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Purification, Characterization, and Immunocytochemical Localization of the Major Basic Protein of Pig Blastocysts[1]

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ABSTRACT

The major basic protein (BP) synthesized and secreted by elongating pig blastocysts was purified from medium of Day 14–17 conceptus cultures. Sequential ion-exchange and gel-filtration chromatographies resulted in isolation of BP as a single polypeptide of M_r = 43,100 or 42,800 under denaturing or native conditions, respectively. BP was found to be a glycoprotein by incorporation of [^{3}H]glucosamine and susceptibility to N-glycopeptidase F. Two BP polypeptides were produced by N-glycopeptidase F (M_r = 39,800 and 36,300). Antiserum to BP immunoprecipitated radiolabeled BP from blastocyst culture medium. BP was not detected in medium from 1–2 mm diameter spherical (Day 10) blastocysts but was found in medium from 3–5 mm spherical (Day 10) and filamentous (<50 cm, Day 12) conceptuses, suggesting that BP synthesis and secretion began at the initiation of trophoblast expansion. With immunocytochemical procedures, BP was located in the apical cytoplasm of trophectoderm cells of Day 11 expanding (5–7 and 10–20 mm) blastocysts. These results suggest that trophoblast epithelium secrete BP apically toward the uterine lumen and that BP may play a role in maternal-fetal interactions during the peri-implantation period.

INTRODUCTION

The porcine conceptus undergoes extensive cellular proliferation and remodeling during the blastocyst stage (Day 5–15 post estrus). Hatching from the zona pellucida occurs on Day 6–7, and the 1–2 mm diameter, spherical blastocyst consists of the inner cell mass (ICM, embryonic disk) and the trophoblast, which contacts the uterine epithelium. On Days 10–11, the blastocyst expands from a 1–2 mm diameter sphere into a 10–50 mm tubular shape (Day 11.5), then to a filamentous structure greater than 150 mm in length (Day 12). The growth rate has been estimated to exceed 45 mm/h due mainly to cellular remodeling, not cell division (Geisert et al., 1982). At this time, the trophoblast synthesizes and secretes estrogen (Gadsby et al., 1976, 1980), which may be the signal for maternal recognition of pregnancy (Frank et al., 1978; Bazer et al., 1984). After Day 12, growth occurs by both hyperplasia and hypertrophy to an overall length in excess of 1 m (Geisert et al., 1982). Trophoblast attachment to the uterine epithelium occurs between Days 16 and 18.

During the expansion and elongation stages, the pig blastocyst synthesizes and secretes several polypeptides of unknown function (Godkin et al., 1982a; Powell-Jones et al., 1984; Whyte et al., 1986-1987). A group of low molecular weight acidic polypeptides have been identified as interferon-α-like proteins (Cross and Roberts, 1988), similar to those secreted by blastocysts of sheep (Godkin et al., 1982b; Imakawa et al., 1987; Stewart et al., 1987) and cattle (Helmer et al., 1987; Imakawa et al., 1988). In sheep, interferon of trophoblast origin is implicated as the embryonic signal for maternal recognition of pregnancy (Godkin et al., 1984a,b). A high molecular weight glycoprotein, similar to one from sheep and cow

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blastocysts (Masters et al., 1984), has been implicated in immune suppression during early pregnancy (Murray et al., 1987).

Between Days 13 and 18, a basic polypeptide (BP) with an approximate molecular weight of 45,000 was an abundant secretory protein whose temporal expression correlated with blastocyst elongation (Godkin et al., 1982a). BP was partially purified from medium of Day 16 blastocyst cultures by a combination of diethylaminoethyl (DEAE)-cellulose, carboxymethyl (CM)-cellulose, and Sephacryl S200 chromatographies.

Although the function of BP is unknown, purification of this major secretory product is a necessary first step towards determining its role in early pregnancy. The objectives of the experiments described in this paper were to complete the purification of BP, to characterize the protein biochemically, and to produce a monospecific antibody in order to study BP synthesis and secretion. BP was isolated by a combination of CM-Biogel and gel-filtration chromatography. An antiserum was raised in rabbits and used to demonstrate the presence of BP in blastocyst trophectoderm cells.

MATERIALS AND METHODS

Materials

Tissue culture medium was purchased from GIBCO Laboratories, Life Technologies, Inc. (Grand Island, NY). L-[4, 5-^{3}H]leucine ([^{3}H]leucine; 55 Ci/mmol), D-[6-^{3}H]glucosamine hydrochloride ([^{3}H]glucosamine; 25 Ci/mmol) and L-[methyl-^{35}S]methionine ([^{35}S]methionine; 800 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA) or New England Nuclear (Boston, MA). Chemicals for electrophoresis were described previously (Roberts et al., 1984). Carboxymethyl-Biogel (CM-Biogel) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) were from Bio-Rad Laboratories (Richmond, CA). Sephacryl S200, Sephadex G75, and Protein-A Sepharose were from Pharmacia-LKB (Piscataway, NJ). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma Chemical Co. (St. Louis, MO) and glycopeptide-N-glycosidase (N-glycopeptidase F) was from Boehringer-Mannheim (Indianapolis, IN). Detection of bound antibodies in tissue sections utilized the Histogen Immunohistology Kit (Peroxidase-Anti-peroxidase System; Bio-Genex Laboratories, San Ramon, CA). All other chemicals were reagent grade or better.

Animals

Gilts were checked daily for estrus in the presence of an intact boar, and the day of the onset of estrus was designated Day 0. Gilts were bred by natural service. Gilts were hysterectomized under asceptic conditions between Days 11 and 17 of pregnancy.

Conceptus Cultures

For purification of BP, conceptuses were collected on Days 14–17 of pregnancy by flushing each uterine horn with 50 ml of Hanks' Balanced Salt Solution (Bazer et al., 1978; Godkin et al., 1982a). One-half of the blastocyst tissue from each horn was incubated for up to 20 h in 50 ml Modified Eagle's Medium (MEM) supplemented with 5 g/l glucose and 5 μCi/ml [^{3}H]leucine (Basha et al., 1980; Godkin et al., 1982a). Blastocysts were incubated in an atmosphere of 50% (v/v) O_2, 45% (v/v) N_2, and 5% (v/v) CO_2 at 37°C in a gas-tight chamber on a rocking platform.

For determination of the onset of BP synthesis, blastocysts were collected on Days 10, 12, 14, and 17 of pregnancy. Day 10 blastocysts were sorted by size (1–2 mm diameter or 3–5 mm diameter) and incubated in 5 ml of medium with 5 μCi/ml of [^{3}H]leucine. Day 12 through 17 conceptuses were incubated in 20 ml of MEM with 5 μCi/ml of [^{3}H]leucine, 15 μCi/ml of [^{35}S]methionine, or 6 μCi/ml of [^{3}H]glucosamine.

Protein Electrophoresis

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) was performed according to the procedure of Laemmli (1970) in 10% (w/v) polyacrylamide gels. Proteins were analyzed using two-dimensional (2D) SDS-PAGE as described by O'Farrell (1975) and modified by Roberts et al. (1984) or by the nonequilibrium pH gradient electrophoresis system (NEPHGE) of O'Farrell et al. (1977). Proteins in gels were fixed and stained with Coomassie Brilliant Blue. Gels were impregnated with 1 M sodium salicylate for fluorography and dried (Chamberlain, 1979). Radiolabeled proteins were detected using Kodak XAR film. Protein standards used were human transferrin (M_r = 78,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 45,000), chymotrypsinogen (M_r = 27,500) and ribonuclease A (M_r = 14,300).

Purification of BP

All procedures were conducted at 4°C. All buffers used contained 0.02% (w/v) sodium azide, 7% (w/v) glycerol, 1 mM PMSF, and 10 mM ethylenediaminetetraacetic acid (EDTA), unless noted otherwise. Individual fractions from column chromatography separations were monitored for radioactivity by scintillation spectrometry, protein by absorbance at 280 nm, and acid phosphatase activity using p-nitrophenylphosphate as substrate at pH 4.5 (Schlosnagle et al., 1974). The concentration of protein in pooled fractions and in purified BP preparations was quantified by the method of Lowry et al. (1951), with bovine serum albumin used as the standard.

Approximately 500 ml of conceptus culture medium ([^{3}H]leucine-labeled, Days 14–17) were dialyzed against 12 l (4 l × 3 changes) of 10 mM sodium acetate, pH 5.4. A precipitate was removed by centrifugation (10,000 × *g*; 20 min), and the supernatant medium was applied to a 2.5 × 10-cm column of CM-Biogel equilibrated in 10 mM sodium acetate, pH 5.4. Bound proteins were eluted with a linear gradient of NaCl (0 to 0.5 M) in the equilibration buffer.

The late-eluting radiolabeled peak from above was pooled, diluted 1:1 with water, concentrated by binding to a 1-ml column of CM-Biogel, and then eluted with 0.5 M NaCl. This fraction was applied to a 2 × 80-cm column of Sephadex G75 superfine containing 0.33 M NaCl and buffered with 10 mM sodium acetate, pH 5.4. The radiolabeled peak was pooled, dialyzed against 10 mM sodium acetate, pH 5.4, and applied to a 0.5 × 5-cm column of CM-Biogel. Bound protein was eluted with a linear gradient of NaCl (0.10–0.35 M) in dialysis buffer.

Glycopeptidase F Treatment

Approximately 4 μg of BP were incubated for 18 h at 37°C with 1 mU of glycopeptidase F in 200 mM potassium phosphate (pH 7.2), 10 mM o-phenanthroline, and 20 mM EDTA (Tarentino et al., 1985). Removal of carbohydrate residues on BP was detected by an increase in the electrophoretic mobility of BP using 1D-SDS-PAGE and fluorography.

Antiserum to BP

Purified BP (100 μg) was separated in a 10% polyacrylamide gel (1D-SDS-PAGE). A companion lane was fixed and stained with Coomassie Brilliant Blue to locate BP. The gel was rinsed with water and the BP-a band was cut out of the unstained gel. The gel slices containing BP-a were macerated, emulsified in Complete Freund's Adjuvant, and injected s.c. into the shoulders and back of a male New Zealand White rabbit (Vaitukaitis et al., 1971). Thirty days later, a second preparation in Incomplete Freunds Adjuvant was injected similarly. Serum was collected 2 wk after the boost injection. Prior to immunization, serum was collected from the rabbit (preimmune serum) to detect nonspecific binding in immunoprecipitation, western blotting, and immunocytochemistry procedures.

The specificity of the antiserum (anti-BP) was determined by immunoprecipitation of radiolabeled BP from culture medium, and by western blotting of BP and the basic glycoprotein of pig endometrium, uteroferrin (Uf). Western blotting of proteins separated by 1D-SDS-PAGE was performed by the method of Towbin et al. (1979). Goat anti-rabbit IgG conjugated with horseradish peroxidase (using 4-chloro-1-naphthol and 0.002% (v/v) hydrogen peroxide as substrates) was used for visualization of bound primary antibody.

Purification of Uf and Anti-Uf Production

Uf was purified from uterine washings of a gilt on Day 45 of pseudopregnancy as described previously (Buhi et al., 1982; Baumbach et al., 1984). Purity of the Uf preparation was assessed by 1D-SDS-PAGE and shown to be a single band at M_r = 35,500. Uf (1 mg) was emulsified in Complete Freund's Adjuvant and injected into a rabbit as described previously (Baumbach et al., 1984) to produce antibody to Uf (anti-Uf). Booster injections were at monthly intervals using Incomplete Freund's Adjuvant.

Immunoprecipitation

Culture medium (100–500 μl) was incubated with 5 μl of anti-BP at 4°C overnight. Immune complexes were collected onto Protein A-Sepharose (100-μl of a 10% [v/v] suspension in 50 mM tris(hydroxymethyl) aminomethane [Tris] acetate, pH 7.5, 0.15 M NaCl, 1 mM PMSF, 2% [v/v]Nonidet P-40 and 0.02% [w/v] sodium azide). The gel matrix was washed five times using 50 mM Tris acetate (pH 7.5), 0.15 M NaCl, 0.5% (v/v) Nonidet P-40, and 0.1% (w/v) SDS, and a final wash in water.

Immunocytochemistry

The immunocytochemical technique of Sternberger et al. (1970) was employed as described by Godkin et al. (1984a). Day 11 blastocyst tissue and Day 11 nonpregnant endometria were fixed at 4°C for 1–2 h in Bouin's fixative, washed in 0.9% (w/v) NaCl, and dehydrated in ethanol. Tissue was embedded in paraffin, cut into 6-μm-thick sections and deparaffinized (Galigher and Kozloff, 1971).

Endogenous tissue peroxidases were blocked with 3% (v/v) hydrogen peroxide. Sections were incubated in normal goat serum. Anti-BP, anti-Uf, or preimmune serum were diluted (1:50, 1:100, 1:200, 1:500, 1:1,000, and 1:2,000) in 0.05 M phosphate (pH 7.4), and 0.14 M NaCl, applied to sections, and allowed to react at 4°C for 18 h. Bound antibody was detected by the peroxidase-anti-peroxidase method (Sternberger et al., 1970) using 3-amino-9-ethylcarbazole and 0.5% (v/v) hydrogen peroxide as substrates. Sections were not counterstained. To detect possible antibody reactivity to Uf, anti-BP (1:100) was incubated at 4°C for 18 h with 100 μg/ml Uf, centrifuged (15,000 × *g*, 15 min), and applied to tissue sections. Alternatively, 2 ml of anti-BP was applied to a 1 × 6-cm column of Uf-Sepharose, as described previously (Baumbach et al., 1984). The unbound fraction was diluted to 200 ml (1:100) and applied to tissue sections.

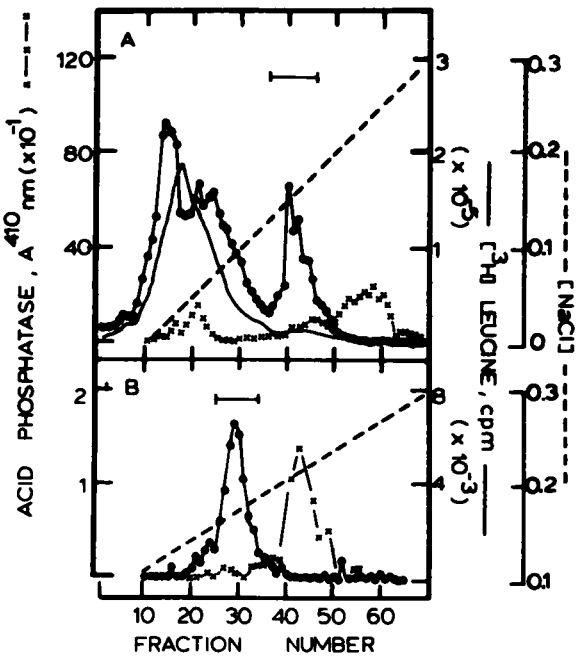

FIG. 1. CM-Biogel fractionation of conceptus secretory proteins radiolabeled with [^{3}H]leucine. Medium pooled from Day 14 to 17 conceptus cultures was applied to a 2.5 × 10-cm column of CM-Biogel at pH 5.4 *(Panel A)*. *Panel B* is the CM-Biogel elution profile of the basic protein (BP)-containing fraction pooled from the Sephadex G-75 fractionation step (see *Fig. 3*). Bound protein was eluted with a linear gradient of NaCl *(dashed line)*. Indicated for each fraction is total protein *(solid line*; A^{280} = absorbance at 280 nm), total radioactivity *(closed circles)* and acid phosphatase activity (*x*–*x*; A^{410} = absorbance at 410 nm). The elution position of BP is indicated by a *bar*.

RESULTS

Purification of BP

Approximately 500 ml of [^{3}H]leucine-labeled medium pooled from blastocyst cultures were used to purify BP. Basic proteins were eluted with a linear gradient of NaCl as shown in Figure 1A. Two radiolabeled protein fractions were detected. The late eluting fraction (0.15 M NaCl) contained one radiolabeled polypeptide with an approximate molecular weight of 43,000 (BP-a; Fig. 2B, Lane 3) and two Coomassie Brilliant Blue-stained polypeptides with molecular weights of 43,000 and 35,000 (Fig. 2A, Lane 3) by 1D-SDS-PAGE. The latter polypeptide (M_r = 35,000) was not radiolabeled (i.e., compare Lane 3, Fig. 2A and 2B). Acid phosphatase activity was detected in column fractions co-eluting with BP (Fig. 1A), suggesting that the nonradiolabeled polypeptide was uteroferrin (Uf), the basic acid phosphatase (M_r = 35,500) secreted by the pig uterus (Buhi et al., 1982; Schlosnagle et al., 1974). Western blotting of 1D-SDS-PAGE-separated proteins from the BP peak with anti-Uf confirmed the identity of the M_r = 35,000 polypeptide as Uf (not shown).

The late-eluting radiolabeled peak from Figure 1A was pooled, concentrated and applied to Sephadex G75 (Fig. 3). A single radiolabeled protein peak was detected having a molecular weight of 43,000. The bulk of the remaining acid phosphatase activity was separated from radiolabeled protein.

The Sephadex G75 peak was applied to a 0.5 × 15-cm column of CM-Biogel. Bound protein was eluted with a linear gradient of NaCl (0.10 to 0.35 M). As shown in Figure 1B, more than 90% of the acid phosphatase activity eluted later than radiolabel. The amount of Uf was reduced greatly when the radiolabeled peak in Figure 1B was pooled as indicated, analyzed by 1D-SDS-PAGE, and then stained with Coomassie Brilliant Blue (Fig. 2A, Lane 4). Approximately 4 μg of this BP preparation was subjected to 1D-SDS-PAGE, transferred to nitrocellulose paper, and probed with anti-Uf (Fig. 4A). At a dilution of 1:700, Uf was not detected in the

preparation of BP (Fig. 4A). Anti-Uf did detect 3 μg of purified Uf in an adjacent lane. The lower limit of detection of Uf by anti-Uf (1:700) was approximately 20–50 ng (results not shown).

Examination of the BP preparation by fluorography revealed the presence of a single polypeptide of M_r = 43,000 by 2D NEPHGE (Fig. 2C) or by 1D-SDS-PAGE (Fig. 2B, Lane 4). In the latter analysis, higher molecular weight, radiolabeled polypeptides were found in addition to BP-a when 2-mercaptoethanol was omitted from sample preparations and the fluorogram was overexposed (Fig. 2B, Lane 4). Addition of the reducing agent resulted in loss of these minor, higher molecular weight polypeptide species (Fig. 5A and B, Lane 1).

Yields of BP varied between preparations. Typically 100–500 μg of BP were isolated from 500 ml of culture medium. One preparation yielded nearly 2 mg.

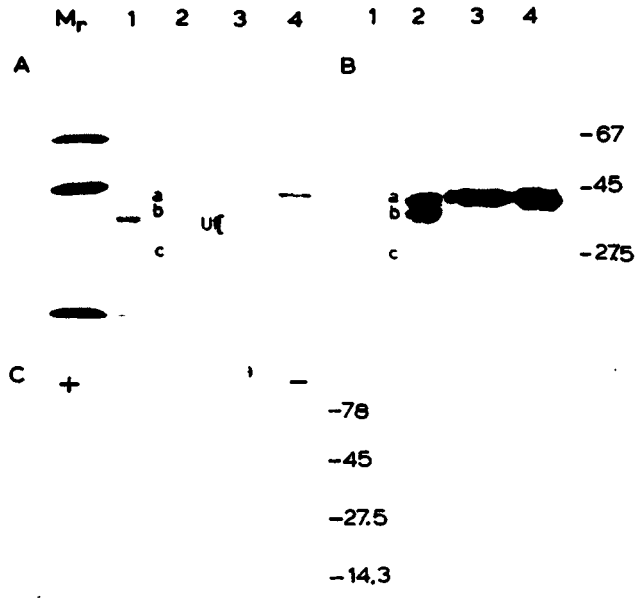

FIG. 2. Electrophoretic analysis of basic protein (BP) fractions during purification. In *Panel A* is a Coomassie Brilliant Blue-stained electropherogram of CM-Biogel fractions containing [^{3}H]leucine-labeled BP. The resulting fluorogram (3-wk exposure, −70°C) is shown in *Panel B* and was intentionally overexposed to reveal minor polypeptides. *Lane 1* contains purified uteroferrin (*Uf*, 3 μg). The pooled Fractions 36–46 from the initial CM-Biogel elution step *(Fig. 1A)* were loaded in *Lanes 2* (6 μg) and 3 (2 μg) from medium prepared in the absence *(Lane 2)* or presence *(Lane 3)* of EDTA and PMSF. The location of Uf is indicated with a *bracket*. The BP polypeptides are labeled *a*, *b*, and *c*. The pooled fraction (26–34) from the second CM-Biogel step was solubilized without 2-mercaptoethanol and loaded in *Lane 4* (3 μg). This same sample was reduced with dithiothreitol and analyzed by 2D-NEPHGE. The resulting fluorogram is shown in *Panel C*. Molecular weight (M_r) standards are shown in the *left lane* of *Panel A* and are indicated ($\times 10^{-3}$) in the *right* margins.

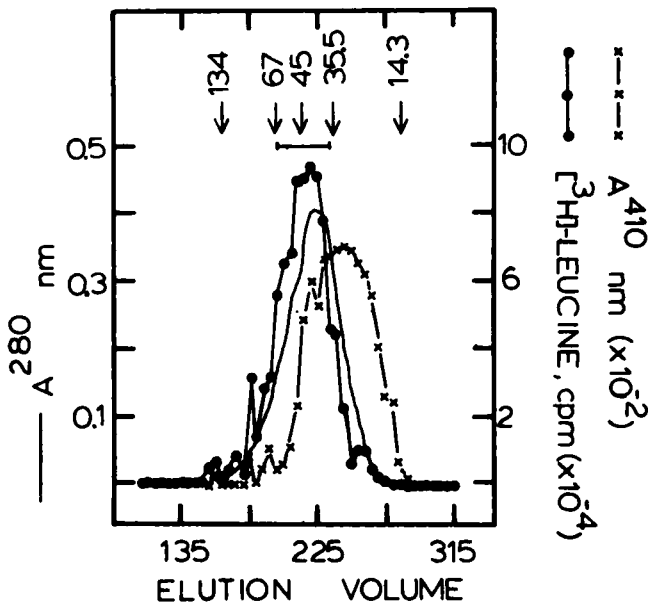

FIG. 3. Sephadex G-75 fractionation of basic conceptus secretory proteins. The basic protein (BP)-containing fractions (36–46) from CM-Biogel, pooled as indicated in *Figure 1A*, were concentrated and applied to a 2 × 80-cm column of Sephadex G-75. Total radioactivity, total protein, and acid phosphatase activity are indicated as described in *Figure 1*. The *bar* indicates fractions pooled for subsequent analyses. *Arrows* indicate the position and molecular weight ($\times 10^{-3}$) of protein standards: dimeric bovine albumin (M_r = 134,000), monomeric bovine albumin (M_r = 67,000), ovalbumin (M_r = 45,000), and ribonuclease A (M_r = 14,300). Also shown is the elution position of purified uteroferrin (*Uf*, M_r = 35,500).

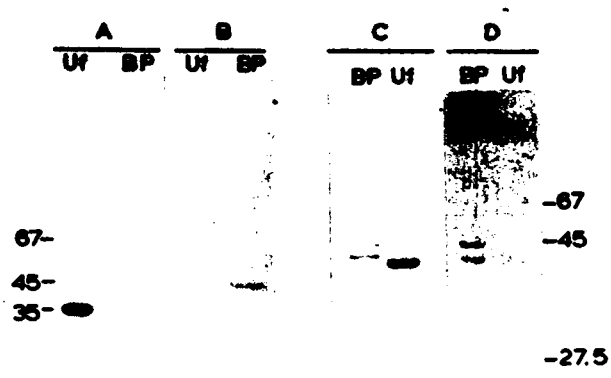

FIG. 4. Western blot analysis of purified basic protein *(BP)*. BP was purified and subjected to 1D-SDS-PAGE, transferred to nitrocellulose and probed with anti-uteroferrin (Uf) *(Panel A)* or anti-BP *(Panels B* and *D)* at dilutions of 1:700. The BP sample (4 μg) used in *Panels A* and *B* was purified in the presence of EDTA and PMSF, whereas the sample in *Panels C* and *D* (6 μg) was purified in the absence of these protease inhibitors. *Panel C* is a Coomassie Brilliant Blue-stained companion gel to the immunoblot in *Panel D*. Purified Uf (3 μg) was applied in adjacent lanes as control.

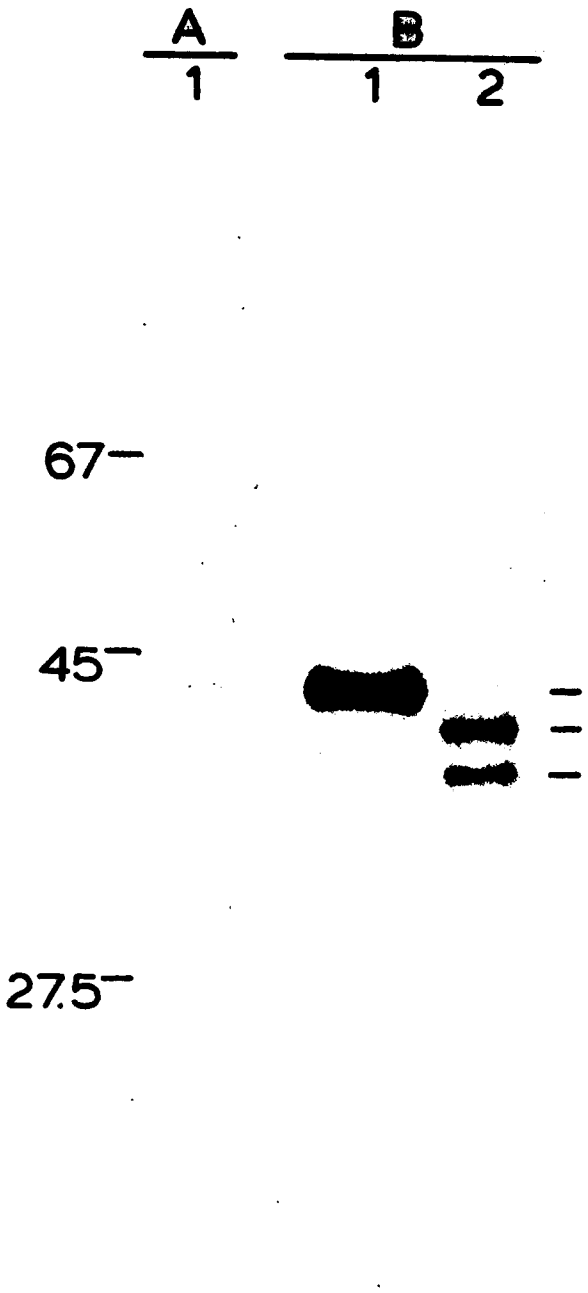

FIG. 5. Electrophoretic analysis of purified basic protein (BP)-a and of deglycosylated BP-a. [^{3}H]Leucine-labeled BP (4 μg; 1000 dpm) was treated with 2 mU of glycopeptidase F for 18 h and subjected to 1D-SDS-PAGE using 2-mercaptoethanol reduction during sample preparation. In *Panel A* is purified BP-a stained with Coomassie Brilliant Blue (untreated control). *Panel B* is the resulting fluorogram of untreated (*Lane 1*) and glycopeptidase F-treated (*Lane 2*) BP-a. Molecular weight is indicated ($\times 10^{-3}$) in the left-hand margin.

Molecular Weight Estimation of BP

The molecular weight of BP was determined under denatured and native conditions. When reduced with 2-merceptoethanol and denatured by boiling in SDS, BP-a had a molecular weight of 43,000 ± 2600 ($\bar{x}$ ± SD; 4 preparations, n = 13 electrophoretic separations) by 1D-SDS-PAGE in a 10% polyacrylamide gel. By gel filtration (Sephadex G75) chromatography at pH 5.4 in 0.33 M NaCl (native conditions), BP had a molecular weight of 42,800 ± 1600 (3 preparations; n = 3 separations).

When PMSF and EDTA were omitted from buffers, additional radiolabeled polypeptides (BP-b and BP-c) were typically present (Fig. 2A and B, Lane 2). By 1D-SDS-PAGE analysis, BP-b and BP-c had molecular weights of 36,400 ± 3500 and 22,300 ± 2800 (2 preparations; n = 8 separations), respectively.

Preparation of Antiserum to BP

The BP polypeptide (BP-a; 100 μg) was excised from an 1D-SDS-PAGE gel and injected into a rabbit. Serum collected 2 wk following a second 100-μg booster of BP was used in the Western blotting procedure. Purified Uf and two different BP preparations (one consisting of only BP-a and a second consisting of both BP-a and BP-b) were subjected to electrophoresis and transferred to nitrocellulose paper. Anti-BP at a 1:700 dilution did not react with 3 μg of Uf but did bind to 4 μg of BP-a (Fig. 4B). Pre-immune serum did not react with BP in the range of dilutions covering 1:700 to 1:100 (not shown). Furthermore, antibody generated against BP-a also reacted with BP-b (Fig. 4C and 4D). This antiserum also reacted with BP-c when sufficient amounts (>10 μg) of certain BP preparations were used. When total medium proteins from a Day 17 conceptus culture were separated by 1D-SDS-PAGE, transferred, to nitrocellulose and probed with anti BP, polypeptides with molecular weights of 43,0000, 36,000, and 22,000 bound antibody (not shown).

BP was immunoprecipitated using anti-BP from [^{35}S]methionine-supplemented medium of Day 17 blastocysts. A single radiolabeled band with a molecular weight equivalent to purified [^{3}H]leucine-labeled BP-a was detected by 1D-SDS-PAGE and fluorography (Fig. 6A, Lanes 1 and 2). Preimmune serum did not react with BP in medium (Lane 3). These

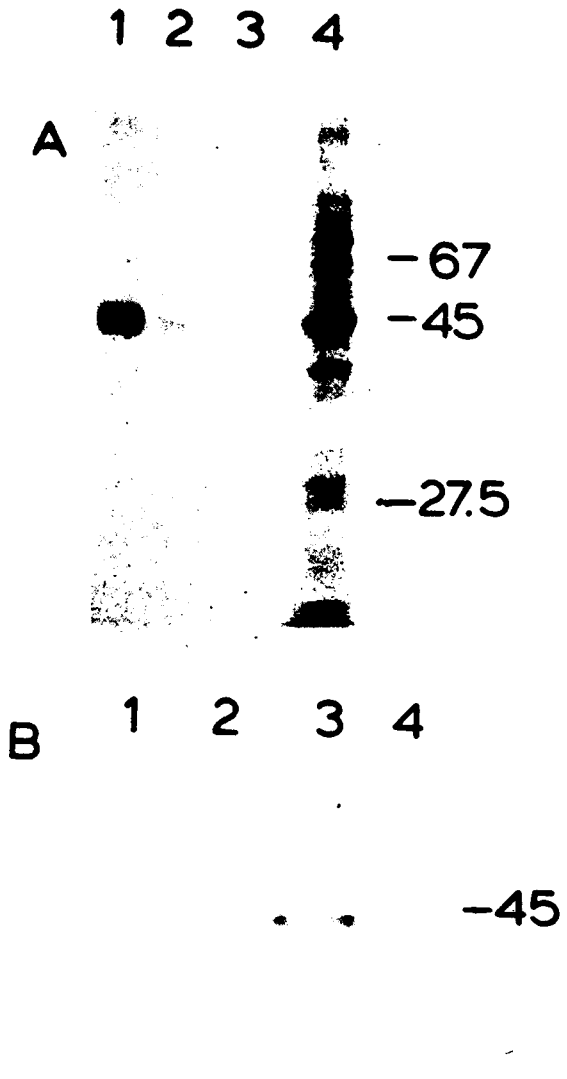

FIG. 6. Immunoprecipitation of radiolabeled basic protein (BP) from conceptus culture medium. Day 14 blastocysts were incubated with [^{35}S]methionine and the medium (500 μl) subjected to immunoprecipitation with anti-BP and Protein A-Sepharose and analyzed by SDS-PAGE and fluorography *(Panel A)*. The complete immunoprecipitation product was loaded in *Lane 1*. Purified, [^{3}H]leucine-labeled BP was loaded in *Lane 2*. In *Lane 3*, preimmune serum was substituted for anti-BP in the immunoprecipitation protocol. Total medium proteins used as the source of BP for immunoprecipitation were loaded in *Lane 4*. In *Panel B* is the resulting fluorogram of 1D-SDS-PAGE separated and immunoprecipitated BP ([^{3}H]leucine-labeled, *Lanes 1–3*; [^{3}H] glucosamine-labeled, *Lane 4*) from medium (500 μl) of eleven 1–2 mm diameter blastocysts (Day 10; *Lane 1*), six 3–5 mm diameter blastocysts (Day 10; *Lane 2*) or early filamentous blastocysts (<50 cm, Day 12; *Lanes 3* and *4*). Molecular weight is indicated ($\times 10^{-3}$) in the *right margin*.

results suggested that a monospecific antiserum was generated against BP.

Synthesis of BP by Blastocysts of Different Developmental Stages

Blastocysts from Day 10 (sorted by size) were incubated in 5 ml of DMEM containing [^{3}H]leucine. BP was immunoprecipitated from medium (25,000 cpm total/sample) using anti-BP and analyzed by 1D-SDS-PAGE and fluorography (Fig. 6B). No radiolabeled BP was detected in medium from eleven blastocysts (Day 10, 1–2 mm diameter, spherical; Lane 1). BP was barely detectable in medium from six expanding blastocysts (Day 10, 3–5 mm diameter, spherical; Lane 2). Day 12 filamentous blastocysts produced immunoreactive BP (Lane 3). These results suggested that BP synthesis and secretion occurred during blastocyst expansion and continued during elongation.

Glycosylation of BP

Day 15 blastocysts were incubated in the presence of [^{3}H]glucosamine and the medium was analyzed by 2D-SDS-PAGE and fluorography. For comparison, a fluorogram of [^{35}S]methionine-labeled conceptus medium proteins is shown in Figure 7A. A basic protein of M_r = 43,000 incorporated [^{3}H]glucosamine (Fig. 7B). That BP incorporated [^{3}H]glucosamine was confirmed by immunoprecipitation of medium with anti-BP followed by 1D-SDS-PAGE and fluorography. The major polypeptide detected was BP-a (Fig. 6B, Lane 4). In some preparations of conceptus medium, a second, abundant, basic glycoprotein was detected by 2D-SDS-PAGE (Fig. 7B; note polypeptide beneath arrow). This polypeptide (BP-b) was immunoprecipitated by anti-BP (not shown).

Glycopeptidase F cleaves N-linked oligosaccharides between the linkage N-acetyl-D-glucosamine and asparagine (Tarentino et al., 1985). Purified, [^{3}H]leucine-labeled BP-a (Fig. 5A) was incubated with glycopeptidase F. Glycopeptidase F treatment generated two radiolabeled polypeptides of M_r = 39,800 and 36,300 (Fig. 5B). This result suggested that BP-a contained at least two oligosaccaharides N-linked to asparagine. When a mixture (4 μg total protein) of BP-a and BP-b were incubated with glycopeptidase F, two additional polypeptides with molecular weights of 32,000 and 29,000 accumulated. Thus, BP-b bore multiple oligosaccharide chains and most likely differed from BP-a in polypeptide mass.

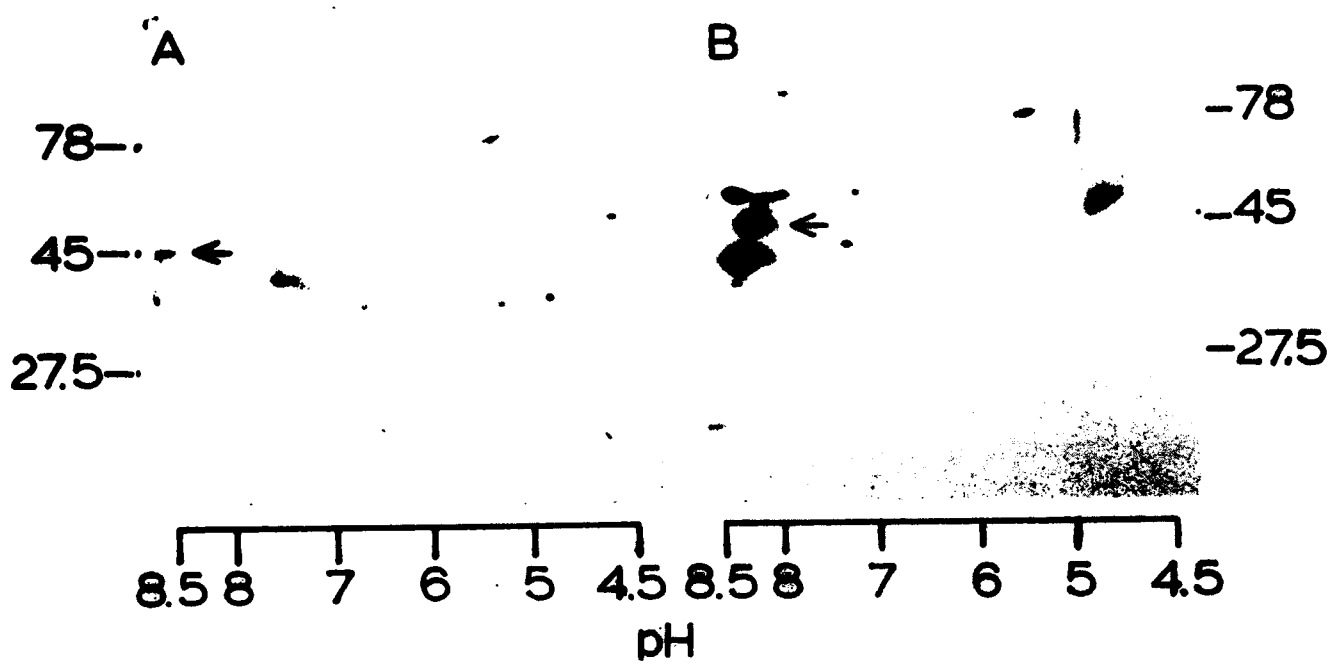

FIG. 7. Two-dimensional electrophoretic analysis of radiolabeled proteins released into medium of preimplantation pig conceptus cultures. Pig conceptuses were incubated for 18 h in the presence of [^{35}S]methionine or [^{3}H]glucosamine. Proteins in medium were separated by 2D-SDS-PAGE using a 10% polyacrylamide gel in the second dimenison. The resulting autoradiogram (*Panel A*; [^{35}S]methionine, 25,000 dpm applied) and flurogram (*Panel B*; [^{3}H]glucosamine; 100,000 dpm applied) are shown. The position of basic protein (BP)-a is indicated with an *arrow*. Molecular weight ($\times 10^{-3}$) is indicated in the *right* and *left* margins. Approximate *pH* is also indicated.

Immunocytochemical Localization

Anti-BP was used to visualize the cell type(s) containing BP in blastocysts using the peroxidase-anti-peroxidase method. Sections of Day 11 (5–7 mm, spherical, and 10–20 mm, tubular) blastocyst tissue were incubated with anti-BP or preimmune serum. Dark-colored reaction product was found in trophectoderm cells of trophoblast treated with anti-BP at dilutions of 1:100 (Fig. 8B and 8D) and 1:200 (not shown). Staining was darkest in the apical cytoplasm. Staining diminished at higher dilutions (1:500 and 1:1000). Endoderm did not react with anti-BP. No staining was observed using preimmune serum in dilutions ranging from 1:1000 to 1:100 (Fig. 8A and 8C).

The staining pattern was unchanged when anti-BP was passed through a column of Uf-Sepharose or when anti-BP (1:100) was incubated with 100 μg/ml of Uf prior to application to tissue sections (results not shown). The specificity of anti-BP was also tested in Day 11 endometrium from a nonpregnant gilt. No staining product was seen in surface epithelium, stroma, or glands using preimmune serum (Fig. 9A) or anti-BP (Fig. 9B) at dilutions of 1:1000 to 1:100. In contrast, glandular epithelial cells stained darkly using anti-Uf (Fig. 9C). Thus, anti-BP did not react with Uf. BP was not detected in endometrium of nonpregnant gilts.

DISCUSSION

BP is an abundant polypeptide synthesized and secreted by elongating pig blastocysts (Godkin et al., 1982a). BP was first identified by 2D-SDS-PAGE analysis of proteins harvested from medium of Day 16 blastocysts cultured in vitro (Godkin et al., 1982a). Incorporation of [^{3}H]leucine into the polypeptide during culture demonstrated that BP was synthesized by conceptus tissues. It had an apparent molecular weight of between 42,000 and 45,000 and an apparent pI greater than 7. BP was partially purified by anion-exchange, cation-exchange, and gel-filtration chromatographies. Here we describe a modified scheme to achieve purity and experiments to characterize the polypeptide.

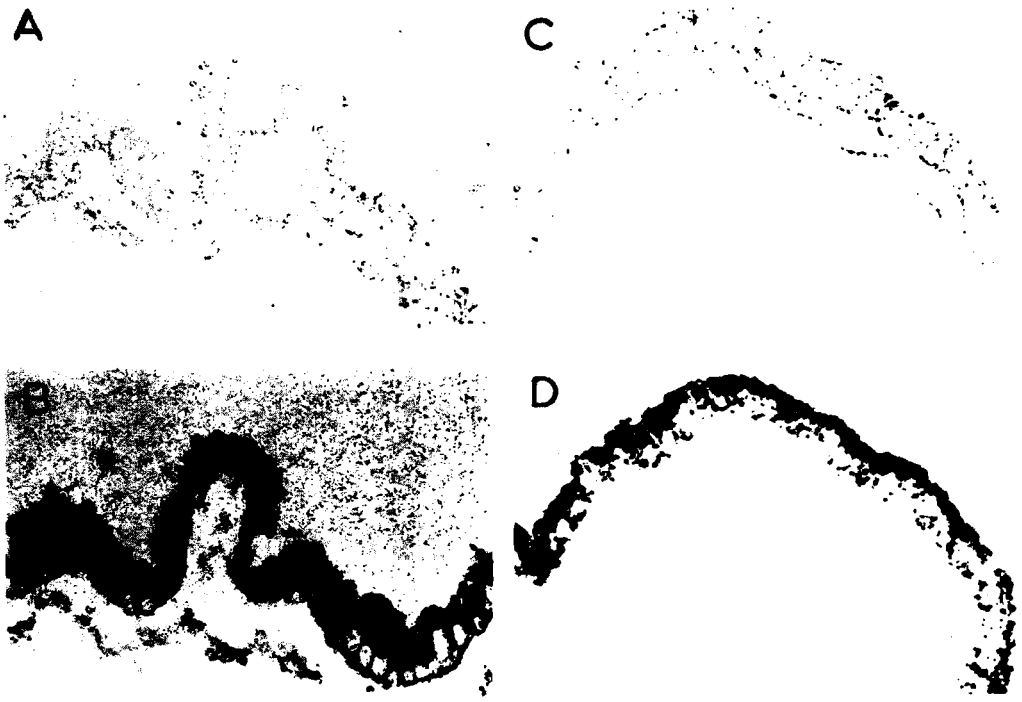

FIG. 8. Immunocytochemical localization of basic protein *(BP)* in blastocysts. Blastocysts were collected on Day 11 of pregnancy, sorted by size, and processed for immunocytochemistry. Tissue sections from 5–7 mm spherical blastocysts (*A* and *B*) and from 10–20 mm tubular blastocysts (*C* and *D*) were treated with the following sera at a dilution of 1:100: *A* and *C*, preimmune; *B* and *D*, anti-BP.

We have purified BP from medium of blastocysts (Days 14–17) cultured in vitro in the presence of [^{3}H]leucine by a combination of CM-Biogel and gel-filtration chromatographies. Purity was assessed by 1D-SDS-PAGE and 2D-NEPHGE analysis. The resultant preparation contained a single Coomassie Brilliant Blue-stained polypeptide with a molecular weight of 43,000. This polypeptide was the only radiolabeled species detected by subsequent flurography of the electropherogram. At this level of sensitivity, this protein was the only conceptus product present in the final preparation. This protein was basic in charge as demonstrated by migration toward the cathode in the first dimension (nonequilibrium pH gradient electrophoresis) of the 2D-NEPHGE procedure.

During development of the purification protocol, Uf was detected in the initial CM-Biogel and Sephadex G75 fractions by enzymatic assay for acid phosphatase and by 1D-SDS-PAGE. With the latter technique, a Coomassie Brilliant Blue-stained polypeptide having a molecular weight of 35,500 was found in the BP preparations. It did not appear in fluorograms of the 1D-SDS-PAGE gels but did react with anti-Uf by Western blotting. Uf is synthesized and secreted by glandular epithelium of the endometrium (Roberts and Bazer, 1980); thus, Uf was not radiolabeled in blastocyst culture medium. Reapplication to CM-Biogel removed greater than 90% of the remaining acid phosphatase activity. The Uf polypeptide was detectable no longer by 1D-SDS-PAGE and by subsequent Western blotting with anti-Uf. We estimate that one percent or less of the protein mass in the final BP preparations was Uf.

Importantly, the use of PMSF and EDTA was necessary to prevent degradation of BP, although no experiments were conducted to determine if just one, or both, inhibitors were necessary. The BP-b polypeptide was most likely a product of protease action. In some preparations, further degradation to a lower molecular weight polypeptide (BP-c) has been observed. The variable yields of BP may reflect the presence of proteases in culture medium.

The molecular weight of the BP polypeptide was approximately 43,000 by both 1D-SDS-PAGE under reducing and nonreducing conditions and by gel-filtration chromatography under nondenaturing conditions. These results strongly suggested that BP was secreted as a monomeric polypeptide without subunit or polymeric contributions. BP is a glycoprotein as

indicated by biosynthetic incorporation of [^{3}H] glucosamine and by susceptibility to glycopeptidase F. The molecular weights of polypeptides generated by glycopeptidase F were 39,800 and 36,300, a result which suggested that BP may have several N-linked oligosaccharide units. The presence of O-seryl (threonyl)-linked oligosaccharides was not investigated.

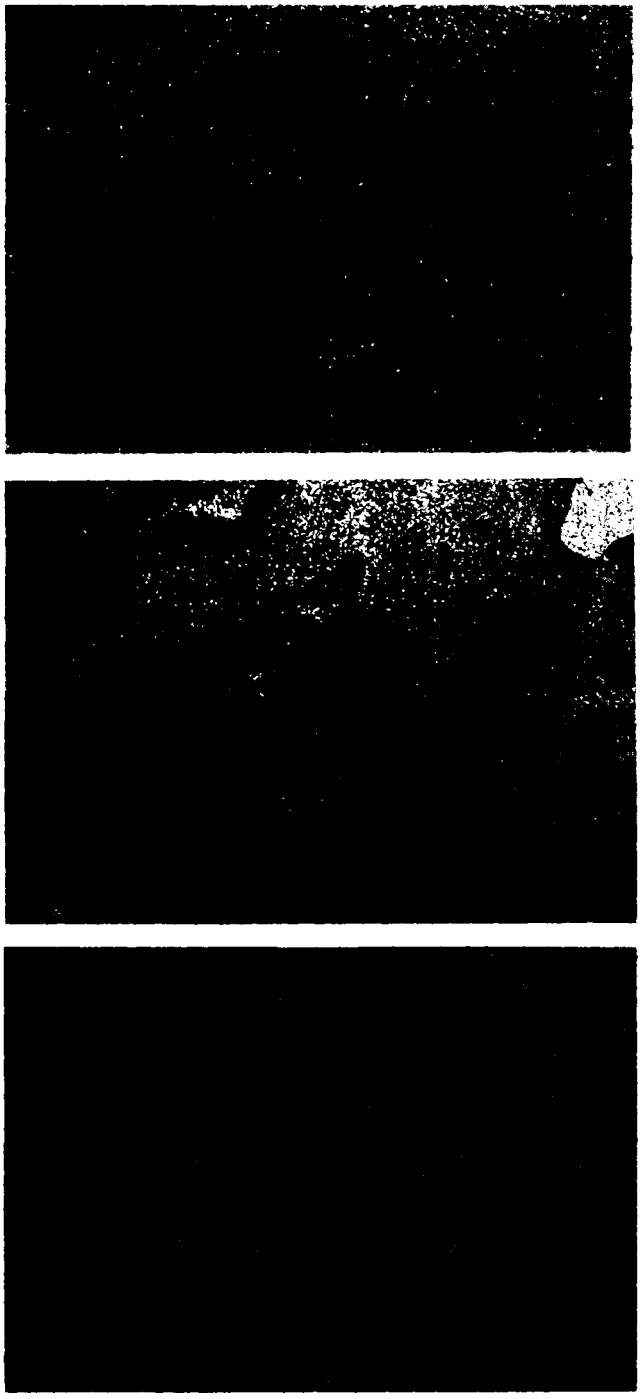

FIG. 9. Immunocytochemical analysis of nonpregnant endometrium. Endometrium collected on Day 11 of the estrous cycle was processed for immunocytochemistry using peroxidase-anti-peroxidase staining. Sections were treated wiht the following sera at a dilution of 1:100: *A*, preimmune; *B*, anti-basic protein; *C*, anti-uteroferrin. Indicated are glands *(g)*, stroma *(s)*, and luminal epithelium *(le)*.

A monospecific antiserum to BP was generated by excising the BP-a polypeptide from electrophoretic gels. This antiserum reacted with BP-a, BP-b, and BP-c from blastocyst medium, but not with Uf. Immunoprecipitation of BP from medium of filamentous (Day 12) blastocysts confirmed previous results whereby BP was detected by 2D-SDS-PAGE in medium of Day 13 filamentous blastocysts (Godkin et al., 1982a). Additionally, we were unable to detect BP in medium of 4 mm diameter (Day 10.5) blastocysts by electrophoretic analysis (Godkin et al., 1982a). However, in the results presented here, BP was detected by immunoprecipitation in medium of 3–5 mm diameter blastocysts and by immunocytochemistry in tissue of 5–7 mm diameter and 10–20 mm tubular blastocysts. These results suggest that BP was synthesized and secreted during expansion and elongation of blastocysts. Future experiments will quantify these results and also determine when BP production is initiated and terminated.

BP was localized to trophectoderm cells of the preimplantation trophoblast by immunocytochemistry. These cells are likely to be the site of synthesis of BP. The presence of BP at the apical membrane region of trophectoderm cytoplasm is interpreted by us to indicate that BP may be secreted from the apical surface toward the uterine lumen. Preliminary experiments not reported here indicated that BP is detectable in uterine flushings on Day 12–14 of pregnancy by Western blotting. Thus BP may be secreted in vivo. It was not surprising that the trophoblast synthesized and secreted BP. In other species such as sheep, trophoblast secretory proteins play important roles in fetal-maternal interactions. Protein secretory products of sheep trophectoderm mediate the phenomenon of maternal recognition of pregnancy (Godkin et al., 1982b; 1984a,b). Thus, BP may interact with the maternal system after secretion and play a role in maternal recognition. Many other processes occur during the time of BP secretion, including expansion and elongation of the blastocyst, initial attachment of trophectoderm to endometrial epithelium, outgrowth of yolk sac, amnion and allantois, growth and vascularization of the uterus, and continued development of the inner cell mass. BP may play an important function in one or more of these developmental processes.

In summary, we have purified BP from conceptus culture medium, determined its molecular weight to be 43,0000, and identified it as a glyoprotein. Antiserum produced to BP was used to detect its presence in trophectoderm of the trophoblast and its secretion into medium by preimplantation blastocysts. Purified preparations of BP and production of a specific antiserum may provide the necessary probes to determine the function of this product, which is a major secretory conceptus protein produced during early pregnancy in pigs.

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REFERENCES

Basha SMM, Bazer FW, Roberts RM, 1980. Effect of the conceptus on quantitative and qualitative aspects of uterine secretions in pigs. J Reprod Fertil 60:41–48

Baumbach GA, Ketcham CM, Richardson DR, Bazer FW, Roberts RM, 1986. Isolation and characterization of a high molecular weight stable pink form of uteroferrin from uterine secretions and allantoic fluid of pigs. J Biol Chem 261:12869–78

Baumbach GA, Saunders PTK, Bazer FW, Roberts RM, 1984. Uteroferrin has N-asparagine-linked high-mannose type oligosaccharides that contain mannose-6-phosphate. Proc Natl Acad Sci USA 81:2985–89

Bazer FW, Marengo SR, Geisert RD, Thatcher WW, 1984. Exocrine versus endocrine secretion of prostaglandin $F_{2\alpha}$ in the control of pregnancy in swine. Anim Reprod Sci 7:115–32

Bazer FW, Roberts RM, Sharp III DC, 1978. Collection and analysis of female genital tract secretions. In: Daniel JC (ed.), Methods in Mammalian Embryology. NY: Academic Press, pp. 503–28

Buhi WC, Gray WJ, Mansfield EH, Chun PW, Ducsay CA, Bazer FW, Roberts RM, 1982. Iron content and molecular weight of uteroferrin and a comparison of its iron and copper forms. Biochim Biophys Acta 701:32–38

Chamberlain JP, 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor sodium salicylate. Anal Biochem 98:132–35

Cross JC, Roberts RM, 1988. An interferon produced by elongating porcine embryos. Biol Reprod 38 (Suppl. 1): 99 (Abstr. 153)

Frank M, Bazer FW, Thatcher WW, Wilcox CJ, 1978. A study of prostaglandin F_2 alpha as the luteolysin in swine: IV. An explanation for the luteotropic effect of estradiol. Prostaglandins 15: 151–60

Gadsby JE, Burton RD, Heap RB, Perry JS, 1976. Steroid metabolism and synthesis in early embryonic tissue of pig, sheep and cow. J Endocrinol 71:45–54

Gadsby JE, Heap RB, Burton RD, 1980. Oestrogen production by blastocysts and early embryonic tissue of various species. J Reprod Fertil 60:409–17

Galigher AE, Kozloff EN, 1971. Essentials of Practical Microtechnique, 2nd ed. Philadelphia: Lea and Febiger

Geisert RD, Brookbank JW, Roberts RM, Bazer FW, 1982. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 27:941–55

Godkin JD, Bazer FW, Lewis GS, Geisert RD, Roberts RM, 1982a. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. Biol Reprod 27:977–87

Godkin JD, Bazer FW, Moffatt J, Sessions F, Roberts RM, 1982b. Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts on days 13–21. J Reprod Fertil 65:141–50

Godkin JD, Bazer FW, Roberts RM, 1984a. Ovine trophoblast protein 1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. Endocrinology 114: 120–30

Godkin JD, Bazer FW, Roberts RM, 1984b. Proteins released by cultured day 15-16 conceptuses prolong luteal maintenance when introduced into the uterine lumen of cyclic ewes. J Reprod Fertil 71:57–64

Helmer SD, Hansen PJ, Anthony RV, Thatcher WW, Bazer FW, Roberts RM, 1987. Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. J Reprod Fertil 79:83–91

Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM, 1987. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature (Lond) 330: 377–79

Imakawa K, Hansen TR, Anthony RV, Polites HG, Marotti KR, Roberts RM, 1988. An embryonic-derived interferon is found in the bovine and ovine species. Biol Reprod 38 (Suppl. 1): 190 (Abstr. 426)

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–85

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–75

Masters RA, Roberts RM, Lewis GS, Thatcher WW, Bazer FW, Godkin JD, 1984. High molecular weight glycoproteins released by expanding, pre-attachment sheep, pig and cow blastocysts in culture. J Reprod Fertil 66:571–83

Murray MK, Segerson ED, Hansen PJ, Bazer FW, Roberts RM, 1987. Suppression of lymphocyte activation by a high molecular-weight glycoprotein released from preimplantation ovine and porcine conceptuses. Am J Reprod Immunol Microbiol 14:38–44

O'Farrell PH, 1975. High resolution two dimensional polyacrylamide gel electrophoresis of proteins. J Biol Chem 250:4007–21

O'Farrell PZ, Goodman HM, O'Farrell PH, 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133–42

Powell-Jones CHJ, Lester JB, Polge C, Hartree AS, 1984. Pattern of medium proteins radiolabeled after culture of day 13 to 16 pig conceptus tissue with [^{3}H]leucine and absence of chorionic gonadotropin-like activity. J Reprod Fertil 71:161–72

Roberts RM, Baumbach GA, Buhi WC, Denny JB, Fitzgerald LA, Babelyn SF, Horst MN, 1984: Analysis of membrane polypeptides by two-dimensional polyacrylamide gel electrophoresis. In: Ventor JC, Harrison LC (eds.), Molecular and Chemical Characterization of Membrane Receptors. NY: Alan R Liss, pp. 61–113

Roberts RM, Bazer FW, 1980. The properties, function and hormonal control of synthesis of uteroferrin, the purple protein of the pig uterus. In: Beato M (ed.), Steroid Induced Uterine Proteins. Amsterdam: Elsevier/North-Holland Biomed Press, pp. 133–49

Schlosnagle DC, Bazer FW, Tsibris JC, Roberts RM, 1974. An iron-containing phosphatase induced by progesterone in the uterine fluids of pigs. J Biol Chem 249:7574–79

Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG, 1970. The unlabeled antibody-enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex. (horseradish peroxidase-antiperoxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–33

Stewart HJ, McCann SHE, Barker PJ, Lee KE, Lamming GE, Flint APF, 1987. Interferon sequence homology and receptor binding antiluteolytic protein. J Endocrinol 115:R13–15

Tarentino AL, Gomey CM, Plummer TH Jr, 1985. Deglycosylation of

asparagine-linked glycans by peptide: N-glycosidase F. Biochemistry 24:4665–71

Towbin H, Staehlin T, Gordon J, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–54

Vaitukaitis J, Robbins JB, Nieshlag E, Ross GT, 1971. A method for producing specific antisera with small doses of immunogen. J Clin Endocrinol 33:988–91

Whyte A, Wooding FBP, Saunders PTK, 1986-1987. Characteristics of cellular structures derived by tissue culture of pre-implantation blastocysts of the pig. Arch Anat Microsc Morphol Exp 75:91–109