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Expression of an inter-α-trypsin inhibitor heavy chain-like protein in the pig endometrium during the oestrous cycle and early pregnancy

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In pigs, changes in an unidentified endometrial glycoprotein, pGP30, are temporally associated with rapid trophoblast elongation and initial placental attachment on day 12 of gestation. Identification of endometrial pGP30 was undertaken through protein purification, NH_2-terminal amino acid sequencing and cDNA sequencing of products generated through reverse transcription–polymerase chain reaction. Sequencing of 35 amino acids from the NH_2-terminal end of pGP30 revealed that the 30 kDa glycoprotein is a cleavage product from the C-terminal region of inter-α-trypsin inhibitor heavy chain 4 (IαIH4), previously known as inter-α-trypsin inhibitor heavy chain-like protein. IαIH4 is unique compared with the three other inter-α-trypsin inhibitor heavy chains as it does not contain a binding site for bikunin that has serine protease inhibitory activity and is sensitive to cleavage by kallikrein. Endometrial gene expression of IαIH4 was detected during the oestrous cycle (days 0–18) and early pregnancy (days 10–18). Gene expression of IαIH4 appeared to be enhanced during the midluteal phase (days 12 and 15) of the oestrous cycle and the period of trophoblast attachment (days 12–18). Expression of IαIH4 was not detected in day 12 conceptus tissue mRNA. Endometrial expression of IαIH4 in pigs may function as an acute phase protein for protection of the uterus from the inflammatory response induced by conceptus attachment to the uterine epithelium.

Introduction

In pigs, conceptus synthesis and release of oestrogen is not only involved with maintenance of the corpora lutea throughout pregnancy, but also provides the signal for initiating conceptus attachment to the uterine epithelial surface (Geisert *et al.*, 1994). Conceptus implantation and placentation in pigs is initiated after rapid elongation of the conceptus trophoblast through the uterine lumen on day 12 of gestation (Geisert *et al.*, 1982; Stroband and Van der Lende, 1990). The non-invasive epitheliochorial placentation of the pig conceptus (King *et al.*, 1982) is associated with a reduction of the glycocalyx present on the uterine epithelial microvilli during interdigitation of the trophoblast to the uterine surface (Dantzer, 1985).

Alterations in the uterine microvillus glycocalyx play a major role in placental attachment between the trophoblast and uterine epithelium in the pig (Dantzer, 1985; Stroband *et al.*, 1986; Keys and King, 1990; Blair *et al.*, 1991). Secretion of oestrogen by the elongating conceptus regulates changes in the uterine epithelial glycocalyx necessary for trophoblast attachment. Keys and King (1992) indicated that administration of oestrogen induces ultrastructural changes in the uterine epithelium similar to that observed during days 10–13 of pregnancy in gilts. Although oestrogen is a major regulator of corpora lutea maintenance and uterine function, it has detrimental effects on conceptus survival if administered before conceptus elongation (days 9 and 10) and oestrogen release (Pope *et al.*, 1986; Morgan *et al.*, 1987; Gries *et al.*, 1989). Premature stimulation of the uterus with oestrogen induces conceptus degeneration between days 14 and 18 of gestation. Conceptus fragmentation is associated with shedding of the uterine epithelial glycocalyx (Blair *et al.*, 1991) and a temporally associated decrease in an unknown 30 kDa endometrial glycoprotein termed pGP30 (Gries *et al.*, 1989; Geisert *et al.*, 1995). Synthesis of the glycoprotein by the uterine epithelium is stimulated by progesterone (Geisert *et al.*, 1995). Evaluation of pGP30 synthesis during early pregnancy indicated that alterations in the M_r of pGP30 released by endometrial explants *in vitro* are temporally associated with the time of conceptus trophoblast elongation, oestrogen release and placental attachment to the uterine surface (Geisert *et al.*, 1995). Alterations in the M_r of pGP30 appear to occur through changes in glycosylation and possibly cleavage of the glycoprotein. The identity and

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function of pGP30 in uterine function is unknown. Therefore, the present study was undertaken to determine the NH_2-terminal amino acid sequence of pGP30, to obtain a partial cDNA sequence of the glycoprotein, and to demonstrate endometrial gene expression of pGP30 during the oestrous cycle and early pregnancy of gilts.

Materials and Methods

Collection of uterine secretory proteins

Sexually mature, cyclic, crossbred gilts were observed for oestrous behaviour in the presence of intact boars on a daily basis. After displaying two oestrous cycles of normal duration (17–22 days), gilts were mated to fertile boars at the onset of oestrus (day 0) and 12 and 24 h later. Endometrium was obtained from gilts (n = 5) on day 14 of pregnancy. Gilts were hysterectomized as described by Gries *et al.* (1989). A surgical plane of anaesthesia was induced with a 5% solution of thiopentone sodium (Abbott Laboratories, North Chicago, IL) administered i.v. Anaesthesia was maintained on a closed circuit system of halothane (2–5% Fluothane; Aveco Company Inc., Fort Dodge, IA) and oxygen (2.0 l min^{-1}). After exposure following mid-ventral laparotomy, the uterine horns and ovaries were surgically removed. The uterus was immediately transported on ice to a sterile horizontal flow hood. The surgical incision was closed in a routine fashion and gilts were treated i.m. with procaine penicillin G (20 000 iu kg^{-1} body weight).

Excised uterine horns were flushed with 20 ml of sterile saline (0.9%) and the flushing examined for the presence of conceptuses to confirm pregnancy. Endometrial tissue was dissected from the myometrium, cut into 3–4 mm explants, and placed in dishes containing 15 ml Eagle's minimum essential medium (MEM; GibcoBRL, Grand Island, NY). Antibiotic and antimycotic solutions (GibcoBRL, Grand Island, NY) containing penicillin (100 000 iu ml^{-1}) and streptomycin (10 mg ml^{-1}) were added to media prior to culture. The endometrial explants (500 mg) were cultured in 15 ml MEM for 3 h at 37°C in an atmosphere of 5% CO_2, 45% N_2 and 50% O_2 to remove initial release of blood from the tissue. After 3 h the medium was removed, replaced with fresh MEM and incubated for an additional 24 h. After the 24 h incubation, culture medium was separated from tissue and centrifuged at 12 000 *g* for 15 min. The supernatant was decanted and stored at –80°C.

Purification of pGP30

Medium collected from short-term explant cultures of pig endometrium obtained on day 14 of pregnancy was pooled and concentrated by ultrafiltration at 4°C in an Amicon Model 8400 stirred cell (Amicon Corp., Danvers, MA) over a YM10 membrane (10 000 M_r cut-off). All chromatographic procedures to isolate pGP30 were conducted as described by Geisert *et al.* (1995).

NH_2-terminal amino acid analysis

Purified pGP30 was NH_2-terminal amino acid microsequenced by pulse liquid phase Edman degradation on a Procise Model 492 protein sequencer (Applied Biosystems, Perkin & Elmer, Foster, CA). The amino acid sequence was analysed for homology to known protein sequences stored in the National Protein and SWISS-PROT databases.

Internal protein sequences of purified pGP30 were determined after proteolytic digestion of the protein with the endoproteinase Clostripain (Sigma, St Louis, MO). Approximately 300 µg pGP30 was incubated with 7.5 µg Clostripain in reaction buffer (50 mmol Tris–HCl l^{-1} (pH 7.5), 2 mmol dithiothreitol l^{-1}, 1 mmol $CaCl_2$ l^{-1}) at 37°C for 16 h. Resulting peptide fragments were separated with a Reliasil C18 HPLC column and selected peptide peaks were subjected to automated protein sequence analysis.

Collection of endometrium from gilts during oestrous cycle and early pregnancy

Endometrium was obtained from gilts (n = 3 per day) on days 0, 5, 10, 12, 15 and 18 of the oestrous cycle and days 10, 12, 15 and 18 of pregnancy as described previously. Immediately after removal of the uterine horns, a 7–10 cm section anterior to the uterine body was excised and opened along the antimesometrial border. Endometrium was removed from the underlying myometrium with scissors, blotted on surgical sponges, and frozen in liquid nitrogen until used for extraction of total RNA.

Isolation of total RNA

Total RNA was isolated from endometrium using the guanidinium–thiocyanate–phenol–chloroform extraction procedure (Puissant and Houdebine, 1990) as previously described for bovine endometrium (Geisert *et al.*, 1991). Total RNA (50 µg) was diluted in 50 µl of 10 mmol Tris–HCl l^{-1} buffer (pH 7.4) (Fischer Scientific, Pittsburgh, PA) and incubated at 37°C for 30 min with 50 µl DNase reaction mix containing 100 U DNase (GibcoBRL, Grand Island, NY) and 4 U RNasin ribonuclease inhibitor (Promega, Madison, WI) as previously described (Sun and Pettinger, 1996). The reaction was terminated with 25 µl DNase stop mix (50 mmol EDTA l^{-1}, 1.5 mol sodium acetate l^{-1} and 1% (w/v) sodium dodecyl sulphate). RNA was recovered through phenol–chloroform–iso-amyl alcohol precipitation. Total RNA was quantified spectrophotometrically by absorption at 260 nm. Final RNA purity was determined from calculations of 260:280 ratios.

Preparation of cDNA

Total RNA was reverse transcribed to cDNA in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler Model 480. The reactions were carried out in 20 µl of 200 U Moloney

murine leukaemia virus reverse transcriptase–Rnase H⁻ (M-MLV-RT), 1.0 μg oligo $(dT)_{15}$ primer, 0.5 mmol l^{-1} each of dATP, dCTP, dGTP and dTTP, 50 mmol Tris–HCl l^{-1} (pH 8.3), 75 mmol KCl l^{-1}, 3 mmol MgCl l^{-1}, 10 mmol dithiothreitol l^{-1}, 20 U RNasin ribonuclease inhibitor, 1 μg total RNA (10 μl volume) and brought to volume with diethylpyrocarbonate (DEPC)-treated double distilled water. The sample was incubated at 22°C for 15 min, followed with a 42 min incubation at 42°C. The reaction was terminated by heating at 95°C for 5 min and quickly cooling to 4°C. The cDNA sample was further diluted with 20 μl sterile DEPC-treated double distilled water and was stored at 4°C. The M-MLV-RT, reaction buffer, RNasin ribonuclease inhibitor and oligo $(dT)_{15}$ primer were obtained from Promega Corporation (Madison, WI).

Reverse transcription–polymerase chain reaction (RT–PCR) and cDNA sequencing of pGP30

The NH_2-terminal amino acid sequence and sequence of the internal fragments of pGP30 released by Clostripain endoprotease digestion revealed significant homology with the C-terminal region of the 120 kDa human inter-α-trypsin inhibitor family heavy chain-related protein (IHRP) isolated from liver (Nishimura *et al.*, 1995; Saguchi *et al.*, 1995). The C-terminal region of pGP30 that is homologous to human IHRP is unique compared with all other inter-α-trypsin inhibitor heavy chains. The region of amino acid homology between pGP30 and human IHRP was used to design PCR 5′ primer 5′TGCCACTGCCTGGGCAGAGT3′ (2336–2355) and 3′ primer 5′TGAAGGTCACTTCGATCCAT3′ (2472–2491) based on the sequence of human cDNA (Nishimura *et al.*, 1995). These primers allowed specific expression of only the IHRP mRNA and excluded the other three heavy chains of the inter-α-trypsin inhibitor family.

All PCR reactions were performed in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler Model 480 in 25 μl volumes covered with 25 μl of mineral oil. Optimal conditions for amplification of primers were determined empirically in the presence of pooled cDNA generated from endometrium collected on days 12, 15 and 18 of gestation. Endometrial cDNA (75 ng) was amplified with 0.6 U *Taq* DNA polymerase in the $MgCl_2$-free buffer supplied (Promega, Madison, WI) and a $3 \times 2 \times 3$ factorial combination of primer concentration (100, 300 or 500 nmol l^{-1} of each primer), deoxynucleotidetriphosphates (dNTPs; 100 or 200 μmol l^{-1} each), and $MgCl_2$ (0.75, 1.5, or 2.25 mmol l^{-1}). All samples were kept on ice until placed in tubes into the 95°C heat block. The first PCR cycle consisted of denaturation at 95°C for 2 min, annealing at 55°C for 1 min and a 2 min extension at 72°C. This was followed by 29 cycles of denaturation at 95°C for 2 min, annealing at 55°C for 1 min and a 1 min extension at 72°C. A final period of extension at 72°C was carried out for 9 min. The PCR product was resolved in a 3% agarose gel at 60 V for 1.5 h, followed by a 30 min staining in ethidium bromide (0.5 g ml^{-1}) and destaining in deionized water for 30 min. Agarose gels were exposed to an ultraviolet light source and photographed with a MP4 Instant Camera System (Fotodyne Inc., Hartland, WI). Optimal conditions were defined as those that generated the strongest single PCR product of the predicted base pair (bp) size. The conditions selected to produce a single 155 bp band for the IHRP cDNA primers were 0.75 mmol $MgCl_2$ l^{-1}, 100 μmol dNTPs l^{-1} and 300 nmol IHRP primers l^{-1} with an annealing temperature of 58°C.

Amplification and sequencing of each cDNA product was duplicated and repeated when discrepancies in base homology were detected. For direct sequence analysis, cDNA was amplified, visualized in a 1% agarose gel and the respective band purified with Qiagen gel purification columns (QIAGEN, Chatsworth, CA). The endometrial cDNA product was sequenced using an Applied Biosystems Automated DNA sequencer (Perkin & Elmer, Foster, CA).

Two additional clones were sequenced after amplification with overlapping primers developed to amplify the 5′ upstream region that corresponded to the area of homology between all inter-α-trypsin inhibitor heavy chains. A 5′ primer, 5′CGTGCCCAGAGAATCTACGA3′, was designed from the pig IαIH1 (GenBank Accession No. D38754) and combined with our initial 3′ primer 5′TGAAGGTCACTTCGATCCAT3′ from the human IHRP (Nishimura *et al.*, 1995). An additional set of primers, 5′ primer 5′TGAAGGCCAAGAACAGTCT3′ and 3′ primer 5′AACGAGTAGGCTGAAGAGGT3′, was developed to confirm and fill in a cDNA region of the previous primers.

Demonstration of endometrial gene expression of IHRP

Total RNA isolated from pig endometrium collected from gilts ($n = 3$ per day) on days 0, 5, 10, 12, 15 and 18 of the oestrous cycle and days 10, 12, 15 and 18 of pregnancy was reverse transcribed as previously described. cDNA was amplified with the 5′ primer 5′TGCCACTGCCTGGGCAGAGT3′ and the 3′ primer 5′TGAAGGTCACTTCGATCCAT3′ designed to the specific 155 bp region of pig IHRP as previously described. The number of cycles for amplification was adjusted to evaluate endometrial IHRP expression on the exponential phase of the PCR reaction.

Total RNA from conceptuses collected on days 10 and 12 of pregnancy was isolated as described by Yelich *et al.* (1997). Conceptus RNA was used to generate cDNA for PCR amplification with primers that are specific for IHRP, 5′ primer 5′TGCCACTGCCTGGGCAGAGT3′ and 3′ primer 5′TGAAGGTCACTTCGATCCAT3′.

Results

NH_2-terminal amino acid analysis of pGP30

The NH_2-terminal 35 amino acids of pGP30 have 50% sequence homology with amino acids (684–718) in the C-terminal region of the 120 kDa human IHRP (Nishimura *et al.*, 1995; Saguchi *et al.*, 1995) and 100% homology with amino acids (703–737) in the C-terminal region of a sequence of pig liver IHRP (Hashimoto *et al.*, 1996) (Fig. 1). Homology of pGP30 and human IHRP increases to 81% when only amino

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hIHRP (684)   M N M K I E E T T M T T Q T P A P I Q A P S A I L P L P G Q S V E R L
pIHRP (703)   D M D S R I I G A T I P P P P A R I Q A P S V I L P L P G Q S V D Q L
pGP30 (1)     D M D S R I I G A T I P P P P A R I Q A P S V I L P L P G Q S V D Q L

hIHRP (719)   C V D P R H R Q G P V N L L S D P E Q G V E V T G Q Y E R E K A G F S
pIHRP (738)   C V D L K H S Q G P V K L L S D P G Q G V E V T G H Y E R E K A R F S
pGP30                                                                 F S

hIHRP (754)   W I E V T F K N P L V W V H A S P E H V V V T R N R R S S A Y K W K E
pIHRP (773)   W I E V T F K H P P L Q V R A S L E H I V V I R N R Q S S A Y K W K E
pGP30         W I E V T F K           A S L E H I V V I R   Q S   A Y K T F E

hIHRP (789)   T L F S V M P G L K M T M D K T G L L L L S D P D
pIHRP (808)   T L Y S V M P G L K I T M D K A G L L L L S S P N
pGP30         T L Y D V M P G L D I T M D K A G L L L L S S P N
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Fig. 1. NH_2-terminal (1–35) and peptide digest analysis of pGP30 glycoprotein from pig endometrium showing amino acid sequence homology (bold letters) to the C-terminal region of human (Saguchi *et al.*, 1995) and pig inter-α-trypsin inhibitor family heavy chain-related protein (Hashimoto *et al.*, 1996).

acid residues 15–35 of the NH_2-terminal sequence are compared. Four internal peptide sequences (total of 52 amino acids) obtained from proteolytic digestion of pGP30 had 81% sequence homology to human IHRP and 96% homology to pig IHRP (Fig. 1).

cDNA analysis of pig endometrial IHRP

The partial cDNA sequence of pig endometrial IHRP is presented in Fig. 2. PCR primers used to amplify a region of cDNA corresponding to pGP30 from endometrial mRNA collected on days 12 and 15 of pregnancy, generated a 155 bp product. The cDNA product encoded the NH_2-terminal amino acid sequence obtained for pGP30 and had significant nucleotide homology with human (89%) (Nishimura *et al.*, 1995; Saguchi *et al.*, 1995) and pig (100%) IHRP (Hashimoto *et al.*, 1996). Since pGP30 had significant homology to the larger human IHRP, we designed additional primers based on the region of homology between human IHRP and pig IαIH1 (Nishimura *et al.*, 1995) since the sequence of pig IHRP had not been published at the time of our study. Amplification of the upstream 5′ region from pGP30 common to all inter-α-trypsin inhibitor heavy chains (Nishimura *et al.*, 1995) confirmed that pGP30 is contained in the C-terminal region of the larger 120 kDa IHRP (Fig. 3) and is highly homologous to the previously reported sequence of pig liver IHRP (Hashimoto *et al.*, 1996). The NH_2-terminal 600 amino acid region of the various inter-α-trypsin inhibitor heavy chains are highly conserved (Salier *et al.*, 1996). However, there is no significant homology between C-terminal 300 amino acids of pig and human IHRP and IαIH1, IαIH2 or IαIH3 (see Fig. 3). The C-terminal 300 amino acids represent the location of pGP30 and therefore indicate that pig endometrium expresses IHRP. In contrast to other inter-α-trypsin inhibitor heavy chains (IαIH1, IαIH2 and IαIH3), pig endometrial IHRP and pig and human liver IHRP lack the consensus DPHFII sequence (Fig. 3) that is necessary for binding to the light chain of the inter-α-trypsin inhibitor family, bikunin. Bikunin possesses all the serine proteinase inhibitory activity for the inter-α-trypsin inhibitory family (Salier *et al.*, 1996).

Pig endometrial and liver IHRP contain one (Phe_{478}) of the three putative kallikrein cleavage sites (Phe_{482}, Phe_{660}, Phe_{687}) contained in human IHRP (Fig. 3). Putative cleavage sites for kallikrein are unique to IHRP as they are absent in the other three inter-α-trypsin inhibitor heavy chains (Salier *et al.*, 1996).

The partial sequence of endometrial IHRP cDNA had complete homology to pig liver IHRP (Hashimoto *et al.*, 1996) with the exception of a 54 bp region encoding 18 amino acids that is present in pig liver cDNA (1902–1956) but is absent from the RT–PCR generated cDNA sequence of endometrial IHRP (Fig. 3). Pig liver cDNA was used to amplify with our PCR primers and compare sequences with the pig endometrium. The 54 bp region was amplified in liver cDNA by our PCR primers (data not shown) indicating that endometrial IHRP is slightly modified from that reported for liver.

Endometrial gene expression of IHRP

Endometrial IHRP gene expression was detected in the pig endometrium during the oestrous cycle and early pregnancy using the specific primers to the IHRP gene (Fig. 4b). IHRP gene expression is enhanced on days 12 and 15 of the oestrous cycle and days 12–18 of pregnancy. However, the present results are only qualitative, as northern blot analyses are necessary to determine quantitative changes in gene expression. The primers used to amplify the 155 bp region of IHRP spanned an intron, as a larger product was amplified with pig genomic DNA. Gene expression of IHRP was not detected in day 10 or day 12 pig conceptus tissues (Fig. 5).

Discussion

The NH_2-terminal analysis and partial cDNA sequencing of pig endometrial pGP30 clearly demonstrated that the 30 kDa glycoprotein is related to IHRP. Hashimoto *et al.* (1996) recently published the entire pig liver cDNA sequence for

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                                                                cgtgcccagagaatctacga-----
------------------TCTGACTCTGCCCTGCAGCTCGAGGACTTCTACCAGGAGGTGGCCAACCCACTGCTGAGG   60
                  S  D  S  A  L  Q  L  E  D  F  Y  Q  E  V  A  N  P  L  L  R
TTGGTGGCCTTTGAGTACCCAAGCAATGCCGTGGAGGCAGTCACGCAGGACAACTTCCGGCTGTTCTTCAAAGGCTCT  138
L  V  A  F  E  Y  P  S  N  A  V  E  F  V  T  Q  D  N  F  R  L  F  F  K  G  S
GAGTTGGTGGTGGCCGGGAAGCTCCGGGACCAGAGCCCCGATGTGCTCTCCGCCAAAGTCAGGGGGCAACTGCACATG  216
E  L  V  V  A  G  K  L  R  D  Q  S  P  D  V  L  S  A  K  V  R  G  Q  L  H  M
GAGAATGTCACCTTCGTAATGGAGTCCAGGGTAGCAGAGCAGGAGGCGGAGTTCCTGAGCCCCAAGTACATCTTCCAC  294
E  N  V  T  F  V  M  E  S  R  V  A  E  Q  E  A  E  F  L  S  P  K  Y  I  F  H
AGCTTCATGGAGAGACTCTGGGCATACCTGACCATCCAGCAACTGTTGGCGCAAACAGTCTCTGCGTCAGATGCTGAG  372
S  F  M  E  R  L  W  A  Y  L  T  I  Q  Q  L  L  A  Q  T  V  S  A  S  D  A  E
AAGAAGGCCCTTGAAGCCCGAGCCCTGAGCTTGTCACTCAACTACAGCTTTGTCACCCCTCTCACATCTATGGTGATC  450
K  K  A  L  E  A  Q  A  L  S  L  S  L  N  Y  S  L  V  T  P  L  T  S  M  V  I
CACCAAACCtgaaggccaagaacagtctCAGGTTGCTGAGAACCCGTGGAAAATGGAAACCGACAGGGGAACACCCAC  528
T  K  P  E  G  Q  E  Q  S  Q  V  A  E  K  P  V  E  N  G  N  R  Q  G  N  T  H
TCAGGTCACTCTTCCTTTCAATTTCATTCTGTGGGAGACAGAACATCCAGACTAACAGCCCAAGGTTTTGAGAAAATG  606
S  G  H  S  S  F  Q  F  H  S  V  G  D  R  T  S  R  L  T  A  Q  G  F  E  K  M
TCCTACCTCCCACCGAGATTAGGACCCCCCGGacctcttcaqcctactcgttTTTCTCATCCGTTTTCCCGTATAACC  684
S  Y  L  P  P  R  L  G  P  P  G  P  L  Q  P  T  R  F  S  H  P  F  S  R  I  T
TTGGACCGGGTGCTGCCAGAGGTGCTATCCGTTCCTGATGAAACGTCACATGACATGGATTCAAGAATCATAGGAGCC  762
L  D  R  V  L  P  E  V  L  S  V  P  D  E  T  S  H  D  M  D  S  R  I  I  G  A
ACGATACCTCCACCCCCTGCCCGCATCCAGGCTCCTTCCGTCATCTtgccactgcctgggcagagcGTGGACCAGCTC  840
T  I  P  P  P  P  A  R  I  Q  A  P  S  V  I  L  P  L  P  G  Q  S  V  D  Q  L
TGTGTGGACCTCAAGCACTCCCAGGGCCCAGTGAAGCTGCTCTCAGACCCTGGCCAAGGGGTTGAAGTGACTGGCCAC  918
C  V  D  L  K  H  S  Q  G  P  V  K  L  L  S  D  P  G  Q  G  V  E  V  T  G  H
TATGAGAGGGAGAAGGCCCGCTTCTCatggatcgaagtgaccttc                                  968
Y  E  R  E  K  A  R  F  S  W  I  E  V  T  F
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Fig. 2. Partial nucleotide sequence of pig endometrial inter-α-trypsin inhibitor family heavy chain-related protein (IHRP) cDNA (first line) and its predicted amino acid sequence (second line). The partial nucleotide sequence is 100% homologous to the 1317–2337 base pair (bp) region of pig liver IHRP cDNA (Hashimoto *et al.*, 1996) with the exception of 54 missing nucleotides between 1902–1956 bp of the liver IHRP. Underlined letters indicate locations of PCR primers used to amplify cDNA. Combinations of primers included PCR 5′ primer 5′TGCCACTGCCTGGGCAGAGT3′ and 3′ primer 5′TGAAGGTCACTTCGATCCAT3′; 5′ primer, 5′CGTGCCCAGAGAATCTACGA3′ and 3′ primer 5′TGAAGGTCACTTCGATCCAT3′; and 5′ primer 5′TGAAGGCCAAGAACAGTCT3′ and 3′ primer 5′AACGAGTAGGCTGAAGAGGT3′.

IHRP. IHRP belongs to the inter-α-trypsin inhibitor family of serine proteinase inhibitors that act as acute phase reactants after trauma (Buchman *et al.*, 1990). Structure, function and nomenclature for the inter-α-trypsin inhibitor family have recently been reviewed (Salier *et al.*, 1996). The inter-α-trypsin inhibitor family of serine proteinase inhibitors contain either two or three chains consisting of a combination of IαIH1, IαIH2 and a single light chain known as bikunin (Jessen *et al.*, 1988); IαIH3 and bikunin (Enghild *et al.*, 1989); or IαIH2 and bikunin (Enghild *et al.*, 1993). IαIH1, IαIH2 and IαIH3 form the various complexes with bikunin by binding to a chrondroitin sulphate chain. Bikunin possesses all the serine proteinase inhibitory activity as it contains two Kunitz-type serine inhibitor domains (Hochstrasser *et al.*, 1981). Bikunin originates from a separate mRNA in which α-1-microglobulin and bikunin are synthesized as a single protein that undergoes proteolytic cleavage (Kaumeyer *et al.*, 1986). The inter-α-trypsin inhibitor heavy chains are translated from four separate genes (Diarra-Mehrpour *et al.*, 1989). Proteolytic cleavage removes N- and C-terminal peptides from the precursor proteins and bikunin is connected to the heavy chains through chondroitin sulphate (Enghild *et al.*, 1989). Three heavy chains (H1, H2, H3) of the inter-α-trypsin inhibitors contain a consensus sequence (DPHFII) that encodes the region for proteolytic cleavage and attachment of chrondroitin sulphate (Saguchi *et al.*, 1995). Pig and human IHRP are now classified as inter-α-trypsin inhibitor heavy chain 4 (IαIH4) (Salier *et al.*, 1996). The novel IαIH4 in human and pig liver, and pig endometrium is unique from other inter-α-trypsin inhibitor heavy chains since it lacks the consensus DPHFII sequence for binding bikunin and has a unique C-terminal 300 amino acids. Absence of a binding site for bikunin indicates that IαIH4 does not provide a carrier for serine proteinase inhibitory activity (Saguchi *et al.*, 1995; Hashimoto *et al.*, 1996).

Since IαIH4 does not bind bikunin, it must have a different function from the other heavy chains. In contrast to the other inter-α-trypsin inhibitor heavy chains, IαIH4 from human and pig plasma is a substrate for the plasma serine protease kallikrein, which is confirmed by the presence of putative cleavage sites in cDNA (Gonzales-Ramon *et al.*, 1995; Nishimura *et al.*, 1995; Hashimoto *et al.*, 1996). Initial characterization of pGP30 with a specific antiserum indicated that in addition to identification of a 30 kDa band, a larger, approximately 110 kDa form was also evident in endometrial cultures and serum (Geisert *et al.*, 1995). We detected a similar size product in endometrial culture

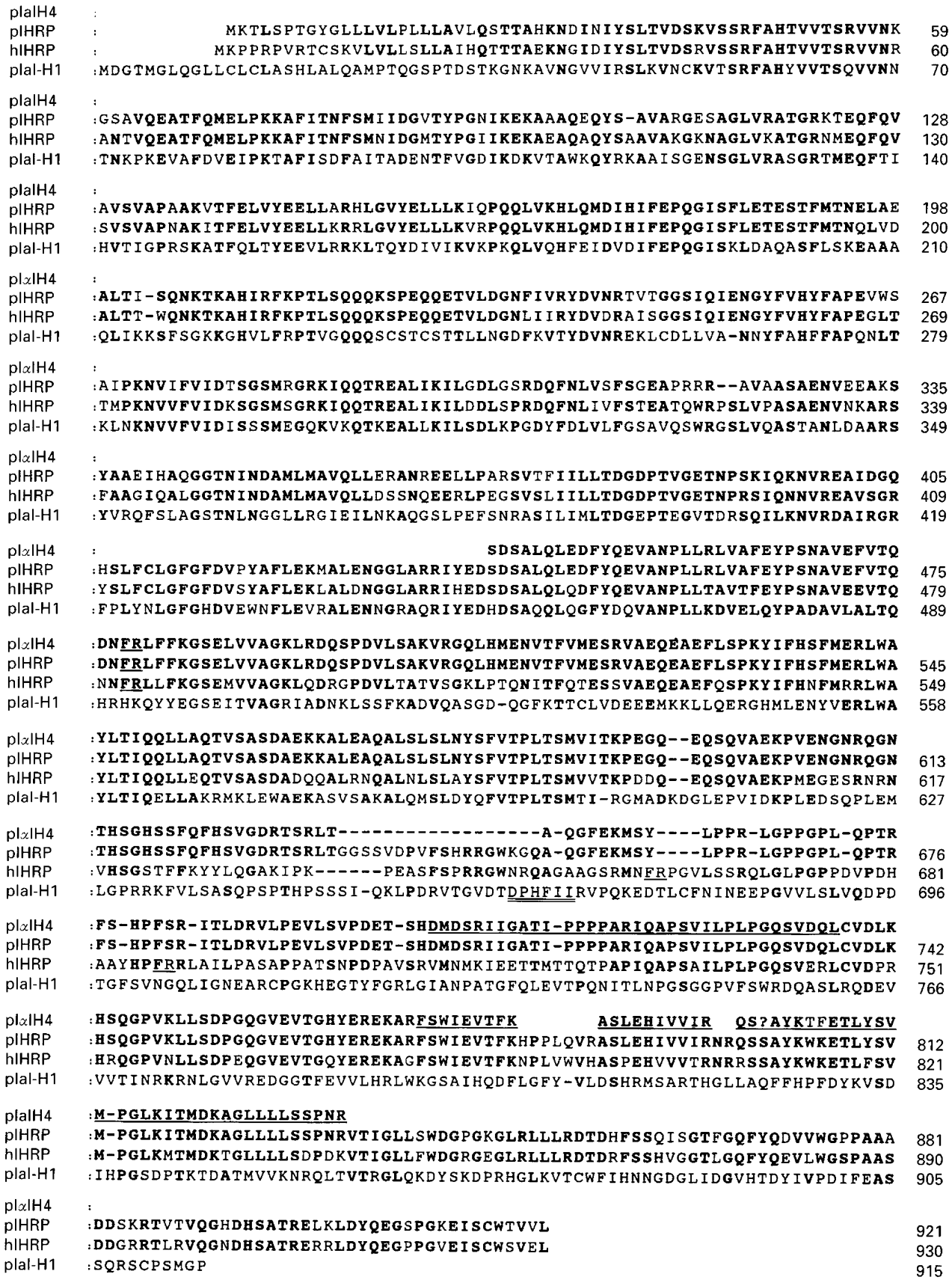

Fig. 3. For legend see opposite.

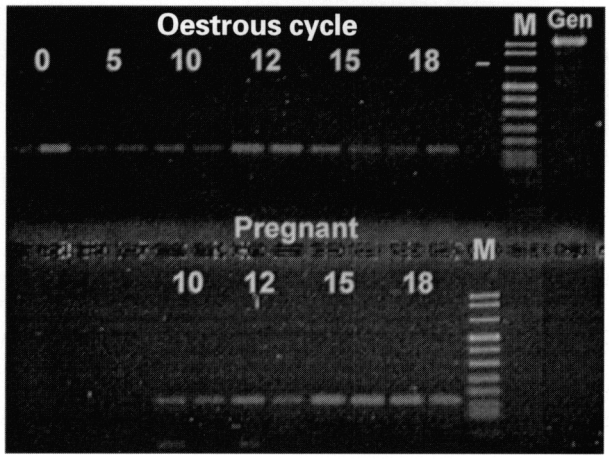

Fig. 4. Reverse transcription–polymerase chain reaction endometrial gene expression for inter-α-trypsin inhibitor family heavy chain-related protein (IHRP) in gilts on days 0, 5, 10, 12, 15 and 18 of the oestrous cycle and days 10, 12, 15 and 18 of early pregnancy (n = 2 gilts per day). IHRP gene expression is enhanced on days 12 and 15 of the oestrous cycle and maintained from day 12 to 18 in pregnancy. A larger gene product was obtained with primer amplification of pig genomic DNA (Gen). Negative control (–) lacked addition of PCR primers. Lane M contains molecular markers (1114, 900, 692, 501, 489, 404, 320, 242, 190 and 147 base pairs from the top of the gel to the bottom).

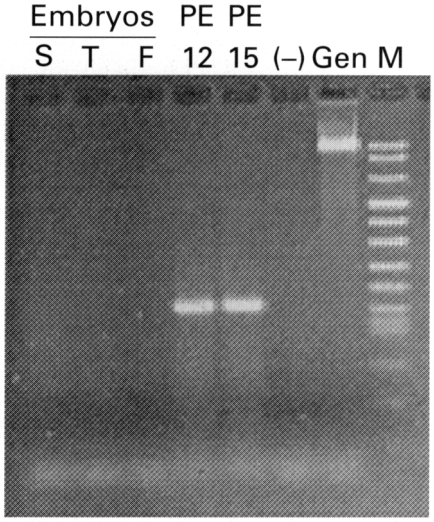

Fig. 5. Reverse transcription–polymerase chain reaction of endometrial gene expression for pig inter-α-trypsin inhibitor family heavy chain-related protein in spherical (S), tubular (T) and filamentous (F) day 11 conceptuses and endometrium obtained from gilts on day 15 of pregnancy (PE). Note the lack of product from early pig conceptuses and the larger base pair size of the genomic product. Negative control (–); pig genomic DNA (Gen); molecular markers (M). Molecular markers 1114, 900, 692, 501, 489, 404, 320, 242, 190 and 147 base pairs from the top of the gel to the bottom.

medium using polyclonal antibody to inter-α-trypsin inhibitor heavy chains (R. D. Geisert and T. Pratt, unpublished data). The larger product could represent intact IαIH4 from which pGP30 is cleaved after kallikrein or other enzymatic digestion within the uterus. Kallikrein enzymatic activity is present within the pig uterus after day 10 of the oestrous cycle and is significantly enhanced by the presence of the conceptus on day 12 of gestation (Vonnahme *et al.*, 1997). Pig plasma IαIH4 is sensitive to cleavage by kallikrein but only contains one (Phe_{478}) of the three putative kallikrein cleavage sites contained in the human IαIH4 amino acid sequence (Salier *et al.*, 1996). Incubation of pig plasma IαIH4 with kallikrein cleaves the polypeptide into 55 and 25 kDa peptides (Hashimoto *et al.*, 1996). The 25 kDa peptide is considerably smaller than the predicted mass of 49 kDa of C-terminal product taken from the amino acid sequence. The NH_2-terminal analyses of the kallikrein cleavage products revealed a sequence of SVPDET and another sequence of GATIPPPPAR, which do not reflect putative cleavage sites for kallikrein (Hashimoto *et al.*, 1996). The two pig IαIH4 cleavage products of kallikrein are contained within the same region (Ser_{697}–Arg_{712}) as the start of our NH_2-terminal sequence (Asp_{703}) for pGP30. Hashimoto *et al.* (1996) suggested that because this region of IαIH4 is rich in proline and is hydrophobic, it is possible that many proteases can cleave in this region. Cleavage of our endometrial IαIH4 with kallikrein needs to be evaluated to determine the products that are formed.

In humans, plasma kallikrein releases a 27 amino acid residue peptide from IαIH4 that has a C-terminal portion similar to bradykinin released from high molecular weight kininogen, the normal substrate for plasma kallikrein. However, there is no evidence of a similar peptide in either pig liver or endometrial IαIH4. Pig endometrial IαIH4 contains an 18 amino acid deletion compared with pig liver IαIH4. It is presumed that this deletion results either from alternate splicing or possibly from another gene which needs to be determined through complete sequencing of the endometrial product.

Synthesis of IαIH4 within the uterus is specific for pig

Fig. 3. Comparison of the partial amino acid sequence of pig endometrial inter-α-trypsin inhibitor heavy chain 4 (IαIH4) with sequences of pig (Hashimoto *et al.*, 1996) and human (Nishimura *et al.*, 1995) liver inter-α-trypsin inhibitor family heavy chain-related protein (IHRP) and pig IαIH1 (GenBank Accession No. D38754). The partial sequence (450–833) of pig endometrial IαIH4 is identical to pig liver IHRP from amino acid residue 450 to 833 with the exception that endometrial IαIH4 has a deletion of 18 amino acids (634–652). Bold letters show amino acid residues that are identical to pig liver IHRP. Amino acid sequences that were confirmed by our NH_2-terminal and peptide digest amino acid analyses are underlined. Three putative kallikrein cleavage sites in human (Phe_{482}, Phe_{660}, Phe_{687}) IHRP and one site (Phe_{478}) in pig IHRP are represented by FR. The consensus sequence DPHFII that encodes attachment of chrondroitin sulphate to bind bikunin in the pig IHRP sequence is double underlined. Note that human and pig IHRP have close homology with the exception of amino acid residues 603 to 753. The NH_2-terminal sequence (upper 600 amino acids) is homologous to all the other heavy chains of the inter-α-trypsin inhibitor family. However, the C-terminal region (bottom 347 amino acids) for both human and pig IHRP is totally unique from the other three heavy chains of the inter-α-trypsin inhibitor family.

endometrium as PCR primers failed to amplify the gene in early conceptus tissue. Homology of pig endometrial IαIH4 with heavy chains of the inter-α-trypsin inhibitors suggests a possible function for IαIH4 during pregnancy. Heavy chains of inter-α-trypsin inhibitors contain calcium-binding sites, potential reactive sites as thiol-protease inhibitors (Salier *et al.*, 1987, 1996) and, most importantly, associate with hyaluronan (Zhao *et al.*, 1995). Numerous studies are now establishing the relevance of hyaluronic acid (HA) binding to inter-α-trypsin inhibitor heavy chains with cell types that display an HA-containing coat (Salier *et al.*, 1996).

All inter-α-trypsin inhibitor heavy chains possess a von Willebrand type-A domain (Salier *et al.*, 1996). Adhesion molecules such as integrins, collagen, proteoglycans and heparin are targets for proteins with von Willebrand domains. Heavy chains 1 and 2 of inter-α-trypsin inhibitors have been shown to bind to HA (Huang *et al.*, 1993). Inter-α-trypsin inhibitor heavy chains are proposed to stabilize the extracellular matrix after release of bikunin (Huang *et al.*, 1993; Chen *et al.*, 1994; Jessen *et al.*, 1994). In arthritis, binding of inter-α-trypsin inhibitor heavy chains to hyaluronate is suggested to protect the joint from inflammatory damage possibly caused by free oxygen radicals (Hutadilok *et al.*, 1988). Pig endometrial IαIH4 synthesis may serve to stabilize the epithelial glycocalyx and inhibit conceptus endometrial invasion. It is clear that the pig endometrium expresses IαIH4-like glycoprotein. We have also cloned the pig IαIH1 (R. D. Geisert and T. Pratt, unpublished data) and bikunin (Diederich *et al.*, 1997) of the inter-α-trypsin inhibitor family. In contrast to IαIH4 from the pig endometrium, IαIH1 contains the binding site for bikunin. These results indicate that both forms of inter-α-trypsin inhibitor heavy chain are expressed in pig endometrium.

The function of the C-terminal peptides that correspond to pGP30 following cleavage of IαIH4 is unknown. We have previously reported that pGP30 is taken up by the developing pig conceptus (Geisert *et al.*, 1995). The function of the unique C-terminal peptide of IαIH4 in uterine function and in the conceptus has not been established. Regulation of cleavage for release of the polypeptide during pregnancy and early conceptus development suggests that it may play a role in conceptus–uterine interactions for the establishment of pregnancy in pigs. Identification of pGP30 as a region of IαIH4 provides exciting information concerning a model for uterine–conceptus interactions that are critical for the establishment of pregnancy and placental attachment in pigs.

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