Identification and cloning of two isoforms of human high-temperature requirement factor A3 (HtrA3), characterization of its genomic structure and comparison of its tissue distribution with HtrA1 and HtrA2

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In the present study, we identified an additional member of the human high-temperature requirement factor A (HtrA) protein family, called pregnancy-related serine protease or HtrA3, which was most highly expressed in the heart and placenta. We cloned the full-length sequences of two forms (long and short) of human HtrA3 mRNA, located the gene on chromosome 4p16.1, determined its genomic structure and revealed how the two mRNA variants are produced through alternative splicing. The alternative splicing was also verified by Northern blotting. Four distinct domains were found for the long form HtrA3 protein: (i) an insulin/insulin-like growth factor binding domain, (ii) a Kazal-type S protease-inhibitor domain, (iii) a trypsin protease domain and (iv) a PDZ domain. The short form is identical to the long

form except it lacks the PDZ domain. Comparison of all members of human HtrA proteins, including their isoforms, suggests that both isoforms of HtrA3 represent active serine proteases, that they may have different substrate specificities and that HtrA3 may have similar functions to HtrA1. All three HtrA family members showed very different mRNA-expression patterns in 76 human tissues, indicating a specific function for each. Interestingly, both HtrA1 and HtrA3 are highly expressed in the placenta. Identification of the tissue-specific function of each HtrA family member is clearly of importance.

Key words: placentation, pregnancy, protease, tissue remodelling.

INTRODUCTION

The technique of RNA differential display PCR (DDPCR) was successfully used to identify genes that are uniquely regulated at implantation in the mouse uterus [1]. We cloned the full mRNA sequence of one novel gene (GenBank® accession number AY156509) and found that the deduced protein sequence was structurally similar [56% amino acid (aa) similarity] to mammalian HtrA1 (high-temperature requirement A1) serine protease [2,3]. This newly identified mouse gene, which we have termed pregnancy-related serine protease (PRSP), was not only uniquely regulated at implantation, but was also dramatically increased during placentation in the mouse (G.-Y. Nie, Y. Li and L. A. Salamonsen, unpublished work).

Placental formation and function is essential for development of the mammalian embryo during pregnancy, and insufficient placentation can lead to foetal growth restriction [4]. Even moderately impaired foetal health correlates with a number of disorders of adult life, including hypertension, diabetes, coronary heart disease and obesity [5]. In addition, a number of gestational disorders such as pre-eclampsia, gestational diabetes and pre-term labour are associated with placental abnormalities [6]. Thus understanding the regulation of genes that are critical for the formation and function of human placenta could provide vital leads to the early diagnosis and alleviation of such disorders. To date, however, very few genes have been identified that are both critical for placental development and clinically useful. We predict that the human homologue of mouse PRSP is important in human pregnancy, particularly during placentation.

The present paper describes in detail the molecular cloning and characterization of the human PRSP, its mRNA and its protein products. Given that this is a newly identified member of

the human HtrA protease family, the similarities and differences between all three known human HtrA proteins in relation to their domain structures and tissue distributions were also investigated.

MATERIALS AND METHODS

Tissue collection and RNA isolation

Adult female mice (Swiss outbred) were mated with fertile males of the same strain and placentae were collected on day 10.5 of pregnancy (where the morning of finding a vaginal plug is day 0). Human endometrial tissues were obtained by curettage from women with regular menstrual cycles and no apparent endometrial dysfunction, who gave informed consent for collection of tissues. Approval was given by the Human Ethics Committee at Monash Medical Centre, Melbourne, Australia. All tissues were snap-frozen in liquid nitrogen and total RNA was extracted as previously described [1].

Reverse transcriptase (RT)-PCR and T/A cloning

RT-PCR was carried out as described in [7]. In brief, 1 µg of DNA-free RNA from human endometrium or mouse day 10.5 placenta was reverse-transcribed with random hexanucleotide primers and avian myeloblastosis virus (AMV) RT (Boehringer Mannheim, Nunawading, Vic., Australia). PCR was performed in a total volume of 40 µl with 1–1.5 µl of the RT reaction with the high-fidelity *Taq* polymerase (Boehringer Mannheim). PCR products were analysed on a 1.5% (w/v) agarose gel and bands of interest were excised, purified with Qiaquick gel extraction kit (Qiagen Pty Ltd, Clifton Hill, Vic., Australia), cloned into a

Abbreviations used: aa, amino acid(s); EST, expressed sequence tag; GI, GenBank® identifier; HtrA, high-temperature requirement factor A; IAP, inhibitor of apoptosis; IGF, insulin/insulin-like growth factor; IGFBP, IGF-binding proteins; MTE, multiple tissue expression; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PBL, peripheral blood leucocyte; poly(A⁺), polyadenylated; PRSP, pregnancy-related serine protease; RD, regulatory domain; RT, reverse transcriptase; UTR, untranslated region.

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Table 1 Primers used in the present study

U, upper primer; L, lower primer.

Primer	Sequence (5' to 3')
PRSP-U	GCG GTT CTG GCT TCA TCA TGT
PRSP-L	GCC GTG ACC TTG AGC GTG T
HtrA1-U	GTG GGT CTG GGT TTA TTG TG
HtrA1-L	GGC GTC GGT CTG GAT G
HtrA2-U	GCC GTG GTC TAT ATC GAG ATC CT
HtrA2-L	TGC CGG ATG TGA TCG TGT
mPRSP-U	GCG GTT CCG GCC TCG GTA T
mPRSP-L	AGC TTT TTC TTG GGG TGG ATT

pGEM-T easy vector (Promega, Madison, WI, U.S.A.) and sequenced.

All primers used for RT-PCR cloning are listed in Table 1. For mouse PRSP, a cDNA fragment of 785 bp (mPRSP-785 bp) containing some of the 5' untranslated region (UTR) and a large part of the open reading frame (ORF) (nt 76–860) of the mouse PRSP mRNA (accession number AY156509) was amplified with the primer pair mPRSP-U and mPRSP-L from mouse placenta. Human HtrA cDNAs were all cloned from human endometrium. A cDNA of 457 bp representing the common region of the newly cloned human PRSP mRNAs (nt 713–1169 of sequence L, see Figure 2) was amplified with the primer pair PRSP-U and PRSP-L. A 356-bp cDNA of HtrA1 containing nt 656–1011 of human HtrA1 mRNA (accession number D87258) with the primer pair HtrA1-U and HtrA1-L, and a 343-bp cDNA of HtrA2 containing nt 495–839 of human HtrA2 mRNA (accession number AF020760) with the primer pair HtrA2-U and HtrA2-L were also amplified. The identities of each amplicon were confirmed by sequencing.

Screening of a human multiple tissue expression (MTE) array

A nylon membrane containing normalized loadings of polyadenylated [poly(A)⁺] RNA from 76 different human tissues (Clontech, Palo Alto, CA, U.S.A.) was hybridized with ³²P-labelled cDNA and washed according to the manufacturer's instructions. It was subsequently stripped (according to the manufacturer's instructions) and used for multiple hybridizations. A mouse PRSP probe of 785 bp (mPRSP-785 bp) was initially used for the screening. After cloning the human HtrA3, the same membrane was hybridized with a human probe of 457 bp. A 356-bp cDNA of human HtrA1 and a 343-bp cDNA of human HtrA2 (Table 1) were used for detection of HtrA1 and HtrA2 respectively. The expression patterns were analysed by densitometric scanning using a Bio-Rad GS-710 Calibrated Imaging Densitometer (Regents Park, NSW, Australia) and Bio-Rad Quantity One software (version 4.1.0).

cDNA Library Screening

A λ cDNA library of human heart cloned uni-directionally into the Uni-ZAP XR vector (Stratagene, La Jolla, CA, U.S.A.) was screened as previously described in [8] using the ³²P-labelled mouse PRSP cDNA probe (mPRSP-785 bp). After tertiary screening, the pBluescript phagemid containing the cloned insert was excised from the positive phages *in vivo* with the ExAssist helper phage of SOLR strain (supplied with the library) according to the manufacturer's instructions. The sequence was determined by an automated sequencer using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit.

Northern blot analysis

Northern blot analysis was performed using a 12-lane MTN Northern blot (Clontech) containing poly(A)⁺ RNA (1 μ g/lane) from 12 human tissues [brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and peripheral blood leucocytes (PBLs)] according to the manufacturer's instructions. Briefly, the membrane was probed at 42 °C overnight with ³²P-labelled cDNA probes generated by random-primer labelling of 25 ng of cDNA with [³²P]dCTP (50 μ Ci/reaction), and washed as described in [7].

Gene analysis by bioinformatics

To search the structure of the human gene and its chromosomal location, the mRNA sequence(s) was compared with the human genome sequence available in the National Center for Biotechnology Information (NCBI) databases using the BLAST program on the server. The gene whose sequence showed perfect alignment with the mRNA(s) was analysed further, and a possible intron–exon structure was then determined using the alignment result.

RESULTS

Tissue distribution of PRSP mRNA in humans

In order to clone the human counterpart of mouse PRSP, it was essential to identify an appropriate tissue source from which to obtain RNA or cDNA libraries. It was also important to establish the organ distribution of PRSP mRNA to assist in the prediction of its function. An MTE array containing RNA from 76 different human tissues was screened (Figure 1 and Figure 7) using a mouse PRSP cDNA fragment of 785 bp (mPRSP-785 bp) as a probe. PRSP is widely expressed in the human body with variable levels of expression in different organs. In general, the highest level of expression was detected in heart tissues, including foetal heart, as well as some reproductive organs, including the ovary, uterus and placenta, although the expression was much lower in the prostate and testis. A high level of expression was also observed in the bladder. Moderate expression was detected in the gastrointestinal tract (highest in the ileum), lung, foetal lung, mammary gland and thyroid gland. Lower expression was seen in the kidney, skeletal muscle, bone marrow, lymph node, trachea and thymus. All brain-related tissues displayed very low levels of expression. No expression was detectable in any of the eight cell lines examined, including HeLa S3 and lung carcinoma A549 (Figure 1 and Figure 7). This result is in accord with the expression pattern of mouse PRSP where the high levels of expression were detected in the heart, placenta and ovary (results not shown). On the basis of these results, a human heart cDNA library was chosen for cloning the human PRSP mRNA.

Cloning the human PRSP mRNAs

A human heart cDNA library was screened with the mouse probe previously used for the MTE array. More than ten positive clones were identified and three were subcloned and fully sequenced. Two of these were identical and contained 2543 nt (sequence L, Figure 2), the other was much shorter and contained 1953 nt (sequence S, Figure 2). Sequences L and S are both 86% identical with mouse probe mPRSP-785, and 80% and 87% identical with the full-length mouse PRSP mRNA respectively, confirming that both human sequences are the true counterpart of mouse PRSP mRNA. The initial 1158 nt of sequence S are identical to nt 86–1243 of sequence L, the remaining nt are

Figure 1 mRNA tissue distribution of human PRSP

An MTE array containing normalized loadings of poly(A⁺) RNA from 76 different human tissues was screened with a 785 bp cDNA of mouse (mPRSP-785) covering part of the 5' UTR and most of the coding region of mouse PRSP. (A) The expression pattern of human PRSP. (B) The tissue layout of (A).

completely different (Figure 2). Sequences L and S each contained a clear ORF, with a perfect Kozak consensus site (GCCGCC-ATGC, with the start codon underlined) for protein initiation. The ORF of sequence L is 1362 nt long with the start codon ATG at nt 193–195 and stop codon TGA at nt 1552–1554; outside the ORF is a 5' UTR G+C-rich (82% G+C) region of 192 nt and a long 3' UTR region of 989 nt (Figure 2). The ORF of sequence S contains 1074 nt with the start codon ATG at nt 108–110 and stop codon TGA at nt 1179–1181; outside the ORF is a 5' UTR G+C-rich (85% G+C) region of 107 nt and a 3' UTR region of 772 nt (Figure 2). Sequences L and S each contained a distinct polyadenylation signal sequence AATAAA starting at nt 2498 and nt 1918 in the L and S sequences respectively, indicating that both represent full-length mRNA sequences. Sequences L and S are consistent with two alternatively spliced mRNA variants of human PRSP with the splicing site close to the 3' end. Sequence L is referred to as the long form and sequence S as the short form mRNA of human PRSP.

The ORF of sequence L translates into an aa sequence of 453 residues (sequence pL, Figure 2), with a predicted molecular mass of 49 kDa and an isoelectric point of 7.04. The ORF of

sequence S translates into an aa sequence of 357 residues (sequence pS, Figure 2), with a predicted molecular mass of 38 kDa and an isoelectric point of 7.85. These two proteins are nearly identical, except that pS is shorter and the last seven C-terminal residues of pS (APSLAVH) are completely different from the corresponding residues in pL (DWKKRFI) (Figure 2). It is apparent that these proteins are long and short isoforms of PRSP. The identical N-terminal ends of both proteins contain a predicted signal peptide (aa 1–17). A search of the NCBI protein structure database showed that the long-form protein contains aa sequences consistent with four other domains (Figure 3): (i) an insulin/insulin-like growth factor (IGF)-binding domain (aa 29–94), (ii) a Kazal-type S protease-inhibitor domain (aa 89–126), (iii) a trypsin protease domain (aa 176–341) and (iv) a PDZ domain (aa 384–440). The short-form protein contains only the first three domains found in the long form and lacks the PDZ domain (Figure 3).

A comparison of the sequences L and S, and pL and pS (Figure 2) with all entries in the GenBank® and SwissProt databases (January 2000) identified a number of expressed sequence tags (ESTs) that were highly homologous. It is of interest that these ESTs were from foetal heart (accession number AA055124, 281 bp), mature heart (accession number C03429, 200 bp), ovarian tumour (accession numbers AA479844, 490 bp and AA411195, 580 bp) and total foetus (accession numbers AA447906, 482 bp, AA441966, 379 bp and AA496125, 518 bp), and that, in all these tissues, high levels of PRSP expression were detected on the MTE array. However, longer, highly homologous mRNA sequences were not found. Candidates with the greatest homology were the mammalian HtrA1 proteins. At the cDNA level, there is 67% identity of the L sequence and 71% identity of the S sequence with the human HtrA1 cDNA sequences (accession numbers D87258, Y07921 and AF097706). At the protein level, there is 61% (pL) and 66% (pS) identity with human HtrA1 protein (accession numbers BAA13322, CAA69226 and AAC97211). The HtrA1 proteins are homologous with the HtrA from bacteria [2,3]. These HtrA proteins belong to a family of serine proteases that possess the aa sequence motif of GNSGGAL (in bacteria) or GNSGGPL (in mammals) in their active sites. In addition, they display a motif of TNAHV residues in the vicinity of GNSGGPL and the triad of histidine, aspartic acid and serine for their catalytic activity [2,3]. All of these motifs are present in both protein pL and pS (Figure 2). The serine protease active site sequence GNSGGPL is at aa 303–309, the TNAHV residues are at aa 188–193, and the catalytic triad histidine, aspartic acid and serine are at aa 191, 229 and 305 respectively. This confirmed that, as for its mouse counterpart, the human PRSP represents an additional member of the human HtrA serine protease family.

During the course of this work, nucleotide and protein sequence databases (mainly GenBank® and SwissProt) were periodically searched. In July 2001, an mRNA sequence of 2551 nt [accession number AY040094, version 1, GenBank® identifier (GI) 14700055] appeared in the database, though the tissue source of cloning this mRNA was not stated. It was predicted to encode a protein of 452 aa (accession number AAK71475, version 1, GI 14700056) called human HtrA3. This mRNA sequence was very similar to our newly identified human PRSP mRNA (99.6% identity). However, the predicted protein from this sequence was not identical with PRSP (only 91.5% identity) as it contains 452 residues whereas PRSP contains 453 residues and shows 42 residues (in the region of aa 252–300) completely different to PRSP (results not shown). However, during the preparation of this manuscript, the above sequence was updated (June 2002) to version 2 (accession number

L	1	GTGCGCTCCCTGCGCCCTGGGGATGCCCTGCCGCCCTGACGCCGCCAGCCTGAGCCACCGGCGCATGTGACCGCGCGT	80
L	81	CCGCCCCAGTCCCATCCGTAGGCGCCCGCGCCCGCCGCGAGCGGCTCGTTGTCCCCGCGGCCCCCGCGGCTCTC	160
S	1	-----	75
L	161	CCGCGCTGCCACCCGCGCCGCCCTGCCGCCATGCAGGCGGAGCGCTGCTCCTGGCGCGTTGGCCGCGCTGGCGCTG	240
S	76	-----	155
pL	1	M Q A R A L L L A A L A A L A L	16
pS	1	- - - - -	16
L	241	GCCCCGGAGCCCCCTGCGGCGCGCTGTCCCGCGCGCTGCGACGTGTCGCGGTGTCCAGCCCCCGCTGCCCGGCGGCTA	320
S	156	-----	235
pL	17	A R E P P A A P C P A R C D V S R C P S P R C P G G Y	43
pS	17	- - - - -	43
L	321	CGTGCCCGACCTCTGCAACTGCTGCCTGGTGTGCGCGCCAGCGAGGGCGAGCCCTGTGGCGGCCCTCTGGACTCGCCTT	400
S	236	-----	315
pL	44	V P D L C N C C L V C A A S E G E P C G G P L D S P C	70
pS	44	- - - - -	70
L	401	GCGGCGAGAGCTGGAGTGCCTGCGCGCCCTATGCCGCTGCCGCTGGTGCACGCCGTGTGTGGCACCAGCGGCACACC	480
S	316	-----	395
pL	71	G E S L E C V R G L C R C R W S H A V C G T D G H T	96
pS	71	- - - - -	96
L	481	TATGCCAACGTGTGCGCGCTGCAGGCGGCCAGCCCGCGCGCTGCAGCTCTCCGGGAGCCCCGTGCCCGAGCTGCAGAA	560
S	396	-----	476
pL	97	Y A N V C A L Q A A S R R A L Q L S G T P V R Q L Q K	123
pS	97	- - - - -	123
L	561	GGGCGCTGCCCGTTGGGTCTCCACCAGCTGAGCAGCCCGCGCTACAAGTTCAACTTCATTGCTGACGTGGTGGAGAAGA	640
S	477	-----	555
pL	124	G A C P L G L H Q L S S P R Y K F N F I A D V V E K I	150
pS	124	- - - - -	150
L	641	TCGCACCAGCCGTGGTCCACATAGAGCTCTTCTGAGACACCCGCTGTTTGGCCGCAACGTGCCCTGTCCAGCGTTCT	720
S	556	-----	635
pL	151	A P A V V H I E L F L R H P L F G R N V P L S S G S	176
pS	151	- - - - -	176
L	721	GGCTTCATCATGTGCAGAGCCGCGCTGATCATCAACATGCCACGTGGTGTCCAGCAACAGTGTGCCCGGGCAGGCA	800
S	636	-----	715
pL	177	G F I M S E A G L I I T N A H V V S S N S A A P G R Q	203
pS	177	- - - - -	203
L	801	GCAGCTCAAGGTGCAGCTACAGAATGGGACTCCTATGAGGCCACCATCAAAGACATCGACAAGAAGTCGGACATTGCCA	880
S	716	-----	795
pL	204	Q L K V Q L Q N G D S Y E A T I K D I D K K S D I A T	230
pS	204	- - - - -	230
L	881	CCATCAAGATCCATCCCAAGAAAAAGCTCCCTGTGTTGTGCTGGGTCACTCGGCCGACCTGCGGCCTGGGGAGTTTGTG	960
S	796	-----	875
pL	231	I K I H P K K K L P V L L L G H S A D L R P G E F V	256
pS	231	- - - - -	256
L	961	GTGGCCATCGGCAGTCCCTTCGCCCTACAGAACACAGTGACAACGGGCATCGTCAGCACTGCCAGCGGGAGGGCAGGGA	1040
S	876	-----	955
pL	257	V A I G S P F A L Q N T V T T G I V S T A Q R E G R E	283
pS	257	- - - - -	283
L	1041	GCTGGGCCTCCGGGACTCCGACATGGACTACATCCAGACGGATGCCATCATCAACTACGGGAACCTCCGGGGGACCACTGG	1120
S	956	-----	1035
pL	284	L G L R D S D M D Y I Q T D A I I N Y G N S G G P L V	310
pS	284	- - - - -	310
L	1121	TGAACCTGGATGGCGAGGTCAATTGGCATCAACACGCTCAAGGTCACGGCTGGCATCTCCTTTGCCATCCCCTCAGACCGC	1200
S	1036	-----	1115
pL	311	N L D G E V I G I N T L K V T A G I S F A I P S D R	336
pS	311	- - - - -	336
L	1201	ATCACACGGTTCTTCACAGAGTTCCAAGACAAGCAGATCAAAGACTGGAAGAAGCGCTTCATCGGCATACGGATGCGGAC	1280
S	1116	-----	1195
pL	337	I T R F L T E F Q D K Q I K D W K K R F I G I R M R T	363
pS	337	- - - - - A P S L A V H *	357

Figure 2 For legend, see facing page

L 1281	GATCACACCAAGCCTGGTGGATGAGCTGAAGGCCAGCAACCCGACTTCCCAGAGGTCAGCAGTGAATTTATGTGCAAG	1360
S 1196	CTCACGTTTCCCCCTCCTCCATGACCCCGTCAGCCAAGCACATGGACCCAGTCAGGCCAAGGCTGGTGCCATGAGGGCT	1275
pL 364	I T P S L V D E L K A S N P D F P E V S S G I Y V Q E	390
L 1361	AGGTTGCGCCGAATTCACCTTCTCAGAGAGGCGGCATCCAAGATGGTGACATCATCGTCAAGGTCAACGGGCGTCTCTA	1440
S 1276	GGTCACATGAAGAGCTGCTGTTGAGGATGCCGCCATTGTTCTTCTGTGTCCATTATGGGAAGACAATCTGGAGCCAGGCA	1355
pL 391	V A P N S P S Q R G G I Q D G D I I V K V N G R P L	416
L 1441	GTGGACTCGAGTGAGCTGCAGGAGGCCGTGCTGACCGAGTCTCTCTCTACTGGAGGTGCGGCGGGGGAACGACGACCT	1520
S 1356	GAGCCTGTCTTTCCCAAAGAAGCTGAAGTCTTCTTCTCTTGAACAGTGGGGACCATCTAATCTCTTGAGCCCTTTTCCTG	1435
pL 417	V D S S E L Q E A V L T E S P L L L E V R R G N D D L	443
L 1521	CCTCTTCAGCATCGCACCTGAGGTGGTCATG TGA GGGGCGCATTCTCCAGCGCCAAGCGTCAGAGCCTGCAGACAACGG	1600
S 1436	TTGGCTTCTAGGAAGCTCAGAGCTAGATTACAGGGGTGCACCCAGACCTGTCTAGCATGCTCCTTTCCCTAATGACCGAG	1515
pL 444	L F S I A P E V V M *	453
L 1601	AGGGCAGCGCCCCCGAGATCAGGACGAAGGACCACCGTCGGTCTCTCAGCAGGGCGGCAGCCTCTCTGGCTGTCCGG	1680
S 1516	TCTTTCTGTGAATTATCCATTCTCCATGGGTGCCTTTGACTTTGGCCTCCTTACTGGAAATTAGCGGAGCTGCTGTT	1595
L 1681	GGCAGAGCGGAGGCTGGGCTTGCCAGGGGCCGAATTTCCGCCTGGGGAGTGTGGATCCACATCCCGGTGCCGGGGAG	1760
S 1596	TGCACACACTGAGCTGTGAGGTGGCTTTCCTTGGAAGTGATGATAGTGTCTCTTCCCTCTTGCCTCTCTCTTTCTCC	1675
L 1761	GGAAGCCCAACATCCCTTGTACAGATGATCTGAAAGTCACTTCCAAGTTCTCCGATATTACAAAACTGCCTTCCAT	1840
S 1676	TGAGACAGGATCCCCCTGGGGCTAGGTTTGTCTCTTGTGTACAGGGGTGTCCAGTTAGTGCCTGACCTACCCAG	1755
L 1841	GGAGGTCCCCTCTCTCTAGCTTCCCGCCTCTGCCCTGTGAACACCCATCTGCAGTATCCCCTGCTCCTGCCCTCCT	1920
S 1756	AACCCCTGGGAAATATCCCTGTCTCAGAGCTGTGTCCCTCCCCAAGGACAGTGCAGACTAAGTGGGAGCCTGATA	1835
L 1921	ACTGCAGGTCTGGGCTGCCAAGCTTCTTCCCCCTGACAAACGCCCACCTGACCTGAGGCCCCAGCTTCCCTCTGCCCTA	2000
S 1836	AACCTTAGCTGCATGGCACACTTGCAATTTTAAATCCTTCTGAAGTTGACTGGTGTTTGTACTTGCTTCTCTTTTAT	1915
L 2001	GGACTTACCAAGCTGTAGGGCCAGGGCTGCTGCCTGCCAGCCTGGGGTCCCTGGAGGACAGGTACATCTGATCCCTTTG	2080
S 1916	TT <u>AATAAA</u> ATCCAATGATCCAAAAA	1953
L 2081	GGGTGCGGGGTGGGGTCCAGCCAGAGCAGGCACTGAGTGAATGCCCCCTGGCTGCGGAGCTGAGCCCCGCCCTGCCAT	2160
L 2161	GAGGTTTTCCTCCCAGGCAGGAGGCGCGGGGAGCACGTGGAAGTTGGCTGCTGCCTGGGGAAGCTTCTCTCC	2240
L 2241	CCAAGCGGCCATGGGGCAGCTGCAGAGGACAGTGGACGTGGAGCTGCGGGGTGTGAGGACTGAGCGGCTTCCCCTTC	2320
L 2321	CCACGCAGCTCTGGGATGCAGCAGCCGCTCGCATGGAAGTGCCGCCAGAGGCATGCAGGCTGCTGGGACCACCCCTC	2400
L 2401	ATCCAGGGAACGAGTGTGTCTCAAGGGGCATTTGTGAGCTTTGTGTAAATGGATTCCAGTGTTGCTTGTACTGTATGT	2480
L 2481	TTCTCTACTGTATGGAA <u>AATAAA</u> AGTTTACAAGCACAGGTTCTCAAAAAA	2543

Figure 2 Full-length mRNA sequences (L and S) and their deduced aa sequences (pL and pS) of human PRSP

Sequences L and pL represent the long-form mRNA and protein respectively. Sequences S and pS are for the short-form mRNA and protein respectively. In sequence S, nt that are identical to those in sequence L are denoted with hyphens (-), only those nt unique to sequence S are indicated. Similarly, in sequence pS, aa that are identical to those of sequence pL are also denoted with hyphens (-), only those aa unique to sequence pS are specified. The Kozak consensus site (GCCGCCATGC) for protein initiation is boxed and the translation start codon (ATG) is in bold and underlined. The stop codon (TGA) is in bold and boxed, and the polyadenylation signal sequence (AATAAA) is in bold and underlined. The active serine protease site GNSGGPL and the additional TNAHV residues are underlined. The catalytic triad aa His¹⁹¹, Asp²²⁷ and Ser³⁰⁵ are boxed. The stop codon is indicated with an asterisk (*).

Figure 3 Schematic representation of the protein domains of pL and pS

SP, signal peptide; IB, IGF-binding domain; Kazal, Kazal-type S protease inhibitor domain; trypsin, trypsin protease domain; PDZ, PDZ domain.

AY040094.2, GI 21326482), with a predicted protein (accession number AAK71475.2, GI 21326483) 100 % identical with human PRSP. Detailed cloning and characterization of these mRNA and protein sequences has not been reported. Also during the

preparation of this manuscript, two additional human sequences, one (2585 nt, accession number BC034390) cloned from a pool of adult testis, lung and brain, and the other (2541 nt, accession number XM_114416) predicted by automated computational analysis (NCBI annotation project), appeared in GenBank® (August 2002). These sequences are 98 % identical with the long-form human PRSP mRNA and their predicted proteins (also 453 aa, accession numbers AAH34390.1 and XP_114416.1) are 100 % identical with the long-form PRSP protein. Both these proteins were named HtrA3, but one is only a predicted sequence, and detailed identification, molecular cloning or characterization of the other is not reported. Given that PRSP is indeed an additional member of the mammalian HtrA protease, we will refer to our newly cloned PRSP as HtrA3 to maintain consistency within the literature. To date, no GenBank® database entries similar to the short form PRSP mRNA or protein have appeared in the databases. Therefore, the alternative splicing of HtrA3 described in this study is novel.

Figure 4 Northern blot analysis of human HtrA3 mRNA expression

A Northern blot membrane containing poly(A⁺) RNA (1 µg/lane) from human PBLs, lung, placenta, small (S) intestine, liver, kidney, spleen, thymus, colon, skeletal (Skel) muscle, heart and whole brain was hybridized with a 457 bp cDNA of human HtrA3 (nt 713–1169 of sequence L representing both forms of HtrA3) as a probe. The 2.8 kb and 2.6 kb bands, representing the long- and short-form transcripts respectively, are indicated.

Northern blot analysis of HtrA3 transcript variants in the human

Following cloning of the full-length mRNA sequences of human HtrA3, a cDNA probe representing both forms was applied to the MTE array and a similar pattern to that shown in Figure 1 was obtained (results not shown). To confirm the alternative splicing, Northern blot analysis was performed on a range of human tissues with the same probe (Figure 4). Two distinct bands of 2.8 and 2.2 kb were observed in the heart, placenta, skeletal muscle and kidney; the 2.8 kb band being predominant in the heart and skeletal muscle, whereas the 2.2 kb band was predominant in the placenta and kidney. Interestingly, only a single faint band at 2.2 kb was detected in the brain, and a single band of 2.8 kb in lung and small intestine. Very faint bands were detected in colon, thymus and spleen, and no bands were detected in PBLs. These findings confirmed that human HtrA3 is alternatively spliced and that both forms are present in a range of human tissues. The relative expression of HtrA3 mRNAs (both forms together) detected by Northern blot analysis is consistent with the previous MTE array data (Figure 1).

Genomic characterization of human HtrA3 gene by bioinformatics

The genomic structure of human HtrA3 was analysed by comparing its mRNA sequences with the human genome sequence available in the NCBI databases. A perfect match was found on contig NT_006307.11 located at p16.1 of chrom-

osome 4. The alignment of mRNA sequences L and S to contig NT_006307.11 is summarized in Table 2. Alignment of sequence L partitioned the long form mRNA into nine distinctive segments (segments L1–L9, Table 2), each being aligned perfectly to a unique region on the gene. Alignment of sequence S partitioned the short mRNA into seven clear segments (segments S1–S7, Table 2), each likewise being aligned perfectly to a unique region on the gene, with segment S7 totally distinct and the rest identical with the equivalent L segments (Table 2). These alignment data clearly indicate that each unique mRNA segment represents an individual exon. Thus, according to the specific location of each mRNA segment and the distance between each segment on the gene, the exon–intron structure of this gene can be predicted as that shown in Figure 5. This gene consists of at least ten exons, exon 10 being the largest and exon 5 being the smallest; exon 1 contains the ATG start codon, and both exon 7 and exon 10 contain the TGA stop codon. This structure also demonstrates the alternative splicing of exons to produce the long and short form mRNAs. The short mRNA (thus the short protein) is produced by utilizing exons 1–7, whereas the long mRNA (thus the long protein) is obtained by utilizing all but exon 7 (Figure 5).

Comparison of HtrA3 protein structure with family member HtrA1 and HtrA2

In order to predict the function of HtrA3, its protein structure was compared with HtrA1 and HtrA2. Although alternative splicing has not been reported for HtrA1, at least four mRNA species of HtrA2 due to alternative splicing have been identified [9], two of which were characterized to encode two protein isoforms (long and short) [10]. At the domain structure level, the long form of HtrA3 is strikingly similar to HtrA1, as both contain a signal peptide, an IGF-binding domain and a Kazal-type S protease inhibitor domain, as well as a trypsin and a PDZ domain (Figure 6A). The short-form HtrA3 is also similar to HtrA1, except that it does not contain the PDZ domain (Figure 6A). HtrA2 is quite distinct, its N-terminus does not contain the signal peptide, the IGF-binding domain or the Kazal-type S protease inhibitor domain that are found in HtrA1 and HtrA3. It has a completely different N-terminal domain [11], called the regulatory domain (RD) (Figure 6A). The two characterized isoforms of HtrA2 protein also differ at the C-terminus, but the difference between them does not resemble that between the HtrA3 isoforms. The short-form HtrA2 contains all domains present in the long form, but the trypsin and the PDZ domain are much shorter. In comparison, the shorter form HtrA3 is identical with the long form except that it lacks the PDZ domain (Figure 6A).

Table 2 Alignment of the L and S mRNA sequences (Figure 2) with the human genomic contig NT_006307.11

Alignment of the L mRNA on the gene				Alignment of the S mRNA on the gene			
Segment	Nucleotides of the L mRNA	Segment length	Nucleotides of the gene	Segment	Nucleotides of the S mRNA	Segment length	Nucleotides of the gene
L1	2–579	578	384957–385534	S1	1–494	494	385041–385534*
L2	577–677	101	397615–397715	S2	492–592	101	Same as L2
L3	676–900	225	401738–401962	S3	591–815	225	Same as L3
L4	899–1095	197	406547–406743	S4	814–1010	197	Same as L4
L5	1096–1129	34	407500–407533	S5	1011–1044	34	Same as L5
L6	1128–1243	116	409265–409380	S6	1043–1158	116	Same as L6
L7	1242–1292	51	425344–425394	S7	1157–1938	782	418576–419357
L8	1291–1388	98	427065–427162				
L9	1387–2525	1139	428852–429990				

* Segment S1 overlaps L1, but is shorter.

Figure 5 Schematic representation of the intron–exon structure of the HtrA3 gene

The gene contains at least ten exons, each sequentially numbered and illustrated by a box on the gene. The sizes of exons and distances between them are illustrated in proportion to their actual sizes. The sequences between exons represent introns. The relative positions of the start codon ATG on exon 1, and the stop codon TGA on exons 7 and 10 are shown. The Figure also illustrates how the long- and short-form mRNAs (and hence the long- and short-form proteins) are produced from the different exons.

Figure 6 Domain structural comparison of human HtrA protein family and alignment of the sequences around the catalytic trypsin residues of HtrA family members

(A) Comparison of the domain structures of all known members of the human HtrA family, including HtrA1, two isoforms of HtrA3 (HtrA3-L and -S) and two isoforms of HtrA2 (HtrA2-L and -S). SP, signal peptide; IB, IGF-binding domain; Kazal, Kazal-type S protease inhibitor domain; trypsin, trypsin protease domain; PDZ, PDZ domain; RD, regulatory domain. For each domain, the percentage of aa identity with HtrA1 is shown. (B) Alignment of aa sequences around the catalytic trypsin region of HtrA1, two isoforms of HtrA2 (HtrA2-L and -S) and two isoforms of HtrA3 (HtrA3-L and -S). The actual residues are shown for HtrA1; for the other sequences, residues identical to HtrA1 are denoted by asterisks (*), only residues unique to each sequence are specified. A dot (.) indicates a gap introduced for alignment. The active serine protease site GNSGGPL and the additional TNAHV histidine residues are boxed. The catalytic triad aa histidine, aspartic acid and serine are in bold and boxed.

Figure 7 Comparison of the expression pattern of three human HtrA family members

The expression patterns of three family members of human HtrA proteins HtrA1, HtrA2 and HtrA3 were analysed by densitometric scanning of the MTE array data shown in Figure 1 and were also screened with a 356 bp cDNA of HtrA1 and a 343 bp cDNA of HtrA2 respectively and compared. For each screening, the greatest level of expression was allocated an arbitrary unit of 100, and the expression level in the rest of the tissues was expressed as a relative percentage. The numbers in the graphs for HtrA2 and HtrA3 relate to the numbered tissues in the graph for HtrA1.

A close comparison of the trypsin domain of all HtrA proteins revealed that it is highly conserved (Figure 6B). All components that are essential for protease activity, especially the active site sequence GNSGGPL, the TNAHV sequence and the catalytic aa triad, are totally conserved among all the members (Figure 6B). However, the short-form HtrA2 is distinct from other family members, as it lacks a stretch of 64 residues between the second and the third triad aa.

Comparison of HtrA3 expression pattern with its family members HtrA1 and HtrA2

Only very limited organ distributions of human HtrA1 and HtrA2 have been reported. The expression patterns of HtrA1 and HtrA2 were determined on the same MTE array that was used for HtrA3, and the expression patterns were compared. To verify the data, the membrane was re-hybridized with the HtrA3 probe at the end of the experiment and the previously detected pattern (Figure 1) was obtained.

Similar to HtrA3, HtrA1 was also widely expressed, but with variable expression levels in different tissues (Figure 7). However, its expression pattern was quite different from that of HtrA3 (Figure 1). HtrA1 expression was much higher in tissues related to the brain, whereas the expression of HtrA3 was relatively low (Figure 1 and Figure 7). All tissues of the heart, except the aorta, displayed a low level of HtrA1 expression, compared with high expression of HtrA3. Placental expression of HtrA1 was much higher than in any other organs examined, whereas HtrA3 expression in placenta was lower than that in the ovary, heart and foetal heart. Apart from the placenta, ovary, aorta and brain-related tissues, HtrA1 expression was low in other organs, whereas HtrA3 was high in a wider range of tissues.

HtrA2 was also widely expressed, but, unlike that of HtrA1 and HtrA3, its relative expression was quite uniform among all tissues examined, the highest level of expression being in the foetal liver (Figure 7), with high expression in all foetal tissues. In adult tissues, relatively higher expression was observed in the kidney, thymus, bone marrow and thyroid gland, whereas the expression in the reproductive tract was highest in the testis and lowest in the placenta.

DISCUSSION

The present study characterized a novel human gene, PRSP or HtrA3, that is strongly expressed in the heart, ovary and placenta. We cloned the full-length mRNA sequences of two alternatively spliced variants, long and short forms of human HtrA3. The genomic structure of HtrA3 explicitly demonstrates how the two mRNA variants are produced through alternative splicing, and further corroborates the accuracy of the two mRNA sequences experimentally cloned in this study, as these mRNA sequences could be perfectly aligned with specific regions on the gene sequence. This, in turn, verified the accuracy of the two protein sequences that could be deduced from these mRNAs. It is of particular interest that, although the sequence of the long-form HtrA3 was found in the GenBank® database without a report of its detailed cloning and characterization, no sequences resembling the short-form HtrA3 have been reported, nor is it predicted by computational analysis. Thus this is the first identification of the accurate genomic structure of this gene and its alternative splicing.

Protein-domain structural comparison of all members of the human HtrA family (HtrA1 [2], HtrA2 [10,11] and HtrA3, including their isoforms), was a powerful tool to predict possible functions of the new members. All members display a highly conserved protease domain, the essential components for protease activity, having 100 % identity among all members. It is of

interest, however, that, although the short form of HtrA2 does contain these essential components, its trypsin domain is lacking a stretch of 65 residues between the second and the third triad aa. This protein isoform has been reported to lack protease activity [10], suggesting that not only the reported essential components but also residues in close physical proximity are necessary for protease activity. Both isoforms of HtrA3 have a well-conserved trypsin domain similar to HtrA1 and the long-form HtrA2; both of these proteins have been confirmed experimentally to display protease activity [3,11]. Therefore, it can be predicted that both isoforms of HtrA3 will possess serine protease activity.

The difference between the two isoforms of HtrA3 is the lack of a PDZ domain in the short form. PDZ domains are found in diverse proteins and are believed to mediate protein–protein interactions [12,13]. Thus it can be predicted that these two isoforms may have differential substrate specificities or other protein–protein interaction differences. It may also be that they are localized in different cell compartments. These predictions still need to be experimentally verified.

Domain structural comparison among the three members of the human HtrA family revealed that the newly identified HtrA3 structurally resembles HtrA1, but that HtrA2 is distinct. The main difference between these proteins lies at the N-terminal end. Whereas both HtrA1 and HtrA3 contain an IGF-binding domain and Kazal-type S protease inhibitor domain, HtrA2 contains a unique RD in the N-terminus. The RD possesses a putative transmembrane region, a potential phosphorylation site for stress-activated kinases and an Src-homology-3-binding domain [11]. At the present time, HtrA2 has been relatively well studied among the family members. It is up-regulated in mammalian cells in response to stress induced by both heat shock and tunicamycin treatment, suggesting an involvement in the mammalian stress-response pathway [14]. The proteolytic activity of HtrA2 is markedly up-regulated in the mouse kidney following ischaemia/reperfusion injury [11]. Recently, several elegant studies established that HtrA2 has an important role in programmed cell death [15–18]. The full-length protein of HtrA2 is imported into the mitochondrion and autoprocessed to form the mature protein by removing the first 133 aa. This autocatalytic cleavage results in a mature HtrA2 protein containing an N-terminal sequence highly homologous with pro-apoptotic Reaper family proteins that can bind to inhibitor of apoptosis (IAP) proteins. These IAPs bind to apoptosis protease caspases and inhibit cell death [19]. In healthy cells, HtrA2 is localized in the mitochondrial intermembrane space. Interestingly, in response to apoptotic stimuli such as UV irradiation, HtrA2 moves into the cytosol and interacts with IAPs, thus eliminating the caspase-inhibitory activity of IAPs and leading to promoting cell death. The recently solved crystal structure of HtrA2 provided further insights into the unique function of HtrA2 in promoting apoptosis [20].

In contrast with HtrA2, the precise function of HtrA1 is still largely unknown. It is reported to be a secreted protein [14], and has been suggested to be involved in the degradation of extracellular matrix proteins that are important in both arthritis and tumour progression and invasion [21]. At the present time, no studies have reported any involvement of HtrA1 in cell death. Given that HtrA1 does not contain the unique N-terminal domain present in HtrA2, it is very likely that these two proteins have different functions. However, further studies are needed to establish the precise function of HtrA1.

Given the striking structural similarity between HtrA1 and HtrA3, the function of HtrA3 is more likely to be similar to that of HtrA1. It is of note that these two proteins display a very different pattern of expression, indicating that the functional

redundancy between these two family members is likely to be minimal. It is particularly interesting that both HtrA1 and HtrA3 are highly expressed in the placenta. One possibility is that both of these proteins also participate in apoptosis during placentation, which involves remarkable cell growth, differentiation and tissue remodelling [22,23]. However, this needs to be proved experimentally. In addition, the presence of an IGF-binding domain in both HtrA1 and HtrA2 is most likely to be of importance. During pregnancy, IGFs are important for growth of both foetal and maternal tissues. Ablation of either the *IGF-I* and/or the *IGF-II* gene has been shown to reduce foetal size [24], and it has been speculated that this may result from impaired placental function. A recent study in which the expression of IGF-II was ablated exclusively in mouse placenta, clearly established that IGF-II is an important modulator of placental and foetal growth [25]. It is also well established that the binding proteins of IGFs (IGFBPs) regulate the bioavailability of IGFs [26], and that proteolytic cleavage of the IGF-IGFBP complex leads to increased levels of bioactive IGFs [27]. Activities of proteases that degrade IGFBPs have been demonstrated within the endometrial/foetal axis during pregnancy [28,29] and in other systems [30,31]. However, the exact identities of proteases involved in regulating the IGF-IGFBP system remain unknown at the present time. We now propose that HtrA1 and HtrA3 may be such proteases. Future studies will establish the intrinsic functions of HtrA1 and HtrA3, especially in the formation and function of the placenta during pregnancy, and will determine whether they are capable of binding IGF or IGF-IGFBP complexes, and whether they can proteolyse any components of the IGF-IGFBP system.

In conclusion, this study has identified an additional member of the HtrA protein family, HtrA3, with highest expression in the heart, ovary and placenta. Both HtrA1 and HtrA3 are likely to be of great importance in the formation and function of the placenta during pregnancy.

We are grateful to Sue Panckridge and Samantha Park for assistance with the preparation of this manuscript. The study was supported by the Rockefeller Foundation Contraceptive 21 Program, the Wellcome Trust (grant 52666), the National Health and Medical Research Council of Australia (RegKeys 143798 to L.A.S. and 983212 and 198705 to J.K.F.) and the Rockefeller/World Health Organization (WHO) Initiative on Implantation.

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