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Haptoglobin expression and release by rabbit oviduct and endometrium, its localization in blastocyst extra-embryonic matrix and fluid during preimplantation time

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BACKGROUND: Evidence is emerging that haptoglobin, an acute phase protein with immunomodulatory properties, is expressed by the endometrium of various species. The present study describes an in-depth investigation of haptoglobin expression and release in the rabbit reproductive tract and in preimplantation embryos. METHODS: The full-length cDNA sequence of rabbit haptoglobin was determined by rapid amplification of cDNA ends PCR. Haptoglobin expression was studied in the oviductal ampull, and isthmus, endometrium and embryos from the time of ovulation up to adhesion. These results were completed by western blot analysis of reproductive tract secretions and embryonic tissues. RESULTS: cDNA sequencing showed a high homology between rabbit and human haptoglobin (84.1%). In oviductal tissues haptoglobin mRNA is clearly expressed from 6 h post-conception (p.c.) to day 3, and in the uterus on days 5 and 6. In the oviductal fluid highest haptoglobin protein content was found between 6 h p.c and day 2, and in the uterine fluid on days 5 and 6 p.c. Embryos do not express haptoglobin mRNA during preimplantation development. However, considerable amounts of maternal haptoglobin protein were detected in the blastocyst coverings and in blastocyst fluid. CONCLUSIONS: Already during periovulatory time and oviductal passage, high amounts of haptoglobin are present in the microenvironment surrounding the oocyte/embryo. Two days before implantation, again, high haptoglobin levels are detectable in the embryo's environment. The incorporation of haptoglobin into the extra-embryonic matrix may be of particular functional significance.

Key words: amino acid sequence/embryo/haptoglobin/oviduct/uterus

Introduction

Haptoglobin, discovered in 1939 (Polonovski and Jayle, 1939), is synthesized in the liver and known to bind free haemoglobin. It is often produced as an acute phase reactant during infection, inflammation, traumatic damage and malignant proliferation. Furthermore, El Ghmati *et al.* (1996) were able to show that haptoglobin binds to leukocyte CD11b receptors, suggesting an immune modulatory function, and recently, Bottini *et al.* (1999) pointed to a significant impact of haptoglobin on the fertility of women.

The active protein is formed by two α- and two β-subunits, resulting in a tetramer. Furthermore, in humans two α-alleles are known (Dobryszycka, 1997). Haptoglobin mRNA is expressed in mice liver already during fetal development, and in the adult organism in lung, spleen, kidney and skin (D'Armiento *et al.*, 1997). The protein is present in most body fluids (e.g. serum, urine, saliva, ascites) (Dobryszycka, 1997). Furthermore, the haptoglobin α-subunit is detectable in human uterine secretions (Beier and Beier-Hellwig, 1998), as well as the haptoglobin β-subunit (Berkova *et al.*, 2001) and haptoglobin in the endometrium (Sharpe-Timms *et al.*, 2000). As Sharpe-Timms *et al.* (2000) detected haptoglobin expression in the endometrial stroma by in-situ hybridization, at least some of the haptoglobin detected in the secretions seems to be of local origin. Hoffman *et al.* (1996) identified the rabbit 42 kDa glycoprotein (GP42) to be β-haptoglobin. GP42 has been shown to be secreted by the rabbit endometrium shortly before implantation (Thie *et al.*, 1984; Anderson *et al.*, 1986). Although haptoglobin expression in the late preimplantation uterus is well established, nothing is known about haptoglobin mRNA expression and protein content in the oviduct, the early preimplantation uterus or in the embryo itself.

In the investigation presented here, the complete rabbit haptoglobin cDNA was sequenced. Another goal of the study was to investigate whether haptoglobin is part of the preimplantation maternal–embryonic signalling. Consequently, the time schedule of haptoglobin mRNA expression and protein distribution in the oviduct and uterus, as well as in the embryo, during the preimplantation period was studied.

To investigate whether haptoglobin is detectable at the embryo–maternal interface like several other functional proteins (Herrler *et al.*, 2002), the extra-embryonic matrices, i.e. the zona pellucida, were analysed for the presence of haptoglobin protein. In addition, haptoglobin mRNA expression and protein synthesis was determined in all preimplantation embryos. In this investigation we did not differentiate between the known isoforms of the α-subunits, which will be part of further functional investigations.

Materials and methods

Determination of the full-length rabbit haptoglobin cDNA sequence

To determine the rabbit haptoglobin cDNA sequence, rabbit liver was used as the RNA source. Samples were collected from does as described below. RNA isolation was performed as described by Chomczynski and Sacchi (1987). Briefly, mRNA was recovered by phenol/chloroform extraction followed by a column-based purification step (Qiagen, Hilden, Germany). To prevent DNA contamination, 2.27 U DNAse per microlitre was added. One microgram of RNA was used for cDNA synthesis. cDNA was stored at −80°C until further use. For the initial sequence analysis two primers were chosen, resulting in a 386 bp transcript spanning the intron between exon 4 and 5 of the human genome sequence: forward 5′-TGTGARGCAGTRTGTGGAAGC-3′ (primer 1; Figure 1) and reverse 5′-CRTARTCYTTGGAWGGYAGGCA-3′ (primer 2; Figure 1; synthesized by MWG Biotech, Ebersberg, Germany) (R = A/G, Y = C/T, W = A/T). As the resulting DNA product spans a 921 bp intron (referring to human haptoglobin, M69197 NCBI), DNA contamination would be detected as a product of 1307 bp. Standard PCR conditions were as following: 10 pmol primer, 35 cycles, 1.5 mM MgCl, annealing temperature 55°C. These primers also were used to investigate haptoglobin mRNA expression by semiquantitative RT–PCR in reproductive tract organs and embryos as described below. Sequence analysis (SeqLab, Göttingen, Germany) of the PCR product confirmed analogy with the known rabbit haptoglobin cDNA sequence (CAB96389). To obtain the 3′ end of the cDNA sequence a second forward primer was generated (5′-AGAGTGATGCCCATTTGCCTAC-3′) (primer 3; Figure 1) and the Not-I (dT_{18}) primer (5′-AACTGGAAGAATTCGCGGCCGCA-GGAAT$_{18}$-3′) (Amersham Pharmacia, Freiburg, Germany; primer 4; Figure 1) was used as reverse primer. As the sequence could not be determined down to the poly(A) end, a further forward primer was generated for using together with the Not-I (dT_{18}) primer: 5′-GTGA-AGACATTCAACATCCTG-3′ (primer 5; Figure 1). Conditions for the Not-I PCR were as following: 10 pmol primer, 35 cycles, 1.5 mM $MgCl_2$, annealing temperature 50°C (sequencing: SeqLab). To obtain the 5′ end, the RLM-RACE Kit (Ambion, Austin, TX, USA) was used. Therefore, two specific primers were created using available rabbit sequence: inner primer, 5′-GAAAACTGCCTTTGG-CGTCCA-3′; and outer primer, 5′-TGTGGCGGGAGACCATCG-TTAG-3′ (primers 6 and 7; Figure 1; PCR conditions as proposed by the kit: 35 cycles, annealing temperature 60°C). Sequencing was performed using SeqLab. Each product was sequenced at least twice. The overlapping cDNA sequences and the putative amino acid sequence were determined using the MBS translator (mbshort-cuts.com/cgi-bin/translator). This sequence was aligned to known human, mouse, rat, golden hamster, dog and bovine haptoglobin sequences (NCBI accession numbers CAA25926, AAB29697, NP_036714, BAA21723, P19006 and CAC00531, respectively). Furthermore, the sequence was compared with the published rabbit sequence of the α-subunit (NCBI accession number CAB96389), as well as to the partial sequence published by Olson *et al.* (1997).

As we have detected previously that in the rabbit endometrium ubiquitin and β-actin are regulated in a pregnancy-dependent manner (Krusche, 1999), ribosomal S26 was used as a control.

mRNA expression analysis

All animal experiments were conducted in accordance with the principles for the care and use of research animals and were run with the permission of the District Government at Cologne, Germany (AC 39 15/95). Liver, oviducts, uteri and embryos were recovered from female New Zealand White rabbits ranging in age from 6 to 8 months. They were housed individually in air-conditioned rooms (25°C, 45% relative humidity) under a 12 h light, 12 h dark cycle (0700 to 1900 h) and were fed with a commercial diet and water *ad libitum*.

To stimulate multiple follicular growth, rabbits received one injection of 100 IU pregnant mare's serum gonadotrophin (PMSG) s.c. (Intergonan®; Intervet, Tönisvorst, Germany), except those animals used for tissue sampling before ovulation (+6 and +9 h). For ovulation induction, 75 IU HCG (Primogonyl®; Schering, Berlin, Germany) were administered i.v. directly after mating by two fertile bucks. To terminate further PMSG activity and follicular growth, Neutra-PMSG® (Intervet) was administered i.v. in parallel to HCG application (i.e. 72 h after PMSG injection). Six hours (6 h), 9 h (9 h), 24 h (day 1), 48 h (day 2), 72 h (day 3), 96 h (day 4), 120 h (day 5) and 144 h (day 6) following mating, samples were collected. The total reproductive tract was removed. Embryos were collected by flushing oviducts (days 1, 2 and 3) and/or uteri (days 3, 4, 5 and 6) with 5–10 ml Dulbeco's phosphate-buffered saline (PBS) (Sigma, Deisenhofen, Germany). Embryos were washed three times in PBS, pooled in 500 µl RNAzol (WAK Chemie, Bad Soden, Germany; days 1 and 2, $n = 40$; days 3 and 4, $n = 20$; days 5 and 6, $n = 10$) and snap-frozen in liquid nitrogen. Oviducts were split into ampulla and isthmus, removing the middle piece and fimbriae. Uteri were opened at the mesometrial side and endometrium was sampled by scraping off the surface tissue. Endometrial as well as oviductal samples were submerged in 500 µl RNAzol (WAK Chemie) and snap-frozen in liquid nitrogen. Samples were stored at −20°C prior to analysis. At least three animals were analysed per day of pregnancy. Liver samples served as positive control. PCRs primers 1 and 2 were used as described above. To determine the size of the PCR

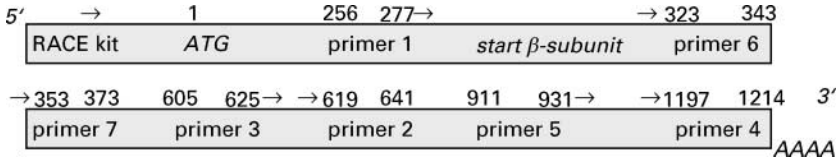

Figure 1. Haptoglobin sequence obtained by sequencing different overlapping PCR products using the presented primer. Primer position is described in accordance with the sequence presented in Figure 2.

products a 100 bp ladder (GeneRuler Fermentas, St Leon-Rot, Germany) was used.

Western blot analysis

Animals were treated as described above. Six to 144 h following mating, uteri and oviducts were flushed with 150 mM ammonium bicarbonate buffer. The first millilitre was collected separately and was centrifuged following embryo recovery (1000 *g*, 10 min). The supernatant was snap-frozen and used for determination of haptoglobin secreted by the oviduct and endometrium.

For dissection, embryos were washed three times in PBS and transferred into a clean culture well. All remaining fluid was removed. The embryo was then punctured and the blastocyst fluid collected. The collapsed embryo was floated in a drop of PBS, and the embryo separated from the embryonic coats. Coats and embryo were washed and sampled separately. For each investigation, five embryos, 10 coats or blastocyst fluid of 20 embryos were pooled separately, snap-frozen and stored at −20°C until further analysis. Tissue samples were collected by separating ampulla and isthmus. The endometrium was scraped off with a scalpel. All samples were snap-frozen directly in liquid nitrogen. Protein content (except of embryonic coats) was determined by DC Protein Assay (Bio-Rad, Munich, Germany) according to Lowry *et al.* (1951). Twenty micrograms of protein of each sample was diluted in loading buffer [5% (v/v) β-mercaptoethanol; 30 mM Tris, pH 6.8; 1.5 M urea; 7.5% (v/v) glycerol; 0.5% (w/v) sodium dodecyl sulphate (SDS); 0.05% (w/v) Bromophenol Blue]. Samples were heated to 100°C for 5 min, centrifuged and subjected to SDS–PAGE using a 5% stacking gel and a 15% resolving gel under reducing conditions. For size determination, a prestained molecular weight marker (Rainbow, high molecular weight range; Amersham Biosciences, Freiburg, Germany) was used. After electrophoretic separation, the proteins were transferred to a polyvinylidene diflouride membrane (Sartorius, Epsom, UK) by semi-dry blotting (Kyhse-Andersen, 1984) for 45 min at 900 mA. Following transfer, the membrane was blocked over night with 5% (w/v) milk powder in PBS/Tween-20 (0.1% w/v) at 4°C. Because of the high homology between rabbit and human haptoglobin sequence, a primary antibody against human haptoglobin was used (goat anti-human haptoglobin; SP1121P; DPC Biermann, Bad Nauheim, Germany; crossreactivity to rabbit haptoglobin has been described by DPC Biermann) at a concentration of 12 μg IgG/ml PBS containing 5% milk powder. The blot was incubated at room temperature for 2 h. After three washes in PBS/0.1% Tween, the second antibody (donkey anti-goat conjugated with horseradish peroxidase, diluted 1:5000 in PBS/0.1% Tween/1% milk powder; Dako, Glostrup, Denmark) was added and left at room temperature for 1 h. After further rigorous washing, detection of the second antibody was performed using enhanced chemiluminescence

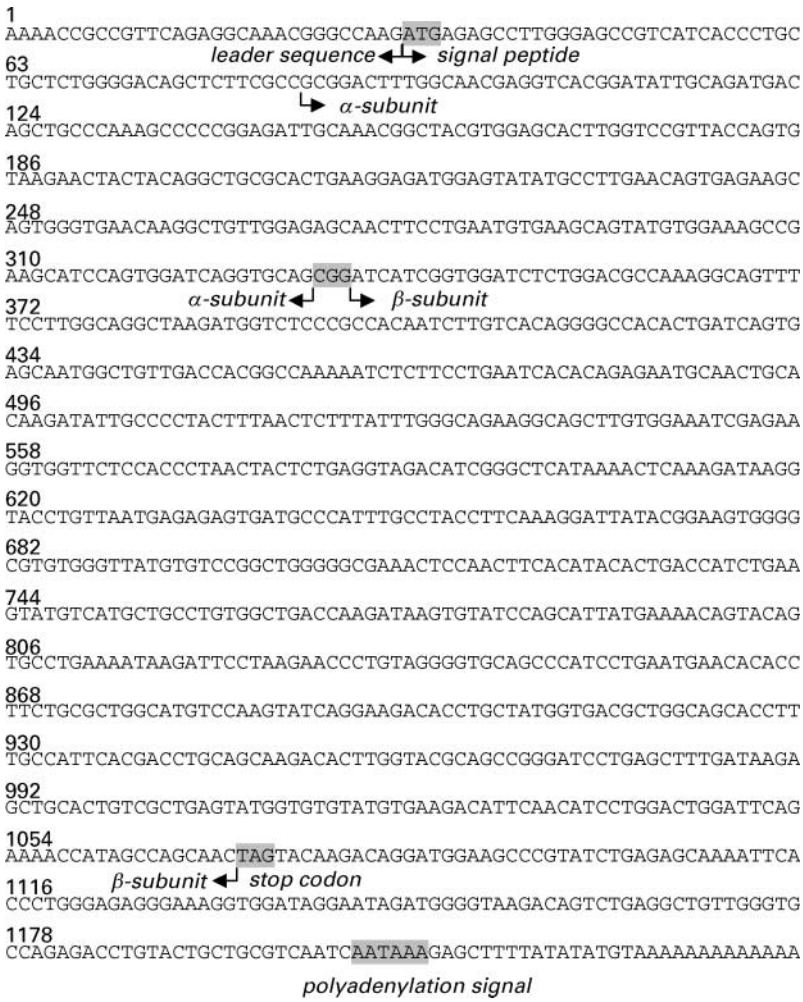

Figure 2. Rabbit haptoglobin cDNA sequence. Haptoglobin mRNA can be subdivided by following codons (shadowed in grey): (**A**) translation start signal site (ATG); (**B**) spacer codon between α- and β-subunit (CGG); (**C**) stop codon (TAG); and (**D**) polyadenylation signal (AATAAA).

(ECL) reagents (Amersham, Little Chalfont, UK; ECL: horseradish peroxidase-induced chemiluminescent reaction) and the blot was exposed to an X-ray film (X-Omat AR; IBI-Kodak Ltd, Cambridge, UK) for 3 min. At least three animals were analysed per day of pregnancy. Rabbit serum served as positive control. To verify the specificity of the antibody it was preabsorbed for 30 min with haptoglobin (60 μg haptoglobin 1-1 plus 60 μg haptoglobin 2-2 plus 12 μg anti-haptoglobin IgG/ml; H 0138 and H 9762; Sigma, St Louis, MO, USA) and applied to day 6 uterine secretions (see Figure 5, negative control).

Results

By successive sequencing, a 1243 bp sequence of rabbit haptoglobin (Figure 2) was obtained and was submitted to the NCBI database (accession number AAM21313). The sequence includes a part of the 5′ end, the signal peptide,

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1  MRALGAVITLLLWGQ LFAADFGNEVTDIAD DSCPKPPEIANGYVE HLVRYQCKNYYRLRT EGDGVYALNSEKQWV NKAVGEQLPECEAVC
2  MRALGAVITLLLWGQ LFAADFGNEVTDIAD DSCPKPPEIANGYVE HLVRYQCKNYYRLRT EGGGVYALNSEKQWV NKAVGEQLPECEAVC
3  --------------- --------------- --------------- --------------- --------------- ---------------
4  MSALGAVIALLLWGQ LFAVDSGNDVTDIAD DGCPKPPEIAHGYVE HSVRYQCKNYYKLRT EGDGVYTLNDKKQWI NKAVGDKLPECEAVC
5  MRALGAVVTLLLWGQ LFAVELGNDAMDFED DSCPKPPEIANGYVE HLVRYRCRQFYRLRA EGDGVYTLNDEKQWV NTVAGHKLPECEAVC
6  MRALGAVVTLLLWGQ LFAVELGNDATDIED DSCPKPPEIANGYVE HLVRYRCRQFYKLQT EGDGIYTLNSEKQWV NPAAGDKLPKCEAVC
7  MRALGAVVTLLLWGQ LFAVDLSNDAMDTAD DSCPKPPEIENGYVE HLVRYRC-QHYRLRT EGDGVYTLNSEKQWV NTAAGERLPECEAVC
8  --------------- ---EDTGSEATNNTE VSLPKPPVIENGYVE HMIRYQCKPFYKLHT EGDGVYTLNSEKHWT NKAVGEKLPECEAVC
9  --------------- --------------- --------------- --------------- --------------- ---------------
10 * *****. ****** ***  .  ..  .  .    **** * .**** * .**.*. .*.*   ****.* ** .* *  *   * .**.*****
       signal peptide ◄──► mature protein

1  GKPKHPVDQVQRIIG GSLDAKGSFPWQAKM VSRHNLVTGATLISE QWLLTTAKNLFLNHT ENATAQDIAPTLTLY LGRRQLVEIEKVVLH
2  GKPKHPVVKVQRIIG GSLDAKGSFPWQAKM VSRHNLVTGATLISE QWLLTTAKNLFLNHT ENATAQDIAPTLTLY LGRRQLVEIEKVVLH
3  --------------- ---DAKGSFPWQAKM VSRHNLVTGATLISE QWLLTTAKNLFLNHT ENATAQDIAPTLTLY LGRRQLVEIKKVVLH
4  GKPKNPANPVQRILG GHLDAKGSFPWQAKM VSHHNLTTGATLINE QWLLTTAKNLFLNHS ENATAKDIAPTLTLY VGKKQLVEIEKVVLH
5  GKPKHPVDQVQRIIG GSMDAKGSFPWQAKM ISRHGLTTGATLISD QWLLTTAKNLFLNHS ETASAKDITPTLTLY VGKNQLVEIEKVVLH
6  GKPKHPVDQVQRIIG GSMDAKGSFPWQAKM ISRHGLTTGATLISD QWLLTTAQNLFLNHS ENATAKDIAPTLTLY VGKNQLVEIEKVVLH
7  GKPKHPVDQVQRIIG GSLDAKGSFPWQAKM VSRHELITGATLISD QWLLTTAKNLFLNHS EDATSKDIAPTLKLY VGKMQPVEIEKVVIH
8  GKPKNPVDQVQRIMG GSVDAKGSFPWQAKM VSHHNLTSGATLINE QWLLTTAKNLFLGHK DDAKANDIAPTLKLY VGKNQLVEVEKVVLH
9  --------------- -----KGSFPWQAKM VSQHNLISGATLINE RWLLTTAKNLYLGHS SDKKAKDITPTLRLY VGKNQLVEVEKVVLH
10 ****.*   **** * *  ************ .* * *  *****.. .******.**.* *      . ** *** ** .*. * **.****.*
   α-subunit ◄──► β-subunit

1  PNYSEVDIGLIKLKD KVPVNERVMPICLPS KDYTEVGRVGYVSGW GRNSNFTYTDHLKYV MLPVADQDKCIQHYE NSTVPENKIPKSPVG
2  PNYSEVDIGLIKLKD KV------------- --------------- --------------- --------------- ---------------
3  PNYSEVDIGLIKLKD KVPVNERVMPICLPS KDYTEVGRVGYVSGW GRNSNFRYTDHLKYV MLPVADQDKCIQHYE NSTVPENKIPKSPVG
4  PNYSQVDIGLIKLKQ KVSVNERVMPICLPS KDYAEVGRVGYVSGW GRNANFKFTDHLKYV MLPVADQDQCIRHYE GSTVPEKKTPKSPVG
5  PNHSVVDIGLIKLKQ RVLVTERVMPICLPS KDYIAPGRVGYVSGW GRNANFRFTDRLKYV MLPVADQDKCVVHYE NSTVPEKKNLTSPVG
6  PERSVVDIGLIKLKQ KVLVTEKVMPICLPS KDYVAPGRMLCVRLG ARNVNFRFTERLKYV MLPVADQEKCELHYE KSTVPEKKGAVTPVG
7  PNRSVVDIGVIKLRQ KVPVNERVMPICLPS KDYIAPGRMGYVSGW GRNANFRFTDRLKYV MLPVADQDSCMLHYE GSTVPEKEGSKSSVG
8  PDYSKVDIGLIKLKQ KVPIDERVMPICLPS KDYAEVGRIGYVSGW GRNSNFNFTELLKYV MLPVADQDKCVQHYE GSTVPEKKSPKSPVG
9  PDHSKVDIGLIKLRQ KVPVNDKVMPICLPS --------------- --------------- --------------- ---------------
10 *  * ****.***.  .*   ..******** ***   **.  * *   **.** .*. **** *******. *  ***  ***** .     **

1  VQPILNEHTFCAGMS KYQEDTCYGDAGSAF AIHDLQQDTWYAAGI LSFDKSCTVAEYGVV VKTFNILDWIQKTIA SN
2  --------------- --------------- --------------- --------------- --------------- --
3  VQPILNEHTFCAGMS KYQEDTCYGDAGSAF AIHDLQQDTWYAAGI LSFDKSCAVAEYGVV --------------- --
4  VQPILNEHTFCAGMS KYQEDTCYGDAGSAF AVHDLEEDTWYATGI LSFDKSCAVAEYGVV VKVTSIQDWVQKTIA EN
5  VQPILNEHTFCAGLT KYQEDTCYGDAGSAF AIHDMEEDTWYAAGI LSFDKTCAVAEYGVV VRATDLKDWVQETMA KN
6  VQPILNKHTFCAGLT KYEEDTCYGDAGSAF AVHDTEEDTWYAAGI LSFDKSCAVAEYGVV VKATDLKDWVQETMA KN
7  VQPILNEHTFCAGMT KYQEDTCYGDAGSAF AIHDLEQDTWYAAGI LSFDKSCSVAEYGVV VKVNSFLDWIQETMA KN
8  VQPILNEHTFCAGMS KFQEDTCYGDAGSAF AVHDQDEDTWYAAGI LSFDKSCTVAEYGVV VKVPSVLAWVQETIA GN
9  --------------- --------------- --------------- --------------- --------------- --
10 ******.******.. *..************ *.**  .***** ** *****.* ******* *.  .   * *. .*  *

1   Oryctolagus cuniculus, new full length sequence (AF372520)
2   Oryctolagus cuniculus, partial sequence (CAB96389)
3   Oryctolagus cuniculus, partial sequence (Olson et al. 1997)
4   Homo sapiens (CAA25926)
5   Mus musculus (AAB29697)
6   Rattus norwegicus (NP_036714)
7   Mesocricetus auratus (BAA21723)
8   Canis familiaris (P19006)
9   Bos taurus, partial (CAC00531)
10  * = 100% identity, . = 100% homology
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Figure 3. Haptoglobin protein sequence derived from translation of the cDNA (Figure 2, start codon to stop codon). Sequence has been compared with known sequences in human, mouse, rat, golden hamster, dog and bovine, as well as with the published partial sequences for the rabbit. Identical amino acids over all species are marked by an asterisk, and amino acids with functional homology by a dot. Differences in amino acids from a published partial sequence of rabbit haptoglobin are highlighted in grey. Argenine (R) in position 102 is the spacer between α- and β-subunit.

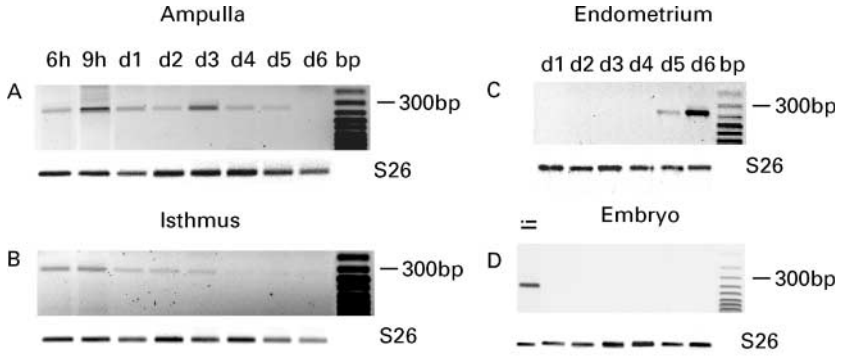

Figure 4. Detection of haptoglobin mRNA expression in pregnant rabbits from 6 h post-mating/hCG to day 6 p.c. in the ampulla (**A**) and isthmus (**B**). For the endometrium (**C**) and embryos (**D**), day 1 to day 6 of early pregnancy were investigated. Liver (D, lane 1) served as positive control. A 100 bp ladder is shown in the far right lane. Equivalent S26 ribosomal housekeeping gene expression is shown underneath each lane. Products are visualized as an inverse picture to improve image clarity.

mature protein sequence and the non-coding 3′ end including the polyadenylation signal and the poly(A) tail. The translation starts at position 32–34 (ATG), and stops at position 1073–1075 (TAG). The protein coding sequence therefore consists of 1041 bp.

Translating the nucleotide sequence into protein sequence results in a 347 amino acid protein (Figure 3). Using the computer program SignalP (www.cbs.dtu.dk), a cut-off for the signal peptide was predicted between amino acid position 18 and 19 (LFA-ADF; $P = 0.953$). Thus, a signal peptide of 18 amino acids results. The α-subunit reaches to the arginine at position 102 (83 amino acids), followed by the β-subunit (245 amino acids).

The rabbit protein sequence shows 84% cDNA identity and 91% amino acid homology to the human sequence (NCBI accession number CAA25926). The cDNA identity and amino acid homology between the rabbit and other species is lower (mouse, AAB29697: 79% identity/88% homology; rat, NP_036714: 76%/86%; golden hamster, BAA21723: 81%/89%; dog, P19006: 78%/88% (Figure 3). The rabbit sequence presented here differs in four amino acids from the previously published sequences (Figure 3): at positions 80 + 81 (Asp-Gln versus Val-Lys), 214 (Thr versus Arg) and 305 (Thr versus Ala) of the mature protein. Comparing the sequences of all species results in an overall identity of 45.8% and functional homology of 60.5%.

Haptoglobin in maternal and embryonic tissues

Haptoglobin mRNA expression has been investigated in ampulla and isthmus from 6 h after mating to day 6 of pregnancy, in the endometrium as well as in preimplantation embryos from day 1 to day 6. Haptoglobin mRNA expression could be detected in the oviduct and in the endometrium (380 bp RT–PCR product; Figure 4). In the oviduct, haptoglobin mRNA expression was detectable at each day during embryo passage (day 0 to day 3). Already 6 h after mating (the first point of time investigated, ~3 h before ovulation) haptoglobin mRNA is expressed in both segments of the oviduct. From day 1 to day 3 post-conception (p.c.), when embryos are passing through the oviduct, haptoglobin mRNA expression was detectable; however, expression was slightly higher in the ampulla than in the isthmus (Figure 4A versus B). By day 4 p.c. haptoglobin mRNA expression decreased to undetectable levels. The haptoglobin mRNA expression pattern in the endometrium differs from the oviduct (Figure 4C). First expression was detected by day 4 p.c. within ~30% of all investigated endometria. By day 5 p.c. haptoglobin mRNA expression was detected in all endometrial samples, and expression reached its maximum by day 6 p.c. (Figure 4C, last day investigated).

In preimplantation embryos no haptoglobin mRNA expression was detectable in any stage investigated (see Figure 4D). Liver from two does served as positive control (day 6 p.c., Figure 4D).

Finally, the presence of haptoglobin protein in the different compartments of the mother and embryo was investigated by western blot analysis. Serum from day 6 pregnant does served as positive control, revealing a very intense staining at 42 kDa (Figure 5F, lane 4). Preabsorbtion of the antibody with haptoglobin decreased the signal significantly, proving the specificity of the antibody (Figure 5F, lane 5). In the tissue of both oviductal segments haptoglobin was detectable (Figure 5A and B). In the tissue of the ampulla 6 h after mating (i.e. 3 h before ovulation) haptoglobin was detectable, persisting up to day 2 p.c. and decreasing thereafter. In the isthmus, the highest haptoglobin tissue levels were reached at the time of ovulation (9 h) and declined to barely detectable signals on day 2 p.c, reflecting the results detected by RT–PCR. In the oviductal fluid, which surrounds the embryo from day 1 to day 3 p.c., haptoglobin was detected in high amounts from 6 h after mating up to day 2 p.c. (Figure 5C). At day 3 p.c., when the rabbit embryos are passing from the oviduct to the uterus, the haptoglobin content of the oviductal fluid clearly decreased, and stayed low until day 5 p.c.

In endometrial tissue samples (Figure 5D) haptoglobin was already detectable from day 1 to day 3 p.c., whereas on day 4 p.c. only very low haptoglobin levels were found. On day 5 p.c. haptoglobin level increased again, reaching maximum levels on day 6 p.c. In parallel with the increased haptoglobin content in endometrial tissue, substantial levels of haptoglobin were detected in uterine secretions on day 5 p.c. (Figure 5E). In the following 24 h the haptoglobin content

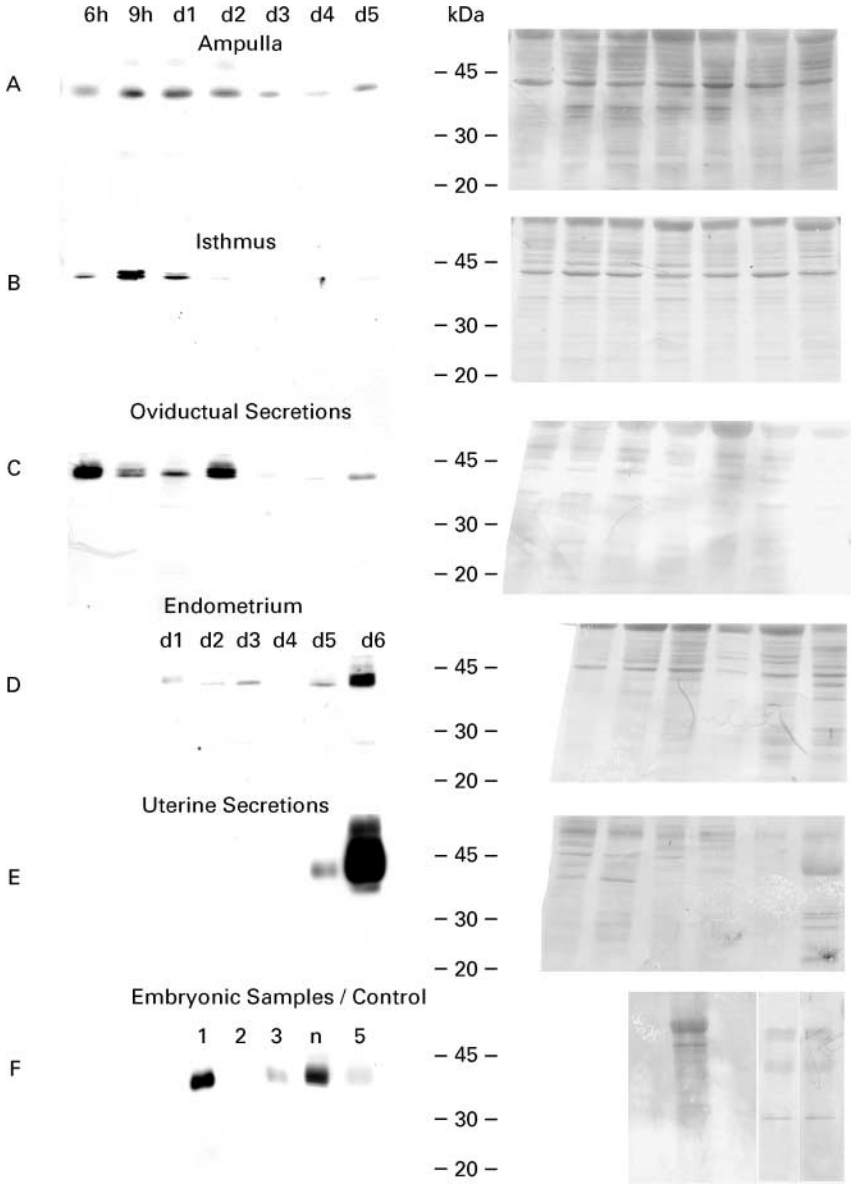

Figure 5. Detection of haptoglobin by western blot analysis. Ampulla (**A**) and isthmus (**B**) were investigated from 6 h p.c to day 5 of pregnancy. The equivalent oviductal secretions are presented in (**C**). Endometrial tissue (**D**) has been investigated from day 1 to day 6 of pregnant rabbits; the equivalent uterine secretions are presented in (**E**). Haptoglobin in rabbit extra-embryonic matrices (1), embryonic tissue (2) and blastocyst fluid (3) of day 6 p.c. are shown in (**F**). A rabbit serum sample served as positive control (F4). For negative control the antibody was preabsorbed with haptoglobin protein (F5). Right panel: loading control, Coomassie-stained blotting membranes used for haptoglobin western blots.

increased remarkably, reaching levels higher than the blood serum level.

Rabbit embryos (day 6 p.c.) were investigated (Figure 5F) for the presence of haptoglobin. Although embryos do not express haptoglobin mRNA, the protein was detected in significant levels in extra-embryonic matrices (Figure 5F, lane 1). In the embryonic tissue almost no haptoglobin was found, but detectable levels were present in the blastocyst fluid (Figure 5F, lane 3).

Discussion

The haptoglobin cDNA sequence of 1243 bp presented here includes the full length of the protein coding region, as well as the 5′ end plus signal peptide and the 3′ end including polyadenylation site. Computational analysis predicted an 18 amino acids signal peptide, similar to the known length of the signal peptide of human haptoglobin. Comparing the amino acid sequence of rabbit haptoglobin with those of other species resulted in an cDNA identities between 76% and 84%, in accordance with the data of Yang *et al.* (1993) and Berkova *et al.* (1997). The highest amino acid homology was found between human and rabbit haptoglobin (84.1%), confirming the results of Olson *et al.* (1997), who previously sequenced parts of the haptoglobin β-subunit.

The major goal of our study was to investigate whether haptoglobin is part of the preimplantation maternal–embryonic

signalling. Therefore, all compartments that are potentially involved in this maternal–embryonic dialogue, i.e. oviductal and endometrial tissues and secretions as well as the embryo and its extra-embryonic matrices, were investigated at each day of the preimplantation time. Haptoglobin mRNA expression is detectable in maternal tissue only. Embryos 1–6 days old never expressed haptoglobin, in agreement with Thomas *et al.* (1990), who found that haptoglobin is expressed in the fetal rat liver not earlier than day 18 of pregnancy.

In the mother, haptoglobin mRNA is expressed by the oviduct already 3 h before ovulation. Ovulation in rabbits occurs 9.5 h following mating/HCG injection (Knobil and Neill, 1988). Haptoglobin mRNA was detected in the ampullar and isthmic part of the oviduct as early as 6 h after mating/HCG (the first point of time investigated), with the highest expression at 9 h p.c. (time of ovulation). At the same time, the highest amount of haptoglobin protein is found in ampullar and isthmic tissue, as well as in the oviductal secretions, and thus is available to both, spermatozoa and oocytes. This demonstrates that haptoglobin is present in the oviductal fluid at a time when spermatozoa undergo capacitation and the fertilization of oocytes takes place. Consequently, it can be hypothesized that haptoglobin might play a role in this fundamental reproductive process. Recently, Lavery *et al.* (2003) published the detection of haptoglobin in bovine oviducts. By immunohistological investigation they detected a more intensive staining in the ampulla relative to the isthmus. This corresponds to the higher expression of haptoglobin in the ampulla of rabbits, as shown in our study. Furthermore, we could demonstrate that haptoglobin is synthesized by the oviduct. Therefore, at least some of the haptoglobin detected in oviductual fluids is not transported from the vascular system (as discussed by Lavery *et al.*, 2003), but expressed and secreted by the oviductal mucosa. The immunohistological pictures presented by Lavery *et al.* (2003) support this by showing apical release of haptoglobin by oviductual luminal epithelial cells. Additionally, the significant amount of haptoglobin in oviductal secretions already 3 h before ovulation (Figure 5C, 6 h) excludes an origin of follicular fluid at this time. Therefore, we conclude that the oviduct expresses and secretes haptoglobin for the entire time that the gametes and embryos are present, which may intermingle with haptoglobin originating from the blood.

Furthermore, the extra-embryonic matrix of preimpantation embryos as well as the blastocyst fluid contain haptoglobin, although the embryo by itself does not express haptoglobin. This implies an uptake of maternal haptoglobin into the extra-embryonic matrices during the passage of the embryo through the oviduct and/or the uterus. On day 2 p.c., when the so-called mucoprotein layer emerges, a second layer of the extra-embryonic matrix, originating from oviductal mucosa fluid, is formed. At the same time, high amounts of haptoglobin are detectable in the oviductal secretions. The mucoprotein layer is built up by glycoproteins (Denker, 1970; Gandolfi, 1995), networked by lectins that are secreted especially in the isthmic part of the oviduct (Biermann *et al.*, 1997). One of these glycoproteins may be haptoglobin, which is glycosylated at several positions and was originally characterized by lectin-binding activity (Thie *et al.*, 1984; Anderson *et al.*, 1986). It might be recruited during the process of mucoprotein layer formation on day 2 and day 3 p.c. This would explain the high amounts of maternal haptoglobin within the extra-embryonic matrices of the embryo.

Within the endometrium the first haptoglobin mRNA expression was found by day 4 p.c in 30% of samples, in accordance with the findings of Hoffman *et al.* (1996). By western blot analysis, already between day 1 and day 3 p.c. a small volume of haptoglobin was detectable in endometrial samples, although at this stage no haptoglobin expression was detectable by RT–PCR in that tissue. This may be explained by high endometrial proliferation during this time, which is accompanied by high vascularization. Therefore, these endometrial samples contain more blood and consequently more haptoglobin. By day 4, the endometrium starts to synthesize haptoglobin itself. The secreted protein is detectable on day 5 and day 6 within the uterine fluid. As in the oviduct, there is a high temporal correlation between the further transformation of the extra-embryonic matrices and haptoglobin expression in the endometrium. On day 5 and day 6 p.c., two new layers, the neozona and the gloiolemma (Denker and Gerdes, 1979), are formed, at least partly by proteins of the uterine secretions. Furthermore, lectins were mainly found in the endometrium around day 5 p.c., in parallel with the formation of the new extra-embryonic matrix layers (Biermann *et al.*, 1997) and haptoglobin expression. As demonstrated by western blot analysis, extra-embryonic matrices of day 6 p.c. embryos contain considerable amounts of haptoglobin. As the embryo itself does not produce any haptoglobin at any stage of preimplantation, development this has to be of maternal origin. Although the presence of an embryo is not prerequisite for endometrial haptoglobin expression, as shown by Olson *et al.* (1997), embryonic development may be supported by the uptake of maternal haptoglobin.

The release of haptoglobin in the rabbit by the endometrial epithelium as shown by Olson *et al.* (1997), the presence of this protein in oviductal and uterine secretions (Hoffman *et al.*, 1996; this work) and the uptake of maternal haptoglobin by the embryo support a reproductive function during preimplantation time. In the human, haptoglobin is also detectable in uterine secretions (Beier and Beier-Hellwig, 1998), with an increase during peri-implantation time (our unpublished data). Furthermore, in the human endometrium, haptoglobin expression and localization was found in the endometrial stroma (Sharpe-Timms *et al.*, 2000) and decidua (Berkova *et al.*, 2001), suggesting a possible function during peri-implantation time, which may be correlated to the deep interstitial implantation of the embryo in primates. The function of this protein in the early embryo–maternal signalling may be an immunomodulatory one, possibly by inducing early maternal immunotolerance. This biological question is under current investigation in our laboratory.

Whether treatment, especially HCG administration, influenced the amount of haptoglobin expression has to be investigated. In general this treatment protocol is widely used in assisted reproduction to induce pregnancies. However, it is

also known that HCG has a longer half live than LH, resulting in increased ovulation rate and progesterone levels (Romeu *et al.*, 1995). Therefore, haptoglobin levels might be differnt in untreated animals.

In summary, haptoglobin is secreted prior to and at the time of ovulation by the oviducts (hereby probably influencing the gametes and fertilization). Two additional peaks of haptoglobin release have been detected in the reproductive tract, which show a temporal correlation to the transformation of the extra-embryonic matrices: in the oviduct on day 2 p.c. and in the uterus on day 5 p.c. Although the embryo itself does not express haptoglobin, high amounts of maternal haptoglobin are incorporated into the extra-embryonic matrices. Whether haptoglobin is secreted by the reproductive tract also at times following implantation or by the non-pregnant reproductive tract has yet to be investigated.

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