Introduction

Successful implantation and subsequent pregnancy require fine co-ordination of both maternal and embryonic factors (for reviews see Rinkenberger et al. (1997) and Carson et al. (2000)). Success depends on the synchronous development of the embryo and the uterus, so that at the appropriate time both participants are ready to interact. As the fertilized egg approaches the uterus it undergoes numerous divisions, culminating in the formation of a blastocyst. Simultaneously, the endometrium is undergoing proliferation in preparation for the arrival of the blastocyst, becoming most receptive to the attachment of trophoblast cells in a brief definitive period called the ‘implantation window’ (Psychoyos, 1973; Paria et al., 1993). In mice, this window of receptivity opens late on day 4 and closes early on day 5. During this tight timing period, the blastocyst must shed its protective proteinaceous sheath, the zona pellucida, to begin fostering a relationship with the uterine deciduvm.

In the mouse model, oestrogen and progesterone are necessary to synchronize the interaction of the embryo and uterus. Remnants of oestrogen released before ovulation stimulate the differentiation of uterine luminal and endometrial epithelia on the first 2 days of pregnancy (Martin et al., 1973). By day 3, increasing concentrations of progesterone prompt stromal cell proliferation, which is aided on the following day by a preimplantation surge of oestrogen (Huet-Hudson et al., 1989). This surge synchronizes implantation by creating the window of receptivity, whereupon the uterus responds to tactile stimuli, either naturally by an embryo or artificially by an oil droplet (Finn, 1966), to develop a decidual cell differentiation response. If this surge does not occur (for example in ovariectomized females) hatched blastocysts are unable to attach and instead lie dormant in the uterus (Paria et al., 1993). Such delayed implantation can be overcome, within 20 days, by the administration of oestrogen if preceded by progesterone priming for 24–48 h (Yoshinaga and Adams, 1966).

In response to global regulation of implantation by oestrogen and progesterone, cytokines show local autocrine and paracrine effects and create a dialogue that operates largely between the endometrial glands, the luminal epithelium and the embryo. This dialogue is mediated through several vital cytokine networks including epidermal growth factor (EGF), leukocyte inhibitory factor (LIF), colony-stimulating factor (CSF) and insulin-like growth factor (IGF) (Regenstreif and Rossant, 1989; Pollard et al., 1991; Stewart et al., 1992; Baker et al., 1993; Das et al., 1994). In the early stages of pregnancy, before the establishment of the placenta, the endometrial glands serve as an important signalling centre producing several of these cytokines and their corresponding receptors.

Under the direction of cytokines, embryonic proteinases and their corresponding uterine inhibitors are regulated carefully to ensure controlled invasion of the deciduvm by the trophoblasts (for review see Bischof et al. (2000)). Urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP-9) were once thought to be critical proteinases in this process (Harvey et al., 1995;
Alexander et al., 1996; Teesalu et al., 1996). However, gene disruption experiments have demonstrated that such proteinases are dispensable and indicate that implantation operates under conditions of genetic redundancy (Carmeliet et al., 1994; Vu et al., 1998). In the search for additional proteinases in implantation we have identified two implantation serine proteinase genes, ISP1 and ISP2, encoding novel and distinct secreted tryptases. O’ Sullivan et al. (2001) provided functional data indicating that ISP1 encodes the embryo-derived hatching enzyme, trypsin, and demonstrated that this enzyme plays an additional role in the initiation of implantation. In the present study, the expression of ISP2 trypsin in glandular epithelium during zona lysis and implantation in vivo was investigated.

Materials and Methods

Animals and treatments

CD1 mice were obtained at the age of 6–7 weeks from Charles River Canada (St Constant, PQ) and maintained in a standard laboratory animal facility with controlled temperature (20°C) and lighting (lights on between 07:00 h and 19:00 h). The maintenance and treatment of the animals were in full compliance with standard laboratory animal care protocols approved by the University of Calgary’s Animal Care Committee.

Natural pregnancies were obtained by pairing female mice with adult males and checking the female mice daily for the presence of a vaginal copulatory plug as an indication of mating. For embryo collection, day 0.5 corresponded to mid-day of the day on which a vaginal plug was detected. Pregnant dams were killed on a specific day by cervical dislocation, after which uteri and oviducts were removed surgically before isolation of embryos, either by dissection or flushing (Hogan et al., 1994).

All surgical procedures were performed after the mice were anaesthetized with an i.p. injection of Avertin (2% (w/v) tribromoethanol; Aldrich Chemical Co, Milwaukee, WI). Ovariectomy was performed by dorsal–lateral incision (Hogan et al., 1994).

Ovariectomized mice were allowed at least 1 week to recover before the induction of deciduomas. All steroid hormones including RU486 were dissolved in sesame oil and injected s.c. At each stage of the experiment, control mice were used that received only oil (0.1 ml per mouse) injections. Three animals were treated and analysed in each group.

The standard regimen for artificial induction of deciduomas (Finn, 1966) was modified to mimic pseudopregnancy more closely (Milligan et al., 1995). Here, the first progesterone treatment was started 2 days after exposure to oestrogen. This modified regimen consisted of 100 ng oestrogen administered once a day starting on day 0, and 1 mg progesterone plus 10 ng oestrogen from day 3 onwards. Deciduomas were induced surgically on day 5 (between 14:00 h and 16:00 h) by injecting sesame oil (10 µl) into the lumen of one uterine horn from its oviductal tip. Injected and uninjected uterine horns were collected 24, 48 or 72 h later for histological sectioning and in situ hybridization analysis.

Delayed implantation was induced and maintained by ovariectomized mice on day 3 of pregnancy, followed by administration of progesterone (2 mg per mouse) on days 4–6. Subsequently, half of these mice were treated with oestrogen (25 ng per mouse) on the morning of day 7, while the other half received the normal progesterone injection. Mice were killed 24 h later for analysis by in situ hybridization.

The effect of steroids on uterine development was examined by ovariectomizing pregnant mice and treating them with hormone injections either immediately or after a 2 week recovery period. Mice received injections of either progesterone (1 mg per mouse), oestrogen (100 ng per mouse), or a combination of both (10 ng oestrogen and 1 mg progesterone per mouse) for 3 days. The mice were then killed on the morning of day 4. Superovulated pregnant and pseudopregnant mice (Hogan et al., 1994) were treated with RU486 (400 µg per mouse) on the morning of day 3 after a vaginal plug was detected to determine further the importance of progesterone on ISP2 gene expression. The mice were killed 24 h later and their uterine horns were collected for in situ hybridization analysis.

Embryo culture

Morulae were collected from oviducts of superovulated day 2.5 pregnant dams in M2 medium (Hogan et al., 1994). For in vitro hatching, morulae (approximately 100) were cultured in microwells for approximately 24 h at 37°C, 5% CO2 in KSOMaa medium (Erbach et al., 1994). For in vitro embryo outgrowth onto extracellular matrix, hatched blastocysts (approximately 100) were cultured for an additional 48 h at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium plus 5% (v/v) fetal bovine serum on microwells coated with extracellular matrix derived from 10% (v/v) Triton X-100-treated mouse embryo fibroblasts (Behrendt et al., 1995).

Embryo RNA preparation

Total RNA was collected from a litter (approximately ten) of embryos plus deciduum (day 6.5 implantation sites), embryos (days 8.5, 11.5 and 13.5 of gestation) and placentae (days 11.5 and 13.5) using Trizol (Life Technologies, Rockville, MD). In vitro hatching blastocysts (at 50% hatch) were collected by centrifugation at 3000 g for 5 min for Trizol RNA preparation (O’Sullivan et al., 2001). RNA from blastocysts outgrowing in vitro was collected by Trizol lysis directly in microwells (O’Sullivan et al., 2001). Poly (A)+ RNA was enriched from day 6.5 embryo plus deciduum total RNA using oligo (dT) cellulose chromatography (Sambrook et al., 1989).

cDNA cloning and sequence analysis

A 478 bp ISP2 PCR subfragment derived previously by active site degenerate PCR (O’Sullivan et al., 2001) was
used to screen $5 \times 10^5$ recombinants from a day 6.5 embryo plus deciduum library in GT10, and resulted in the identification of two cDNA clones bearing a 1.2 kb insertion. The 1.2 kb EcoRI fragment was subcloned into pBKCMV (Stratagene, La Jolla, CA) for cycle sequencing (PE Biosystems, Foster City, CA). The nucleotide sequence was translated into protein sequence using the Swiss Protein ExPasy tool (http://expasy.cbr.nrc.ca/tools/dna.html). Nine serine proteinase peptides identified from a BLAST identity search were aligned with ISP2 using Clustal W (Higgins, 1994; http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and compared to develop a dendrogram using the protein parsimony method (http://bioweb.pasteur.fr/seqanal/phylogeny/phyllip-uk.html).

Expression analysis

Poly (A)$^+$ RNA (5 μg) from day 6.5 murine embryo plus deciduum was subjected to electrophoresis through a 1.2% (w/v) formaldehyde–agarose gel alongside an RNA high molecular mass ladder (Life Technologies). After transfer to Hybond N$^+$ (Amersham Pharmacia, Piscataway, NJ), the membrane was probed with the 1.2 kb $^{[32]}$P-labelled ISP2 cDNA clone.

The presence of ISP2 transcripts in embryos and placentae was monitored using RT–PCR. Total RNA (1 μg) was reverse-transcribed and amplified using ISP2-specific primers (ISP2$\text{for}$: 5'–TGTGAGCCGGGTCATCATCC–3' and ISP2$\text{rev}$: 5'–GGCATTGTGGTACATCTCCT–3') and 40 rounds of thermal cycling (1 min at 94°C, 2 min at 60°C and 2 min at 72°C). The RT–PCR amplification of GAPDH (as a control for mRNA loading) was performed as described by Arcellana-Panlilio and Schultz (1993). The predicted 360 bp and 175 bp amplification products were separated on a 2% (w/v) agarose gel.

Whole embryo and sectioned RNA in situ hybridization using digoxigenin-labelled RNA probes were performed essentially as described by Rancourt and Rancourt (1997). Uteri from nonpregnant, pregnant and pseudopregnant mice (see above) were fixed in 4% (w/v) paraformaldehyde, embedded in paraffin wax and cut into sections (7 μm thickness). The ISP2 probe comprised the 478 bp RT–PCR subclone in pBSKS+. The antisense probe was synthesized using T3 polymerase after plasmid linearization with EcoRI. The sense probe was synthesized using T7 polymerase after plasmid linearization with BamHI. All experiments were performed in parallel with the sense RNA probe to detect non-specific hybridization.

Results

ISP2 represents another novel branch of the tryptase subfamily of S1 proteinases

After evidence of genetic redundancy within the implantation proteinase cascade was found we initiated experiments to identify additional serine proteinases that may play a vital role in implantation. Active site RT–PCR of material from day 6.5 implantation sites was used to reveal two genes encoding novel implantation serine proteinases, ISP1 and ISP2 (O'Sullivan et al., 2001). A 478 bp ISP2 cDNA fragment derived from active site RT–PCR was used to screen a day 6.5 mouse embryo plus deciduum cDNA library (O’Sullivan et al., 2001) and a 1.2 kb cDNA clone was isolated. On the basis of prior analysis of ISP1, which also gave rise to a 1.2 kb cDNA clone, it was suspected that a full length cDNA clone for ISP2 had also been isolated. This idea was confirmed by northern blot analysis of day 6.5 embryo plus deciduum poly(A)$^+$ RNA (5 μg) revealed a mRNA species of 1.3 kb when hybridized with a random prime-labelled 1.2 kb ISP2 cDNA clone. (b) ISP2 (360 bp; upper panel) and GAPDH (175 bp; lower panel) expression in embryogenesis as detected by RT–PCR. ISP2 gene expression is moderate in placental RNA from day 11.5 (lane 4) and day 13.5 (lane 6) pregnancies, and in embryo RNA from day 13.5 (lane 5) pregnancy. It is not detected in RNA from hatching (lane 7) or outgrowing (lane 8) blastocysts.
identity; Lutzelschwab et al., 1997). Other mast cell proteases showed similar degrees of sequence identity. The next closest subfamilies contain chymotrypsins and elastases with approximately 34% shared amino acid identity. Like ISP1, the relationship of ISP2 to the S1 peptidase family is clear, as it shares the conserved His and Ser active site moieties (IHPQW and GDSGGPL), in addition to the common N-terminal sequence (IVVG) of mature trypases (Fig. 2; Smyth et al., 1996). Maximum parsimony analysis (Higgins, 1994) indicates that, based on the low degree of similarity between the ISPs and their nearest neighbours within the mast cell trypase family, the ISPs represent a distinct branch of the S1 proteinase superfamily that diverged from the elastase/chymotrypsin and mast cell proteinase clusters at approximately the same time (dendrogram not shown).

**Temporal expression of ISP2 during gestation**

RT–PCR was used to characterize the expression of ISP2 throughout gestation (Fig. 1b). High expression was observed in day 6.5 embryo plus deciduum RNA consistent with the expression observed using northern blotting. Lower expression was also observed in placental RNA isolated...
ISP2 expression was not observed in RNA from the embryo proper at days 8.5 and 11.5; a residual amount of expression was detected at day 13.5. This pattern of expression for ISP2 resembled that previously identified for ISP1 (O’Sullivan et al., 2001). On the basis of the role of ISP1 in blastocyst hatching and outgrowth onto extracellular matrix, it was investigated whether ISP2 is expressed in the blastocyst and plays a similar role to ISP1. However, RT–PCR of RNA isolated from blastocysts hatched or outgrown in vitro indicated that ISP2 was not expressed in early embryos (Fig. 1b). This result was confirmed by additional in situ hybridization experiments performed on morulae and blastocysts, which indicated that ISP2 is not expressed (data not shown). On the basis of these expression results, it was concluded that the function of ISP2 was distinct from that of ISP1 and it was hypothesized that the function of ISP2 resided within the uterine deciduum during implantation.

Expression of ISP2 in glandular epithelium during implantation

In situ hybridization analysis of sagittal sections was performed to confirm that ISP2 gene expression originated from the deciduum. Throughout the peri-implantation period, intense ISP2 mRNA staining was observed specifically within endometrial gland epithelium (Fig. 3). Expression was first identified in sagittal sections of day 6.5 implantation sites (not shown) and subsequently in implantation sites of day 7.5 and day 8.5 pregnancies (Fig. 3a,b). At day 6.5, ISP2 mRNA staining was also observed between implantation sites lying remote from the embryo (Fig. 3c). These results first indicated the possibility that ISP2 gene expression might not be dependent upon decidua surrounding the implantation site. Interestingly, ISP2 mRNA staining was not observed in nonpregnant uterus (Fig. 3d), indicating that ISP2 gene expression occurred specifically in response to pregnancy. ISP2 gene expression was also not observed on day 2.5 (Fig. 3e), when the morula is oviductal, or on day 3.5 (Fig. 3f), when the blastocyst enters the uterus. However, ISP2 mRNA staining was observed at days 4.5 and 5.5 (Fig. 3g,h), when the implantation window is opened. These results indicate that ISP2 expression occurs either in response to the implantation reaction or is regulated hormonally in synchrony with implantation.

ISP2 gene expression in pseudopregnancy

The potential role of hormones and the decidualization reaction in regulating ISP2 gene expression was investigated further by inducing artificial pregnancies in ovariectomized females using uterine oil injections after progesterone and oestrogen priming (Finn, 1966; Milligan et al., 1995). As part of the experimental design, oil was introduced into only one uterine horn to ensure that the decidualization reaction occurred on one side of the animal only. The other side served to control for the potential role of hormonal treatments on ISP2 gene expression. After in situ hybridization, ISP2 mRNA staining was observed in both uterine horns, indicating that ISP2 gene expression also occurs during artificial pregnancy and in the absence of decidualization (Fig. 4).

Induction of ISP2 gene expression by progesterone

The influence of steroid hormones was examined using models of pregnancy and pseudopregnancy. In delayed implantation experiments, ISP2 gene expression was abrogated by ovariectomy (Fig. 5a). However, when progesterone was administered to maintain pregnancy, normal ISP2 expression was observed (Fig. 5b). A similar maintenance of ISP2 gene expression was not observed in the presence of oestrogen only (Fig. 5c). These results indicate that there is probably a requirement for progesterone in maintaining ISP2 gene expression during pregnancy.

Mice were treated with RU486 on day 3 of pregnancy or pseudopregnancy and analysed for ISP2 gene expression in uterine sections to confirm the requirement of progesterone for uterine ISP2 gene expression. When mice were killed on the following day, normal ISP2 mRNA staining was observed in vehicle-treated control mice (Fig. 6a,c).
However, in mice treated with the antiprogestin, ISP2 mRNA staining was not observed (Fig. 6b,d). These results established the necessity of progesterone for maintaining ISP2 gene expression in pregnancy.

Interestingly, ISP2 gene expression could be induced by progesterone after the cessation of pregnancy by ovariectomy (Fig. 5d). In the absence of progesterone maintenance after ovariectomy, ISP2 gene expression was not observed (data not shown). However, when pregnancy failure was induced by ovariectomy, ISP2 gene expression could still be induced up to 14 days after ovariectomy (Fig. 5d). These results confirmed that after ovariectomy and a long absence of progesterone signalling, the uterus remains responsive to progesterone, and indicates that ISP2 gene expression may be induced by progesterone.

Similar experiments also demonstrated that oestrogen did not have a stimulatory or inhibitory effect on ISP2 gene expression. After ovariectomy in the absence of progesterone maintenance, it was observed that oestrogen did not have a stimulatory effect on ISP2 gene expression (Fig. 5f). Moreover, administration of oestrogen in combination with progesterone after induced pregnancy failure resulted in no significant alteration of ISP2 gene expression (Fig. 5d) compared with that of progesterone treatment only. These results indicate that progesterone alone is necessary and sufficient to bring about maximal ISP2 gene expression.

**Discussion**

In previous studies, active site RT–PCR was used to identify a serine proteinase of the subtilisin lineage, SPC6, which functions in decidua during implantation (Rancourt and Rancourt, 1997). In the present study and in an accompanying study (O’Sullivan et al., 2001), active site RT–PCR was used to identify novel serine proteinases of the chymotrypsin lineage that are involved in implantation or embryonic development. Before the beginning of this work, urokinase and tissue type plasminogen activator had both been characterized in implantation and embryogenesis (Strickland et al., 1976). Our PCR strategy showed that four additional serine proteinases are expressed during implantation, granzyme D and F, the functions of which are currently unknown (Smyth et al., 1996; Allen and Nilsen-Hamilton, 1998) and two novel implantation serine proteinases, ISP1 and ISP2. O’Sullivan et al. (2001) demonstrated that ISP1 encodes a novel embryo-derived trypsin that functions in blastocyst hatching and initiation of invasion into extracellular matrix. The aim of the present study was to characterize ISP2 structure and expression during implantation.

DNA sequencing of a full-length cDNA clone of ISP2 derived from a day 6.5 embryo plus deciduum cDNA library demonstrated that the ISP2 gene also encodes a novel serine proteinase related to trypsinases. Although the ISPs have hallmark signatures of trypsinases, maximum parsimony analysis indicates that the ISPs represent a distinct lineage of the SP1 superfamily, having first diverged from the mast cell proteinase and elastase/chymotrypsin clusters at approximately the same time. The evolution of these trypsinases is consistent with the suggestion that the ISPs play overlapping roles in zona lysis and implantation (see below).

The data obtained in the present study indicate that during gestation the ISP2 gene is expressed predominantly during implantation, although residual expression is observed in the developing placenta. Unlike ISP1, the ISP2 gene is not expressed in the pre-implantation embryo. Instead, ISP2 gene expression was observed in endometrial gland epithelium throughout the peri-implantation period (days 4.5–8.5). During implantation, it was observed that ISP2 gene expression occurs initially in glands throughout the decidua, including regions proximal to the embryo, but is progressively restricted when the glands decrease in size and move to the periphery of the uterine crypt during deciduomata regression and placentation.

*In situ* hybridization experiments revealed that ISP2 gene expression might be regulated by progesterone. Hybridization of ISP2 mRNA within glandular epithelium lying between implantation sites first indicated the possibility that ISP2 gene expression might not be dependent on the presence of the embryo. This possibility was confirmed when oil-induced deciduomata were established in hormonally treated pseudopregnant females, whereas ISP2 mRNA was observed within the glands of non-decidualized control horns. Further investigation using ovariectomy and models of delayed implantation demonstrated that ISP2 gene expression was dependent on progesterone administration only, and that oestrogen showed no effect either on its own or in combination with progesterone. ISP2 gene expression was abrogated in both pregnancy and pseudopregnancy in the presence of the
anti-progestin, RU486. These data indicate that glandular ISP2 gene expression is regulated positively by progesterone. At this time, it is unclear whether the effect of progesterone on ISP2 gene expression is direct.

A key feature of successful implantation is the synchrony between embryonic and endometrial development. This synchrony is achieved through timely preparation, regulated first by hormones and, after zona lysis, by cytokine signalling between the endometrium and the embryo. Only on day 4 of pregnancy, as progesterone concentrations increase, does the glandular epithelium differentiate and become secretory (Duc-Goiran et al., 1999; Paria et al., 1999). The in situ hybridization experiments performed in the present study have demonstrated that ISP2 mRNA is not detected at stages before the endometrial gland secretory phase. These observations indicate that ISP2 secretion into the glandular and uterine lumen may occur as a consequence of progesterone-induced epithelial differentiation.

In the pregnancy setting, the endometrial gland acts as a ‘command centre’, sending and receiving cytokine dispatches that support implantation. LIF, for example, is secreted from the endometrial gland into the uterine lumen, where it is thought to interact with luminal LIF receptors and result in the presentation of EGF receptors that are necessary for apposition of the embryo (Song et al., 2000). As observed for ISP2, the LIF gene is not expressed after RU486 administration (Danielsson et al., 1997; Ghosh et al., 1998; Liu et al., 1999). RU486 has a profound effect on preventing the differentiation of secretory glandular epithelium, which probably accounts for its effect on LIF expression and in preventing implantation (Greb et al., 1999). However, LIF secretion is distinct from ISP2 in that it is also oestrogen-dependent (Song et al., 2000). Although oestrogen appears to co-ordinate expression of LIF during the ‘window of implantation’, a morphologically normal endometrial gland is necessary for secretion into the lumen. This role for
The ISP2 proteinase protein is part of the function of ISP-2 protein and represents 500 kDa. No ISP2 mRNA staining is observed in the vehicle-treated pregnant uterus. (b) No ISP2 mRNA staining is observed in the RU486-treated pregnant uterus. (c) ISP2 mRNA staining is observed in the vehicle-treated pseudopregnant uterus. (d) No ISP2 mRNA staining is observed in pseudopregnant RU486-treated uterus. Scale bar represents 500 µm.

Implantation serine proteinase (ISP2) mRNA is not detected in pregnant and pseudopregnant mouse uterus after RU486 treatment. Uteri from (a,c) vehicle- and (b,d) RU486-treated (a,b) pregnant and (c,d) pseudopregnant dams were sectioned sagittally and hybridized in situ with a digoxigenin-labelled ISP2 probe. (a) ISP2 mRNA staining is observed in the vehicle-treated pregnant uterus. (b) No ISP2 mRNA staining is observed in the RU486-treated pregnant uterus. (c) ISP2 mRNA staining is observed in the vehicle-treated pseudopregnant uterus. (d) No ISP2 mRNA staining is observed in pseudopregnant RU486-treated uterus. Scale bar represents 500 µm.

Progestin in generating a fully functional endometrial gland explains why progesterone priming is required before the oestrogen pulse in delayed implantation. As ISP2 gene expression is independent of the oestrogen spike and occurs during the progesterone-priming phase, it is likely that the first proteolytic role of ISP2 may precede implantation.

In addition to ISP2, the matrix metalloproteinase, MMP-9, and the serine proteinase, kallikrein 1, are also expressed in glandular epithelium during implantation and are found in uterine luminal fluid (Jeziorska et al., 1996; Corthorn et al., 1997). Although these proteinases are presumed to participate in the extracellular matrix remodelling that occurs during implantation, the role of ISP2 at this time is unclear. As MMP-9 is activated by tryptases *in vivo* (Keski-Oja et al., 1992; Lohi et al., 1992), part of the function of ISP-2 protein may lie in activating luminal MMP-9. In addition, a direct role for ISP2 in matrix remodelling is also possible. O’Sullivan et al. (2001) demonstrated that ISP1 tryptase participates in the extracellular matrix degradation that occurs during blastocyst outgrowth. Accordingly, the potential matrix remodelling activity of ISP2 might also be direct.

It is not known how ISP2 may be recruited to participate in matrix degradation at the implantation site. One mechanism may be that ISP2 encodes a second enzyme associated with lysis of the zona pellucida (see O’Sullivan et al., 2001). The ISP1 gene encodes the enzyme, trypsinogen, which is associated with the focal hatching of blastocysts *in vitro*. However, there is increasing evidence to indicate that the ‘focal’ hatching observed *in vitro* is distinct from the zona ‘lysis’ that occurs *in vivo* (Gonzales and Bavinster, 1995; Montag et al., 2000; Gonzales et al., 2001). Accordingly, it has been suggested that a distinct ‘lysin’ protein occurs in luminal fluid before implantation. Interestingly, lysis activity is dependent upon an intact ovary and progesterone (Joshi and Murray, 1974; Denker, 1977), yet does not require the presence of an embryo within the uterus (Orsini and McLaren, 1967). Although it has been suggested that kallikrein 1 may participate in zona lysis, this hypothesis is inconsistent with the expression profile of kallikrein 1, which is downregulated by progesterone and dependent on the presence of the embryo. Instead, based on the striking similarity in expression profiles between lysis and ISP2, in addition to the relationship of ISP2 tryptase to the embryo-derived hatching enzyme strypsin, it is possible that the ISP2 gene may encode a lysis proteinase.

On the basis of the observation that tryptases multimerize for activity (Huang et al., 2000), O’Sullivan et al. (2001) suggested that ISP1 proteinase (approximately 27 kDa) must multimerize to yield active strypsin (approximately 70 kDa). Zymography has also revealed a 70 kDa proteinase doublet in uterine flushings of pregnant hamsters (Gonzales et al., 2001). Although it has been suggested that this doublet may represent strypsin, as strypsin is only observed in blastocysts, this suggestion is inconsistent with the presence of this doublet in uterine flushings of pseudopregnant females. Instead, this 70 kDa species may represent multimers of ISP2. Nonetheless, this suggestion still does not account for the observation of a second doublet (45 kDa), which is expressed in a narrow window around the time of zona lysis. While this doublet may represent a smaller form of the 70 kDa species (an ISP2 dimer), another proteinase associated with zona lysis may yet be found.

The observation that two related tryptases are derived separately from the embryo and uterus to effect zona lysis reiterates the hypothesis that genetic redundancy has evolved to ensure successful implantation. Given that hatching constitutes a serious block in assisted reproduction (De Vos and Van Steirteghem, 2000), the further characterization of the roles of ISP1 and ISP2 tryptases in hatching and implantation is of utmost importance.

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