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Biochemistry

Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family

(binucleate cell/trophoblast)

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ABSTRACT **Pregnancy in cattle and sheep can be diagnosed by the presence of a conceptus-derived antigen in maternal serum that is secreted by trophoblast and placental tissue primarily as an acidic component of M_r 67,000. Molecular cloning of its cDNA reveals that the antigen belongs to the aspartic proteinase family and has >50% amino acid sequence identity to pepsin, cathepsin D, and cathepsin E. The inferred sequences of the ovine and bovine polypeptides show ≈73% identity to each other. Critical amino acid substitutions at the active site regions suggest that both proteins are enzymatically inactive. The antigen is a product of trophoblast binucleate cells that invade maternal endometrium at implantation sites.**

Proteins released by the trophoblast of mammals have long been recognized as likely endocrine factors involved in maternal recognition of pregnancy (1) and can also provide a potential means for determining the presence of a viable conceptus (2). Unlike primates, early conceptuses of domestic ruminant species, such as cattle, are not considered to secrete chorionic gonadotropins, however (3). Implantation in ruminant species (4, 5) occurs later (≈3 weeks after ovulation) than in primates (≈1 week after ovulation) and is accompanied by the migration of secretory trophoblast binucleate cells into the endometrium (5). Binucleate cells have numerous dense granules and continue their secretory activity even after they become resident in maternal tissue (6, 7). In 1982 Butler *et al.* (8) recognized two antigens, pregnancy-specific proteins (PSPs) A and B, after injecting rabbits with bovine placental extracts. PSP-A was identified as α-fetoprotein. The second antigen PSP-B, however, appeared to be a product of binucleate cells (9, 10) and could be employed as the basis of a useful pregnancy test in both cattle (11) and sheep (12), since it was detectable in maternal serum at or about the third week of pregnancy. Originally thought to have a molecular weight of 47,000–53,000 (8), PSP-B has since been reported to be considerably larger (13). Zoli *et al.* (14) have been able to purify several isoforms of pregnancy-associated glycoprotein (PAG) of M_r 67,000 from fetal bovine cotyledonary tissue that may correspond to PSP-B. Although an NH_2-terminal amino acid sequence was obtained (14), it showed no obvious similarity to sequences in any other known protein. In the present paper we show that the antigens belong to the aspartic proteinase family of proteins, although key substitutions in the active site regions of the molecules make it unlikely that either is proteolytically active.

MATERIALS AND METHODS

Animals. Bovine conceptuses were obtained from uteri removed at a local slaughterhouse on specific days after breeding. Ages of older conceptuses were estimated by measuring crown-rump length of the fetuses (15). Preimplantation and postimplantation ovine conceptuses were recovered surgically at specific times after breeding ewes (16, 17).

Tissue Culture and Metabolic Labeling. Bovine and ovine placental tissues were cut into small (≈1 mm^3) pieces and washed three times in L-methionine-free Dulbecco's modified Eagle's medium (DMEM) containing penicillin (200 units/ml), streptomycin (200 μg/ml), and Fungizone (0.5 μg/ml). The tissue (≈100 mg) was incubated at 37°C under 5% CO_2/95% air in 1 ml of L-methionine-free DMEM containing L-[^{35}S]methionine (100 μCi/ml; 1151.4 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN). At the time of harvest, the tissue and medium were separated by centrifugation.

Antisera. oPAG and bPAG were purified from ovine and bovine placental cotyledonary tissues, respectively (14, 18). Antisera were raised in rabbits (14), lyophilized, reconstituted in water, and stored at 4°C in presence of 0.05% sodium azide.

Immunoprecipitation. Placental tissue was lysed in 1 ml of triple-detergent lysis buffer (19). Tissue extracts and media (1 ml) were first incubated with rabbit preimmune serum (5 μl) overnight at 4°C. Any immune complexes were allowed to adsorb to protein A-Sepharose CL-4B and collected by centrifugation. The precleared tissue extracts and media were then incubated with 1.5 μl of anti-oPAG or anti-bPAG antisera at 4°C overnight. The immunocomplexes were adsorbed to protein-A Sepharose CL-4B for 2 h, washed three times in NET-gel buffer (50 mM Tris Cl, pH 7.5/150 mM NaCl/0.1% Nonidet P-40/1 mM EDTA, pH 8.0/0.25% gelatin/0.02% sodium azide), and analyzed by SDS/PAGE and fluorography.

Immunocytochemistry. Tissues were fixed in 4% (wt/vol) paraformaldehyde and embedded in paraffin (20). Immunohistochemical localization for oPAG or bPAG was carried out on 6-μm sections with an indirect peroxidase method (ICN) with anti-bPAG antiserum used at a 1:40,000 dilution. Sections were finally counterstained briefly with Giemsa stain.

Screening cDNA Libraries and DNA Sequencing. The preparation of the conceptus λgt11 cDNA libraries of bovine origin (day 21–22) and ovine origin (day 15) has been described (21, 22). Libraries of bovine (day 260 of pregnancy) and ovine (day 100 of pregnancy) cotyledonary tissues were constructed from polyadenylylated RNA in λZAPII (Stratagene) (17).

Initially, conceptus cDNA libraries were screened with anti-oPAG and anti-bPAG rabbit antisera. The cDNA were subcloned and sequenced in both directions by the dideoxynucleotide chain-termination method (19).¶

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Abbreviations: PAG, pregnancy-associated glycoprotein; bPAG, bovine PAG; oPAG, ovine PAG; PSP, pregnancy-specific protein.
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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M73961 and M73962).

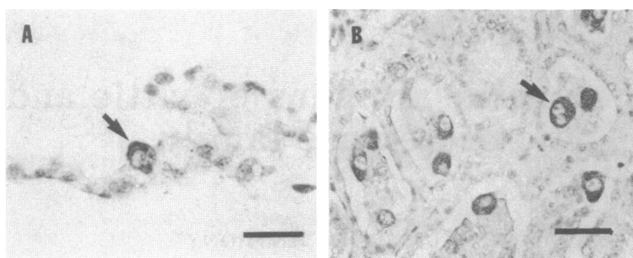

FIG. 1. Immunohistochemical localization for PAG in trophoblast and placenta. bPAG staining was detected in binucleate cells (indicated by arrow) within trophectoderm of day 18 bovine conceptus (*A*) and in bovine placenta of midgestation fetal cotyledonary villi (*B*) that interdigitate with maternal uterine tissue. No specific staining was observed in negative control sections when the antiserum was substituted with normal rabbit serum (data not shown). (Bars = 50 μm.)

Cotyledonary cDNA libraries were screened with ^{32}P-labeled cDNA isolated from the conceptus libraries under stringent conditions (19, 21). Purified clones that screened positively for both probes were converted into phagemids from λZAPII by the *in vivo* excision procedures (23) and sequenced.

RNA Isolation and Northern Blot. Tissue RNA was isolated by guanidinium thiocyanate extraction and purified on cesium chloride density gradients (19). RNA (20 μg from sheep cotyledons and 4 μg from bovine conceptuses) was separated by electrophoresis on 1.2% (wt/vol) agarose gels containing formaldehyde and transferred onto nylon membranes. Specific mRNA was detected by hybridization with random-primed ^{32}P-labeled full-length cDNA under the conditions described above for screening cotyledonary cDNA libraries.

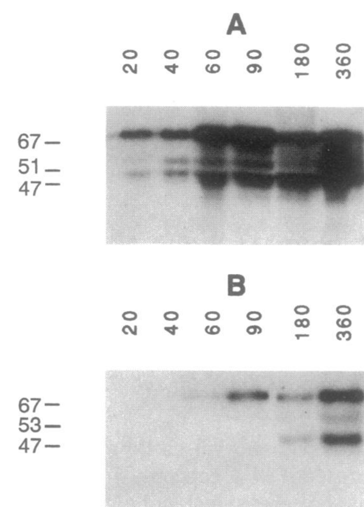

FIG. 2. Time course of oPAG biosynthesis in ovine placental explants *in vitro*. Trophoblast tissue from a day 25 conceptus was incubated for the time periods (in min) shown above the lanes. Immunocomplexes from tissue extract (*A*) and medium (*B*) were analyzed in 10% polyacrylamide gels containing SDS and radioactive polypeptides were detected by fluorography. Molecular weights on the left ($\times 10^{-3}$) were calculated from standards electrophoresed on each side of the gel.

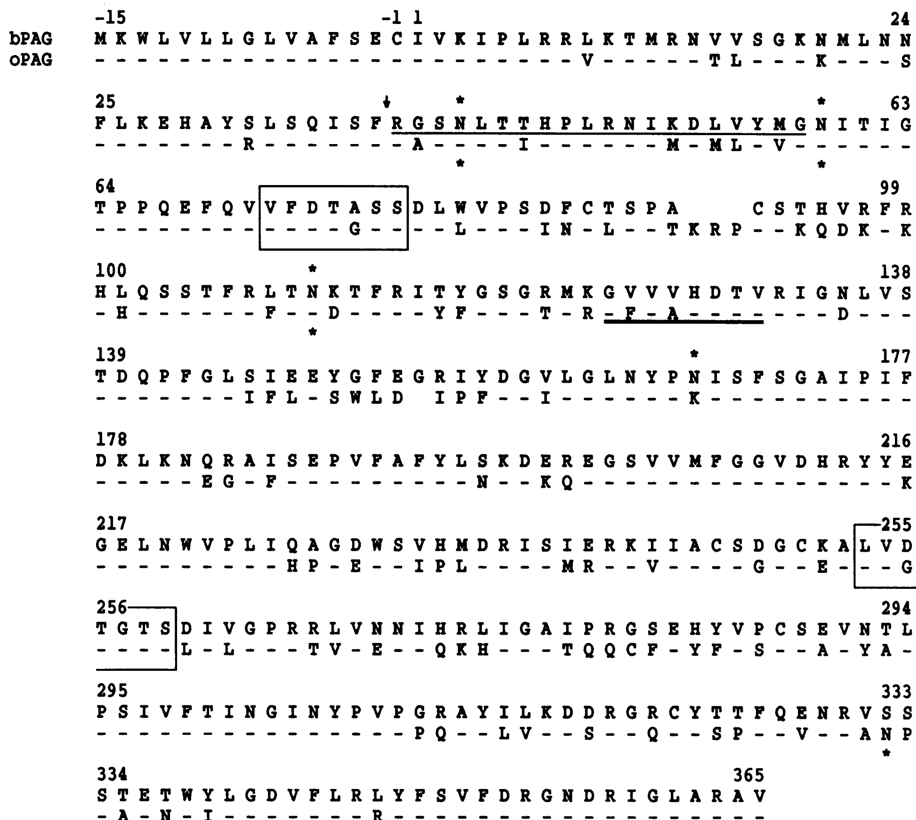

FIG. 3. Inferred amino acid sequences of bPAG and oPAG. A dash indicates a residue present in oPAG that is identical to the residue in bPAG. Gaps are shown by open spaces. Differences are indicated with the inclusion of the amino acid (single letter code). The putative signal peptide is numbered −15 to −1. The region of the protein corresponding to the NH_2 terminus of bPAG, as determined by amino acid sequencing (14), is underlined. This sequence (RGH–LTPGPLRNFKDLVYMG) differs at four positions (boldface type) from that inferred from the bp314 cDNA. The site at which the putative proform is cleaved is indicated by an arrow. The two active site segments are boxed and the second conserved aspartic domains of each lobe are double underlined. Potential glycosylation sites are marked by asterisks and include Asn-4 that appeared as a blank during cycle 4 of amino acid sequencing.

		NH_2-Terminus							COOH-Terminus						
Pepsin	(human)	V	F	D	T	G	S	S	I	V	D	T	G	T	S
Cathepsin E	(human)	I	F	D	T	G	S	S	I	V	D	T	G	T	S
Cathepsin D	(human)	V	F	D	T	G	S	S	I	V	D	T	G	T	S
Chymosin	(bovine)	L	F	D	T	G	S	S	I	V	D	T	G	T	S
Renin	(human)	V	F	D	T	G	S	S	L	V	D	T	G	A	S
PAG	(bovine)	V	F	D	T	A	S	S	L	V	D	T	G	T	S
PAG	(ovine)	V	F	D	T	G	S	S	L	V	G	T	G	T	S

FIG. 4. Comparison of the amino acid sequences in bPAG and oPAG with several aspartic proteinases at the regions flanking the aspartic acid residues considered to be essential for catalytic activity of pepsin. The two aspartyl side chains are located within the substrate-binding cleft and, though present on opposite lobes of the pepsin molecule, are within hydrogen-bond distance of each other. Sequences were inferred from cloned cDNA for human pepsin (30), human cathepsin E (32), human cathepsin D (34), bovine chymosin (33), and human renin (35). The NH_2 terminus refers to the sequences in the NH_2-terminal lobes, and COOH terminus refers to those in the COOH-terminal lobes.

Modeling of Active Site Regions. Residues of PAG that were sequentially homologous to the active site of pepsin were modeled in the context of a porcine pepsin (24), entry 4PEP, obtained from the Protein Data Bank (25). Residues 30–36 and 213–219 of porcine pepsin were replaced with the side chains of residues 72–78 and 255–261 of oPAG and residues 72–78 and 253–259 of bPAG by using PSFRODO (26). The structures were energy minimized by means of the program AMBER Version 3.0 (27). All modeling was carried out in the Protein Studies Program, Oklahoma Medical Research Foundation (Oklahoma City) by Jordan Tang.

Proteinase Assay. The assay was based on that of Lin *et al.* (28) and employed 0.01 μCi of reductively alkylated hemoglobin (methemoglobin; DuPont/NEN; 0.02 mCi/mg). Porcine pepsin (50 ng to 5 μg) was used as a standard.

RESULTS

Localization of PAG to Binucleate Cells. The antiserum to bPAG was used to immunolocalize PAG on sections of day 19 bovine trophoblast and bovine cotyledonary tissue at implantation sites (placentomes) around midpregnancy. In trophoblast, staining was confined to scattered binucleate cells in the trophectoderm (Fig. 1*A*). In the older placental tissues numerous positively stained binucleate cells were associated with fetal cotyledonary villi that had interdigitated with maternal uterine tissue (Fig. 1*B*). A similar localization of PAG to binucleate cells in both trophoblasts and mature placenta has been noted in ovine tissues (data not shown).

Synthesis of PAG by Placental Explant Tissue. The same antiserum used for immunostaining was employed to immunoprecipitate products synthesized by explants of ovine and bovine placental tissue. Fig. 2 shows data for day 25 ovine trophoblast after explants had been cultured in presence of L-[^{35}S]methionine. Clearly the antiserum recognized an array of molecules ranging in size from M_r 67,000 to M_r 47,000. The former was the dominant product in both tissue extracts and medium in the early stages of culture, but with time there was a major increase in the relative amount of the M_r 47,000 form. However, no clear-cut precursor–product relationship has been established between the various sizes of molecule.

Cloning of cDNAs for bPAG and oPAG. Conceptus cDNA libraries were screened with antisera, and the cDNA inserts of several positive clones were sequenced. None of these cDNA were larger than 300 base pairs (bp), but the bovine and ovine cDNA were closely similar in regions that were superimposable (≈80% sequence identity).

Sequenced ^{32}P-labeled cDNA were then used to screen cDNA libraries from day 260 bovine and day 100 ovine cotyledonary tissue. The ovine clone isolated (op201) was 1266 bp and consisted of a 20-bp untranslated 5′ segment, a 1146-bp open reading frame, and a 100-bp 3′ end (Fig. 3). The longest bovine clone (bp314) started at the thymidine within the putative ATG initiation codon and possessed a complete 3′ terminus. The 5′ upstream region for the bPAG mRNA was, therefore, derived from a second clone (bp319) that was identical in sequence to bp314 where the two overlapped. The cDNA for oPAG and bPAG, op201 and bp314, shared 86% nucleotide sequence identity and potentially coded for polypeptides 382 and 380 amino acids long (Fig. 3) with molecular weights of 42,985 and 42,852, respectively. The first 15 amino acids of each PAG appeared to constitute a signal peptide (29). Overall, oPAG and bPAG showed about 73% identity in amino acid sequences. The NH_2 terminus of bPAG began with Arg-39 (Fig. 3), suggesting that further proteolytic processing after signal peptide cleavage had occurred.

A GenBank search (July 24, 1991, for homology at the level of nucleotide and amino acid sequences) revealed that the PAG belonged to the aspartic proteinase family and clearly resembled human (30), hog (28), and monkey (31) pepsinogens (60% nucleotide sequence identity), human cathepsin E (32) (60% identity), chymosins (33) (57% identity), and cathepsin D (34) (58% identity). These similarities were reflected in their respective amino acid sequences (50% identity to pepsinogens), especially in the two active site regions of the aspartic proteinases where the amino acid residues flanking the aspartic acid are highly conserved (Fig. 4).

Expression of PAG mRNA During Pregnancy. Northern blot analysis of mRNA isolated from preimplantation conceptuses (Fig. 5*A*) and from placentae of later-stage fetuses

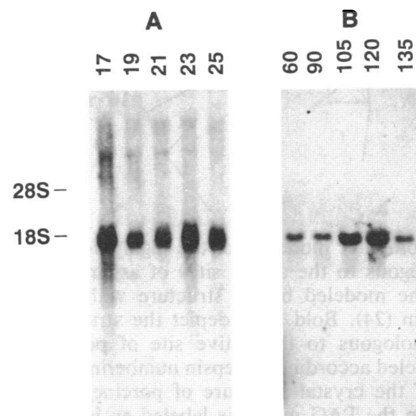

FIG. 5. Northern blot analysis of the RNA present in preimplantation bovine conceptuses and in cotyledonary tissue from postimplantation ovine conceptuses. RNA (4 μg) isolated from bovine conceptuses (*A*) and RNA (20 μg) from cotyledonary tissue of ewes (*B*) at various days of pregnancy as indicated above lanes (gestation is ≈145 days) are shown. The positions of 28S and 18S rRNA are shown to the left.

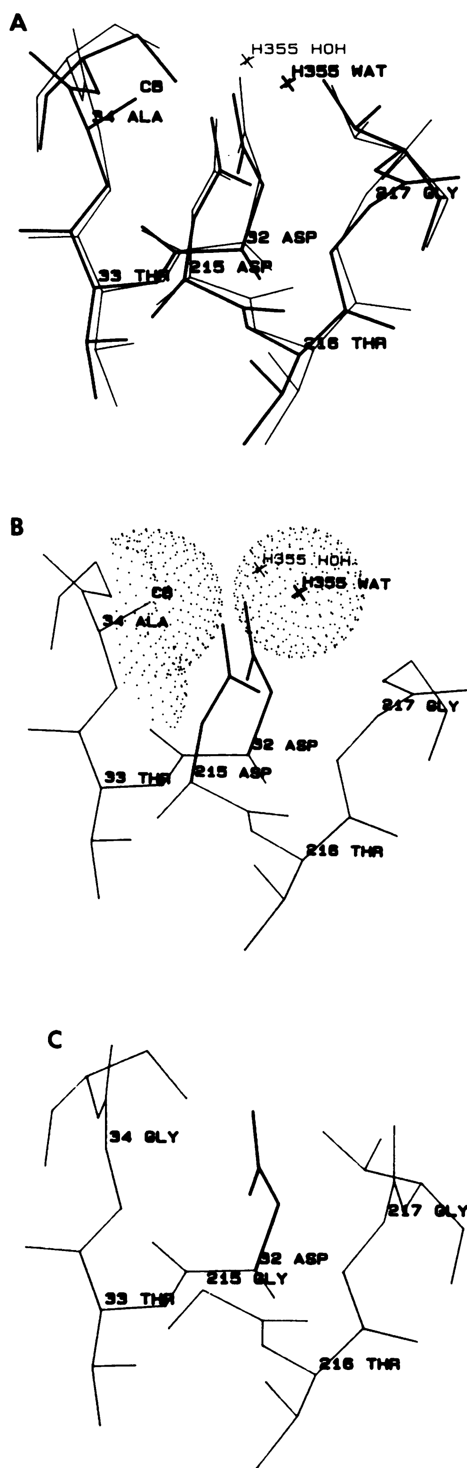

FIG. 6. Computer modeling of the highly conserved regions of PAG, homologous to the active sites of aspartic proteinases. (*A*) Overlay of the modeled bPAG structure with the active site of porcine pepsin (24). Bold lines depict the structure of the bPAG residues homologous to the active site of porcine pepsin, with α-carbons labeled according to pepsin numbering. Thin lines are the active site of the crystal structure of porcine pepsin. The water molecule from the PAG model is labeled as H355 WAT, and the solvent from the crystal structure of porcine pepsin is labeled as H355 HOH. (*B*) Computer model of the elements of bPAG homologous to the active site of pepsin. The "active-site" Asp-32 and Asp-215 (pepsin numbering) are plotted with bold lines. The van der Waals surface of water molecule H355 WAT and Ala-34 from the PAG model are depicted as stippled areas. The native position of H355 HOH from the crystal structure of porcine pepsin is drawn and labeled with light lines. The native position of the solvent is altered by the presence of the methyl group of the Ala-34 β-carbon, labeled CB. (*C*) Model of the elements of oPAG homologous to the active site of porcine pepsin. Heavy lines depict the "active-site" Asp-32 (pepsin numbering). Note the absence of the active-site Asp-215.

(Fig. 5*B*) revealed that PAG mRNA (size, ≈1.7 kilobases) was abundant and expressed in a relatively uniform manner throughout gestation. There was no evidence for size heterogeneity that might account for the differences in molecular weights of forms noted in Fig. 2.

Modeling of Active Site Regions of PAG. The active sites of bPAG and oPAG were modeled in the context of a porcine pepsin. The bovine PAG model, analogous to a G34A mutation in pepsin, revealed a subtle change in the van der Waals surface of water molecule H355 and Ala-34 in the "active site" determined from the energy minimization (Fig. 6 *A* and *B*). In the active site of crystal structures for known aspartic proteinases, a water molecule is situated medially between the two active site aspartic acids, Asp-32 and -215 (36). This highly conserved solvent molecule is probably displaced by the β carbon of the Ala-34 (pepsin numbering) of bPAG.

The structure of the oPAG, analogous to a D215G mutation in pepsin (28), showed little difference from the expected structure of a pepsin lacking one of the active site aspartic acids (Fig. 6*C*).

Lack of Proteolytic Activity of PAG. Up to 1 μg of purified bPAG was unable to hydrolyze denatured hemoglobin in a standard assay for aspartic proteinases that was sensitive to 100 ng of porcine pepsin. Moreover, crude culture medium from ovine cotyledonary tissue and partially purified oPAG also lacked pepsin-like activity in the standard assay.

DISCUSSION

These experiments show that a major secretory product of trophoblast binucleate cells of cattle and sheep throughout pregnancy is a glycoprotein with a primary structure resembling that of aspartic proteinases. The ovine and bovine molecules are closely similar in sequence and immunologically related to each other. Whether placental products homologous to the PAG are to be found outside the domestic ruminant species is not yet known.

Although the PAGs clearly belong to the aspartic proteinases, they are probably not active proteolytically. Catalytic activity of pepsin is dependent upon two conserved aspartic acid residues, at positions 32 and 215, that are in close contiguity in the center of the substrate-binding cleft (36–38), even though they are in opposite lobes of the molecule. Each lobe itself exhibits twofold symmetry and possesses a second conserved region centering around an aspartic acid. The active site segments and the secondary conserved regions are clearly present in the two PAGs (Figs. 3 and 4). Furthermore, both putative catalytic aspartic acids are present in bPAG, and the flanking regions are also largely conserved, except Ala-76 replaces Gly in the Asp-Thr-Gly-Ser sequence (Fig. 4). This change would be analogous to a G34A mutation in pepsin. An overlay of the modeled bPAG structure in this region shows that it is indeed closely similar to that of pepsin (Fig. 6*A*), except for a subtle change in the placement of the water molecule that normally is situated medially between the two active site aspartic acids. During catalysis, this solvent molecule is proposed to be activated and to make a nucleophilic attack on the substrate bond carbonyl (36). Although a G34A mutation in pepsin has not been tested, such improper positioning might be expected to thwart the nucleophilic capability of the water molecule and render the protein catalytically inactive. A computer model of the bPAG site showing how the van der Waals surface of this water molecule is altered by the proximity of the methyl

group of Ala-76 (Ala-34 by porcine pepsin numbering) illustrates this point (Fig. 6*B*).

For oPAG, the aspartic acid (Asp-257) at the catalytic domain is replaced by Gly (Fig. 6*C*) and would, therefore, be analogous to a D215G mutation in pepsin, which again has not been tested. However, a comparable change in pepsin, where the active site Asp-32 of the first lobe is replaced by Ala (a D32A mutation), abolishes all activity, even though the molecule is still capable of binding pepstatin A with high affinity (28).

Thus these observations explain why both bPAG and oPAG are likely to be proteolytically inactive. However, the data also suggest that the deep substrate-binding clefts would be largely unaffected by the substitutions and still might be capable of accommodating a peptide chain of appropriate conformation.

The molecular weights of PAG produced by placental extracts (Fig. 1) are considerably higher than the value (≈37,000) anticipated from the inferred sequences of their polypeptide chains. It seems unlikely that N-linked carbohydrate chains alone can account for this discrepancy since there are only four potential sites for N-glycosylation on the molecule, and the total neutral carbohydrate content of bPAG has been estimated to be less than 10% (14). Conceivably additional molecular mass is acquired from other processing events so far undefined. The relationship between the molecular weight forms of oPAG shown in Fig. 2 is likewise unclear. Pulse–chase and continuous labeling studies have not yet enabled us to establish a clear precursor–product relationship between these size classes of molecule.

In conclusion, the PAG antigens that are first produced by binucleate cells during the very early phases of trophoblast attachment and implantation are a heterogeneous group of molecules with considerable amino acid sequence identity to pepsin. They do not, however, appear to be enzymatically active, although the substrate-binding clefts on the molecules are probably conserved. Clearly such molecules may still be capable of binding polypeptide ligands. Their relative abundance and localized expression in the placental cells that encroach most aggressively into maternal tissues at implantation sites suggest that they have an important, though as yet unresolved, function during pregnancy.

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