against recombinant pPAG2 proteins (titer, 1:100), prepared on a basis of cDNA in a bacterial system (anti-RpPAG2), were immunologically very weak due to post-translational glycosylation of native pPAG proteins (Figs. 3I and 4I). Adsorption of different pPAG sera was completed when the antisera did not bind endometrial proteins or BSA, both non-specific for gestation (Fig. 4). Finally, pregnancy-non- specific binding (to BSA) of anti-d17 sera (Fig. 4A) and polyvalent antisera (Fig. 4J) has also been removed by additional adsorptions (data not shown). All endometrial proteins introduced into adsorbed antisera (during the purification procedure) were removed and did

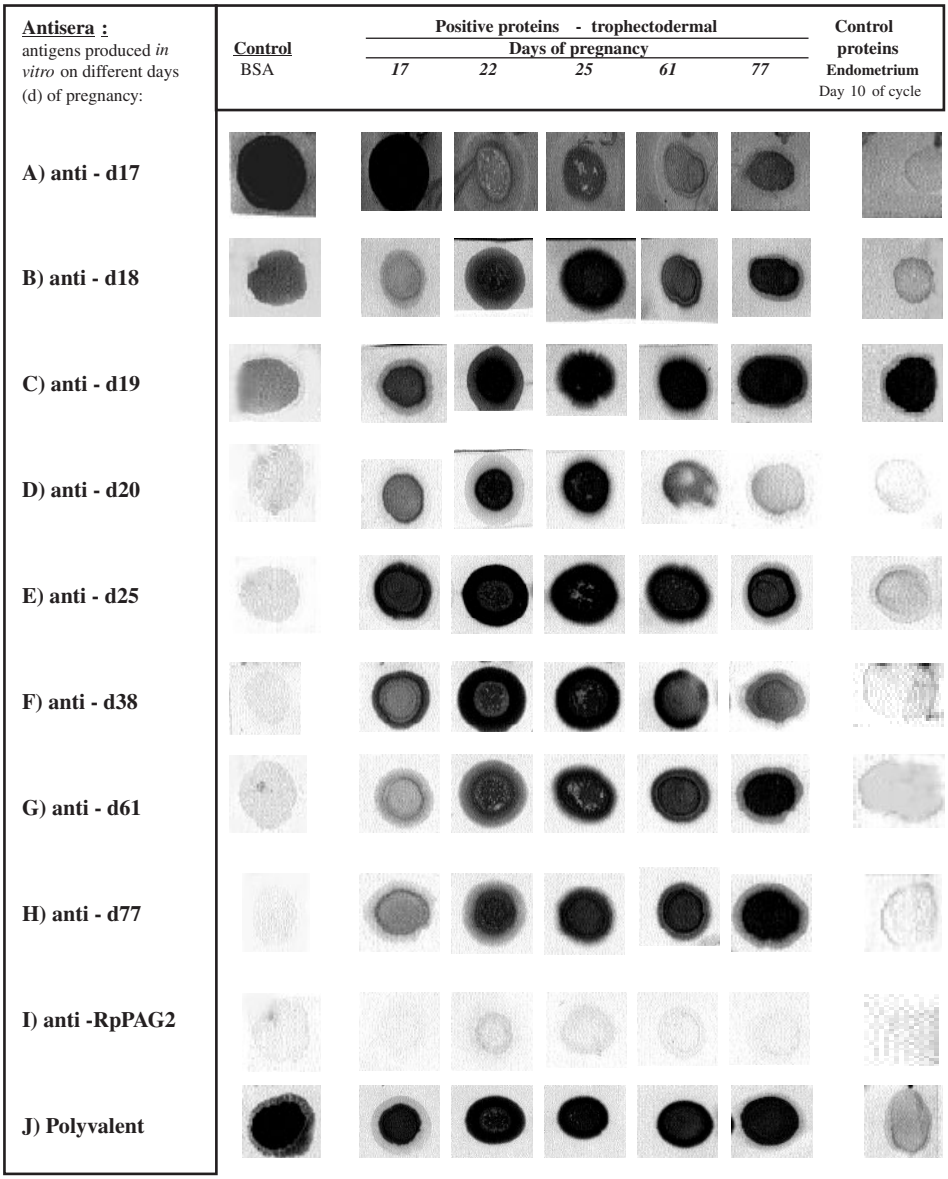

Figure 3. Western dot-blot analysis of the pPAG proteins produced in vitro (5 μg·sample^{-1}) or control proteins (BSA and END10) with crude polyclonal antisera (in titer 1:300) obtained from rabbits immunised against different porcine antigens: native trophectodermal proteins (**A–H**), or recombinant pPAG2 protein (**I**) and mixed "diagnostic" polyvalent antisera (**J**).

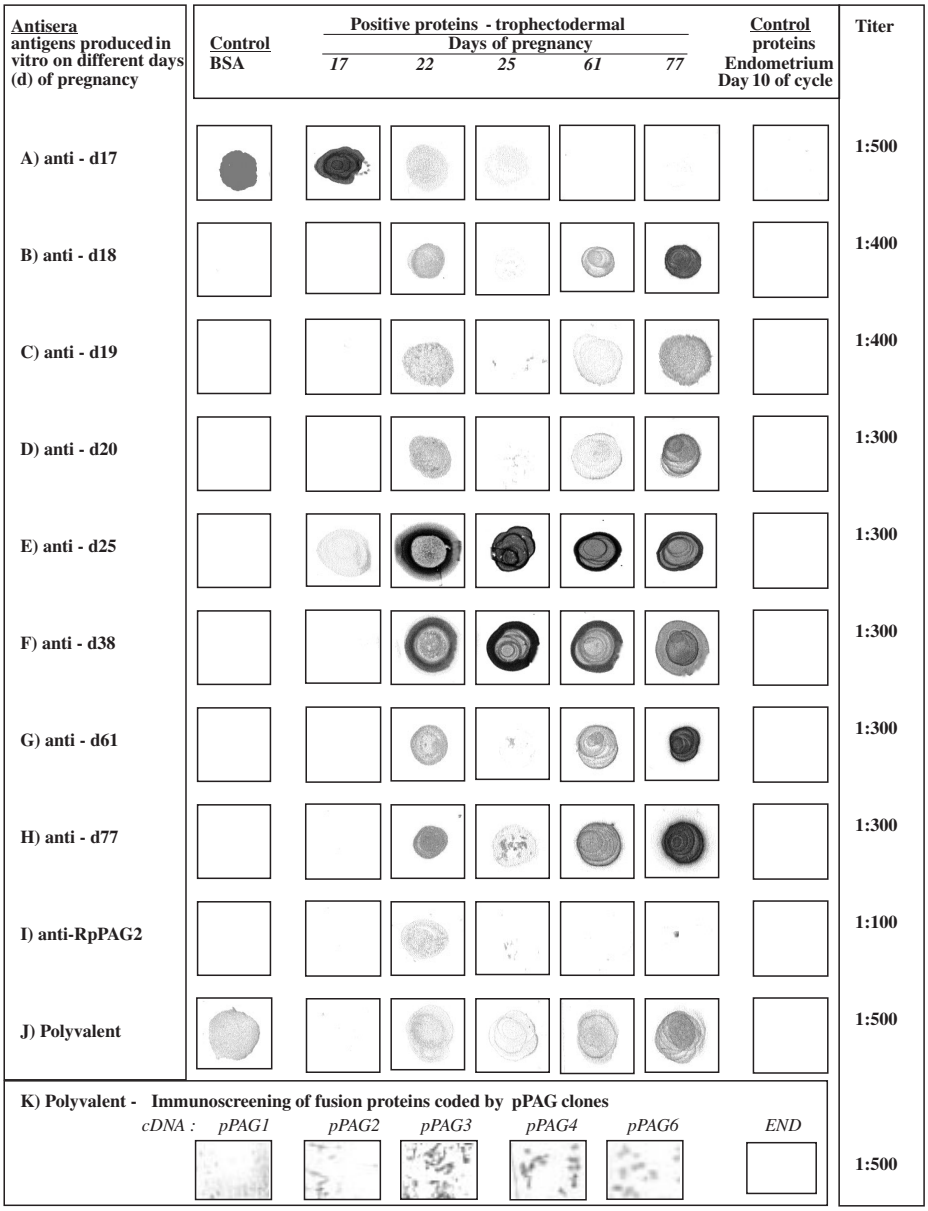

Figure 4. The cross-reactions of adsorbed polyclonal antisera (**A**–**J**, in titers from 1:100 up to 1:500) with different (5 μg·sample^{-1}) porcine native trophectodermal proteins produced in vitro (from Days 17, 22, 25, 61 and 77) or control proteins (BSA and END10) analysed by Western dot-blotting. Unspecific bounding of each antiserum has been removed by adsorption with endometrial tissues or endometrial proteins produced in vitro. (**K**) Specificity of purified polyvalent anti-pPAG serum identified by immunoscreening of various pPAG cDNA clones isolated from a porcine trophoblastic cDNA library in contrast to the lack of cross-reactions to endometrial cDNA library.

not effect positive (chorionic) antigen blotting on nitrocellulose membranes. The results of Western dot-blots obtained after several adsorptions have proven the effectiveness of the pregnancy-specific antisera purification procedure applied (compare Figs. 3 and 4).

Purified anti-porcine sera (by removing of antibodies to antigens non-specific for gestation) contained remaining immunoglobulins specific for trophectodermal proteins. The specificity of anti-pPAG sera was examined by immunoblotting of fusion pPAG proteins released by several clones isolated from the cDNA library. These pPAG clones, plaque-purified and their cDNA sequenced (deposited in the GenBank database), definitely represented pPAG proteins. These immunoscreening data provide strong evidence for the binding of pPAG proteins by our polyvalent anti-pPAG sera (Fig. 4K). Possibly, our anti-pPAG sera can also bind some other pregnancy-specific proteins, however such chorionic proteins (similar in M.W.) have still not been identified or characterised in the pig.

3.3. Heterogeneity of pPAG proteins throughout pregnancy examined by PAGE and homologous Western blotting

Antisera purified by adsorption were tested by 1D-PAGE-Western analysis for their cross-reactivity with various separated porcine trophectodermal proteins produced in vitro on different days of pregnancy and compared to negative control endometrial proteins (from cyclic and pseudopregnant pigs) that are not pregnancy-specific (Figs. 5 to 8). The de novo-synthesis of porcine trophectodermal proteins released by cultured explants into the medium (Fig. 5) was confirmed by the results obtained during long-term studies (9–239 h) with anti-pPAG sera (anti-d25 and anti-d38). Secretory trophectodermal proteins identified by homologous Western blotting with antisera raised against porcine antigens (anti-pPAG sera: anti-d17, -d18, -d19, -d25, -d38, -d61 and anti-d77) were very similar in their M_r to forms of pPAG proteins detected by heterologous Western blotting with anti-bPAG sera, raised against purified ~67 kDa electrophoretically homogenic bovine PAG protein (compare Figs. 1 and 2 and Figs. 6 and 7). Homologous Western blotting with each anti-pPAG serum revealed single or multiple immunoreactive forms of trophectodermal proteins throughout porcine gestation. Only anti-d17 sera, raised against porcine antigens produced in vitro by trophoblastic explants on Day 17 of pregnancy, mainly recognised the immunogen used, with M_r of ~68 kDa, however, some other proteins were also weakly detected (Fig. 6A). Other antisera (except anti-d25) did not bind trophoblastic proteins specific for Day 17 of pregnancy (Figs. 6 and 7). The multiple pPAG forms with lower M_r were identified by anti-d18 serum and their forms had increased expression with the progress of pregnancy (Fig. 6B). Serum anti-d19 detected two forms of proteins, ~68 and ~43 kDa (Fig. 6C). Serum anti-d25 recognised one major group of proteins (68–70 kDa) that were present in all of the samples, although some minor ~95 and 35 kDa proteins were also detected (Fig. 6D). Antisera anti-d38, -d61, and -d77 bound two groups of immunoreactive proteins, a larger dominating form of ~70 kDa, and multiple smaller proteins possessing a M_r close to 35–45 kDa. The latter M_r forms increased with the progress of pregnancy (Fig. 7A–C).

All separately produced and purified polyclonal antisera were mixed together, and a polyvalent serum was created for general diagnostic purposes of various trophectodermal protein examination throughout pregnancy (Fig. 8). According to the results obtained with each antiserum (compare Figs. 4 and 6A), such “diagnostic” mixed polyclonal/polyvalent anti-pPAG serum allowed the comparison of molecular masses of several pregnancy-time dependent trophectodermal proteins during the same

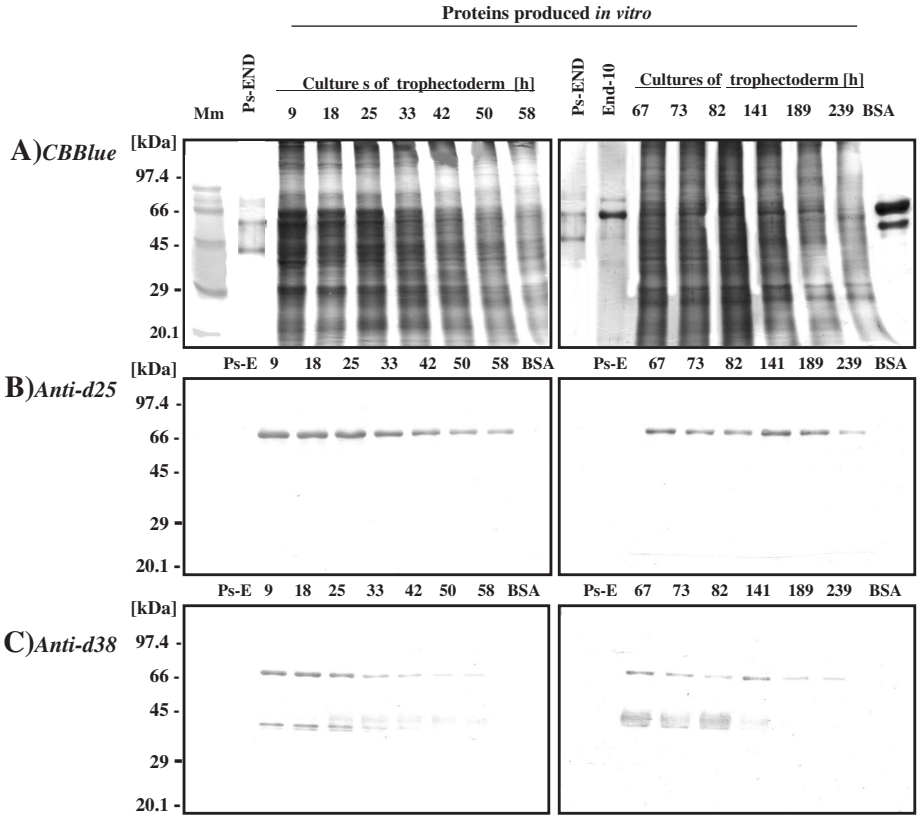

Figure 5. The heterogeneous products of the pPAG gene family examined during long-term studies of trophectodermal explants harvested on day 24 of pregnancy and cultured for 239 h. (**A)** CBB-stained proteins (1D-SDS-PAGE) separated in 12.5% gel. Secretory proteins produced in vitro (10 μg·sample^{-1}) by trophectodermal explants during 9–239 h and negative control proteins: endometrial explants of cyclic (End10) and pseudopregnant (Ps-END) gilts or BSA (non-specific for pregnancy) were compared. (**B-C)** Duplicates of proteins were transferred onto membranes that were blotted with different antisera raised against porcine antigens (anti-d25 and anti-d38). Molecular markers (Mm) are on the left.

blotting (Fig. 8). This polyclonal/polyvalent anti-pPAG serum indicated the difference in M_r of dominant proteins: ~43 kDa specific for implantation (Day 16) with a sensitivity of 5–10 μg (Fig. 8B), ~68.1 kDa specific for early pregnancy (Days 17–25) and ~72.3 kDa for later stages (Days 31–77) of pregnancy (Fig. 8C and D). During early placentation times (Days 20 and 25), some additional big immunoreactive proteins (~97 kDa), probably dimers, were observed (Fig. 8C). The second group of smaller pPAG proteins (from ~35.4 up to ~47.2 kDa) was not generally detected during placenta formation but they markedly increased from Day 38. Their M_r varied up to ~11.8 kDa (difference between 35.4 and 47.2 kDa) on Day 77 of pregnancy (Fig. 8D).

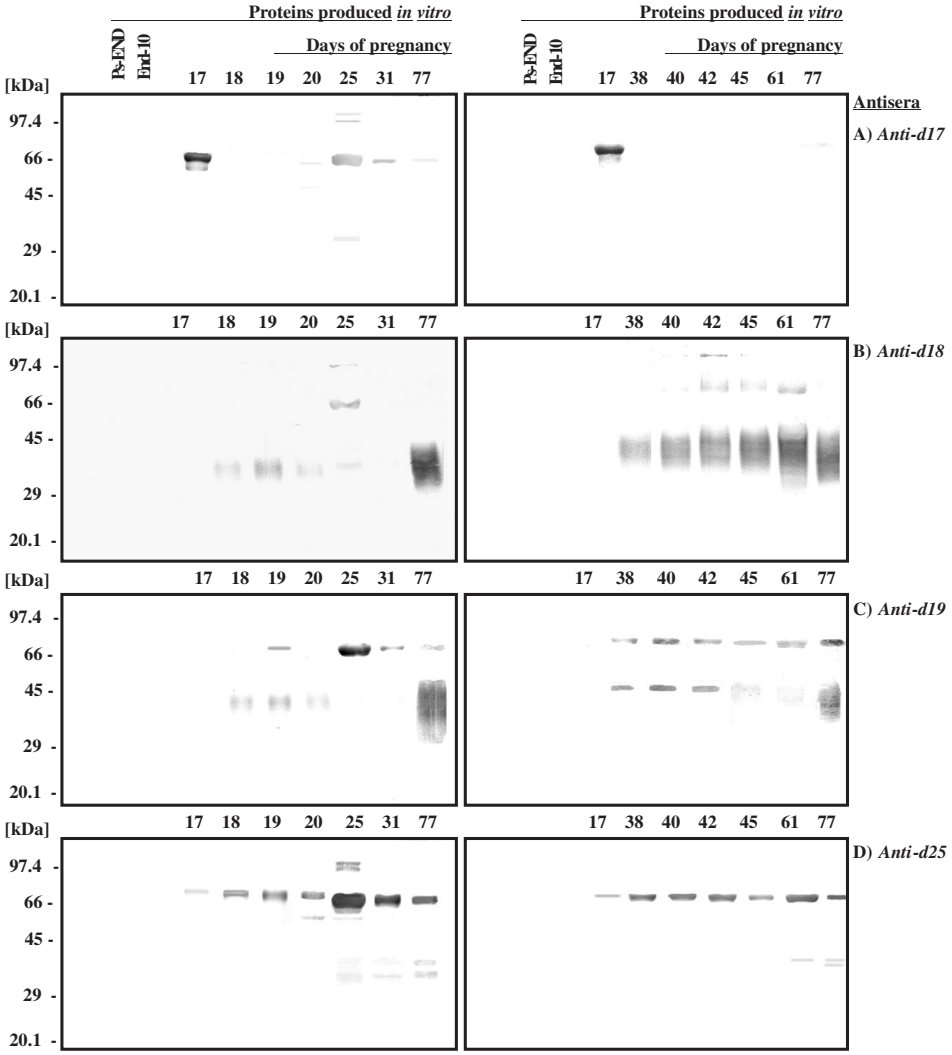

Figure 6. The heterogeneous products of the pPAG gene family examined during various stages of embryonic and foetal developments throughout pregnancy. (**A–D)** Western blot (1D-SDS-PAGE) of secretory proteins produced in vitro (10 μg·sample^{-1}) by trophectodermal explants (Days 17–77) and control endometrial explants of cyclic (End10) and pseudopregnant (Ps-END) gilts. Membranes were blotted with different purified antisera raised against various porcine immunogens (anti-d17, anti-d18, anti-d19 and anti-d25 sera). Molecular markers (Mm) are on the left.

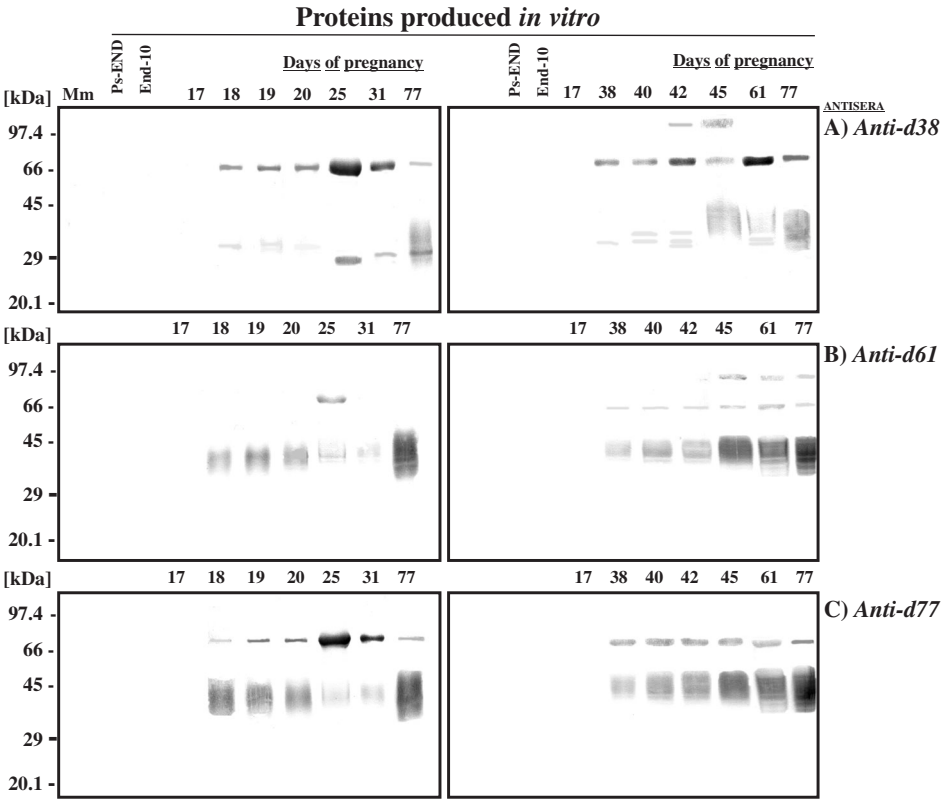

Figure 7. The heterogeneous products of the pPAG gene family examined during various stages of embryonic and foetal developments throughout pregnancy. **(A–C)** Western blot (1D-SDS-PAGE) of secretory proteins produced in vitro (10 μg·sample^{-1}) by trophectodermal explants (Days 17–77) and control endometrial explants of cyclic (End10) and pseudopregnant (Ps-END) gilts. Membranes were blotted with different purified antisera raised against various porcine immunogens (anti-d38, anti-d61 and anti-d77 sera). Molecular markers (Mm) are on the left.

4. DISCUSSION

This paper presents the heterogeneity (M_r from ~35 up to ~72 kDa) of the porcine pregnancy-associated glycoprotein family (pPAGs) produced in vitro throughout embryonic and foetal development in the pig. These chorionic proteins revealed similar molecular masses during initial hetero- and final homologous immunodetection (antisera against bovine and porcine antigens, respectively). The antisera against ruminant PAG (raised against purified and sequenced cotyledonary PAG proteins as immunogens) allowed for primary heterologous (cross-species) detection that definitely indicated several forms of porcine PAG proteins, which were required for porcine antigen preparation (Figs. 1 and 2). These heterologous anti-bPAG and anti-oPAG sera indicated that the production of the pPAG antigens, as proteins secreted in vitro by porcine chorionic tissues into the media, was a more effective method of protein harvesting in comparison to limited amounts of pPAG proteins remaining in these tissues

(Fig. 1). Previously, anti-bPAG2 sera recognised several purified PAG molecules with apparent masses ranging from 45 kDa to 70 kDa [27] and were also used to discover bPAG2 cDNA (as fusion proteins) during immunoscreening of the bovine cDNA library [7]. Similar immunoscreening employed with anti-bPAG1 antisera, permitted the identification of the bPAG1 precursor [6], which shares 76% of identity to bPAG4 [10]. Unfortunately, even such antisera raised against single purified native PAG antigens (bPAG1, bPAG2) or recombinant proteins (oPAG1), were able to bind several related forms of oPAG proteins, purified by chromatography from the media of cultured ovine cotyledon explants [41]. Cross-reacting antigens for the anti-bPAG and anti-oPAG sera were not detectable in non-pregnant animals; both anti-PAG sera have been used previously for routine pregnancy detection in various ruminants (see below). In

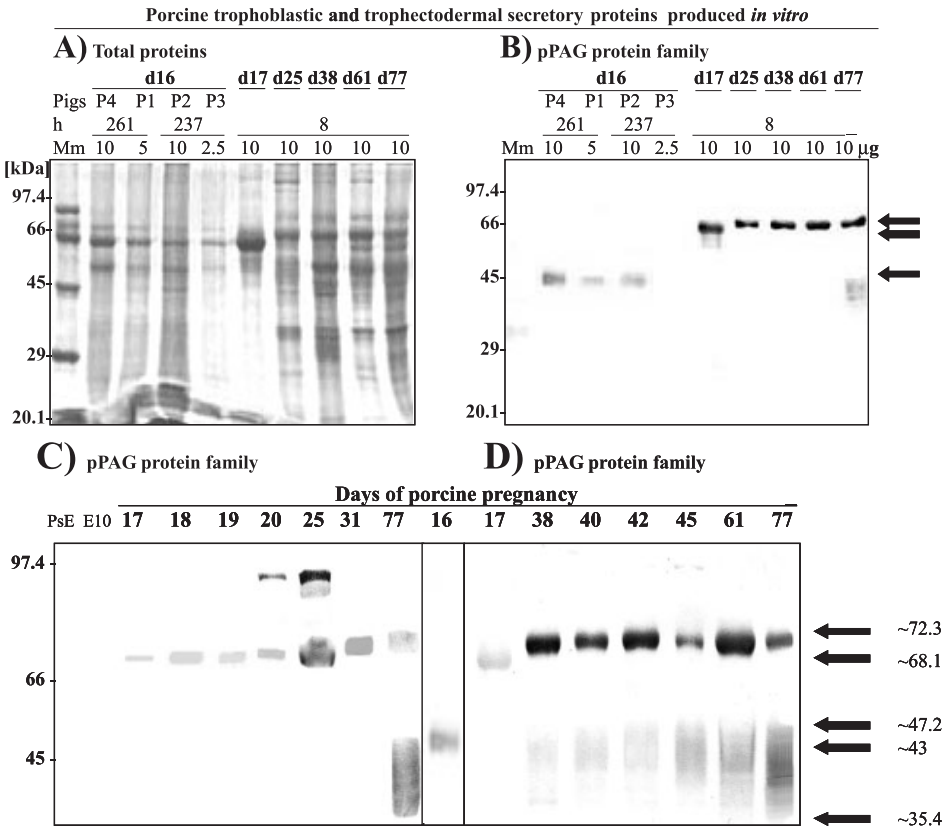

Figure 8. Production efficiency and immunodetection sensitivity of total proteins (2.5–10 μg·sample^{-1}) secreted in vitro by trophoblastic explants (237–261 h) recovered from pigs on day 16 (P1–P4) and by trophectodermal explants (8 h) recovered on various days (d17–d77) of pregnancy. (**A**) SDS-PAGE of total chorionic proteins stained by CBB dye. (**B–D**) Western blot of secretory pPAG proteins during early embryonic and foetal development throughout porcine pregnancy detected by polyvalent "diagnostic" anti-pPAG serum. Arrows on the right indicate apparent M_r (kDa) of the pPAG proteins.

the present study, the same heterogeneous antisera were also able to recognise similar immunological epitope(s) of PAG proteins in the pig. However, these anti-ruminant PAG sera were able to recognise antigens that are not pregnancy-specific, such as porcine endometrial proteins of pseudopregnant pigs (Fig. 2), thus the production of homologous antisera against various porcine antigens was required to make progress in the study of the pPAG protein family.

Presently produced homologous antisera (with porcine immunogens), purified by multiple adsorption (Figs. 3 to 4), allowed for the detection of various products of the diverse pPAG gene family expression during embryonic and foetal development in the pig (Figs. 5 to 7). The adsorption-purification procedure appropriately eliminated immunoglobulins that are not porcine pregnancy-specific: several antibodies against antigenic proteins dominantly expressed in uterine endometrial tissue and antigens expressed in both trophectodermal and endometrial tissues, e.g. anti-uteroferrin, anti-serpin, anti-Kunitz-type proteinase inhibitors, anti-lysosyme or anti-retinol binding proteins (see: [1, 4, 5, 29–33]). The M_r of immunoreactive porcine trophectodermal proteins produced in vitro (35–72 kDa), examined with the use of newly produced anti-pPAG sera (specific for pPAG fusion proteins during immunoscreening of cDNA clones), were very similar to forms of pPAG proteins detected initially by antisera against heterologous ruminant PAG antigens. The profile of the pPAG proteins in vitro secretion was pregnancy-stage-dependent. The polyclonal/polyvalent, mixed diagnostic antisera, provided direct evidence of various M_r (~43 and ~68.1 kDa) during implantation, and a larger group of pPAG proteins (~72.3 kDa) during placentation and as pregnancy advanced (Fig. 8).

Porcine PAG proteins, known also as the pBP-porcine basic proteins, have been identified as major secretory multiple 43–70 kDa proteins dominating during early pregnancy [29, 31], which are very similar in size to pBP proteins immunodetected in the serum of a fetal pig at Day 60 of gestation [30]. Unfortunately, only one dominant native ~43 kDa pBP protein has been purified and subjected to N-terminal micro-sequencing [33]; it appears to be an internal fragment of the pPAG2 precursor [1]. Moreover, presently identified ~43 kDa (Day 16) and additionally ~68.1 kDa (Days 17–20) pPAG proteins detected during implantation time (Fig. 8) possibly represent the processing of the pPAG family precursors (~72.3 kDa), by removal of their propieces, similar to the proteolytic degradation of the pepsinogen family into active pepsins. Most of the pPAG precursors are at first reduced by 15 amino acid (aa) signal peptides (1.66 kDa), and then by a 37 aa-propiece that is identical (4.38 kDa) in pPAG2, pPAG4 and pPAG6 to several smaller presently immunodetected secretory forms of native pPAG proteins. The N-terminus of the pBP protein [33], allowed for identification of a reduction of pPAG2-like precursors (by removal of 52 aa), and a N-terminal LSK sequence of three so far identified, pPAG2-like precursor subfamilies [1, 4, 12]. However, it seems that purified pBP [33] represented another similar but distinct member of the pPAG2-like protein, because their Asp was substituted to Val in pBP [1, 4, 12]. Although, the N-terminus of mature pPAG2-like proteins begins with the LSK sequence, the possible N-terminus of mature pPAG1-like proteins is still unknown because a similar sequence is not present in the pPAG1-like subfamily, including pPAG1, pPAG3 and pPAG5 [1, 13, 14]. We can, however, presume that members of this subfamily can be similarly processed to other members of the pPAG precursor family.

The post-translational processing of PAG precursors by proteolytical degradation provides different forms of mature PAG proteins. In sheep, oPAG proteins of ~70 kDa have been autoradiographically indicated to be precursors of smaller forms of 53–61 and 47 kDa [6, 9], while the

calculated masses of oPAG3 and oPAG7 polypeptide portions of precursors are 42.49 and 42.93 kDa, respectively [10, 41]. Similarly in the pig, several pPAG proteins which have M_r of ~72 kDa, appear approximately twice as big in mass as the calculated peptide portion of pPAG2-like precursors, possessing 37.52 kDa [1, 12]. The common size of the propiece is 38 aa in the oPAG precursors and the relatively conserved N-terminus of mature oPAG proteins starts with the RG/DS sequence [41]. A similar RGD tri-peptide sequence, such as the major binding sites, is present in a variety of integrin ligands [42]. The family of integrins belong to cell adhesion receptors and can play an important role in early embryonic attachment [43, 44]. During implantation, embryonic differentiation is also connected with changes in the extracellular matrix pattern [45]. However, the relatively conserved tri-peptide sequence R G/D S in bovine PAG proteins is highly variable in other species. In the horse, micro-sequencing has indicated two cleavage sites (stronger at T^{34} and the second between L^{22} and K^{23}), suggesting two different propieces (33 and 22 aa) in identified so far ePAG [16]. A similar underestimation occurred in the oPAG family, because the N-terminus of one purified native oPAG (65 kDa) protein indicates full identity to the internal fragments of both oPAG3 and oPAG7 precursors, encoded by distinct cDNA with 84% sequence identity over their full lengths [10, 41]. Thus, our results obtained in the pig and the cited results demonstrate the diversity of the purified PAG protein family from the placenta, since multiple products of the numerous PAG gene family are expressed at different stages of pregnancy in several species.

Presently, it remains unknown, whether the heterogeneity in the M_r of the PAG family is entirely due to the degradation of their distinct precursors or/and to post-translational precursor processing, involving carbohydrate chain modifications. The PAG epitope(s) can correspond partially to carbohydrate chains [26] and partially to seven hypervariable loops exposed on the surface of the PAG molecules [23]. These hypervariable regions represent surface domains, where occurring amino acid substitutions in different members of the PAG family [10, 12] can have some influence on the structural surface integrity of PAG molecules. In sheep, the number of the oPAG family members was enlarged by the use of the monoclonal SBU-3 antibody, recognising carbohydrate chains, unique to the trophectoderm only, with bi-, tri- and tetra-antennae structures [26]. Their micro-sequences are only partially similar to other identified oPAG proteins [41]. Different numbers of potential N-glycosylation sites (N-x-S/T) have been found in sequences of the PAG precursors: up to seven in ruminant PAG [6, 7, 10] and at least three in porcine PAG [1, 4, 12]. However, only one potential N-glycosylation site, located on the C-terminus, is present in equine PAG, which does not occur in zebra PAG [16]. In the pig, the multiple pPAG proteins have been shown as relatively variable in their M_r (Figs. 1, 2 and 5 to 8), possibly also due to their glycosylation that is presently under intense examination.

Previously, a similar method of crude PAG antisera production and their purification was utilised. Initial polyclonal antisera against PAG/PSPB proteins were obtained by injecting rabbits with extracts of cotyledons or crude placental proteins and then non-specific antibodies were removed by their adsorption with endometrial tissues of non-pregnant cows [24, 27]. After such purification, antisera were very useful for the isolation of different PAG/PSPB proteins that were employed for the measurements of PAG proteins in the blood circulation of some domestic ruminants [25, 28]. Several forms of the purified native bPAG/bPSPB proteins were recognised as being antigenically unrelated to alpha-fetoprotein, fetuin, bovine placental lactogen [24], BSA, and human pregnancy-specific beta 1 glycoprotein [27]. These initial antisera were helpful for the isolation of several

distinct PAG/PSPB/SBU-3 proteins (M_r from 31 up to 90 kDa). These proteins, isolated from placental tissues of the goat [46], zebu [47], moose and elk [48] represent mostly PAG precursors, while proteins produced in vitro and isolated from the media represent dominating secretory forms of the mature PAG protein family in cattle [7] and sheep [9, 41]. Among many purified native placental PAG proteins and their multiple Ip variants, only a few of them – especially very limited in amounts during early pregnancy – were obtained in the values required for N-terminal sequencing [26, 33, 41, 46]. But, only two micro-sequence fragments of native PAG-like proteins, oPAG (65 kDa) and pBP (43 kDa), corresponded to the sequences of PAG precursors: oPAG3 or oPAG7 and the pPAG2-like subfamily, respectively. However, the purified native PAG proteins, mostly bovine and ovine PAG, have been applied with different cross-reacting anti-PAG sera for radioimmunologic assays (RIA) as routine diagnostic tests of pregnancy in domestic ruminants [25, 28, 49, 50], as well as in various wild ruminants, including the bison [51], moose [52], elk [53], Alpine goat [54] and several deers [55–57]. These RIA tests were also used to recognise twins or single pregnancy [58] and the sex of the fetus [59]. The pregnancy diagnosis by RIA tests, mainly performed with bovine antigens (as standard and tracer) in combination with different heterogeneous or homologous antisera, provided up to 99% of accuracy [60]. This high rate of early determination of pregnancy status has obvious economical advantages.

In conclusion, the pPAG-proteins were initially identified as major positive bands (35–72 kDa) by polyclonal antisera raised against ruminant PAG antigens. This is the first paper reporting the heterogeneity of pPAG proteins that were immunodetected by different homologous antisera raised against multiple porcine antigens produced in vitro. The pPAG proteins specific for early pregnancy correspond to processed forms of the pPAG precursor family. These processed and glycosylated heterogeneous multiple immunopositive forms of pPAG proteins can have an important role during embryonic and foetal development in the pig, especially during implantation, when the trophoblast grows faster than any other tissue, even the fastest growing tumors.

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