

Reports

Application of the wheat-germ cell-free translation system to produce high temperature requirement A3 (HtrA3) proteases

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Mammalian high temperature requirement A3 (HtrA3) is a serine protease of the HtrA family. It is an important factor for placental development and a tumor suppressor. The biochemical properties of HtrA3 are uncharacterized. One critical step in biochemical characterization is overexpressing and purifying the full-length recombinant protein. However, utility of cell-based expression systems is limited for a protease because of autocleavage. The wheat-germ cell-free translation system is highly efficient at producing “difficult” eukaryotic multidomain proteins and is easily modifiable for protein synthesis at different temperatures. In this study, we evaluated the potential of the wheat-germ cell-free translation system for producing human HtrA3. HtrA3 underwent autocleavage when synthesized at 17°C. When the synthesis temperature was lowered to 4°C, full-length HtrA3 was successfully produced and proteolytically active. Catalytic site serine substitution with alanine (S305A) stabilized HtrA3 while abolishing its protease activity. This mutant was readily synthesized and stable at 17°C. When used with glutathione *S*-transferase (GST) pull-down assay, S305A HtrA3 was a valuable bait in searching for endogenous HtrA3 binding proteins. Thus, we demonstrated the unique utility of the wheat-germ cell-free translation system for producing and characterizing human HtrA3. These strategies will be likely applicable to a wide range of proteases.

The high temperature requirement A (HtrA) proteases are a family of serine proteases identified in organisms ranging from bacteria to mammals (1). They are typically characterized by the presence of a serine protease domain and one or two carboxy (C)-terminal PDZ (postsynaptic density of 95 kDa, discs large, and zonula occludens) domains (2). To date, four mammalian HtrAs are identified in the genome (2). The first three members (HtrA1, HtrA2/Omi, HtrA3) have been cloned, and their expression patterns reported (3); the fourth one (HtrA4) is known only by the cDNA sequence deposited in GenBank (2).

While bacterial HtrAs act as proteases and chaperones to participate in key aspects of protein quality control (1), the mammalian HtrAs have evolved to exert diverse functions, including cell proliferation, migration, and apoptosis, and their altered expression is associated with severe diseases, including cancer, arthritis,

neurodegenerative and neuromuscular disorders, and age-related macular degeneration (1,3). This highlights the need to specifically characterize each mammalian HtrA.

HtrA3 was initially identified in the developing placenta both in the mouse and human as a serine protease associated with pregnancy (4–7). HtrA3 is now known to negatively regulate trophoblast invasion during placental development (8,9), and abnormal levels of HtrA3 during early pregnancy in women are associated with risks of developing preeclampsia (a severe pregnancy-specific disorder) (10,11). HtrA3 is also down-regulated in a number of cancers (ovary, uterus, lung) (12–15) and promotes etoposide- and cisplatin-induced cytotoxicity in lung cancer cell lines (15). HtrA3 is thus proposed to be a tumor suppressor and a potential therapeutic target in cancer treatment (3,15). It has been suggested to

inhibit the transforming growth factor- β (TGF- β) signaling (16).

However, to date, the biochemical properties of HtrA3 are largely unknown. Unlike other HtrAs, HtrA3 has two isoforms [long (HtrA3-L) and short (HtrA3-S)] resulting from alternative splicing (4,5). Both isoforms contain a signature serine protease domain following an N-terminal insulin-like growth factor binding domain and a Kazal protease-inhibitor domain. They differ only by the presence in HtrA3-L and absence in HtrA3-S of a C-terminal PDZ domain (4,5). HtrA3-S thus presents a unique naturally occurring HtrA lacking the C-terminal PDZ domain (2). While HtrA3-L is predominantly expressed in the mouse, both HtrA3-L and HtrA3-S are expressed in human tissues especially in the placenta (5,6). It is unknown whether HtrA3-L and HtrA3-S are biochemically distinct.

One critical step in biochemical characterization of a protein is to overexpress and purify the full-length recombinant protein. Cell-based expression systems present challenges for protease expression because of autocleavage of the product. Indeed multiple bands were obtained when human HtrA1 was expressed in either insect or mammalian cells (17), while no distinct bands were detected for HtrA2 expressed in either *Escherichia coli* or insect cells due to autodegradation during overexpression (18,19). Thus the truncated forms of HtrA1 and HtrA2 are often used for functional analysis (19–22).

In recent years, significant advances have been made in the area of cell-free *in vitro* transcription/translation systems to express “difficult” proteins (23). One such approach is the wheat-germ cell-free translation system, which is highly efficient in producing eukaryotic multidomain proteins in a folded state for functional characterization (23–25).

In this study, we evaluated the potential of the wheat-germ cell-free translation system for producing human HtrA3-L and HtrA3-S in both wild-type (WT) and mutant (MT) forms and explored their potential for further characterizing human HtrA3.

Materials and methods

Production of human HtrA3 protein and its MT using the wheat-germ cell-free translation system

The long (HtrA3-L) and short (HtrA3-S) isoforms of human HtrA3 were produced using wheat-germ cell-free technology (23,24,26). In brief, the open reading frames of the human HTRA3-L and HTRA3-S cDNA (4) were cloned into the pEU-E01-GST expression vector (27) (CellFree Sciences, Matsuyama, Japan) using standard methods. The engineered constructs for producing N-terminal glutathione *S*-transferase (GST)-tagged HtrA3 were transformed into DH5 α cells (Invitrogen, Mulgrave, Vic, Australia), and the plasmids were purified using Qiagen Plasmid Maxi kit (Qiagen, Doncaster, Vic, Australia).

Transcription and translation were performed using a CFS-TRI-1240G kit (CellFree Sciences) as per the manufacturer’s instructions. The transcription was carried out at 37°C for 6 h with 250 μ L containing 100 ng/ μ L plasmid, 1 U/ μ L SP6 RNA polymerase, 1 U/ μ L RNase inhibitor, 2.5 mM NTPs, and 1 \times transcription buffer. To test different synthesis conditions, small-scale trans-

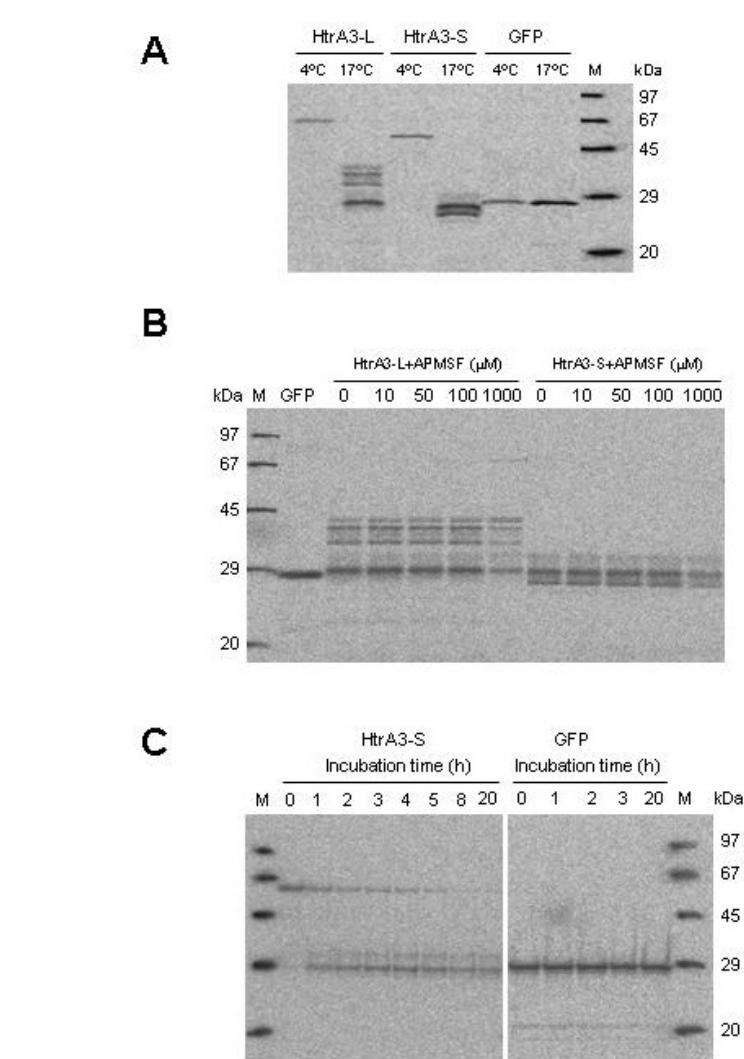


Figure 1. Autoradiographic analysis of HtrA3 protein synthesis using the wheat-germ cell-free system. (A) Synthesis of HtrA3-L and HtrA3-S at 4°C and 17°C. GFP was used as a control. (B) Synthesis of HtrA3-L and HtrA3-S at 17°C in the presence of different concentrations of APMSF. (C) Incubation (0–20 h) of HtrA3-S and GFP at 17°C following synthesis at 4°C. M, protein marker (APRO-Marker; APRO Science Inc., Tokushima, Japan).

lation was carried out in standard flat-bottomed 96-well plates as a bilayer reaction. Briefly, 206 μ L 1 \times SUB-AMIX containing all 20 amino acids (300 μ M each) in a proprietary buffer were transferred into the well, and the transcription mixture (10 μ L transcription product, 0.8 μ L 1 mg/mL creatine kinase, 10 μ L WEPRO1240G) and 2 μ L 14 C-Leu (50 μ Ci) were then carefully pipetted into the bottom of the same well to form a bilayer. The plate was sealed with Parafilm and incubated at 4°C or 17°C for 16 h. The resultant translation products (6 μ L) were analyzed by denaturing SDS-PAGE, stained with Coomassie Blue, and scanned with a phosphorimaging system (Storm Molecular Imager, Molecular Dynamics, Sunnyvale, CA, USA). To produce MT HtrA3 proteins, the catalytic site serine

(S305) was mutated into alanine by site-directed mutagenesis with a PCR overlap extension strategy (28). The MT constructs were confirmed by sequencing, and the corresponding proteins were produced as for the WT counterparts.

To purify HtrA3, the translation was scaled up as per the manufacturer’s instructions using 6-well plates. Briefly, 5.5 mL 1 \times SUB-AMIX was transferred into the well, the transcription mixture (250 μ L transcription product, 1 μ L 20 mg/mL creatine kinase, and 250 μ L WEPRO1240G) was then pipetted into the bottom of the well, and the plate was incubated at 4°C (WT) or 17°C (MT) for 16 h. The synthesized proteins were purified at 4°C (WT) or room temperature (MT) using Glutathione Sepharose 4B gel (GE Healthcare Bio-Sciences,

Rydalme, NSW, Australia) and analyzed by SDS-PAGE to confirm purity.

In vitro protease activity assay

Protease activities of both HtrA3-L and HtrA3-S and their S305A MT forms were detected using casein labeled with fluorescein isothiocyanate (FITC) as a generic substrate as published (29) with minor modifications. In brief, the reaction contained 10 μ L HTRA3-L or HTRA3-S (WT or MT; 500 μ g), 20 μ L assay buffer (20 mM sodium phosphate buffer with 150 mM sodium chloride, pH 7.6, at 37°C), and 20 μ L 0.01% (w/v) FITC-casein (both from Sigma-Aldrich, St. Louis, MO, USA) in a microcentrifuge tube. After mixing, the solution was incubated at 37°C (0–120 min), acidified by gently adding 150 μ L 0.6 N trichloroacetic acid solution (Sigma-Aldrich), and incubated for an additional 60 min at 37°C. The mixture was then centrifuged (1 min at 100 \times *g*), a 60- μ L aliquot of the supernatant was neutralized to 400 μ L with 500 mM Tris buffer (pH 8.5), and 200 μ L of this was transferred to a 96-well plate for fluorescence measurement (490/525 nm, Wallac Victor2 spectrophotometer; PerkinElmer, Boston, MA, USA). In every assay, 0.001% (w/v) trypsin and ultrapure water were used as positive control and blank, respectively.

Using MT HtrA3 as bait in a GST pull-down assay to identify HtrA3 binding proteins

A GST pull-down assay (ProFound pull-down GST protein-protein interaction kit; Pierce, Rockford, IL, USA) was modified to include the MT HtrA3 as bait to search for endogenous HtrA3 binding proteins. In brief, MT HtrA3-L (containing an N-terminal GST-tag, ^{GST}HtrA3-L-MT) or GST alone (negative control) were immobilized to glutathione affinity resin as per the manufacturer's instructions and served as the bait. Prey protein lysates were from a day (d) 10.5 mouse placenta (which expresses high levels of HtrA3; approval was obtained from the Animal Ethics Committee at Monash Medical Centre, Melbourne, Australia). The bait and prey were incubated at 4°C for 16 h with gentle rocking, the resulting mixture was washed five times, and the binding proteins were eluted with 100 mM glutathione elution buffer.

The eluted proteins were analyzed by SDS-PAGE, bands of interest were excised from the gel, and protein identifications were determined by mass spectrometry

as previously described (30). For further validation, the elution products from ^{GST}HtrA3-L-MT or GST resins were analyzed by Western blot analysis using specific antibodies (myosin-9 antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA; HtrA3 antibody as published, Reference 11). Coimmunoprecipitation (co-IP) was used to further validate the identified proteins as endogenous HtrA3 binding proteins. The candidate proteins were immunoprecipitated from the placenta, and the resulting IP products were analyzed by Western blot analysis for HtrA3 co-IP.

Results and discussion

Synthesis of full-length HtrA3 requires low temperature

We first attempted to synthesize human (HtrA3-L) and short (HtrA3-S) at 17°C using a standard protocol and including GFP as a control. While a single band corresponding to the expected size of GFP was detected, multiple bands of smaller than expected HtrA3 proteins were consistently observed (Figure 1A). This suggests that HtrA3-L and HtrA3-S were unstable at 17°C. Presentation of discrete bands for both HtrA3-L and HtrA3-S indicates autocleavage rather than random degradation, consistent with previously reported features of human HtrA1 (17).

We next investigated conditions that could stabilize HtrA3 proteins. When the protein synthesis temperature was lowered to 4°C, single bands of expected sizes (~72 and ~61 kDa, respectively) were obtained for both HtrA3-L and HtrA3-S (Figure 1A), demonstrating that HtrA3 stability is temperature-dependent. The bands produced at 4°C were noticeably less intense than those produced at 17°C (especially GFP), reflecting a reduced protein yield at the lower temperature. With the small-scale translation, the average yield at 4°C was ~0.2 μ g/well.

We determined whether including serine protease inhibitors during the 17°C synthesis would stabilize HtrA3 (by inhibiting HtrA3's own proteolytic activity). When 4-amidinophenylmethane sulfonyl-fluoride (APMSF), a widely used serine protease inhibitor, was included during the synthesis, the resulting band pattern was essentially the same between those containing no (control) or different concentrations of APMSF for either HtrA3-L or HtrA3-S (Figure 1B). Although an intact HtrA3-L band was just visible when APMSF was

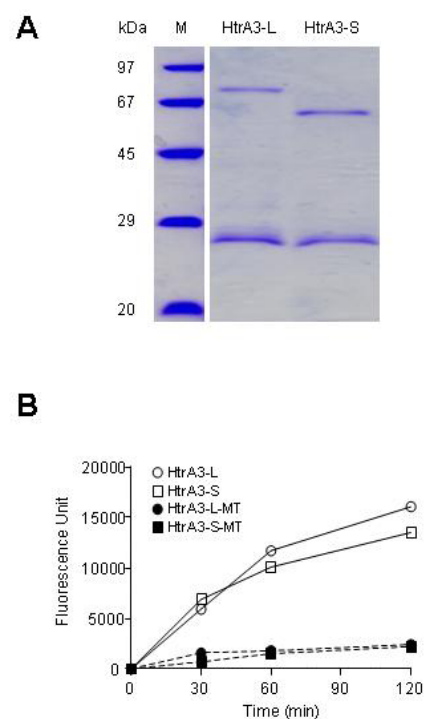


Figure 2. Synthesis and purification of HtrA3-L and HtrA3-S at 4°C and assessment of their proteolytic activity. (A) SDS-PAGE analysis of HtrA3-L and HtrA3-S following synthesis and purification at 4°C. M, protein marker (APRO-Marker; APRO Science Inc., Tokushima, Japan). (B) Representative progressive curve of proteolytic activity of purified HtrA3-L and HtrA3-S. The activity of MT HtrA3-L (HtrA3-L-MT) and HtrA3-S (HtrA3-S-MT) is also presented in comparison to the WT counterparts.

at 1 mM (Figure 1B), indicating slight effect on the stability, overall APMSF had no significant effect on HtrA3 stability. Probably higher concentration of inhibitor is required, because the extract has a high concentration of intrinsic proteins.

We then established the stability of HtrA3 after synthesis at 4°C. Intact HtrA3 proteins were synthesized at 4°C, then incubated at 17°C for 0–20 h. While GFP showed no visible changes in the band pattern over 20 h, both HtrA3-L and HtrA3-S gradually converted from a single intact band to discrete multiple smaller bands (Figure 1C, HtrA3-S; similar data for HtrA3-L, not shown). The conversion was clear at 1 h, and no intact band was visible by 8 h (Figure 1C). Inclusion of APMSF during the 17°C incubation had no effect (data not shown).

HtrA3 produced at 4°C is enzymatically active

As 4°C synthesis was successful in providing full-length HtrA3 proteins,

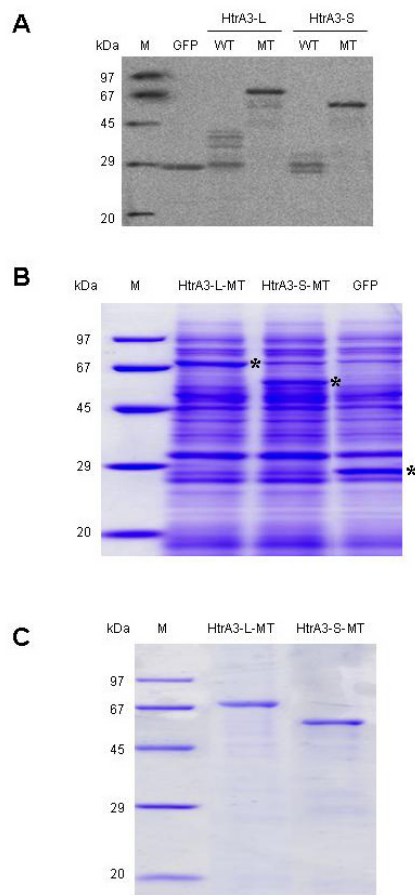


Figure 3. Synthesis and purification of MT HtrA3. (A) Autoradiographic analysis of WT and MT HtrA3-L and HtrA3-S synthesis at 17°C. GFP was used as a control. (B) SDS-PAGE and Coomassie blue staining of 17°C products of HtrA3-L-MT, HtrA3-S-MT, and GFP. Asterisks (*) represent the specific protein bands. (C) Analysis of purified HtrA3-L-MT and HtrA3-S-MT following synthesis in panel B. M, protein marker (APRO-Marker; APRO Science Inc., Tokushima, Japan).

we determined whether the 4°C synthesized and purified HtrA3 proteins were enzymatically active. Their purity was confirmed by SDS-PAGE analysis (Figure 2A). As their natural substrates are unknown, a generic *in vitro* protease activity assay using FITC-labeled casein as a substrate was used to assess the protease activity. Both HtrA3-L and HtrA3-S progressively cleaved casein over time, with typical protease characteristics (Figure 2B).

Thus, the wheat-germ system is useful to synthesize intact and active HtrA3 proteins for future downstream characterization. HtrA3-S, which naturally contains no C-terminal PDZ domains, had activity equal to that of HtrA3-L, suggesting the PDZ domain is not essential for HtrA3 enzymatic activity, which is consistent with a recent

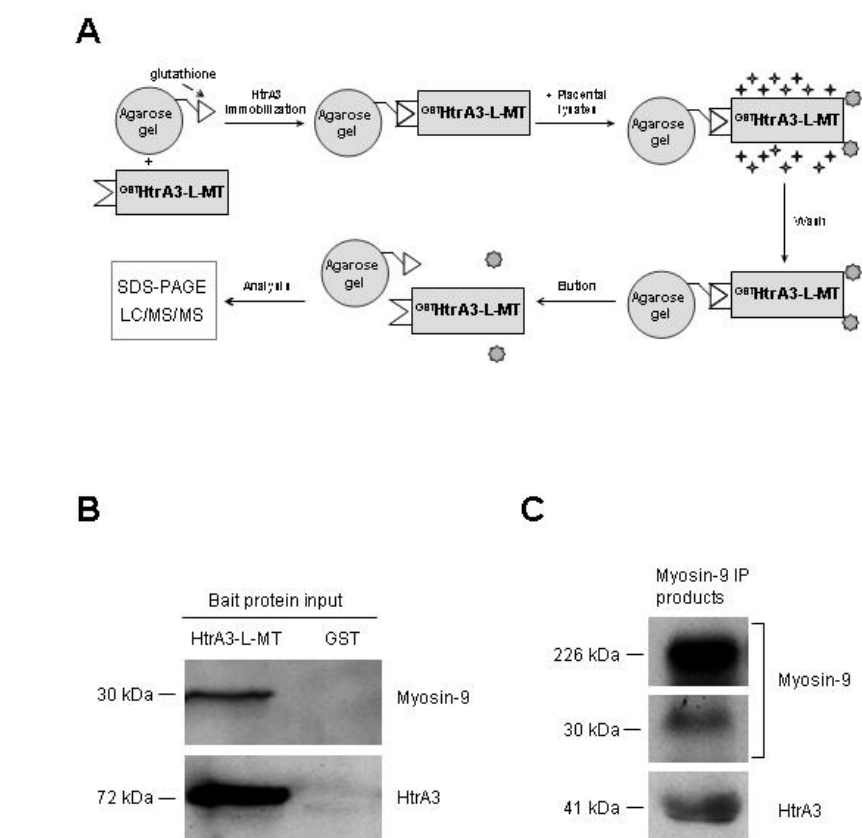


Figure 4. Using HtrA3-L-MT as bait combined with a GST pull-down approach to identify HtrA3 binding proteins. (A) Schematic illustrating the main steps involved in the pull-down assay. (B) Western blot analysis of the elution products from GSTHtrA3-L-MT or GST resins with myosin-9 and HtrA3 antibodies. (C) Western blot analysis, using myosin-9 and HtrA3 antibodies, of myosin-9 IP products from placental lysates.

report that deletion of the PDZ domain in human HtrA1 does not affect its enzymatic activity (31). These observations are in clear contrast to nonmammalian HtrAs, which require the PDZ domain to activate the proteases (32,33). Thus, through evolution, mammalian HtrAs may have lost the contribution of the PDZ domain to enzymatic activity. It is still possible that the PDZ domain in mammalian HtrAs may modulate protein-protein interactions within the cell, thereby allowing differential cellular actions for HtrA3-L versus HtrA3-S.

Minimal S305A mutation stabilizes HtrA3 but abolishes the protease activity

For a serine protease, the serine residue in the catalytic triad at the active site is critical for catalysis. We thus determined whether mutating the catalytic site serine (S305) would stabilize HtrA3. Serine S305 was substituted with alanine (S305A) in both HtrA3-L and HtrA3-S, and the protein band patterns when synthesized at 17°C were compared between the MT and

the corresponding WT HtrA3 proteins (Figure 3A). While multiple bands were detected for WT proteins, a major single band of the expected size was observed for both MT HtrA3-L and HtrA3-S (Figure 3A), demonstrating that the mutation stabilized the protein. Due to the increased stability of the MT proteins, their yield was also enhanced, resulting in a single prominent band corresponding to the MT HtrA3-L or HtrA3-S, clearly visible on a stained gel (marked as asterisks in Figure 3B). The average yield of MT production at 17°C using the large-scale translation was ~20 µg/well.

The MT HtrA3 proteins were purified and analyzed by SDS-PAGE to confirm purity (Figure 3C). When these were tested for activity, no clear proteolysis was detected by either MT HtrA3-L or HtrA3-S (Figure 2B), confirming that the S305A mutation inactivated the protease as expected. This is consistent with early reports that substitution of the catalytic site serine with alanine abolishes the protease activity of HtrA1 (17). These results demonstrate that S305A mutation

stabilized HtrA3 proteins through inactivation of its protease activity, which in turn suggests that HtrA3 instability is likely by self-proteolysis.

MT HtrA3 is a valuable bait to identify binding proteins using pull-down assays

Because the S→A substitution is a minimal mutation, the resulting proteolytically inactive MT proteases should retain many properties of the WT counterparts and thus provide unique tools for characterizing WT proteins (31). Further, the S305A MT HtrA3 proteins can be produced more easily and abundantly than the WT forms. We thus explored the potential utility of the MT HtrA3 proteins as research tools.

The first test application was to use the MT HtrA3 proteins as bait to identify endogenous HtrA3 binding proteins, since MT HtrA3 should bind similarly as WT to endogenous binding proteins. Indeed, human HtrA1 and its MT (similar active site S→A mutation) show an equal binding affinity to their endogenous binding proteins (34). Catalytically inactive MT-proteases have been successfully used in the yeast two-hybrid system to search for protease binding proteins and to trap potential substrates (35). Taking an approach similar to the commonly used GST pull-down assay, we searched for HtrA3 binding proteins in tissues using MT HtrA3-L as bait (Figure 4A). Day 10.5 mouse placenta, which expresses high levels of HtrA3 (5), was used as a test tissue. GST-HtrA3-L-MT or GST alone (negative control) was immobilized to glutathione affinity resin (agarose beads) as bait, then incubated with the placental lysates to allow binding of the putative binding proteins. After washing, the protein complexes (bait and bound proteins) were eluted with glutathione, the resulting proteins analyzed by SDS-PAGE, and the identities of potential binding proteins determined by mass spectrometry (Figure 4A).

One of the candidates identified as a novel HtrA3 binding protein was myosin-9. For validation, the elution products from GST-HtrA3-L-MT or GST resins were analyzed by Western blot analysis. Using a myosin-9-specific antibody, a single band of ~30 kDa corresponding to a cleaved form of myosin-9 (full-size myosin-9 is ~226 kDa) was detected in the GST-HtrA3-L-MT but not the GST control elution (Figure 4B); demonstrating myosin-9 coeluted specifically with HtrA3. Using an HtrA3-specific antibody, a single band of ~72 kDa corresponding to GST-HtrA3-

L-MT was detected only in the GST-HtrA3-L-MT elution (Figure 4B), confirming that HtrA3 and myosin-9 coexisted in the same elution.

Co-IP was performed to further validate myosin-9 as an endogenous binding protein of WT HtrA3 in the placenta. Myosin-9 was immunoprecipitated from the placental lysates, and the resulting IP products were analyzed by Western blot analysis. With a myosin-9 antibody, an ~30-kDa band (a cleaved form of myosin-9), similar to that seen in the pull-down elution, and an ~226-kDa band of full-length myosin-9 were detected (Figure 4C). This also demonstrates that the placenta contains both the full-length and the cleaved form of myosin-9. No myosin-9 was immunoprecipitated with control IgG (data not shown), confirming IP specificity. When the myosin-9 IP products were probed with an HtrA3 antibody, a specific band of ~41 kDa corresponding to the mature native HtrA3-L was detected (Figure 4C), confirming that HtrA3 was coimmunoprecipitated with myosin-9.

These results provide strong evidence that the MT HtrA3 proteins can be utilized to pull down endogenous HtrA3 binding proteins in tissues. Since only the cleaved form of myosin-9 bound to HtrA3, future studies will investigate whether myosin-9 is an HtrA3 substrate.

Taken together, these studies demonstrate the potential of the wheat-germ cell-free translation system to produce and characterize human HtrA3 proteases. As it is a cell-free *in vitro* system, it is amenable for protein synthesis at different temperatures; this unique attribute allowed successful production of full-length human HtrA3 proteins at 4°C that had not been previously possible in cell-free systems. This is the first report demonstrating the feasibility of obtaining a full-length mammalian HtrA *in vitro*. The system also allows the production of MT HtrA3 with high efficiency; such MTs have the potential utility for characterizing the WT proteases. Such strategies will be applicable to studies of a wide range of proteases.

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Competing interests

The authors declare no competing interests.

References

- Clausen, T., M. Kaiser, R. Huber, and M. Ehrmann. 2011. HTRA proteases: regulated proteolysis in protein quality control. *Nat. Rev. Mol. Cell Biol.* 12:152-162.
- Clausen, T., C. Southan, and M. Ehrmann. 2002. The Htra family of proteases. Implications for protein composition and cell fate. *Mol. Cell* 10:443-455.
- Chien, J., M. Campioni, V. Shridhar, and A. Baldi. 2009. Htra serine proteases as potential therapeutic targets in cancer. *Curr. Cancer Drug Targets* 9:451-468.
- Nie, G.-Y., A. Hampton, Y. Li, J.K. Findlay, and L.A. Salamonsen. 2003. Identification and cloning of two isoforms of human Htra3, characterisation of its genomic structure and comparison of its tissue distribution with Htra1 and Htra2. *Biochem. J.* 371:39-48.
- Nie, G.-Y., Y. Li, H. Minoura, L. Batten, G.T. Ooi, J.K. Findlay, and L.A. Salamonsen. 2003. A novel serine protease of the mammalian Htra family is up-regulated in mouse uterus coinciding with placentation. *Mol. Hum. Reprod.* 9:279-290.
- Nie, G., Y. Li, K. Hale, H. Okada, U. Manuelpillai, E.M. Wallace, and L.A. Salamonsen. 2006. Serine protease HTRA3 is closely associated with human placental development and is detectable in pregnancy serum. *Biol. Reprod.* 74:366-374.
- Nie, G., Y. Li, H. He, J.K. Findlay, and L.A. Salamonsen. 2006. Htra3, a serine protease possessing an IGF-binding domain, is selectively expressed at the maternal-fetal interface during placentation in the mouse. *Placenta* 27:491-501.
- Singh, H., Y. Endo, and G. Nie. 2011. Decidual Htra3 negatively regulates trophoblast invasion during human placentation. *Hum. Reprod.* 26:748-757.
- Singh, H., S.i. Makino, Y. Endo, and G. Nie. 2010. Inhibition of HTRA3 stimulates trophoblast invasion during human placental development. *Placenta* 31:1085-1092.
- Than, N.G., R. Romero, R. Hillermann, V. Cozzi, G. Nie, and B. Huppertz. 2008. Prediction of preeclampsia—a workshop report. *Placenta* 29:83-85.
- Li, Y., M. Puryer, E. Lin, K. Hale, L.A. Salamonsen, U. Manuelpillai, S. Tong, W. Chan, et al. 2011. Placental Htra3 is regulated by oxygen tension and serum levels are altered during early pregnancy in women destined to develop preeclampsia. *J. Clin. Endocrinol. Metab.* 96:403-411.
- Bowden, M.A., L. Di Nezza-Cossens, T. Jobling, L.A. Salamonsen, and G. Nie. 2006. Serine proteases HTRA1 and HTRA3 are down-regulated with increasing grades of human endometrial cancer. *Gynecol. Oncol.* 103:253-260.
- Beleford, D., Z. Liu, R. Rattan, L. Quagliuolo, M. Boccellino, A. Baldi, J. Maguire, J. Staub, et al. 2010. Methylation induced gene silencing of Htra3 in smoking-related lung cancer. *Clin. Cancer Res.* 16:398-409.
- Bowden, M.A., A.E. Drummond, P.J. Fuller, L.A. Salamonsen, J.K. Findlay, and G. Nie. 2010. High-temperature requirement factor A3 (Htra3): a novel serine protease and its potential role in ovarian function and ovarian cancers. *Mol. Cell. Endocrinol.* 327:13-18.
- Beleford, D.T., R. Rattan, J. Chien, and V. Shridhar. 2010. High-temperature requirement A3 (Htra3) promotes etoposide- and cisplatin-induced cytotoxicity in lung cancer cell lines. *J. Biol. Chem.* 285:12011-12027.
- Tocharus, J., A. Tsuchiya, M. Kajikawa, Y. Ueta, C. Oka, and M. Kawaichi. 2004. Developmentally regulated expression of mouse Htra3 and its role as an inhibitor of TGF-beta signaling. *Dev. Growth Differ.* 46:257-274.
- Hu, S.-I., M. Carozza, M. Klein, P. Nantermet, D. Luk, and R.M. Crowl. 1998. Human Htra, an evolutionarily conserved serine protease identified as a differentially expressed gene product in osteoarthritic cartilage. *J. Biol. Chem.* 273:34406-34412.
- Savopoulos, J.W., P.S. Carter, S. Turconi, G.R. Pettman, E.H. Karran, C.W. Gray, R.V. Ward, O. Jenkins, and C.L. Creasy. 2000. Expression, purification, and functional analysis of the human serine protease Htra2. *Protein Expr. Purif.* 19:227-234.
- Seong, Y.-M., H.-J. Park, G.-H. Seong, J.-Y. Choi, S.-J.K. Yoon, B.-R. Min, S. Kang, and H. Rhim. 2004. N-terminal truncation circumvents proteolytic degradation of the human Htra2/Omi serine protease in *Escherichia coli*: rapid purification of a proteolytically active Htra2/Omi. *Protein Expr. Purif.* 33:200-208.
- Grau, S., P.J. Richards, B. Kerr, C. Hughes, B. Caterson, A.S. Williams, U. Junker, S.A. Jones, et al. 2006. The role of human Htra1 in arthritic disease. *J. Biol. Chem.* 281:6124-6129.
- Tsuchiya, A., M. Yano, J. Tocharus, H. Kojima, M. Fukumoto, M. Kawaichi, and C. Oka. 2005. Expression of mouse Htra1 serine protease in normal bone and cartilage and its upregulation in joint cartilage damaged by experimental arthritis. *Bone* 37:323-336.
- Cilenti, L., Y. Lee, S. Hess, S. Srinivasula, K.M. Park, D. Junqueira, H. Davis, J.V. Bonventre, et al. 2003. Characterization of a novel and specific inhibitor for the pro-apoptotic protease Omi/Htra2. *J. Biol. Chem.* 278:11489-11494.
- Endo, Y. and T. Sawasaki. 2006. Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotechnol.* 17:1-8.
- Sasaki, T., T. Ogasawara, R. Morishita, and Y. Endo. 2002. A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA* 99:14652-14657.
- Takai, K., T. Sawasaki, and Y. Endo. 2010. Practical cell-free protein synthesis system using purified wheat embryos. *Nat. Protocols* 5:227-238.
- Endo, Y. and T. Sawasaki. 2003. High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *Biotechnol. Adv.* 21:695-713.
- Tsuboi, T., S. Takeo, H. Iriko, L. Jin, M. Tsuchimochi, S. Matsuda, E.-T. Han, H. Otsuki, et al. 2008. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect. Immun.* 76:1702-1708.
- Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51-59.
- Twining, S.S. 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal. Biochem.* 143:30-34.
- Stephens, A.N., N.J. Hannan, A. Rainczuk, K.L. Meehan, J. Chen, P.K. Nicholls, L.J.F. Rombauts, P.G. Stanton, et al. 2010. Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. *J. Proteome Res.* 9:2438-2449.
- Truebestein, L., A. Tennstaedt, T. Mönig, T. Krojer, F. Canellas, M. Kaiser, T. Clausen, and M. Ehrmann. 2011. Substrate-induced remodeling of the active site regulates human HTRA1 activity. *Nat. Struct. Mol. Biol.* 18:386-388.
- Wilken, C., K. Kitzing, R. Kurzbauer, M. Ehrmann, and T. Clausen. 2004. Crystal structure of the DegS stress sensor: how a PDZ domain recognizes misfolded protein and activates a protease. *Cell* 117:483-494.
- Krojer, T., J. Sawa, R. Huber, and T. Clausen. 2010. Htra proteases have a conserved activation mechanism that can be triggered by distinct molecular cues. *Nat. Struct. Mol. Biol.* 17:844-852.
- Oka, C., R. Tsuchimoto, M. Kajikawa, K. Koshihara-Takeuchi, J. Ina, M. Yano, A. Tsuchiya, Y. Ueta, et al. 2004. Htra1 serine protease inhibits signaling mediated by Tgfbeta family proteins. *Development* 131:1041-1053.
- Lopez-Otin, C. and C.M. Overall. 2002. Protease degradomics: a new challenge for proteomics. *Nat. Rev. Mol. Cell Biol.* 3:509-519.

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