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Multiple Pregnancy-Associated Glycoproteins Are Secreted by Day 100 Ovine Placental Tissue[1]

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ABSTRACT

Pregnancy-associated glycoprotein (PAG)-1 (PAG1) and pregnancy-specific protein B are either identical or closely related antigens released by trophoblast binucleate cells of placentas of cattle. Sheep and other ruminants produce similar products. There is evidence, however, that these antigens, which are related structurally to the pepsinogens and other aspartic proteinases, are not single gene products but members of an extensive family. Here, the sequential use of ammonium sulfate precipitation and Sepharose blue, anion-exchange, and cation-exchange chromatographies, as well as isoelectric elution from a Mono P column, has allowed several PAG1-related molecules to be purified from the medium after culture of explants from Day 100 sheep placentas. Each of these PAGs cross-reacted to a varying extent with a panel of three different anti-PAG1 antisera. Four of them, all of which were major secretory products of the placenta, were subjected to amino-terminal microsequencing. Although each was related to ovine (ov) PAG1, none was identical. Reverse transcription-polymerase chain reaction was then used to amplify PAG1-related cDNA from Day 100 placental RNA. Seven novel full-length cDNA, all distinct from ovPAG1, were identified from 25 cDNA selected for sequencing. Only two of these (ovPAG3 and ovPAG7) encoded polypeptides identical in sequence at their inferred amino termini to one of the PAGs ($ovPAG_{65}$) purified from explant cultures. Even so, they were only 84% identical in overall sequence. The remaining five cDNA were unique. In situ hybridization analysis revealed that expression of ovPAG3 and ovPAG7, like that of ovPAG1, is confined to trophoblast binucleate cells. The data confirm that at Day 100 of pregnancy the ovine placenta produces many different PAGs, which differ considerably in sequence and immunological cross-reactivity.

INTRODUCTION

Pregnancy-associated glycoprotein (PAG)-1 (PAG1) [1], also known as pregnancy-specific protein B (PSPB) [2], was identified by two different groups of investigators who used essentially comparable approaches in their experiments. Both obtained an initial antiserum by injecting rabbits with a crude mixture of bovine placental proteins and by adsorbing out nonrelevant antibodies on tissue extracts and red blood cells from nonpregnant cows. The resulting preparations were used to monitor the purification of the pregnancy-associated antigens themselves from placental extracts, which in turn were employed to generate more specific "second-generation" antisera useful for detection of pregnancy in cattle [3–6]. In the case of the pioneering studies of Sasser and colleagues, it has become clear that the PSPB used to immunize the rabbits was only partially purified [2]. In addition, a series of molecular weights have been published, ranging from ~50 000 [2] to 78 000 [3], and Western blotting has been reported to show several bands of immunoreactive protein [5]. Independently, Mialon et al. [7, 8] described a 60-kDa placental protein that was also pregnancy specific and presumed to be distinct from PSPB. The PAG1 antigen purified by Zoli et al. [1] had a molecular weight of 67 000 and appeared pure as judged by Edman sequencing. Nevertheless, it was composed of several isoelectric variants believed to be the result of a varying content of sialic acid. Moreover, the amino-terminal sequence obtained was similar, but not identical, to that inferred from the bovine (bo) PAG1 cDNA described below.

A cDNA for boPAG1 was cloned [9] by screening a placental library with the antiserum prepared by Zoli et al. [1], but the same procedure also identified a less common cDNA encoding a related polypeptide too distinct in sequence to be an allele of PAG1 [10]. At about the same time, an antiserum raised against yet another placental protein identified a third bovine PAG [11], and Southern genomic blotting strongly suggested that there were multiple PAG genes in cattle [10]. Most surprising was the discovery that the PAGs were members of the aspartic proteinase gene family and were closely related in structure to the pepsins [9–11]. Despite this similarity to a proteolytic enzyme, mutations within or near the active sites of bovine PAG would likely have rendered the bovine PAG catalytically inactive [9, 12].

PAG-related antigens have been reported in several ruminant ungulate species other than cattle [13–17], and PAG cDNA have been cloned from both the sheep [9, 18], a ruminant, and the pig [19], a monogastric species. In both the latter species, at least two distinct cDNA were identified.

Together, the results outlined above suggest that the PAGs may exist as a more extensive family than originally suspected and that the various antisera that have been generated have not necessarily been equivalent in cross-reactivity. Such suspicions have been strengthened by the observation of Atkinson et al. [20], who examined the specificity of the monoclonal antibody SBU-3, which had been selected on the basis of its ability to stain ovine trophoblast binucleate cells. This antibody recognizes a carbohydrate epitope of as yet unknown structure but was also able to form immune complexes with several different proteins in placental extracts that had amino-terminal sequences similar to that of ovine (ov) PAG1. OvPAG1 was not itself immunoprecipitated.

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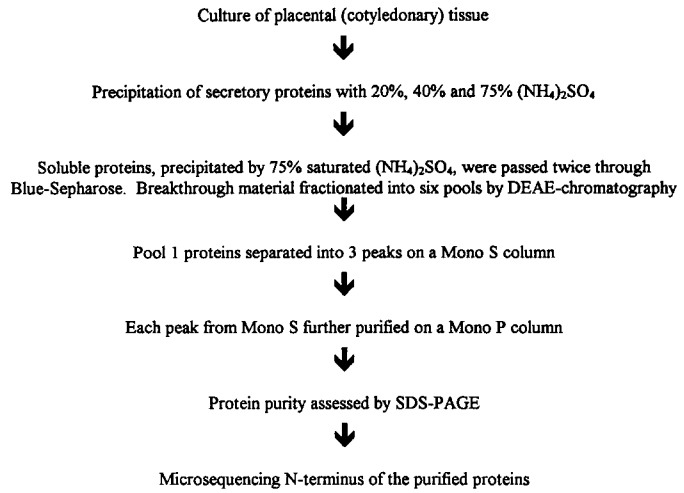

FIG. 1. A schematic outline of ovPAG purification. Secretory proteins from Day 100 ovine placenta-conditioned medium were first precipitated with ammonium sulfate. The material collected in the 40–70% ammonium sulfate fraction was dialyzed in 10 mM Tris (pH 8.0), concentrated, and further analyzed by DEAE-Sepharose chromatography. The fractions containing materials cross-reactive to anti-PAG antisera were pooled and further separated on Mono S and Mono P HPLC columns. The purified ovPAGs were sequenced at their amino termini.

The present experiments were conducted to verify that multiple kinds of PAGs are secreted by the sheep placenta. They were also designed to provide an estimate of the amounts of PAG protein secreted, since the frequency of PAG transcripts in cDNA libraries, including those from the pig [19], seemed unusually high [9, 11, 21]. Finally, it was of interest to know whether the complement of PAGs produced at Day 100 of the 145-day pregnancy were among those already identified in the preimplantation period [18], in midpregnancy [20], and closer to term [9].

MATERIALS AND METHODS

Culture of Placental Tissue

Uteri were removed surgically from three Day 100 pregnant mixed-breed ewes and dissected open to expose the placenta in a tissue culture hood. Cotyledons were removed from the placenta as cleanly as possible and minced into small pieces (~5 mm^3). These explants were washed three times in Dulbecco's Modified Eagle's medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.5 μg/ml) and cultured in the same medium at 37°C under 5% CO_2:95% air for 8 h. A total of 852 g wet tissue was cultured in a total of 5 L of DMEM. The tissue and medium were separated by centrifugation after 8 h of incubation.

Purification of ovPAG from Cotyledon-Conditioned Medium

The purification of ovPAG is shown schematically in Figure 1. All procedures were carried out at 4°C except high-performance chromatography, which was performed rapidly at room temperature. Between purification steps, samples were stored at −20°C in buffers containing a cocktail of proteinase inhibitors (0.5 mM EDTA, 0.5 mM PMSF, and 0.4 μg/ml leupeptin). Before the beginning of each chromatographic procedure, all samples were dialyzed against the start buffer used to equilibrate the column and concentrated in Centriprep-30 cartridges (cutoff 30 kDa; Amicon, Beverly, MA). Protein concentrations were determined by the Bradford procedure [22].

$(NH_4)_2SO_4$ precipitation. Proteins from 4 L of the conditioned medium were precipitated sequentially by addition of $(NH_4)_2SO_4$ to 20%, 40%, and 75% saturation. The pelleted proteins were resuspended and dialyzed against 10 mM Tris-HCl, pH 8.0. The majority of the protein (11.48 g) was precipitated between 40% and 75% saturated $(NH_4)_2SO_4$, while smaller amounts were recovered at 20% (290 mg) and 40% (5.8 g) $(NH_4)_2SO_4$. OvPAGs detected by immunoblotting and ELISA were present predominantly in the 40% to 75% $(NH_4)_2SO_4$ fraction.

Blue Sepharose chromatography. A portion (405 mg) of the protein that had been precipitated at 75% $(NH_4)_2SO_4$ saturation was applied to a Blue Sepharose column (20 × 1.3 cm; Sigma Chemical Co., St. Louis, MO) that had been equilibrated with 0.1 M sodium acetate, pH 7.0. This procedure was employed in order to remove much of the albumin present in the culture medium. The unabsorbed, breakthrough fractions (243 mg) contained all of the immunoreactive materials and were pooled for further purification of PAG. Proteins bound to the Blue Sepharose were eluted with a buffer containing 0.5 M NaCl in 0.10 M sodium acetate, pH 4.0.

Anion-exchange chromatography. The unabsorbed material from the Blue Sepharose column was loaded onto an open DEAE-Sepharose column (2.5 × 92 cm; Pharmacia, Kalamazoo, MI) that had been equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was washed extensively with the same buffer until the 280-nm absorbency reached baseline. Proteins were eluted from the column by a combination of a gradient of NaCl in 10 mM Tris, pH 8.0. Step 1 consisted of elution with a 1-liter linear gradient (1 mM to 100 mM NaCl). This was followed by washing of the column with three bed volumes of 100 mM NaCl. Step 2 was a 1-liter linear gradient (100–200 mM NaCl) followed by a wash with three bed volumes of 200 mM NaCl. In the final step, a third linear gradient (200–300 mM NaCl) was used. The column was then washed with 300 mM NaCl as described above. The eluate was collected in 9.5-ml fractions, monitored for absorbance at 280 nm, and assayed for the presence of PAG by ELISA. Immunopositive fractions were combined into 5 pools according to their absorbance at 280 nm and immunoreactivity. Only the analyses of pool 1 (D1) and pool 3 (D3) (see Figs. 2 and 3) are reported here.

Cation-exchange HPLC. The DEAE pools were separately analyzed by chromatography on a Mono S HR5/5 column (Pharmacia). The column was washed with 20 mM sodium acetate (pH 5) at a flow rate of 1 ml/min for 30 min. The proteins were then eluted by using a linear gradient (0–1 M NaCl in 20 mM sodium acetate, pH 5) at a flow rate of 1 ml/min. The fractions (1 ml/tube) were collected and assayed for ovPAG by using an ELISA or Western blotting.

Chromatofocusing HPLC. Immunoreactive pools from the Mono S column were subjected to isoelectrofocusing on a Mono P HR5/20 column (Pharmacia). The column was equilibrated with start buffer (0.025 M *bis*-Tris, pH 6.3). Proteins were eluted with polybuffer 74, pH 4.0 (Pharmacia), at a flow rate of 0.5 ml/min. Each protein peak was analyzed by Western blotting after proteins were separated by SDS-PAGE.

N-Terminal Amino Acid Sequence Analysis

The purified ovPAG was transferred to polyvinylidene difluoride (PVDF) membranes with a ProSpin device (Ap-

plied Biosystems, Foster City, CA) and sequenced by the Edman degradation method on an Applied Biosystems Model 470 protein sequencer with online analysis for phenylthiohydantoin derivatives (Protein Core, University of Missouri-Columbia, Columbia, MO). One fraction (see lane 14 in Fig. 7) contained two separable immunoreactive bands. Proteins in this fraction were separated by SDS-PAGE, stained with Coomassie blue, and then transferred to a PVDF membrane for sequencing.

Anti-PAG Antisera

The preparation of anti-native ovPAG1 [9, 23] and anti-native boPAG1 antisera [1, 6] has been described previously. The anti-recombinant ovPAG1 serum was raised in rabbits against ovPAG1, which had been produced in *Escherichia coli* strain BL21 (DE3)pLysS as previously described [24]. Briefly, the coding region of ovPAG1 (without the signal peptide) was amplified by polymerase chain reaction (PCR) and engineered to contain in-frame, flanking ndeI restriction sites for cloning into the pET11a vector (Invitrogen, San Diego, CA). Recombinant ovPAG1 expression was induced with 1 mM isopropyl ß-D-thiogalactopyranoside. The protein was produced as insoluble inclusion bodies that were isolated by centrifugation from French-pressed cells. The inclusions were washed two times in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 μM PMSF, and 1% Triton X-100 and solubilized in 8 M urea, 20 mM Tris-HCl (pH 8.0), 100 mM 2-mercaptoethanol, and 1 mM EDTA. The turbid solution was then cleared by centrifugation. The solubilized proteins (250 μg) were mixed with 0.5 ml Freund's complete adjuvant (Gibco, Chagrin Falls, OH) and injected s.c. into two New Zealand white rabbits [25]. The rabbits were reimmunized at 5-wk intervals with 100 μg of the recombinant protein in Freund's incomplete adjuvant. Antiserum was collected from the central ear vein 2 wk after each immunization [25].

Detection of ovPAGs by Western Blotting and on Dot Blots

To denature proteins before electrophoresis, the samples were boiled for 5 min in loading buffer that contained 1% SDS and 1% 2-mercaptoethanol. The denatured proteins were separated in a 10% polyacrylamide gel containing 0.1% SDS and then either stained with Coomassie blue (loading: 10 μg/lane) or transferred to a nitrocellulose membrane by electroblotting (loading: either 2 or 0.5 μg/lane). For dot blotting, proteins (1 μg) were directly spotted onto nitrocellulose membranes. Both kinds of blots were then exposed to 5% (w:v) milk proteins, washed, and reacted with either preimmune serum, anti-native ovPAG1, anti-native boPAG1, or anti-recombinant ovPAG1 antiserum [25]. Visualization of the antigen-antibody complexes on both dot blots and Western blots was achieved with the use of alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies in an immunoblotting kit purchased from Promega (Madison, WI).

ELISA

The assay was a variation of an antibody capture assay to detect and provide a semiquantitative assay for the relative amount of ovPAGs in samples [25]. Briefly, samples (3 μl) were applied to the bottom of a polyvinylchloride microtiter plate. The plates were washed twice with PBS and blocked with 3% BSA, and an anti-PAG antiserum (1:50 000, each antiserum) was added to the wells. Unbound protein was removed by four washes with PBS. An alkaline solution (0.1 ml) containing alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (0.2 μg/ml) was then added to the microplates. After washing, the complexes of antigen-primary antibody-secondary antibody were detected by adding *p*-nitrophenyl phosphate (1 mg/ml; 20 μl) to quantitate bound enzyme [25]. The microplates were read at 405 nm to provide a measure of the amount of PAG in the original sample. The positive control in the assay was 3 μl of the diluted 40–75% $(NH_4)_2SO_4$ fraction containing 240 ng of protein.

Hemoglobin Proteinase Assay

The hemoglobin proteinase assay was performed as described previously [24, 26]. Briefly, a 100-μl solution containing purified ovPAG (1 μg) and [^{14}C]methyl-hemoglobin (6 μg; 0.04 μCi in 150 mM acetate buffer, pH 4.5) was incubated at 37°C for up to 48 h. Protein was then precipitated with 0.2 ml of 10% (w:v) trichloracetic acid. Supernatant solution (0.2-ml samples) containing ^{14}C-labeled peptides liberated by the enzyme was counted in a liquid scintillation counter. Controls included negative and positive assays run in the absence or presence of porcine pepsin (Sigma), respectively.

Cloning of ovPAG Transcripts from Day 100 Ovine Placenta

PAG transcripts from Day 100 ovine placenta were cloned by reverse transcription (RT) and PCR procedures [27]. In brief, cellular RNA extracted from a Day 100 placenta was first reverse transcribed into cDNA and amplified by PCR with a pair of well-conserved primers (ovPAG forward 5′AGGAAAGAAGCATGAAGTGGCT3′, ovPAG reverse 3′ATTTACGAACCTGAACAAGTCCT5′; see Fig. 8). The RT-PCR products were cloned into TA cloning vectors (Invitrogen). Plasmid DNA was prepared from individual colonies and sequenced.

In Situ Hybridization

Riboprobes (cRNA) were prepared by using the Riboprobe Preparation System (Promega). Briefly, a fragment (407 base pairs [bp]) of ovPAG3 and 7 cDNA, corresponding to exons 3, 4, and 5, was amplified by PCR with two well-conserved primers (forward 5′TGGGTAACATCACCATTGGAA3′, reverse 3′AAAGACTCGGACAAAAACGG5′; see Fig. 8). PCR products were subcloned into the TA cloning vectors (Invitrogen). The orientation and sequence of the inserts were confirmed by sequencing. The subcloned cDNA fragments were then transcribed in vitro into cRNA in the presence of [^{35}S]CTP. The probes (each $\sim 1.25 \times 10^9$ cpm/μg) were used within 3 days of preparation.

Day 100 ovine placental tissue was sectioned (14 μm) at −18°C on an IEC cryostat (International Equipment Co., Needham Heights, MA) and mounted onto prechilled microscope slides. The sections were then fixed in 4% formaldehyde in PBS for 5 min, washed in double-strength SSC (single-strength SSC: 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 2 min, acetylated in 0.25 (v:v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, rinsed twice in double-strength SSC, and dehydrated sequentially through 70%, 80%, 95%, and 100% ethanol. To increase the accessibility of the target mRNA, the tissue was soaked to remove lipid membranes in 100% chloroform for 5 min,

rehydrated in 100% ethanol for 2 min and in 95% ethanol for 2 min, and air dried. In situ hybridization was performed according to procedures described previously [28] after application of 0.2 ml probe solution (4×10^6 cpm) to cover each section. Exposure was for 1–4 wk at 4°C. After development, the slides were counterstained with hematoxylin and eosin and examined microscopically.

RESULTS

Characteristics of Antisera Used in Immunodetection of PAGs during Purification

The anti-boPAG1 antiserum [1] was raised against purified boPAG1 extracted from fetal cotyledons, but it clearly recognizes an array of different ovPAG molecules [9, 29] (see Figs. 3 and 6). Presumably, different PAGs, even those belonging to different species, possess similar epitopes as a result of their sequence similarities.

The antigen used to generate the antiserum against native ovPAG1 [23] was prepared against protein fractionated as described by Zoli et al. [1] from bovine fetal cotyledons pooled from several different stages of pregnancy [23]. At the time of antiserum preparation it was believed that only a single PAG type existed. In retrospect, the antigen probably contained a mixture of potential antigens. In the context of the present experiments, the antiserum was anticipated to have relatively broad immunological cross-reactivity.

The third antiserum used was generated by immunizing rabbits against urea-solubilized, recombinant ovPAG1. Three lines of evidence suggested that this nonglycosylated product was largely denatured. First, when the urea was removed from the preparation, much of the protein precipitated from solution; second, the protein that remained soluble eluted with the void volume on a Superose 12 HPLC column; third, the protein failed to bind to a pepstatin affinity column (data not shown). The antiserum raised against this recombinant protein was likely, therefore, to recognize certain epitopes possibly present on improperly folded but not native protein and to have some value in recognizing a range of PAGs on Western blots.

When used in combination, it seemed likely that these antisera would be capable of detecting a broad array of PAGs produced by Day 100 placental tissue.

Fractionation of ovPAGs from Cultured Placental Explants

Proteins that had been released by explants of Day 100 placental tissue during an 8-h culture in serum-free medium were initially fractionated by addition of ammonium sulfate (Fig. 1). Little material that cross-reacted on Western blots with any of the anti-PAG antisera was detected in either the 20% or 40% ammonium sulfate fractions, which contained 0.29 g and 5.80 g of protein, respectively. The protein (11.5 g) that was precipitated at between 40% and 75% ammonium sulfate saturation contained all but a trace of the immunopositive material (data not shown).

After dialysis against 0.1 M sodium acetate (pH 5.5), a portion of this protein fraction (405 mg) was passed twice through a Blue Sepharose column to remove much of the serum proteins, particularly serum albumin, that contaminated the preparation. Bound protein, which could be subsequently eluted in 0.5 M NaCl, contained no ovPAG1-immunoreactive material as determined by immunoblotting. Instead, the PAGs were all present in the initial breakthrough fraction (243 mg).

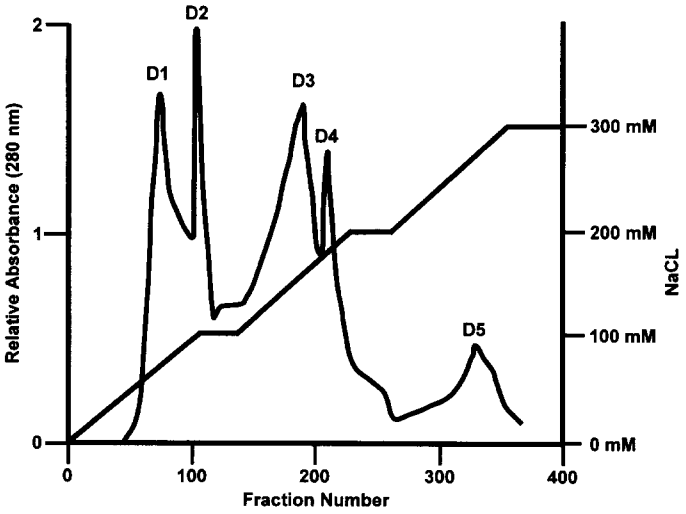

FIG. 2. Anion-exchange chromatography of protein fractionated by precipitation with ammonium sulfate on a column of DEAE-Sepharose. Proteins precipitated with 40–70% saturated ammonium sulfate were resuspended in 10 mM Tris (pH 8.0), dialyzed, and then passed three times through a Blue Sepharose column. The proteins in the final breakthrough from the Blue Sepharose column were concentrated, loaded onto a DEAE-Sepharose column (25 × 92 cm), and washed extensively with 10 mM Tris, pH 8.0. The column was eluted by a combination of step gradient and linear gradient of 0–300 mM NaCl in 10 mM Tris, pH 8.0. The eluate was collected in 9.5-ml fractions whose content of protein was assessed by their absorbance at 280 nm. Each fraction was analyzed for immunoreactivity by using dot blotting and ELISA. Five pools were generated, and pool 1 (D1) was divided into two subpools (D1A and D1B).

Anion-exchange chromatography on DEAE-cellulose at pH 8.0 was then used to purify the PAGs further (Fig. 2). The unbound, flow-through fraction contained about one quarter of the 243 mg protein loaded, but no detectable material that reacted with the antisera (Fig. 3, A and B, lane 3). Subsequent elution with a gradient of NaCl yielded five major pools (Fig. 2; D1–D5). Pool D1 was arbitrarily divided into two subpools (D1A, leading peak; D1B, the trailing shoulder). Both contained material that reacted strongly with the antisera to boPAG1 (Fig. 3C, lanes 4 and 5) and anti-recombinant ovPAG1 (Fig. 3D) but relatively weakly with native ovPAG1 antiserum (Fig. 3B).

In contrast, pool D3 reacted most strongly with anti-native ovPAG1 antiserum and not at all with anti-native boPAG1 (Fig. 3B, lane 7). Although pools 2, 4, and 5 all contained immunoreactive protein (Fig. 3, B–D, lanes 6, 8, and 9, respectively), these were not studied further. Instead, the effort was to purify the PAGs in pools D1 and D3.

Purification of PAGs from Pool D1A

Pool D1A (fractions 50–71, Fig. 2; 19.4 mg protein) was subjected to high-performance cation-exchange chromatography on a Mono S column. All immunoreactive material bound to the column, and there was very little protein (< 1 mg) in the flow-through. Salt elution provided three further pools of PAGs (S1, 4.4 mg; S2, 5.7 mg; S3, 5.0 mg; Fig. 4A). After electrophoresis, a broad band of Coomassie blue-staining material was evident in each pool (Fig. 4B) that cross-reacted with the antiserum to boPAG1 (Fig. 4C).

Further fractionation of a portion (2.8 mg) of pool S1 on a Mono P column, in which proteins are separated according to their isoelectric points, gave five further pools of protein (S1P1-S1P5; pI 5.54, 5.19, 4.06, 4.00, and 3.56, respectively) (Fig. 5). Each contained a protein of apparent

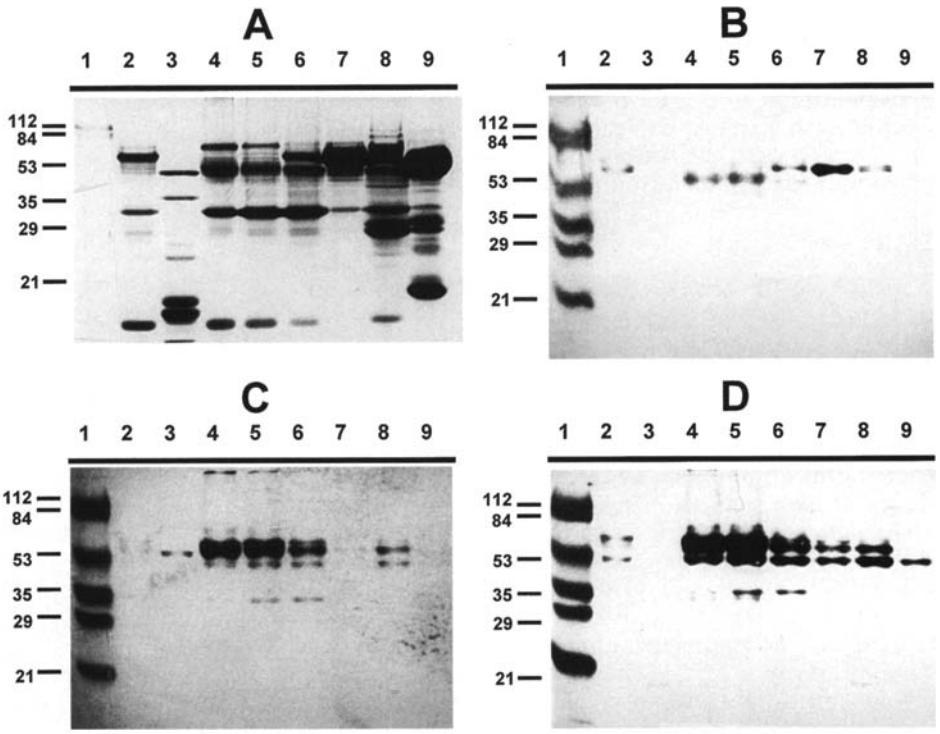

FIG. 3. Electrophoretic analyses and Western immunoblotting of proteins fractionated by DEAE-Sepharose. Samples were subjected to electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Lane 1, molecular weight standards (scale on left $\times 10^{-3}$); 2, total proteins initially loaded on the DEAE-Sepharose column; 3, proteins in the unbound fraction. Lanes 4 to 9 correspond to pooled materials of D1A, D1B, and D2 through D5, respectively (see Fig. 2). In **A**, proteins (10 μg loaded) were visualized by staining the gel with Coomassie brilliant blue. **B**, **C**, and **D** are Western blots (2 μg loaded per lane) analyzed with either anti-native ovPAG1 (**B**), anti-native boPAG1 (**C**), or anti-recombinant ovPAG 1 antisera (**D**).

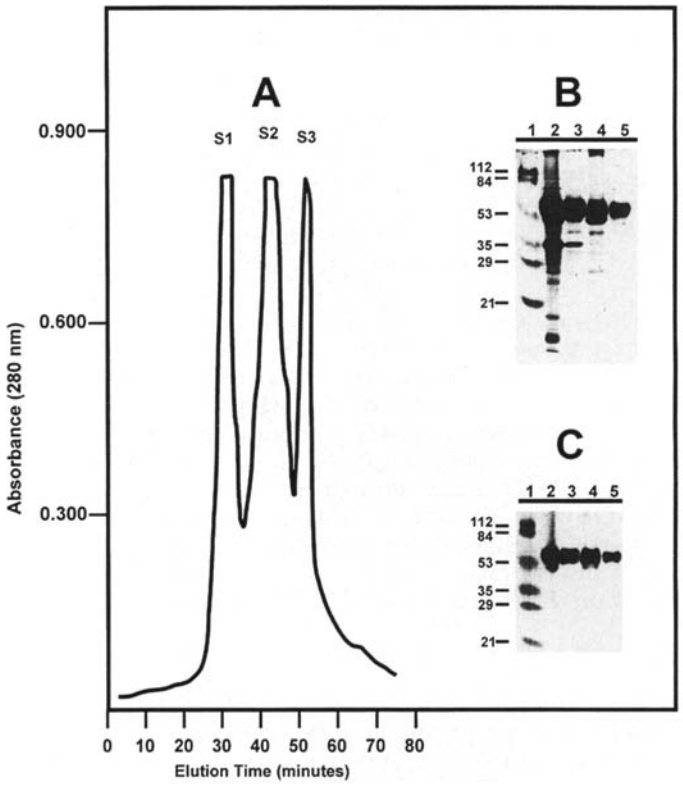

FIG. 4. Analysis of proteins from DEAE-Sepharose fraction D1 by Mono S chromatography. **A**) Proteins from the D1 fraction were loaded onto a Mono S column. The column was washed extensively with 20 mM sodium acetate (pH 5.0) and eluted with a gradient of 0.1 M NaCl in 20 mM sodium acetate (pH 5.0) at a flow rate of 1 ml/min. The eluate (1 ml/fraction) was assayed for ovPAG by dot blotting and ELISA. The fractions in pools S1, S2, and S3 were combined separately and analyzed by SDS-PAGE (**B** and **C**). Lane 1, molecular weight standards (scale on left $\times 10^{-3}$); 2, total protein initially loaded on the Mono S column; lanes 3, 4, and 5 correspond to pooled materials of S1, S2, and S3. Proteins were visualized either by staining with Coomassie blue (10 μg/sample) (**B**) or by immunoblotting (2 μg/sample) with anti-native boPAG1 (**C**).

mass ~65 kDa (Fig. 6, lanes 7–11). This band cross-reacted weakly with anti-ovPAG1 (Fig. 6B, lanes 7–11) but strongly with anti-recombinant ovPAG1 (Fig. 6D). Fractions S1P1 and S1P2 were also positive against anti-boPAG1 (Fig. 6C).

Material in two of these pools (S1P2 and S1P4) was selected for sequencing. The two (designated $ovPAG_{65}$ in Fig. 7) were identical over the first 21 amino acids (aa) of amino-terminal sequence.

Pools S2 and S3 from Figure 3 were also fractionated further on the Mono P column. Only two peaks were generated for each sample (data not shown). The proteins in the two peaks (S2P1, 0.8 mg; S2P2, 0.02 mg) from the S2 pool had isoelectric points of 5.68 and 4.0, respectively, and an apparent molecular mass of 61 kDa (Fig. 6A, lanes 12 and 13). A band of slightly higher mobility (apparent mass 55 kDa), which cross-reacted strongly with the anti-

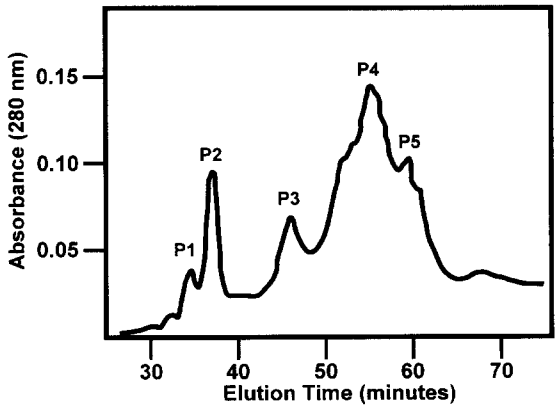

FIG. 5. Isoelectric point analysis of ovine placental proteins in fraction S1 by chromatography on a Mono P column. **A**) Proteins from the Mono S fraction S1 (Fig. 4) were loaded onto a Mono P column equilibrated with start buffer (0.025 M *bis*-Tris, pH 6.3). The column was eluted with polybuffer 74 (Pharmacia) at a flow rate of 1 ml/min. Samples were collected as five pools (P1, P2, P3, P4, and P5, respectively).

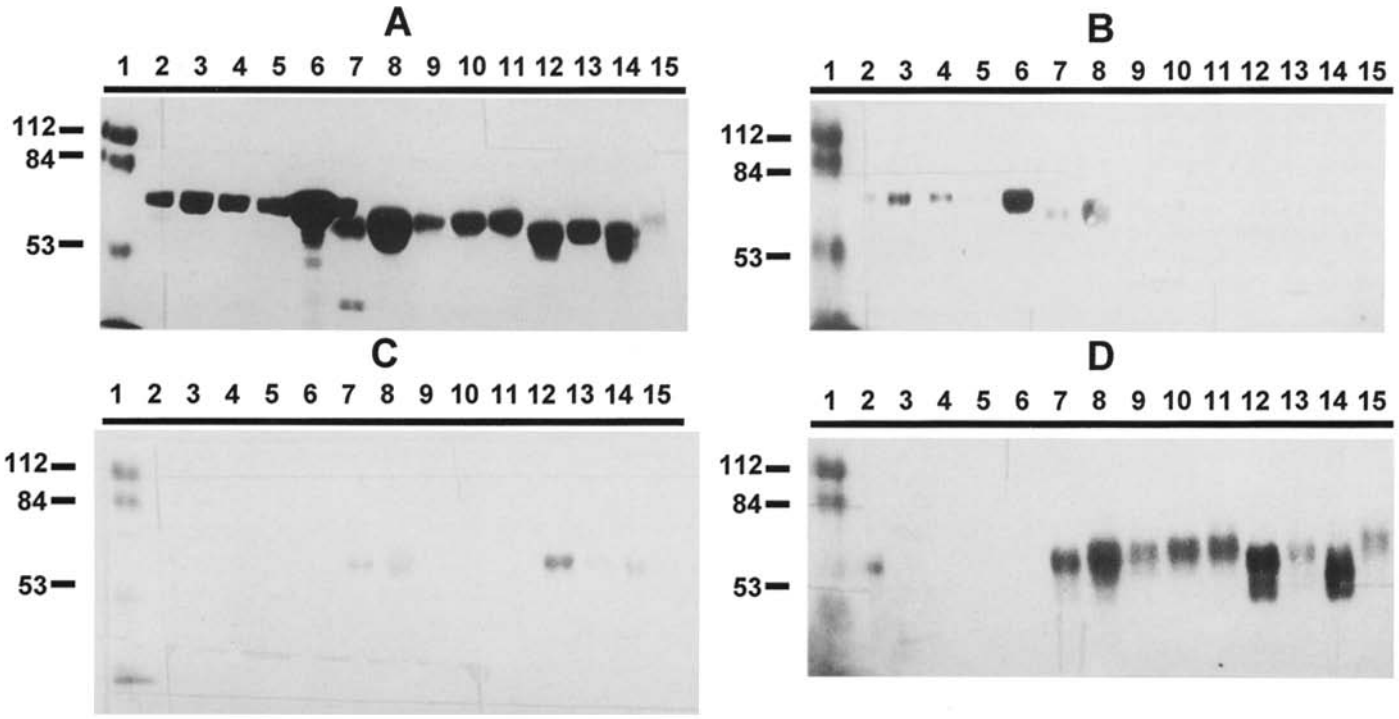

FIG. 6. Electrophoretic and Western immunoblot analyses of proteins fractionated by Mono P chromatography. Samples were subjected to electrophoresis on polyacrylamide gels containing 0.1% SDS. Lane 1, molecular weight standards (scale on left $\times 10^{-3}$). Lanes 2–6 were samples derived by further fractionation of the DEAE-Sepharose D3 pool (see Fig. 2). This was first separated into two fractions by Mono S chromatography (data not shown). Only the Mono S fraction 1 contained immunoreactive materials and was further separated by use of Mono P chromatrophy into three peaks. Lanes 2 and 3 correspond to the leading and trailing ends of Mono P peak 1; lanes 4 and 5 to the leading and trailing edges of peak 2; lane 6 represents peak 3 from the Mono P column. Lanes 7–11 correspond to fractions P1 through P5 from Figure 5, respectively. Fraction S2 (Fig. 4) was separated into two subfractions by Mono P chromatography; lanes 12 and 13 represent subfractions. Fraction S3 (Fig. 4) was also separated into two fractions by Mono P chromatography; lanes 14 and 15 were samples from subfractions 1 and 2, respectively. In **A**, gels were stained by Coomassie blue and were loaded with 5 μg protein per lane. **B–D** were immunoblots containing 0.5 μg protein per lane. These immunoblots were developed with either anti-native ovPAG1 (**B**), anti-native boPAG1 (**C**), or anti-recombinant ovPAG1 (**D**).

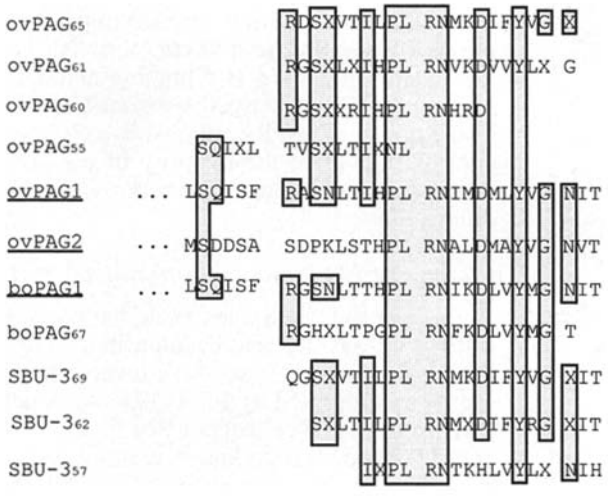

FIG. 7. Comparison of the NH_2-terminal amino acid sequences of four purified ovPAGs with other PAG-related proteins that were purified from placental tissues and with three PAG that were cloned but not purified. $OvPAG_{65}$ and $ovPAG_{61}$ correspond to the samples shown in Figure 6, lanes 8 and 12, respectively. $OvPAG_{55}$ corresponds to the lower band and $ovPAG_{60}$ to the slower-mobility band in Figure 6, lane 14. The subscript for each ovPAG denotes its apparent molecular mass (in kDa). The purified PAGs were subjected to microsequencing. Sequences obtained were compared with those of three glycoproteins isolated by immunopurification on the SBU-3 monoclonal antibody [20], the $boPAG_{67}$ sequence reported by Zoli et al. [1], and the sequences inferred for ovPAG1 [9] and ovPAG2 [18] from their cDNA. Regions of strongest sequence conservation are shaded. X indicates a blank cycle and probably corresponds to a glycosylated asparagine residue. The cDNA for ovPAG1 (GenBank accession no. M73961), ovPAG2 (U30251), and boPAG1 (M73962) encode both the signal sequence and the propeptide. The existence of additional amino acids at the amino termini is represented by dots.

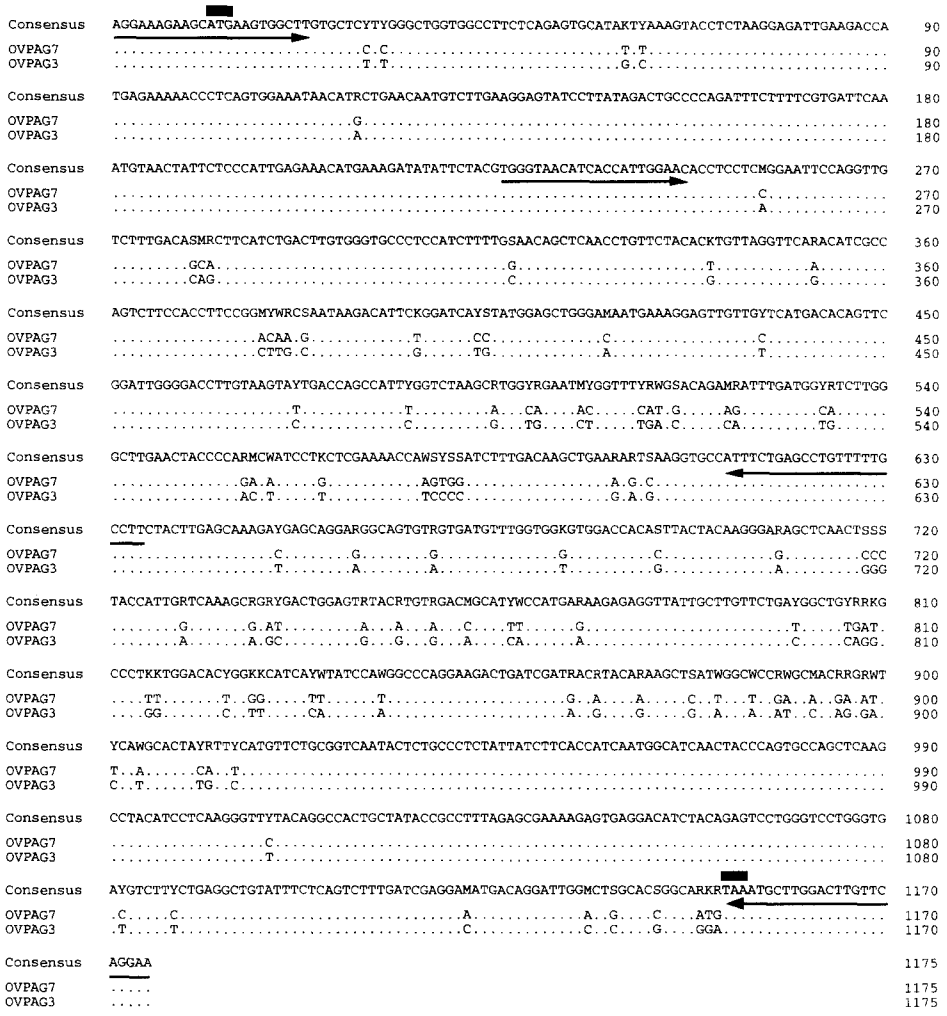

FIG. 8. Nucleotide sequence of cDNA clones ovPAG3 and ovPAG7 identified by RT-PCR from RNA isolated from Day 100 cotyledonary RNA. The arrows at the beginning and end of the sequences correspond to those chosen to provide probes for the initial PCR amplification of reverse-transcribed RNA. The two internal arrowed sequences are those selected to provide the probe used for in situ hybridization. The two solid rectangles mark the initiation and stop codons, respectively.

recombinant ovPAG1 antiserum, was also detected in the S2P1 material (Fig. 6D). However, sequencing of the total material in S2P1 was consistent with the presence of only a single polypeptide. If a mixture of PAGs had been present, more than one signal might have been evident during at least some of the cycles. The sequence ($ovPAG_{61}$) obtained was again unique.

The two Mono P peaks from the S3 pool (S3P1, pI 5.54, 0.24 mg; S3P2, pI 4.00, 0.2 mg) contained proteins that differed in apparent mass (Fig. 6, lanes 14 and 15). S3P1 itself also contained at least two bands of material (60 kDa and 55 kDa) when it was analyzed electrophoretically (Fig. 6, lane 14). Each was subjected to "off-blot" analysis. The two ($ovPAG_{60}$ and $ovPAG_{55}$) differed markedly in their amino-terminal sequences (Fig. 7). The polypeptides in fraction S3P2 have not been analyzed further.

Further Analysis of Polypeptides in Pool D3

Again the sequential use of chromatography on Mono S and Mono P columns provided fractions that appeared relatively homogeneous in composition (Fig. 6, lanes 2–6). These protein bands (mass ~70 kDa) reacted weakly with anti-native ovPAG1 but not with the other two antisera. Sequencing failed to reveal the presence of PAG. Instead, the data indicated that most of the protein in pool D3 was serum albumin (data not shown) whose signal was strong enough to mask that of any PAGs.

Purified PAGs Lack Proteolytic Activity Toward Denatured Hemoglobin

In order to examine whether any of the PAGs so far purified possessed proteolytic activity, 1 μg of each was incubated with 6 μg [^{14}C]methyl-hemoglobin for as long as 48 h at 37°C. No hydrolytic activity was evident in any of the preparations. By contrast, 1 μg of pepsin, the positive control, converted the majority of the protein to trichloracetic acid-soluble peptides within 1 h under identical conditions.

Cloning of PAG Transcripts from Day 100 Placenta

In order to isolate transcripts that possibly matched some of the ovPAGs that had been purified (Fig. 7), mRNA from Day 100 placental tissue was reverse transcribed and subsequently amplified by PCR. The oligonucleotides used for PCR represented sequences just 5′ to the ATG start codon and 3′ to the stop codon in regions well conserved in all PAG transcripts so far cloned [9–12, 18, 19]. A total of 25 cDNA, distinct from but closely related to ovPAG1 and ovPAG2 in sequence, were sequenced, and these could be classified into seven different groups. They were named ovPAG3 to ovPAG9 based on the order in which they were sequenced to completion (Genbank accession numbers 494789 to 494795, respectively). Two of the seven cDNA (ovPAG3 and ovPAG7) encoded a polypeptide that contained sequence corresponding to the amino terminus of

```
          -1         -15
Consensus MKWLVL.GLV AFSECI.KVP LRRLKTMRKT LSGNN.LNNV LKEYPYRLPQ ISHRDSNVTI   60

ovPAG3    ......F... ......V... .......... .....I.... .......... ..........   60
ovPAG7    ......L... ......F... .......... .....M.... .......... ..........   60

Consensus LPLRNMKDIF YVGNITIGTP P.EFQVVFD. .SSDLWVPSI F.NSSTCST. VRF.HRQSST  120

ovPAG3    .......... .......... .Q.......T A......... .C.......R ...R......  120
ovPAG7    .......... .......... .P.......S T......... .W.......L ...K......  120

Consensus FR..NKTF.I .YGAG.MKGV V.HDTVRIGD LVS.DQPFGL S..E.GF..R .FDG.LGLNY  180

ovPAG3    ..LA....G. M....K.... .V........ ...T...... .VV.S..EH. Q...V.....  180
ovPAG7    ..TT....W. T....T.... .A........ ...I...... .MA.Y..MD. R...I.....  180

Consensus P..S.SKT.. IFDKLK..GA ISEPVFAFYL SKDEQEGSVV MFGGVDH.YY KG.LN..PL.  240

ovPAG3    .NL.F...IP ......NE.. .......... .......... .......S.. ..K..WV..I  240
ovPAG7    .RQ.C...KW ......SQ.. .......... .......... .......T.. ..E..SL..V  240

Consensus KA.DWS..VD .I.M.REVIA CSDGC.AL.D TG.S.I.GPG RLID..QKLI G.......HY  300

ovPAG3    ..G...VR.. S.T.K..... .....R..V. ..S.H.Q... ....NV.... .TMPQGSM..  300
ovPAG7    ..D...IH.. R.S.R..... .....D..L. ..A.F.H... ....DI.... .SEQRDFK..  300

Consensus ..CSAVNTLP SIIFTINGIN YPVPAQAYIL KG.TGHCYTA FRAKRVRTST ESWVLGDVFL  360

ovPAG3    VP........ .......... .......... ..F....... .......... ..........  360
ovPAG7    IS........ .......... .......... ..S....... .......... ..........  360

Consensus RLYFSVFDRG .DRIGLA.A.   380

ovPAG3    .......... H......R.G   380
ovPAG7    .......... N......P.M   380
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FIG. 9. The inferred amino acid sequences of ovPAG3 and ovPAG7. The inferred signal sequence of 15 aa is shown. The shaded rectangle corresponds to the region of apparent identity between ovPAG3, ovPAG7, and $ovPAG_{65}$ (from Fig. 7). The asterisks mark asparagines that are potentially glycosylated. The open rectangles designate the sequences immediately surrounding the two aspartic acid residues (D) that are normally involved in catalysis. The signal sequence of 15 aa is numbered separately. The propeptide region is between the end of the signal sequence and the shaded rectangle.

$ovPAG_{65}$ shown in Figure 7. Despite this similarity at their 5′ termini (Fig. 8), the two were clearly distinct in nucleotide sequence (Fig. 8), and it was clear that they encoded different proteins (Fig. 9). None of the other cDNA (ovPAG4, 5, 6, 8, and 9) encoded any of the protein sequences shown in Figure 7.

The lengths of the polypeptides encoded by ovPAG3 and ovPAG7 (each 380 aa) were the same, but the sequences showed only 84% identity overall (Fig. 9). After the signal peptide (15 aa) and propeptide (38 aa) regions had been removed, the mature ovPAG3 and ovPAG7 proteins had theoretical molecular weights of 36 281 and 36 680, respectively. The additional mass of the proteins is presumably the result of posttranslational processing, particularly of the addition of carbohydrate, as has previously been reported for ovPAG1 [9, 29]. Both ovPAG3 and ovPAG7 possess four conserved asparagine residues in the context Asn X Thr/Ser, which could be targets for *N*-glycosylation. In addition, ovPAG3 contains a fifth such site (Fig. 9). Edman sequencing of $ovPAG_{65}$ (Fig. 7) provided no signal on cycles 4 and 21, which correspond to Asn57 and Asn74 on both ovPAG3 and ovPAG7, suggesting that the residues at these positions are indeed glycosylated.

Aspartic proteinases that are enzymatically active characteristically possess conserved sequences of amino acids around the two catalytic aspartic acid residues that face each other across the peptide-binding cleft separating the two lobes of the molecule (unshaded boxes in Fig. 9). In ovPAG3, glycine in the usually conserved triad DTG in the amino-terminal lobe [9, 19, 21] is replaced by an alanine. A similar mutation is believed to be inactivating in the case of boPAG1 [9, 12]. In ovPAG7, the same DTG triad is replaced by DST (Fig. 9) and again is not anticipated to support enzymatic activity.

Localization of ovPAG3 and ovPAG7 mRNA to Trophoblast Binucleate Cells

In situ hybridization was performed on sections of Day 100 placentomes with probes representing relatively poorly conserved regions of ovPAG3 and ovPAG7 transcripts (Fig. 8). Positive hybridization signals were restricted to the scattered binucleate trophoblast cells (Fig. 10) that make up about 25% of the cells of trophectoderm [30]. This localization is clearly observed near the tips of the chorionic villi close to the edges of the placentome (Fig. 10A) but is also evident in the meshwork of chorionic villi in the central region of the placentome (Fig. 10B). There, the villi

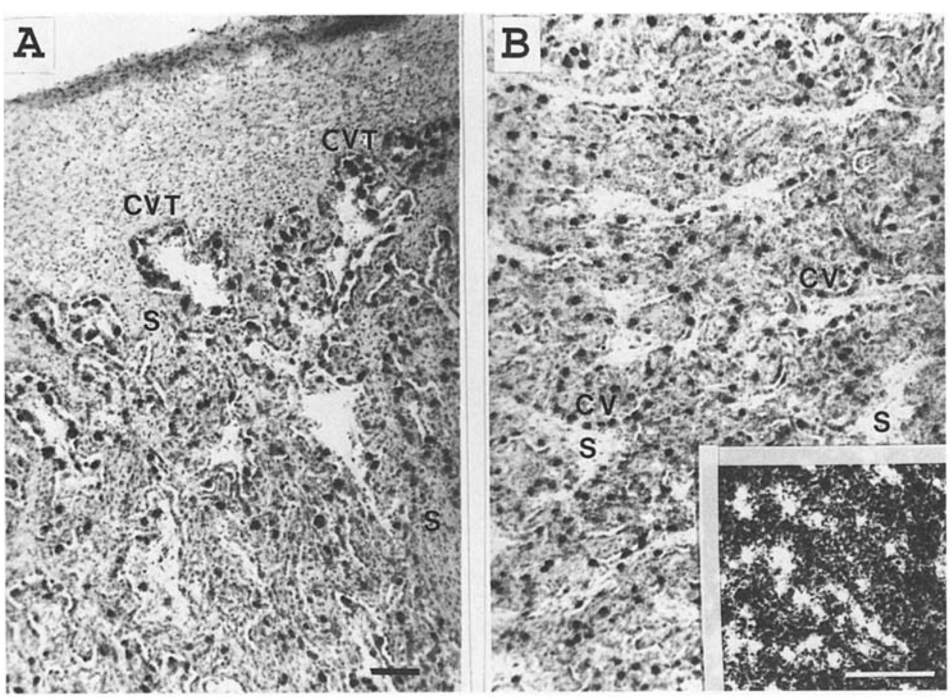

FIG. 10. Localization of the mRNA for ovPAG7 and ovPAG3 in Day 100 ovine placental tissue by in situ hybridization analyses. In situ hybridization was performed with ^{35}S-labeled probes on transverse sections across a placentome removed from a sheep at Day 100 of pregnancy. Two sections are shown. **A**) A section near the edge of the placentome showing the tips of cotyledonary villi with the strongly positive binucleate cells heavily labeled with silver grains from the ovPAG7 probe. **B**) A section within the central region of the placentome showing cross sections of cotyledonary villi as islands between thin septae of maternal caruncular tissue. Again, all the label from the ovPAG7 probe is confined to binucleate cells. The inset shows a similar section hybridized to an ovPAG3 cDNA but photographed under dark-field illumination. Magnification is about twice that in the main figure. Silver grains appear white. CVT, cotyledonary villous tips; CV, cotyledonary villi in cross section; S, caruncular septae protruding between cotyledonary villi. Bars = 100 μm.

appear as islands separated by thin septa of maternal tissue [30, 31]. This restricted distribution of PAG mRNA appears identical to that observed for ovPAG1 ([9] and unpublished results) and different from that shown by ovPAG2, whose expression is strongest in mononucleated cells [18].

DISCUSSION

These data confirm earlier suspicions that the PAGs are the products of many genes [10, 21]. In our initial studies, we had observed that anti-ovPAG1 antisera could recognize several molecules in medium from cultured trophoblast, these molecules ranging in apparent mass from 45 kDa to 70 kDa; but we attributed much of the complexity to heterogeneous processing of an initial single form [9]. As other PAGs were discovered, this simplistic view had to be amended. Here, a combination of protein purification, microsequencing, and cDNA cloning has provided evidence for at least 12 different ovPAGs at around Day 100 of pregnancy in the ewe. The fact that several immunoreactive pools of protein from ion-exchange chromatography were not chosen for further analysis, and that only 25 of several hundred positive cDNA clones were sequenced, suggests that many more PAGs remain to be discovered. The fact that the PAGs recognized by the SBU-3 monoclonal antibody [20] were all different from the ones reported here and from ovPAG1 [9] and ovPAG2 [18] tends to confirm this likelihood.

Even amino acid sequencing can underestimate PAG complexity. Here we described two distinct cDNA (Figs. 8 and 9) each of which could have encoded one of the PAGs ($ovPAG_{65}$) that had been column purified (Fig. 7). In fact, ovPAG3 and ovPAG7, although expressed simultaneously and in the same binucleate cell type, have only 84% sequence identity over their full lengths. Their similarities at their amino termini could have arisen by a recent gene-conversion event.

These interpretations of the data are not meant to imply that posttranslational modifications do not also contribute to PAG heterogeneity. These molecules are glycosylated at asparagine residues and probably at serines and threonines as well [29]. Some, at least, are phosphorylated [29], and the loss of higher molecular weight forms during pulse-chase experiments suggests that proteolytic processing also occurs [9, 29].

Our data confirm that even an antiserum raised against a homogeneous PAG product binds several different PAGs on Western blots (Figs. 3 and 6). On the other hand, not all PAGs can be recognized on such blots by a single antiserum. Such antigenic variability is not surprising, as the molecules demonstrate a considerable range of sequence identities. Antisera may show rather more selectivity in screening of cDNA libraries than in blotting experiments. The anti-PAG antisera of Zoli et al. [1, 23], for example, were remarkably specific for fusion proteins encoded by PAG1 inserts [9]. A second antiserum recognized boPAG2 fusion proteins exclusively [11]. Conceivably, much of the antigenicity of the fully processed molecules is provided by carbohydrate, absent on bacterial fusion proteins. The work of Atkinson et al. [20] predicted that trophoblast binucleate cells, which synthesize many PAGs, express a structurally unusual carbohydrate antigen that is specifically recognized by the SBU-3 monoclonal antibody.

The PAGs are clearly major secretory products of the ovine placenta, although their exact contribution cannot be easily assessed from our purification scheme because the starting solution was undoubtedly contaminated with serum leached from the tissue capillaries of the cotyledonary explant cultures. Indeed, if the initial Blue Sepharose step was not included in the purification protocol, albumin contaminated, to some extent at least, every subsequent protein pool (data not shown). Nevertheless, except for some additional albumin in pool D3, which had escaped entrapment on the Blue Sepharose, PAGs seemed to be the dominant components of the fractions pooled from the initial DEAE-cellulose column steps (Fig. 2) and subsequent fractionation (Fig. 4).

None of the four ovPAGs examined had proteolytic activity when tested against denatured hemoglobin. One explanation is that, like renin, they have narrow specificity. However, the lack of consensus peptide sequence in the catalytic centers of ovPAG3, ovPAG7, and several other PAGs identified earlier [12, 19, 21] strongly suggests that these particular molecules do not serve a proteolytic function. Unfortunately there is little to indicate what actual roles these curious molecules might play [12, 32]. A region of propeptide, which in pepsinogens fits tightly into the substrate-binding cleft and prevents catalytic activity until it has been removed [33], has been excised from the PAGs, presumably by some active proteinase, by the time they have been purified. Although this processing is not uniform (see Fig. 7), the event seems not to be random. In three of the ovPAG group purified here, for example, proteolytic cleavage of the propeptide appears to be associated with a relatively conserved ISF↓RG/DS sequence, where the arrow indicates the site of peptide band cleavage (Fig. 7). Members of the SBU-3 group, on the other hand, are processed at a different site.

Removal of the propeptide exposes the cleft formed between the two lobes of the molecules [12] and might well provide the PAGs the ability to associate with peptides without hydrolyzing them [32]. It seems unlikely that the PAGs are mere biological curiosities, as they have been found in all hoofed animals so far examined and encompass an evolutionary distance that is probably greater than 80 million years [12].

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