Changes in major proteins in the embryonic capsule during immobilization (fixation) of the conceptus in the third week of pregnancy in the mare

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Abstract

During the third week of pregnancy, the equine conceptus is enclosed within a capsule, the glycan composition of which changes at around day 16 (ovulation = day 0) when the conceptus becomes immobilized (fixed) in the uterine lumen. Our objective was to characterize the process of fixation by identifying changes in major capsule-associated proteins. Individual equine conceptuses (n=55) were collected transcervically by uterine lavage between days 13.5 and 26.5. Major proteins extracted from capsules were compared with those in fluids from the uterus and yolk sac by SDS–PAGE. Until day 14, a major capsule-associated protein that migrated at ~10 kDa was identified by N-terminal sequencing as equine β2 microglobulin (β2M). During fixation, β2M in the capsule underwent limited proteolysis to an ~8 kDa form lacking nine amino acids from the N terminus, and was subsequently degraded. Expression of β2M mRNA was detected in the yolk-sac wall tissues and endometrium between days 13.5 and 17.5. During this period, β2M in the capsule was evidently not part of a complex with major histocompatibility complex class 1 heavy chain bands because these were undetectable in the capsule and uterine lavage. Uterocalin (p19) was detected in uterine lavage and capsule throughout fixation, but in yolk-sac fluid only before fixation. These studies indicate that intact β2M is a major protein associated with the embryonic capsule before fixation, after which it undergoes limited proteolysis to a truncated ~8 kDa form that remains in the capsule after the conceptus is immobilized.

Introduction

Pregnancy maintenance in the mare depends upon the transitory appearance of an acellular, mucin-like, glycoprotein capsule around the blastocyst as it expands in the uterus during the second and third weeks of gestation (Marrable & Flood 1975, Betteridge 1989, Oriol et al. 1993a, 1993b). For the first two-thirds of its existence, the capsule is resilient and favors extensive migration of the spherical conceptus within the uterus until, at about day 16–17, migration ceases and the conceptus becomes ‘fixed’ at the site of subsequent placentation (Ginther 1983, 1985). Pre-fixation mobility is essential for pregnancy maintenance (McDowell et al. 1988), allowing signals to be exchanged between the conceptus and the entire endometrium.

The capsule is essential for survival of the early embryo, protects it while it is mobile (Stout et al. 2005) and provides a regulatory interface between the trophoblast and the luminal epithelium of the uterus. Pregnancy failure due to embryonic loss is of great economic importance in horse breeding and many of the losses are incurred during the period that the capsule is present (Ball 1988, Baker et al. 1993, Carnevale et al. 2000, Morris & Allen 2002). In particular, Morris & Allen (2002) have shown that 16–17% of pregnancies diagnosed by ultrasound at about day 15 are subsequently lost, 60% of them between days 15 and 35. The concept that maternal secretions (‘uterine milk’) nourish the early mammalian embryo is a very old one (Needham 1667, Harvey 1847, Marshall 1910, Ewart 1915, Amoroso 1952) that still underlies current research into the constituents of the secretion (Gray et al. 2005). The capsule might regulate the transfer of secreted uterine substances to the equine embryo. Selective binding of some proteins to the capsule supports the concept that the capsule is like a ‘mailbox’ for delivery of various substances required by the embryo (Herrler et al. 1998, 2000b). For example, maternal p19/uterocalin

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binds to the capsule but is implicated mainly in delivery of lipids to the equine conceptus (Stewart et al. 1995, 2000, Crossett et al. 1996, 1998, Suire et al. 2001). Equine yolk-sac wall tissues before fixation express unusually large amounts of the GM2-activator protein (GM2AP) implicated in transport and catabolism of glycolipids (Quinn et al. 2006). They also produce insulin-like growth factor-binding protein 3 that binds insulin-like growth factor which is important for embryonic development (Herrler et al. 2000a). At the same time, a selectively permeable capsule composed largely of polysaccharides might shield embryonic antigens from immune rejection (Betteridge 1989) before non-classical major histocompatibility complex (MHC) class 1 expression and other immunoregulatory mechanisms prevent the rejection of paternal alloantigens in the invading trophoblast (Hunt et al. 2005, Trowsdale & Betz 2006).

Changes in the capsule and the endometrium might have functional roles in the process of fixation or its failure. Coincidently with fixation, the capsule becomes flaccid (Betteridge & Waelchli 2005) and loses sialic acid (Oriol et al. 1993a, 1993b, Chu et al. 1997). Loss of terminal sialic acid from mammalian glycans can increase binding of some proteins to exposed galactose or mannose residues, and increased expression of some uterine lectins have been demonstrated in early pregnancy (Gray et al. 2005). In the horse, various proteins in and around the early conceptus have been described in uterine fluid (Zavy et al. 1982, McDowell et al. 1990, Müller-Schöttle et al. 2002), the zona pellucida (Miller et al. 1992), the capsule (Stewart et al. 1995, Herrler & Beier 2000, Herrler et al. 2000b), and yolk-sac fluid (Crossett et al. 1996), but both the range of proteins characterized and definition of their developmental times of appearance and disappearance in the conceptus itself remain limited. To further investigate the role of the capsule in the success and failure of pregnancy in horses, we have started to identify proteins that are associated with the capsule and change during the process of fixation at about day 16. We have focused on major changes in the protein profiles within the capsule itself before and after fixation, and compared these profiles with those in contemporaneous uterine fluid (lavage), yolk-sac fluid, and yolk-sac wall tissues between days 13.5 and 26.5 of pregnancy. A preliminary account of some of these data has been reported previously (Quinn et al. 2005).

Materials and Methods

Sample collection and preparation

Standardbred and Thoroughbred mares (n = 11) from the research herd at the University of Guelph were used to recover conceptuses under animal care and research protocols approved by the University of Guelph Animal Care Committee. Ovulation was diagnosed by transrectal ultrasonography and was considered to have occurred (day 0) midway between the detections of an intact follicle followed by a corpus luteum. Because ultrasonographic monitoring was at 1–3 days interval, the ages of conceptuses, counted from day 0, were subject to an error ranging from ±0.5 to ±1.5 days. Conceptuses were collected from mares by transcervical uterine lavage with PBS (pH 7.4) using a technique based on the one described previously (Waelchli & Betteridge 1996). The mares were sedated with xylazine hydrochloride (Rompun; Bay-Vet, Etobicoke, ON, Canada) administered i.v. usually 0.3 mg/kg body weight. The uterine lavage fluid (1000 ml) containing the conceptus was collected into a beaker by gravity flow. Immediately after recovery of the conceptus, an endometrial biopsy sample was obtained transcervically using a pulling biopsy punch (Lane Medical Co., Boulder, CO, USA) as described by Kenney (1975). These endometrial samples were bisected; one half was flash-frozen and stored in liquid nitrogen, the other half was stored in RNAlater (Ambion, Austin, TX, USA).

The conceptus was transferred to a Petri dish containing PBS where it was punctured after aspirating the PBS into a syringe and drying the dish with absorbent paper. The yolk-sac fluid released into the dry dish was collected by aspiration through a 20G needle. Puncturing the conceptus resulted in a split in the capsule through which yolk-sac wall (omphalopleure) tissues could be gently withdrawn after re-addition of PBS to the dish. After separation, the whole capsule and yolk-sac wall tissues were either stored frozen in PBS (capsule) or divided equally (tissues) for flash-freezing in liquid nitrogen or immersion in RNAlater before storage at −70 °C. The lavage and yolk-sac fluids were centrifuged to remove cells and particulates. Thirty-nine conceptuses and uterine samples were collected over one breeding season, ranging in age from 13.5 to 26.5 days. Additional conceptuses (n = 16) from previous breeding seasons ranging in age from 14 to 22 days were also examined.

A 100 ml aliquot of the uterine lavage fluid (1000 ml) was centrifuged to remove cells and particulates and then soluble proteins were concentrated 50 times, by centrifugal ultrafiltration at a 5 kDa cutoff (Amicon Ultra; Millipore Corporation, Bedford, MA, USA) before analysis. The yolk-sac fluids were similarly processed and concentrated 10 times. Uterine biopsies and yolk-sac wall tissues were homogenized in 0.5 ml PBS containing proteinase inhibitors (Complete Mini, Roche Diagnostics). Protein concentrations were determined by the Bio-Rad protein assay (Bradford 1976). The capsule was collected on a filter (Whatman 1; Fisher Scientific, Ottawa, ON, Canada) washed thrice in PBS and cut into sections for analysis. A section representing ~50% of the total capsule was boiled for 5 min in 100 μl 2× SDS sample buffer (0.2 M Tris–HCl, pH 6.8 containing 2% SDS, 8% mercaptoethanol, and
24% glycerol), then diluted with 100 µl PBS. The capsule structure remained intact visually throughout the elution procedure.

**Electrophoresis and protein identification**

Proteins in samples were first characterized by 15% one-dimensional SDS–PAGE (with a 4% stacking region) in reducing conditions (Laemmli 1970) using the Bio-Rad modular Mini-Protean II system (Bio-Rad). Prestained broad range markers (Bio-Rad or New England Biolabs P7701S) were used to identify apparent molecular weights of isolated proteins. Electrophoresis was carried out at 4 °C for 1.5 h at 150 V.

The previously described equine uterine proteins p19/uterocalin (Stewart et al. 1995) and uteroglobin (Müller-Schöttle et al. 2002) were tentatively identified by their apparent molecular mass on SDS–PAGE and confirmed by immunoblots. Proteins were electrotransferred to a nitrocellulose membrane (Trans-Blot, Bio-Rad) in a 25 mM Tris–HCl buffer, containing 100 mM glycine and 20% methanol, at 4 °C for 1 h at 150 V, followed by 2 h at 100 V and were incubated overnight in PBS containing 1% BSA to block non-specific binding. Specific binding was detected by incubation with rabbit antisemur against recombinant equine p19/uterocalin from which the glutathione S-transferase fusion partner had been removed (Kennedy 2005) or rabbit anti-rat uteroglobin/Clara cell secretary protein (CCSP; generously provided by Drs Paula Katavolos and Dorothee Bienzle, University of Guelph). To identify immuno-reactive MHC class 1 heavy chains, we used a mouse monoclonal IgG2a against horse MHC class 1 (Abcam, ab23491) followed by peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) H + L (Cedarlane, Hornby, ON, Canada). The major capsule bands identified as β2 microglobulin (β2M) were also detected by immunoblotting with primary rabbit anti-human β2M antibody (Sigma). Immunoblots were washed twice for 15 min in PBS containing 0.01% Tween 20 (PBS/Tween) and incubated for 45 min in a 1:5000 diluted secondary antibody, peroxidase-labeled goat polyclonal anti-rabbit IgG H + L (Biomedra Corp., Foster City, CA, USA). All antibodies were diluted in PBS, 0.01% Tween 20 and 1% BSA. After washing four times for 15 min in PBS/Tween, the signal was revealed using a chemiluminescence substrate (ECL, Amersham Biosciences Inc.) following the manufacturer’s recommendations. Eluted capsule proteins, yolk-sac wall tissue, and endometrial homogenates were loaded at 10 µl/well and concentrated lavage and yolk-sac fluids were loaded at 20 µl/well for visualizing by silver staining (Oakley et al. 1980) and Western blot.

For N-terminal amino acid sequencing, proteins were loaded at 40 µl/well to 15% SDS–PAGE reduced gels, electrotransferred to PVDF membrane Immobilon-PSQ (Millipore Corporation) at 4 °C for 1 h at 150 V, followed by 2 h at 100 V in 25 mM Tris, 100 mM glycine, 20% methanol and stained with Coomassie G 250 stain (Bio-Rad). The bands of interest were cut from the membrane and analyzed (Biotechnology Laboratory, University of British Columbia, Canada).

For mass fingerprint analysis, proteins of interest were loaded at 40 µl/well to 15% SDS–PAGE reduced gels and the bands were excised and digested with trypsin. The gel slices were destained in 15 mM ferricyanide and 50 mM sodium thiosulfate for 20 min in the dark and washed with water until samples were clear. Gel slices were then dehydrated in 100% methanol in 100 mM NH4HCO3 at 50 °C for 30 min, followed by alkylolation in 55 mM iodoacetamide in 100 mM NH4HCO3 for 60 °C min, at room temperature. Samples were then washed in 100 mM NH4HCO3, followed by a wash in 50 mM NH4HCO3 in 50% acetonitrile, then dehydrated in 100% acetonitrile and dried in a vacuum. Samples were rehydrated in digestion buffer containing 50 mM NH4HCO3 and 3 ng/µl of sequencing grade trypsin (Sigma) and incubated overnight at 37 °C. The digestion products were collected into a small volume of 2% trifluoroacetic acid (TFA). Peptides were further extracted from the gel slices with two successive volumes of 0.1% TFA and 50% acetonitrile followed by one volume of 100% acetonitrile. The collected volumes were pooled and concentrated in a vacuum centrifuge. The peptide solutions were desalted using a C18 reverse phase chromatography matrix (ZipTipC18, Millipore) according to manufacturers recommended protocol. The peptide mass spectra were obtained by MALDI-TOF mass spectrometry with a Bruker reflex IV mass spectrometer (MALDI-TOF Mass Spectroscopy Facility, Department of Molecular Biology and Genetics, University of Guelph). The NCBI non-redundant protein sequence database was searched initially using the MS Fit algorithm (Clauser et al. 1999) for proteins matching mass spectra within the taxonomic category Equus caballus. If no matches were found, further searches were performed to include all species.

**Gene expression**

Tissue expression of MHC class 1 α chain and β2M was detected qualitatively by RT-PCR using endometrial biopsies and yolk-sac wall tissues previously stored in RNAlater. Total RNA was isolated using TRIzol reagent (Ambion), based on the phenol–guanidine isothiocyanate protocol (Chomczynski & Sacchi 1987). cDNA was synthesized using the Thermoscript RT-PCR system according to manufacturer’s recommended protocol (Invitrogen) using oligo dT primers, an incubation step of 60 °C for 60 min and the optional RNase H step. cDNA amplification was performed using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer’s recommendations, with a primer
concentration of 1 μM. Primers and conditions were designed following a previously described protocol (Bacon et al. 2002). For β2M, a forward primer (5’ GGTTTCTAGTACTACGTG 3’) and a reverse primer (5’ CACACATTGAGTGAAGT 3’) were used with amplification conditions of 94 °C (1 min), 50 °C (1 min), for 34 cycles and a final extension at 72 °C (10 min). For MHC class I, amplification conditions were as above, using a forward primer (5’ CGTGCGGTTGATGCACG 3’) and a reverse primer (5’ GTGAAACATCTGCATC 3’). PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Results

Under reducing conditions, seven major bands were observed at various developmental stages in capsule extracts subjected to 15% SDS–PAGE and silver staining (Fig. 1). Some unidentified protein extracted from the capsule remained in the wells and did not enter the gels. An ~10 kDa band (p10) was identified as equine β2M by N-terminal amino acid sequencing (Fig. 2), trypsin mass fingerprints, and immunoblotting (Fig. 3). The N-terminal sequencing also indicated that the 10 kDa band contained a minor amount of another form of β2M (Ellis & Martin 1993) lacking two N-terminal amino acids. After day 16, the p10 β2M band became less distinct while a prominent ~8 kDa band (p8) appeared until day 19. This band was consistent by N-terminal sequencing (Fig. 2) and immunoblots (Fig. 3) with a form of β2M from which nine amino acids had been removed from the N-terminus. By day 19, all p10 and p8 forms of β2M in capsule were lost but subsequently a smaller band ~5 kDa appeared (Fig. 1) that was weakly immunoreactive with the anti-β2M antiserum (Fig. 3). However, in some capsule extracts containing the p5 band, no immunoreactive β2M was observed. No acceptable match for its peptide mass fingerprint and no N-terminal amino-acid sequence could be obtained for the p5 bands in capsule extracts and it was negative in immunoblots with antisera to uteroglobin and p19/uterocalin.

To investigate the possible origin and disposition of β2M and other major proteins in the capsule, we examined the uterine lavage fluid (Fig. 4), and compared immunoreactive β2M in the yolk-sac fluid, yolk-sac wall tissues, endometrium, and uterine flush (Fig. 5). No prominent p10, p8, or p5 bands were observed in stained gels of uterine lavage fluids (Fig. 4) but faint immunoreactive β2M bands were detected in lavage fluid and yolk-sac fluid (Fig. 5) indicating that β2M was relatively more concentrated in the capsule. Protein concentrations in the yolk-sac fluid increased markedly through the fixation period (Fig. 6), with several changes in the bands present (Fig. 7). Faintly stained bands were sometimes apparent in the ~43–45 kDa region in reduced gels of capsule (Fig. 1) but not lavage fluids (Fig. 4). However, these were not in proportion with the more abundant β2M bands and were not immunoreactive with anti-horse MHC-1 α chains (not shown), suggesting that β2M in the capsule was not part of an MHC class 1 complex. Transcripts of equine β2M (Fig. 8) and MHC-1 (not shown) were found in proportional amounts by RT-PCR in all yolk-sac wall tissues and

**Figure 1** Silver-stained 15% SDS–PAGE reducing gel of proteins eluted from equine embryonic capsules collected between days 15.5 and 23 of gestation. Major bands identified are p19 (p19/uterocalin), p17 (phospholipase A2), p14 (α-hemoglobin), p13 (β-hemoglobin), p10 (β2-microglobulin), and p8 (truncated β2-microglobulin). Fixation of the previously mobile conceptus occurs at about day 16.

**Figure 2** N-terminal amino-acid sequence of p10 and p8 bands eluted from equine embryonic capsules, in comparison with the sequence of secreted equine β2M (Ellis & Martin 1993). Major and minor residues over the initial six amino acids of p10 indicated the presence of the major form (1) predicted from the nucleotide sequence (Ellis & Martin 1993) and minor form (2) lacking the first two amino acids. The first nine amino acids were missing from p8.

**Figure 3** Immunoblot of proteins eluted from the equine embryonic capsules incubated with primary polyclonal anti-human β2M. The abundant intact form (p10) present in the capsule before fixation is mostly converted to the p8 form by day 16 and appears to be further converted to an ~5 kDa form by day 19 when the capsule is detaching from the trophoblast.
endometrial biopsies, so it was not possible to locate the origin of β2M in the capsule.

We also noted several other proteins that changed in the capsule, uterine lavage, yolk-sac wall tissues, and yolk-sac fluids during the day 13.5–26.5 period of gestation. In the yolk-sac wall tissues before day 18, two bands of 23 and 23.5 kDa consistent with GM2AP (Quinn et al. 2006) were prominent (not shown). A prominent 19 kDa band was observed in similar amounts in the capsule between the days 15 and 23 (Fig. 1). This corresponded to the most prominent band in the uterine lavage fluids from pregnant mares (Fig. 4). The p19 bands in capsule, yolk-sac fluid, and uterine lavage fluid were each identified as p19/uterocalin (Stewart et al. 2000) by MALDI mass spectra of their trypsin-digested peptides, and also by immunoblotting with anti-p19/uterocalin antiserum (Fig. 9). P19/uterocalin was detected in the yolk-sac fluid until day 16, but subsequently diminished markedly (Fig. 7) and it was not detected in the uterine lavage of anoestrous non-pregnant mares (Fig. 4).

A prominent band at ~6 kDa (p6) in all uterine lavage samples (Fig. 4) was identified as uteroglobin/CCSP (Beier 2000) by immunoblots with anti-rat-CCSP antibody (Fig. 10). Immunoreactive uteroglobin was detected in yolk-sac fluid at day 15.5 but not in later stage yolk-sac samples (Fig. 10) suggesting that uteroglobin, like uterocalin/p19, transferred into the cavity of conceptus proteins and fixation in mares

Figure 4 Silver-stained 15% SDS–PAGE reducing gel of proteins in concentrated uterine lavage fluid collected between days 13.5 and 23 of gestation. The major band p19, identified as p19/uterocalin by trypsin mass fingerprint and immunoblotting, is present throughout this period with some reduction by day 20, but is not detected in uterine lavage fluids collected in October from non-cycling non-pregnant mares (NP). By comparison, the band p6 identified as uteroglobin by immunoblots was consistently detected in similar amounts in pregnant and NP mares. The doublet at p13–14 is consistent with hemoglobins, the band at 66 kDa is consistent with albumin, and the bands at 55 and 25 kDa are likely immunoglobulins.

Figure 5 Immunoblots using anti-β2M primary antibody applied to transfers of 15% SDS–PAGE reducing gels of samples of concentrated uterine lavage fluid, yolk-sac fluid, yolk-sac wall tissue homogenates, and endometrial homogenates. While the p10 band is inapparent in silver stained gels (see Fig. 4), it is detected in small amounts in uterine lavage fluids and yolk-sac fluids but not in homogenates of yolk-sac wall tissue or endometrium. By comparison, β2M in both p10 and p8 forms is preferentially associated with the capsule in amounts too large to be explained by passive contamination from the adjacent tissues and secretions.
the yolk sac before the capsule was modified during the fixation phase (Fig. 11).

A 17 kDa band was also observed in the capsule from day 15 to 16 coincidently with the 10 kDa form of intact β2M. At later stages, p17 was not visible in silver-stained gels of the capsule extracts (Fig. 1). Similar p17 and p10 bands were seen as early as day 10 in archived samples of capsule (not shown). Attempts to determine the N-terminal sequence for p17 were unsuccessful but electrophoretic mobility was most consistent with a uterine phospholipase A2 first reported by Beier-Hellwig et al. (1995). Partial amino acid sequences of p17 determined by tandem mass spectrometry, and also the N-terminus reported by Beier-Hellwig et al. (1995), were predicted by the same cDNA which we obtained from equine endometrium (manuscript in preparation). The band at 66 kDa is consistent with serum albumin, and those at 55 and 25 kDa are probably immunoglobulin heavy and light chains. By day 19, two bands that variably appeared in the capsule extracts at 13 kDa (p13) and 14 kDa (p14; Fig. 1) were respectively identified as equine α-hemoglobin and β-hemoglobin by MALDI mass spectra of their trypsin-digested peptides. Hemoglobins in the capsule increased after day 19 consistent with the development of the vasculature of the conceptus. In some mares, similar hemoglobin p13 and p14 bands were sporadically present in the uterine lavage fluids at earlier stages (Fig. 4) when they were not visible in the capsule (Fig. 1).

Discussion

These studies demonstrate that β2M is a major protein associated with the equine embryonic capsule and it undergoes stepwise proteolysis while associated with the capsule. We also show that the conversion of β2M in the capsule from the intact form to a cleaved form (i.e. ΔN9-β2M lacking nine amino acids from the N-terminus) occurs at the stage around day 16 when the conceptus becomes fixed in the uterine lumen. The relatively large amounts of β2M in the capsule but not in the uterine lavage suggest that some constituent of the capsule has the ability to preferentially bind both the intact and partially cleaved forms of β2M. These observations raise the possibility that β2M, without an associated MHC class 1 heavy chain, has a role in the capsule, either as the intact form before
fixation, and/or as the cleaved form after fixation. However, it is equally possible that equine embryonic capsule has the unusual ability to bind β2M, an ubiquitous protein, without any functional role therein.

The origin of the β2M in the capsule could not be determined in our studies but since the capsule is acellular, it is most likely derived from the endometrium or yolk-sac wall tissues. We detected expression of β2M in both by non-quantitative RT-PCR using primers described by others (Bacon et al. 2002). However, β2M is expressed in most tissues from which it is continuously released as a small protein that is cleared by the kidney, and free β2M has a tropism for some extracellular matrix substances, so the β2M in the capsule might originate elsewhere. For example, β2M without associated MHC class 1 heavy chains can be deposited in the connective tissue as amyloid in chronic renal disease (Monti et al. 2005, Myers et al. 2006). Intact and partially cleaved forms of β2M can bind to connective tissue matrix components including collagen (Giorgetti et al. 2005) and glycosaminoglycans (Ohashi et al. 2002, Yamaguchi et al. 2003) in a manner that involves conformational changes induced by low pH (Monti et al. 2005) or copper ions (Villanueva et al. 2004, Deng et al. 2006). Limited proteolysis to the ΔN6-β2M form of human β2M alters binding in amyloid but is not required (Monti et al. 2005).

Sialic acid is removed from the capsule during fixation (Oriol et al. 1993a, 1993b, Chu et al. 1997) and the capsule is degraded within the following week. The N-terminal nine amino acid peptide is located on the surface of human β2M in a position that might mask an internal functional site. Accordingly, proteolytic removal of the N-terminal fragment from β2M in the capsule might be a necessary modification rather than a stage of capsule degradation. However, it is also plausible that equine monomeric β2M initially binds to the capsule in a manner that limits its susceptibility to proteolysis. Experimental digestion of human monomeric β2M with pepsin and other proteases yields various peptides but, under some conditions (e.g., pH 2.5), pepsin cleaves β2M in a single location at valine nine (Myers et al. 2006). This cut yields a product (ΔN9-β2M) that lacks nine amino acids from the N-terminus equivalent to the p8 form identified in the capsule in our studies. Such limited proteolysis has been explained on the basis of the limited access of proteases to β2M embedded in connective tissue matrix or amyloid deposits (Monti et al. 2005). A similar explanation might apply to the degradation of β2M in the capsule, because the 8 kDa form (ΔN9-β2M) subsequently decreased in the capsule while there was an increase in a ~5 kDa band.

Intact monomeric β2M might have an earlier role in the capsule before it is degraded. The terminal nine amino acid peptide is located on the surface of β2M where it might provide a function to the capsule that is lost when β2M is degraded. It appears that the N-terminal portion is not involved in binding to the capsule, since the ΔN9-β2M form remains bound. Structural models of β2M in association with MHC α-chains in hemochromatosis factor (HFE; Lebrón et al. 1998), MHC-1 (Reiser et al. 2002), and immunoglobulin transporter Fc γ-receptor (Martin et al. 2001) all indicate that the nine N-terminal amino acids of β2M and the underlying polypeptide are located away from the surfaces that associate with the MHC-1 heavy chain partners.

Known functions of β2M during early pregnancy are those of various MHC class 1 complexes in which β2M is the light chain complexed non-covalently with a heavy α-chain of ~43 kDa. However, the relative abundance of β2M in the capsule without an equivalent amount of a MHC class 1 α-chain partner suggests that β2M might have some other role independent of those of the MHC class 1 proteins. The horse β2M gene is highly conserved among equids which has regulatory regions similar to those in the human and murine β2M genes and it is usually co-expressed with various MHC class 1 α-chains (Tallmadge et al. 2003). During in utero development in mammals, β2M can be co-expressed with various MHC class 1 α-heavy chain partners. These include the classical polymorphic MHC class 1 antigen presentation complex (Leitner et al. 2002), the non-classical MHC class 1 proteins implicated in blocking NK-cell-mediated rejection of the trophoblast (Morales et al. 2003, Hunt et al. 2005), the iron uptake protein HFE (Gruper et al. 2005), and the class of Fc receptors involved in placental or enteric transport of immunoglobulins or the recycling of albumin (Ellinger et al. 2005). Between days 14 and 20 of pregnancy in sheep, endometrial expression of β2M is induced under the influence of maternal progesterone and interferon τα from the conceptus but the β2M and MHC-1 proteins are expressed mainly in the uterine glands and stroma away from the conceptus (Choi et al. 2003, Gray et al. 2006). Expression of classical highly polymorphic antigen-presenting MHC class 1 proteins is typically reduced in embryonic tissues in mammals, including horses, to shield the paternally derived antigens from the maternal immune system (Bacon et al. 2002). However, classical and non-classical MHC class 1 α-chains and β2M are focally induced between days 30 and 36 in the invasive chorionic girdle region of the equine trophoblast (Kydd et al. 1991, Vagnoni et al. 1996a, 1996b, Bacon et al. 2002, Tallmadge et al. 2005). MHC-class 1 α-chain genes expressed in invasive human trophoblast are mainly the non-classical HLA-C, -E and G class involved in regulating NK-cell responses involved in implantation and placentation (Morales et al. 2003, Hunt et al. 2005, Trowsdale & Betz 2006). All of the expressed MHC class 1 α-chain genes in the cluster on chromosome 20q21 have membrane-binding domains (Tallmadge et al. 2005). However, some forms of human HLA-G that associate with β2M, are soluble and lack the membrane association domain (Hunt et al. 2005). Expressed soluble MHC class 1 complexes (ESC1) analogous to the Q10 molecule, which is expressed in...
the liver and yolk sac of the mouse, have been reported in the serum of horses (Lew et al. 1986).

Other major proteins present during the encapsulation phase such as p19/uterocalin and uteroglobin in the uterus, and the GM2AP in the trophoblast (Quinn et al. 2006) are lipid-binding proteins with transport roles that are likely necessary for the uptake of lipids through the hydrophilic capsule glycan. A lipid provisioning role has been proposed for p19/uterocalin that is secreted in abundance under the influence of progesterone, and that is transferred into the yolk sac of encapsulated embryos (Suire et al. 2001, Kennedy 2005). P19/uterocalin was first identified in the capsule (Stewart et al. 1995), but whether it is there in large amounts as it is bound or in transit is still unclear. We observed that p19/uterocalin was present in consistent amounts both in the capsule and in the uterine lavage fluids throughout the fixation period. By comparison, amounts of p19/uterocalin in the yolk-sac fluid were greatest before fixation, while β2M was the most abundant protein in the capsule. This suggests that either the entry of p19/uterocalin into the yolk-sac fluid is transient until fixation or that it does not accumulate later because it is catabolized. A similar situation was noted for uteroglobin that was always plentiful in the uterine lavage fluids but present in the yolk-sac fluid only before fixation. Uteroglobin/CCSP has a phospholipid-binding pocket (Beier 2000, von der Decken et al. 2005) and it may also serve some role in the delivery of lipid ligands through the capsular glycan in the horse. Uteroglobin is a progesterone-dependent uterine secretory protein in various mammals including those in which the conceptus is not encapsulated (Beier 2000), and the abundant uteroglobin did not bind to the capsule. By comparison, p19/uterocalin has not been demonstrated in non-equids and its presence in the yolk sac declines as the capsule is degraded.

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